Characterisation of the Auxin Signalling Pathway in *Vitis vinifera* L. cv. Shiraz

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Abstract

Auxin is a key regulator of plant growth, mechanistically controlled by a finely tuned transcriptional system. The auxin signalling pathway consists of the transcription factors, auxin response factors (ARFs), the transcriptional repressors, auxin/indole-3-acetic acid (Aux/IAAs), and the transport inhibitor response 1/auxin signalling F-box (TIR1/AFB) proteins that form a co-receptor complex with the Aux/IAA proteins. These proteins modulate the plant's response to auxin, mediating a myriad of downstream responses. Auxin is involved in most, or all, developmental processes and in responses to environmental cues. It has been found to be a repressor of ripening in grapes, and a decrease in berry auxin concentration is required to allow the initiation of ripening.

To investigate the role of the auxin signalling pathway in grape, 19 *VviARF* transcription factors, 23 *VviIAA* repressors and six *VviAFB* receptor family members were identified in *Vitis vinifera* sp. The conserved domains were analysed in each group of proteins and their presence or absence related to possible function. Phylogenetic trees demonstrated the relationship of the *Vvi* genes with apple, Arabidopsis, poplar, and tomato family members, some of which have proven function in fruit development.

Expression analysis across a 16 week *V. vinifera* L. cv. Shiraz berry developmental series suggested that 39 of the 48 auxin signalling transcripts were highly expressed pre-veraison (before the onset of ripening) and were down-regulated from veraison and throughout berry ripening, correlating with the high concentration of auxin pre-veraison. The varied expression patterns of these genes suggest participation in a range of developmental processes at different stages during development. The change in expression of a large proportion (39 of 48) of auxin signalling genes at veraison indicates that it is a key change-point in berry development. Those genes expressed early in development may play roles in cell division and cell expansion. Two *VviARF* transcripts, *VviARF27* and *2b*, and five *VviIAA* transcripts, *VviIAA15b, 19, 31, 38* and *40*, were highly up-regulated post-veraison suggesting that they may play roles in fruit ripening. ARF proteins have also been found to play roles in fruit ripening through interactions with other proteins such as MYBs and bHLHs. The expression patterns of the 48 auxin signalling genes in a nine stage leaf developmental series could be clustered into 12 groups. Interestingly, very few auxin signalling pathway genes were expressed in leaves with a pattern that correlated to the pattern of IAA or IAA-Asp accumulation. Various transcripts had high transcript expression in flowers, roots and/or tendrils again suggesting a diversity in the roles these genes play.

Yeast 2-hybrid and bimolecular fluorescence techniques showed that VviARF4-VviIAA19, VviARF27-VviIAA19 and VviARF27-VviIAA27 protein pairs interact and have nuclear localisation. The ARF

activator, *VviARF27*, and *VviIAA19* have overlapping expression patterns, in post-veraison berries and flowers, suggesting the interaction between these two proteins may occur *in planta* and play a role in flowering and berry ripening. From their expression patterns, VviARF4 and VviIAA19 may interact in tendrils, and VvARF27 and VviIAA27 may interact in flowers.

Ex planta berry treatments suggested that auxin and ethylene/abscisic acid (ABA) have antagonistic effects on the auxin signalling pathway in grape berries. Auxin treatment up-regulated *VviIAA* transcripts pre- and post-veraison whilst the enhancers of ripening, ethylene, in the form of Ethrel, and ABA, down-regulated some auxin signalling transcripts, both pre- and post-veraison. ABA may play an important role in ripening by switching off the vegetative pathways pre-veraison, such as photosynthesis, while ethylene enhances ripening factors post-veraison.

A model explaining the role of auxin signalling during berry development is proposed where the majority of VviARF and VviIAA proteins function during pre-veraison berry development, when the levels of IAA are high. These are switched off towards veraison, and allow the transition to ripening. During fruit ripening, a select number of VviARF and VviIAA proteins may interact to affect the ripening process – potentially through interactions with other protein families. ABA may down-regulate the auxin signalling pathway pre-veraison, and ethylene down-regulates some members of the pathway post-veraison. These findings support the existence of a fruit specific, complex hormonal network that works in concert to modulate and ensure grape berry growth and ripening.

This research represents the most in-depth analysis of the auxin signalling pathway components in *V. vinifera* to date and highlights the pleiotropic roles the candidates play throughout plant development. These findings may aid in the development of strategies to manipulate berry ripening and identifies areas for future research.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Abbreviations

Standard abbreviations were included in this work as detailed by Plant Physiology (https://pphys.msubmit.net/html/Abbreviations.pdf) for the common abbreviations. Any abbreviation that occurs three or more times within this document are included in the tables below. Commonly abbreviated gene names that were only used one or two times were left in their abbreviated forms.

General

Term	Definition
°Brix	Degrees Brix
2, 4-D	2, 4-dichlorophenoxyacetic acid
35S	35S constitutive promoter from the Cauliflower Mosaic Virus
A	Activator
aa	Amino acid
ABA	+cis trans abscisic acid, abscisic acid
AbA	Aureobasidin A
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ABP1	Auxin binding protein 1
ABRE	Abscisic acid-responsive element
AFB	Auxin signaling F-box
AGRF	Australian Genome Research Facility
ARF	Auxin response factors
ARF+	ARF activator proteins
ASK	ARABIDOPSIS SKP1 HOMOLOGUE
At	Arabidopsis
Aux/IAA	Auxin/indole-3-acetic acid
auxin_resp	Auxin response factor domain
AuxRE	Auxin responsive elements
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BEAUTi	Bayesian Evolutionary Analysis Utility
bHLH	Basic helix-loop-helix
BiFC	Bimolecular fluorescence
BL	24-epibrassinolide
BLASTP	Basic Local Alignment Search Tool protein
BMGE	Block Mapping and Gathering using Entropy

Term	Definition
BR	Brassinosteroid
BTOA	Benzothiazole-2-oxyacetic acid
CA	California
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CEB1	Cell Elongation bHLH
CFP	Cyan fluorescent protein
Chr.	Chromosome
COOH	Carboxyl-terminus of a protein
C-terminus	Carboxyl-terminus of a protein
cYFP	Yellow fluorescent protein C-terminus end, the gene of interest is fused at the N-terminus of the YFP fragment
DAPI	4', 6-diamidino-2-phenylindole
DBD	B3 DNA binding domain
DD	Dimerization domain
DHS	DNase I hypersensitivity
E value	Expect value
E. coli	*Escherichia coli*
epi-BL	Epi-brassinolide
ER	Endoplasmic reticulum
gFW-1	Per gram of fresh weight
GH3	Gretchen Hagen 3
GRIP	Grape ripening-induced proteins
GTR	Generalised time reversible
HCL	Hierarchical clustering
IAA	Indole-3-acetic acid
IAA-Asp	IAA-Aspartate
IAA-Glu	IAA-Glutamine
IAA-Trp	IAA-Tryptophan
iP	6-(γ,γ-Dimethyl-allylamino)-purine, isopentenyladenine
LB medium	Luria-Bertani broth medium
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LRR	Leucine rich repeats
Md	*Malus domestica*
MeV	MultiExperiment Viewer

Term	Definition
MF	Multiplication factor
miRNA	MicroRNA
MR	Middle region
MUSCLE	Multiple Sequence Comparison by Log-Expectation
MYB	Myeloblastosis
N.D.	Not detected
NAA	1-naphthaleneactic acid
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
NH	Amino-terminus of a protein
N-terminal	Amino-terminus of a protein
nYFP	Yellow fluorescent protein N-terminus end, the gene of interest is fused at the N-terminus of the YFP fragment
ORF	Open reading frame
PB1	Phox and Bem1 domain
Pfam	Protein family
PIN1	PIN-FORMED 1
PlantPAN	Plant promoter analysis navigator
Pt	*Populus trichocarpa* (poplar)
QDO/X/AbA	Quadruple drop out, X-α-Gal, aureobasidin A
qPCR	Quantitative PCR
Q-rich	Glutamine-rich
RIN	Ripening-inhibitor
RNAseq	RNA sequencing
RT-qPCR	Real-time quantitative PCR
SAUR	SMALL AUXIN UP RNA
SCF complex	S-PHASE KINASE-ASSOCIATED PROTEIN-CULLIN-F-Box complex
$SCF^{TIR1/AFB}$	S-PHASE KINASE-ASSOCIATED PROTEIN-CULLIN-F-Box transport inhibitor response 1/auxin signaling F-box complex
SD	Sucrose deficient
siRNA	Small interfering RNA
SKP	S-PHASE KINASE-ASSOCIATED PROTEIN
Sl	*Solanum lycopersicum* (tomato)
SNAP33	Soluble N-ethylmaleimide-sensitive factor adapter protein 33
sp.	Species
TAIR	The Arabidopsis Information Resource

Term	Definition
TBLASTN	Translated nucleotide Basic Local Alignment Search Tool
TIR1	Transport inhibitor response 1
Tm	Melting temperature
TPL	TOPLESS
TSS	Total soluble solids
t-Z	Trans-zeatin
USA	United States of America
UTR	Untranslated region
V. vinifera	*Vitis vinifera*
Vvi	*Vitis vinifera*
WPF	Weeks post flowering
XET	Xyloglucan endotransglycosylases
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-Bromo-4-chloro-1H-indol-3-yl β-D-glucopyranosiduronic acid
Y2H	Yeast 2-hybrid
YFP	Yellow fluorescent protein
YFPc	Yellow fluorescent protein C-terminus end, the gene of interest is fused at the C-terminus of the YFP fragment
YFPn	Yellow fluorescent protein N-terminus end, the gene of interest is fused at the C-terminus of the YFP fragment
Zm	*Zea mays*

Chapter 1 Introduction

1.1 Introduction

1.1.1 Grapes and the grape industry

Grapevine is a woody perennial that belongs to the genus *Vitis* in the *Vitaceae* family (Iland *et al.*, 2011). The ancestors of grapevine were thought to be present 181 million years ago during the Jurassic period (Thomas & van Heeswijck, 2004; Iland *et al.*, 2011). Since that time more than 800 grapevine cultivars belonging to the species *Vitis vinifera* (*V. vinifera*) have been domesticated and are grown for wine, table grapes and dried fruit. Most of the species in the *Vitis* genus flower in a monoecious manner, characterised by small flowers bearing both male and females organs that hang in inflorescences and later form the characteristic bunches of grape berries (May, 2000; Iland *et al.* 2011).

Wine, table grapes and dried fruit are an important part of the Australian economy. The Australian Table Grape Association Inc. states that table grapes were worth AUD$330 million annually in 2008. The wine industry is the largest grape industry in Australia, and Australia is the fourth largest wine exporter in the world (Australian Bureau of Statistics, 2012). The Australian Bureau of Statistics reported that for the financial year of 2011–12 the Australian Wine and Grape Industry was worth AUD$2.49 billion in domestic wine sales and Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES) predicted that 2016–17 wine export sales would reach AUD$2.37 billion (Australian Bureau of Statistics, 2013; ABARES, 2017).

Since grape cultivation began in 7000–5000 B.C. many techniques have been developed to manipulate grape size and composition including trunk girdling, rootstock selection, deficit irrigation and pruning (Coombe, 1960a; Roby *et al.*, 2004; Roby & Matthews, 2004; Walker *et al.*, 2005; Williams & Ayars, 2005; Iland *et al.*, 2011). The molecular mechanisms that mediate grape berry development and composition in grapevine are still poorly understood, however, great progress has been made in recent years (Kohno *et al.*, 2012; Kuhn *et al.,* 2014; Castellarin *et al.,* 2016; Pilati *et al.,* 2017).

1.1.2 Grape berry development

Berry ripening can be divided into three stages: a phase of rapid berry growth, followed by a lag phase and finally the second phase of rapid berry growth and fruit ripening (Figure 1.1) (Kohno *et al.,* 2012). Most of the cell division in the flesh (mesocarp, Figure 1.2) of the grape berry occurs during a period from 5–10 days (d) prior to anthesis to 5–10 d after anthesis (Coombe, 1960a; 1960b; Harris *et al.,* 1968). Cells also expand during this time, but the berries remain firm in texture and green due to the presence of chlorophyll. The sugar content of the berries is low during the first stage of berry development, and organic acids, such as tartaric acid and malic acid, begin to accumulate (Coombe,

1960a; 1960b; Pratt, 1971). The onset of berry ripening (veraison) and is determined by the commencement of sugar accumulation (Davies & Robinson, 1996). Prior to veraison is the second stage of development that is known as the lag phase when berry growth slows before resuming again in the third stage. During the second stage the berries are firm, organic acid concentrations reach their highest levels, and the loss of chlorophyll begins. In the third stage of development berry growth resumes as cell expansion and ripening begins. Ripening is characterised in red varieties by colour development in the form of anthocyanins. The berries soften, chlorophyll is lost and sugar and aroma and flavour compounds accumulate (Coombe & McCarthy, 2000). Berry softening begins at veraison approximately 45–60 d after anthesis, depending on the cultivar, and involves a decrease in turgor pressure followed by cell wall changes that allow berry expansion to occur (Coombe, 1960a; 1960b; Ishimaru *et al.*, 2007; Schlosser *et al.*, 2008; Castellarin *et al.*, 2016). Sugar is transported as sucrose through the plant and is cleaved into the hexose sugars, glucose and fructose, within the berry for storage in the vacuole (Coombe, 1992; Davies & Robinson, 1996; Zhang *et al.,* 2006; Shiraishi *et al.,* 2010). The amount of tartaric acid is largely maintained across berry development but the concentration decreases due to rapid berry growth. Malic acid concentration decreases, in part from respiration and enzymic degradation as well as dilution. Many factors influence ripening in grape, including the grape variety, viticultural management and climate.

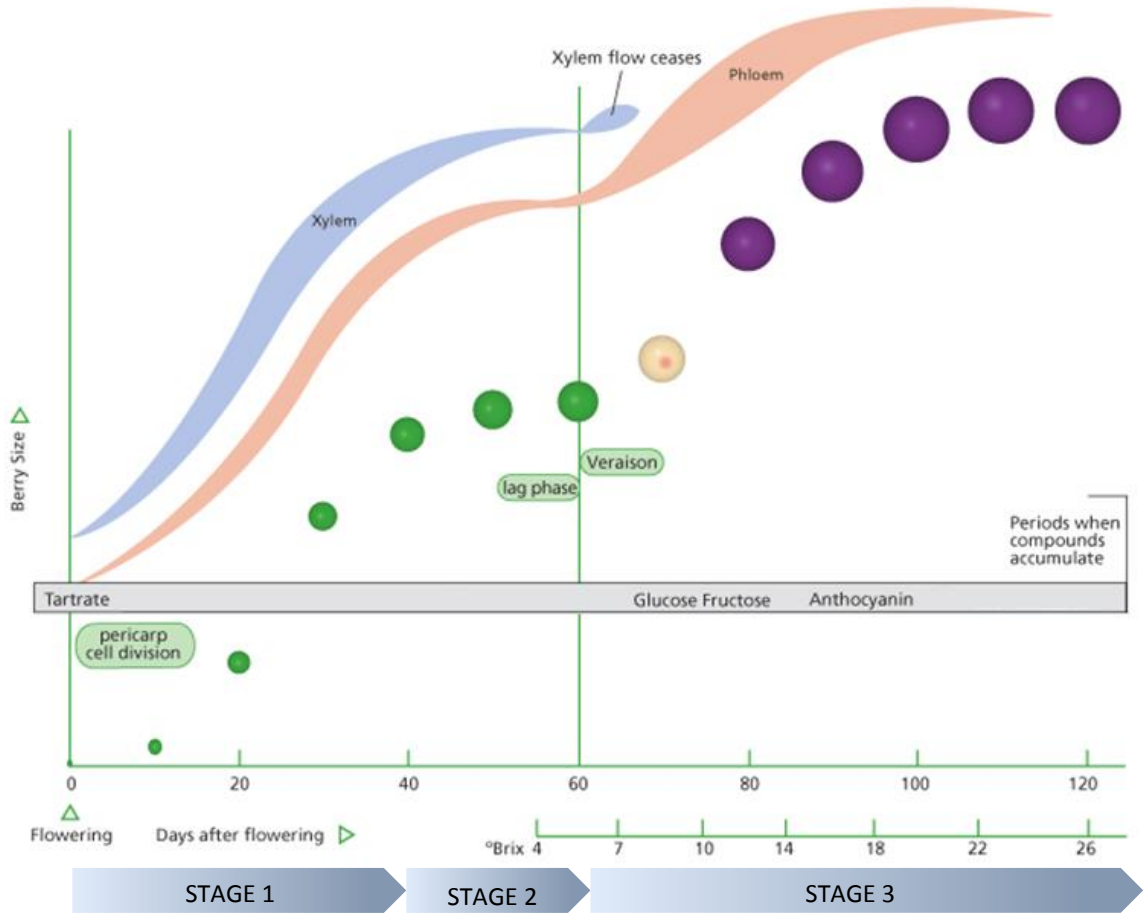


Figure 1.1 A schematic diagram of grape berry development.

Grape berry development begins with flowering and can be divided into three stages. Stage one is characterised by rapid berry growth due to cell division and expansion, the organic acid tartrate accumulates early in development. Stage two is a lag phase that ends at veraison while stage three incorporates a second phase of rapid berry growth through cell expansion and berry ripening. During ripening the concentration of sugars (°Brix), glucose and fructose, increase within the berry. Secondary metabolites, including anthocyanins mainly accumulate during ripening. Sugar and water flow into the berry are mediated through the xylem and phloem in stage one and two, with xylem flow ceasing at the beginning of stage three and only phloem flow remaining. Adapted from Coombe (1987) and Kennedy (2002), Jordan Koutroumanidis (2002, original figure illustrator).

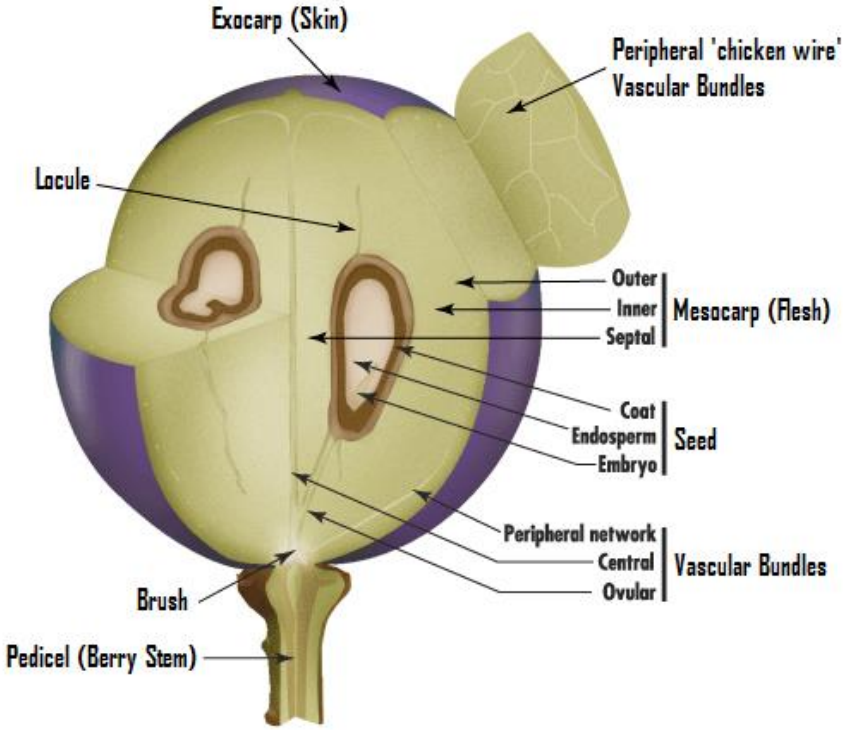


Figure 1.2 A schematic diagram of the anatomy of a mature grape berry.

Together the exocarp and mesocarp tissues form the pericarp of the berry. Taken from Kennedy (2002), Jordan Koutroumanidis (2002, original figure illustrator).

1.1.3 Transcriptional changes accompany berry ripening

Coordinated transcriptional changes occur at veraison, where a large number of gene transcripts are up- or down-regulated. The proteins encoded by these transcripts may play roles in controlling the changes associated with ripening including: (1) cell wall changes through pectin-modifying enzymes, extensins and expansins, grape ripening-induced proteins (GRIPs) and xyloglucan endotransglycosylases (XETs) (Robinson & Davies, 2000; Pilati *et al.,* 2007; Kohno *et al.,* 2012; Ishimaru *et al.,* 2007; Schlosser *et al.,* 2008); (2) increased sugar uptake, and the osmotic stress involved with this sugar uptake; and (3) the formation of secondary metabolites, such as anthocyanins via the phenylpropanoid pathway (Coombe & McCarthy, 2000; Pilati *et al.,* 2007). Transcriptional changes within the berry may be developmentally controlled, but also may occur in response to external factors including light stimuli, involving proteins such as CONSTANS-like family transcription factors and phytochrome-associated proteins, stress responses, such as disease, and seasonal variation. These coordinated transcriptional changes suggest that master regulators, such as phytohormones, may be involved, allowing global transitions to occur within the berry (Böttcher & Davies, 2012).

1.1.4 The role of phytohormones in fruit development

Phytohormones, including auxins, abscisic acid (ABA), cytokinins, brassinosteroids (BR), gibberellic acids and ethylene, form a complex network of interactions (Figure 1.3) that allows for a highly responsive system to regulate transcript levels throughout fruit development in a range of plant species (Paponov *et al.*, 2008; Santner & Estelle 2009; Jaillais & Chory 2010; Böttcher & Davies, 2012; Karlova *et al.*, 2014). The perception and transduction of these signals and the ability to respond to phytohormones is thought to vary throughout plant development, and also depends on external stimuli (Davies & Böttcher 2009; Böttcher *et al.*, 2013a). It has been suggested that phytohormones regulate the same plant processes through non-overlapping transcriptional responses dependent on the developmental stage (Nemhauser *et al.*, 2006). McAtee *et al.* (2013) divided fruit development in a range of species including; grape, citrus, strawberry, kiwifruit, melon, tomato, apple and banana, into two stages; fruit maturation and fruit ripening. It was suggested that auxins and cytokinins playing key roles in regulating fruit maturation and ethylene and ABA are responsible for promoting fruit ripening. In grape, the exact mechanisms that control the initiation of berry development and the transition to ripening have not been well characterised, however, the physiological effects of phytohormones have been widely studied. ABA, BR, ethylene, cytokinin and auxin have been selected here for discussion in the context of grape berry development. The concentration of these phytohormones vary throughout grape berry development (Figure 1.4) and a body of evidence indicates that they play key roles in berry development and ripening.

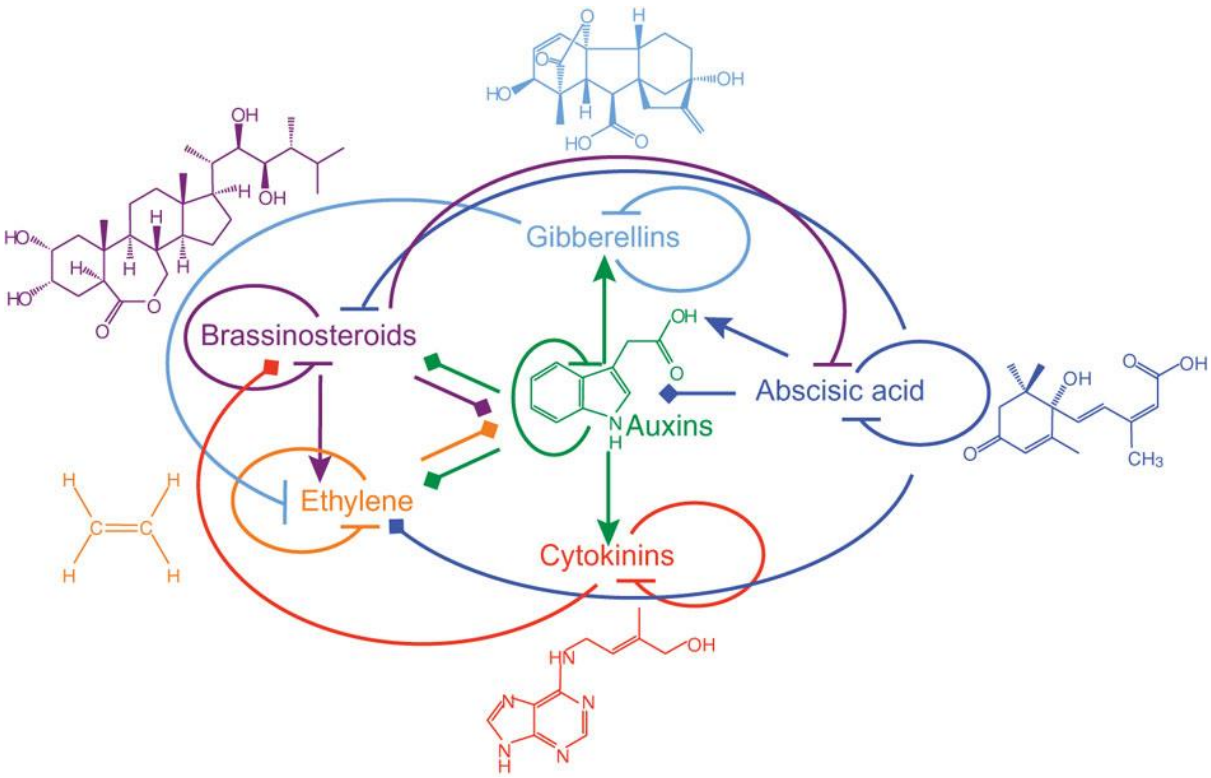


Figure 1.3 The interplay of auxins, abscisic acid, cytokinins, ethylene, brassinosteroids and gibberellins.

Lines with arrowheads represent the up-regulation of hormone biosynthetic genes or up-regulation of genes involved in hormone inactivation. Lines with blocked ends represent the down-regulation of genes involved in hormone biosynthesis or up-regulation of genes involved in inactivation of a hormone. Lines with diamond arrowheads represent changes in gene expression with an ambiguous outcome. Taken from Jaillais & Chory (2010).

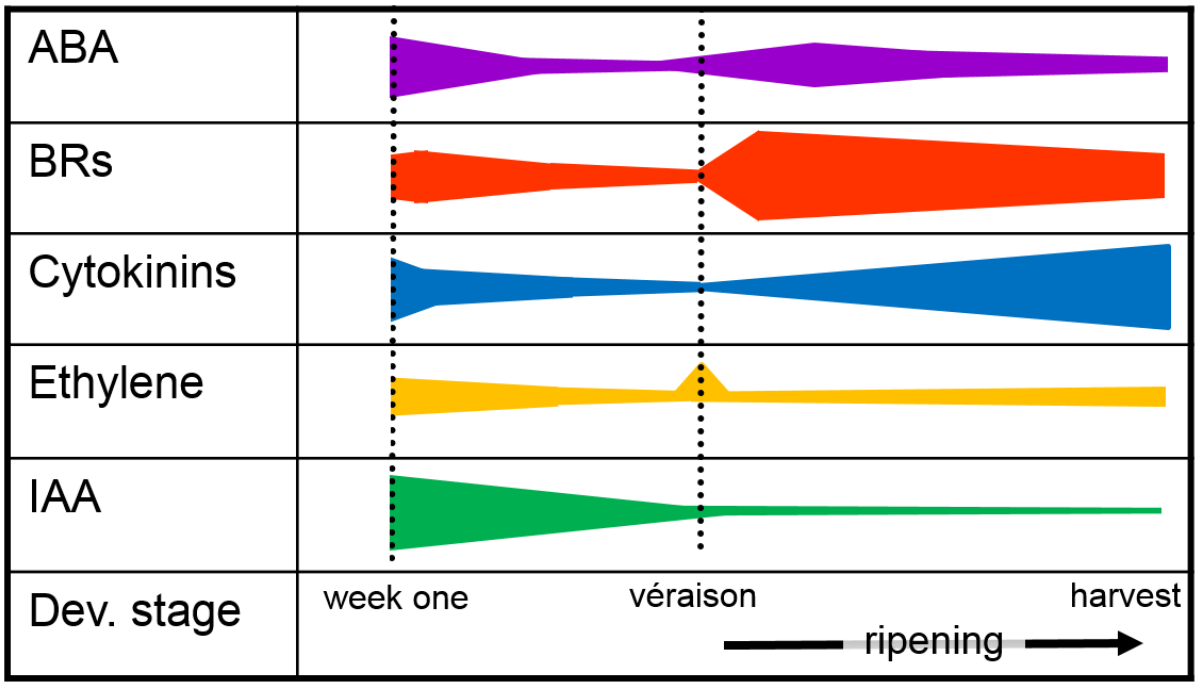


Figure 1.4 The levels of four hormones throughout grape berry development.

The levels of abscisic acid (ABA), brassinosteroids (BRs), cytokinins, ethylene and auxin – indole-3-acetic acid (IAA) are shown across grape berry development with week one of berry development, veraison, ripening and harvest indicated in the box marked developmental stage (dev. stage). Edited from Böttcher & Davies (2012), additional information from Alleweldt & Koch (1977).

1.1.4.1 Ethylene

Ethylene is a well-known hormone that is classically linked to ripening and is associated with fruit softening, colour change and an increase in energy-rich compounds, such as sugars, sugar alcohols or lipids, in some fruit (Porritt, 1951; Burg & Burg, 1962; Böttcher & Davies, 2012). However, the ripening process can be markedly different depending on the plant species. Climacteric fruits, including tomato, banana, apple and peach, have a peak of ethylene and respiratory activity linked with fruit ripening and high levels of ethylene are required for fruit ripening (Bapat *et al.*, 2010; Paul *et al.*, 2012; McAtee *et al.*, 2013). Non-climacteric fruit have a declining respiratory activity during fruit development and do not produce high levels of ethylene, ethylene is therefore thought to be involved in ripening to a lesser degree (Setha, 2012; McAtee *et al.*, 2013). As the ethylene detection systems have improved, ethylene has been detected in a wide range of fruits and it is now believed ethylene plays key roles in both climacteric and non-climacteric fruits to varying degrees, however, the exact role that ethylene plays in the ripening of non-climacteric fruits is unclear (McAtee *et al.*, 2013; Böttcher *et al.*, 2013b). This may leave a gap for other hormones, such as ABA, to play key roles in ripening in place of ethylene either through the promotion of ripening or repression of inhibitory pathways, for example via decreasing the levels of a hormone such as auxin (Setha, 2012; McAtee *et al.*, 2013).

There are mixed reports about ethylene production in grape berries depending on the method used to measure ethylene (Böttcher & Davies, 2012). Inaba *et al.* (1976) and Weaver & Singh (1978) measured ethylene production from flowering to post-veraison within grape berries, identifying a peak at flowering but no detectable ethylene at veraison (Figure 1.3). Alternatively, Alleweldt & Koch (1977) showed an ethylene peak in berries at veraison and Chervin *et al.* (2004) measured an ethylene peak just before veraison, however, the levels in these reports were too low to merit the 'climacteric fruit' classification. Together these reports suggest that there may be low levels of ethylene produced during grape berry development, most likely with a small peak around veraison (Figure 1.4) (Böttcher & Davies, 2012). The transcript level of the enzyme responsible for the last stage of ethylene biosynthesis, *1-aminocyclopropane-1-carboxylate (ACC) oxidase*, was highest during the pre-veraison lag phase before declining for the remainder of development, and the ethylene pathway activation appears to occur in the three weeks prior to veraison (weeks 6–8) when ethylene levels were at their highest in Cabernet Sauvignon (Chervin *et al.*, 2004; Deluc *et al.*, 2007). Fortes *et al.* (2011) noted that several *ACC oxidase* transcripts were down-regulated during ripening, whilst one was up-regulated, supporting the idea that the peak of activity occurs before veraison. However, some isoforms are also active post-veraison, matching previous findings in watermelon (Wechter *et al.,* 2008).

The exogenous application of ethylene has been found to stimulate changes in grape berry development (Böttcher *et al.*, 2013b). The treatment of Cabernet Sauvignon berries with ethylene led to an increase in berry expansion through increased water-exchange via aquaporins and by altering cell wall modifying genes, such as cellulose synthases and expansins, suggesting ethylene plays a role in controlling berry size in grape (Chervin *et al.*, 2008). Szyjewicz *et al.* (1984) summarised a range of ethylene-mediated responses including an increase in the respiratory rate of berries and enhancing colour development in response to exogenous application of an ethylene-releasing compound. Previous studies suggested that the timing of the ethylene treatment was crucial, with both auxin and ABA concentrations implicated in differential responses to ethylene throughout fruit development (Hale *et al.*, 1970; Coombe & Hale, 1973). Coombe & Hale (1973) hypothesised that endogenous ABA mediates the response to exogenous application of ethylene-releasing compounds, however ABA must have passed a threshold before ABA and ethylene are able to promote ripening. If the application of ethylene is prior to this threshold, ripening is delayed (Coombe & Hale, 1973). Interestingly, the treatment of Shiraz berries with the ethylene-releasing compound Ethrel some weeks before veraison led to an increase in auxin biosynthetic gene transcript levels, subsequently increasing the auxin indole-3-acetic acid (IAA) and the IAA conjugate, IAA-Aspartate (IAA-Asp) concentrations within the fruit and delaying ripening (Böttcher *et al.*, 2013a). This supports the concept that the removal of auxin is necessary for the onset of ripening in grapes and may provide a basis for why ethylene alone is unable to induce ripening in non-climacteric fruits (Davies *et al.*, 1997; Chervin *et al.*, 2004; 2008; Giribaldi *et al.*, 2010a; Böttcher *et al.*, 2011b; 2013b).

1.1.4.2 Abscisic acid (ABA)

ABA has many functions within the plant, including stress responses, preparation for dormancy and fruit development (Seo & Koshiba, 2002; Leng *et al.*, 2014). ABA appears critical in fruit ripening in both climacteric and non-climacteric fruits, playing roles in sugar signalling, anthocyanin accumulation and cell wall changes (Leng *et al.*, 2014). Unlike ethylene, ABA is present at readily detectable levels in grape berries. ABA has a biphasic accumulation pattern, and a high concentration post-veraison suggests ABA is a candidate for the induction of ripening (Figure 1.3) (Coombe, 1973; Coombe & Hale, 1973; Scienza *et al.*, 1978; Davies *et al.*, 1997; Owen *et al.*, 2009; Wheeler *et al.*, 2009; Böttcher & Davies, 2012). A recent review of fruit development supports this model as does the effects arising from the treatment of grapes with ABA (McAtee *et al.*, 2013). ABA treatments increased anthocyanin accumulation in Kyoho grapes (Ban *et al.*, 2003; Jeong *et al.*, 2004) and in Merlot grapes decreased the levels of chlorophyll (Gény *et al.*, 2004). Gény *et al.* (2004) showed that ABA application decreased IAA levels within Merlot berries. Giribaldi *et al.* (2010b) treated grape berries with ABA at three time points; before veraison, early and mid-veraison. They found that berries were most responsive to ABA

application prior to veraison, leading to an increase in the number of coloured berries at veraison and an increase in sugar content (Giribaldi *et al.*, 2010a). The application of ABA to grape bunches at three pre-veraison time points enhanced fruit ripening, seen as an increase in average berry weight and higher anthocyanin concentrations and an increase in ABA and sugar-related transcripts (Wheeler, 2006; Wheeler *et al.*, 2009; Gambetta *et al.,* 2010). Fortes *et al.* (2011) discussed previous findings that ABA is able to induce *MYB (myeloblastosis)* transcription factors involved in the synthesis and accumulation of anthocyanins in addition to sugar uptake and accumulation. The treatment of pre-veraison berries with ABA followed by RNA sequencing (RNAseq) of the berry skin indicated ABA triggers its own biosynthesis and initiates a cascade of transcript changes associated with ripening (Pilati *et al.,* 2017). These transcriptional changes, related to effects on acidity, sugar and anthocyanin accumulation, the loss of chlorophyll, and the high concentration of ABA post-veraison suggest ABA plays a crucial role in grape berry ripening (Palejwala *et al.*, 1985; Ban *et al.*, 2003; Gény *et al.*, 2004; Jeong *et al.*, 2004; Giribaldi *et al.*, 2010a; Pilati *et al.,* 2017).

1.1.4.3 Brassinosteroids (BRs)

BRs have been associated with cell expansion and elongation, accelerating senescence, protection against various plant stresses and have been reported to play a key role in determining the onset of ripening in fleshy fruits, including tomato, potentially through enhancing ethylene production (Clouse & Sasse, 1998; Vardhini & Rao 2002; Haubrick & Assmann, 2006; Böttcher & Davies, 2012). Brassinosteroids accumulate in a biphasic pattern during grape berry development (Figure 1.4) (Symons *et al.*, 2006). Exogenous ABA application rapidly inhibits BR signalling outputs, and it is proposed that ABA and BR signalling cascades intersect after BR perception but before transcriptional activation (Zhang *et al.*, 2009). Many BR-responsive genes are also ABA responsive, further highlighting the complexity of the phytohormone network (Zhang *et al.*, 2009). Symons *et al.* (2006) applied a synthetic BR, epi-brassinolide, and a BR biosynthesis inhibitor, brassinazole, to grape bunches at four time points across berry development. Epi-brassinolide enhanced fruit ripening, whilst brassinazole delayed ripening, indicating that BRs enhance grape ripening (Symons *et al.*, 2006). The exact mechanism by which BR enhances grape ripening is unknown.

1.1.4.4 Cytokinins

Cytokinins are involved in a range of physiological functions within plants, including playing roles in cell proliferation and differentiation, light responses and circadian rhythm, senescence, the transition to flowering and stress responses (reviewed in Hwang *et al.*, 2012). Interestingly, cytokinin responses involve a signal transduction pathway similar to that of auxin, however, there is a high dependency on phosphorylation mediating the transfer of the cytokinin signal (Hwang *et al.*, 2012). In grape,

cytokinins are thought to play roles in berry set and promoting berry growth (Davies & Böttcher, 2009). Zhang *et al.* (2004) measured the concentrations of trans-zeatin (*t*-Z) in 'Kyoho' (*Vitis labrusca* L. x *Vitis vinifera* L.) and determined that they were high in early berry development then decreased towards veraison then becoming undetectable. However, the more recent analysis in Böttcher *et al.* (2015) described the concentration of the cytokinins *t*-Z, *trans*-zeatin-*O*-glucoside and isopentenyladenine (iP) during weeks 4 to 16 of Shiraz berry development. *t*-Z was detected at low concentrations in weeks 10, 14 and 16, and *trans*-zeatin-*O*-glucoside displayed an increase in concentration between weeks 14 and 16. They also found that in Cabernet Sauvignon, Riesling and Pinot Noir levels of the cytokinin iP increased from veraison, increasing from weeks 8 to 14, with a drop in concentration at week 16 (Böttcher *et al.* 2015). An increase in iP concentration was also found in the fleshy fruit strawberry, kiwifruit and tomato implicating this increase in fruit ripening (Pilkington *et al.*, 2013). These results suggest, in contrast to Zhang *et al.* (2003), that after veraison the levels of some cytokinins are seen to increase throughout berry ripening and until grape harvest (Figure 1.4). As iP concentration is similar to the accumulation of hexose sugars, Böttcher *et al.* (2015) suggested that the iP concentration may be involved in the accumulation of hexoses or may result from the osmotic stress caused by the accumulation of hexose. It is also thought that cytokinins may play roles in post-veraison cell expansion, potentially through the induction of cell wall changes (Böttcher *et al.*, 2015). This is supported by the increase in berry size seen in a range of seedless and seeded grape cultivars following the treatment with a synthetic cytokinin (Zabadal & Bukovac, 2006).

1.1.4.5 Auxin

Auxins have been found to play roles in cell division and expansion, apical dominance, tropisms, in root and shoot architecture, organ patterning, vascular development, fruit set and development (Went & Thimann, 1937; Guilfoyle *et al.*, 1998; Friml *et al.*, 2003; Davies, 2004; Woodward & Bartel, 2005; Jain *et al.*, 2006a; 2006b; 2006c; Mockaitis & Estelle, 2008; Çakir *et al.*, 2013). Apart from the most prevalent form of auxin, IAA, there are other natural auxins as well as synthetic auxins. Synthetic auxins such as 1-naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) have been found to have auxin-like activity (Woodward & Bartel, 2005).

Auxin levels are high early in fruit development when cell division and expansion are occurring in the fruit of a range of climacteric and non-climacteric species. Auxin levels then decline to low levels at the onset of ripening and remain low for the rest of berry development in Cabernet Sauvignon, with excess IAA thought to be readily conjugated by the IAA-amido synthetase Gretchen Hagen 3 (GH3) proteins (Figure 1.4, Figure 1.5) (Staswick, 2002; Staswick & Tiryaki, 2004; Böttcher *et al.*, 2010b; Böttcher *et al.*, 2011a). Cawthon & Morris (1982) reported that fruit set of grape berry was correlated

with an increase in IAA concentration in grape berries, followed by an observed decrease in IAA concentration prior to veraison. As with a range of other species including, banana (Vendrell, 1968; Purgatto *et al.*, 2002), kiwifruit (Fabbroni *et al.*, 2006), tomato (Cohen, 1996) and strawberry (Given *et al.*, 1988), the application of auxin, synthetic auxins and auxin-like substances to unripe fruit or pre-veraison grape berries delays the onset of ripening (Figure 1.8) (Weaver, 1962; Hale, 1968; Davies *et al.*, 1997; Yakushiji *et al.*, 2001; Jeong *et al.*, 2004; Fujita *et al.*, 2006; Böttcher *et al.*, 2010b; Böttcher *et al.*, 2011b; Davies *et al.*, 2015). This is seen as a delay in the accumulation of sugars and anthocyanins, a delay in the decrease of acids and chlorophyll levels, and a delay in the post-veraison phase of berry expansion (Davies *et al.*, 1997; Böttcher *et al.*, 2010a; Böttcher *et al.*, 2011b). Interestingly, the application of IAA does not delay ripening, while the synthetic auxins NAA and benzothiazole-2-oxyacetic acid (BTOA) do, with BTOA having the strongest effect. This is due to the fact that IAA is readily conjugated by GH3 proteins to form IAA-Asp, which is not active as a form of auxin, while NAA, and particularly BTOA, are poor substrates and therefore have longer lasting effects (Böttcher *et al.*, 2011a). Interestingly, the size of NAA-treated berries was larger than control fruit at harvest and sugar accumulation was more synchronous (Böttcher *et al.*, 2010b). BTOA-treated berries were smaller than both control and NAA berries at harvest (Böttcher *et al.*, 2011b). Figure 1.6 illustrates the potent ripening inhibition that is possible when pre-veraison berries are treated with BTOA, with treated berries retaining their chlorophyll and appearing smaller than the untreated berries due to decreased cell expansion (Davies *et al.*, 1997; Robinson & Davies, 2000). BTOA is not transported systemically and maintains the berries in a pre-veraison state thereby inhibiting ripening. This supports the concept that the removal of auxin from the grape berry may be a prerequisite for ripening to occur (Frenkel & Dyck, 1973; Given *et al.*, 1988; Buta & Spaulding, 1994; Purgatto *et al.*, 2002; Böttcher *et al.*, 2010a; Böttcher & Davies, 2012). Therefore, in opposition to ABA and BR enhancing grape berry ripening, auxin is thought to play a key role as a negative regulator of ripening (Davies & Böttcher, 2009).

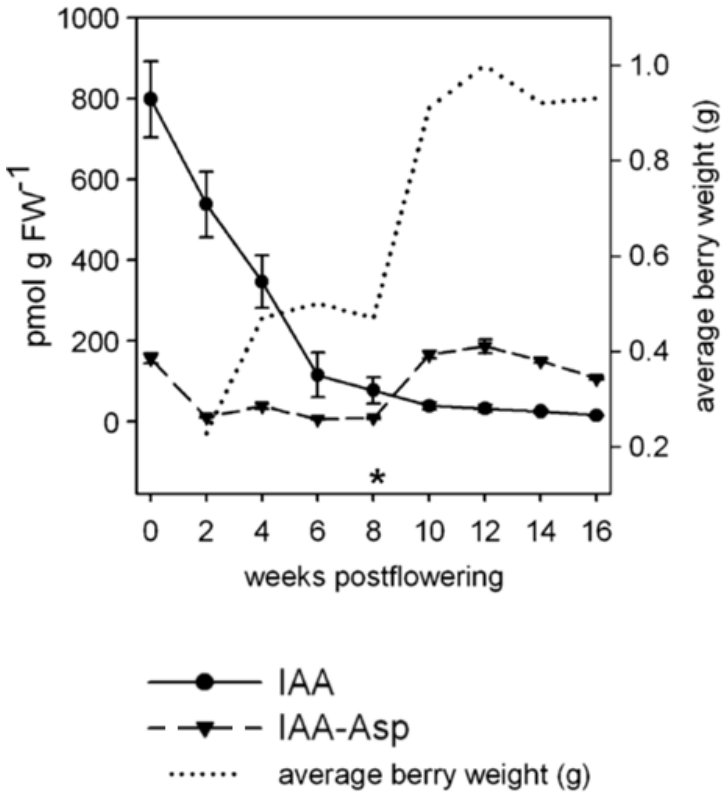


Figure 1.5 The concentration of IAA and IAA-Aspartate in *V. vinifera* L. cv. Cabernet Sauvignon berries sixteen weeks post flowering in field-grown plants compared to average berry weight.

Free IAA and IAA-Aspartate conjugate were measured in picomoles per gram of fresh weight (FW) and average berry weight was calculated by dividing the total berry weight by the number of berries (100–150) sampled at each time point. Taken from Böttcher *et al.* (2010b).

Figure 1.6 The inhibition of grape berry ripening by benzothiazole-2-oxyacetic acid (BTOA) treatment.

The lower half of a bunch of grapes was treated with 20 parts per million BTOA prior to veraison, the top half of the bunch remained untreated, indicated by the red line. The untreated berries at the top of the bunch ripened normally while the ripening was delayed by two weeks in the BTOA treated berries in the bottom half of the bunch. Taken from Robinson & Davies (2000).

The delay in the initiation of ripening by auxin leads to questions about how auxin specifically regulates fruit ripening. As the removal of auxin from the berry may be key to the onset of ripening, the focus of this study will be understanding the role of auxin and the auxin signalling pathway in grape berry development. At the onset of this research, the involvement of auxin and the roles it plays in fruit development were, in general, poorly characterised. Since this time a number of studies have been published on the auxin signalling pathway candidates and their expression patterns across fruit development in a range of species.

1.1.5 Auxin – What is known?

Auxin is initially perceived within the cell by auxin receptors resulting in a signalling cascade, covered in more detail in Section 1.1.5.3. Of note, the short-lived auxin/indole-3-acetic acid (Aux/IAA) proteins with the transcription factors, auxin response factors (ARFs), and the transport inhibitor response 1 (TIR1)/auxin signaling F-box (AFB) receptor proteins form the auxin signalling pathway. Aux/IAA proteins are involved in the primary response to auxin mediating the downstream transcriptional responses to auxin through interactions with the ARF proteins, detailed in Section 1.1.5.4 (Abel & Theologis, 1996).

1.1.5.1 Biosynthesis of auxin

Auxin is synthesised in young developing leaves, the shoot apical meristem, in the meristematic tissue of primary root tips and in the tips of lateral roots (Pollmann *et al.*, 2006). Two methods of IAA biosynthesis have been suggested: the tryptophan-dependent pathway and the tryptophan-independent pathway (Mano & Nemoto, 2012). Currently, only a single biosynthetic pathway has been fully elucidated, the indole-3-pyruvic acid pathway, which uses the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 enzyme to convert tryptophan to IPyA, then YUCCA enzymes convert IPyA to IAA (Stepanova *et al.*, 2008; Tao *et al.*, 2008; Yamada *et al.*, 2009; Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011; Dai *et al.*, 2013; Enders & Strader 2015).

Various mechanisms for controlling auxin levels are active within the cell and help to mediate the auxin response. These mechanisms include the GH3 proteins which appear to dampen the auxin signal and maintain auxin homeostasis by conjugating auxin to amino acids thus altering their activity (Staswick, 2002; Staswick & Tiryaki, 2004; Böttcher *et al.*, 2011a). It is proposed that conjugation allows for the transport, storage, compartmentalization of auxin, and prevention of auxin toxicity (Woodward & Bartel, 2005). Nine *GH3* genes have been identified in grapevine (Böttcher *et al.,* 2010b; Böttcher *et al.,* 2011a). *GH3-1* (Böttcher *et al.*, 2010b) and *GH3-2* (Böttcher *et al.*, 2011a) are both induced by auxin, *GH3-2* follows a similar expression pattern to IAA concentrations, and GH3-1 has been associated with grape berry ripening.

1.1.5.2 Transport of auxin within the plant and cells

IAA is transported basipetally (apex to the base) and suppresses lateral growth through apical dominance (Woodward & Bartel, 2005). Basipetal and acropetal (base to the apex) movements occur within the roots. The influx of IAA into cells and the directional transport of IAA between cells are mediated by AUX1/LAX proteins that are located asymmetrically in the plasma membrane of cells (Marchant *et al.*, 1999; Woodward & Bartel, 2005; Enders and Strader, 2015). The efflux of IAA from cells is mediated by the long PIN-FORMED 1 (PIN1) transporter proteins and the ATP-BINDING CASETTE SUBFAMILY B (ABCB) which are located in the plasma membrane of cells. PIN1 transporter proteins are asymmetrically located allowing for polar auxin efflux (Hayashi, 2012; reviewed by Remy & Duque, 2014). After reorientation of the tissue, PIN1 proteins are relocated from the old lateral walls to the new lateral walls (Woodward and Bartel, 2005). Short PIN1 proteins are localised to the endoplasmic reticulum (ER) and mediate the transfer of IAA from the cytoplasm into the endoplasmic reticulum (reviewed in Enders & Strader, 2015; Mravec *et al.*, 2009; Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Sawchuk *et al.*, 2013). Some ABCB family members have been found to be involved in the influx or efflux of auxin, depending on the cytoplasmic concentration of auxin, importing auxin upon low cytoplasmic concentrations and exporting auxin upon high cytoplasmic concentrations (reviewed in Enders & Strader, 2015; Yang & Murphy, 2009; Kamimoto *et al.*, 2012; Kubeš *et al.*, 2012). Currently little is known about the transport of auxin precursors and auxin conjugates.

1.1.5.3 Perception of auxin

Three potential auxin receptors have been identified: auxin binding protein 1 (ABP1), the TIR1/AFB F-box proteins, and S-phase kinase associated protein 2A (SKP2A) proteins (Napier, 2001; Timpte, 2001; Dharmasiri *et al.*, 2005a; Dharmasiri & Estelle, 2004; Kepinski & Leyser, 2005; Wan *et al.*, 2010). Until recently, the ABP1 receptors were hypothesised to mediate rapid non-genomic effects of auxin, potentially by regulating the distribution of the PIN1 proteins (Robert *et al.*, 2010; Xu *et al.*, 2010; Chen *et al.*, 2012; Wang *et al.*, 2013; Chen *et al.*, 2014; Xu *et al.*, 2014). However, Gao *et al.* (2015) identified *abp1* mutants indistinguishable from wild-type plants suggesting that they may not play a key role in plant development in Arabidopsis in normal growth conditions. SKP2A proteins have been found to bind IAA and 2,4-D and are required for auxin-regulated cell division, however, currently as very little is known about this pathway and the role of SKP2A and auxin in the cell cycle they are considered possible receptors only (Jurado *et al.*, 2010; Enders & Strader 2015).

The SCF complex is an E3 ligase protein complex comprised of an S-phase kinase associated protein 1 (SKP1, ARABIDOPSIS SKP1 HOMOLOGUE, or ASK in plants), CULLIN1, a RING-BOX1 (RBX1), and a substrate adapter protein called an F-box protein. F-box proteins are named due to the presence of

one or more F-box motifs that mediate protein-protein interaction, and they act as receptors (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Pickart, 2001). The F-box proteins provide substrate specificity, recruiting substrates to the SCF complex, promoting ubiquitination and often resulting in the degradation of the target protein by the proteasome (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Pickart, 2001). In plants, the TIR1/AFBs have been identified as F-box family proteins that perceive and bind auxin, prior to stimulating a signalling cascade through the proteasome-mediated protein degradation pathway (Dharmasiri *et al.*, 2005a; Mockaitis & Estelle, 2008). TIR1/AFB proteins bind the Aux/IAA proteins as substrates, with auxin binding to the leucine rich repeats (LRRs) domain of the TIR1/AFB protein and acting as a 'molecular glue' strengthening the interaction with the Aux/IAA proteins and promoting their ubiquitination (Figure 1.6) (Worley *et al.*, 2000; Ramos *et al.*, 2001; Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005; Tan *et al.*, 2007; Parry *et al.*, 2009; Lee *et al.*, 2009).

1.1.5.3.1 The TIR1/AFB family of receptors

The signalling cascade triggered upon the perception of auxin by TIR1/AFBs has been well characterised in Arabidopsis, where six family members have been identified and AtTIR1, AtAFB1, 2, 3 and 5 have been found to function as auxin receptors (Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Kepinski & Leyser, 2005; Parry *et al.*, 2009; Calderon Villalobos *et al.*, 2012). In Arabidopsis, *TIR1/AFBs* are almost ubiquitously expressed throughout the plant, especially in areas of cell division and expansion, and have overlapping functions (Dharmasiri *et al.*, 2005b; Parry *et al.*, 2009). Gene expression analysis of roots treated with auxin illustrated that *TIR1/AFB* mRNA levels are not rapidly changed by the application of auxin (Parry *et al.*, 2009). Mutant studies conducted by Parry *et al.*, (2009) show that the different *TIR1/AFBs* appear to vary in their auxin responses and have different specialised functions, with TIR1 having the strongest activity that is irreplaceable by the AFB proteins even when they were expressed under the *TIR1* promoter (Parry *et al.*, 2009). The TIR1 and AFB2 groups are thought to act as positive regulators of auxin signalling through the promotion of Aux/IAA protein degradation (Dharmasiri *et al.*, 2005b). AtAFB4 and 5 also act as auxin receptors, however, they exhibit selective auxin binding and bind the picloram family of synthetic auxinic herbicides (Calderon Villalobos *et al.*, 2012; Prigge *et al.*, 2016). Binding assays suggest that the TIR1/AFB-Aux/IAA interaction varies depending on auxinic substances, the auxin affinity is determined largely by the Aux/IAA protein (Calderon Villalobos *et al.*, 2012). The Aux/IAA proteins appear to show differences in degradation rates dependent on the specific TIR1/AFB receptor they are bound to (Havens *et al.*, 2012). The exact function of each protein remains to be elucidated, as do the specific functions of the Aux/IAAs that each individual TIR1/AFB targets.

In grapevine, two transcriptome profiling studies have illustrated potential differences in the expression of the *TIR1/AFB* homologues during berry ripening. *AFB2/3* (*VviAFB9*) appears to be up-regulated during ripening, whilst *TIR1* (*VviAFB8*) appears to be down-regulating during ripening (Fortes *et al.*, 2011; Lijavetzky *et al.*, 2012). In a study of *TIR1/AFB* phylogenies, six potential grapevine *TIR1/AFB* homologues were identified; two similar to *TIR1*, two similar to *AFB2* and one in the *AFB4* and *AFB6* clades (Parry *et al.*, 2009), however, these need to be confirmed by further bioinformatic analysis. TIR1/AFBs have been found to be regulated by microRNA (miRNA) pathways, specifically miRNA393, and potentially small interfering RNAs (siRNAs) indicating that although *TIR1/AFBs* may not be rapidly induced by auxin, there is a complex method of regulation of *TIR1/AFB* (Navarro *et al.*, 2006). The relationship between miRNA and siRNA with auxin in berry ripening has yet to be explained in grapevine.

1.1.5.4 The auxin response and auxin signalling pathway

Perception of auxin by plants results in up-regulation of genes from the *GH3, SMALL AUXIN UP RNA (SAUR)*, and *Aux/IAA* protein families (Theologis *et al.*, 1985; Abel & Theologis, 1996; Chapman & Estelle, 2009). The auxin-responsive genes can vary significantly between cell types, highlighting the complexity of the transcriptional response to auxin (Bargmann *et al.*, 2014; Salehin *et al.*, 2015). The GH3s, as mentioned earlier, are involved in the conjugation of auxin and specific *GH3s* are rapidly induced upon the application of auxin (Böttcher *et al.*, 2010b). The high levels of *GH3* transcripts in early berry development, when IAA levels are high, may aid in the control of auxin homeostasis (Böttcher *et al.*, 2011a). As free IAA can comprise only up to 25% of total IAA, conjugation with esters or amino acids plays a major role in auxin regulation (Ludwig-Müller, 2011). Some IAA conjugates are able to be hydrolysed back to free IAA, whilst the GH3 generated IAA-Trptophan (IAA-Trp), IAA-Asp and IAA-Glutamine (IAA-Glu) conjugates are thought to permanently remove IAA from the free IAA pool. IAA-Trp is thought to be an inhibitor of auxin action and IAA-Asp and IAA-Glu are intermediates in the degradation pathway (Ludwig-Müller, 2011). IAA-Asp has recently been suggested to affect the response to abiotic stress in Pea (*Pisum sativum* L.) indicating that the conjugate may play additional functional roles within plants (Ostrowski *et al.*, 2016). The SAUR proteins are currently poorly characterised, with over 70 *SAURs* in Arabidopsis, with a range of functions including roles in cell elongation downstream of auxin, leaf senescence, and increased vegetative biomass (Markakis *et al.*, 2013; Li *et al.*, 2015b). There is also an increase in some Aux/IAA protein levels as part of the auxin signalling pathway, detailed below.

Upon the introduction of auxin to a cell, auxin is bound to TIR1/AFB which then recruits Aux/IAA proteins for targeted poly-ubiquitination via the SCF protein complex (Gray & Estelle, 2000; Maraschin

et al., 2009). Aux/IAAs are 20-35 kDa proteins that are quickly induced, nuclear located and short-lived (Figure 1.7) (Abel & Theologis, 1996; Dreher *et al.*, 2006; Hagen, 2015). The polyubiquitinated Aux/IAA target is recognised by the 26S proteasome and is degraded (Figure 1.7). Aux/IAA proteins contain four domains, I, II, III and IV. Domain I contains one or more LXLXL (L = leucine, X = any other amino acid) repression motifs that enhance the repression of ARF proteins through the interaction with TOPLESS (TPL)/TOPLESS-RELATED proteins, which recruit chromatin remodelling enzymes that stabilise the gene repression (Tiwari *et al.*, 2004; Szemenyei *et al.*, 2008; Lee *et al.*, 2009; Causier *et al.*, 2012; Hagen, 2015). Domain II is responsible for the short-lived nature of the Aux/IAA proteins as it contains a 13 amino acid degron that acts as the site of interaction with the TIR1/AFB proteins conferring protein instability (Worley *et al.*, 2000; Ramos *et al.*, 2001; Lee *et al.*, 2009). Domains III and IV form the protein-protein interaction domains that allow the interaction of the Aux/IAA with ARF activator proteins, and facilitate the homo and hetero-dimerization between multiple Aux/IAA proteins (Abel *et al.*, 1994; Kim *et al.*, 1997; Ulmasov *et al.*, 1997; Ulmasov, 1997; Tiwari *et al.*, 2001; Tiwari *et al.*, 2004; Lee *et al.*, 2009; Çakir *et al.*, 2013). Most *Aux/IAA* transcripts in Arabidopsis are induced by the presence of auxin; the homodimerization of Aux/IAA proteins is thought to help mediate the speed at which the auxin response occurs and the absence of Aux/IAA homodimers is thought to decrease the speed of the induction of transcription (Paponov *et al.*, 2008; Farcot *et al.*, 2015).

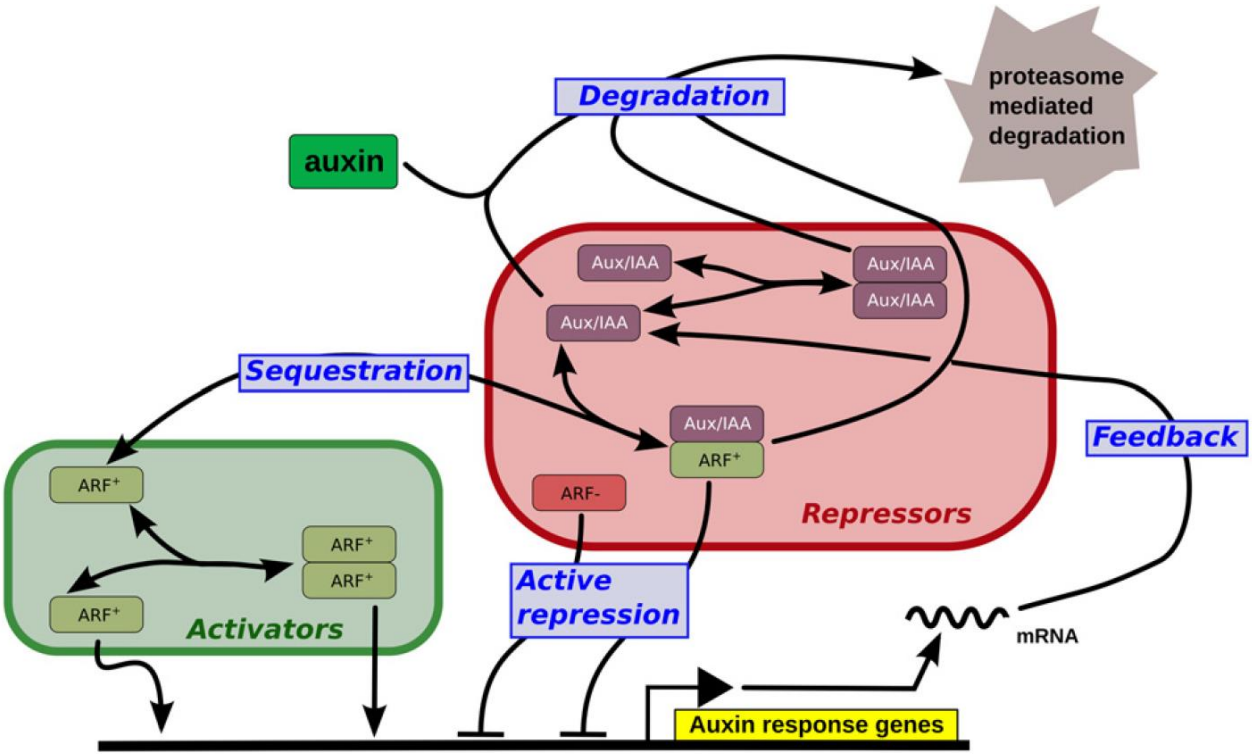


Figure 1.7 A schematic representation of the auxin signalling pathway.

Under low concentrations of auxin, ARF activator activity is repressed by multimerisation with Aux/IAA repressor proteins, sometimes Aux/IAA proteins are also bound to TOPLESS or TOPLESS-related co-repressors. This multimerisation represses gene transcription via ARF proteins. In the presence of auxin, Aux/IAA and TIR1/AFB proteins form a co-receptor complex and the Aux/IAA protein is polyubiquitinated by the $SCF^{TIR1/AFB}$ complex and targeted to the proteasome for degradation. This Aux/IAA protein degradation relieves the ARF activators of their repression allowing auxin-responsive gene transcription. Active repression also occurs directly through the binding of ARF repressors to DNA. Aux/IAA proteins and ARF activators are able to form homo- and heterodimers in addition to multimerisation. Auxin-responsive gene transcription can form a feedback loop, up- or down-regulating the transcript levels of members of the auxin signalling pathway. Taken from Farcot *et al.* (2015).

The ARF proteins are 70-130 kDa, stable, nuclear localised proteins that act as transcription factors (Hagen, 2015). At their N-terminus they have a conserved B3 DNA-binding domain (DBD) that is used to bind specific auxin responsive elements (AuxRE) which have the sequence TGTCTC and are typically found in promoter regions of auxin responsive genes (Quint & Gray, 2006; Hagen , 2015). Recent structural analysis of interacting ARF proteins has identified the dimerization domain (DD) that exists within the DBD and facilitates the interaction between two ARF proteins bound to DNA (Boer *et al.,* 2014; Pierre-Jerome *et al.,* 2016). The number of AuxRE motifs, their proximity to each other, and their orientation can strongly influence the activation potential of ARF proteins as these factors help to determine the type of dimerization complexes that can form (Pierre-Jerome *et al.,* 2016). The middle region of the ARF protein contains non-conserved amino acid sequences that determine whether the ARF acts as an activator or repressor of auxin signalling (Quint & Gray, 2006). At the C-terminus they have a protein-protein interaction Phox and Bem1 (PB1) domain that shares homology with domains III and IV in Aux/IAA proteins. These domains allow the interaction of ARF proteins with Aux/IAA proteins in homodimers, heterodimers or large oligomers. They interact in a front-to-back manner through electrostatic interactions between the acidic and basic residues at either end of the domains (Quint & Gray, 2006; Korasick *et al.,* 2014; Nanao *et al.,* 2014; Hagen, 2015). Similarly to TIR1/AFB proteins, members of the ARF family in Arabidopsis are regulated by miRNAs; AtARF17 is regulated by miR160 (Mallory *et al.*, 2005) and AtARF6 and AtARF8 are regulated by miR167 (Wu *et al.*, 2006).

The roles of ARF activators are well characterised, and there are two proposed modes of action. The first is the dimerization model, where Aux/IAA proteins bind to and repress ARF activator proteins that sit bound to gene promoters and in the absence of auxin, the auxin responsive genes remain untranscribed (Figure 1.7) (Farcot *et al.*, 2015). However, when auxin is perceived and the repression of ARFs by Aux/IAA is lifted, through the degradation pathway described above, the ARF proteins are able to regulate transcription of auxin responsive target genes in a positive or negative manner depending on the ARF, the promoter sequence of the target gene and the interaction with additional coactivators or corepressors (Lee *et al.*, 2009; Farcot *et al.*, 2015). The second is the sequestration model where ARF-Aux/IAA dimers are able to sequester ARF activators away from promoters, and upon the perception of auxin this repressive function is lifted, allowing the ARFs to be active on the auxin-responsive promoters (Figure 1.7) (Farcot *et al.*, 2015). There is some support for the hypothesis that rather than ARF and Aux/IAA proteins interacting in dimerization pairs they may act as larger repression complexes, incorporating different ARF and Aux/IAA proteins to allow for the fine tuning of the auxin response (Korasick *et al.*, 2014; Nanao *et al.*, 2014; Enders & Strader, 2015). The mode of action of the ARF repressors which make up the majority of the ARF family is less clear (Hagen, 2015).

There is limited evidence for their interaction with Aux/IAA proteins and it is thought that they may compete with ARF activators for AuxRE motif binding sites in gene promoters or that they may act through recruiting additional repressor proteins (Ulmasov *et al.*, 1999; Boer *et al.*, 2014; Franco-Zorrilla *et al.*, 2014; Farcot *et al.*, 2015; Hagen , 2015). Unlike Aux/IAA proteins, very few ARFs are thought to be auxin-inducible (Okushima *et al.*, 2005; Guilfoyle & Hagen, 2007; Paponov *et al.*, 2008; Lau *et al.*, 2011).

In Arabidosis 29 Aux/IAA proteins and 23 ARF proteins have been identified, in addition to the six TIR1/AFBs previously mentioned (Ulmasov *et al.*, 1997; Guilfoyle *et al.*, 1998; Parry *et al.*, 2009). Efficient binding of auxin requires both the TIR1/AFB and Aux/IAA proteins, as a co-receptor complex (Calderon-Villalobos *et al.*, 2012). Different auxinic compounds have different affinities for different co-receptor complexes, leading to many potential interactions and regulation mechanisms (Calderon Villalobos *et al.*, 2012; Prigge *et al.*, 2016). Additionally, each Aux/IAA potentially mediates different responses within the plant dependent on the ARFs they bind to and what genes the ARFs transcriptionally activate or repress (Tatematsu *et al.*, 2004; Tashiro *et al.*, 2009; Fujita *et al.*, 2012). A single Aux/IAA protein can mediate changes in expression of a large number of genes; for example, Arabidopsis AtIAA1 was found to up-regulate 148 genes and down-regulate 59 genes with roles in transcriptional control, metabolism and signal transduction (Lee *et al.*, 2009). Interestingly, loss-of-function mutations in Aux/IAA and ARF proteins in Arabidopsis often fail to produce clear mutant phenotypes suggesting that functional redundancy exists (Remington *et al.*, 2004; Overvoorde *et al.*, 2005; reviewed in Reed, 2001; Lee *et al.*, 2009). In tomato however, both Aux/IAA and ARF loss-of-function mutants have clear phenotypes, indicating less functional redundancy (reviewed in Salehin *et al.*, 2015).

Specific ARFs have been associated with flowering and fruit development in Arabidopsis and tomato, with mutations in one ARF in tomato leading to abnormal fruit ripening and modifications to cell and tissue structures (Jones *et al.*, 2002; Goetz *et al.*, 2007; Guillon *et al.*, 2008; Kumar *et al.*, 2011; Breitel *et al.,* 2016). DR12 (SlARF4) down-regulation in tomato causes a delayed loss of chlorophyll in fruit, suggesting the gene is involved in fruit development (Jones *et al.*, 2002; Guillon *et al.*, 2008; Legland *et al.*, 2010; Sagar *et al.*, 2013). High expression of SlARF4 in the pericarp tissue of immature fruit declines at the onset of ripening, which correlates with an increase of sugars. It is suggested that SlARF4 is a negative regulator of genes and enzymes activities involved in starch biosynthesis (Jones *et al.*, 2002). The *Aux/IAA SlIAA9* gene in tomato is expressed in many organs and throughout plant development, SlIAA9 anti-sense plants produced single instead of compound leaves and parthenocarpic fruits (Wang *et al.*, 2005). SlIAA9 is thought to be a negative regulator of auxin

responses, and in fruit development it may prevent ovary development prior to pollination (Wang *et al.*, 2005). Strawberry Aux/IAAs, *FvIAA1* and *IAA2*, are expressed early in fruit development and are both induced by NAA treatment (Liu *et al.*, 2011). A mutation in AtARF8 is able to cause parthenocarpic fruit in both Arabidopsis and tomato (Goetz *et al.*, 2007). Similarly, silencing of SlARF7 leads to parthenocarpic fruit development. Lower levels of SlARF7 lead to larger cell sizes in the mesocarp and endocarp layers suggesting that down-regulation of SlARF7 down-regulates cell division and up-regulates cell expansion (de Jong *et al.*, 2009). Overexpression of SlARF2A caused patches of accelerated ripening in tomato fruit and early ethylene production. Ethylene inhibition delayed the ripening phenotype, and the down-regulation of SlARF2A and 2B within tomato plants produced less ethylene. Additionally, yeast 2-hybrid assays found that SlARF2A interacted with ABA STRESS RIPENING protein (Hao *et al.,* 2015; Breitel *et al.,* 2016). Together these results suggest that SlARF2A, and potentially SlARF2B, are involved in fruit ripening and connect several hormonal pathways. Breitel *et al.,* (2016) designed a model where SlARF2A may repress an unknown ripening-repressor allowing for the activation of ripening regulators and down-stream ripening genes in an ethylene-dependent manner, potentially with some form of feedback loop impacting SlARF2A expression. This correlates well with AtARF2, the SlARF2 homolog, which has been linked with ethylene and plays roles in plant aging, including leaf senescence, floral abscission and silique ripening (Ellis *et al.,* 2005).

1.1.5.4.1 What is known about the auxin signalling pathway in *V. vinifera*?

Three studies have been completed on *Aux/IAAs* in grape, two prior to the start of this study in 2012 (Fujita *et al.*, 2012; Kohno *et al.*, 2012; Çakir *et al.*, 2013). Fujita *et al.* (2012) characterised *VvIAA9* (*VviIAA9*) from *V. vinifera* L. cv. Chardonnay, which encodes the same protein as *VvAux/IAA4* in Çakir *et al.* (2013). *VvIAA9* is highly expressed within leaf and berry tissue with transcription up-regulated pre-veraison and down-regulated after veraison. Expression of the *VvIAA9* transcript was increased upon the application of auxin to grape leaves. This study also suggested that VvIAA9 is an auxin responsive promoter of growth and maturity that promotes meristem transition (Fujita *et al.*, 2012). Kohno *et al.* (2012) characterised *VvIAA19* (*VviIAA19*) from grapevine and found that it was highly expressed in berries, with low expression levels pre-veraison and was up-regulated from veraison with high expression levels maintained until the end of ripening. Unlike *VvIAA9*, exogenous auxin application to grape leaves did not induce *VvIAA19* expression, suggesting that it is not auxin responsive. Similar to VvIAA9, VvIAA19 may be playing a role as a regulator of plant growth, however, it is not auxin responsive and a role in grape berry development and ripening has yet to be elucidated (Kohno *et al.,* 2012). Çakir *et al.* (2013) used genome-wide analysis to identify the whole *Aux/IAA* family in grape, identifying 26 candidates. A single *Aux/IAA* gene, *VvAux/IAA4 (VviIAA9),* was shown via expressed sequence tag (EST) data to be the most highly expressed *Aux/IAA* transcript, with 104

ESTs deposited in NCBI, most frequently from inflorescence, fruit, leaf and bud (NCBI, 2012). *VvAux/IAA4* is auxin-inducible in leaves and shows high expression levels in young leaves, roots and throughout berry development, decreasing from pre-veraison to harvest in *V. vinifera* cv. Sultanine. Down-regulation of *VvAux/IAA4* expression after drought, salicylic acid treatment, and in response to salt stress, indicates there is crosstalk between auxin and multiple hormone or stress related signalling pathways.

During this study, a publication described the identification and characterisation of 19 *ARFs V. vinifera* L. cv. Cabernet Sauvignon, fewer than the 23 identified in Arabidopsis (Wan *et al.*, 2014). All 19 grape ARFs contained the B3 DNA binding domain and all but two proteins, VvARF8 (VviARF3) and 17 (VviARF17), had full or truncated domains III and IV for protein-protein interaction. Wan *et al.* (2014) used quantitative real-time PCR (qPCR) analysis to determine the expression pattern of nine *VvARF* transcripts in Cabernet Sauvignon berries at 20, 40, 70, 90 and 100 d after full bloom, covering small pea sized berries, veraison and mature berries. Their results were compared directly to the nine *VvARF* transcripts identified in the Deluc *et al.* (2007) microarray experiment. They determined that four of the transcript patterns were consistent between the microarray and qPCR analysis, and the remaining five transcripts were not (Wan *et al.*, 2014). The authors suggest this may be due to different environmental growth conditions of the grapes. In addition, the authors suggest that high levels of the transcriptional activators, *VvARF3 (VviARF8)* and *11 (VviARF28)* in small pea-sized berries, and *VvARF5 (VviARF4)* and 15 *(VviARF24)* in mature berries, means that these proteins may be involved in cell division and berry ripening, respectively (Wan *et al.*, 2014).

1.1.6 Protein-protein interactions

The protein-protein interaction between ARF activator proteins (ARF+) and Aux/IAA proteins (IAA), denoted by ARF+-IAA, is well characterised, with interacting partners having been studied in Arabidopsis through yeast two-hybrid analysis and bimolecular fluorescence (Tiwari *et al.,* 2004; Szemenyei *et al.*, 2008; Lee *et al.*, 2009; Causier *et al.*, 2012; Piya *et al.*, 2014; Farcot *et al.*, 2015; Hagen, 2015). Piya *et al.* (2014) conducted a comprehensive yeast two-hybrid assay of all of the ARF and Aux/IAA proteins in Arabidopsis (Figure 1.8). Their analysis was able to generate an interaction network of all AtARF and AtIAA candidates, with a total of 213 interactions from a possible 551. The five ARF activators AtARF5, 6, 7, 8, and 19 and a single ARF repressor AtARF4 interacted with all of AtIAAs, with the exception of AtARF7 and AtIAA7 (Figure 1.8). The interactions ranged from weak to strong, with the AtARF activators consistently interacting strongly with AtIAA1–11, 13–19, and largely weaker interactions with the remaining AtARF–AtIAA combinations. Interestingly, despite AtARF4 not containing the Q-rich middle region that is characteristic of ARF activators, it interacted strongly with

28 of the 29 AtIAA proteins with the exception of AtIAA34, with which it interacted weakly. AtIAA32–34 all interacted more extensively with the AtARF proteins than the other AtIAAs, potentially due to mutations or truncations in the Aux/IAA sequences. Additionally, AtARF18 had strong interactions with ten of the 29 AtIAAs. Bimolecular fluorescence (BiFC) with split-YFP was used to validate these results, ten AtARF-AtIAA partners weakly or strongly interacting in yeast were confirmed to interact within the nuclei of onion cells. As negative controls, four AtARF-AtIAA partners that did not interact in yeast also did not interact within the BiFC system (Piya *et al.*, 2014). Although now well characterised in Arabidopsis, limited information is available on ARF-IAA interactions in other species. Similarly, the homo- or hetero-dimerization of Aux/IAA and ARF proteins, have not been characterised in-depth.

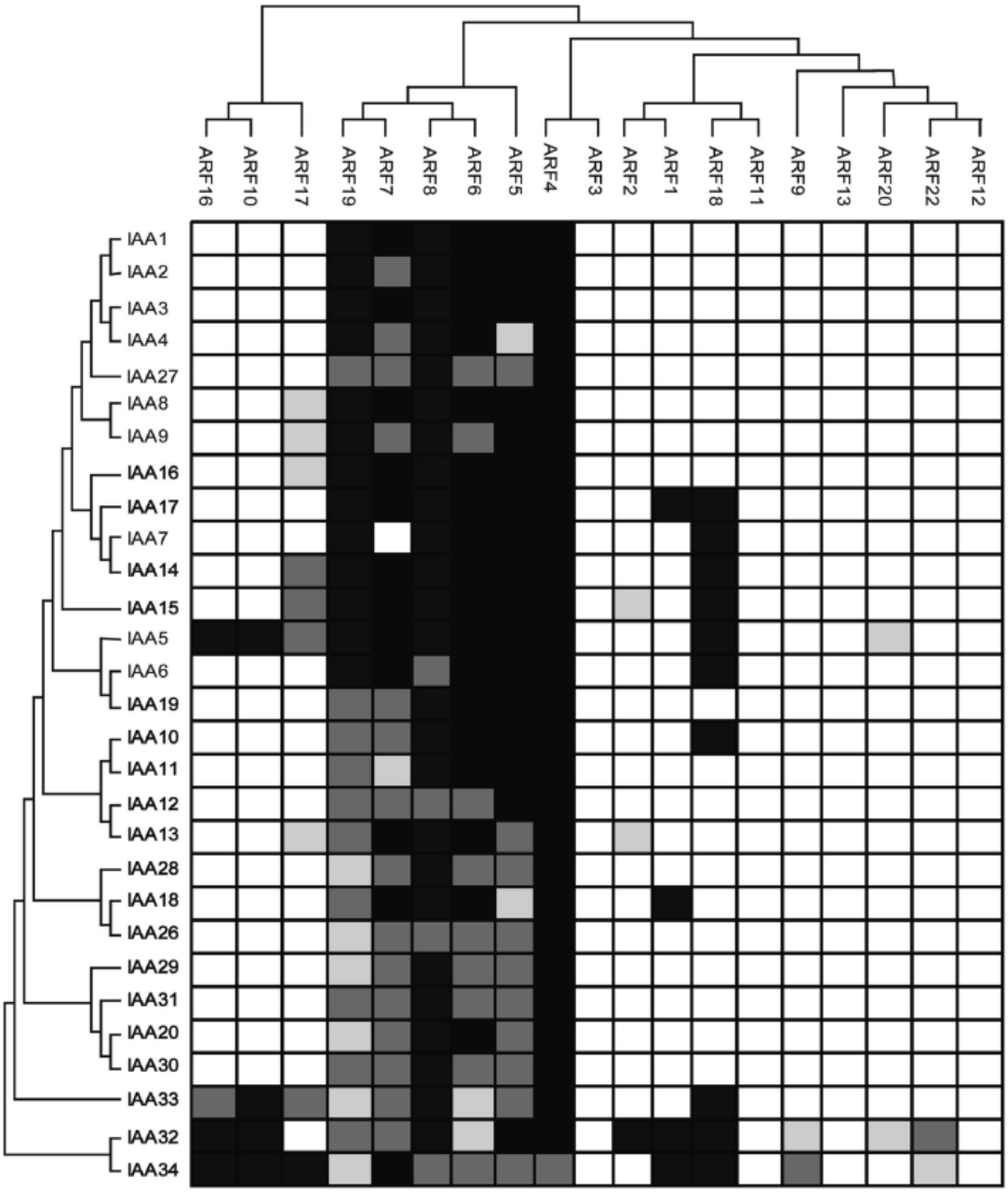


Figure 1.8 A schematic diagram of the SCF^{TIR1} complex involved in the auxin response based on yeast two-hybrid interactions.

In the absence of auxin the auxin/indole-3-acetic acid (Aux/IAA) proteins are bound to auxin response factors (ARFs) and repress the transcription of auxin response genes by the ARFs. Yeast 2-hybrid analysis can be used to determine which of the ARF and Aux/IAA proteins (IAA) interact. The ARF and Aux/IAA protein sequences were aligned using ClustalX and arranged in neighbour-joining trees based on their protein sequence similarity. White boxes indicate no interaction between the ARF and IAA proteins, gray boxes indicate a weak interaction, and black boxes indicated a strong interaction. Taken from Piya *et al.* (2014).

1.2 Summary

Characterisation of the auxin signalling pathway during fruit development has been undertaken in a number of species including Arabidopsis (Hagen & Guilfoyle, 2002; Remington *et al.*, 2004; Dharmasiri *et al.*, 2005b; Okushima *et al.*, 2005; Overvoorde *et al.*, 2005), tomato (Kumar *et al.*, 2011; Audran-Delalande *et al.*, 2012) and apple (Devoghalaere *et al.*, 2012). Auxin signalling in grape has not been comprehensively researched, although recent studies have investigated the *Aux/IAA* (Çakir *et al.*, 2013; Fujita *et al.*, 2012; Kohno *et al.*, 2012), *ARF* (Wan *et al.*, 2014), *ABP1* (Wan *et al.*, 2010) and *GH3* (Böttcher *et al.*, 2011a) gene families. An in-depth study into the members of the auxin signalling pathway that are expressed in grape berries, including the *Aux/IAAs, ARFs,* and *TIR1/AFBs*, and their interactions during grape development would provide novel information relating to auxin signalling in grape. This in turn may aid the improvement of strategies used in the vineyard to control the timing of veraison and to enhance berry composition and production.

1.3 Aims

The main aim of this work was to identify and characterise the members of the auxin signalling pathway in grape and use phylogenetics, transcriptional expression patterns and phytohormone responsiveness to infer the potential roles that they may play in grape development. An additional aim was to identify signalling proteins that may interact *in planta* as interactions between Aux/IAA and ARF proteins are key mechanism in the auxin control of grape development. These aims were met by completing the following four objectives:

- Identify and characterise the TIR1/AFB, ARF, and Aux/IAA candidate genes from the auxin signalling pathway in *V. vinifera* using bioinformatics analysis
- Determine the transcriptional expression patterns of all *TIR1/AFB, ARF,* and *Aux/IAA* candidates in *V. vinifera* L. cv. Shiraz berry development, leaf development and within flowers, roots, and tendrils using quantitative real-time PCR
- Identify protein-protein interactions between a subset of ARF-Aux/IAA proteins using yeast two-hybrid and bimolecular fluorescence
- Determine the hormone responsiveness of members of the auxin signalling pathway and identify motif sequences within the *Aux/IAA* and *ARF* candidate promoters

At the outset of this study RNA was available for a *V. vinifera* L. cv. Shiraz berry developmental series, flowers, roots, and tendrils. In addition, yeast cDNA libraries had been generated and a method for

berry assays and the quantification of IAA and IAA-Asp had been development. All of these resources were kindly made available for use in this work.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, enzymes, buffers, reagents, solutions and media

See Appendix A for all details.

2.1.2 Oligonucleotide primers

See Appendix B for all primer sequences and details.

2.1.3 Vectors

See Appendix C for all plasmid and construct details.

2.1.4 Plant tissue samples

2.1.4.1 Expression analysis

Fruit was collected from *Vitis vinifera* L. cv. Shiraz vines over the 2010–2011 season from a commercial vineyard in Willunga (Chalk Hill Wines, Willunga, South Australia, 35.263489, 138.550406). The berries were collected weekly for 16 weeks after flowering between 09:30 and 11:30. Approximately 100–200 whole berries were collected for weeks one to three, and 60–100 berries were collected and deseeded from week four onwards. All berries were immediately frozen in liquid nitrogen and stored at -80°C. Three biological replicates were sampled at each time point. These samples were collected by the Davies lab members prior to the start of this work.

Böttcher *et al.* (2013a) determined the berry weight, sugar content and anthocyanin accumulation for the Shiraz 2010/2011 berry developmental series that was used in this study (Figure 2.1). The initial measurements began at three weeks post-flowering. Degrees Brix (°Brix) is measured by refractive index and is a measure of total soluble solids (TSS). Before veraison this is mainly malic and tartaric acids. Veraison is the time-point immediately prior to a significant increase in °Brix, and the point at which red berries begin to accumulate anthocyanins (Coombe, 1992; Davies & Robinson, 1996). After veraison sugars, such as glucose and fructose, increasingly comprise a greater proportion of the TSS. The °Brix remained at five from weeks three to eight pre-veraison, there was a rapid increase from veraison with the levels increasing from week eight onwards and the highest levels at week 16 at ~23 °Brix (Figure 2.1). Berry weight increased from week three (~0.25 g) throughout development being ~1.0 g at veraison at week eight and ~1.5 g at harvest at week 16 (Figure 2.1). The levels of anthocyanins were measured every two weeks from week four onwards (Figure 2.1). Pre-veraison there are minimal levels of anthocyanins. The anthocyanin levels increase from week 10 at ~8 g FW^{-1} to ~38 g FW^{-1} at week 16.

Root samples were harvested from *V. vinifera* L. cv. Shiraz canes grown in the glasshouse in 50:50 Perlite:vermiculite mix until young roots emerged from the base. The roots were washed, root tips (1-2 cm) were excised and frozen in liquid nitrogen by the Davies lab members prior to the commencement of this study; no biological replicates were available. The tendril samples (single biological replicate) and three biological replicates of *V. vinifera* L. cv. Shiraz flower samples were collected by Dr C. Davies at 50% cap-fall from the commercial vineyard in Willunga (as described above 05/11/2013) and frozen in liquid nitrogen.

A leaf developmental series was collected from *V. vinifera* L. cv. Shiraz vines during the course of this study by Dr C. Davies and myself from the commercial vineyard in Willunga (as described above; 18/11/2013). A total of nine leaf stages were collected based on their size and position on the growing shoot (Figure 2.2). Leaves at stage one were small unexpanded leaves closest to the growing shoot tip, and leaves at stage nine were fully expanded leaves in the ninth position from the growing shoot tip. Leaves were pooled from shoots from a number of vines on both sides of the canopy, providing a single collection for each developmental stage representing a single biological replicate.

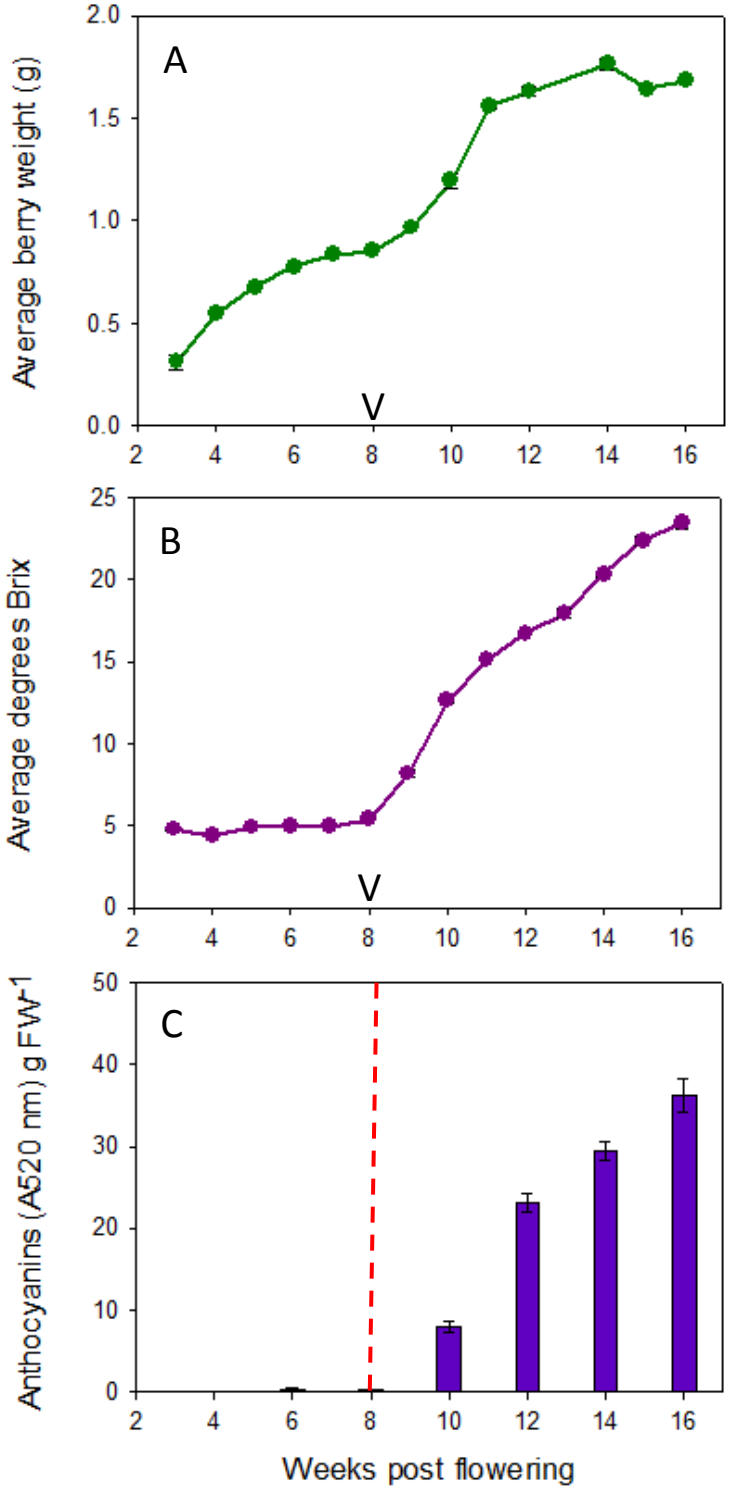


Figure 2.1 The berry weight, degrees Brix and anthocyanin accumulation in the Shiraz 2010/2011 developmental series.

(A) The average berry weight in grams from weeks three to 16 post flowering, (B) the average degrees Brix from weeks three to 16, (C) the fortnightly anthyocyanin levels from week four to 16 measured as absorbance at 520 nm in grams fresh weight $^{-1}$, the bars represent standard error. V or dashed line = veraison, defined as the sample time immediately before a change in colour or degrees Brix within the berry (Coombe, 1992). Redrawn from Böttcher *et al.* (2013a).

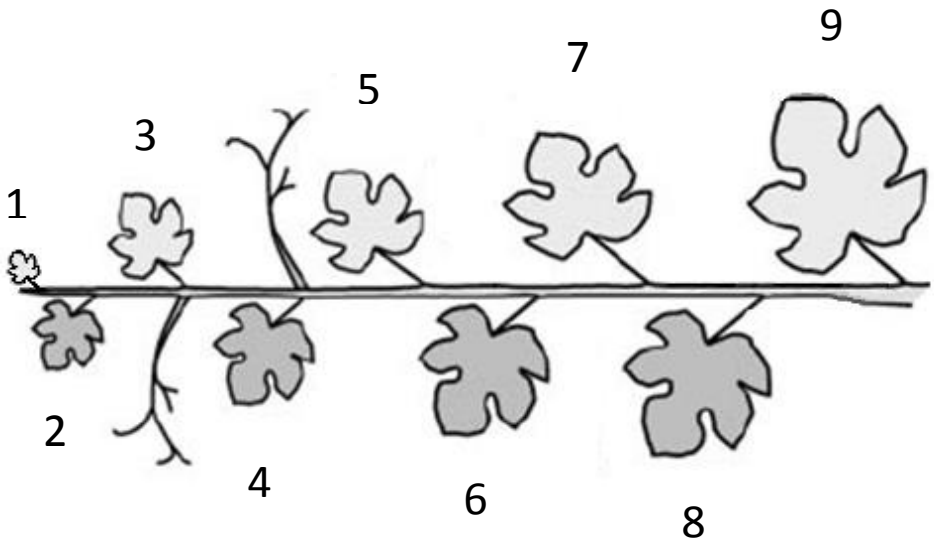


Figure 2.2 A schematic of the origin of the nine leaf samples collected from *V. vinifera* L. cv. Shiraz as a leaf developmental series.

Leaves 1 to 9 are labelled. Adapted from Carmona *et al. (*2002).

2.1.4.2 *Ex planta* analysis

Fruit was harvested from *V. vinifera* L. cv. Shiraz vines for two consecutive seasons (2013-2014 and 2014-2015) from a vineyard in the Adelaide Hills (Nepenthe Wines, Hahndorf, South Australia, -35.018223, 138.838220). Samples from 2013-2014 were used for the NAA *ex planta* analysis and the 2014-2015 samples were used for the multiple hormone *ex planta* analysis. There were two sampling time points each year, 6 weeks after flowering (pre-veraison) and 12 weeks after flowering (post-veraison) (Figure 2.3). All berries were collected at approximately 09:30 AM on the day of sampling by Dr C. Davies and kept on ice until used. The number of bunches collected was sufficient for three biological replicates and bunches were collected from approximately 35 vines. Total soluble solids (°Brix) were measured for each sample using an RFM710 digital refractometer (Bellingham Stanley, Kent, UK); 2014 – week 6, 8/1/14 (4.3 ^{o}Brix) and week 12, 4/3/14 (17.2 ^{o}Brix), 2015 – week 6 7/1/15 (4.3 ^{o}Brix) and week 12, 11/2/15 (17.2 ^{o}Brix).

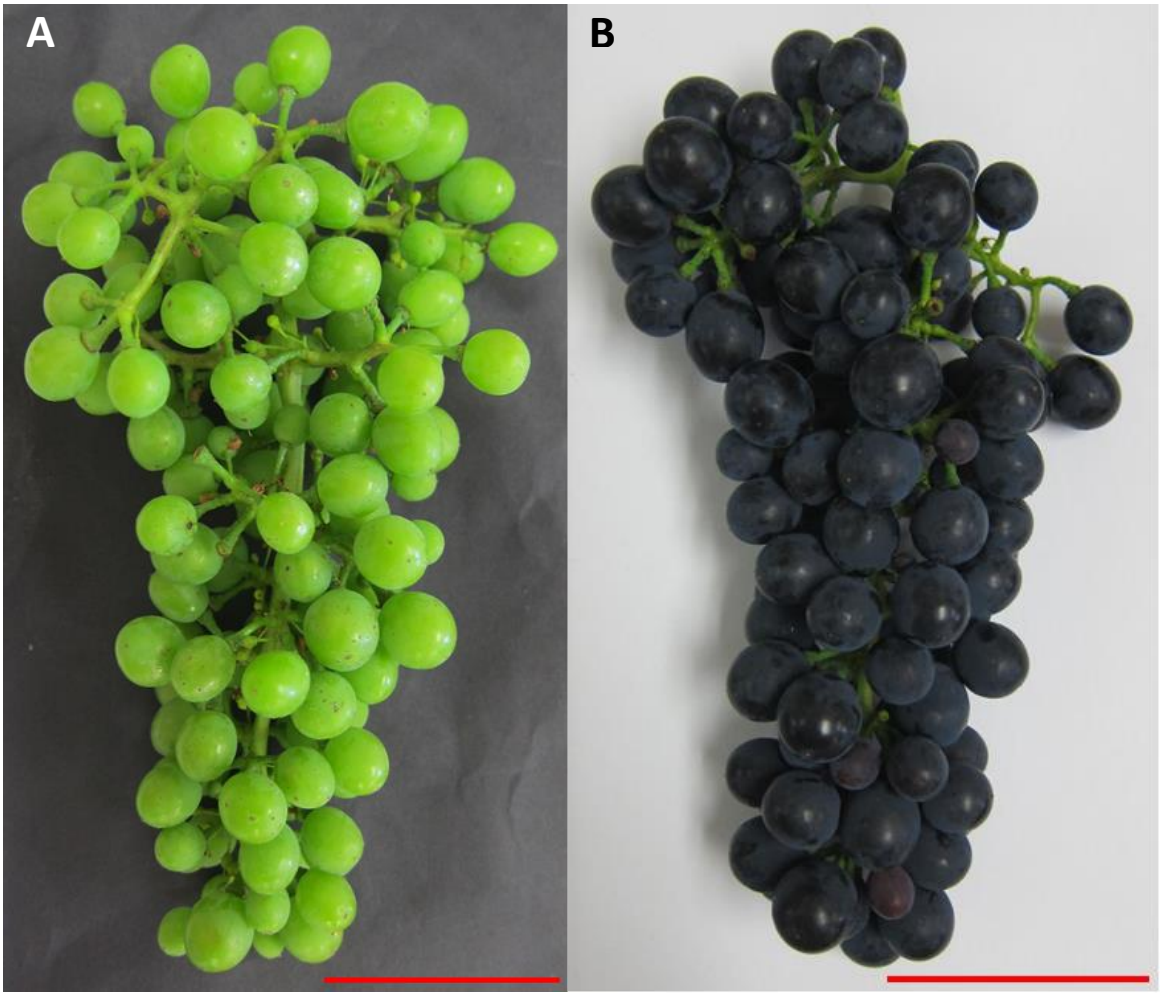


Figure 2.3 *V. vinifera* L. cv. Shiraz berry samples used within the *ex planta* experiments.

Pre-veraison berries six weeks post-flowering. B) Post-veraison berries 12 weeks post-flowering (5 cm scale).

2.1.5 RNA samples

2.1.5.1 Gene expression analysis

Total RNA was extracted from the 16 week *V. vinifera* L. cv. Shiraz berry developmental series, root and tendril samples (Section 2.2.5.2) prior to the commencement of this study by the Davies lab members and stored at -80°C. Total RNA was extracted from the *V. vinifera* L. cv. Shiraz flower samples and the leaf developmental series within this work using the method as described in Section 2.1.4.1.

2.1.5.2 *Ex planta* analysis

Total RNA was extracted from all *V. vinifera* L. cv. Shiraz *ex planta* samples within this work using the method as described in Section 2.2.5.2.

2.1.6 cDNA samples

For real-time quantitative PCR (RT-qPCR) experiments cDNA was generated for the *V. vinifera* L. cv. Shiraz berry developmental series, leaf developmental series, flower, root and tendril samples and all *ex planta* samples using the method as described in Section 2.2.5.3.

2.1.7 Bacterial strains

2.1.7.1 *Escherichia coli (E. coli)* strains

For the cloning of gene fragments for the generation of standard curves for RT-qPCR, pCR®-Blunt (Life Technologies, Carlsbad, CA, USA) and BiFC constructs, DH5α, XL1 or Top10 *E. coli* cells were used. These cells were purchased from Invitrogen (Carlsbad, CA, USA). For the cloning of pGBKT7 and pGADT7 constructs for use in yeast work, Stellar cells (Clontech Laboratories, Inc. Mountain View, CA, USA) were used.

2.1.7.2 Yeast strains

Both yeast strains used within this work were sourced from Clontech. Y2H Gold cells were used for both pGBKT7 bait vector transformations and the co-transformations of the pGBKT7 bait vector and pGADT7 prey vector. Y187 cells were used for the transformation of prey vectors into yeast to test their viability and auto-activation.

2.2 Methods

2.2.1 Sequence storage, analysis and annotation software

Geneious Pro 8.0.5 (Biomatters Ltd., Auckland, New Zealand) was used to manage all promoter, gene and protein sequences. Sequence alignments and motif annotation were performed using applications contained within Geneious using the default settings. Vector maps were also generated in Geneious.

2.2.2 Bioinformatic techniques

To identify genes from the *ARF, Aux/IAA* and *TIR1/AFBs* families, a range of bioinformatic techniques (see the summary in Figure 2.4 and Sections 2.2.2.1 and 2.2.2.2) were used across *V. vinifera* (grape), *Arabidopsis thaliana* (Arabidopsis), *Populus trichocarpa* (poplar), *Malus domestica* (apple) and *Solanum lycopersicum* (tomato).

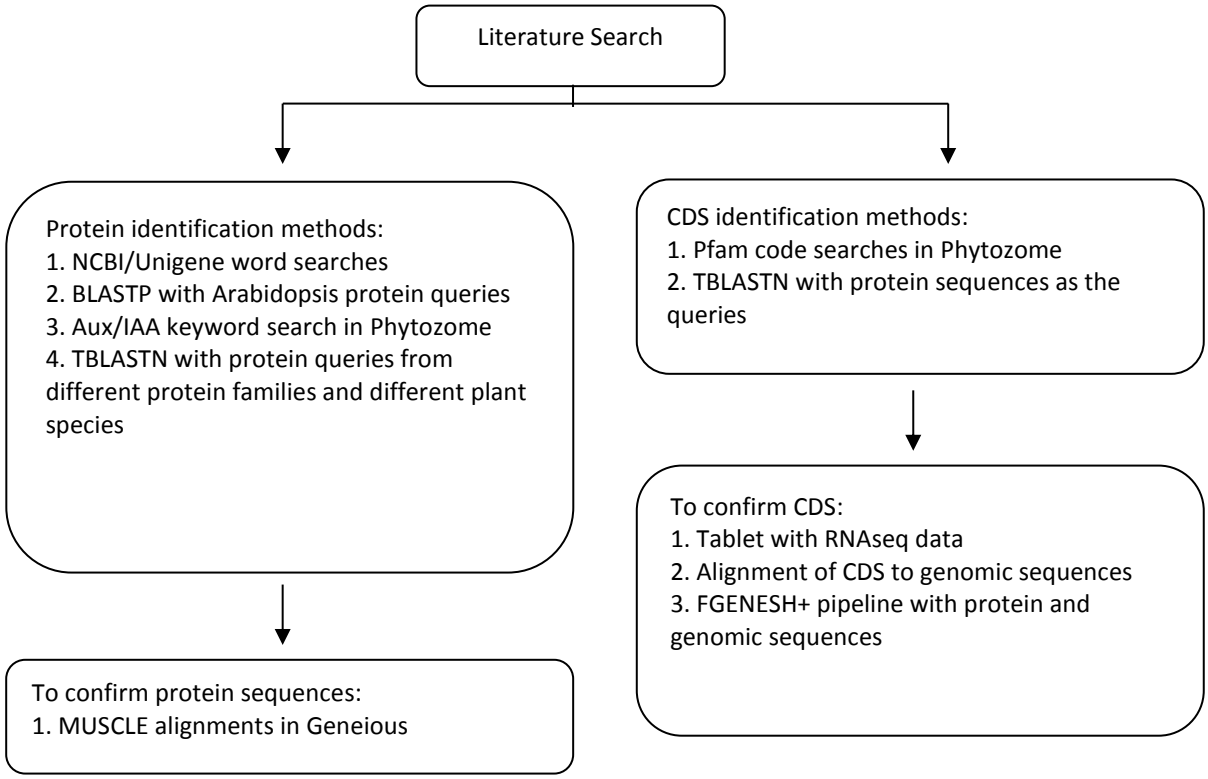


Figure 2.4 Overview of sequence identification and confirmation for bioinformatic analysis.

2.2.2.1 Gene mining and identification – protein sequences

Initially a literature search was used to isolate previously identified ARF, Aux/IAA and TIR1/AFBs amino acid sequences from grape, Arabidopsis, poplar, apple and tomato (Table 2.1). All family members that had been previously described were extracted from NCBI (2012) (http://www.ncbi.nlm.nih.gov/) or the respective species genomic websites (Arabidopsis (Lamesch *et al.*, 2011; TAIR, https://www.arabidopsis.org/), poplar (Goldstein *et al.*, 2012, Phytozome http://www.phytozome.net/), apple (Jung *et al.,* 2008, Genome Database for Rosaceae - http://www.rosaceae.org/), tomato (Tecle *et al.*, 2010, Sol Genomics Network - http://solgenomics.net/) and grape (Grape Genome Browser, 2012, http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) and transferred to Geneious. During the course of this work publications were released that detailed the auxin-related gene families and these were compared to the sequences initially identified in this study in late 2012.

Table 2.1 The publications (website) that aided in identifying protein sequences for TIR1/AFB, ARF and Aux/IAA protein families.

Species	Gene family	Publication
Arabidopsis	TIR1/AFB	TAIR[1]
Grape		Parry *et al.*, 2009
Poplar		Parry *et al.*, 2009
Apple		Devoghalaere *et al.*, 2012
Tomato		Parry *et al.*, 2009
Arabidopsis	ARF	TAIR
Grape		Finet *et al.*, 2012; Wan *et al.*, 2014
Poplar		Kalluri *et al.*, 2007
Apple		Devoghalaere *et al.*, 2012
Tomato		Zouine *et al.*, 2014
Arabidopsis	Aux/IAA	TAIR
Grape		Çakir *et al.*, 2013
Poplar		Kalluri *et al.*, 2007
Apple		Devoghalaere *et al.*, 2012
Tomato		Audran-Delalande *et al.*, 2012

[1]TAIR - https://www.arabidopsis.org/ (Lamesch *et al.*, 2011)

Note: Sequences were initially obtained from these papers and confirmed or corrected based on sequences isolated from Phytozome and genomic websites.

To identify the remaining amino acid sequences in Phytozome, multiple methods were used (Figure 2.4). First, word searches were completed in NCBI and UniGene using each gene family name, 'grape'

and 'auxin' as the word queries. All relevant sequences were downloaded into Geneious. Second, NCBI was used within Geneious to perform individual BLASTP searches using the previously identified Arabidopsis proteins for each gene family as the query sequences. All hits that had an E value (expect value) < 1e-40 were considered and downloaded into Geneious. The amino acid sequence matches were subsequently downloaded for Arabidopsis, apple, tomato, grape, and poplar. Third, a keyword search of '*Aux/IAA* family' was used in Phytozome to extract all *ARF* and *Aux/IAA* CDS, genomic and protein sequences. These sequences were used in an NCBI TBLASTN (translated nucleotide) search to isolate any previously characterised protein sequences and CDS. The best matches had 100% sequence similarity to the query, although large insertions or deletions were included to allow for mis-annotations in Phytozome. The full-length protein and CDS were imported into Geneious. Finally, TBLASTN searches were completed in Phytozome with protein queries from the different protein families and different plant species. The best matches with an E value < 1e-50 were selected and all sequence homologs were downloaded via Biomart and imported into Geneious. This included the protein sequences, CDS and genomic sequences (including 5000 bp upstream and downstream) from Arabidopsis, apple, tomato, grape, and poplar. The molecular weight (MW) and isoelectric point (pI) were calculated using the pI/Mw tool on the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics, 2012, http://web.expasy.org/compute_pi/).

All protein sequences were compared within Geneious and know protein domains were detected using InterProScan (Quevillon *et al.*, 2005). Searches were carried out using domains present in each family to ensure all family members were isolated. In addition, the sequences were compared using Multiple Sequence Comparison by Log-Expectation (MUSCLE) sequence alignments to check for sequence similarity, and compared to the orthologs from other species to check for the conservation of the sequences (Edgar *et al.,* 2004).

2.2.2.2 Gene mining and identification – coding sequences

The CDS for each predicted gene was obtained for translational alignments and tree building. A protein family (Pfam) search was completed within the Phytozome keyword search to isolate the *Aux/IAA* and *ARF* gene families (Figure 2.4). The Pfam codes used were Aux/IAA family PF02309, present in both ARFs and Aux/IAAs, B3 DNA binding domain PF02362, present in ARFs, and auxin response factor PF06507, present in ARFs. All sequences identified were extracted using BioMart in Phytozome and imported into Geneious. In Geneious the sequences were assessed to determine if they were in-frame and contained no stop codons. To group the sequences into the *ARF* and *Aux/IAA* families all sequences were aligned using the translational alignment in Geneious, which uses MUSCLE alignment (Edgar *et al.,* 2004), and sorted them based on the domains present and the size of the genes. This

method did not identify any *ARF* or *Aux/IAA* sequences that were truncated and/or lacked one or more of the protein domains. Where the CDS were not identified by the original CDS search, TBLASTN in Phytozome was used with the protein sequences as the search query and the CDS results were then transferred to Geneious. This technique was used to isolate the TIR1/AFB sequences as a Pfam search was not possible.

Three methods were used to confirm the CDS. First, Tablet software (Milne *et al.,* 2013) was used to view RNAseq data isolated from three developmental stages (post-setting, veraison and ripening) and aligned to the Pinot Noir 40024 genome (Zenoni *et al.,* 2010). The putative translation initiation and termination codons were identified and the structure of the introns and exons examined. Second, alignments of the genomic sequences, obtained from Phytozome, and the CDS were aligned using MUSCLE alignment to check the intron/exon structure. Third, the genomic sequences and the protein sequences were introduced into a FGENESH+ pipeline (Schwerdt *et al.,* 2015) (Solovyev, 2007, http://linux1.softberry.com/berry.phtml) and predicted CDS and protein sequences were determined. These three techniques were used in combination to ensure the correct CDS regions were used in the phylogenetic analysis. The alignments of corrected CDS to genomic DNA were used to design both qPCR and full-length primers for further gene analysis.

The CDS sequences that were isolated for yeast analysis (Section 2.2.6.1) (VviARF4, 24, 27 and VviIAA19, 27, 41) were fully sequenced by the Australian Genome Research Facility Ltd. (AGRF, Adelaide, Australia) (Section 2.2.4.11) and the updated sequences were included in Geneious and used for bioinformatic analysis.

2.2.2.3 Mapping the auxin related genes onto chromosomes

The chromosome number and location of each auxin-related gene in *V. vinifera* was determined through Phytozome, however, the data provided by Phytozome is originally from Genoscope. The gene prediction software used within Genoscope was GAZE which predicts and annotates genes on the chromosomal sequences (Howe *et al.*, 2002). When searching for each gene within Phytozome, chromosomal positioning is provided but if the contig that contains the gene has not been mapped to a specific chromosome the location is listed as chromosome unknown. Each gene was then mapped onto a schematic of the 18 *V. vinifera* chromosomes based on the chromosomal information provided by Genoscope on the size and number of chromosomes.

2.2.2.4 Sequence alignments

All CDS alignments were initially constructed in Geneious using the translational alignment tool and default settings. The alignments were then exported to Mobyle @Pasteur which uses Block Mapping

and Gathering using Entropy (BMGE) (Criscuolo & Gribaldo, 2010, http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::BMGE) to isolate the conserved CDS regions for phylogenetic analysis. The sequence structure was set to "codons" and the default settings were used. The resulting alignments were imported into Geneious to assess their quality. For amino acid alignments the conserved CDS alignments were converted to protein sequences in Geneious.

2.2.2.5 Phylogenetic tree construction

To construct phylogenetic trees, CDS and protein sequence alignments were generated using the method in Section 2.2.2.1. These alignments were imported into Bayesian Evolutionary Analysis Utility (BEAUTi) (Drummond *et al.,* 2012, http://beast.bio.ed.ac.uk/beauti). BEAUTi was used to set the parameters for tree building. The parameters used were as follows: the GTR substitution model, estimated base frequencies, gamma site heterogeneities, partitioning into three separate codon positions with unlinked substitution models, and a Yule tree prior (Schwerdt *et al.,* 2015). Files were generated with a strict clock prior and then a relaxed clock prior (relaxed uncorrelated log normal) to test the fit of the data (Schwerdt *et al.,* 2015). Protein alignments had the same priors except that a WAG (Whelan and Goldman) substitution model was used. BEAUTi was used to generate XML (extensible markup language) files that were needed for running in Bayesian Evolutionary Analysis Sampling Trees (BEAST) (Drummond *et al.,* 2012, http://beast.bio.ed.ac.uk/Main_Page). BEAST uses posterior probability to identify the most likely tree and trees are generated every 1000 iterations. While running, Tracer v1.5 was used to determine the progress of the analysis. Tracer v1.5 shows the number of states that have been explored at that point in the analysis. After the analysis had run to completion or sufficient states had been explored TreeAnnotator v1.7.5 (Rambaut & Drummond, 2010; http://beast.bio.ed.ac.uk/treeannotator) was used to set the burn-in value, which removed the first trees generated (the number of trees removed is determined by the burn-in value). The remaining trees were then compared and a consensus tree generated. The consensus tree was opened in FigTree v1.4.0 (Rambaut, 2016; http://tree.bio.ed.ac.uk/software/figtree/). The nodes were annotated with the posterior probability values which suggest the likelihood of each node occurring. During the tree building process the 'rootedness' of each node is tested, the final tree therefore has a root which signifies the point of origin of all sequences within the phylogenetic tree. The final trees were then exported as PDFs.

Initially, the phylogenetic tree building was used during the protein and CDS identification period, using multiple species to help identify any gene models that may be present in Arabidopsis, apple, tomato and poplar but that are missing in grape, or that are present in grape but are not present in other species. Once all of the candidates had been identified, final multiple species phylogenetic trees

were generated for each gene family using the method described above. The conserved coding sequences were used as they are provide a deeper level of detail on the phylogenetic history of the sequences. These phylogenetic trees contained the candidates from grape, tomato, Arabidopsis, poplar and apple.

2.2.2.5.1 Construction of protein trees for gene nomenclature

Grimplet *et al.* (2014) described a new nomenclature for grape gene candidates, all with the new prefix '*Vvi*' instead of the original '*Vv*' to further distinguish grape naming from other species, such as the bacteria *Vibrio vulnificus*. To identify these names, three phylogenetic trees were generated containing the Arabidopsis and grape protein sequences for ARF, Aux/IAA and TIR1/AFB candidates, respectively. Within Geneious, multiple sequence alignments were generated using MUSCLE and maximum likelihood trees constructed using the PhyML (Phylogenetic inferences using maximum likelihood) plug-in (Guindon *et al.,* 2005). The JTT (Jones, Taylor and Thorton) matrix-based model was used and 100 bootstraps generated to infer evolutionary history, any branch nodes with less than 70% support were collapsed. Where a one-to-one relationship existed between an Arabidopsis and grape sequence, the grape sequence was given the same gene number as the Arabidopsis gene. Where two grape sequences where present on the same branch as a single Arabidopsis gene, the grape sequences were given the same number and an additional letter to distinguish between them, such as VviARF2a and VviARF2b. Where there were multiple Arabidopsis genes paired with multiple grape genes or no Arabidopsis gene in close proximity, the grape sequences were given a number higher than the highest Arabidopsis gene number in a top to bottom order within the tree. In the case of the AFB tree, the AFB6 clade has been identified and described in Parry *et al.* (2009) and no homolog has been identified in Arabidopsis. Based on this previous characterisation, it was beneficial to continue with this naming system and name VviAFB6 based the sequence which was a clear outlier in the AFB nomenclature tree.

2.2.2.6 Co-expression analysis

2.2.2.6.1 Heatmap construction

Heatmaps representing normalised gene expression across data sets were generated using MultiExperiment Viewer (MeV) (Saeed *et al.,* 2003). All data was normalised by scaling between 0 and 1 within the data set represented in the heatmap. Text files containing the data were loaded into MeV. The colour scale limits were set to 0.0, 0.5 and 1.0, with blue as the colour minimum and green as the colour maximum with a blue-white-green colour gradient.

2.2.2.6.2 Cluster analysis

The hierarchical clustering (HCL) function of MeV was used to identify transcripts with similar expression patterns across data sets. The following parameters were used: 'gene tree selection' was used for tree selection, 'optimise by gene leaf order' was used for ordering optimisation, 'Euclidean distance' was used as the distance metric selection, and 'average linkage clustering' was used as the linkage method selection. Euclidean distance was selected to ensure clustering based on expression patterns irrespective of expression values, aided by the normalisation of the expression data (Yeung & Ruzzo, 2001; D'haeseleer, 2005). Images were saved as PNG image files. The resulting clusters were used to generate graphs in SigmaPlot 12.5 (San Jose, CA, USA) as an alternative method of representing the data.

2.2.2.7 Promoter Analysis

For promoter analysis, the 5'-region upstream of the predicted start codon was isolated either using a keyword search with the transcript number or by BLAST searches using sequences from Geneious, within Phytozome. The promoter region was deemed to be within a 2000 bp region upstream from the 5'-UTR or start codon if a 5'-UTR was not present, while the 5'-UTR sequence was included in addition to the 2000 bp region if present. The sequences were imported into Geneious and additionally into promoter analysis software. To identify motifs the plant promoter analysis navigator program, PlantPAN (Chow *et al.*, 2016, http://plantpan.mbc.nctu.edu.tw/), which incorporates motif analysis from multiple species and databases including PLACE (Higo *et al.*, 1998, http://www.dna.affrc.go.jp/PLACE/). Motif searches were completed on the promoter and 5'-UTR sequences of all *ARF* and *Aux/IAA* genes. These were compared to genes that did not appear to be hormone responsive and other unrelated genes, and subsequently assessed for the presence/absence of motifs that may be playing a role in hormonal regulation.

PlantPAN is an internet-based program that is able to identify DNA motifs within a query sequence. Each promoter sequence was entered into PlantPAN and an output file was created, which contained any known motifs that were identified within the sequence. In addition to PlantPAN, manual searches were used on the promoter sequences stored within Geneious using the 'Search Function' to identify motifs that were not present within the PlantPAN database, the specific motifs searched for are included in Chapter 6. These results give an indication about the type of regulation occurring on promoter sequences for discussion and comparison with experimental results, however, more in-depth analysis is always required on each gene family to find species-specific motifs and was unfortunately not within the scope of this work.

2.2.2.8 Primer design

Primer sequences were generated using techniques specific to each experiment. Primers were obtained from GeneWorks (Thebarton, SA, Australia; 40 nmol per tube, PCR/sequencing quality, desalted) as dried pellets that were resuspended as 100 µM stocks and diluted for use.

2.2.2.8.1 Primers for qPCR analysis and cloning of standards

Primers for the amplification of standards and for qPCR analysis were designed to the 3'-end of the sequences and included part of the 3'-UTR if possible. The primers were designed using the Primer3 software within Geneious with the following parameters: 70–150 bp fragment size, 17–27 bp primer size (optimal 20 bp), Melting temperature (Tm) 57–65°C (optimal 60°C), %GC 40–60% (optimal 50%), 1 GC clamp, and default settings were used for the maximum hairpin score, primer dimer score, Poly-X (the maximum allowable length of a mononucleotide repeat in a primer) and 3'-stability. All primer pairs were generated with a maximum Tm difference of 2°C.

Primers were screened for suitability and one or two sets were selected for each gene. For some genes more sets had to be designed due to difficulties with cloning or qPCR analysis, including the presence of primer dimers or no product amplification.

2.2.2.8.2 Primers for yeast cloning

Primers for the yeast transformation experiments were generated to include the whole CDS sequence of each *ARF* and *Aux/IAA* sequence. The primers were designed to begin at the start codon and end at the stop codon using the same parameters as the primers in Section 2.2.2.8.1 except the fragment size was dependent on the CDS size. Fragments were cloned into pCR®-Blunt (Life Technologies) plasmids and confirmed by sequencing. New primers were subsequently designed with In-Fusion Advantage overhangs, as detailed in the In-Fusion Advantage PCR cloning kit user manual (Clontech). These primers included 24 bp homology to the CDS and 15 bp homology to the pGBKT7 or pGADT7 plasmids, with an additional base pair to ensure the restriction enzymes sites were maintained. After initially cloning full-length sequences, truncated *ARF* sequences were later generated that did not contain their DNA binding domains.

Primers were also generated to confirm that entire sequences were correct before use in experimental work. These were designed using the same parameters as above and were designed to ensure full coverage of each gene.

2.2.2.8.3 Primers for BiFC Gateway cloning

Primers for the BiFC experiments were generated to include the whole CDS sequence of each *ARF*, *Aux/IAA* and sequences identified through the yeast two-hybrid library screening. The stop codons

were removed from each CDS to ensure the continued translation into the YFPn or YFPc sequence. The primers were designed as outlined for Gateway® Cloning (Life Technologies). For negative controls *VviARF4, VviARF27, VviIAA19* and *VviIAA27* were truncated to remove Domains III and IV using new reverse primers.

2.2.2.9 Expression analysis figures

The figures for cluster analysis and expression analysis were generated using SigmaPlot 12.5.

2.2.3 Molecular biology techniques

2.2.3.1 DNA and RNA quantification

The concentration of DNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). One microlitre of MilliQ water was used to initiate the Nanodrop, followed by one microlitre of the diluent (such as water, TE, or elution buffer) as a blank. One microlitre of each DNA or RNA sample was placed on the Nanodrop, the pedestal automatically adjusted to the optimal path length of between 0.05-1 mm, the OD was measured at 280/260 nm and the concentration recorded (Thermo Fisher Scientific). The stage was wiped between each sample and the method was repeated with all of the samples.

2.2.3.2 Agarose gel electrophoresis

2.2.3.2.1 DNA quality analysis via gel electrophoresis

Nucleic acid fragments (such as PCR products or restriction enzyme digests) were separated by agarose gel electrophoresis (using Bio-Rad tanks and combs); (Hercules, CA, USA). The 1–2% agarose gels were made by dissolving Ultra Pure™ Agarose (Invitrogen) in 1 X TBE buffer using a microwave. SYBR Safe™ stain (Invitrogen) was added to the agarose mixture at 1 X concentration once cooled and prior to setting. The nucleic acid solution was mixed with 0.2 volumes of electrophoresis loading dye (Appendix A, Table A.2). Gels were electrophoresed at 100–125 V for 40–120 min in 1 X TBE buffer, until the loading dye had migrated close to the end of the gel. The nucleic acids were visualised using the Quantum-ST4 1120/Blue transilluminator system and Quantum-Capt software (Montréal Biotech Inc., Dorval, Quebec, Canada). All gels were run with 1 kb Plus DNA Ladder™ (Invitrogen) loaded with loading dye as specified by Invitrogen. The ladder allowed the sizing of linear, double-stranded DNA fragments between 100 bp and 12 kb.

2.2.3.2.2 RNA quality analysis via gel electrophoresis

Gel electrophoresis was used to assess the quality of total RNA after extraction. Gel electrophoresis trays, comb and tank were soaked in 0.2 M NaOH for 30 min prior to gel electrophoresis to remove any RNases followed by a wash with sterile distilled water before use. Agarose gels (1%) were made

as described in Section 2.2.3.2.1. All samples were diluted to ensure 0.5 µg was loaded in a final volume of 8 µL with 4 µL of loading buffer and sterile water. Gels were run at 120 V for approximately 45 min and the 23 S and 16 S ribosomal RNA bands were visualised using the transilluminator (Section 2.2.3.2.1). All samples that had two clear ribosomal RNA bands were considered suitable for cDNA synthesis.

2.2.3.3 PCR master mix and reaction

2.2.3.3.1 Standard amplification using Platinum® Taq

The standard PCR reaction was used for diagnostic purposes to establish the presence of the correct insert within bacteria in colony PCRs (Section 2.2.3.3.2) and for the amplification of fragments for the generation of standard curves in qPCR analysis (Section 2.2.5.4). When multiple reactions were performed, a master mix was made up for the total number of reactions. The standard master mix for the total number of reactions had a final concentration of 1 X PCR buffer, 0.2 mM of dNTP mixture, 1.5 mM $MgCl_2$, 0.2 µM of forward primer specific to the target DNA, 0.2 µM of reverse primer specific to the target DNA, 1 unit Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) and autoclaved MilliQ water to volume. Eighteen µL of the master mix was pipetted into reaction tubes and the DNA added. MilliQ water was added to a final volume of 20 µL. The concentration of template DNA was dependent on the purpose of the PCR (Sections 2.2.3.3.2 and 2.2.3.3.3).

PCR was carried out in a Bio-Rad S1000 thermal cycler PCR machine. After the initial denaturation at 94°C for 2 min, the amplification was performed at 94°C for 20 s, 56°C for 30 s, 72°C for 1 min per kb; the number of amplification cycles was dependent on the origin of the DNA product (Sections 2.2.3.3.2 and 2.2.3.3.3). A final elongation step at 72°C for 5 min completed the PCR run. All products were then analysed on agarose gels (Section 2.2.3.2.1) to determine the fragment size.

2.2.3.3.2 Colony screening PCR

Colony screening PCR was undertaken on bacterial colonies after transformation to confirm the insertion of the desired plasmid. Single bacterial colonies were picked from plates using sterile 10 µL pipette tips and placed in 30 µL of Luria-Bertani (LB) broth medium (Appendix A, Table A.3). Two µL of the resulting mixture was added to 18 µL of master mix (Section 2.2.3.3.1). Thirty two cycles of PCR were performed on the samples with an extension time appropriate for the size of the desired fragment.

2.2.3.3.3 PCR amplification for interaction analysis

PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) was used for the amplification of fragments for the yeast and BiFC interaction analysis. Each reaction had a final concentration of 1 X PCR buffer, 0.2 mM of dNTP mixture, 0.2 µM of forward primer specific to the

target DNA, 0.2 µM of reverse primer specific to the target DNA, 1 U PfuUltra II Fusion HS DNA Polymerase and autoclaved MilliQ water to volume. All components were pipetted into reaction tubes and the DNA added. MilliQ water was added to make a final volume of 25 µL. The concentration of template DNA was dependent on the origin of the material being amplified, 2 µL of undiluted cDNA or 1 µL of previously isolated plasmid. The PCR was carried out in a Bio-Rad S1000 thermal cycler PCR machine. After the initial denaturation at 95°C for 2 min, the amplification was performed at 95°C for 20 s, 56°C for 20 s, 72°C for 45 s; repeated 35 times. A final elongation step at 72°C for 3 min completed the PCR run. All products when then analysed on agarose gels (Section 2.2.3.2.1) to determine the fragment size and for gel extraction.

2.2.3.4 PCR product gel purification

The Purelink TM Quick gel extraction kit (Life Technologies, Carlsbad, CA, USA) was used to purify fragments excised from agarose gels as described in the manufacturers protocol. Digested pGBKT7 and pGADT7 plasmids and the PCR products for use in yeast work were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) as described in the manufacturer's protocol.

2.2.4 Cloning

There were four cloning strategies used within this work. The methodology and purpose of each is described below.

2.2.4.1 pDRIVE cloning

The pDRIVE (Qiagen, Venlo, Netherlands) plasmid was used to check the sequences of standards for qPCR analysis. As the qPCR standards are 70–150 bp in size it was often difficult to obtain good sequence reads from direct PCR product sequencing. Therefore, after PCR amplification (Section 2.2.3.3.1) of each standard, samples were electrophoresed on an agarose gel (Section 2.2.3.2.1), gel extracted (Section 2.2.3.4) and ligated into pDRIVE using the manufacturer's protocol (Qiagen PCR cloning kit). After ligation the ligation mix was transformed into DH5α cells (Section 2.1.7.1), individual colonies were checked for the presence of the correct insert by colony PCR (Section 2.2.3.3.2). If a band of the correct size was present, overnight cultures (Section 2.2.4.7) were inoculated and plasmids extracted. The plasmids were sent for sequencing (Section 2.2.4.11) and if the sequences were accurate based on the predicted gene sequence in Geneious, the original gel extract was then tested via qPCR analysis for suitability as a standard.

2.2.4.2 pCR-Blunt cloning

The pCR®-Blunt (Life Technologies) plasmid was used to check the CDS of each gene prior to cloning into the pGBKT7 or pGADT7 plasmids for yeast work. Fragments were amplified using PCR as described in Section 2.2.3.3.3. These were electrophoresed on an agarose gel (Section 2.2.3.2.1), gel extracted (Section 2.2.3.4), and ligated into pCR®-Blunt using the manufacturers protocol (Life Technologies Zero Blunt® PCR Cloning Kit). After ligation the ligation mix was transformed into DH5α cells (Section 2.1.7.1), individual colonies were assessed for the presence of the correct insert by colony PCR (Section 2.2.3.3.2). If a band of the correct size was present, overnight cultures (Section 2.2.4.7) were inoculated and plasmids extracted. The plasmids were sent for sequencing (Section 2.2.4.11) and if the sequences were accurate when compared to the predicted gene sequence in Geneious, the plasmids were used as the template for PCR to include the overhangs for the pGBKT7 or pGADT7 plasmids (Section 2.2.2.8.2).

2.2.4.3 In-Fusion reaction for transformation into pGBKT7 and pGADT7

The In-Fusion Advantage PCR cloning kit (Clontech) was used to insert the CDS of genes of interest into either the bait plasmid, pGBKT7, or the prey plasmid, pGADT7. After the initial PCR described in Section 2.2.3.3.3 the In-Fusion reactions were carried out as described in the manufacturer's protocol. After the completion of the In-Fusion reaction, 2.5 µL of the mixture was transformed into Stellar cells as described in Section 2.2.4.5.1.

2.2.4.4 Gateway® cloning for BiFC analysis

The generation of the constructs for BiFC was a two-step procedure, as detailed in the Gateway® Technology cloning manual (Life Technologies). Initially, a BP reaction inserts the CDS insert into the pDONR221 plasmid and this is subsequently followed by an LR reaction to insert the CDS insert into the pSITE vectors. A single modification was made to both the BP and LR reaction steps. After the BP and LR reactions were completed as described in the manufacturers protocol, 100 µL of n-butanol was added, samples were vortexed and then centrifuged at 14,000 rpm in a microcentrifuge for 10 min. The supernatant was removed and the pellets were washed with 70% ethanol. Samples were dried using a centrifugal vacuum evaporator and resuspended in 5 µL of sterile water. After this purification 3 µL of the resuspension mixture was transformed into DH5α cells as described in Section 2.2.4.5.2.

2.2.4.5 Transformation of *E. coli*

2.2.4.5.1 Heat-shock

The transformation of plasmids into *E. coli* (Section 2.1.7.1) was achieved using the heat-shock method. *E. coli* cells were removed from the -80°C freezer and placed immediately on ice. Once thawed, 50 µL aliquots of the cells were pipetted into 1.5 mL Eppendorf tubes and 2.5 µL of the In-

Fusion mixture was added and gently mixed with the pipette tip. The cell and plasmid mixtures were left on ice for half an hour. The tubes were then placed in a 42°C water bath for 45 s and then placed on ice for 1 min. The cells were then removed from the ice and 450 µL of LB medium was added (Appendix A, Table A.3). The tubes were placed in a 37°C shaker for one hour to recover. After recovery, the cells were plated out in a laminar flow cabinet onto LB plates containing the appropriate antibiotic selection (Appendix A, Table A.1) using 50 µL and 200 µL aliquots. Once dry, the plates were placed upside down at 37°C for *E. coli* growth.

2.2.4.5.2 Electroporation

Transformation of plasmids into *E. coli* (Section 2.1.7.1) was achieved through electroporation using 1 mm electroporation chambers, which were placed on ice prior to the transformation. The *E. coli* cells (Section 2.1.7.1) were removed from the -80°C storage and placed immediately on ice for thawing. Once thawed, 50 µL aliquots of *E. coli* cells were pipetted into microcentrifuge tubes and 1 µL of the desired plasmid DNA was added and gently mixed with the pipette tip. The cells and plasmid mixture were left to sit on ice for 30 mins before being pipetted into the pre-cooled electroporation chambers. The BioRad Gene Pulser TM electroporator was set to 200 Ω, 25 µF and 1.8 kV. After electroporation 450 µL of LB media (Appendix A, Table A.3) was added to the cells as a recovery broth. The *E. coli* and recovery broth mixture was pipetted into a 1.5 mL Eppendorf tube and placed in the 37°C shaker for one hour. After recovery, the culture was plated out onto LB plates (Appendix A, Table A.3) containing the appropriate antibiotic selection using 50 µL and 200 µL aliquots. Once dry, the plates were placed upside down at 37°C for *E. coli* growth.

2.2.4.6 Transformation of yeast

Prior to yeast library screens the pGBKT7 bait vectors containing the CDS of interest were transformed into Y2H Gold cells using the manufacturers protocol (Clontech).

2.2.4.6.1 Co-transformations of Y2H Gold yeast cells

For testing the interaction of the CDS sequences contained within the bait and prey plasmids, Y2H gold cells were co-transformed with both plasmids. The manufacturers protocol (Clontech) was followed, however, 50 ng of each of the bait and prey plasmids was used in the transformation and a selection media was used that allowed the growth of both plasmids.

2.2.4.7 *E. coli* overnight cultures

Five mL aliquots of LB medium (Appendix A, Table A.3) containing 50 µg/mL of the appropriate antibiotic were aseptically inoculated with single bacterial colonies containing the desired plasmid. Cultures were incubated in a shaker at 37°C for *E. coli*.

2.2.4.8 Plasmid isolation from *E. coli*

Prior to the plasmid isolation, overnight cultures (Section 2.2.4.7) were centrifuged at 4000 g at room temperature. Either the AxyPrep plasmid mini prep kit™ (Axygen Scientific Inc., Union City, CA, USA) or the PureLink™ quick plasmid mini prep kit (Invitrogen) were used for plasmid isolation from *E. coli* using the protocols provided by the manufacturers. All samples were eluted in 50 µL of the elution buffer provided in the kit.

2.2.4.9 Extraction of plasmids from yeast

Plasmids were extracted from yeast colonies using the method as described by the manufacturer in the Easy Yeast Plasmid Isolation Kit (Clontech).

2.2.4.10 Glycerol stocks

2.2.4.10.1 E. coli

In a cryogen tube, 900 µL of the culture and 200 µL of 80% sterile glycerol were mixed together with a pipette and were placed at -80°C for long-term storage.

2.2.4.10.2 Yeast

As described in the manufacturers protocol (Clontech) 500 µL YPDA, 500 µL 50% glycerol and one yeast colony were vortexed and placed at -80°C for long-term storage.

2.2.4.11 DNA sequencing

Sequencing was completed by AGRF using Sanger Sequencing. For the pDRIVE plasmids a BigDye PCR (Applied Biosystems, Carlsbad, CA, USA) reaction was completed using the manufacturer's protocol and the M13 forward and reverse primers. The PCR products were purified by adding 80 µL 75% isopropanol to each PCR reaction in a 1.5 mL Eppendorf tube and incubated at room temperature for 20 min. The samples were then centrifuged at 13,000 rpm in a benchtop microcentrifuge for 20 min. The supernatant was subsequently removed and 250 µL of 75% isopropanol was added. The samples were vortexed and centrifuged again at 13,000 rpm for 5 min at room temperature. The supernatant was removed and samples were placed in a centrifuged vacuum evaporator to remove any remaining isopropanol. When dry, the pellets were supplied to AGRF for capillary separation. Alternatively, for all other cloning, sequencing was completed by AGRF who were provided with 600–1200 ng of plasmid/PCR product and 1 µL of 10 µM primer and made up to 12 µL with sterile water. AGRF sequence files were imported into Geneious for analysis.

2.2.4.12 Digestion of DNA by restriction enzymes

Restriction enzyme digests were performed to determine if the correct CDS insert was present in a plasmid and also prior to the In-Fusion reactions on the pGBKT7 and pGADT7 plasmids (Clontech).

Restriction enzyme (1–5 U) and the corresponding reaction buffers supplied by New England Biolabs (NEB) were added to the DNA or plasmid and incubated at 37°C for 2 h. Where multiple restriction enzymes were required, a reaction buffer that allowed the optimal activity of all the restriction enzymes was used. This information was obtained from the NEB Double Digest Finder (http:/ssa/www.neb.com/nebecomm/DoubleDigestCalculator.asp). After digestion, 5 µL of the mixture was electrophoresed on an agarose gel to ensure the digestion was successful and to determine the digest pattern. The digested pGBKT7 and pGADT7 plasmids were purified using the method described in Section 2.2.3.4 prior to use in the In-Fusion reactions.

2.2.5 Gene expression analysis

2.2.5.1 Plant tissue preparation

All samples were frozen in liquid nitrogen prior to processing. Leaf and flower samples were ground using a mortar and pestle and berry samples were ground using an electric IKA A11 analytical grinding mill (IKA, Staufen, Germany) until they became a fine powder. The mortar and pestle and grinding mill were cleaned with 100% ethanol between samples. All samples were kept frozen during processing. All samples were pre-ground and stored at -80°C prior to use in RNA extractions.

2.2.5.2 RNA extraction

RNA extractions were completed using the protocol as described in Böttcher *et al.* (2013a), based on Davies & Robinson (1996). As described in Symons *et al.* (2006) a clean-up step was included where the RNA was processed using an RNeasy mini kit (Qiagen) and the manufacturer's protocol. An additional step was added between the two RW1 washes where all samples were treated with RNase-Free DNase (Qiagen). A volume of 10 µL DNase stock solution was added to 70 µL of RNase-free Buffer RDD (Qiagen), the samples were mixed by inversion and pipetted onto the RNeasy mini-columns provided in the Qiagen RNeasy mini kit and incubated at room temperature for 15 minutes prior to the second wash with the RW1 buffer. All samples were quantified using a NanoDrop (Thermo Fisher Scientific) to ensure a 260/280 ratio > 2.0 and a 260/230 ratio of > 1.4 and electrophoresed on an agarose gel to check their quality (Section 2.2.3.2.1). All samples were then stored at -80 °C.

2.2.5.3 cDNA synthesis

Total RNA was used as a template for cDNA synthesis prior to RT-qPCR. The Transcriptor Reverse Transcriptase (Roche Holding AG, Basel, Switzerland) and an oligo $(dT)_{15}$ primer were used following the manufacturers protocol. After the final step, 380 µL of sterile water was added to each 20 µL reaction for use in RT-qPCR.

2.2.5.4 Standards for RT-qPCR analysis

To determine the copy number of transcripts in RT-qPCR, standards of a known sequence and concentration were used. The gene fragments were generated by PCR (Section 2.2.3.3.1) using specifically designed primers as described in Section 2.2.2.8.1 and examined by gel electrophoresis. Bands of the correct size were gel purified (Section 2.2.3.4), quantified using a Nanodrop (Section 2.2.3.1) and ligated into pDRIVE (Section 2.2.4.1). The ligations were transformed into DH5α *E. coli* cells for blue-white selection and plated on Ampicillin (Amp)/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)/Isopropyl β-D-1-thiogalactopyranoside (IPTG) plates for overnight growth. Individual white colonies were selected and colony PCR was completed to ensure colonies contained the desired insert (Section 2.2.3.3.2). The plasmids were then extracted (Section 2.2.4.8) and sequenced (Section 2.2.4.11). The sequence results were analysed using Geneious.

Gel-extracted DNA samples that corresponded to a positive sequencing result were quantified using the QuantiFluor® dsDNA System (Promega, Madison, Wisconsin, USA) to determine the molecule number for each sample; this was achieved by submitting a 5 µL sample containing 5–10 ng of DNA to Dr P. Gooding at AGRF. Results were returned as Quantifluor concentrations in ng/µL. Using the initial dilution factor (to ensure a concentration between 5–10 ng) an adjusted concentration in ng/µL was calculated (quantifluor concentration x dilution factor).

To calculate the exact copy number of each double stranded transcript the molecular weights of the forward and reverse PCR fragments were calculated by entering their sequences into http://www.encorbio.com/protocols/Nuc-MW.htm.

$$\frac{\text{g/mol}}{6.022\text{x}10^{23}\ \text{molecules/mole}^{*}} = \text{g/molecule}$$

*= Using Avogadro's number, which is approximately 6.022×10^{23} molecules/mole, this can be converted into g/molecule

This g/molecule value can then be used with the DNA concentration (ng/µL) to determine the precise number of molecules in a known volume.

$$\frac{\text{DNA concentration (ng/µL)}}{\text{g/molecule}} = \text{molecules/µL}$$

molecules/µL x volume of template used in PCR = transcript copies per reaction

To test the suitability of a standard for use in RT-qPCR analysis, dilutions (10^{-4} to 10^{-9}) of the gel extract were tested with four technical replicates in the LightCycler® 480 II machines (Roche). Standards were accepted if they had uniform melt curves and a primer efficiency of between 1.8–2.

2.2.5.5 Robot used for pipetting samples for RT-qPCR analysis

The Zephyr (Caliper Life Sciences, Hopkinton, Massachusetts, USA) robot and Caliper Life Sciences Maestro Workstation software was used to pipette all 384 well plates prior to RT-qPCR analysis. All programs used within this work were written by Dr C. Burbidge. The plates containing the master mix and cDNA samples were in 96 wells and were pipetted by hand.

2.2.5.6 Real time quantitative PCR

All RT-qPCR analysis was conducted using LightCycler® 480 II machines (Roche). The 384 well plate layout was labelled and replicates were made in the LightCycler (R) 480 SW 1.5.1 program and the concentrations of each standard were added (Section 2.2.5.4).

Two main programs were used for RT-qPCR. The standard program had an initial denaturation step at 95°C for 5 minutes, followed by the cycle of 95°C for 20 seconds, annealing at 58°C for 20 seconds and extension at 72°C for 20 seconds which was repeated for 45 cycles. This was followed by a hold period at 72°C for 5 minutes. The melt curve was then determined by heating the samples to 95°C for 15 seconds, and cooling to 50°C for 45 seconds followed by continuous heating to 95°C at 0.11°C s^{-1}. Alternatively, the entire program remained the same however the annealing temperature was increased from 58°C to 62°C and this was dependent on the primer pair used (Appendix B).

Four technical replicates were included for each of four dilutions (e.g. 10^{-5}, 10^{-6}, 10^{-8} and 10^{-9}) of each gene standard to generate a standard curve for quantifying gene expression. After the completion of the RT-qPCR run a standard curve was generated using absolute quantification (Abs Quant)/2^{nd} Derivative Max and used to quantify the gene expression. Any expression that was detected below the standard curve was listed as not detected (N.D.) or zero within heatmaps. The melt curve was generated using Tm calling in the LightCycler software. The gene expression values were exported into Microsoft Excel for further calculations (Section 2.2.5.7).

2.2.5.7 Calculation of expression levels

All expression data was initially placed into Microsoft Excel (Microsoft, Redmond, Washington, USA) to calculate the copy number. The results were normalised against VviActin2 (AM465189), which was amplified for each set of cDNA used for qPCR analysis. The maximum expression value for the actin samples was designated to be 1; the maximum expression value was then divided by all of the other values. This generated a multiplication factor (MF) to normalise any inter-cDNA variation. All

expression data was multiplied by the MF to obtain the final copy number used for the expression analysis.

Two to four technical replicates were run for each biological replicate (two technical replicates for berries and flowers, and four technical replicates for leaves, roots and tendrils). These technical replicates were averaged, and the values for each biological replicate were then averaged (the mean) as representative copy number for each sample. The standard error was calculated in Excel as STDEV(data)/(SQRT(number of samples)) (standard deviation, square root).

2.2.6 Investigating protein-protein interactions

2.2.6.1 Yeast analysis

All yeast work was completed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). Prior to library screens and interaction analysis all bait vectors were tested for auto-activation (Section 2.1.7.2).

2.2.6.1.1 Yeast library screens

The yeast prey cDNA libraries made from week 4 and week 12 post-flowering Shiraz berry RNA used within this work were generated by Dr C. Davies and Dr C. Böttcher according to the manufacturer's protocol. Library screens were completed using the manufacturers protocol (Clontech) with VviARFs of interest.

2.2.6.1.2 Yeast 2-hybrid

For yeast 2-hybrid analysis, pGBKT7 bait and pGADT7 prey vectors containing genes of interest were co-transformed into Y2H Gold cells as described in Section 2.2.4.6.1. These were grown on selection media. Individual colonies that resulted were resuspended in 60 µL of 0.9% (w/v) sodium chloride by vortexing, 5 µL of the cell resuspension was pipetted on double drop out plates (-tryptophan/-leucine) containing 5-Bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid (X-Gluc) and also on quadruple drop out plates containing X-Gluc and the toxin aureobasidin A (AbA). When dry, these plates were placed at 30°C for two nights for yeast growth. Plates were photographed after one and two nights of incubation.

2.2.6.2 BiFC experiments

2.2.6.2.1 Bombardment

The bombardment of onion for BiFC experiments was completed using the protocol as described in Selth *et al.* (2005). A combination of *VviARF* and *VviIAA* candidates were used, as well as two genes that were found to be interacting with a VviARF in a yeast library screen. These genes were cloned into pSITE-EYFP (enhanced yellow fluorescent protein) vectors (all variations in Appendix C, Table C.3,

TAIR) and combinations of N'- and C'-terminal fusions were bombarded into onion cells. The pART7-35S-VviSNAP33-CFP (soluble N-ethylmaleimide-sensitive factor adapter protein 33, cyan fluorescent protein) vector was co-bombarded as a positive control, VviSNAP33 is expressed throughout the cell, including the nucleus and plasma membrane.

2.2.6.2.2 Microscopy

To detect the nuclei of the onion cells, 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, Missouri, USA) was used at a final concentration of 2 µg/mL in water. Samples were mounted on slides in the DAPI solution, incubated in darkness for 10–20 min and observed by microscopy. A Zeiss Imager M1 Axio microscope (Zeiss, Oberkochen, Germany) was used to examine the bombarded onion cells, using bright-field, DAPI, CFP and yellow fluorescent protein (YFP) filters. Images were captured using a Zeiss AxioCam MRm T2-C 1,0x and stored for editing in AxioVision Rel. 4.7 (Zeiss). The DAPI, CFP and YFP iamges were overlayed to produce the final composite images displayed in Chapter 5 and the Appendices.

2.2.7 *Ex planta* berry induction assays

To determine the hormone responsiveness of the auxin-related genes, *ex planta* assays were carried out following the method as described in Böttcher *et al.* (2013a), with three biological replicates per treatment (Figure 2.5). Sampling was completed at time zero, 3 h, 24 h, and 48 h. The exception to this is that no time zero was collected within the NAA experiment at the week six time point in 2014. At each sampling time point all harvested berries were deseeded and frozen in liquid nitrogen. The week 12 NAA *ex planta* experiment (2013-2014) and the two multiple hormone *ex planta* experiments, at six and 12 WPF (2014-2015) were conducted within this work as detailed in Section 2.1.4.2. The week 6 NAA *ex planta* experiment (2013-2014) was conducted by members of the Davies lab; samples were frozen in liquid nitrogen and stored at -80°C. The same method was used for the additional three experiments.

The following additives (final concentrations) were added for each treatment 0.54 µM of 1-naphthaleneactic acid (NAA), 25 µM +cis trans abscisic acid (ABA), 25 µM 6-(γ,γ-Dimethyl-allylamino)-purine (iP) (Sigma-Aldrich), 25 µM 24-epibrassinolide (BL) (Mikonik Technologies Ltd., New York, USA), and Ethrel (150 µL/L) and no sucrose. All plates containing Ethrel were stored separately.

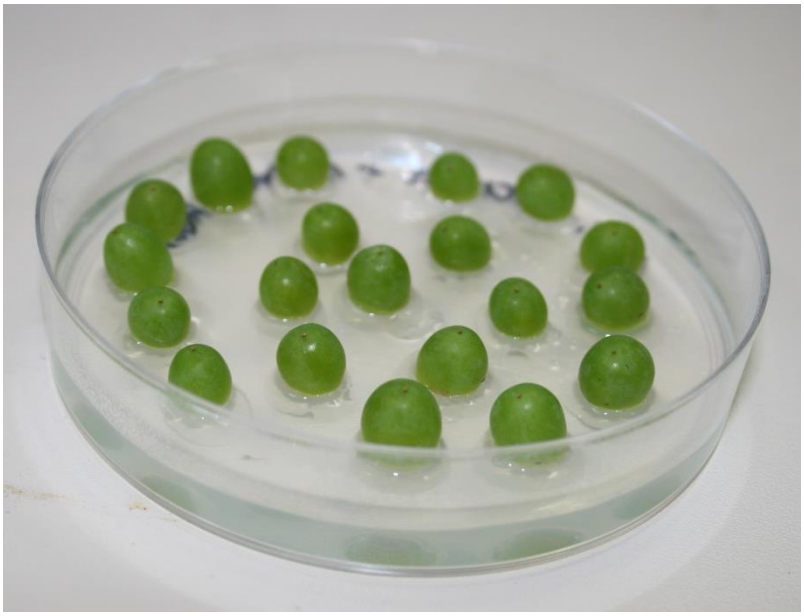


Figure 2.5 *V. vinifera* L cv. Shiraz berry samples used within the *ex planta* experiments.

A) Four week post-flowering, pre-veraison, berries on media. Plates were 100 mm wide and 20 mm deep (Photo courtesy of Dr. C Davies).

2.2.8 Analytical chemistry

2.2.8.1 Auxin extractions

The endogenous auxin of interest, IAA, and its conjugate IAA-aspartate (IAA-Asp), were extracted within this work using a method originally described by Kowalczyk & Sandberg (2001) and modified by Böttcher *et al.* (2010b). Due to the polyphenolics present in berry tissues, different ground sample weights were tested to maximise extraction of IAA and IAA-Asp from the samples. Originally 100 mg was used for all leaf and berry samples, and the measurements worked well in the leaf tissue with this weight. However, it appeared that the measurements were inhibited at 100 mg in berries, especially in the early stages of berry development. For this reason 10 mg of tissue was used for weeks one to five of berry development, and 50 mg of tissue was used for weeks six to 16. Three biological replicates were tested for the berries and the single biological replicate for the leaf developmental series was divided into three batches in place of three biological replicates.

With the 50 mg samples a single 6 h extraction period was used. Samples were centrifuged and the supernatant transferred to a 2 ml Eppendorf tube. A volume of 1 ml of the extraction buffer was added to the pellet and the sample was resuspended and centrifuged again. The supernatant was added to the original 1 mL in the 2 mL Eppendorf tube and the protocol was continued as described in Böttcher *et al.* (2010b).

The 10 mg samples (week one) were resuspended in 30 µL methanol/water/acetic acid (60:39:1, volume/volume/volume) and weeks two to eight were resuspended in 25 µL. The 50 mg samples were resuspended in 30 µL and 100 mg samples were resuspended in 40 µL. The samples were then centrifuged at full speed in a microcentrifuge for 2.5 min and 10 µL was used for LC-MS/MS analysis.

2.2.8.2 Liquid chromatography-liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to detect the IAA and IAA-Asp concentration as described by Böttcher *et al.* (2010b). This was carried out by Dr C. Böttcher and the peaks were quantified within this work using the Agilent MassHunter Optimizer software. The standards d5-IAA and d5-IAA-Asp were quantified and used in the calculation for determining the concentration of IAA and IAA-Asp present. Calibration curves were provided by Dr C. Böttcher with known concentrations of IAA or IAA-Asp (pmol/µL) on the Y axis and the ratio of peak values for IAA or IAA-Asp / standard (d5-IAA or d5-IAA-Asp) as the X axis.

To determine the concentration of IAA the following calculation was used:

((Area of the IAA peak/Area of the d5-IAA peak) – the intercept of the calibration curve)

The slope of the calibration curve

= IAA concentration pmol/µL

IAA conc. pmol/µL (from above) x sample weight (mg) x (1000/resuspension volume in µL)

= IAA concentration pmol g/FW

The mean was calculated for three technical replicates (for leaves) or three biological replicates (for berries) and the standard error calculated in Microsoft Excel (STDEV(samples)/SQRT(sample number))(standard deviation, square root). These values were then graphed. This process was repeated for IAA-Asp by substituting in the IAA-Asp and d5-IAA-Asp values in place of the IAA and d5-IAA values.

To determine the concentration in leaves, IAA-Asp and IAA were extracted from 100 mg of leaf tissue for all stages 1–9. For berries, IAA-Asp and IAA were extracted from 10 mg of tissue for weeks 1–5 and 50 mg of tissue for weeks 6–16.

2.2.9 Statistical analysis

Statistical significance between the *VviAFB*, *VviARF* and *VviIAA* copy numbers for the developmental and organ qPCR data has not been included in this study as it was deemed to not provide any further information about the significance of the results and would unnecessarily add to the complexity of the interpretation of the results.

2.2.9.1 Filtering for correlation analysis

To determine the correlation between gene expression patterns, a method from Dr Neil Shirley (University of Adelaide) was used based on the analysis toolpack in Microsoft Excel. This was completed using correlation function and the formula IF(CELL<1,IF(CELL>FILTER,CELL,””),””). Conditional formatting was used as a filter to determine the significance of the results, highlighting the high co-expression results only.

2.2.9.2 Student’s t-test

The Student t-test was used to determine the significance of the results from the hormone treatment experiments. This was conducted in Microsoft Excel, and confirmed using IBM SPSS Statistics software (IBM, Armonk, New York, USA). This was completed using the formula TTEST(data set 1, data set 2,

number of tails, variance). The number of tails used was 2 as it was unknown which treatment would be higher or lower, and the variance was type 2 as the variance is equal. It was possible to determine the significance of the up- or down-regulation in response to the hormone treatments with 95-99% probability.

2.2.10 Equipment and facilities

All experiments were carried out at CSIRO Agriculture, Waite Campus, Adelaide, South Australia.

Chapter 3 Identification and analysis of the ARF, Aux/IAA and TIR1/AFB families in *Vitis vinifera*

3.1 Aim

The aim of this work was to identify all ARF, Aux/IAA and TIR1/AFB family members from *V. vinifera* and use sequence analysis to investigate their possible roles.

3.2 Introduction

The *ARF, Aux/IAA* and *TIR1/AFB* gene families are the major gene families involved in auxin responsive gene regulation (reviewed in Salehin *et al.,* 2015). The specific interactions between these family members contribute to their roles in auxin signalling, and understanding their transcriptional profiles during grape development may help to infer interacting partners. Characterisation of the ARF, Aux/IAA and TIR1/AFB families is an ongoing area of research in a range of species, aided in recent years by whole genome-analysis with the numbers of *ARF* and *Aux/IAA* family members identified in a range of species listed in Table 3.1 and Table 3.2. The large variation in gene numbers between species is often due to differences in ploidy levels and/or the number of whole genome duplication events that have occurred within each species and the level of gene loss and neofunctionlization that occurred after these events (Panchy *et al.*, 2016). Finet *et al.* (2012) completed an in-depth analysis of the evolution of the *ARF* family in a number of species, identifying 19 *V. vinifera* genes. The *Aux/IAA* gene families tend to be slightly larger in size than the *ARF* families. Parry *et al.* (2009) characterised the *TIR1/AFB* receptor family in a range of plant species, identifying six family members in *V. vinifera*. Fewer studies have been completed on *TIR1/AFB* candidates, with only individuals or pairs being studied in depth, including *Zea mays* ZmAFB2 (Yang *et al.,* 2013), CsTIR1/AFB2 in cucumber (*Cucumis sativus*, Cui *et al.,* 2014), PtTIR1 in poplar (*Populus trichocarpa*, Shu *et al.*, 2015), and PslTIR1 in plum (*Prunus salicina* L., El-Sharkawy *et al.,* 2016).

Table 3.1 The number of auxin response factors (ARF) genes reported in selected plant species.

Species	Gene Number	Reference
Ananas comosus (pineapple)	20	Su *et al.,* 2017
Arabidopsis thaliana (Arabidopsis)	23*	Okushima *et al.*, 2005; Guilfoyle & Hagen, 2007
Brassica rapa	31	Mun *et al.*, 2012
Carica papaya	11	Liu *et al.*, 2015
Citrus sinensis (sweet orange)	19	Li *et al.*, 2015a

Species	Gene Number	Reference
Eucalyptus grandis	17	Yu *et al.*, 2014
Glycine max (soybean)	51	Ha *et al.*, 2013
Gossypium raimondii (cotton)	35	Sun *et al.*, 2015
Malus domestica (apple)	29–39	Devoghalaere *et al.*, 2012; Luo *et al.*, 2014; Hui-Feng *et al.*, 2015
Medicago truncatula	24	Shen *et al.*, 2015
Musa acuminata L. (banana)	47	Hu *et al.*, 2015
Oryza sativa (rice)	25	Wang *et al.*, 2007
Populus trichocarpa (poplar)	39	Kalluri *et al.*, 2007
Prunus mume Sieb. et Zucc (Japanese apricot)	17	Song *et al.*, 2015
Prunus persico L. Chunxue (peach)	18	Li *et al.,* 2016
Solanum lycopersicum (tomato)	22	Kumar *et al.*, 2011; Zouine *et al.*, 2014
Sorghum bicolor	24	Paterson *et al.*, 2009
Zea mays (maize)	31–36	Xing *et al.*, 2011; Wang *et al.*, 2012

* - One of the genes is a pseudogene

Table 3.2 The number of Aux/IAA genes reported in selected plant species.

Species	Gene Number	Reference
Arabidopsis thaliana (Arabidopsis)	29	Overvoorde *et al.,* 2005
Cicer arietinum (chickpea)	22	Singh & Jain, 2015
Cucumis sativus (cucumber)	27	Wu *et al.,* 2014
Eucalyptus grandis	24	Yu *et al.,* 2015
Glycine max (soybean)	63	Singh & Jain, 2015
Medicago truncatula	17	Shen *et al.,* 2014
Oryza sativa (rice)	31	Jain *et al.,* 2006a
Populus trichocarpa (poplar)	35	Kalluri *et al.,* 2007
Solanum lycopersicum (tomato)	26	Audran-Delalande *et al.,* 2012
Solanum tuberosum (potato)	26	Gao *et al.,* 2016
Triticum aestivum L. (wheat)	34	Qiao *et al.,* 2015
Zea mays (maize)	34	Wang *et al.,* 2010; Ludwig *et al.,* 2013

The sequencing of the *V. vinifera* Pinot Noir genome, released in 2007, and the availability of RNAseq data allows for gene mining and annotation corrections (Jaillon *et al.,* 2007). Prior to the commencement of this work, no *V. vinifera*-specific reports had been published characterising any of the three families, however, two papers characterising the *Aux/IAA* and *ARF* families in *V. vinifera* have since been published. Çakir *et al.* (2013) characterised the *Aux/IAA* family in *V. vinifera*, identifying 26

family members, and Wan *et al.* (2014) published a genome-wide identification of the *ARF* family in *V. vinifera*, identifying 19 family members. Within the current study, a range of bioinformatics tools were used to identify and characterise the *V. vinifera* gene candidates in the *TIR1/AFB, ARF* and *Aux/IAA* gene families (Figure 2.4). The Çakir *et al.* (2013) and Wan *et al.* (2014) publications have provided a resource for direct comparison to the family members identified within this work. In addition to *V. vinifera,* other plant species were included for comparison, including Arabidopsis, tomato and apple as fruit models, and poplar as a perennial tree model.

3.3 New gene nomenclature system for *V. vinifera*

Grimplet *et al.* (2014) recently discussed the *V. vinifera* nomenclature and introduced a new naming system when identifying and characterising grapevine genes. This has been used within the current study to ensure the naming system is at an international standard. Due to the confusion a two letter species identifier can cause, and as the bacterial species *Vibrio vulnificus* has been given '*Vv*' as its identifier*,* '*Vvi*' has been adopted as the species identifier for *V. vinifera*. When genes have been previously identified and the correct nomenclature used, these gene names must be used. Alternatively, incorrectly named genes need to be renamed according to the new nomenclature rules. This can be determined through the construction of a protein phylogenetic tree containing both Arabidopsis and *V. vinifera* proteins, with all branches having below 70% bootstrap support being collapsed (detailed in Chapter 2, Section 2.2.2.5.1). If there is a direct one-to-one relationship between an Arabidopsis gene and a *V. vinifera* gene, the *V. vinifera* gene is given the same name as the Arabidopsis gene. When there is a single Arabidopsis gene to two or more *V. vinifera* genes, they are given the same number and a single alphabetical letter to distinguish between them. Finally, if there are multiple Arabidopsis to one or more *V. vinifera* genes, a new number is given to the *V. vinifera* gene. This number needs to be higher than the existing numbers used in both Arabidopsis and *V. vinifera*.

3.4 Results

3.4.1 Identification of the auxin signalling pathway members

The auxin signalling pathway members were identified using a variety of methods as described in Section 2.2.2 (Figure 2.4). Nineteen family members were identified for the *ARFs*, 23 for the *Aux/IAAs* and six for the *TIR1/AFBs*. There were differences between these sequences and those identified in other papers, most notably between this work and Çakir *et al.* (2013). Of the 26 genes identified in Çakir *et al.* (2013), eight were *Aux/IAA* sequences and 18 were *ARF* sequences (Appendix D, Figure D.1). The *TIR1/AFB* sequences were the same as those identified in Parry *et al.* (2009) (Appendix D,

Figure D.2). Upon publication, the *ARF* sequences were compared to the Wan *et al.* (2014) sequences. The candidate numbers were consistent, however some sequence differences were observed (Section 3.4.2 below, comparisons in Appendix D, Figure D.3). The FASTA format of all *VviAFB, VviARF* and *VviIAA* sequences and the promoter sequences of the *VviARF* and *VviIAA* are in Appendix D, Figure D.6.

Table 3.3 The number of TIR1/AFB, ARF, Aux/IAA family members in Arabidopsis, grape, tomato, apple, and poplar.

Plant species	Gene number		
	TIR1/AFB	ARF	Aux/IAA
Arabidopsis thaliana (Arabidopsis)	6	23	29
Malus domestica (Apple)	8	39	41
Populus trichocarpa (Poplar)	8	39	34*
Solanum lycopersicum (Tomato)	4	22	25
V. vinifera (Grape)	6	19	23

* - this was listed as 35, however there was a duplication in Kalluri *et al.* (2007) with regards to naming.

3.4.2 Characterisation of the auxin signalling pathway members

The NCBI database was used to identify the locus tags, and the mRNA and protein accession numbers of *ARF, Aux/IAA* and *TIR1/AFB* candidates (Section 2.2.2). Phytozome was used to identify the transcript numbers; the sequence predictions from Phytozome and Genoscope appear to be consistently inaccurate so the transcript numbers are simply included to correspond to the genomic position of the genes. In some cases transcript numbers were not identified, indicating that the annotation of the *V. vinifera* gene models is currently incomplete within Phytozome and Genoscope. The open reading frame (ORF) and protein lengths in Table 3.4, Table 3.5 and Table 3.6 may differ from Phytozome and NCBI as full-length cloning and the use of Tablet® to compare the genome to RNAseq data predicted different ORF and protein sequences in some cases. The molecular weights of the proteins were predicted using the software described in Section 2.2.2.1.

For the *VviAFB* sequences, the multiple species sequence alignments, genomic and mRNA sequence alignments and RNAseq data in Tablet® determined that the NCBI sequences were correct and are used within this work (Table 3.4). The gene sizes are similar between the six genes and the translational alignment shows a high sequence conservation between the six sequences, with between 50.6–73.6% identity at the nucleotide level (Appendix D, Figure D.7). The protein sequences vary in size by 29 amino acid (aa), from VviAFB9 with 572 aa to VviAFB11 with 601 aa. *VviAFB8* has not been assigned a UniGene number, this may be due to a lack of Expressed Sequence Tag (EST) data for this transcript. Transcript numbers were not available for *VviAFB7* and *8*, and the Phytozome and Genoscope predicted sequences were truncated in comparison to the NCBI sequences, so the transcript numbers listed do not describe identical sequences. The original *VviAFB9* NCBI accessions are now obsolete as a result of the standard genome annotation processing update in NCBI (2017) (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/process/), however the original accession numbers have been included as they are linked with the UniGene information.

Table 3.4 VviTIR1/AFB gene and protein information.

							Deduced polypeptide			
Gene name	**Locus tag[1]**	**mRNA accession[1]**	**UniGene number**	**Transcript number[2]**	**ORF length (bp)**	**Chr.**	**Protein accession[1]**	**Length (aa)**	**MW (kDa)**	**pI**
AFB6	LOC100263734	XM_002271376.2	Vvi.6450	GSVIVT01003183001	1752	Un	XP_002271412.2	584	65.48	5.46
AFB7	LOC100264085	XM_002272814.2	Vvi.15859	-	1743	14	XP_002272850.1	581	65.33	7.76
AFB8	LOC100233127	XM_002269091.3	-	-	1728	7	XP_002269127.1	576	64.61	7.13
AFB9	LOC100263524	XM_010662393.1 XM_002274856.2*	Vvi.6777	GSVIVT01033011001	1716	14	XP_010660695.1 XP_002274892.1*	572	64.39	8.21
AFB10	LOC100252378	XM_002262820.2	Vvi.14646	GSVIVT01000962001	1761	1	XP_002262856.2	587	65.42	9.03
AFB11	LOC100245501	XM_002283891.2	Vvi.12588	GSVIVT01009126001	1803	18	XP_002283927.2	601	67.94	5.76

[1]Locus tags, mRNA and protein accessions correspond to the closest NCBI matches to these sequences
[2]Transcript numbers correspond to Phytozome and Genoscope genome browsers
Messenger ribonucleic acid (mRNA), Opening reading frame (ORF), Chromosome (Chr.), base pairs (bp), amino acid (aa), molecular weight (MW), kilo Daltons (kDa), isoelectric point (pI)
*Protein accession removed from NCBI due to standard genome annotation processing, 'obsolete version' sequences are still visible
Un – unknown chromosome location

Fifteen of the 19 *VviARF* candidates have differences between the sequences in NCBI and the predicted sequences based on multiple species sequence alignments, genomic and mRNA sequence alignments and RNAseq data in Tablet®. This information is shown within Table 3.5 with a comparison of the amino acid length predicted within this work and the NCBI amino acid predicted length shown in brackets underneath. The ORF lengths range from 1,617–3,486 bp between the 19 genes and the translational alignment shows a range of sequence conservation between the sequences, with between 22.1–69.2% identity at the nucleotide level (Appendix D, Figure D.8). These sequence differences were seen as insertions and deletions, potentially due to the mis-annotation of splice sites, cultivar differences, or truncations at the 5' or 3'-ends of the candidates, and alignments of these are shown in Appendix D, Figure D.5. All transcripts were initially identified in 2012 and NCBI accession numbers were identified at that time. The NCBI accession numbers were checked again in 2017 and in some instances the sequences associated with these numbers had been updated based on new genome annotation software. These updates had corrected the sequences to those predicted in this work, suggesting that the new corrected genome annotations in NCBI are more accurate. The proteins predicted in this work range from 538–1,161 aa in size. VviARF30 was spread across two transcript numbers, suggesting it had been misannotated as two genes instead of one complete transcript.

Table 3.5 VviARF gene and protein information.

							Deduced polypeptide			
Gene name	**Locus tag[1]**	**mRNA accession[1]**	**UniGene number**	**Transcript number[2]**	**ORF length (bp)**	**Chr.**	**Protein accession[1]**	**Length (aa)**	**MW (kDa)**	**pI**
ARF1a	LOC100264303	XM_002268312.2	Vvi.1078	GSVIVG01023149001	2,037	12	XP_002268348.2	678	75.39	5.75
ARF1b	LOC100263592	XM_002266911.2	Vvi.9855	-	1,932	18	XP_002266947.1	643 (645)	71.68	6.23
ARF2a	LOC100268072	XM_002284507.3	Vvi.28844	GSVIVG01008639001	2,589	17	XP_002284543.1	862	96.13	6.20
ARF2b	LOC100250592	XM_002268813.3	Vvi.17139	GSVIVG01004942001	2,304	1	XP_002268849.1	767 (769)	86.05	6.43
ARF3	LOC100245251	XM_010657162.1 XM_002273365.2*	Vvi.26711	GSVIVG01021128001	2,106	10	XP_010655464.1 XP_002273401.2*	701 (739)	76.36	6.46
ARF4	LOC100243320	XM_002284983.3	Vvi.20194	GSVIVT01025159001	2,397	6	XP_002285019.2	798	88.15	5.89
ARF5	LOC100254074	XM_003634334.1	Vvi.1321	GSVIVG01009865001	2,709	18	XP_003634382.1	902 (947)	99.45	5.14
ARF8	LOC100258129	XM_002266642.3	Vvi.12020	-	1,617	4	XP_002266678.2	538 (846)	60.18	7.00
ARF16	LOC100251645	XM_002281450.2	Vvi.2941	GSVIVG01025691001	1,902	8	XP_002281486.1	633 (701)	70.36	6.70
ARF17	LOC100255673	XM_002284292.3	Vvi.5833	GSVIVG01008950001	1,653	18	XP_002284328.2	550 (593)	60.78	6.00
ARF24	LOC100265555	XM_010663654.1 XM_002264036.2*	Vvi.5670	GSVIVG01027166001	2,106	15	XP_010661956.1 XP_002264072.2*	701 (701 new, 764 old)	77.75	6.52
ARF25	LOC100247833	XM_002265126.2*	Vvi.17636	GSVIVG01019566001	2,049	2	XP_002265162.2*	682 (693)	75.68	6.05
ARF26	LOC100246055	XM_002270250.2*	Vvi.32095	GSVIVG01011008001	2,091	7	XP_002270286.2*	696 (806)	77.7	5.48
ARF27	LOC100257618	XM_002276601.2*	Vvi.31726	GSVIVG01015035001	3,486	11	XP_002276637.1*	1,161 (1084)	130.30	6.25
ARF28	LOC100263801	XM_002266567.2*	Vvi.4620	GSVIVG01032251001	3,105	11	XP_002266603.2*	1,034 (1117)	114	6.02
ARF29	LOC100260866	XM_002282794.2*	Vvi.31613	GSVIVG01020805001	2,907	12	XP_002282830.2*	968 (891)	107.19	6.67
ARF30	LOC100242923	XM_002279772.2	Vvi.12192	GSVIVG01021552001/ GSVIVG01021553001	2,478	10	XP_002279808.1	825 (908)	91.47	6.11

							Deduced polypeptide			
Gene name	**Locus tag[1]**	**mRNA accession[1]**	**UniGene number**	**Transcript number[2]**	**ORF length (bp)**	**Chr.**	**Protein accession[1]**	**Length (aa)**	**MW (kDa)**	**pI**
ARF31	LOC100256989	XM_002282401.2	Vvi.20162	GSVIVG01025198001	1,866	6	XP_002282437.1	621 (711)	68.36	7.93
ARF32	LOC100265118	XM_002273554.2	Vvi.18826	GSVIVG01016266001	2,052	13	XP_002273590.1	683	75.27	6.43

[1]Locus tags, mRNA and protein accessions correspond to the closest NCBI matches to these sequences
[2]Transcript numbers correspond to Phytozome and Genoscope genome browsers
[3]The length of the NCBI sequences are listed in brackets
Messenger ribonucleic acid (mRNA), Opening reading frame (ORF), Chromosome (Chr.), base pairs (bp), amino acid (aa), molecular weight (MW), kilo Daltons (kDa), isoelectric point (pI)
*Accession removed from NCBI due to standard genome annotation processing, 'obsolete version' sequences are still visible

Of the 23 *VviIAA* sequences, 14 of the original NCBI sequences differed from the final sequences that were predicted within this work (shown as below with amino acid differences in Table 3.6). The differences are shown in Appendix D, Figure D.4. The *VviIAA* candidates are much smaller than the *VviARF* and *VviAFB* candidates, with the gene sizes ranging from 507–1,164 bp between the 23 genes and the translational alignment shows highly variable sequence conservation between the sequences, with 18.8–80.3% identity at the nucleotide level (Appendix D, Figure D.9). In the original BLAST searches VviIAA34b matched to a predicted probable LRR receptor-like serine/threonine-protein kinase. The BLAST match was 889 aa long and the predicted 227 aa VviIAA34b sits at the 5'-end of the BLAST match. The most likely explanation for this is that the *VviIAA34b* coding sequence resides next to a probable LRR receptor-like serine/threonine-protein kinase on the chromosome and they were mis-annotated together as a single gene. This supports the need for regular updates of NCBI which has led to this accession being deemed obsolete. New accessions show XP_010648817.1 as a 812 aa predicted probable LRR receptor-like serine/threonine-protein kinase and XP_010648672.1 as the 227 aa VviIAA34b (predicted: auxin-responsive protein IAA28).

Table 3.6 VviAux/IAA gene and protein information.

							Deduced polypeptide			
Gene name	**Locus tag[1]**	**mRNA accession[1]**	**UniGene number**	**Transcript number[2]**	**ORF length (bp)**	**Chr.**	**Protein accession[1]**	**Length (aa)**	**MW (kDa)**	**pI**
IAA9	LOC100232909	NM_001281241.1	Vvi.137	GSVIVT01009238001	1,080	18	NP_001268170.1	359	38.85	8.35
IAA11	LOC100244630	XM_002269886.2	Vvi.29605	GSVIVT01028432001	699	7	XP_002269922.2	232 (296)	25.14	7.82
IAA13	LOC100256286	XM_002285447.3	Vvi.13701	GSVIVT01027921001	1,113	5	XP_002285483.2	370 (321)	39.17	7.73
IAA15a	LOC100247336	XM_002284825.3	Vvi.9611	GSVIVT01015449001	780	11	XP_002284861.1	259 (224)	28.6	8.68
IAA15b	LOC100258296	XM_002280488.3	Vvi.24588	GSVIVT01017159001	885	9	XP_002280524.2	294 (210)	32.45	8.23
IAA19	LOC100854934	NM_001281157.1	Vvi.8142	GSVIVT01017158001	579	9	NP_001268086.1	192	21.75	6.34
IAA26	LOC100266914	XM_002283552.3	Vvi.5203	GSVIVT01016972001	1,164	9	XP_002283588.2	387 (364)	43.05	8.55
IAA27	LOC100254204	XM_002284082.2	Vvi.1665	GSVIVT01015350001	912	11	XP_002284118.1	303 (320)	32.39	7.60
IAA31	LOC100244346	XM_002275479.2	Vvi.32312	GSVIVT01017711001	597	5	XP_002275515.1	198	21.39	8.79
IAA33	LOC100244496	XM_002268780.1	Vvi.29766		507	11	XP_002268816.1	168	18.43	9.35
IAA34a	LOC100266398	XM_002285318.2	Vvi.3810	GSVIVT01015573001	555	11	XP_002285354.1	184 (224))	20.84	9.28
IAA34b[4]	LOC100249164	XM_010650370.1 XM_002282675.2*3	Vvi.29544	GSVIVT01035866001	684	4	XP_010648672.1 XP_002282711.2*3	227 (8893)	25.36	8.39
IAA35	LOC100250231	NM_001281107.1	Vvi.466	GSVIVT01036283001	513	14	NP_001268036.1	170 (238)	18.91	8.88

							Deduced polypeptide			
Gene name	**Locus tag[1]**	**mRNA accession[1]**	**UniGene number**	**Transcript number[2]**	**ORF length (bp)**	**Chr.**	**Protein accession[1]**	**Length (aa)**	**MW (kDa)**	**pI**
IAA36	LOC100254530	XM_002284097.3	Vvi.1351	GSVIVT01018101001	714	5	XP_002284133.1	237 (243)	26.1	7.46
IAA37	LOC100259693	XM_002281735.3	Vvi.963	GSVIVT01000721001	789	7	XP_002281771.1	262 (244)	29.05	6.77
IAA38	LOC100262403	XM_002279919.3	Vvi.6424	GSVIVT01021779001	600	14	XP_002279955.1	199	22.42	8.60
IAA39	LOC100264878	XM_002281660.2	Vvi.13054	GSVIVT01000720001	561	7	XP_002281696.1	186	21.01	5.47
IAA40	LOC100259648	XM_002284085.2	Vvi.9164	GSVIVT01018099001	579	5	XP_002284121.1	192	21.23	6.42
IAA41	LOC100253148	XM_002284246.2	Vvi.15129	GSVIVT01017046001	1,032	9	XP_002284282.1	343	36.66	6.96
IAA42	LOC100254204	XM_002284082.2	Vvi.1665	GSVIVT01022048001	582	14	XP_002284118.1	193 (320)	21.58	9.57
IAA43	LOC100247345	XM_002277762.3	Vvi.26515	GSVIVT01035295001	618	4	XP_002277798.1	205 (345)	22.13	5.82
IAA44	LOC100257765	XM_010666576.1 XM_002279834.2*	Vvi.32681	GSVIVT01011841001	594	1	XP_010664878.1 XP_002279870.2*	197 (221)	22.46	4.98
IAA45	LOC100241721	XM_002281109.2	Vvi.15904	GSVIVT01028242001	528	7	XP_002281145.1	175	19.68	6.43

[1]Locus tags, mRNA and protein accessions correspond to the closest NCBI matches to these sequences
[2]Transcript numbers correspond to Phytozome and Genoscope genome browsers
[3]The length of the NCBI sequences are listed in brackets
[4]VviIAA34b – Originally the first half matched to a predicted probable LRR receptor-like serine/threonine-protein kinase
Messenger ribonucleic acid (mRNA), Opening reading frame (ORF), Chromosome (Chr.), base pairs (bp), amino acid (aa), molecular weight (MW), kilo Daltons (kDa), isoelectric point (pI)
*Accession removed from NCBI due to standard genome annotation processing, 'obsolete version' sequences are still visible

3.4.3 Chromosomal locations of *ARF, Aux/IAA* and *TIR1/AFB* family members

The genes are present on all chromosomes except for 3, 16 and 19. Chromosome 7 has the highest number of genes, with four *Aux/IAAs*, one *AFB* and one *ARF*, clustered towards the top half of the chromosome. *AFB8* is present on chromosome 7 while *AFB7* and *AFB9* are both on chromosome 14. *AFB10* is on chromosome 1 and *AFB11* is on chromosome 18. *AFB6* has not been assigned to a chromosome (chromosome unknown).

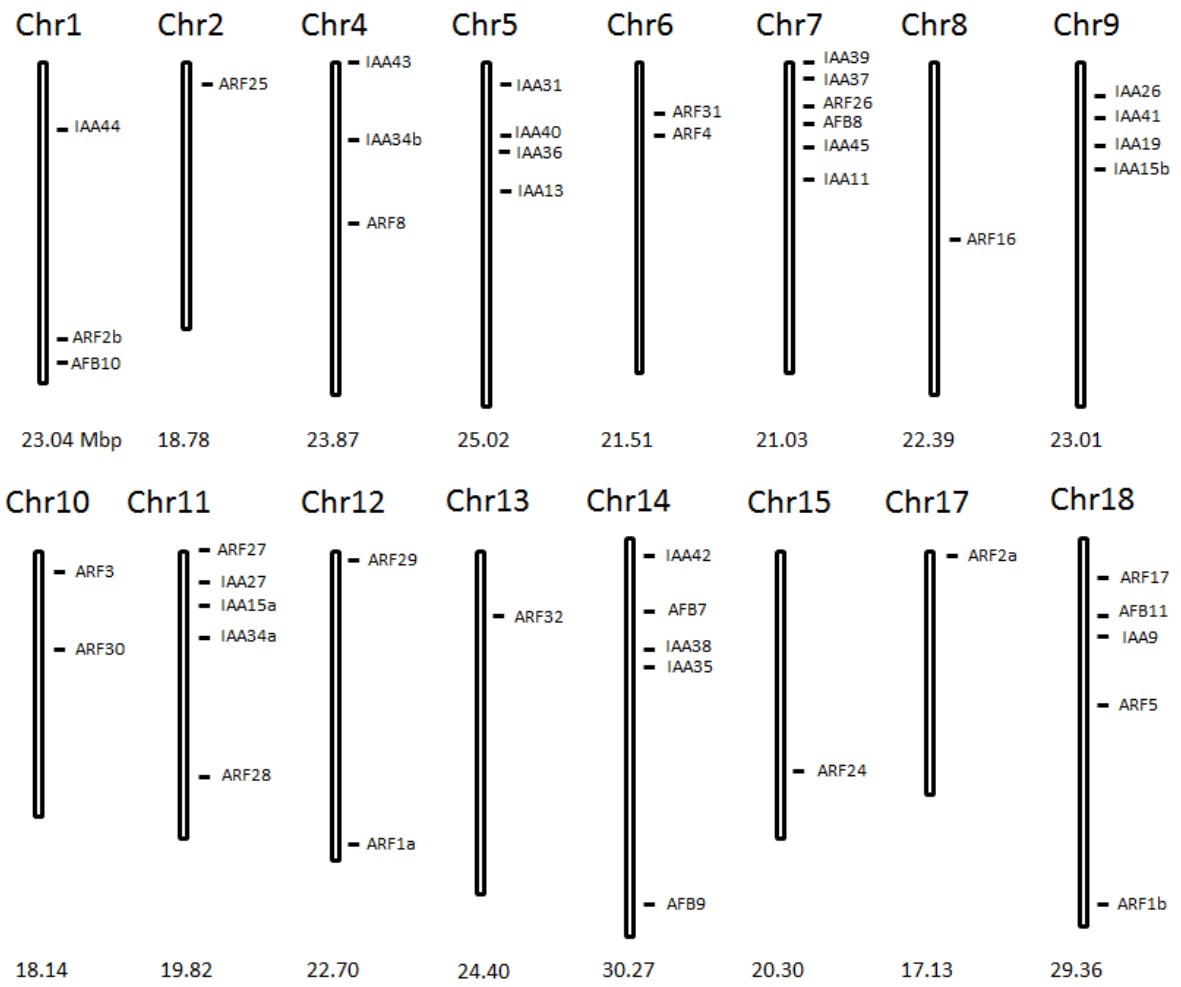


Figure 3.1 The location of all TIR1/AFB, ARF, and Aux/IAA family members on the *V. vinifera* chromosomes. AFB6 = ChrUN (chromosome unknown). IAA33 = chromosome 11, exact location unknown. Chromosome sizes from Genoscope (12x).

3.4.4 Renaming of the ARF, Aux/IAA and TIR1 gene families

The naming of all *Vitis ARF, Aux/IAA* and *TIR1/AFB* was initially completed based on gene names present in Finet *et al.* (2012), Çakir *et al.* (2013) and Parry *et al.* (2009) respectively. However, after the publication of Grimplet *et al.* (2014) they were renamed in line with the new guidelines. Wan *et al.* (2014) candidate names were based on chromosomal positioning and therefore were also not suitable. Protein trees were constructed based on the parameters described in Grimplet *et al.* (2014) described in Section 3.3.

Figure 3.2 shows that there are no one-to-one or two-to-one *Vitis* to Arabidopsis homologues present with the TIR1/AFB sequences. *AFB* is the accepted name for these genes in Arabidopsis and is used here in place of *TIR1*, which describes a mutant phenotype (Ruegger *et al.,* 1998). The VviAFB sequences are given their names, firstly from VviAFB6, then from top to bottom of the phylogenetic tree. The names then continued sequentially from the highest numbered Arabidopsis gene, AtAFB5.

Figure 3.3 shows that VviARF1a and VviARF1b are equally similar to AtARF1, and VviARF2a and VviARF2b to AtARF2 and for this reason they are distinguished by the addition of an alphabetical letter. VviARF3, 4, 5, and 17 have a one to one relationship with an Arabidopsis homologue and are given the same name. The remaining VviARF sequences are given their names from top to bottom of the phylogenetic tree, from ARF24 onwards as the 'highest' known Arabidopsis homologue is named AtARF23.

Figure 3.4 shows that VviIAA15a and VviIAA15b are equally similar to AtIAA15 and VviIAA34a and VviIAA34b to AtIAA34. These are distinguished from each other by the addition of an alphabetical letter. VviIAA9, 31, and 33 have a one to one relationship with an Arabidopsis homologue and are given the same name as the Arabidopsis genes. The remaining VviIAA sequences are given their names from top to bottom of the phylogenetic tree, from IAA35 onwards as the 'highest' known Arabidopsis homologue is named AtIAA34. All Aux/IAA candidates are named VviIAA for simplicity and consistency with more recent publications and the nomenclature system of Grimplet *et al.* (2014).

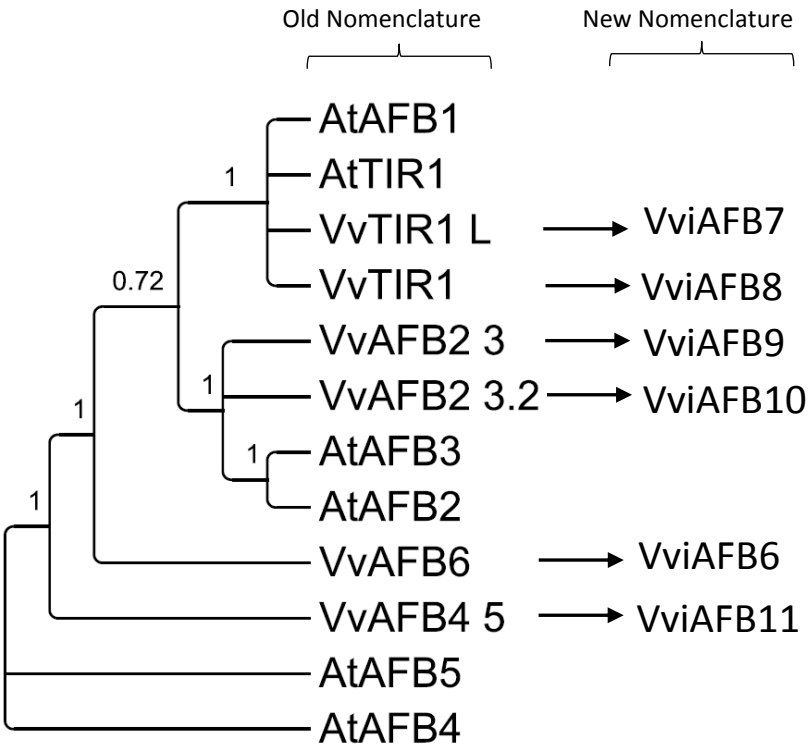


Figure 3.2 The consensus phylogenetic tree of Arabidopsis and grape TIR1/AFB proteins sequences with the original and new nomenclature of the TIR1/AFB *Vitis* proteins based on Grimplet *et al.* (2014).

The maximum likelihood method was used with 100 bootstraps to determine the evolutionary history of the Arabidopsis and grape TIR1/AFB proteins, and a consensus tree was generated. Nodes are numbered with values between 0–1 representing the support of each node occurring, any nodes with less than 70% support (<0.70) were collapsed. The *Vitis* proteins have been renamed based on the guidelines in Grimplet *et al.* (2014).

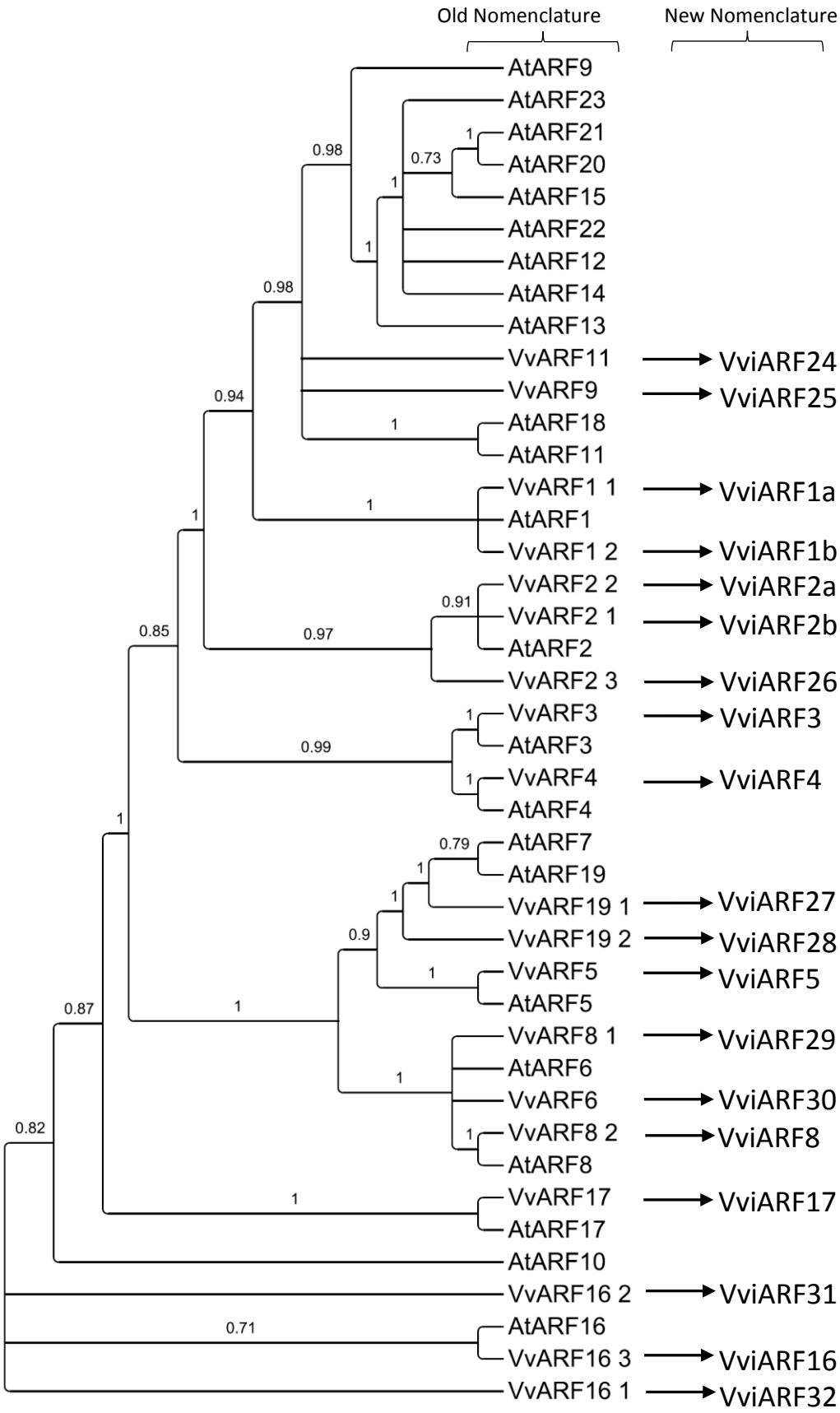


Figure 3.3 The consensus phylogenetic tree of Arabidopsis and grape ARF proteins sequences with the original and new nomenclature of the ARF *Vitis* proteins based on Grimplet *et al.* (2014).

See Figure 3.2 legend for the details of tree construction.

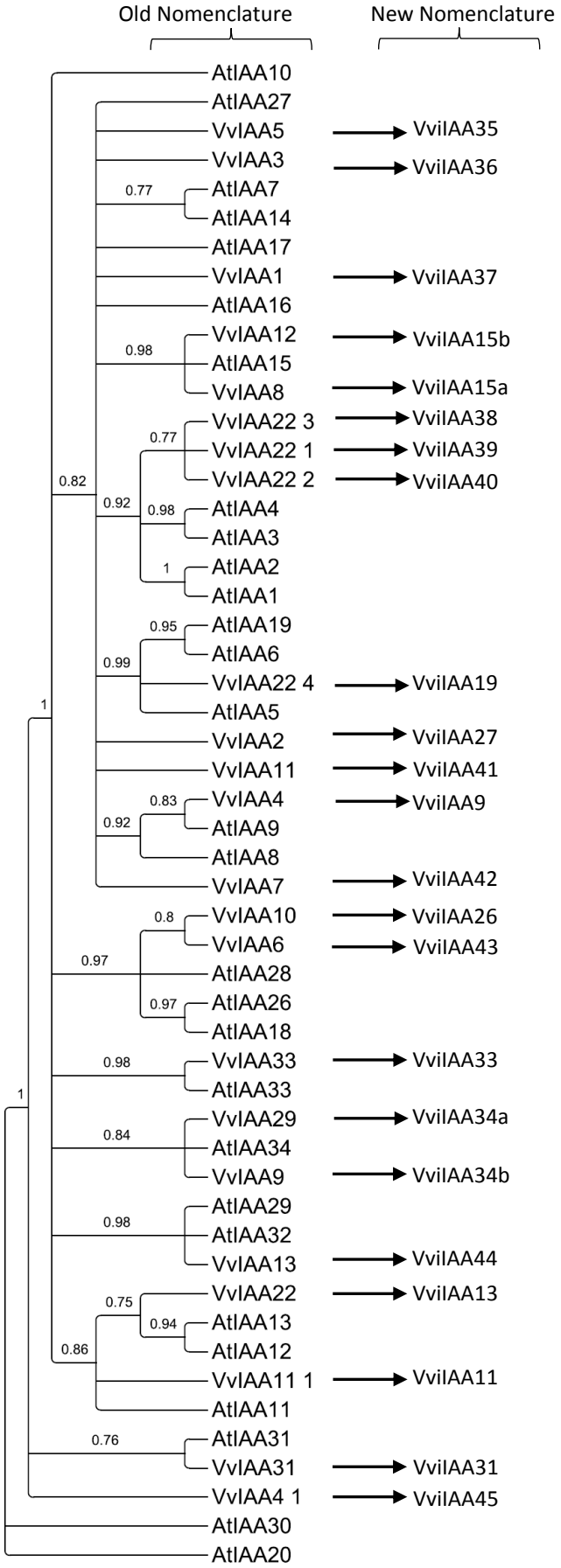

Old Nomenclature
New Nomenclature
AtIAA10
AtIAA27
VvIAA5
VviIAA35
VvIAA3
VviIAA36
0.77
AtIAA7
AtIAA14
AtIAA17
VvIAA1
VviIAA37
AtIAA16
VvIAA12
VviIAA15b
0.98
AtIAA15
VvIAA8
VviIAA15a
VvIAA22 3
VviIAA38
0.77
VvIAA22 1
VviIAA39
0.82
VvIAA22 2
VviIAA40
0.92
0.98
AtIAA4
AtIAA3
1
AtIAA2
AtIAA1
0.95
AtIAA19
AtIAA6
0.99
VvIAA22 4
VviIAA19
1
AtIAA5
VvIAA2
VviIAA27
VvIAA11
VviIAA41
0.83
VvIAA4
VviIAA9
0.92
AtIAA9
AtIAA8
VvIAA7
VviIAA42
0.8
VvIAA10
VviIAA26
VvIAA6
VviIAA43
0.97
AtIAA28
0.97
AtIAA26
AtIAA18
0.98
VvIAA33
VviIAA33
AtIAA33
1
VvIAA29
VviIAA34a
0.84
AtIAA34
VvIAA9
VviIAA34b
AtIAA29
0.98
AtIAA32
VvIAA13
VviIAA44
0.75
VvIAA22
VviIAA13
0.94
AtIAA13
0.86
AtIAA12
VvIAA11 1
VviIAA11
AtIAA11
0.76
AtIAA31
VvIAA31
VviIAA31
VvIAA4 1
VviIAA45
AtIAA30
AtIAA20

Figure 3.4 The consensus phylogenetic tree of Arabidopsis and grape Aux/IAA proteins sequences with the original and new nomenclature of the Aux/IAA *Vitis* proteins based on Grimplet *et al.* (2014).

See Figure 3.2 legend for the details of tree construction.

3.4.5 The ARF, Aux/IAA and TIR1/AFB protein domains

InterProScan was used in Geneious to confirm the presence and absence of known protein domains in the three auxin signalling families (Section 2.2.2.1).

3.4.5.1 The two *Vitis* AFB protein domains

Two types of domains were identified within the AFB protein family members; the F-box domain and the leucine rich repeat (LRR) domain (Figure 3.5). The F-box domain was present in all proteins. In VviAFB6 and 10 the F-box domains were not detected using InterProScan, however, upon examination of the sequences the F-box domains were present with some residue differences to the other four sequences. The LRR domain is present in all six AFB family members and each AFB protein contains between five and seven of these repeat element annotations. In the sequence alignments, VviAFB10 had a 12 aa insertion not within a domain and VviAFB11 had a 4 aa insertion within the F-box domain (Figure 3.5).

3.4.5.2 The three *Vitis* ARF protein domains

Three types of domains were present within the ARF protein family members; the B3 DNA binding domain (DBD), the auxin response factor (auxin_resp) domain, and the protein-protein interaction Phox and Bem1 (PB1) domain (Figure 3.6). At the N-terminus, all 19 proteins contained a B3 DNA-binding pseudobarrel domain and the Auxin_resp domain (Figure 3.6). At the C-terminus is the PB1 domain, which is similar to Domains III and IV in Aux/IAA proteins, and it facilitates the interaction with Aux/IAA proteins and hetero- or homodimerisation with ARF proteins. These domains were not present in VviARF3, 8, and 17. VviARF5, 27–30 all contained glutamine-rich (Q-rich) middle regions, characteristic of ARF activator proteins, suggesting all remaining ARF candidates are ARF repressors (Figure 3.6).

3.4.5.3 The four *Vitis* Aux/IAA protein domains

Two types of domains were identified in the Aux/IAA proteins; the AUX_IAA domain, and the PB1 domain (Figure 3.7). The AUX_IAA domain spans Domains I–IV; I in black, II in blue, III in green, and IV in red in Figure 3.7. The PB1 domain, detailed above, is shown here as Domains III and IV. Although Domains III and IV are present within the AUX_IAA domain annotation, the PB1 domain results are also included to ensure consistency with the PB1 domain annotation for both ARF and Aux/IAA candidates. Eleven Aux/IAA proteins have complete Domain I regions and a further three have Domain I with non-conserved amino acid residues. All 23 have variations of the complete Domain II (19 complete), 22 have complete Domain III, and 22 have variations of the complete Domain IV, with VviIAA33 having no Domain I, and Domains II, III and IV with some non-conserved amino acid sites.

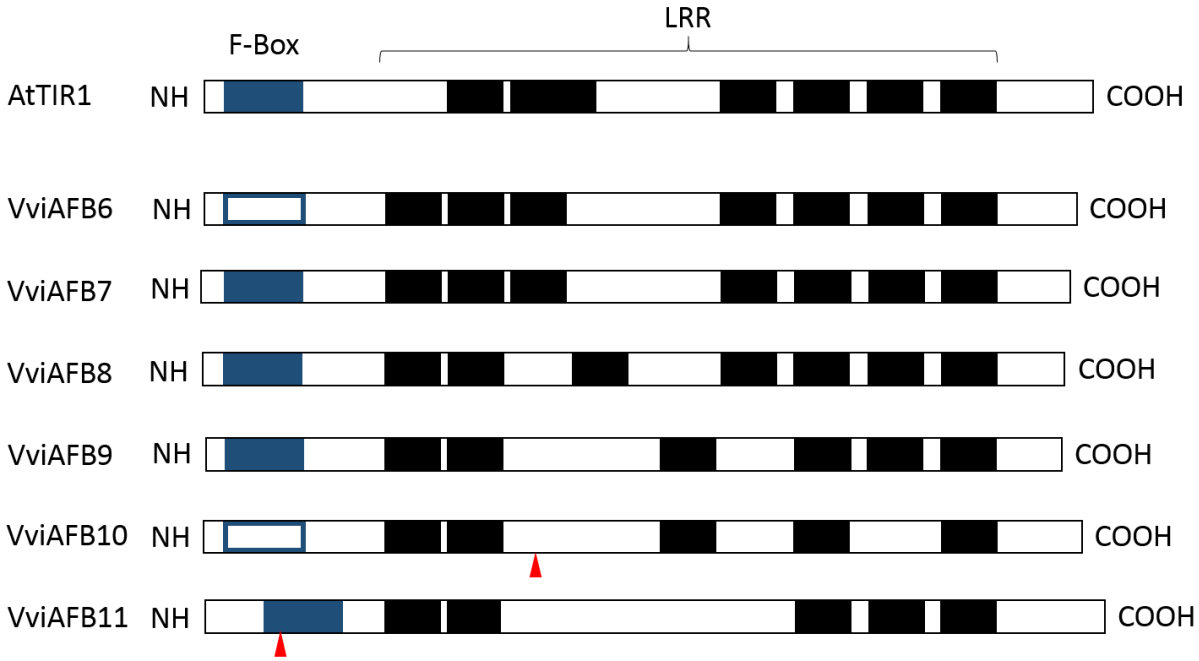


Figure 3.5 A schematic of the protein domains present in AFB proteins as determined by InterProScan in Geneious.

The blue F-box domain was identified by SMART and PFAM with the InterPro domain ID IPR001810, the empty blue boxes represent F-boxes that contain residues not recognised by InterProScan. The black leucine rich repeats (LRR) domains were identified by SMART with the InterPro domain ID IPR006553. The proteins and domains are all shown to scale. The red arrows represent insertions, 12 aa in VviAFB10 and 4 aa in VviAFB11. Arabidopsis TIR1 (AtTIR1) is used as a representation of the standard domains present within the TIR1/AFB protein sequences.

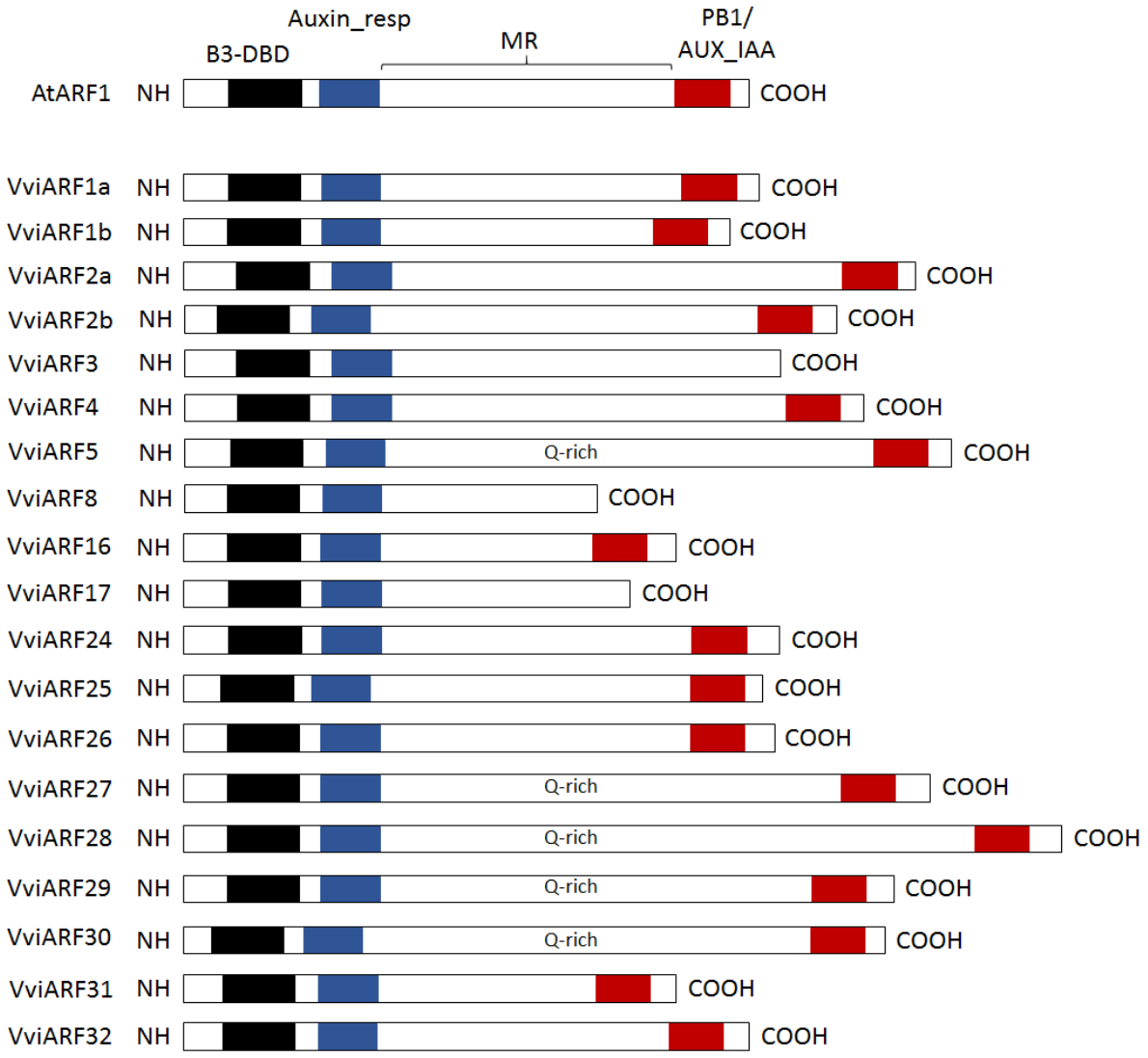


Figure 3.6 A schematic of the protein domains present in ARF proteins as determined by InterProScan in Geneious.

The black B3-DNA binding domain (DBD) is identified by Prosite and PFAM with the InterPro domain ID IPR003340. The blue Auxin_resp domain is identified by PFAM with the InterPro domain ID IPR010525. MR – middle region, if this region is glutamine rich (Q-rich) the protein is an ARF activator. The red protein-protein interaction Phox and Bem1 (PB1) domain/AUX/IAA domain binds with domains III and IV of the Aux/IAA proteins and was identified by Prosite and PFAM with the InterPro domain ID IPR000270. The proteins and domains are all shown to scale. Arabidopsis ARF1 (AtARF1) is used as a representation of the standard domains present within the ARF protein sequences.

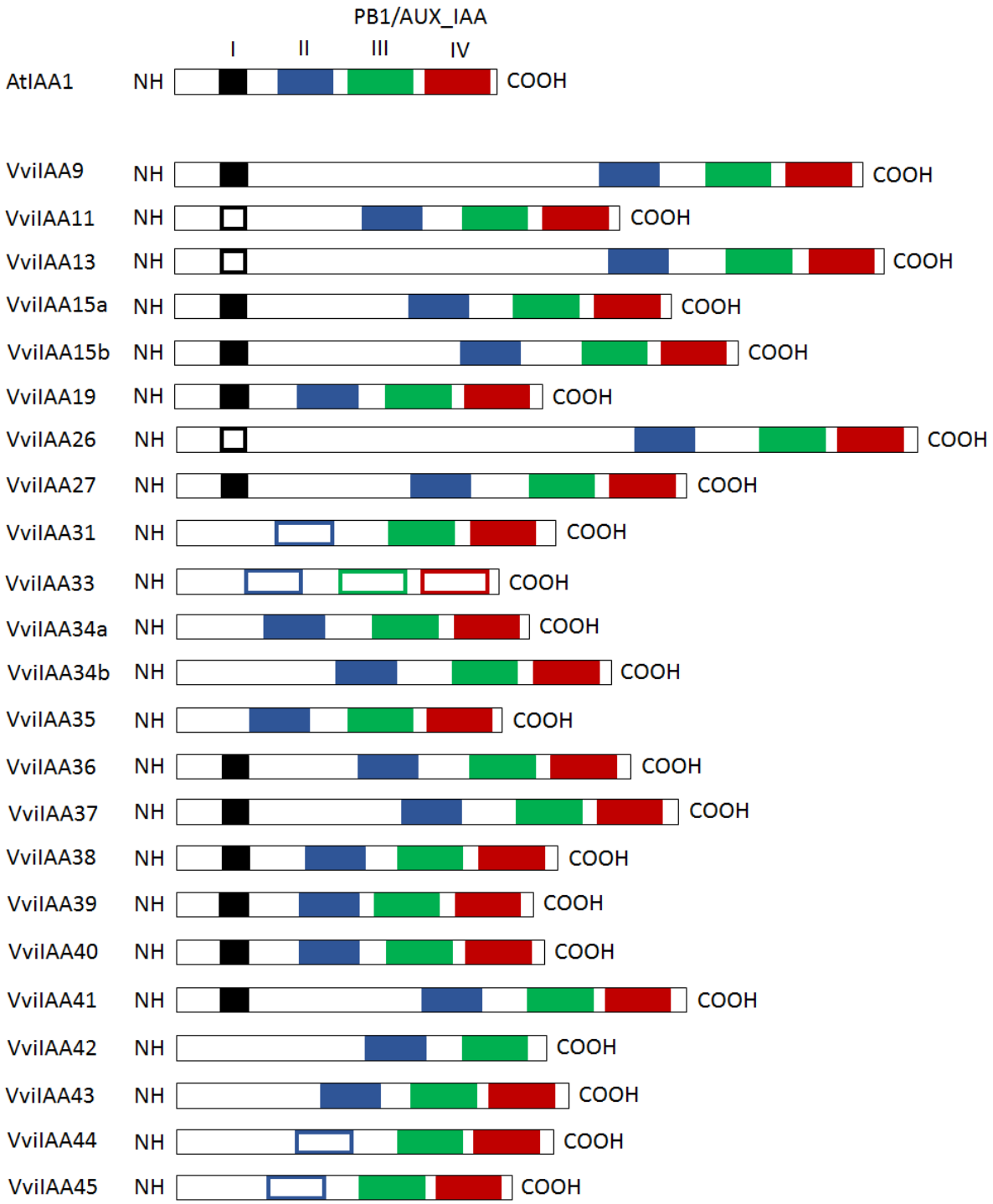


Figure 3.7 A schematic of the protein domains present in Aux/IAA proteins as determined by InterProScan in Geneious.

The AUX_IAA domain is identified by PFAM with the InterPro domain ID IPR003311 and spans domains I, II, III, and IV. Domain I in black, domain II in dark blue, domain III in green and domain IV in red. The protein- protein interacting Phox and Bem1 (PB1) domain is identified by Prosite with the InterPro domain ID IPR000270, this is represented with domains III and IV. The empty coloured boxes represent protein domains that contain most but not all amino acid residues, representing truncated or altered motif sequences. The proteins and domains are all shown to scale. Arabidopsis IAA1 (AtIAA1) is used as a representation of the standard domains present within the IAA protein sequences.

3.4.6 Phylogenetic relationships between the auxin signalling pathway genes of *V. vinifera* and other plant species

Phylogenetic trees were generated using the method described in Section 2.2.2.5, for each of the three auxin signalling pathway gene families. The coding sequences were aligned and only conserved regions were retained for tree building. BEAST generated rooted trees and node values that represent the posterior probability of each node occurring, between 0–1, with any node value above 0.7 considered to be highly supported.

3.4.6.1 The *TIR1/AFB* family contains four distinct clades

There are six *TIR1/AFB* genes in *V. vinifera*, *VviAFB6* to *VviAFB11*. Clade 2 contains *VviAFB7* and *VviAFB8*, the two Arabidopsis genes, *AtTIR1* and *AtAFB1*, as well as two family members from tomato, apple and poplar. Similarly, Clade 3 contains two Arabidopsis genes, *AtAFB2* and *AtAFB3*, two *V. vinifera* genes, *VviAFB9* and *VvAFB10*, and two family members from apple and poplar. There does not appear to be a tomato candidate in this clade. Clade 1 contains the *AFB6* genes present in *V. vinifera*, tomato, apple and poplar, with both poplar and apple having two genes within the clade and tomato and *V. vinifera* only containing a single gene. Arabidopsis does not contain a gene that fits within this clade. The most distant clade is Clade 4, which contains two Arabidopsis genes, *AtAFB4* and *AtAFB5*, apple and poplar genes, and single *V. vinifera* (*VviAFB11*) and tomato genes. The posterior probability node support is high, above 0.78 for all but two nodes, which are 0.63 and 0.62. These are within the *AtAFB4-AFB5* and the *AtTIR1-AFB1* clades.

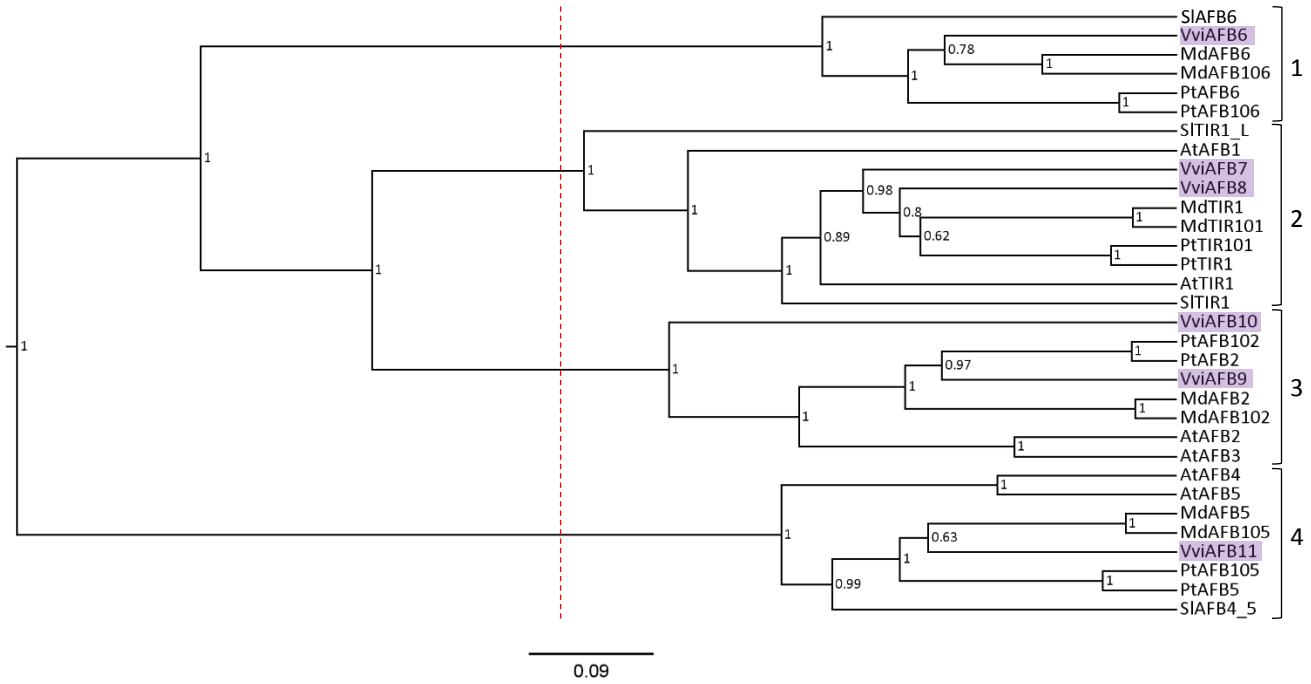


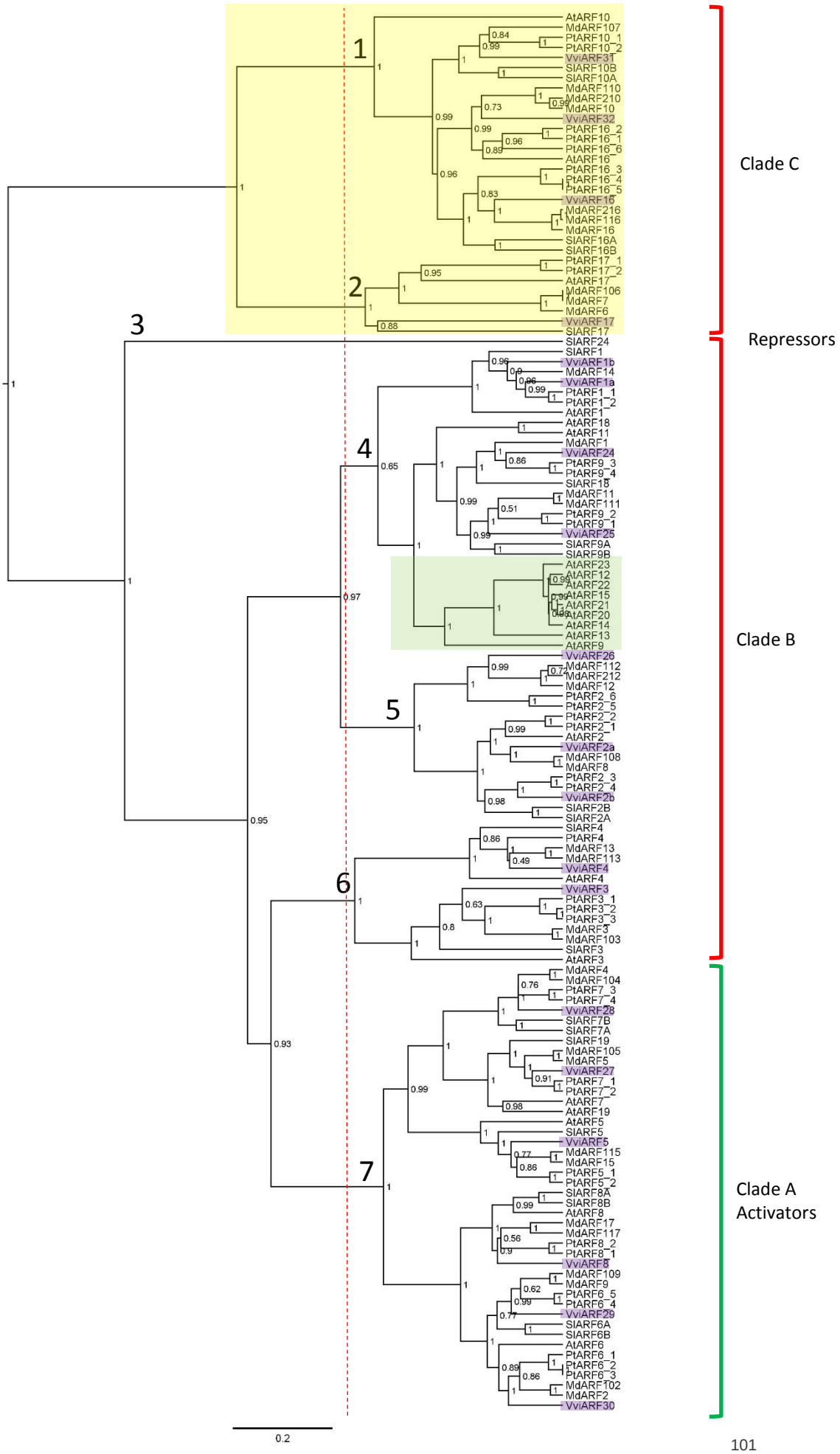
Figure 3.8 A consensus phylogenetic tree of the *TIR1/AFB* family members from *V. vinifera* compared to those from Arabidopsis, apple, tomato and poplar generated using the conserved regions of the coding sequence in BEAST.

A translational alignment of coding sequences was generated in Geneious and conserved alignments produced using BMGE with "codons" and the default settings. Baysian Inference through BEAST was used for tree building with the GTR substitution model, estimated base frequencies, gamma site heterogeneities, partitioning into three separate codon positions with unlinked substitution models, and a Yule tree prior. Trees were generated with a strict clock prior and building was run until completion. The node values represent the posterior probabilities of the nodes occurring with 1 being the highest probability. The scale bar represents the number of base pair substitutions per site. Accession numbers and/or references for the sequences can be found in Appendix D, Table D.1 and Table 2.1. Purple boxes highlight the *V. vinifera* sequences. Four clades are marked. The red dashed line represents the cut-off point for each clade to be designated as a clade.

3.4.6.2 Activators and repressors in the *ARF* family are distinguishable by nucleotide sequence

Two main branches were identified from the root of the *ARF* tree (Figure 3.9). The posterior probability values show a high level of support for each node, with the majority of nodes having over 0.8 support. There are more *ARF* family members in *Malus* and poplar compared to Arabidopsis, *V. vinifera* and tomato, with there being on average two *Malus* and poplar genes for every one Arabidopsis, *V. vinifera* and tomato gene, sometimes increasing to three *Malus* and poplar genes for a single *V. vinifera* gene. This is most apparent in the *ARF* activator clade with six *V. vinifera*, five Arabidopsis and eight tomato activators to 12 *Malus* and 13 poplar genes. Some clades lack any Arabidopsis homologues (Figure 3.9).

Based on Finet *et al.* (2012) the *ARF* phylogeny is divided into three clades: Clades A, B, and C, and within the current work further divisions were made with each clade containing a number of classes, as determined by a set distance indicated by the red dashed line in Figure 3.9. The exception is Clade A which only contains Class 7 sequences. Clade A contains *ARF* activators, whilst Clades B and C contain *ARF* repressors. Clade C, highlighted in yellow, contains two repressor classes: Classes 1 and 2. Class 1 contains *VviARF16, 31,* and *32, VviARF16* shows similarity to *ARF16* sequences from poplar, apple, and tomato. *VviARF31* showed closest similarity to *ARF10* candidates from Arabidopsis, poplar and tomato, whilst *VviARF32* was closer to *MdARF10* candidates. Class 2 contains *VviARF17* and *ARF17* candidates from poplar, Arabidopsis, and tomato and *MdARF6, 7,* and *106* (Figure 3.9). The lower branch of the tree (Clades B and C) contains five classes; four repressor classes and a single activator class (Figure 3.9). *SlARF24* is a clear outlier, forming Class 3. Class 4 is Arabidopsis-specific and contains nine of the 23 Arabidopsis *ARFs*, highlighted in a green box. Additionally, Class 4 contains sub-classes containing *VviARF1a* and *1b, 24* and *25*. *VviARF1a* and *1b* share similarities with *ARF1* candidates from tomato, poplar, and Arabidopsis as well as *MdARF14*. *VviARF24* shows similarity to poplar *PtARF9_3* and *9_4,* and *MdARF1*, whilst *VviARF25* shows similarity to *PtARF9_1* and *9_2* and *MdARF11* and *111*. Class 5 can be separated into two sub-classes: *VviARF26* shares similarities with three apple sequences and two poplar sequences; *VviARF2a* classes with *AtARF2, PtARF2_1* and *2_2*, and apple *MdARF8* and *108*; and *VviARF2b* groups with *PtARF2_3* and *2_4* and tomato *SlARF2A* and *2B*. Class 6 contains two sub-classes, *VviARF3* shows similarity to *ARF3* candidates in all five species, whilst *VviARF4* shows similarity to *ARF4* in Arabidopsis, poplar, tomato and *MdARF13* and *113* in apple. Class 7 contains all *ARF* activators, including the Q-rich *VviARF5, 27, 28, 29* and *30*, and *VviARF8* despite the absence of a Q-rich middle region. *VviARF5* is in a sub-class with *ARF5* candidates in all five species. *VviARF8* groups with *ARF8* candidates from Arabidopsis, tomato, poplar and *MdARF17* and *117* in apple (Figure 3.9).


1
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3
4
5
6
7
AtARF10
MdARF107
PtARF10_1
PtARF10_2
VviARF31
SlARF10B
SlARF10A
MdARF110
MdARF210
MdARF10
VviARF32
PtARF16_2
PtARF16_1
PtARF16_6
AtARF16
PtARF16_3
PtARF16_4
PtARF16_5
VviARF16
MdARF216
MdARF116
MdARF16
SlARF16A
SlARF16B
PtARF17_1
PtARF17_2
AtARF17
MdARF106
MdARF7
MdARF6
VviARF17
SlARF17
SlARF24
SlARF1
VviARF1b
MdARF14
VviARF1a
PtARF1_1
PtARF1_2
AtARF1
AtARF18
AtARF11
MdARF1
VviARF24
PtARF9_3
PtARF9_4
SlARF18
MdARF11
MdARF111
PtARF9_2
PtARF9_1
VviARF25
SlARF9A
SlARF9B
AtARF23
AtARF12
AtARF22
AtARF15
AtARF21
AtARF20
AtARF14
AtARF13
AtARF9
VviARF26
MdARF112
MdARF212
MdARF12
PtARF2_6
PtARF2_5
PtARF2_2
PtARF2_1
AtARF2
VviARF2a
MdARF108
MdARF8
PtARF2_3
PtARF2_4
VviARF2b
SlARF2B
SlARF2A
SlARF4
PtARF4
MdARF13
MdARF113
VviARF4
AtARF4
VviARF3
PtARF3_1
PtARF3_2
PtARF3_3
MdARF3
MdARF103
SlARF3
AtARF3
MdARF4
MdARF104
PtARF7_3
PtARF7_4
VviARF28
SlARF7B
SlARF7A
SlARF19
MdARF105
MdARF5
VviARF27
PtARF7_1
PtARF7_2
AtARF7
AtARF19
AtARF5
SlARF5
VviARF5
MdARF115
MdARF15
PtARF5_1
PtARF5_2
SlARF8A
SlARF8B
AtARF8
MdARF17
MdARF117
PtARF8_2
PtARF8_1
VviARF8
MdARF109
MdARF9
PtARF6_5
PtARF6_4
VviARF29
SlARF6A
SlARF6B
AtARF6
PtARF6_1
PtARF6_2
PtARF6_3
MdARF102
MdARF2
VviARF30
0.2
Clade C
Repressors
Clade B
Clade A
Activators

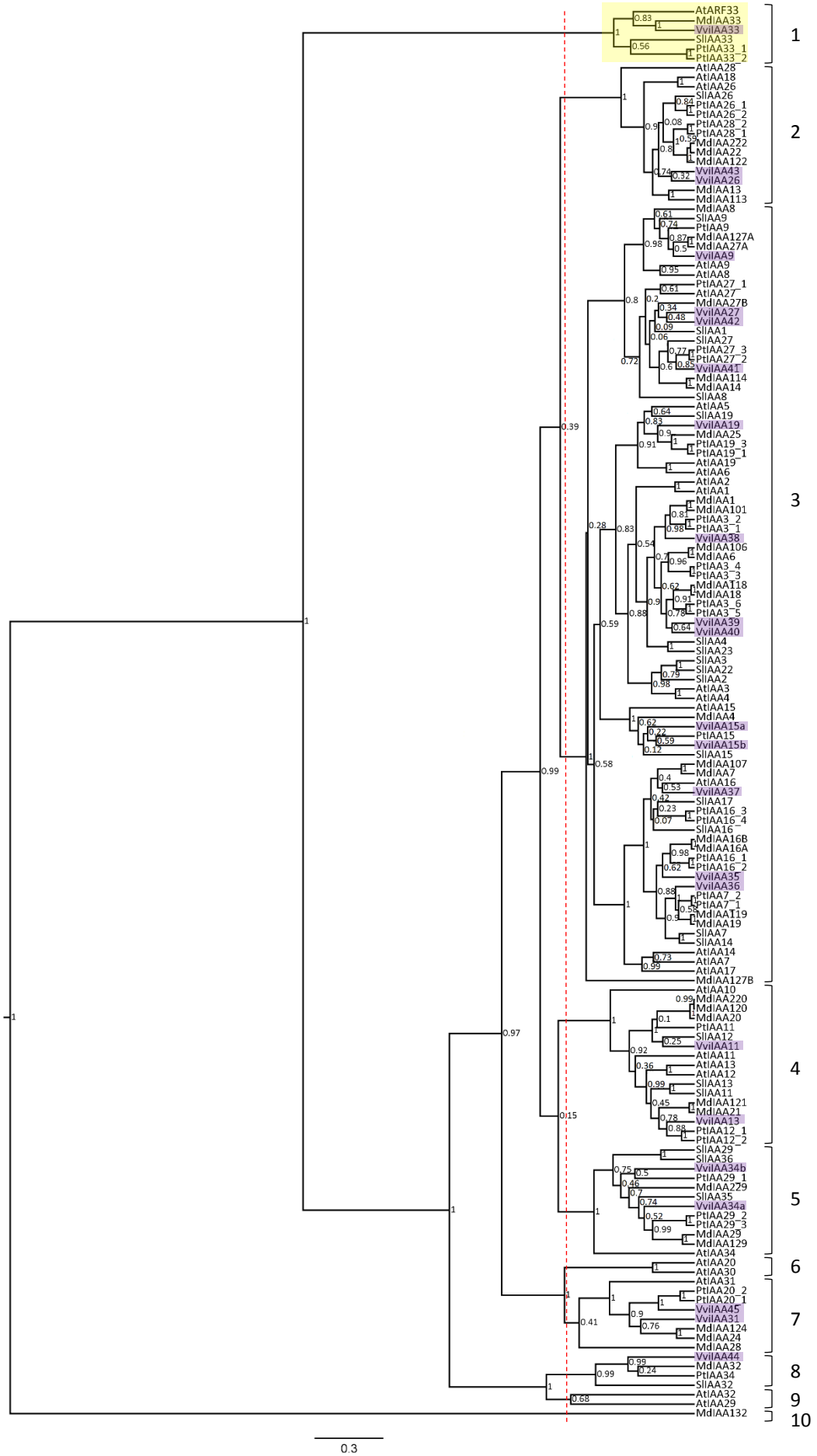
Figure 3.9 A consensus phylogenetic tree of the *ARF* family members from *V. vinifera* compared to Arabidopsis, apple, tomato and poplar generated using the conserved regions of the coding sequence in BEAST.

The tree was constructed using a translational alignment of coding sequences in Geneious and conserved alignments were produced using BMGE with "codons" and the default settings. Baysian Inference through BEAST was used for tree building with the GTR substitution model, estimated base frequencies, gamma site heterogeneities, partitioning into three separate codon positions with unlinked substitution models, and a Yule tree prior. Trees were generated with a strict clock prior and building was run until completion. The activator and repressor clades are labelled. The node values represent the posterior probabilities of the nodes occurring with 1 being the highest probability. Clades A, B, and C were assigned based on Finet *et al.* (2012), and classes one to seven were assigned based on the red dashed line representing the cut-off point for each class to be designated as a class. The seven classes are marked by their node and the three classes are labelled by the branch tips. The *ARF* repressor clades are marked with red brackets and the *ARF* activator clade with a green bracket. The yellow box highlights the repressor clade which is separate from the main activator and repressor clades. The green box highlights the Arabidopsis specific class. The scale bar represents the number of base pair substitutions per site. Accession numbers and/or references for the sequences can be found in Appendix D, Table D.2 and Table 2.1. Purple boxes highlight the *V. vinifera* sequences.

3.4.6.3 The *Aux/IAA* family has conserved domain sequences

The *Aux/IAA* phylogenetic tree has a more compact branching structure compared to the *ARFs*, with *MdIAA132* as a clear outlier from the main *Aux/IAA* clade forming its own clade, Clade 10. Clade 1 is also distinct, containing all *IAA33* candidates, including *VviIAA33*, these proteins are truncated and either contain non-conserved amino acid residues within the domains or lack the domains entirely (Figure 3.10 highlighted in yellow). The posterior probability support for the nodes have lower values in the *Aux/IAA* tree compared to the *ARF* tree ranging from 0.06 to 1, with the majority of nodes above 0.5. The *Aux/IAA* sequences are shorter and resolution of the nodes is more difficult. There are more *Aux/IAA* genes than *ARFs* in every species analysed (Table 3.3). Some clades have the same pattern that was seen with the *ARFs* with two or more *Malus* and Poplar genes for single Arabidopsis, *Vitis* and tomato genes. However, it appears to be more common to have larger sub-clades that contain multiple genes from each of the five species (Figure 3.10).

Clade 2 contains *VviIAA43* and *26*, which appear to be very similar and are 47.5% identical based on sequence identity (Appendix D, Figure D.9), however the node support is low, and Arabidopsis *AtIAA18, 26* and *28, SlIAA26*, four poplar candidates and five apple candidates. Clade 3 is the largest clade, containing 13 of the 23 *VviIAA* sequences. *VviIAA9* is in a sub-clade with *AtIAA8* and *9, SlIAA9* and *PtIAA9*, and three apple candidates. *VviIAA27* shares similarity with *IAA27* candidates from poplar, Arabidopsis, apple and tomato, as well as *VviIAA41* and *42, SlIAA1*, and *MdIAA14* and *114. VviIAA19* is in a sub-clade with *IAA19* candidates from tomato, poplar and Arabidopsis, as are *VviIAA15a* and *15b* with *IAA15* candidates from the same species. Clade 4 contains candidates *AtIAA10* and *IAA11-IAA13* from all species, except for *V. vinifera* that was represented by only two candidates; *VviIAA11* and *13*. Clade 5 contained *AtIAA34, VviIAA34a* and *34b*, and three candidates from each of the other three species. Clades 6 and 9 each contained only Arabidopsis candidates, *AtIAA20* and *30* and *AtIAA29* and 32, respectively. Clade 7 contained *VviIAA31* and *45*, three apple candidates, *PtIAA20_1* and *20_2*, and *AtIAA31*. Clade 8 contained *VviIAA44*, *MdIAA32*, *PtIAA34*, and *SlIAA32*.


AtARF33
MdIAA33
VviIAA33
SlIAA33
PtIAA33_1
PtIAA33_2
AtIAA28
AtIAA18
AtIAA26
SlIAA26
PtIAA26_1
PtIAA26_2
PtIAA28_2
PtIAA28_1
MdIAA222
MdIAA22
MdIAA122
VviIAA43
VviIAA26
MdIAA13
MdIAA113
MdIAA8
SlIAA9
PtIAA9
MdIAA127A
MdIAA27A
VviIAA9
AtIAA9
AtIAA8
PtIAA27_1
AtIAA27
MdIAA27B
VviIAA27
VviIAA42
SlIAA1
SlIAA27
PtIAA27_3
PtIAA27_2
VviIAA41
MdIAA114
MdIAA14
SlIAA8
AtIAA5
SlIAA19
VviIAA19
MdIAA25
PtIAA19_3
PtIAA19_1
AtIAA19
AtIAA6
AtIAA2
AtIAA1
MdIAA1
MdIAA101
PtIAA3_2
PtIAA3_1
VviIAA38
MdIAA106
MdIAA6
PtIAA3_4
PtIAA3_3
MdIAA118
MdIAA18
PtIAA3_6
PtIAA3_5
VviIAA39
VviIAA40
SlIAA4
SlIAA23
SlIAA3
SlIAA22
SlIAA2
AtIAA3
AtIAA4
AtIAA15
MdIAA4
VviIAA15a
PtIAA15
VviIAA15b
SlIAA15
MdIAA107
MdIAA7
AtIAA16
VviIAA37
SlIAA17
PtIAA16_3
PtIAA16_4
SlIAA16
MdIAA16B
MdIAA16A
PtIAA16_1
PtIAA16_2
VviIAA35
VviIAA36
PtIAA7_2
PtIAA7_1
MdIAA119
MdIAA19
SlIAA7
SlIAA14
AtIAA14
AtIAA7
AtIAA17
MdIAA127B
AtIAA10
MdIAA220
MdIAA120
MdIAA20
PtIAA11
SlIAA12
VviIAA11
AtIAA11
AtIAA13
AtIAA12
SlIAA13
SlIAA11
MdIAA121
MdIAA21
VviIAA13
PtIAA12_1
PtIAA12_2
SlIAA29
SlIAA36
VviIAA34b
PtIAA29_1
MdIAA229
SlIAA35
VviIAA34a
PtIAA29_2
PtIAA29_3
MdIAA29
MdIAA129
AtIAA34
AtIAA20
AtIAA30
AtIAA31
PtIAA20_2
PtIAA20_1
VviIAA45
VviIAA31
MdIAA124
MdIAA24
MdIAA28
VviIAA44
MdIAA32
PtIAA34
SlIAA32
AtIAA32
AtIAA29
MdIAA132
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0.3

Figure 3.10 A consensus phylogenetic tree of the *Aux/IAA* family members from *V. vinifera* compared to Arabidopsis, apple, tomato and poplar generated using the conserved regions of the coding sequence in BEAST.

The tree was constructed using a translational alignment of coding sequences generated in Geneious and conserved alignments produced using BMGE with "codons" and the default settings. Baysian Inference through BEAST was used for tree building with the GTR substitution model, estimated base frequencies, gamma site heterogeneities, partitioning into three separate codon positions with unlinked substitution models, and a Yule tree prior. Trees were generated with a strict clock prior and building was run until completion. The node values represent the posterior probabilities of the nodes occurring with 1 being the highest probability. *IAA33* clade is highlighted in yellow. The scale bar represents the number of base pair substitutions per site. Accession numbers and/or references for the sequences can be found in Appendix D, Table D.3 and Table 2.1. Purple boxes highlight the *V. vinifera* sequences. The red dashed line represents the cut-off point for each clade to be designated as a clade.

3.5 Discussion

Within this chapter *VviARF, VviIAA* and *VviAFB* gene candidates were identified and characterised using bioinformatics analysis. The sequences were predicted based on gene models, sequence alignments and RNAseq data. It is possible that there are differences that exist between the predicted sequences and those that exist *in planta* due to varietal differences, allelic differences (Finet *et al.,* 2012) and errors in prediction methods. This was addressed for six of the gene candidates by isolating the cDNA sequences in Chapter 5, however it was not possible to clone and sequence all family members. Any sequence differences may alter the presence/absence of specific protein domains in the putative translational sequences and could alter the predicted function of these protein. For these reasons the statements within this Discussion would need be confirmed by cDNA isolation and sequence analysis for all auxin signalling candidates.

3.5.1 Chromosome mapping of genes suggests a lack of gene duplications

Members of the *VviARF, VviIAA* and *VviAFB* gene families are spread across 16 of the 19 *V. vinifera* chromosomes (Figure 3.1). Gene clusters are present, such as chromosome 7 having six genes in total; four *VviIAAs*, one *VviAFB* and one *VviARF*, located towards the top half of the chromosome. The spread of genes across the chromosomes and the distances between the adjacent genes on the chromosomes in the phylogenetic trees suggests that the *V. vinifera VviAFB*, *VviARF* and *VviIAA* genes have arisen separately or alternatively arose through whole genome duplication and subsequent gene diversification. The *V. vinifera* genome is thought to contain contributions from three ancestral genomes, potentially through a hexaploidization event or successive rounds of whole genome duplication (Jaillon *et al.,* 2007). Therefore, the multiple family members may have arisen from these diverse ancestors.

Whole genome duplication events have been identified in all of the species included in the bioinformatics analysis. The Arabidopsis genome is thought to have undergone at least one whole genome duplication event, and potentially up to three events (Ku *et al.,* 2000; The Arabidopsis Genome Initiative, 2000; Jaillon *et al.,* 2007). This gives rise to the potential for functional redundancy, such as that seen with AtARF6 and 8, and it also allows for functional divergence and gene loss (reviewed in Reed 2001; Remington *et al.*, 2004; Overvoorde *et al.*, 2005; Lee *et al.*, 2009). The tomato genome has a lineage that is thought to have undergone two consecutive genome triplications (The Tomato Genome Consortium, 2012). However, tomato is unusual in that it is comprised of largely low-copy DNA, suggesting that the triplication events were followed by large scale gene loss. This is supported by the strong phenotypes seen in knock-out mutants, suggesting a lack of functional

redundancy in tomato auxin signalling (reviewed in Salehin *et al.,* 2015). Comparison of the tomato and grape genomes supports the idea that an ancient triplication event occurred in a shared ancestor in the rosid lineage (The Tomato Genome Consortium, 2012). The *Populus trichocarpa* and *Malus domestica* genomes are both predicted to have undergone an additional two whole-genome duplication events (Tuskan *et al.,* 2006, Velasco *et al.,* 2010). A slower rate of protein-evolution, and thus slower gene loss, in poplar and apple may be the reason behind the larger gene families in these two families in comparison to Arabidopsis, tomato and *V. vinifera* (Table 3.3). These results suggest that functional redundancy in auxin signalling components occurs in Arabidopsis, apple and poplar, but less so in tomato. It is currently unclear whether functional redundancy exists in *V. vinifera*, but one way to assess this might be through gene knock-out mutant analysis.

3.5.2 The *V. vinifera* AFB family contains six genes

Consistent with Parry *et al.* (2009), six *VviAFB* candidates were identified in *V. vinifera* using data from NCBI and Phytozome databases. The NCBI sequences were deemed to be the most accurate sequences based on the comparison of RNAseq data, the genome sequence and the examination of the UTR sequences and intron/exon boundaries (Table 3.4). Despite having already been named (Parry *et al.*, 2009), the VviAFB names were corrected using the Grimplet *et al.* (2014) method (Figure 3.2). *V. vinifera* contains an *AFB6* candidate which has reportedly been lost in both the Brassicaceae and Poaceae, however, the exact function of AFB6 genes has yet to be elucidated and provides an interesting area of future research (Parry *et al.,* 2009; Hayashi, 2012). VviAFB10 contained a 12 aa (or 36 bp) insertion where the other six sequences did not (Figure 3.5). This insertion did not map to a domain containing region of the protein sequence, making it unclear as to the functional significance. In VviARF11, the F-box domain is annotated as intact despite a 4 aa insertion (Figure 3.5). This insertion was also observed in AtAFB4 and 5, the closest homologues, suggesting it is a conserved sequence difference that may influence the expression and functionality of the proteins.

The AFB protein sequences were analysed for the presence of protein domains using InterProScan in Geneious (Figure 3.5), identifying LRR domains and F-box domains. F-box domains are characteristic of F-box proteins and allow for the binding of the AFB genes to the SKP-CULLIN protein complex (Bai *et al.,* 1996; Skowyra *et al.,* 1997). The typical F-box domain was present in VviAFB7, 8, 9 and 11, suggesting that they are able to bind with the SCF complex. VviAFB6 and 10 had F-box motifs that were not recognised by InterProScan, suggesting that although they appear to have F-box domains they may be mechanistically different, leading to altered functionality or a different mode of action. Between five and seven LRR domains were detected in all six AFB proteins using InterProScan. TIR1/AFB proteins characteristically contain 16–18 repeats (Kobe & Kajava, 2001; Tan *et al.*, 2007;

Salehin *et al.,* 2015), highlighting the difficulties of relying on prediction software for the annotation of domains. The LRR domains are involved in protein folding and conformation, leading to formation of a pocket that specifically binds auxinic molecules, contributing to the specificity of protein-protein interactions with Aux/IAA proteins in the formation of a co-receptor complex (Krek, 1998; Craig & Tyers, 1999). The varying numbers of LRR domains suggests that, similar to other species, the VviAFB candidates may preferentially interact with different auxinic compounds, such as the interaction of AtAFB4 and 5 with picloram (Calderon Villalobos *et al.,* 2012; Prigge *et al.,* 2016).

To investigate the relatedness of *VviAFB* genes to homologues in other species a phylogenetic tree was constructed using Bayesian inference (Section 2.2.2.5, Figure 3.8). The branch nodes represent the posterior probability of each node occurring, which were over 0.8 with the exception of three nodes suggesting that there is strong support for the structure of the tree. Tomato and grape appear to have the same number of genes in each clade with the exception of the clade containing *VviAFB9* and *10*. The conservation of the four clades and gene numbers, as well as conserved insertions in the *VviAFB11* sequence and its homologues, suggest that the functionality of the proteins is well conserved across plant species for the AFB protein family.

3.5.3 The DNA binding capacity is likely to be conserved in the *Vitis* ARF family

Nineteen *V. vinifera ARF* gene sequences were identified using a combination of bioinformatics tools, as detailed in Section 2.2.2.1 and 2.2.2.2, consistent with both Finet *et al.* (2012) and Wan *et al.* (2014). The sequence differences between the NCBI sequences, those from Finet *et al.* (2012), Wan *et al.* (2014), and those predicted within this work, listed in Table 3.5 and Appendix D indicate the variation that can exist between different gene prediction methods. It appears the best way to obtain the correct sequences is to isolate them from cDNA samples as differences may exist due to alternative splicing or allelic or cultivar differences. The chromosomal positions of these genes shows that all are distant from each other, suggesting that none are likely to have arisen from recent duplication events (Figure 3.1). The VviARF names were determined using the Grimplet *et al.* (2014) method (Figure 3.3) as neither Wan *et al.* (2014) nor previous microarray studies correctly named the *V. vinifera ARF* genes with the *Vvi* prefix and Finet *et al.* (2012) used the *Vvi* prefix but the incorrect identifiers (Deluc *et al.,* 2007; Pilati *et al.,* 2007; Fortes *et al.,* 2011).

InterProScan was used to identify protein domains in the VviARF proteins (Figure 3.6). All 19 proteins contained the Auxin_resp_domain, which is a region conserved in ARF proteins that was identified in rice (Sato *et al.,* 2001; Liscum and Reed, 2002). The B3 DBD represents the site at which ARF proteins interact with the AuxRE motif in gene promoters (Suzuki *et al.,* 1997) and was present in all 19 VviARF

candidates, suggesting they all have the potential to bind DNA and function as characteristic ARF proteins in mediating transcription. The PB1 domain is located near the C-terminus of ARF proteins and mediates hetero-dimerisation or homo-oligomerisation between ARF and Aux/IAA proteins and was present in 16 of the VviARF proteins (Terasawa *et al.,* 2001; Hirano *et al.,* 2005; Sumimoto *et al.,* 2007; Korasick *et al.,* 2014; Nanao *et al.,* 2014; Guilfoyle, 2015). The domain was not present in VviARF3, 8 and 17, suggesting that these three family members are unable to form either hetero-dimers or homo-oligomers and would not be regulated by Aux/IAA proteins. The absence of PB1 domains has been reported in other species, such as banana (Hu *et al.,* 2015) and Arabidopsis (Simonini *et al.,* 2016; 2017). As *VviARF8* falls within the *ARF* activator clade, the absence or truncation of this domain suggests that the VviARF8 candidate may be unable to bind and be regulated by Aux/IAA proteins and may be constitutively activating auxin responsive genes. Recent studies suggest that dimerization between ARF proteins requires both the PB1 domain and a dimerization domain (DD) located near the DBD, however more support for this is required (Pierre-Jerome *et al.,* 2016). Analysis of the middle regions of the VviARF proteins identified that VviARF5, 27, 28, 29 and 30 were glutamine-rich (Q–rich) and therefore may act as ARF activators; they formed a distinct clade within the phylogenetic tree (Ulmasov *et al.,* 1999; Figure 3.6, Figure 3.9). The activator clade contains a sixth gene, VviARF8, which has a small middle region with fewer Q-residues compared to the other proteins in the activator clade so the protein may or may not act as an activator.

Within the multiple species *ARF* phylogenetic tree, there is a clear pattern with a single Arabidopsis, tomato and *V. vinifera* gene for every two or more poplar and apple genes, with the exception of the Arabidopsis-specific class that contains nine genes including the *AtARF23* pseudogene (Figure 3.9) (Okushima *et al.,* 2005; Guilfoyle & Hagen, 2007). *AtARF2* has been linked with seed and ethylene response (Wang *et al.,* 2011) and *VviARF2a and 2b* are the closest homologs. *AtARF3* and *4* are associated with patterning in developing vegetative and reproductive tissues, and are most similar to *VviARF3 and VviARF4*, respectively (Pekker *et al.,* 2005; Simonini *et al.,* 2016; 2017). *AtARF6* and *AtARF8* have been linked with cell division related to fertilisation and fruit development, stamen elongation and floral maturation, which matches with the high levels of expression in flowers and early in fruit development while auxin is present (Nagpal *et al.,* 2005; Goetz *et al.,* 2006). *AtARF6* and *8* fall into the ARF activator clade, and cluster with *VviARF8, 29* and *30*. *AtARF16* is closest to *VviARF16* and has been linked with root cap cell differentiation (Wang *et al.,* 2005), and the homologues of *AtARF10* and *AtARF16* in woody species, including poplar and *Eucalyptus grandis,* are thought to be involved in wood cell differentiation (Yu *et al.*, 2014). *DR12* (*SlARF4*) is a negative regulator of genes encoding enzymes activities involved in starch biosynthesis (Jones *et al.,* 2002; Guillon *et al.,* 2008; Legland *et al.,* 2010; Sagar *et al.,* 2013). The closest homolog in *V. vinifera* to *SlARF4* is *VviARF4*. Down-regulation

of *SlARF7* has been found to deregulate cell division and up-regulate cell expansion, leading to larger cells in the fruit (Vriezen *et al.,* 2008; de Jong *et al.,* 2009; 2011). The closest homologues in *V. vinifera* are *VviARF27* and *28*, suggesting that, if the functionality is conserved across species, one or both of these candidates may play a role in the balance between cell division and cell expansion, potentially through promoting cell division. As grape and tomato are both fleshy fruit that arise from the ovary the function of these genes may be conserved between species, what impact the fact tomato is climacteric and grape non-climacteric has in unclear at this stage (Kumar *et al.*, 2014).

3.5.4 An improved understanding of the Aux/IAA family in *V. vinifera*

Using a combination of methods, 23 *Aux/IAA V. vinifera* gene sequences were identified in this study. This finding was inconsistent with the 26 *Aux/IAA* genes identified previously (Çakir *et al.,* 2013). These authors divided the candidates into two subfamilies, subfamily A and subfamily B, which contained longer and shorter candidates respectively. Upon sequence analysis it was established that none of the 18 sequences in subfamily A contain Domains I and II, characteristic of the Aux/IAA proteins. The presence of a DBD and Domains III and IV suggested these sequences in fact encoded ARF proteins, which was confirmed by BLAST searches which identified the closest matches to these sequences to be ARF sequences. As a results eight of the 26 *Aux/IAA* genes identified by Çakir *et al.* (2013) were confirmed as *Aux/IAA* genes in this study (Appendix D, Figure D.1). The remaining 15 sequences within this work contained some differences in comparison with the NCBI sequences listed in Appendix D, Figure D.4. Five *VviIAA* candidate genes were given names based on previous publications: *VvIAA2* was renamed *VviIAA27* (Pilati *et al.,* 2007; Grimplet *et al.,* 2007; Fortes *et al.,* 2011); *VvIAA4* became *VviIAA9* (Grimplet *et al.,* 2007; Kobayashi *et al.,* 2009; Fujita *et al.,* 2012); *VvIAA10* became *VviIAA26* (Pilati *et al.,* 2007); *VvIAA22* became *VviIAA13* (Grimplet *et al.,* 2007; Fortes *et al.,* 2011); and *VvIAA22.4* became *VviIAA19* (Fortes *et al.,* 2011; Kohno *et al.,* 2012; Lijavetzky *et al.,* 2012). The names for all other candidates were determined using the Grimplet *et al.* (2014) method, all with the prefix VviIAA for simplicity and consistency.

InterProScan was used to identify Domains I – IV in Aux/IAA proteins (Figure 3.7). Domain I enhances the repression of ARF proteins by binding the TPL co-repressor; Domain II contains a degron sequence bound by the TIR1/AFB proteins allowing for targeted Aux/IAA polyubiquination and degradation; Domains III and IV allow for protein-protein interactions between Aux/IAA and ARF proteins allowing for heterodimerisation or homo-oligomerisation between ARF and Aux/IAA proteins and the formation of complex dimers (Abel *et al.*, 1994; Kim *et al.*, 1997; Ulmasov *et al.*, 1997; Ulmasov, 1997; Hardtke & Berleth, 1998; Terasawa *et al.,* 2001; Tiwari *et al.*, 2001; Liscum & Reed, 2002; Tiwari *et al.*,

2004; Hirano *et al.,* 2005; Sumimoto *et al.,* 2007; Szemenyei *et al.*, 2008; Lee *et al.*, 2009; Korasick *et al.,* 2014; Nanao *et al.,* 2014). Eleven of the 23 VviIAA proteins contain Domain I, suggesting that they have the capacity to strongly repress ARF proteins in complex with TOPLESS proteins. Of the 23 VviIAA proteins, 20 have Domain II indicating that the majority of VviIAA proteins are degraded by the SCF-complex. Finally, 22 have Domains III and IV suggesting that the majority of proteins are able to form hetero- and homodimers and may act as complexes. The absence of Aux/IAA domains for specific candidates has been reported in other species, including tomato and potato (Wu *et al.,* 2012) and maize (Ludwig *et al.,* 2013). Some sequences consistently lacked certain domains across the range of species analysed, such as IAA33, which does not contain Domains I and II, moreover the Domains III and IV are present in a domain search but their sequences are dissimilar to Domains III and IV in other family members. It is unclear whether the IAA33 proteins are mutated IAAs with a conserved alternative functionality; they may be constitutively bound to ARF proteins due to the absence of Domains I and II. The conservation of IAA33 across all five species suggests they may be functional.

Wu *et al.* (2012) created a multi-species tree containing Aux/IAA protein sequences from tomato, maize, rice and Arabidopsis and divided the phylogeny into ten distinct clades. Similarly, in this work the multi-species tree containing grape, tomato, Arabidopsis, apple and poplar was also divided into ten clades (Figure 3.10). *MdIAA132* was an outlier, forming Clade 10, and is clearly dissimilar to all other *Aux/IAA* sequences. Within the main body of the tree, Clade 1 (highlighted in yellow) is also distinct and contains all *IAA33* sequences. The clades contain two or more *Malus* and poplar genes for a single Arabidopsis, *V. vinifera* and tomato gene and also some larger sub-clades that contain multiple genes from each of the five species. Limited functional data is available for Aux/IAA candidates. In tomato, SlIAA9 acts as a transcriptional repressor of auxin-induced gene expression impacting leaf morphology, fruit set and development, apical dominance and other aspects of vegetative and reproductive growth (Wang *et al.,* 2005; 2009). The closest *V. vinifera* sequence to *SlIAA9* is *VviIAA9*. Silencing SlIAA9 causes upregulation of SlIAA3, suggesting a complex interplay between Aux/IAA family members (Wang *et al.,* 2005, 2009). The closest homologues of *SlIAA3* are *VviIAA38, 39* and *40*. Further analysis would be required to determine if functions are conserved between the genes with a close phylogenetic relationship.

3.5.5 Transcriptional analysis of candidate genes is needed to assess potential function

The bioinformatic characterisation of the *VviARFs*, *VviIAAs* and *VviAFBs* isolated a total of 48 genes across the three families. In some cases these genes showed high levels of similarity to sequences from Arabidopsis and other plant species. The lack of homologues in some clades indicates

diversification between the species investigated and the variation in the predicted protein domains between family members suggests specialisation. As Aux/IAA and ARF candidates have been found to play roles in fruit development in other species, it is likely that *V. vinifera* genes play similar roles. The diversification between species suggests that some homologues may have developed species-specific functions and phylogenetic relationships alone are not sufficient evidence on which to accurately infer protein function. Therefore, it is important to determine transcriptional behaviour of the three families during berry development to further narrow down the list of candidates that may play a key role in development and ripening.

Chapter 4 Transcriptional profiles of *ARF*, *Aux/IAA* and *AFB* genes in *Vitis vinifera* L. cv. Shiraz

4.1 Aim

The aim of this work was to determine the temporal and spatial transcription patterns of gene families involved in the auxin signalling pathway in a range of *Vitis vinifera* L. cv. Shiraz organs.

4.2 Introduction

To date, comprehensive studies detailing the transcriptional profiles of all *TIR1/AFBs, ARFs*, and *Aux/IAAs* in *V. vinifera* have not been reported. Multiple studies have used microarrays and RNAseq to examine global transcript profiles across several stages of development in different *V. vinifera* varieties (Grimplet *et al.,* 2007; Pilati *et al.,* 2007; Deluc *et al.,* 2007; Zenoni *et al.,* 2010; Fortes *et al.,* 2011). Across these studies, a limited number of auxin signalling pathway family members have been identified as being differentially expressed during berry development. The most comprehensive analysis was completed by Deluc *et al.* (2007) using *V. vinifera* L. cv. Chardonnay mRNA from seven stages of berry development. The authors found that five *Aux/IAA* and seven *ARF* transcripts were down-regulated at veraison, and that two *Aux/IAA* transcripts were up-regulated. One *TIR1/AFB* (*VviAFB8*) transcript was down-regulated during ripening, whilst another was up-regulated (*VviAFB9*). Although Wan *et al.* (2014) also identified 19 *VviARF* candidates, they only completed qPCR analysis on nine *VviARF* transcripts across five time points to compare with the microarray data from Deluc *et al.* (2007). They found consistency with four of the nine *ARF* transcript patterns between the two studies and attributed the differences to different environmental growth conditions of the grapes. Therefore, the currently available transcriptional data does not provide a clear pattern of the expression of the auxin signalling pathway candidates across berry development. To address this deficiency, qPCR analysis (Section 2.2.5.6) was completed on a developmental series encompassing 16 weeks of berry development, nine stages of leaf development, and flower, tendril and root samples from *V. vinifera* L. cv. Shiraz (Section 2.1.4.1) for all six *VviAFB*, 19 *VviARF*, and 23 *VviIAA* genes identified in Chapter 3.

This chapter details the temporal and spatial transcription patterns for the 48 auxin signalling pathway members in order to gather further information on the roles they might play in grape development. In addition to this, the concentrations of free IAA and IAA-Asp were determined in a *V. vinifera* L. cv., Shiraz, for the berry and leaf developmental series to act as a direct comparison to the transcriptional

data and existing data available for *V. vinifera* L. cv. Cabernet Sauvignon (Chapter 1, Figure 1.5, Böttcher *et al.*, 2010b). By studying the temporal and spatial dynamics of transcripts involved in auxin signalling the following questions were addressed: are the candidates expressed, and if so, which organs are they expressed in? Are there distinct developmental patterns present in reproductive and vegetative tissues? Which of the candidates have the most prominent expression patterns? Do the candidates form expression clusters that may identify interacting partners? Do these patterns relate to the concentration of auxin within reproductive and vegetative tissues?

4.3 Results

4.3.1 Free IAA and IAA-Asp conjugate levels during berry and leaf development in Shiraz

Free IAA and IAA-Asp concentrations were measured across the 16 week berry developmental series and across the nine stage leaf developmental series (Figure 4.1). In berries, the concentration of IAA and IAA-Asp were inversely related, with IAA concentration at ~500 pmol/gFW (picomoles per gram of fresh weight) at week one decreasing during development to be at low concentrations from veraison onwards. The concentration of IAA-Asp conjugates was low during early development and increased rapidly from veraison with a peak from week 14 to 16 at ~900 pmol/gFW. In leaves, the changes in concentration of IAA and IAA-Asp followed a similar pattern across the leaf stages. Both peaked at leaf stage three, at ~1,400 and 250 pmol/gFW respectively. The concentration of both subsequently decreased with the lowest concentration being present in the oldest leaves.

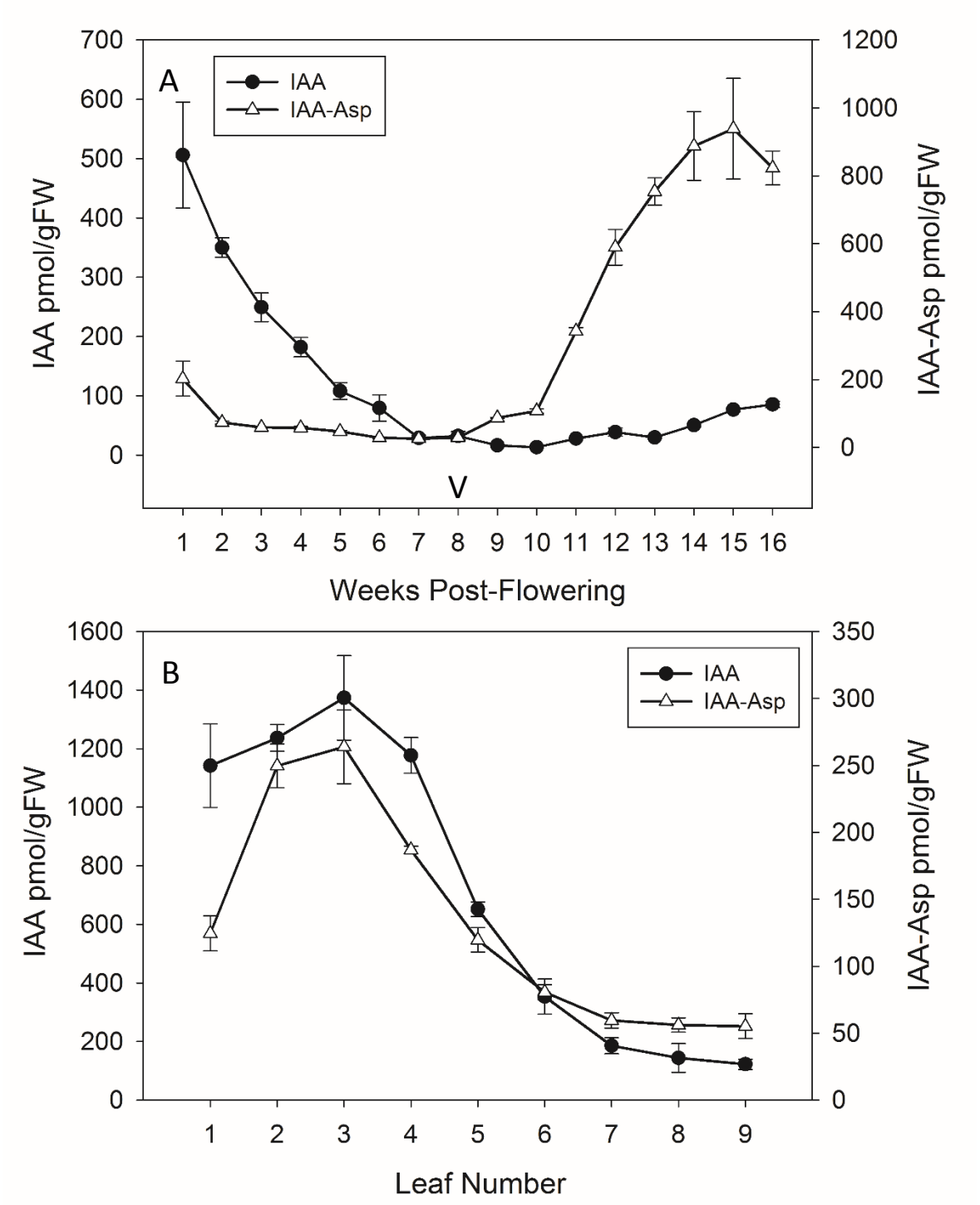


Figure 4.1 The concentration of indole-3-acetic acid and its aspartic acid conjugate in *V. vinifera* L. cv. Shiraz across a sixteen week Shiraz berry developmental series and nine stage leaf series.

(A) Opposing patterns are seen with the indole-3-acetic acid (IAA) and IAA-aspartic acid (IAA-Asp) concentrations shown in picomoles per gram of fresh weight (pmol/gFW) across the 16 weeks post flowering. V = veraison. (B) Similar patterns are seen with IAA and IAA-Asp concentrations shown in pmol/gFW across the nine stage leaf series. Error bars = standard error.

4.3.2 The expression patterns of *VviAFB, VviARF* and *VviIAA* gene families

Gene transcript levels were assessed using qPCR (Section 2.2.5.6) for all *VviAFB*, *VviARF*, and *VviIAA* family members across a 16 week Shiraz berry developmental series and a tissue series, including a nine stage leaf developmental series, flowers, tendrils and roots. For simplicity the expression levels are detailed for each gene family in this section with a brief comment on significant features for each data set and the main discussion of the expression patterns is presented in Section 4.3.3 using cluster analysis of the three gene families.

4.3.2.1 All *Vitis AFB* candidates are expressed during berry development

All six *VviAFBs* were expressed in developing berries (Figure 4.2). *VviAFB7, 8* and *11* were determined to be highly expressed (>10,000 copy number), and *VviAFB6, 9* and *10* were expressed at lower levels (<10,000 copy number). All transcripts were expressed at a relatively high abundance throughout berry development. All six *VviAFB* genes were most abundant in week one berries and the levels remained high during the two week period of cell division in the berries. The error bars are large for all *VviAFB* candidates at week one, except for *VviAFB9*, potentially due to rapid cellular changes in at this stage of development. Only *VviAFB6* and *9* show evidence of increased transcript levels post-veraison.

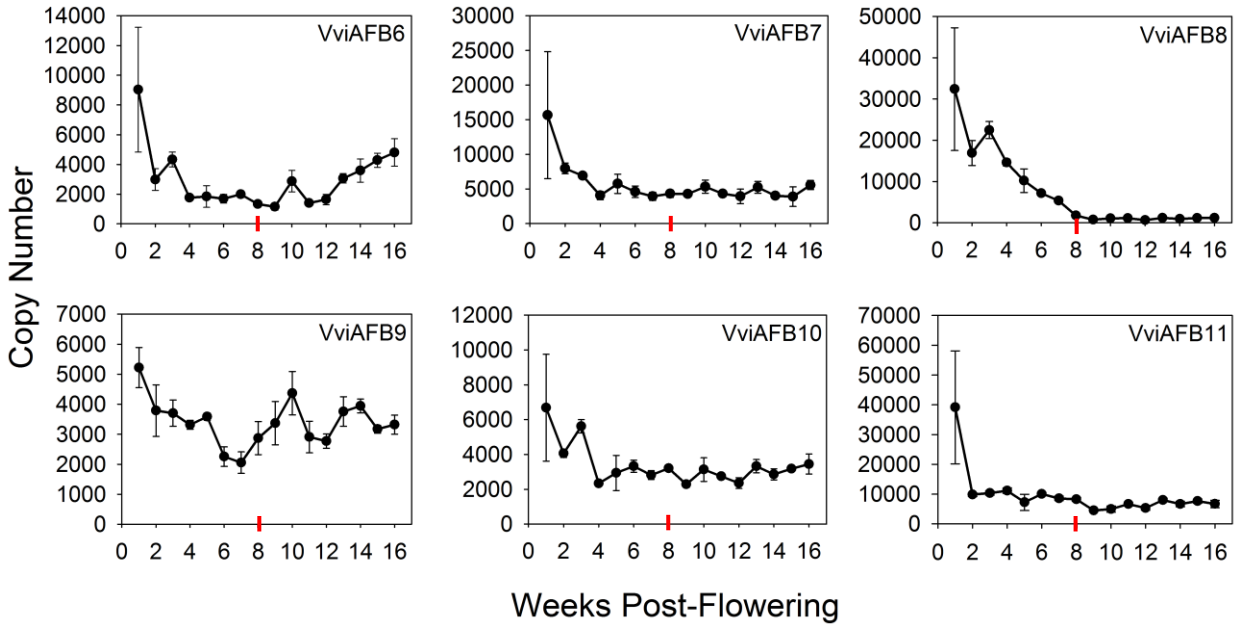


Figure 4.2 The transcriptional profiles of the six *V. vinifera auxin signaling F-box* candidates across sixteen weeks of *V. vinifera* L. cv. *Shiraz* berry development.

The transcriptional profiles of the six *V. vinifera auxin signaling F-box (AFB)* candidates were determined using qPCR on three biological replicates and two technical replicates of a 16 week berry developmental series. The transcript levels are measured in number of copies with the error bars representing the standard error. The red dash indicates veraison.

4.3.2.2 Transcriptional dynamics of *VviAFB* candidate genes during leaf development and in other organ types

High levels of expression were detected for all *VviAFBs* in all organ types (Figure 4.3). *VviAFB6, 8, 10* and *11* were determined to be highly expressed (>10,000 copy number), and *VviAFB7* and *9* were expressed at lower levels (<10,000 copy number). Again, all transcripts were detected at a relatively high abundance throughout all organs and throughout leaf development. In the leaf developmental series the lowest levels of expression were detected in leaf one (the youngest leaf sampled) for all candidates.

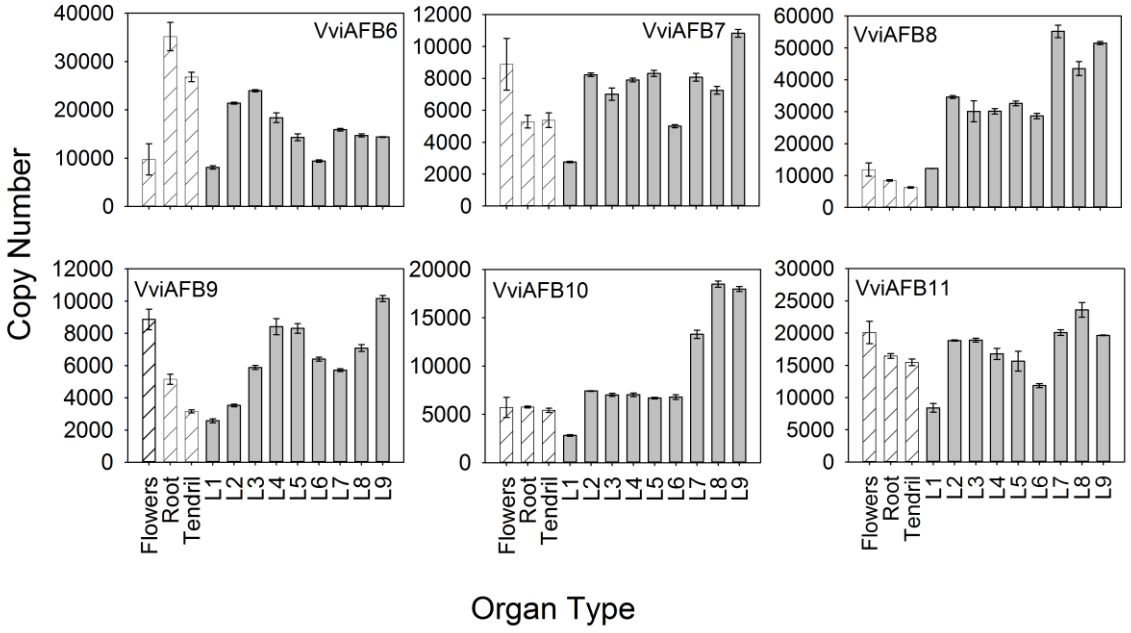


Figure 4.3 The transcriptional profiles of the six *V. vinifera auxin signaling F-box* candidates in plant tissues including the flowers, roots, tendrils and nine leaf stages in *V. vinifera* L. cv. Shiraz.

The transcriptional profiles of the six *V. vinifera auxin signaling F-box (AFB)* candidates were determined using qPCR on three biological replicates and two technical replicates of the flowers, and on one biological replicate and four technical replicates for the nine stage leaf series, root and tendril samples. The transcript levels are measured in number of copies with the error bars representing the standard error. The white dashed boxes represent the organ flowers, roots and tendrils, and the gray boxes are the leaf developmental series. L1-L9 represent leaves one to nine.

4.3.2.3 Many *VviARF* candidate genes are highly expressed pre-veraison

Transcripts for all 19 *VviARFs*, except *VviARF26*, were detected in the berries with a variety of patterns across the 16 weeks of development (Figure 4.4). Half of the genes were determined to be highly expressed (>10,000 copy number) including *VviARF1a, 4, 8 (A), 17, 24, 25,* and *30 (A),* with *VviARF8 (A)* having the highest expression levels. The remainder were determined to be expressed at lower levels (<10,000 copy number), with *VviARF28 (A)* having the lowest expression levels. Fifteen of the *VviARF* candidates were mostly highly expressed at week one, when the free IAA concentration is also at its highest. Veraison (at week eight) appears to be a pivotal stage in development for the expression of *VviARF* genes with 12 changing expression at this stage, and only five expressed significantly post-veraison. Twelve of the 18 *VviARF* candidates were expressed at low levels at, or shortly after, veraison and until harvest (repressors *VviARF3, 4, 8, 16, 17, 24, 25, 31, 32,* and activators (A) *28, 29, 30*). In contrast, *VviARF1a, 1b, 2a, 5(A),* and *27(A),* were detected pre- and post-veraison. *VviARF2b* had a distinct expression pattern, showing a slight increase in transcript levels across berry development.

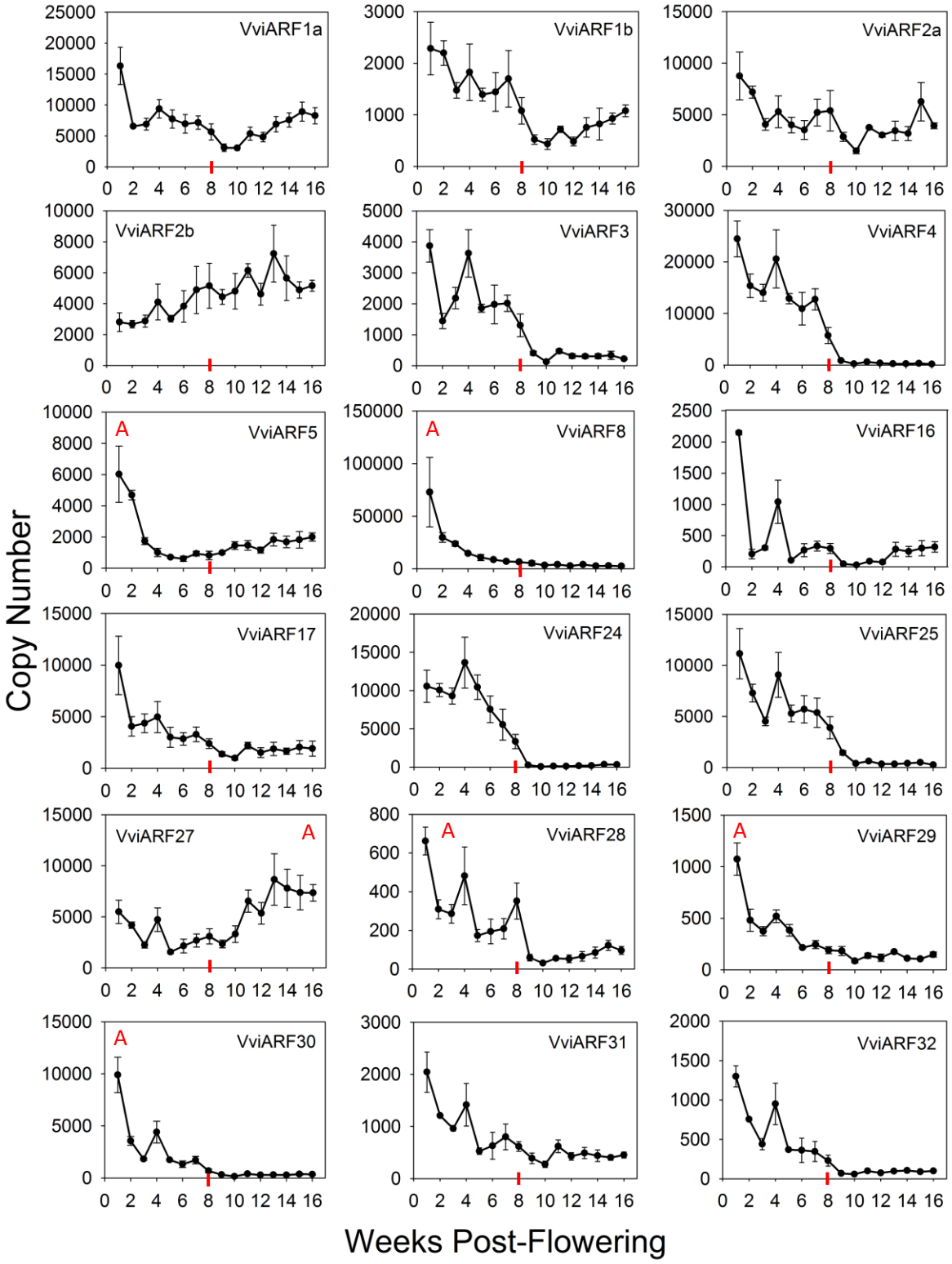


Figure 4.4 The transcriptional profiles of the eighteen *V. vinifera auxin response factor* candidates across sixteen weeks of *V. vinifera* L. cv. Shiraz berry development.

The transcriptional profiles of 18 *V. vinifera auxin response factor (ARF)* candidates were determined using qPCR on three biological replicates and two technical replicates of a 16 week berry developmental series. The transcript levels are measured in number of copies with the error bars representing the standard error. The candidate *VviARF26* did not have detectable expression in the berries as the expression fell outside of the standard curve and is not displayed here. The red dash indicates veraison. Red A represents the *ARF* activators based on which *ARFs* fell within the *ARF* activator clade in Figure 3.9.

4.3.2.4 All *VviARF* candidate genes are expressed during leaf development or in other organs

All 19 of the *VviARFs* had some level of expression in the different organ types (Figure 4.5). The expression in flowers, tendrils and roots varied considerably between the different genes. There was a general trend in 12 of the *VviARFs* towards having higher transcript levels at the end of leaf development. Interestingly, the transcript levels for *VviARF2b, 3, 4,* and *30 (A)* alternated between higher and lower levels of expression during leaf development in a cyclical pattern. Eight of the genes were determined to be highly expressed (>10,000 copy number) in the diverse tissue set, including *VviARF1a, 2a, 4, 5 (A), 8 (A), 25, 27 (A)* and *30 (A)*, with *VviARF8 (A)* having the highest expression levels. The remaining 11 were expressed at lower levels (<10,000 copy number), with *VviARF26* having the lowest expression levels. *VviARF26* transcripts were undetectable in berries, and were expressed at low levels in the tissue series, with the maximum expression being ~600 copies in the roots. Low levels were detected in the flowers and leaves one and two with no detectable expression in leaves 3–9.

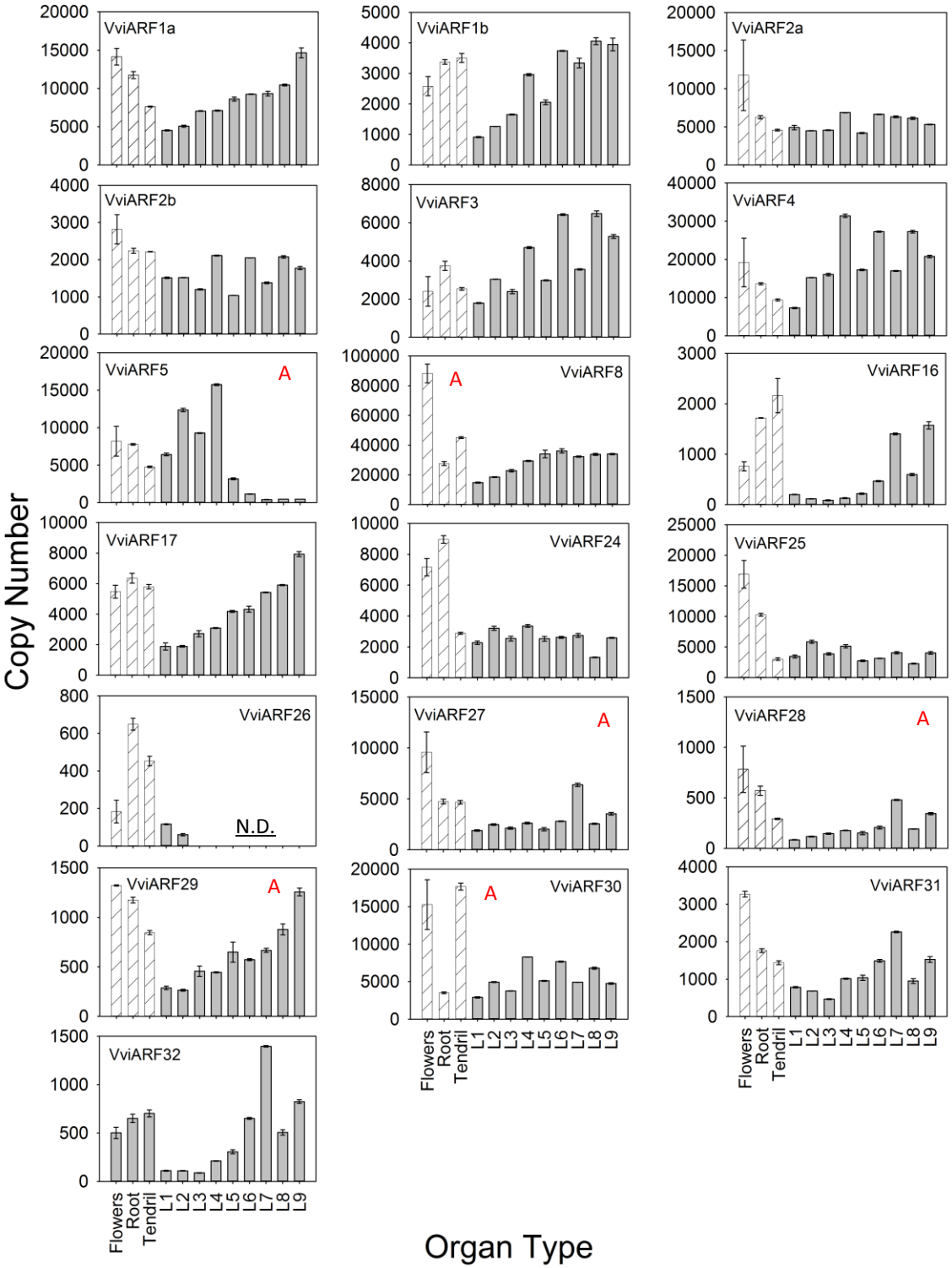


Figure 4.5 The transcriptional profiles of the nineteen *V. vinifera auxin response factor* candidates in plant tissues including the flowers, roots, tendrils and nine leaf stages in *V. vinifera* L. cv. Shiraz.

The transcriptional profiles of the six *V. vinifera auxin response factor (ARF)* candidates were determined using qPCR on three biological replicates and two technical replicates of the flowers, and on one biological replicate and four technical replicates for the nine stage leaf series, root and tendril samples. The transcript levels are measured in number of copies with the error bars representing the standard error. Red A represents the *ARF* activators based on which *ARFs* fell within the *ARF* activator clade in Figure 3.9. The white dashed boxes represent the organ flowers, roots and tendrils, and the gray boxes are the leaf developmental series. L1-L9 represent leaves one to nine.

4.3.2.5 All *VviIAA* candidate genes show low expression at veraison

Twenty-two of the 23 *VviIAA* were expressed within berries, while *VviIAA44* was not detected at any stage of berry development (Figure 4.6). Thirteen of the genes were determined to be highly expressed (>10,000 copy number) including *VviIAA15b, 19, 26, 27, 35, 36, 37, 38, 40*, and *41* with *VviIAA15b* and *19* having the highest expression levels. The remaining nine genes were expressed at lower levels (<10,000 copy number), with *VviIAA45* having the lowest expression levels. Eight of the *VviIAA* candidates had their highest copy numbers at week one, with most having a drop in expression around veraison. Thirteen of the *VviIAAs* expressed during berry development were down-regulated or not expressed, after veraison in week eight, including *VviIAA9, 13, 15a, 26, 27, 34a, 34b, 35, 36, 37, 41, 42, 43*, and *45*, suggesting that veraison is a crucial time point for *VviIAA* expression. Nine of these 13 *VviIAAs* were expressed after veraison, with only two of the nine not showing a decrease in expression during the lag phase. The remaining genes, *VviIAA11, 15b, 19, 31, 33, 38, 39,* and *40*, had higher expression levels post-veraison, often after a rapid decrease in expression during the lag phase.

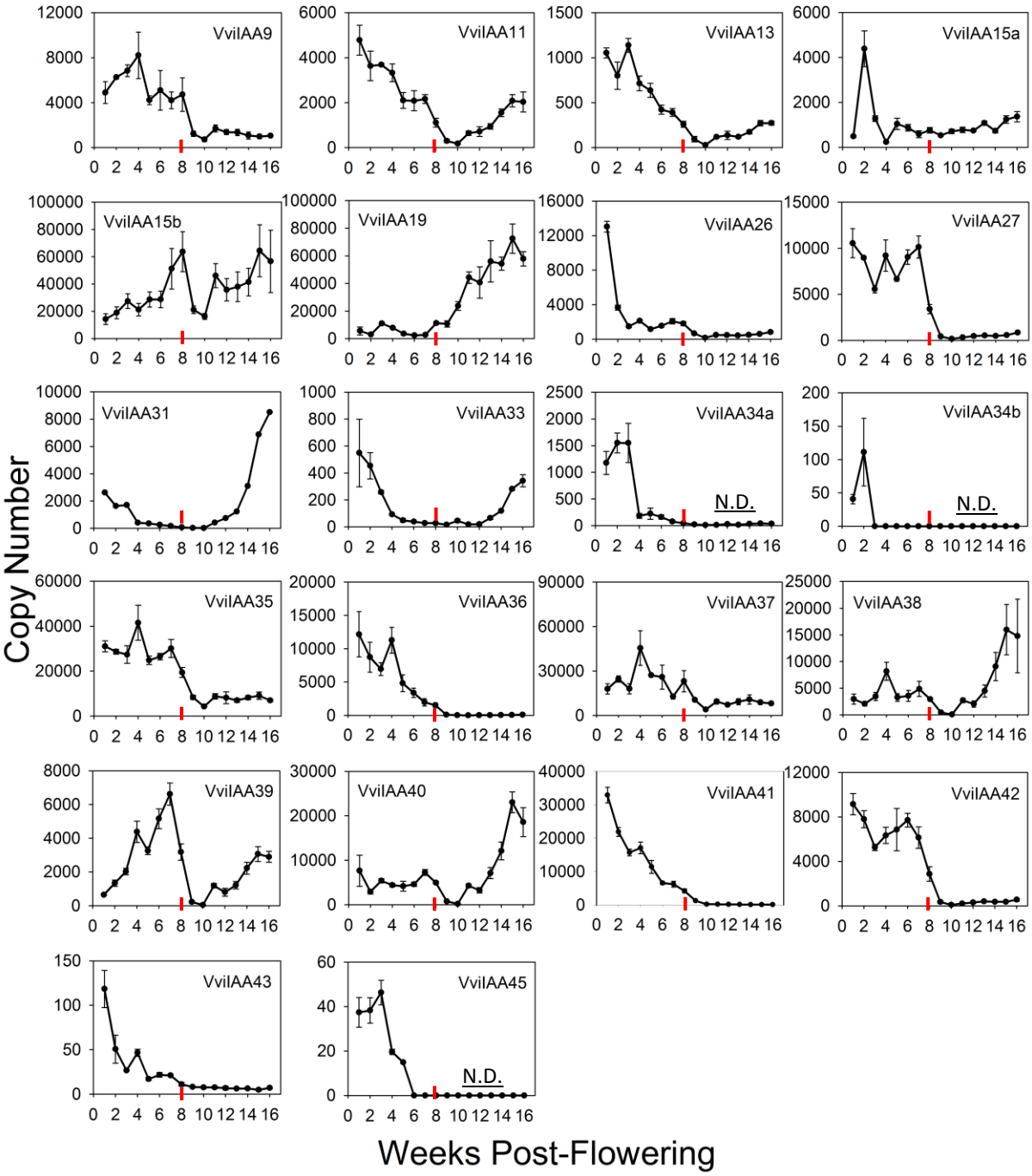


Figure 4.6 The transcriptional profiles of the twenty-two *V. vinifera auxin/indole-3-acetic acid* candidates across sixteen weeks of *V. vinifera* L. cv. Shiraz berry development.

The transcriptional profiles of 22 *V. vinifera auxin/indole-3-acetic acid (Aux/IAA)* candidates were determined using qPCR on three biological replicates and two technical replicates of a 16 week berry developmental series. The transcript levels are measured in number of copies with the error bars representing the standard error. The candidate *VviIAA44* did not have detectable expression in the berries as the expression fell outside of the standard curve and is not displayed here, additionally *VviIAA34a* weeks 9–10, *VviIAA34b* weeks 3–16, and *VviIAA45* weeks 6–16 also fell outside the standard curve and are shown as not detected (N.D.). The red dash indicates veraison.

4.3.2.6 Organ expression patterns of *VviIAA* candidate genes are distinct

Distinct patterns of expression were identified for the *VviIAA* transcripts in flowers, tendrils, roots and the leaf series (Figure 4.7). Fifteen of the genes were determined to highly expressed (>10,000 copy number) in this tissue series including *VviIAA11, 15a, 15b, 19, 26, 27, 33, 34b, 35, 36, 37, 38, 39, 40, 41* and *42* with *VviARF36* having the highest expression levels. The remaining seven were determined to be expressed at lower levels (<10,000 copy number) including *VviIAA9, 13, 31, 34a, 43, 44* and *45* with *VviIAA45* having the lowest expression levels. There were a larger number of genes in the *VviIAA* gene family that had relatively low expression levels throughout leaf development, and low expression in one or more of the other organs. Organ specificity was seen in some of the *VviIAA* candidates. *VviIAA9* was most abundant within flowers, roots and tendrils, *VviIAA15b, 19, 27, 41* and *42* in flowers, *VviIAA31, 34a, 36,* and *44* in both roots and tendrils, and *VviIAA15a, 34b, 37, 38,* and *43* in tendrils.

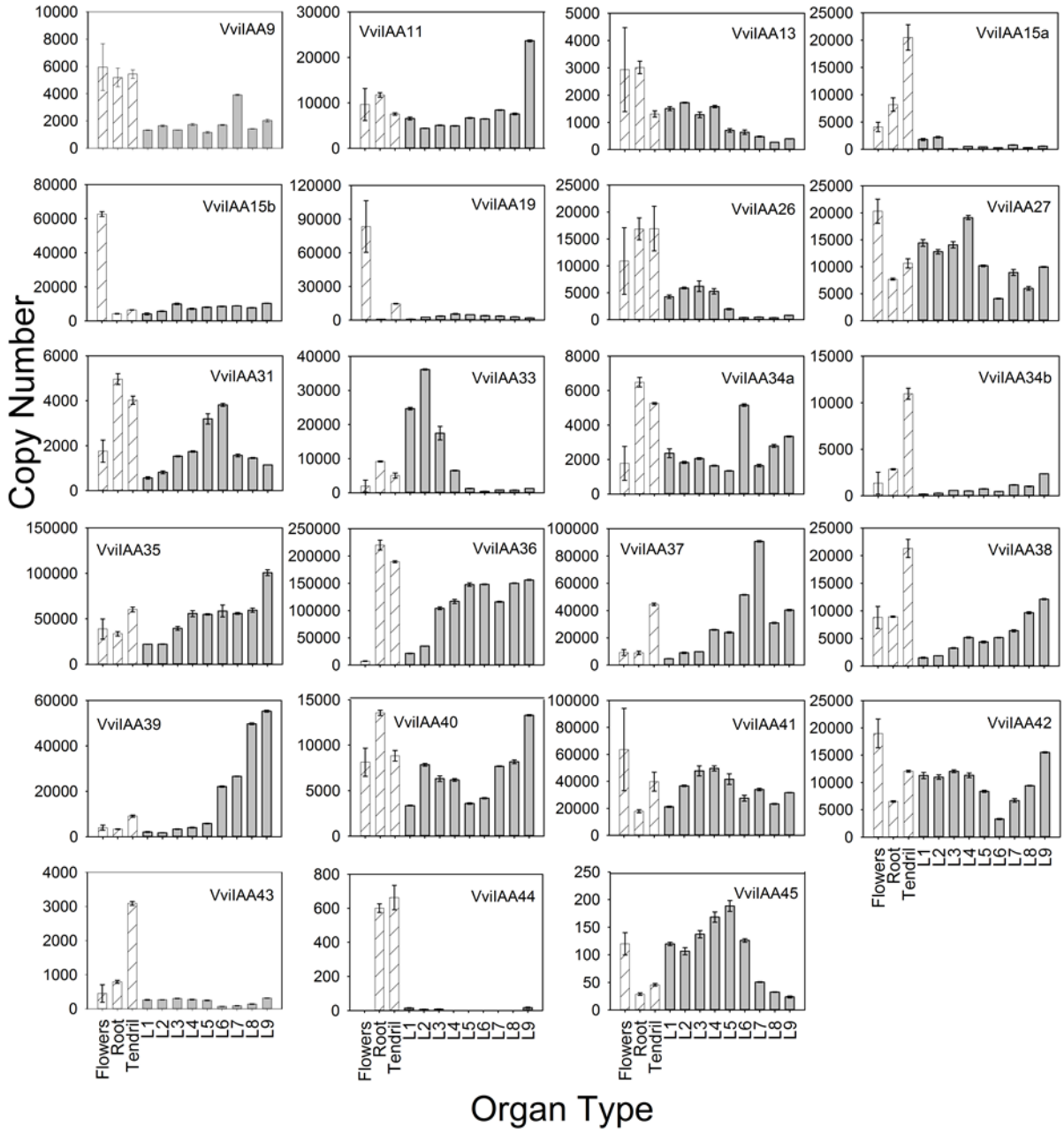


Figure 4.7 The transcriptional profiles of the twenty-three *V. vinifera auxin/indole-3-acetic acid* candidates in plant tissues including the flowers, roots, tendrils and nine leaf stages in *V. vinifera* L. cv. Shiraz.

The transcriptional profiles of the 23 *V. vinifera auxin/indole-3-acetic acid (Aux/IAA)* candidates were determined using qPCR on three biological replicates and two technical replicates of the flowers, and on one biological replicate and four technical replicates for the nine stage leaf series, root and tendril samples. The transcript levels are measured in number of copies with the error bars representing the standard error. The candidate *VviIAA44* did not have detectable expression in leaves 4–8 as the expression fell outside of the standard curve and are shown as not detected (N.D.). The white dashed boxes represent the organ flowers, roots and tendrils, and the gray boxes are the leaf developmental series. L1-L9 represent leaves one to nine.

4.3.3 Heatmaps and cluster analysis of all *VviAFB*, *VviARF*, and *VviIAA* gene expression data

The heatmap and cluster data were generated using the method as described in Section 2.2.2.6.1, to allow the discussion of the expression patterns that were present across the three gene families and to identify gene candidates that share similar expression patterns across the developmental series and the different organ types. Auxin signalling candidates that clustered together in both the berries and leaves are discussion in Section 4.4.2.4.

4.3.3.1 There are eight auxin-related expression pattern clusters during berry development

A heatmap generated with the berry expression data for all *VviARF, VviIAA* and *VviAFB* genes can be divided into eight clusters across the 16 week developmental series (Figure 4.8 and Figure 4.9). Most of the genes are up-regulated early in berry development, including Clusters 4, 5, 6, 7 and 8, and are down-regulated during the ripening phase. However, some genes in Cluster 7 are up-regulated a little later in ripening. The remainder of the genes in Clusters 1, 2 and 3 are up-regulated during ripening, particularly later in ripening, but the single gene in Cluster 1, *VviAFB9*, is also expressed early in development. Cluster 2 contains *VviARF2b* and *VviIAA15b* which were the only candidates that showed high levels of up-regulation during the lag phase and at veraison. The largest cluster is Cluster 6, containing nine *VviARF*, five *VviIAA* and four *VviAFB* candidates. Five of the six *ARF* activators are present in Cluster 6, *VviARF27* is the only ARF activator in a separate cluster having a peak in transcript levels post-veraison. The presence of nine *VviARF*, five of which are *ARF* activators, and five *VviIAA* within Cluster 6 represents a large number of potential interacting partners. Cluster 7 is the second largest cluster, containing seven *VviARF*, seven *VviIAA*, and one *VviAFB*.

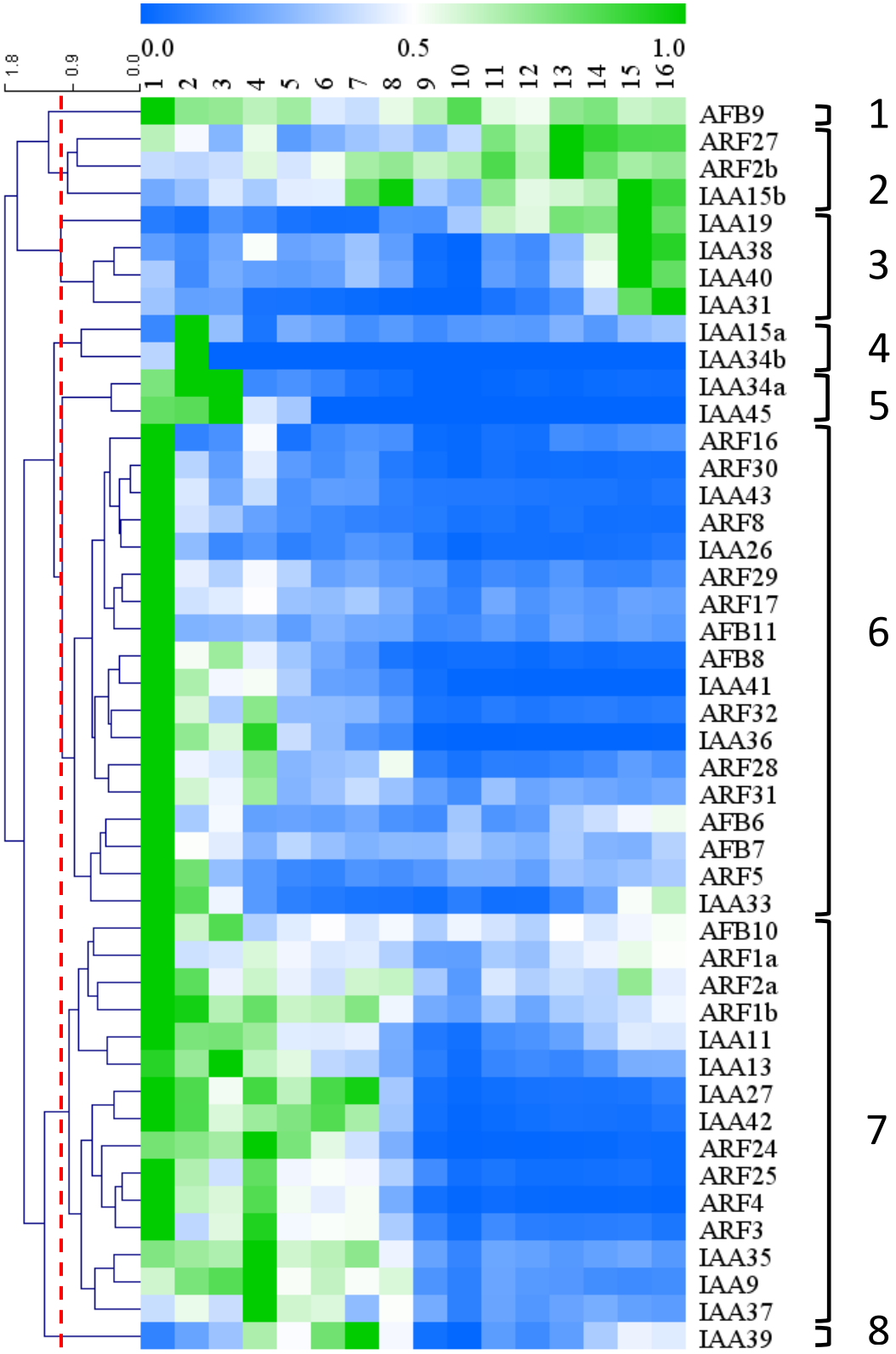
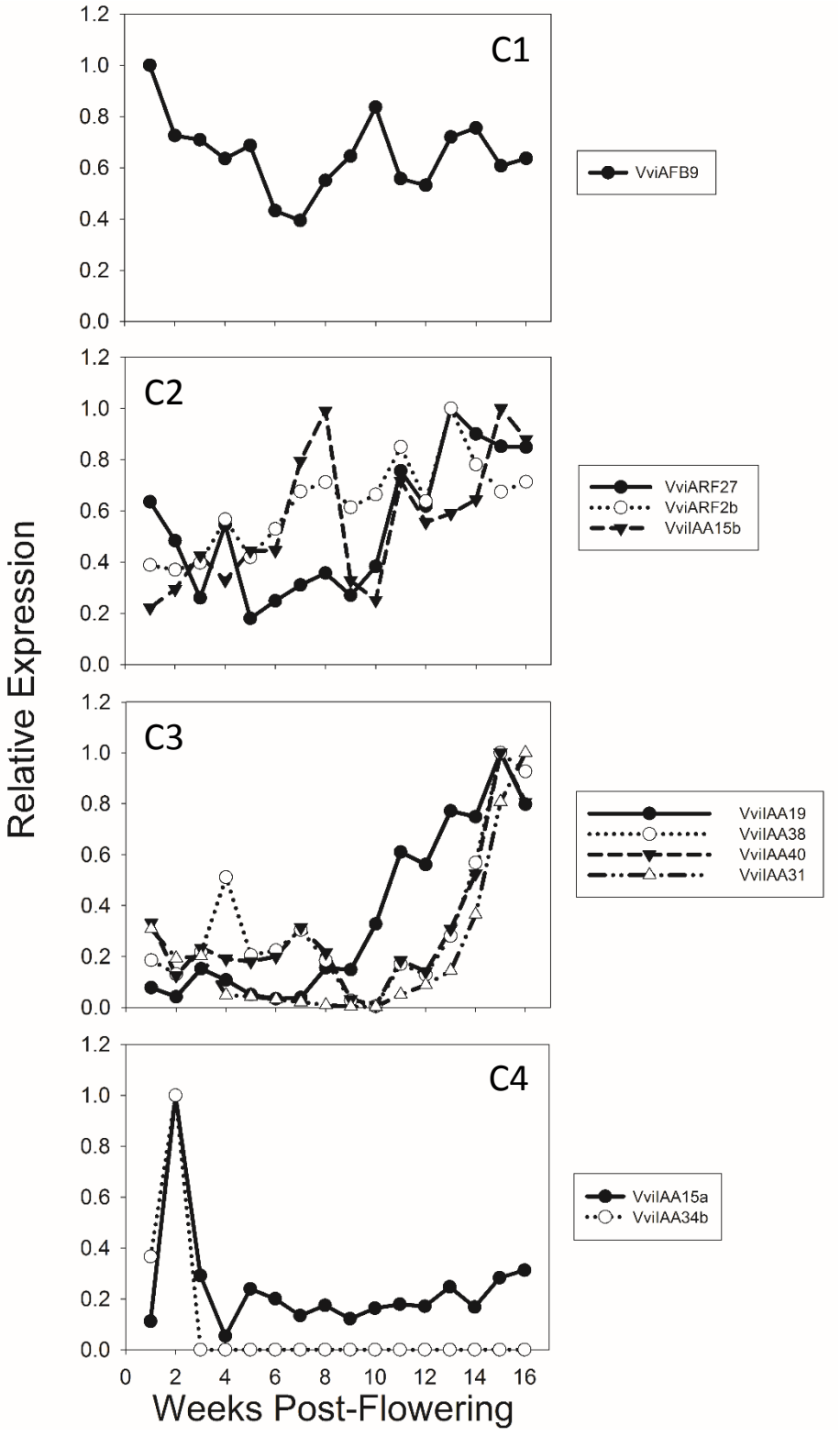


Figure 4.8 Hierarchical clustering tree and heatmap of all *VviAFB*, *VviARF*, and *VviIAA* transcript profiles normalised between zero and one in *V. vinifera* L. cv. Shiraz berries across sixteen weeks post flowering.

The hierarchical clustering tree is shown on the left generated using MultiExperiment Viewer (Saeed *et al.*, 2003), using *Gene tree selection* for tree selection, *optimise by gene leaf order* for ordering optimisation, *Euclidean distance* was used as the distance metric selection, and *average linkage clustering* was used as the linkage method selection. The values above the tree indicate the distance between transcriptional profiles, computed as distance

linkage. Clusters are determined as those that branch below a linkage distance of ~1, as indicated by the red dashed line and are labelled on the far right. The colour scale labelled 0.0 to 1.0 represents the normalised transcript values, with blue indicating low levels and green high levels of relative expression.


C1
C2
C3
C4
Relative Expression
Weeks Post-Flowering
VviAFB9
VviARF27
VviARF2b
VviIAA15b
VviIAA19
VviIAA38
VviIAA40
VviIAA31
VviIAA15a
VviIAA34b

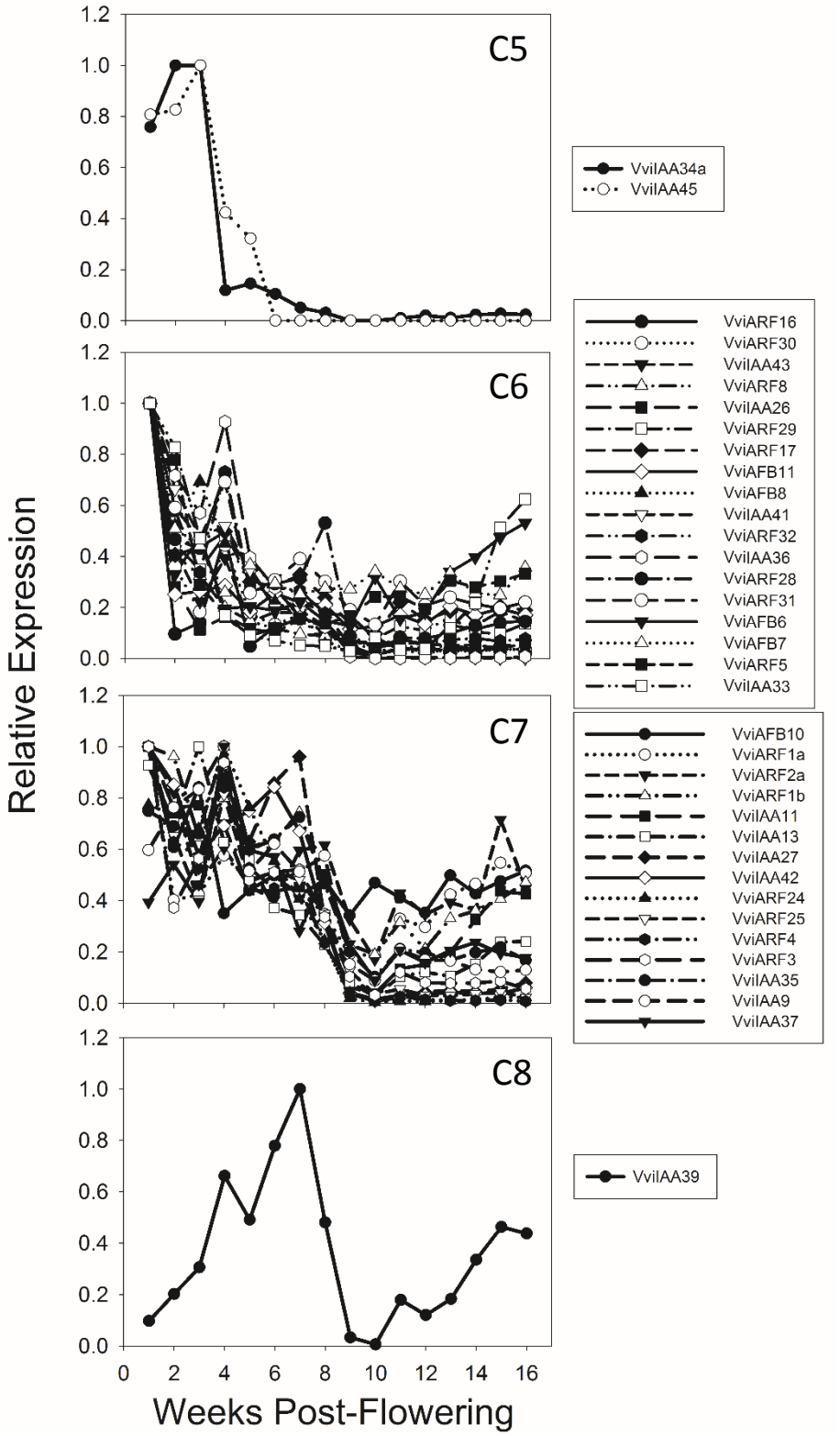


Figure 4.9 The *VviAFB*, *VviARF*, and *VviIAA* transcriptional profiles clusters within Figure 8 hierarchical clustering in MultiExperiment Viewer across a *V. vinifera* L. cv. Shiraz sixteen week berry developmental series.

The transcriptional profiles were normalised by scaling between 0 and 1.

4.3.3.2 There are twelve diverse leaf expression pattern clusters

The heatmap generated with the leaf expression data can be divided into 12 main clusters across leaf development (Figure 4.10 and Figure 4.11). Clusters 1 and 2 contain transcripts that were mostly highly expressed in most stages between leaves 6 – 9. Clusters 3, 4 and 5 contain transcripts that were most abundant in the middle of the leaf developmental series. Transcripts in Clusters 5, 6, 7 and 8 were expressed across most developmental leaf stages. Cluster 8 contains six *VviARF* candidates characterised by a unique expression pattern, with a cyclical pattern alternating low expression in leaves one, three, five, seven and nine and high expression in leaves two, four, six and eight. Transcripts in Clusters 9, 10, 11 and 12 were mainly expressed in leaf stages 1 – 4. The single transcript in Cluster 12 also had a second peak of expression in the leaf 9.

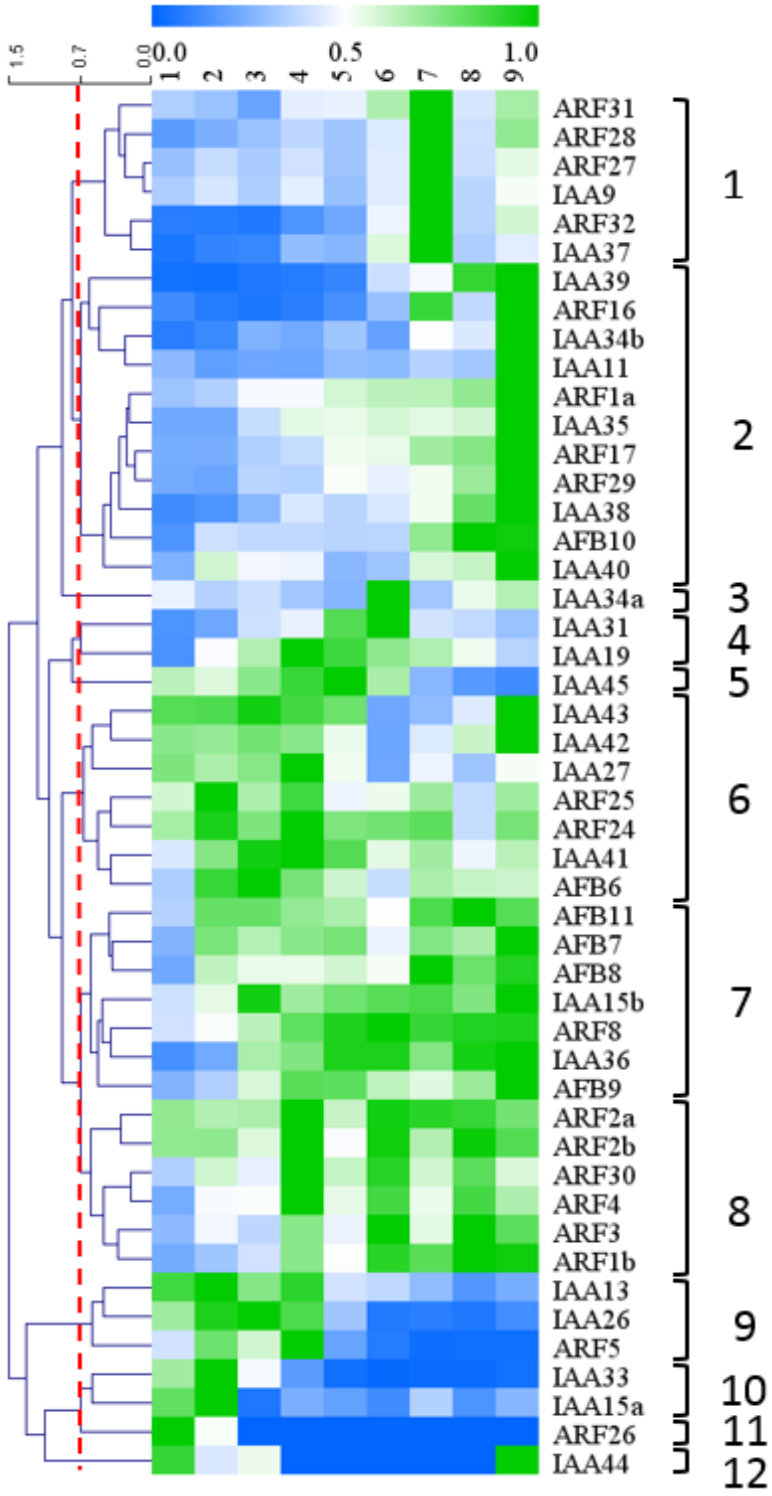
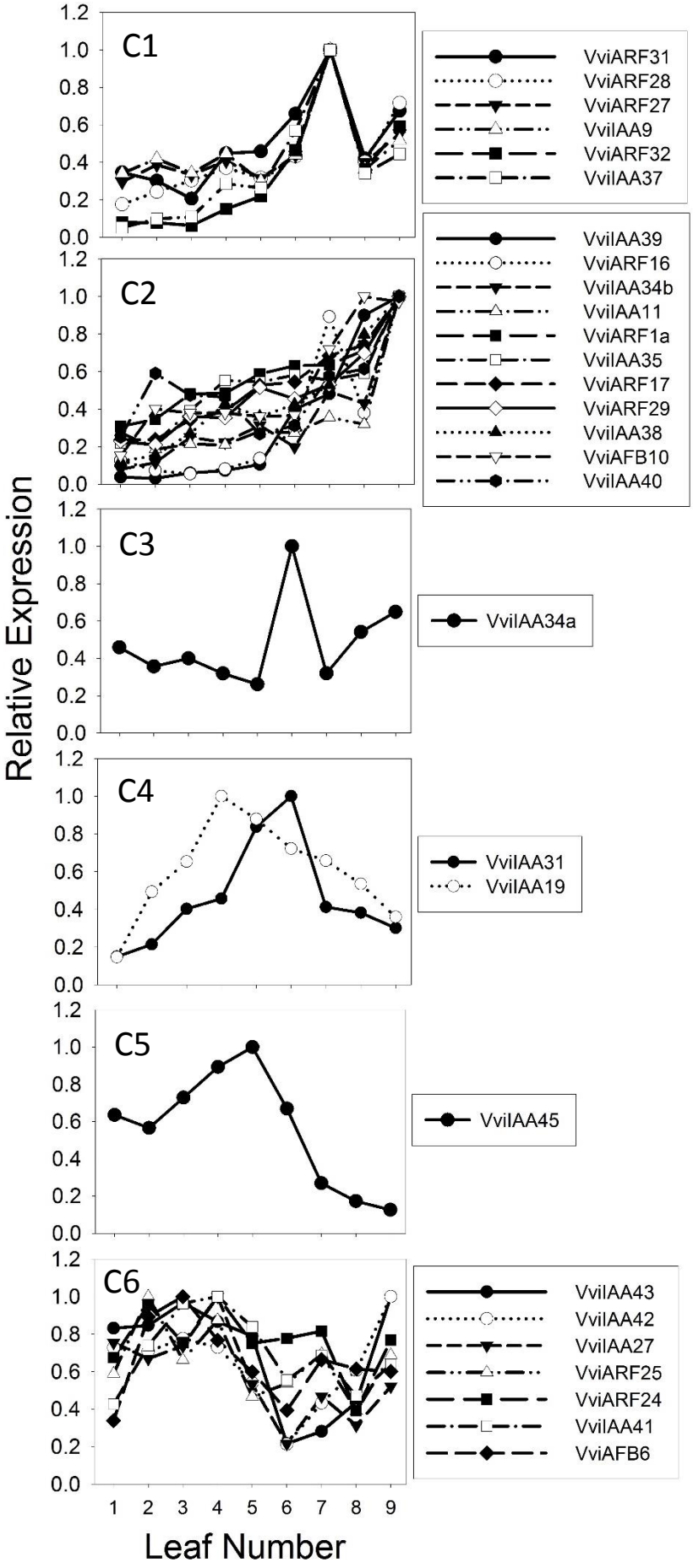


Figure 4.10 Hierarchical clustering tree and heatmap of all *VviAFB*, *VviARF*, and *VviIAA* transcript profiles normalised between zero and one in *V. vinifera* L. cv. Shiraz leaves across nine leaf stages.

Refer to Figure 4.8 for figure construction. Clusters are determined as those that branch below a linkage distance of ~0.8, as indicated by the red dashed line.


C1
VviARF31
VviARF28
VviARF27
VviIAA9
VviARF32
VviIAA37
C2
VviIAA39
VviARF16
VviIAA34b
VviIAA11
VviARF1a
VviIAA35
VviARF17
VviARF29
VviIAA38
VviAFB10
VviIAA40
C3
VviIAA34a
C4
VviIAA31
VviIAA19
C5
VviIAA45
C6
VviIAA43
VviIAA42
VviIAA27
VviARF25
VviARF24
VviIAA41
VviAFB6
Relative Expression
Leaf Number
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1 2 3 4 5 6 7 8 9

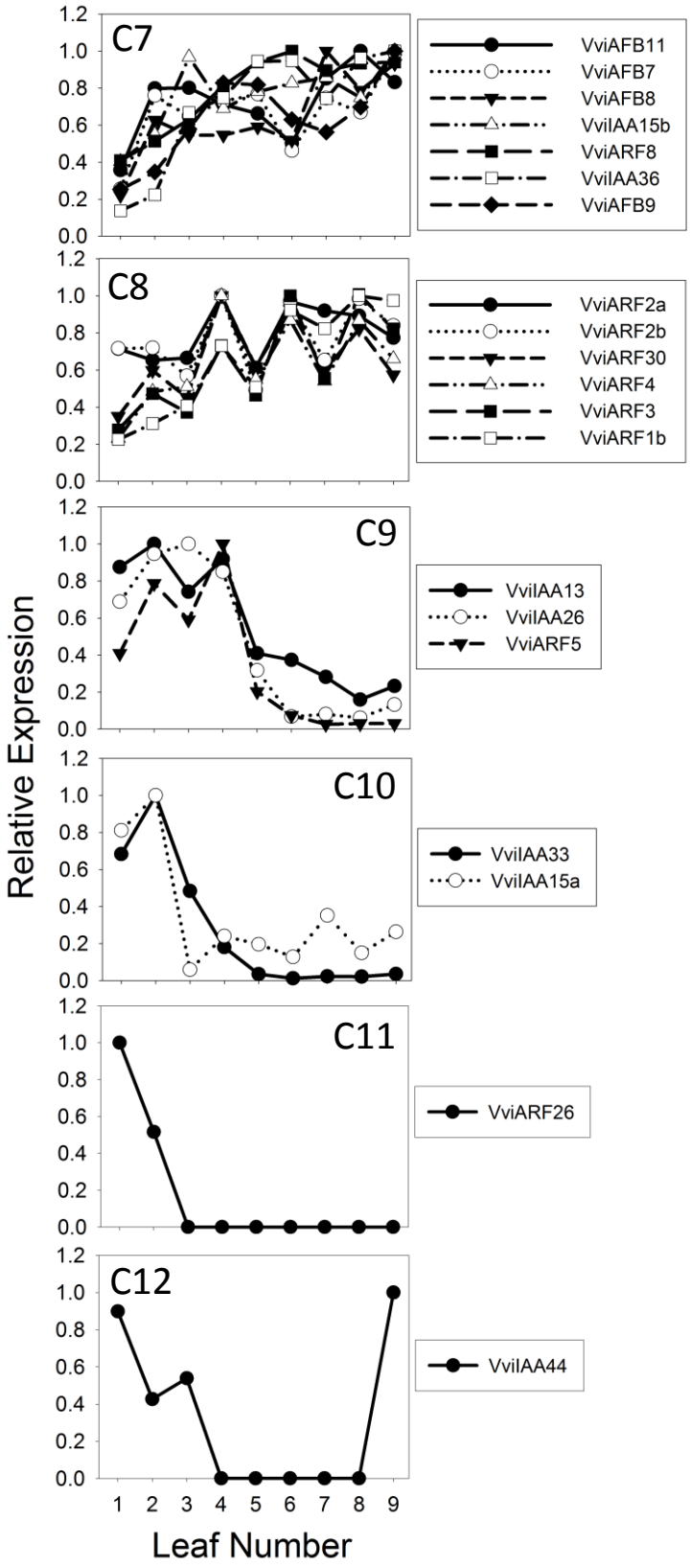


Figure 4.11 A selection *VviAFB*, *VviARF*, and *VviIAA* transcriptional profiles that form clusters within Figure 10 hierarchical clustering in MultiExperiment Viewer across a *V. vinifera* L. cv. Shiraz leaves across nine leaf stages.

The transcriptional profiles were normalised by scaling between 0 and 1. Clusters containing three or more candidates with a cluster linkage distance of <0.8 on the hierarchical tree are shown.

4.3.3.3 Expression patterns of auxin signalling genes in *V. vinifera* flowers, tendrils and roots

The heatmap generated with the transcriptional profiles from the flowers, tendrils and roots identified seven clusters (Figure 4.12). The data was reordered from the data above (flowers, roots, then tendrils) to flowers, tendrils then roots to group flowers and tendrils together, as tendrils are modified flowering shoots and are homologous organs (Srinivasan & Mullins, 1978). Clusters 1 and 2 contain all transcripts that were most highly expressed within the flower. Cluster 3 contains transcripts that are most abundant in the flowers and roots. Cluster 4 contains candidates that were highly expressed in flowers, tendrils and roots. Cluster 5 contains seven candidates which had high expression in the tendrils and roots. Cluster 6 contains eight candidate genes all with high expression levels in the tendrils. Interestingly, the smallest cluster, Cluster 7, contained the *ARF* activator *VviARF30* which was the only gene highly expressed in both flowers and tendrils but not in roots. Most of the genes were preferentially expressed in flowers with 16 candidates having the highest transcript levels in flowers.

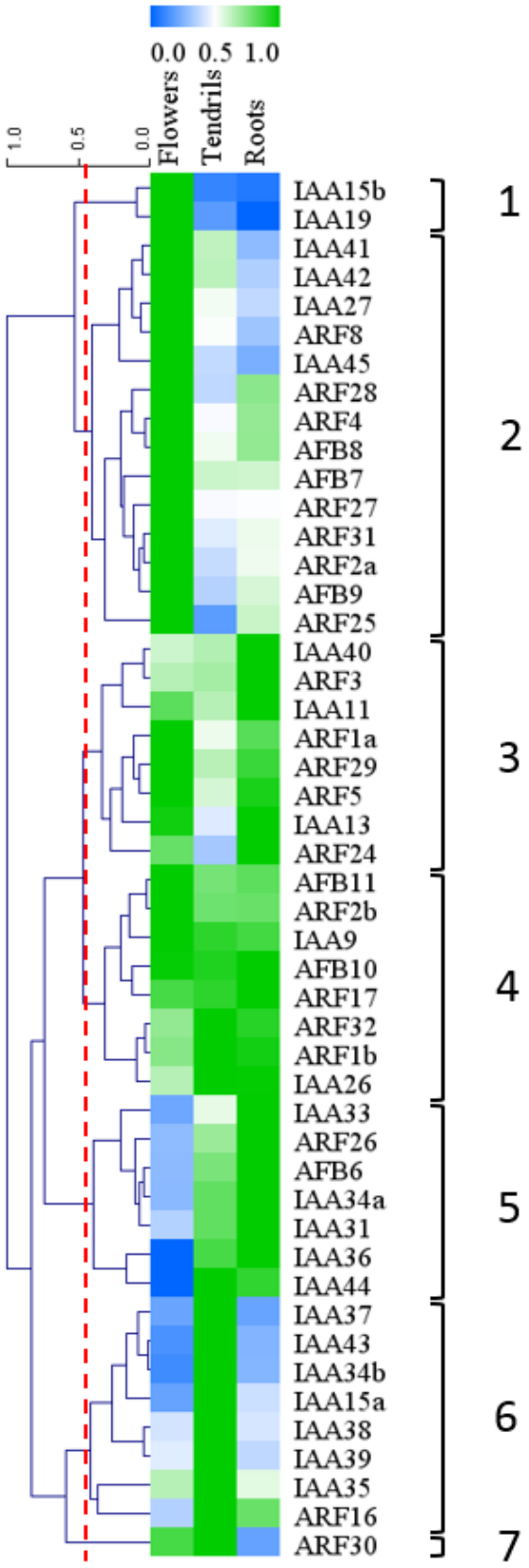


Figure 4.12 Hierarchical clustering tree and heatmap of all *VviAFB*, *VviARF*, and *VviIAA* transcript profiles normalised between zero and one in *V. vinifera* L. cv. Shiraz flowers, tendrils and roots.

Refer to Figure 4.8 for figure construction. Clusters are determined as those that branch below a linkage distance of ~0.5, as indicated by the red dashed line.

4.4 Discussion

Across many studies that identify and characterise *ARF* and *Aux/IAA* candidates through whole genome analysis (Chapter 3, Section 3.2) often only a subset of the candidates were selected for gene expression analysis, or the analysis was only completed on a few organs types, often not whole developmental series. Within this work, the expression patterns were determined for all *VviARF*, *VviIAA*, and *VviAFB* candidates across berry and leaf developmental series, and within three additional organ types – the flowers, tendrils and roots, providing a comprehensive description of the transcript profiles within grape. It is important to note however, that the sampling of whole organs leads to a mixture of tissues and cell types present within each sample, including exocarp (skin), mesocarp (flesh), seeds in the berries (weeks 1–3) and vasculature in berries and leaves, as well as a mixture of cell types within leaves. Therefore, gene transcripts that were found to be lowly expressed within this work may play important roles in a tissue or cell type that comprises only a small portion of the whole organ and may therefore, have been diluted in the sampling within this study. Further analysis would be required to establish tissue-specific functions of the auxin signalling pathway family members. It is also important to note that transcriptional regulation is only part of the complex regulatory process as other factors such as protein synthesis and stability are also important but were not tested in this work.

The sequences used for designing the qPCR primers were based on predicted gene sequences. However, as indicated in Chapter 5 there were differences between the predicted and actual gene sequences in a select number of the auxin signalling transcripts. One candidate has two SNPs located in the 3' region where the qPCR primers are often localised, indicating that it is possible that sequence differences may have influenced the products isolated by qPCR analysis. Without isolating all sequences, including splice variants, it is difficult to determine what influence these differences would have on the transcript expression data. Future RNAseq data would help to determine the accuracy of the qPCR data. It is also important to note that qPCR primer sets were optimised to amplify single products. PCR products that were used as the standards for each gene were sequenced and compared to the predicted gene sequences, and all 48 products were as predicted. SNPs would have been detected via sequencing or in variations in the qPCR melt curves. The presence of any splice variants which may have altered the expression profiles of the transcripts was not determined. Future investigation into splice variants may add an additional layer of complexity and regulation to the auxin signalling pathway.

4.4.1 IAA and IAA-Asp concentrations are inversely correlated in berries and positively correlated in leaves

Böttcher *et al.* (2010b) reported a high IAA concentration at fruit set decreasing towards veraison and remaining low through ripening and an IAA-Asp concentration that was low in pre-veraison berries, increasing at veraison reaching maximum concentration at 12 WPF then slowly declining again towards harvest in *V. vinifiera* L. cv. Cabernet Sauvignon (Figure 1.5). The same patterns of IAA and IAA-Asp concentrations were seen in this work with inversely correlated concentrations across development. However, the concentration of IAA was lower in Shiraz berries compared to Cabernet Sauvignon, and the opposite was the case with IAA-Asp concentrations, where Shiraz was higher than Cabernet Sauvignon (Böttcher *et al.,* 2010b). These results suggest that the patterns of IAA and IAA-Asp accumulation are the same across grape varieties, but the concentrations vary and need to be independently addressed in each variety. Symons *et al.* (2006) stated that the IAA concentration was relatively low and constant during berry development, which is in contrast to that reported in Böttcher *et al.* (2010b) and within this work. The low concentration of IAA measured by Symons *et al.* (2006) could be attributed to the different extraction process used to isolate IAA. During the extraction process in this work it became clear that the measurements of IAA and IAA-Asp can be inhibited in the early stages of berry development, perhaps due to high levels of tannins. For this reason, lower amounts of tissue were used for samples from early stages enabling the IAA and IAA-Asp concentration to be quantified (Section 2.2.8.1).

IAA concentrations are high pre-veraison, when cell division and cell expansion are known to occur, and IAA-conjugation occurs post-veraison, increasing the concentration of IAA-Asp within the berries and leading to the observed decrease in IAA concentration. Böttcher *et al.* (2010b) suggested that the ratio between IAA and its conjugated forms, such as IAA-Asp, may be important for the regulation of ripening. However, *VviGH3-1* levels are also high pre-veraison between 0–2 WPF in Cabernet Sauvignon berries when free IAA concentration are high and IAA-Asp levels are low, suggesting that the role of the GH3 proteins and the IAA-Asp conjugate appear more complex than simply reducing free IAA concentration (Böttcher *et al.*, 2010b). Additional roles of IAA-Asp in IAA inactivation or as a biologically active molecule, remain to be fully elucidated, although oxidation of IAA-Asp and subsequent catabolism of IAA has been reported (Östin *et al.,* 1998; Staswick *et al.,* 2005). It is possible that the conjugate is catabolised before veraison but not after. This further indicates the complexity of phytohormone regulation and suggests a complex role for auxin and its conjugates in berry development.

In berries, the pattern of IAA accumulation clustered with Cluster 6 (Figure 4.8 and Figure 4.9), which contains nine *VviARF* candidates, including five of the six *ARF* activators (*VviARF5, 8, 28, 29,* and *30*) and *VviARF16, 17, 31* and *32*, five *VviIAA* candidates *(VviIAA26, 33, 36, 41,* and *43*), and four *VviAFB* candidates (*VviAFB6, 7, 8*, and *11*) (Appendix E, Figure E.1). The VviIAA candidates may regulate the VviARF activators within this cluster, and also be interacting with the VviAFB candidates to form auxin co-receptors. As all transcripts in Cluster 6 had their highest expression levels at one WPF, these transcripts may be involved in auxin-responsive cell division, and cell expansion that is also occurring during this time. Similarly, *AFB* transcripts were also seen to correlate with IAA accumulation in young plum fruit (El-Sharkawy *et al.,* 2014). The IAA-Asp concentration in berry development clustered with Cluster 3 which contains four *VviIAA* candidates, *VviIAA19, 31, 38* and *40* (Figure 3.8 and Figure 3.9, Appendix E, Figure E.1). These transcripts had high levels of expression post-veraison. *VviARF27* exhibited an expression pattern similar to these four VviIAA candidates, and as an ARF activator, it can be hypothesised that these VviIAA candidates may interact with and regulate VviARF27 and that IAA-Asp concentrations may contribute to their function.

In contrast to the berries, the patterns of IAA and IAA-Asp in leaves followed the same pattern across the nine leaf stages and the concentration of IAA was higher than IAA-Asp (maximum concentration of ~1400 pmol/gFW^{-1} and ~250 pmol/gFW^{-1}, respectively Figure 4.1_B). Both IAA and IAA-Asp peaked in leaf three before decreasing towards leaf nine. Sharing this pattern with IAA and IAA-Asp were *VviIAA13, 26* and *27* (Appendix E, Figure E.2), even though the specific clusters were not identical. *VviIAA26* also clustered with IAA levels within the berry developmental series, suggesting that it may be playing similar roles in both organ types. The clustering of these *VviIAA* candidates with the IAA concentration suggests that they may interact together in the formation of an auxin co-receptor. The putative *ARF* activator *VviARF5* shares a similar expression pattern to IAA and IAA-Asp, and clustered with *VviIAA13* and *36* in Cluster 9 (Figure 4.10 and Figure 4.11) and it is possible that these VviIAA candidates regulate VviARF5 activity, potentially during cell division and cell expansion in leaf development.

4.4.2 Relating transcriptional expression patterns to biological functions

All *VviAFB* candidates were highly expressed in berries, leaves, flowers, tendrils and roots (Figure 4.2 and Figure 4.3). Similarly, all *VviARF* and *VviIAA* candidates were expressed across leaf development or within flowers, tendrils and roots (Figure 4.5 and Figure 4.7). However, within berry development 18 of the 19 *VviARF* candidates were expressed (with the exception of *VviARF26*), and 22 of the 23 *VviIAA* candidates were expressed (with the exception of *VviIAA44*, Figure 4.4 and Figure 4.6). In this

chapter the transcript accumulation patterns were analysed to indicate possible gene function however, it is acknowledged that transcriptomics are only part of a larger story and that this putative data would need to be confirmed by both protein analysis and functional data. Unfortunately, based on the number of gene candidates analysed within this work, an in-depth approach to confirm protein functionality was not possible.

4.4.2.1 Auxin signalling pathway candidate transcripts can be linked to biological functions within berry development

As described in Chapter 1, grape berry development can be divided into three stages (Section 1.1.2, Figure 1.1) (Coombe, 1987; Kennedy, 2002). Within stage one, cell division is rapid and all berry cells are established within the fruit two weeks after flowering. Berry expansion occurs over an approximately eight week pre-veraison period, increasing berry size, and corresponding to high levels of free IAA (Figure 3.5) (Coombe, 1992; Böttcher *et al.,* 2010b). By examining the expression profiles of the auxin signalling pathway candidates during this period we may be able to identify key genes that are targeted and regulated by the high IAA concentrations during this time. Auxin signalling pathway candidates were predominantly expressed during stage one, with 37 of the 46 candidates having their highest peak of expression during this phase of berry development (Figure 4.8). Furthermore, these candidates tended to have high levels of expression pre-veraison, with minimal or low expression post-veraison, as illustrated in Clusters 4, 5, 6, and 7 (Figure 4.9). Candidates in Clusters 4, 5, and 6 reached a peak in expression prior to four WPF, suggesting that these candidates could be playing roles in auxin-mediated cell division and/or cell expansion. Candidates in Cluster 7 had elevated expression for the entire pre-veraison period, rapidly decreased expression between weeks eight and nine and remained low from this point onwards. This may indicate a role in pre-veraison berry expansion, potentially through the modification of xyloglucans or the decrease in arabinogalactans from pre- to post-veraison to allow for later berry softening (Nunan *et al.,* 1998; Deluc *et al.,* 2007). Xyloglucans make up ~10% of the cell wall composition in berries, with depolymerisation of xyloglucans being associated with fruit softening (Nunan *et al.,* 1998). Deluc *et al.* (2007) found that the majority of xyloglucan endotransglycosylases (XET) that hydrolyze and transglycosylate xyloglucans were found to be highly expressed in stage one of berry development before declining, suggesting that modification of xyloglucans is involved in cell wall expansion during this developmental stage (Fry *et al.*, 1993; Nunan *et al.,* 1998). Some candidates in Clusters 6 and 7 declined steadily across development. This is somewhat similar to proteins associated with photosynthesis-related functions that are highly expressed in stage one, with a steady decline across berry development (Deluc *et al.,* 2007). Malate and tartrate are synthesized and reach their maximum concentrations by the end of stage one, tartrate synthesis transcripts are found most highly in the

seeds and the pulp around the seeds which are present within this study only in berries one–three WPF (Possner & Kliewer, 1985; Deluc *et al.,* 2007; Grimplet *et al.,* 2007). The biosynthesis of tannins in the exocarp and hydroxycinnamates (the precursors for phenolic volatiles) occurs primarily in stage one. Pilati *et al.* (2007) also found that stage one of berry development was enriched in genes that are involved in regulatory mechanisms, suggests strong cell reprogramming taking place in berry cells up to veraison.

The second stage of berry development is the pre-veraison lag phase where there is little or no increase in berry size (Coombe, 1992). All transcripts, excluding *VviAFB9* and *VviARF2b*, showed a decrease in expression during the lag phase and many transcripts were expressed at low levels from this stage onwards. In Stage two there are a number of changes occurring within the berry, including the negative regulation of genes involved in cell division and the loss of photosynthetic capacity (Pilati *et al.,* 2007). The *VviIAA15b, 19, 31, 38, 39, 40, VviARF27 (A)* and *VviAFB9* transcripts from Clusters 1, 2, 3, and 8 showed a decrease in expression during this lag phase, before increasing again post-veraison (Figure 4.9). As cells are expanding pre-veraison and post-veraison, with minimal growth at veraison, these candidates may be involved in cell expansion.

Stage three is the post-veraison stage in which berry ripening occurs. Clusters 2, and 3 all contain transcripts up-regulated during the post-veraison period (Figure 4.8), and they may play roles in regulating ripening processes. Berry ripening is characterised by an increase in the accumulation of the hexose sugars, glucose and fructose, the synthesis and accumulation of anthocyanins, transport and synthesis of the metabolites responsible for volatile aroma and flavour compounds, such as terpenes, benzenoids, and phenylpropanoids, and the second phase of cell expansion and berry softening (Coombe, 1960a; 1960b; Coombe, 1987; Coombe, 1992; Coombe, 1995; Pratt, 1971; Coombe & McCarthy, 2000; Kennedy, 2002; Deluc *et al.,* 2007; Pilati *et al.,* 2007). Cluster 3 contains four *VviIAA* transcripts, *VviIAA19, 31, 38* and *40*, which peaked in expression post-veraison and whether this is ARF-dependent or independent needs to be established. Cluster 2 contains the *ARF* activator *VviARF27*, which shares a similar expression profile to these transcripts and may be their target and the *ARF* repressor, *VviARF2b,* which also has a peak of expression post-veraison. *VviARF2b* homologues in tomato, *SlARF2A* and *2B* also show an increase in transcript accumulation throughout tomato fruit ripening, highlighting the potential conservation of some genes between plant species (Liu *et al.,* 2015). *VviIAA15b* is also present in Cluster 2 and may interact with these VviARF. The expression pattern of *VviIAA19* follows the accumulation of anthocyanins and would be an interesting transcript for further research. As the Cluster 3 transcripts were highest post-veraison, they may be involved in post-veraison specific functions. Interestingly, the synthetic auxin 2,4-D has been

associated with anthocyanin production in strawberry cell cultures (Mori *et al.,* 1994), and IAA, NAA and 2,4-D are thought to increase anthocyanin accumulation in mutant Arabidopsis calli dependent on the concentration (Liu *et al.*, 2014). This suggests that the low concentrations of IAA in post-veraison berries could be associated with anthocyanin accumulation, possibly through *VviIAA15b, 38*, and *40*, which were all up-regulated by NAA post-veraison (Chapter 6), but further studies would need to confirm this in grape. Transcripts in Cluster 1 and 8 were also expressed during Stage 3, as were certain transcripts in Clusters 6 and 7, indicating that although the majority of auxin signalling transcripts are likely to be functioning pre-veraison, whilst free IAA levels are high, they may also have roles post-veraison. Transcripts in Clusters 6 and 7 may be involved in post-veraison cell expansion. Berry softening is thought to occur largely due to loss of turgor and cell wall loosening, the high levels of expansins in stage three suggest that the cell wall loosening during fruit ripening is occurs through non-enzymatic mechanisms, unlike cell wall changes in stage one (Nunan *et al.,* 1998; Thomas *et al.,* 2006; Deluc *et al.,* 2007).

4.4.2.2 Auxin signalling pathway candidates display a diverse range of transcription patterns during leaf development

There are two major phases of leaf growth, the initial cell division phase followed by a phase of cell expansion, occurring early in leaf development while auxin levels are high (reviewed in Kalve *et al.,* 2014). Auxin is thought to play roles in leaf initiation, cell division and expansion, cytoplasmic growth, vascular differentiation, and marginal patterning and leaf serration within the leaves (Kalve *et al.,* 2014; Bar & Ori, 2014). Auxin has been observed to decrease in concentration during leaf expansion, consistent with the pattern seen within this work (Figure 4.1_B) (Kalve *et al.,* 2014). The levels of photosynthesis are low in recently unfolded leaves, but increase during leaf expansion as the leaves transition from sink to source, the nature of CO_2 fixation also changing depending on leaf age (Kriedemann *et al.,* 1970). The internal structure of leaves changes with age, as cells become less-densely packed potentially allowing for higher rates of CO_2 exchange.

IAA concentration during leaf development clustered with *VviIAA13, 26,* and *27* in Clusters 6 and 9 (Figure 4.9, Appendix E, Figure E.2), suggesting that these candidates may play roles in IAA-mediated cell division and expansion. Arabidopsis AtARF5 (MONOPTEROS) is thought to mediate the activity of auxin in organ initiation (Hardtke & Berleth, 1998; Yamaguchi *et al.*, 2013) and AtARF5's closest homolog, *VviARF5*, is also highly expressed early in leaf development, in stages 1 to 4 in Cluster 9. The high peak of expression in leaf 2 seen in Cluster 10 is reminiscent of the patterns seen across berry development, and the *VviIAA15a* and *33* candidates in this cluster may also be involved in cell division. Candidates in Cluster 2 and 7 have a general increasing trend across leaf development, suggesting they may have roles in leaf maturity, sugar synthesis and export, and photosynthesis. In Cluster 8, six

VviARF candidates have a very unique alternating low-high pattern occurring across leaf development, which to our knowledge has not been reported previously. This cyclical pattern of expression suggests some specialised role, possibly in phyllotaxy. The phyllotaxy in grapevines alters as the vines age; in mature vines the leaves are produced alternately, with single leaves at each node on alternating sides of the shoot (180° angles) (Keller, 2015). In Cluster 1, there was a general trend of increasing transcript levels toward leaf seven, followed by a decrease in expression in four *VviARF (VviARF27 (A), 28 (A), 31,* and *32*) and two *VviIAA* (*VviIAA9* and *37*) candidates, suggesting functional significance of this pattern. The closest Arabidopsis homologues to *VviARF27*, AtARF7 and 19, have been found to act redundantly in controlling leaf expansion (Wilmoth *et al.,* 2005), while Tomato SlIAA9, closest in homology to VviIAA9, is thought to inhibit the auxin response to restrict lamina growth between developing leaflets (Figure 3.9 and Figure 3.10) (reviewed in Bar & Ori, 2014). In Clusters 4 and 5, *VviIAA19, 31,* and *45* have a peak of expression around the middle of leaf development, these may be involved in the transition of leaves from sinks to sources which potentially occurs halfway through the developmental series (Figure 4.11). Measurements of the physiological states, including sink/source status of the leaves, and linking these to the leaf developmental stages would be necessary to support the previous statements about the potential function of the auxin signalling pathway genes.

4.4.2.3 The comparison of *V. vinifera* auxin signalling candidates in flowers, tendrils and roots with known functions in other species

Flowers, tendrils and roots transcript data can be compared and contrasted to other species to infer potential functions. Arabidopsis AtARF6 and AtARF8 have been linked with cell division related to fertilisation, stamen elongation and floral maturation (Nagpal *et al.,* 2005; Goetz *et al.,* 2006) and show homology to ARF activators *VviARF8, 29* and *30* (Figure 3.9). All three ARF candidates exhibited high expression levels in flowers and it is possible that the function in Arabidopsis flowers is conserved and these ARFs may also be involved in cell division during flower development and maturation (Nagpal *et al.,* 2005; Goetz *et al.,* 2006). In tomato, SlARF7 has been found to be a negative regulator of fruit set, with high expression in flowers and pollination causing the down-regulation of gene expression (Vriezen *et al.,* 2008; de Jong *et al.,* 2009; 2011). Mutant analysis suggests that SlARF7 may play roles in enhancing cell division and repressing cell expansion (Vriezen *et al.,* 2008; de Jong *et al.,* 2009; 2011). The closest homologues in *V. vinifera* are *VviARF27* and *28*, both of which are present in Cluster 2 and were highly expressed in both flowers and roots, suggesting that they may also have roles in cell division in these organs. The tomato Aux/IAA SlIAA9 has been identified as a repressor of auxin-induced gene expression, and like SlARF7, has been found to play roles in fruit set (Wang *et al.,* 2005, 2009). SlIAA9 shares closest homology to *VviIAA9*, which is present in Cluster 4 and had high expression across flowers, tendrils and roots, suggesting a potentially conserved function. Arabidopsis

AtARF16 has been found to play roles in root cap cell differentiation (Wang *et al.,* 2005), and has the closest homology to *VviARF32* that had consistently high levels across flowers, tendrils and roots in Cluster 4. Flower-enriched expression was the most common organ specificity with 16 candidates having flower expression as their highest organ expression. This correlates with the fact that auxin is produced and released by floral meristems and controls the formation and differentiation of flowers, as well as inducing the development of their vascular tissue (Keller, 2015).

4.4.2.4 Are the auxin signalling pathway candidates playing the same roles in multiple organ types and clustering together?

The transcription patterns were compared across the three sets of data to see if any of the candidates might play a similar role across multiple organs, based on expression data alone. Based on their transcript profiles, VviIAA15a, 26, 33, 41, and 43, the ARF activators VviARF5, 8, 29, and 30, and VviAFB6 may play roles in cell division and expansion in two or more organs, including berry development, leaf development and/or flowers and VviIAA15b, 38, and 40 possibly play roles in maturity in both the berry and leaf developmental series. The berry and leaf clusters were compared to identify candidates that were co-clustered in both series (Figure 4.9 and Figure 4.11). Eleven sets of candidates were co-clustered in both berries and leaves: *VviARF28 (A), 31* and *32*; *VviIAA9* and *37*; *VviIAA27* and *42*; *VviARF1b* and *2a*; *VviARF3* and *4*; *VviARF24* and *25*; *VviIAA38* and *40*; *VviARF1a* and *VviAFB10*; *VviARF17* and *29 (A); VviIAA36* and *VviAFB7, 8, 11;* and *VviIAA19* and *31*. With the exception of *VviARF1a* and *VviAFB10* and *VviIAA36* and *VviAFB7, 8, 11*, all candidates that were co-clustered in both berries and leaves were candidates within the same protein families. This suggests that there may be some functional redundancy or interaction between family members. The co-clustering of *VviIAA36* and *VviAFB7, 8* and *11* suggests that these candidates may act together as a co-receptor complex. Only *VviARF28 (A)* and *31*, and *VviIAA27* and *42* are co-clustered in berry and leaf developmental series and within the flower, tendril and root samples, indicating that the auxin signalling pathway tends not to have modules of interacting genes that are used for the same role e.g. controlling cell expansion in a range of organs.

4.4.3 Overlap between this work and previously reported *V. vinifera* transcript levels

Fujita *et al.* (2012) and Kohno *et al.* (2012) completed in-depth analyses on *VviIAA9* and *VviIAA19*, respectively, in *V. vinifera* cv. Chardonnay. *VvAux/IAA4* described in Çakir *et al.* (2012) represents the same transcript as *VviIAA9*. Despite differences in the length of the developmental phases the patterns of gene expression of both *VviIAA9* and *VviIAA19* during the berry developmental series in these publications and this work were consistent (Figure 4.6). Deluc *et al.* (2007) and Wan *et al.* (2014) reported the transcriptional patterns for nine of the 19 VviARFs. Wan *et al.* (2014) found consistency

between the microarray results and qPCR analysis for four of the nine genes described by Deluc *et al.* 2007, meaning the remaining five were inconsistent and this was attributed to environmental differences in the growing conditions. However, there was a high level of consistency with the Deluc *et al.* (2007) results and the expression data within this work. *VviARF4, 8* and *24* all decreased in expression level across berry development, *VviARF2a* and *2b* were expressed through berry development. Whilst *VviARF5* was expressed throughout berry development the transcript levels were not particularly high. *VviARF1b*, *3* and *28* were reported to have no significant change across berry development. Although this was not the pattern seen within this work, it must be noted that if only five time points are selected, these three *VviARF* transcripts undergo periods of fluctuating transcript levels. It appears that the time points for Deluc *et al.* (2007) may have been at stages when the transcripts are particularly low, which may make the pattern appear to be unchanged across development. The inconsistencies between the expression data sets suggests that Deluc *et al.* (2007) and this work isolated the same products using microarray data and qPCR analysis, respectively, however Wan *et al.* (2014) may have isolated different qPCR products for five of the nine genes. It would be necessary to compare the sequences from all three sources to confirm this and highlights the possibility of probe ambigituies between studies.

Vitis Affymetrix GeneChip® microarrays have been used in previous studies to determine the mRNA expression profiles from four different grape varieties: Cabernet Sauvignon, Pinot noir, Trincadeira and Muscat Hamburg (Deluc *et al.,* 2007; Pilati *et al.,* 2007; Fortes *et al.,* 2011; Lijavetzky *et al.,* 2012). The expression of all *Aux/IAA*, *ARF* and *AFB* transcripts identified in these studies was consistent with the Shiraz results in this work. Interestingly, *VviARF2b* was reported to be down-regulated post-veraison in Fortes *et al.* (2011), which is contradictory to the findings in this work, however, examination of their supplementary data suggests they may have misinterpreted their results and that they are consistent with this work. These results indicate that there is a high conservation of transcript expression across these five different *V. vinifera* cultivars and the consistency between these results and the qPCR transcript expression in this work suggests a lack of probe ambiguities. However, this would need to be confirmed by comparing the microarray probe sequences and the sequences predicted in this work.

4.4.4 Previously reported phytohormone concentrations compared to berry transcriptional clusters

The accumulation pattern of a selection of phytohormones are shown in Chapter 1, Figure 1.4 (Böttcher & Davies, 2012). ABA and BR have been associated with fruit ripening in grape (Coombe, 1973; Coombe & Hale, 1973; Scienza *et al.*, 1978; Davies *et al.*, 1997; Clouse & Sasse, 1998; Vardhini

& Rao, 2002; Haubrick & Assmann, 2006; Owen *et al.*, 2009; Wheeler *et al.*, 2009; Böttcher & Davies, 2012), and the application of ABA and BR promotes ripening in grape (Chapter 1, Section 1.1.4.2 and 1.1.4.3) (Ban *et al.,* 2003; Jeong *et al.,* 2004; Gény *et al.,* 2004; Symons *et al.,* 2006; Wheeler, 2006; Wheeler *et al.*, 2009; Giribaldi *et al.,* 2010a; Karlova *et al.,* 2014). The expression pattern of the six transcripts in berry development *VviAFB10*, *VviARF1a, 1b, 2b*, and *VviIAA11* and *13*, are similar to the pattern of ABA and BR accumulation indicating a potential relationship between them (Figure 4.8, Figure 4.9, Chapter 1, Figure 1.4). Ethylene concentrations are high early in berry development, decrease, and then show a small peak at veraison Alleweldt & Koch (1977). *VviAFB9* and *VviARF2b* transcripts levels are high during the ethylene peak at veraison, with four additional transcripts; *VviIAA19*, *VviAFB7* and *10*, and *VviARF2a* also expressed at this time, a relationship may exist between them. Determining the responsiveness of these transcripts to phytohormones is necessary to support inferences of linked functionality in grape development and this is reported in Chapter 6.

4.4.5 Linking transcriptional profiles to phylogenetic information

There is a correlation between the transcriptional profiles of some gene transcripts and their location on the phylogenetic trees in Chapter three, Figure 3.8, Figure 3.9 and Figure 3.10. *VviIAA11* and *13* are in the same clade in Figure 3.10, and their expression clustered together in the berry developmental series but not in the leaf series (Figure 3.8 and Figure 3.10), this is also the case for *VviIAA35* and *27*, *VviIAA26* and *43*, *VviIAA27* and *42*, *VviARF16, 31,* and *32, VviARF1a* and *1b*. *VviARF3* and *4*, and *VviIAA38* and *40*, clustered together in both berry and leaf series. *VviARF8* and *29* are present in the same clade and their transcript levels were high in berries and flowers. *VviARF2a* and *2b*, and *VviARF31* and *32* clustered together in leaves. Other closely related genes had different expression patterns in berries, such as *VviARF27* and *28*, however, they have similar patterns within the tissue series. These differences may suggest that the conservation of specific promoter elements with some candidates, and variation in others, leads to differential expression and phytohormone responses in different organ types. The similar transcript profiles and close phylogenetic relationship suggests that there may be some conservation of function between the species included in the phylogeny.

4.4.6 Why are *AFBs*, ARFs and Aux/IAAs co-expressed? Identifying potential interacting pairs

All gene transcripts were present within multiple organs in this work, with a multitude of transcriptional patterns highlighting the complexity of the auxin signalling pathway and suggesting that they are differentially regulated and may play unique roles. In some cases, transcripts could be correlated with phytohormone levels other than auxin suggesting that the transcripts may act downstream of a different stimulus. TIR1/AFB, ARFs and Aux/IAAs are co-expressed as they function

as a regulatory network within the auxin signalling pathway. Aux/IAA and ARF activator proteins interact to repress the auxin responsiveness of the ARF activator, in addition both families are thought to form hetero- and homo-dimers (Tiwari *et al.,* 2004; Szemenyei *et al.*, 2008; Lee *et al.*, 2009; Causier *et al.*, 2012; Piya *et al.*, 2014; Korasick *et al.*, 2014; Nanao *et al.*, 2014; Farcot *et al.,* 2015; Hagen, 2015; Enders & Strader, 2015). TIR1/AFB proteins bind auxin, recruiting Aux/IAA proteins forming co-receptor complexes facilitating the polyubiquitination and degradation of the Aux/IAA proteins (Worley *et al.*, 2000; Ramos *et al.*, 2001; Dharmasiri *et al.,* 2005b; Kepinski & Leyser 2005; Tan *et al.*, 2007; Lee *et al.*, 2009; Parry *et al.,* 2009). Distinct clusters of *VviARF*, *VviIAA* and *VviAFB* transcriptional profiles were identified in this study, leading to questions as to whether co-expressed transcripts are interacting as proteins. These cluster patterns may in some cases occur due to simple chance, however, there is also the potential that partners exist within these clusters that are functionally related. Alternatively, some proteins may be produced at one point of development and remain undegraded allowing them to interact later on in development, such as Aux/IAA proteins lacking the degron sequence in Domain II; these protein partners would be missed using this analysis.

Interactions between candidates can be tested using protein-protein interaction analysis, such as yeast 2-hybrid (Y2H) or bimolecular fluorescence complementation (BiFC) (Tiwari *et al.,* 2004; Szemenyei *et al.*, 2008; Lee *et al.*, 2009; Causier *et al.*, 2012; Piya *et al.*, 2014; Farcot *et al.,* 2015; Hagen, 2015). AtARF5 and AtIAA12 interact in both Y2H and pull-down assays, they are suggested to play roles in embryo axis formation and vascular tissue differentiation and RNAseq data confirms that they are both co-expressed in Arabidopsis embryos (Nanao *et al.*, 2014; Piya *et al.*, 2014). AtARF7 and AtIAA19 are co-expressed in Arabidopsis roots and interact in Y2H, and AtARF7 has been found to play a role in lateral root formation (Korasick *et al.*, 2014; Piya *et al.*, 2014). The comprehensive BiFC interaction analysis in Vernoux *et al.* (2011) showed that AtARF6 and AtIAA8 interact, they are co-expressed in flowers and have been linked with functions in flower and fruit development (Nagpal *et al.,* 2005; Goetz *et al.,* 2006; Piya *et al.,* 2014). These studies indicate that proteins that are co-expressed have the capacity to interact and have been identified to play key functional roles within these organs, similar interactions will almost certainly be taking place in grape. To further the analysis of co-expressed transcripts and make steps towards determining what functions they play within grapevine, three *VviARF* and three *VviIAA* candidates were selected based on their transcript profiles within berries and used for interaction analysis in Chapter 5. Interaction analysis, phytohormone treatments and the comparison of *V. vinifera* candidates to candidates in other species may provide additional insight towards determining what roles the AFB, ARF and Aux/IAA proteins are playing in grape development.

Chapter 5 Protein-protein interaction analysis

5.1 Aim

The aim of this work was to investigate the interactions between VviARF and VviIAA candidates that have overlapping transcript expression patterns and use yeast 2-hybrid screening of grape berry libraries to identify novel binding partners of VviARF proteins.

5.2 Introduction

ARF and Aux/IAA proteins are nuclear-localised proteins that have been shown to regulate gene activation and repression through multiple modes of action (Abel *et al.,* 1994). Farcot *et al.* (2015) described ARF activators as singular ARFs or complexes of ARFs that interact by enhancing the expression of target genes through an interaction with DNA. Transcriptional repression is proposed to occur through two modes; ARF repressor proteins either function alone, and/or ARF activators are bound in complexes with Aux/IAA proteins, thus repressing the activating capacity of the ARF activator until the Aux/IAA protein is poly-ubiquitinated and degraded. Both the Aux/IAA and ARF proteins need to be present in the nucleus where they can interact with the DNA. The PB1 domain is the crucial domain for correct Aux/IAA-ARF protein-protein interactions whilst the DBD and DD domains are necessary for DNA binding and the dimerization of ARF proteins (Pierre-Jerome *et al.,* 2016).

A range of methods have been developed to directly test interactions between proteins. The Clontech yeast two-hybrid (Y2H) system involves cloning the sequences of interest into bait and prey plasmids, and transforming them into yeast. If the bait and prey interact the yeast will grow on selective media that lacks specific amino acids and contains the toxin Aureobasidin A. The strength of the interaction can be measured by the concentration of α-galactosidase produced in the presence of X-α-Gal in the media. Yeast library screening is a modification of this technique, which uses a bait protein of interest that is mated with another yeast strain containing a cDNA library. Successful interaction between the bait protein and a prey protein encoded within the library provides a capacity to sustain yeast growth on the selective media and the prey cDNA is identified through isolation and sequencing. Additionally, bimolecular fluorescence complementation (BiFC) can be used *in planta* to assess protein-protein interaction and localisation. BiFC utilises two halves of a fluorescent reporter, such as YFP, fused to the proposed interacting partners, when the proteins of interest interact, the two halves of the reporter are able to assemble, making an active fluorophore (Hu *et al.,* 2002; Kerppola, 2006). In this study, Y2H and BiFC analysis were used to assess the interactions between a selection of VviARF and VviIAA candidate proteins and yeast library screening was also used to identify novel VviARF binding partners.

5.3 Results

5.3.1 Selection of candidates for interaction analysis

The cluster analysis in Chapter 4 identified a range of VviARF and VviIAA proteins that had similar transcript profiles during berry development. Transcriptional co-expression during a developmental stage may indicate that the encoded proteins function together in a complex within the nucleus, influencing auxin signalling. Three pairs of *VviARF* and *VviIAA* candidates were selected based on their expression patterns (Figure 1): *VviARF27* and *VviIAA19* which were up-regulated post-veraison and shared a similar pattern to IAA-Asp conjugate concentration; *VviARF24* and *VviIAA27*, which were up-regulated during pre-veraison and down-regulated post-veraison; and *VviARF4* and *VviIAA41*, which show a general decreasing trend towards veraison with minimal expression post-veraison, similar to free IAA concentration (Figure 4.1). Three *VviARFs* (*VviARF4, 24* and *27*) and *VviIAAs* (*VviIAA19, 27* and *41*) were selected for use within the Y2H and BiFC systems. As protein-protein interactions have not been studied for these families in grape, the results can be compared and contrasted to the findings of Piya *et al.,* (2014) in Arabidopsis to determine the conservation of the ARF-IAA interactions across species (Figure 1.8). Although VviARF27 is the only activator ARF selected here, the closest homolog in Arabidopsis to VviARF4 is AtARF4, which interacts widely with AtIAA proteins in Piya *et al.* (2014). VviARF24 was closest in similarity to AtARF11 and 18; AtARF18 interacts with ten of the 29 AtIAA proteins, whilst AtARF11 does not interact with any (Piya *et al.*, 2014).

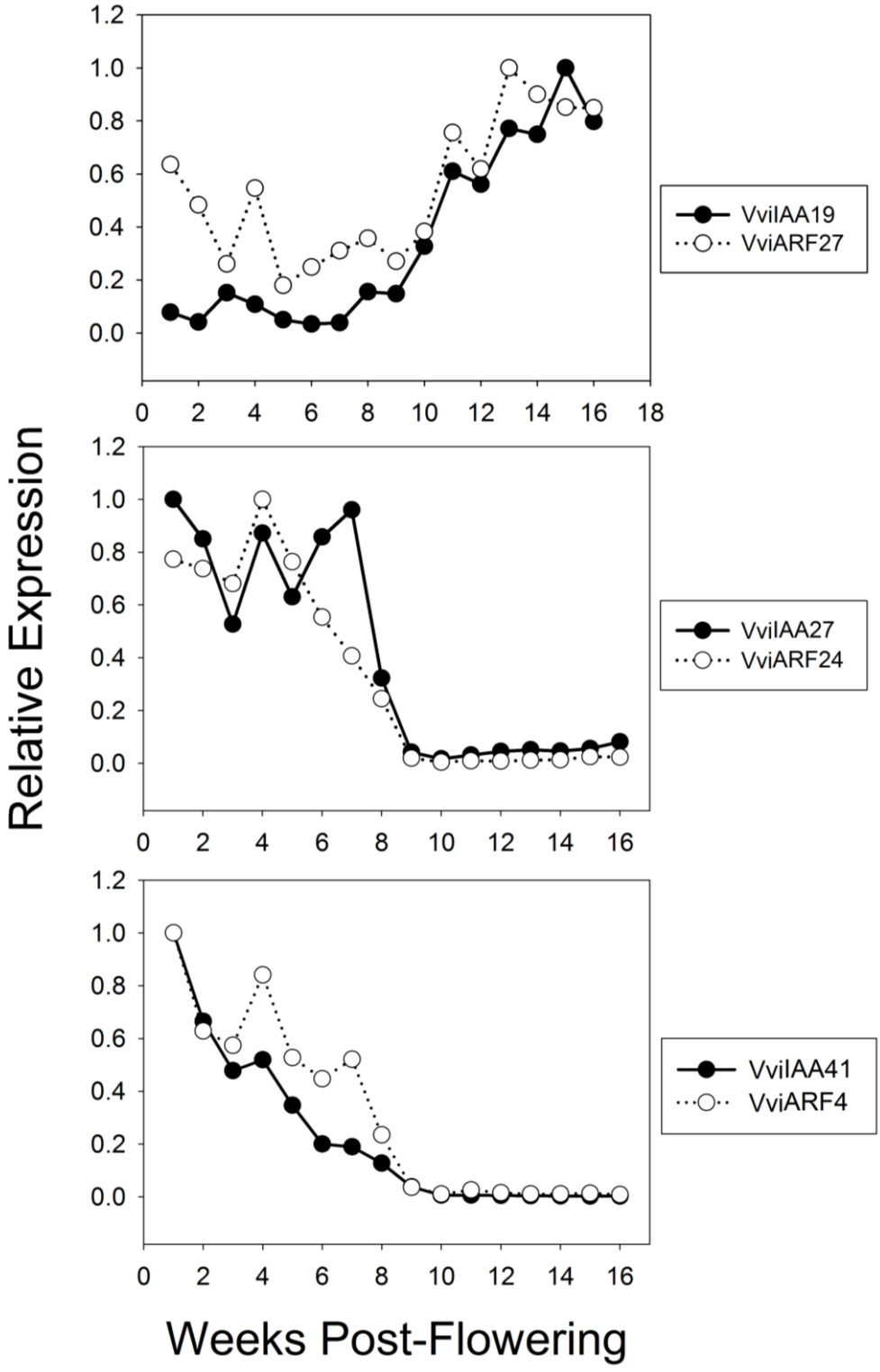


Figure 5.1 The relative expression patterns of the three pairs of *VviARF* and *VviIAA* candidates selected for protein-protein analysis.

The expression patterns shown are from the three biological replicates of berry expression data as detailed in Chapter 4.

5.3.2 Full-length sequences vary from database predictions

The full-length sequences for *VviARF4, 24, 27* and *VviIAA19, 27, 41* were cloned from *Vitis vinifera* L. cv. Shiraz berry cDNA, sequenced and compared to the sequences predicted in Section 3.4.2 which were derived from the RNAseq/genomic DNA FGENESH+ predictions, NCBI, Phytozome and previous publications. Five of the six sequences varied from the predictions, potentially due to the reference sequences being from Pinot Noir compared with Shiraz within this study. With regards to the *VviIAA* candidates none of the changes were present in the predicted functional domains, *VviIAA27* contained a single base pair difference that led to an amino acid change, *VviIAA41* had two insertions, one 63 bp and one 90 bp and *VviIAA19* was as predicted. *VviARF4* had two base pair changes that led to amino acid changes, one of which was in the DBD. *VviARF24* contained an insertion of 69 bp in the middle region and a 3 bp deletion leading to the loss of a lysine in the PB1 domain, as well as a base pair difference that led to a change in an amino acid also in the PB1 domain, while *VviARF27* had 5 bp differences that led to amino acid changes, a 6 bp deletion and a 112 bp insertion none of which were in the protein functional domains. All protein sequences were still full-length with no premature stop codons. A schematic diagram of the *VviIAA41*, *VviARF24* and *27* sequences is shown in Figure 5.2 to demonstrate the largest differences between the predicted sequences and the cDNA isolated from *V. vinifera* L. cv. Shiraz. The sequences derived from the sequencing of the full-length cDNA clones were used for all future work.

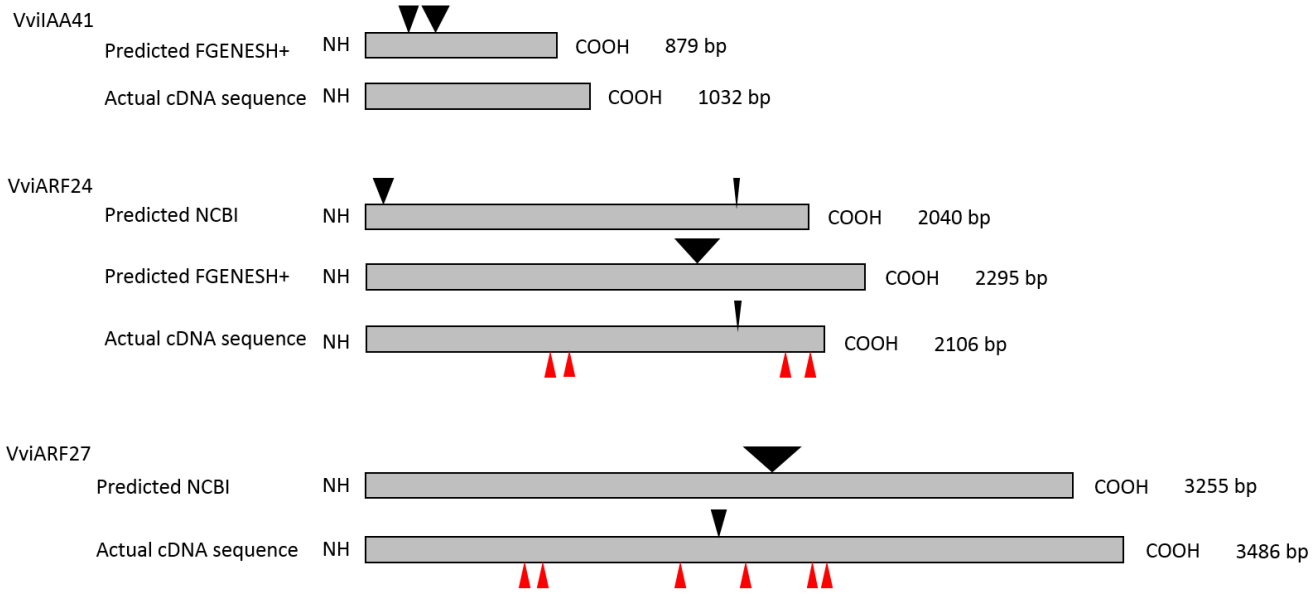


Figure 5.2 A schematic diagram of the differences between the predicted and sequenced cDNA for *VviIAA41, VviARF24* and *VviARF27*.

Gene candidates were isolated from NCBI, Phytozome or publications and analysed by FGENESH+ to determine their predicted coding sequences, details of which are listed in Table 3.5 and Table 3.6 in Section 3.4.2. When full-length cDNA was isolated from *V. vinifera* L. cv. Shiraz the sequences were compared to the predicted sequences, three of the six sequences isolated contained notable differences including insertions, deletions and single nucleotide polymorphisms as shown above. Grey boxes represent the coding sequencing of the gene candidates, black triangles represent deletions or insertions in the comparative sequence, and red triangles represent single nucleotide differences. NH – sequence N-terminus, COOH – sequence C terminus, bp = base pairs, cDNA = complementary DNA, NCBI = National Center for Biotechnology Information.

5.4 Auto-activation of VviARF proteins

The selected *VviARFs* were initially transformed into Y2H gold cells and tested for auto-activation using the method described in Section 2.2.4.6. All three ARF proteins showed auto-activation and were re-cloned without their DNA-binding domain (DBD; Section 2.2.2.8.1). The new vectors were tested and the auto-activation no longer occurred in VviARF4ΔDBD and VviARF24ΔDBD, but was still apparent at a reduced level for VviARF27ΔDBD. The weak interaction for VviARF27ΔDBD was distinguishable from the stronger auto-activation using the unmodified clone or positive interactions which were dark blue in colour with blue halos in the surrounding media. All three ARF proteins were used in the library screening process, VviARF4ΔDBD and VviARF24ΔDBD with a Shiraz berry Week 4 library and VviARF27ΔDBD with a Shiraz berry Week 12 library, in line with their expression profiles during berry development (Chapter 4).

5.4.1.1 VviARF4ΔDBD Week 4 library screen

The ARF4ΔDBD library screen with the Week 4 library was completed using the protocol as described in Section 2.2.8. The mating efficiency was calculated using the method described in the Clontech manual. The efficiency of the screen was 2.6%, which is within the 2-5% optimal efficiency range, indicating at least 1 million diploids were able to be screened. On the 60 plates, approximately 1000 colonies were identified that were initially white in colour. After two to three days of growth approximately 300 colonies turned blue or had halos of blue in the media, while after four to six days of growth, the majority of the colonies had turned blue. A total of 238 colonies that were blue after three days of incubation were streaked onto quadruple drop out, X-α-Gal, aureobasidin A (QDO/X/ABA) plates for continued selection. A total of 28 plasmids were recovered and sequenced using the T7 FWD primer (Appendix B, Table B.4). The sequencing results identified a number of known and uncharacterised *Vitis* proteins (Table 5.1). Of the 28 sequences, one contained a full-length CDS, sample 23 detailed below.

Table 5.1 The VviARF4-DBD Week 4 cDNA yeast library screen plasmid sequencing results.

	Colony	Top match	Closest Arabidopsis match
1	2#25	*V. vinifera* uncharacterized (LOC100261837), mRNA	Octicosapeptide/Phox/Bem1p domain-containing protein - NP_567290.1
2	2#47	*V. vinifera* L-ascorbate oxidase homolog-like (LOC100252389), mRNA	SKU5 similar 5 (sks5), mRNA - NM_106265.4
3	3#22	*V. vinifera* uncharacterized (LOC100255290), mRNA	EMB514 (DUF3223) mRNA, NM_125638.6
4	1#17	*V. vinifera* uncharacterized (LOC100243155), mRNA	Transmembrane protein (DUF616) mRNA, NM_001335148.1

	Colony	Top match	Closest Arabidopsis match
5	1#10	*V. vinifera* nucleolin (LOC100267377), transcript variant X2, mRNA, XM_010653579.2	RNA-binding (RRM/RBD/RNP motifs) family protein mRNA, NM_001339581.1
6	4#23	*V. vinifera* uncharacterized (LOC100243155), mRNA	Transmembrane protein (DUF616) mRNA, NM_001335148.1
7	2#14	*V. vinifera* subsp. *caucasica* chloroplast DNA, complete genome, cultivar: Meskhuri Mtsvane, AB856291.1	Chloroplast DNA, complete genome, ecotype: Columbia, AP000423.1
8	3#12	*V. vinifera* actin cytoskeleton-regulatory complex protein PAN1 (LOC100854676), mRNA	Calcium-binding EF hand family protein mRNA, NM_001332524.1
9	3#41	*V. vinifera* vacuolar protein sorting-associated protein 32 homolog 2-like (LOC100242412)	SNF7 family protein (SNF7.1), mRNA, NM_001084999.2
10	4#50	*V. vinifera* GDSL esterase/lipase At5g33370-like (LOC100243401), mRNA	Li-tolerant lipase 1 (LTL1), mRNA, NM_111300.4
11	3#29	*V. vinifera* COP9 signalosome complex subunit 7-like, transcript variant 3 (LOC100261627)	Proteasome component (PCI) domain protein (FUS5), mRNA, NM_100089.3
12	2#7	*V. vinifera* heavy metal-associated isoprenylated plant protein 33 (LOC100261454), mRNA, XM_002277618.4	Heavy metal transport/detoxification superfamily protein mRNA, NM_001343587.1
13	5#16	*V. vinifera* accelerated cell death 11 (LOC100258392), transcript variant X1, mRNA, XM_002281528.4	Glycolipid transfer protein (GLTP) family protein (ACD11), mRNA, NM_129023.5
14	3#18	*V. vinifera* serine carboxypeptidase-like 40-like (LOC100248271), mRNA, XM_002272925.3	Serine carboxypeptidase-like 40 (scpl40), mRNA, NM_116212.3
15	3#47	*V. vinifera* S-adenosylmethionine synthetase 4 (METK4), partial mRNA	S-adenosylmethionine synthetase (At1g02500) mRNA
16	1#29	*V. vinifera* actin cytoskeleton-regulatory complex protein PAN1 (LOC100854676), mRNA	Calcium-binding EF hand family protein mRNA, NM_001332524.1
17	1#28	*V. vinifera* very-long-chain 3-oxoacyl-CoA reductase 1 (LOC100257681), mRNA	Beta-ketoacyl reductase 1 (KCR1), mRNA NM_105441.3
18	1#6	*V. vinifera* kynurenine formamidase-like (LOC100253780), misc_RNA	Cyclase family protein mRNA, NM_119688.4
19	4#10	*V. vinifera* catalase isozyme 1 (LOC100853165), mRNA, XM_003631877.3	Catalase 2 (CAT2), mRNA NM_119675.4
20	1#9	*V. vinifera* soluble inorganic pyrophosphatase-like (LOC100258490), mRNA	Pyrophosphorylase 4 (PPa4), mRNA NM_115222.3
21	3#19	*V. vinifera* chlorophyll a-b binding protein CP29.2, chloroplastic-like (LOC100266604), mRNA	Putative chlorophyll a/b-binding protein (At3g08940) mRNA, AY081608.1

	Colony	Top match	Closest Arabidopsis match
22	1#40	*V. vinifera* catalase isozyme 1-like (LOC100853165), mRNA	Catalase 2 (CAT2), mRNA, NM_119675.4
23	2#8	*V. vinifera* 30S ribosomal protein S10, chloroplastic-like (LOC100248042), mRNA	Ribosomal protein S10p/S20e family protein mRNA, NM_001338033.1
24	1#14	*V. vinifera* uncharacterized (LOC100267569), mRNA	Structural maintenance of chromosomes domain protein mRNA, NM_112336.5
25	1#1	*V. vinifera* probable WRKY transcription factor 28-like (LOC100267688), mRNA, XM_002283567.1	WRKY DNA-binding protein 71 (WRKY71), mRNA, NM_102726.3
26	1#11	*V. vinifera* uncharacterized (LOC100243155), mRNA, XM_002274035.2	Transmembrane protein (DUF616) mRNA, NM_001335148.1
27	1#19	*V. vinifera* heavy metal-associated isoprenylated plant protein 33 (LOC100261454), mRNA, XM_002277618.4	Heavy metal transport/detoxification superfamily protein mRNA, NM_001343587.1
28	1#25	*V. vinifera* L-ascorbate oxidase homolog-like (LOC100252389), mRNA	SKU5 similar 5 (sks5), mRNA - NM_106265.4

5.4.1.2 VviARF24ΔDBD Week 4 library screen

The ARF24ΔDBD Week 4 library screen was unsuccessful in producing single blue colonies, and instead resulted in a lawn of only white yeast colonies.

5.4.1.3 VviARF27ΔDBD Week 12 library screen

The VviARF27ΔDBD Week 12 library screen resulted in >2000 colonies across the 60 screening plates. The mating efficiency of the screen was 4.5%, which is within the optimal 2-5% efficiency range. Due to the light blue coloured auto-activation that was occurring with the VviARF27ΔDBD plasmid, only the colonies that showed a deep blue colour after one to three days were streaked for further screening. A total of ~884 colonies were streaked onto QDO/X/ABA plates and were scored for their colour and growth. Plasmids were isolated from 11 colonies and sequenced using the T7 FWD primer (Appendix B, Table B.4). The sequencing results identified a number of uncharacterised proteins and a selection of known *Vitis* proteins (Table 5.2). Of the 11 sequences two contained full-length CDSs.

Table 5.2 The VviARF27ΔDBD + Week 12 cDNA yeast library screen plasmid sequencing results.

	Colony	Top match	Closest Arabidopsis match
1	16#1	*V. vinifera* uncharacterized (LOC100257932), mRNA	Wound-responsive family protein, NP_849355.1
2	16#2	*V. vinifera* proline-rich cell wall protein-like (GRIP4), mRNA *V. vinifera* mRNA for putative proline-rich cell wall protein (grip3 gene)	Extensin (atExt1) gene, U43627.1
3	16#3	*V. vinifera* mRNA for putative proline-rich cell wall protein (grip3 gene) *V. vinifera* proline-rich cell wall protein-like (GRIP4), mRNA	Extensin (atExt1) gene, U43627.1
4	16#6	*V. vinifera* uncharacterized (LOC100257168), mRNA *V. vinifera* polyubiquitin-A-like (LOC100267431), mRNA	Polyubiquitin 10 (UBQ10), mRNA, NM_178968.5
5	16#8	*V. vinifera* uncharacterized (LOC100258493), mRNA *V. vinifera* uncharacterized (LOC100854650), mRNA	Hepatocyte growth factor activator, putative (DUF3527) mRNA, NM_125292.4
6	16#9	-*	-
7	1#26	*V. vinifera* thaumatin-like protein VVTL1 mRNA, complete cds	mRNA for osmotin precursor like protein, complete cds, AK228271.1
8	2#43	*V. vinifera* trans-2,3-enoyl-CoA reductase-like (LOC100266766), mRNA	3-oxo-5-alpha-steroid 4-dehydrogenase family protein (CER10), mRNA, NM_115394.4
9	2#51	*V. vinifera* uncharacterized (LOC100245999), mRNA *V. vinifera* proline-rich cell wall protein-like (GRIP4), mRNA	Beta glucosidase 8 (BGLU8), mRNA, NM_001340178.1
10	2#55	*V. vinifera* chromatin structure-remodeling complex subunit RSC1-like (LOC100252013), mRNA	PHD finger family protein / bromo-adjacent homology (BAH) domain-containing protein (SHL1), mRNA, NM_120070.3
11	3#75	*V. vinifera* ATP-citrate synthase beta chain protein 2-like (LOC100267071), mRNA	ATP citrate lyase subunit B 2 (ACLB-2), mRNA, NM_001344844.1

* - the sequence was not a whole CDS and there was no significant BLAST match

5.4.2 Yeast colony PCRs

Due to the large number of colonies and the absence of any *VviIAA* sequences in the isolated plasmids, yeast colony PCR was tested as a more effective large scale screening strategy. A forward primer was designed within the conserved domain IV of the *VviIAA* sequences and was predicted to bind to the majority of the *VviIAA* sequences. When the domain IV forward primer was combined with the 3'AD reverse primer, which binds to the backbone of the pGADT7 vector, fragments of about ~270 bp were

expected if any *VviIAA* gene was present in a yeast colony. PCR fragments were successfully amplified from 10 colonies and sequencing showed that eight of the fragments appeared to be derived from *VviIAA* sequences (Table 5.3). The sequenced fragments matched *VviIAA11, 19*, and *41*. This indicates that even though full-length fragments encoding these VviIAA proteins were not identified after single colony purification, interactions between the ARF proteins with VviIAA sequences were likely taking place in the yeast system.

Table 5.3 Sequence matches from gel extracts from yeast colony PCRs from the VviARF4-DBD and VviARF27 yeast library screens.

	Construct	Colony	Top match	Match	Closest Arabidopsis match
1	ARF4ΔDBD	1#39	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27
2	ARF4ΔDBD	1#42, 1#21	No matches	-	-
3	ARF4ΔDBD	2#3, 2#27	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27
4	ARF27ΔDBD	16#2	Plasmodium sp. P21 cytochrome b (cytb) gene, partial cds; mitochondrial	-	-
5	ARF27ΔDBD	16#3	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27
6	ARF27ΔDBD	1#26	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27
7	ARF27ΔDBD	2#43	*V. vinifera* auxin-induced protein 22A-like (LOC100854934), mRNA	VviIAA19	Indole-3-acetic acid inducible 19 , NP_188173.1
8	ARF27ΔDBD	2#51	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27
9	ARF27ΔDBD	2#55	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA	VviIAA41	Phytochrome-associated protein 2, NP_194637.1, IAA27

	Construct	Colony	Top match	Match	Closest Arabidopsis match
10	ARF27ΔDBD	3#75	*V. vinifera* auxin-responsive protein IAA27-like, transcript variant 2 (LOC100253148), mRNA but also matched *V. vinifera* auxin-responsive protein IAA11-like (LOC100244630), mRNA	VviIAA41 VviIAA11	Phytochrome-associated protein 2 , NP_194637.1, IAA27 Indole-3-acetic acid inducible 11, NP_194593.1

5.5 Yeast two-hybrid co-transformations

5.5.1.1 Confirmation of yeast library screening results

To confirm the interaction of prey isolated from yeast library screening, co-transformation of bait and prey vectors into yeast was performed using the method as described in Section 2.2.4.6. Three prey were selected from both the VviARF4ΔDBD and VviARF27ΔDBD library screens.

For the VviARF4ΔDBD co-transformation, the sequences selected were a *V. vinifera* uncharacterized LOC100261837 (prey 2#25) that matched closely to an Arabidopsis octicosapeptide/Phox/Bem1p domain-containing protein, a *V. vinifera* uncharacterized LOC100243155 fragment (prey 1#17), and the *V. vinifera* COP9 signalosome complex subunit 7-like transcript variant 3 LOC100261627 (prey 3#29). None of the prey interacted with the empty pGBKT7 bait vector (Figure 5.3_A). After co-transformation with ARF4ΔDBD bait, a light blue colour was seen for prey 2#25, however, the intensity was not bright enough to suggest a true interaction (Figure 5.3_A). For prey 1#17 small colonies of a light blue colour were visible, however, once again the intensity did not suggest that this was a true interaction (Figure 5.3_A). Finally, with prey 3#29 the yeast growth and blue colour intensity suggests that there may be an interaction between ARF4ΔDBD and the COP9-signalosome subunit (Figure 5.3_A).

For the VviARF27ΔDBD co-transformations the sequences selected were a *V. vinifera* uncharacterized LOC100257932 fragment (prey 16#1), a full-length *V. vinifera* putative proline-rich cell wall (GRIP3) protein (16#3) and a full-length *V. vinifera* trans-2,3-enoyl-CoA reductase-like LOC100266766 protein (2#43). All three prey did not interact with the empty pGBKT7 bait vector, and all had darker blue yeast growth than the auto-activating VviARF27ΔDBD yeast (Figure 5.3_B). The prey also had halos present within the media suggesting that there is an interaction between preys 16#1, 16#3 and 2#43 and VviARF27ΔDBD (Figure 5.3_B).

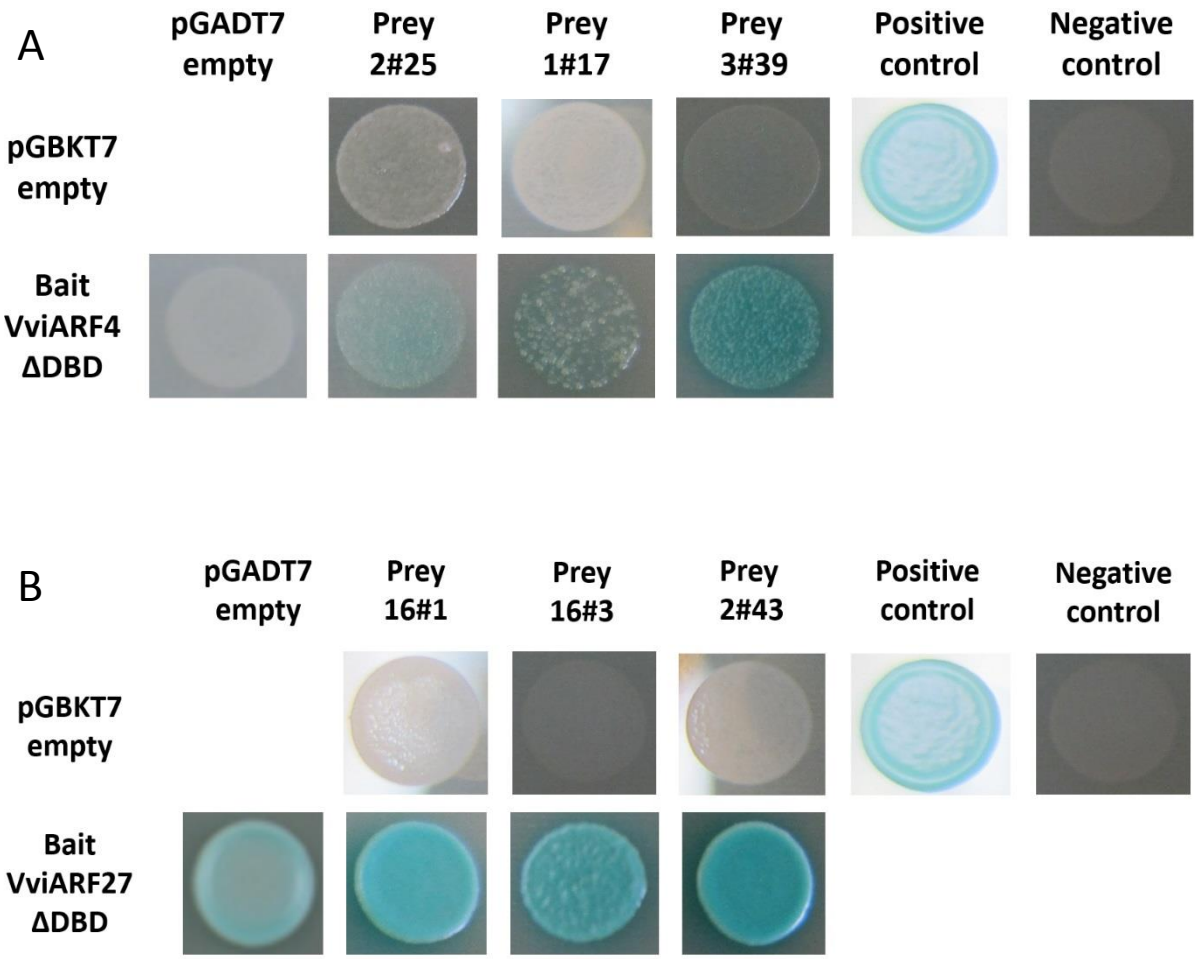


Figure 5.3 Co-transformations of VviARF candidates and prey matches isolated from the yeast library screening.

(A) ARF4ΔDBD + Week 4 yeast library screen interactors tested using yeast 2-hybrid analysis on QDO/X/ABA plates. (B) VviARF27ΔDBD + Week 12 yeast library screen interactors tested using yeast 2-hybrid analysis on QDO/X/ABA plates.

5.5.1.2 Interactions between VviARF and VviIAA pairs

VviARF4ΔDBD, VviARF24ΔDBD and VviARF27ΔDBD were used directly for yeast 2-hybrid with selected VviIAA sequences. Three *VviIAA* sequences were selected based on similarities between their expression patterns to one or more of the *VviARF* candidates, and their high expression levels. These pairs were *VviARF4* and *VviIAA41*, *VviARF24* and *VviIAA27*, and *VviARF27* and *VviIAA19*. VviIAA41 was unable to be transformed into yeast, and for this reason only VviIAA27 and VviIAA19 were able to be used for mating experiments. VviIAA19 and VviIAA27 did not interact with the empty pGBKT7 bait vector (Figure 5.4). VviARF4ΔDBD interacted with VviIAA19 but not VviIAA27, as shown by blue yeast growth and no yeast growth, respectively (Figure 5.4). VviARF24ΔDBD did not interact with either VviIAA19 or VviIAA27 as shown by no yeast growth (Figure 5.4). VviARF27ΔDBD interacted with both VviIAA19 and VviIAA27 as shown by dark blue colony growth with a halo in the surrounding media (Figure 5.4).

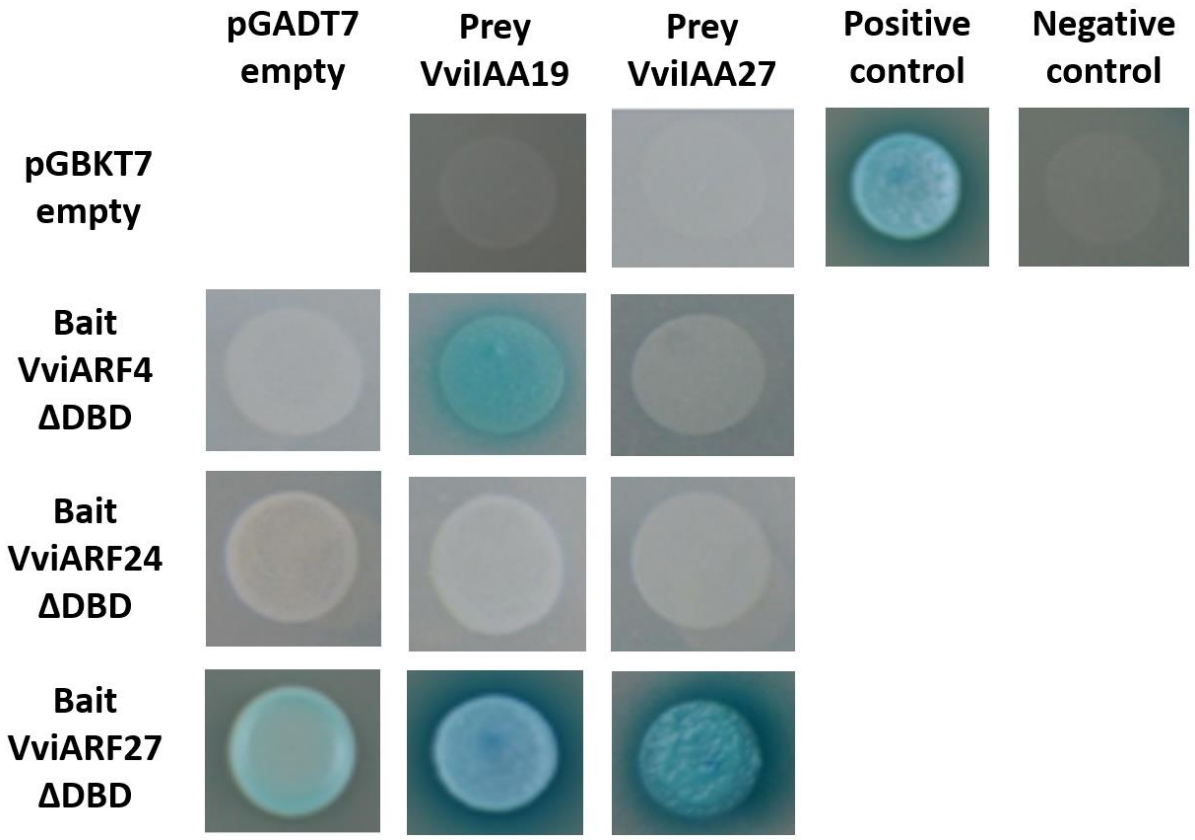


Figure 5.4 Yeast two-hybrid analysis to test the interaction between ARF and Aux/IAA proteins on QDO/X/ABA plates.

5.6 Split YFP (BiFC)

BiFC analysis in onion epidermal cells was used to support the Y2H analysis results. The combinations tested included VviARF4 with VviIAA19, and VviARF27 with the full-length prey matches VviGRIP3 and VviTrans-2,3-enoyl-CoA reductase-like (Table 5.2), and VviIAA19 and VviIAA27. Bombardment of the empty pSITE vectors (Appendix C, Table C.3), which carried the two halves of YFP, resulted in no YFP expression. Six different pSITE vector combinations were tested to establish whether or not the ARF and Aux/IAA proteins were interacting *in planta* (Figure 5.5).

Photographs were taken using bright light, DAPI, YFP and CFP filters (Figure 5.6). The pART7-35S-VviSNAP33-CFP vector was used as a positive transformation control, with all transformed cells first being visualised with CFP then examined for YFP expression. Photographs were only taken of cells that were positive for CFP. The separate channels were overlaid to show the localisation of the nucleus and the YFP expression within each onion cell.

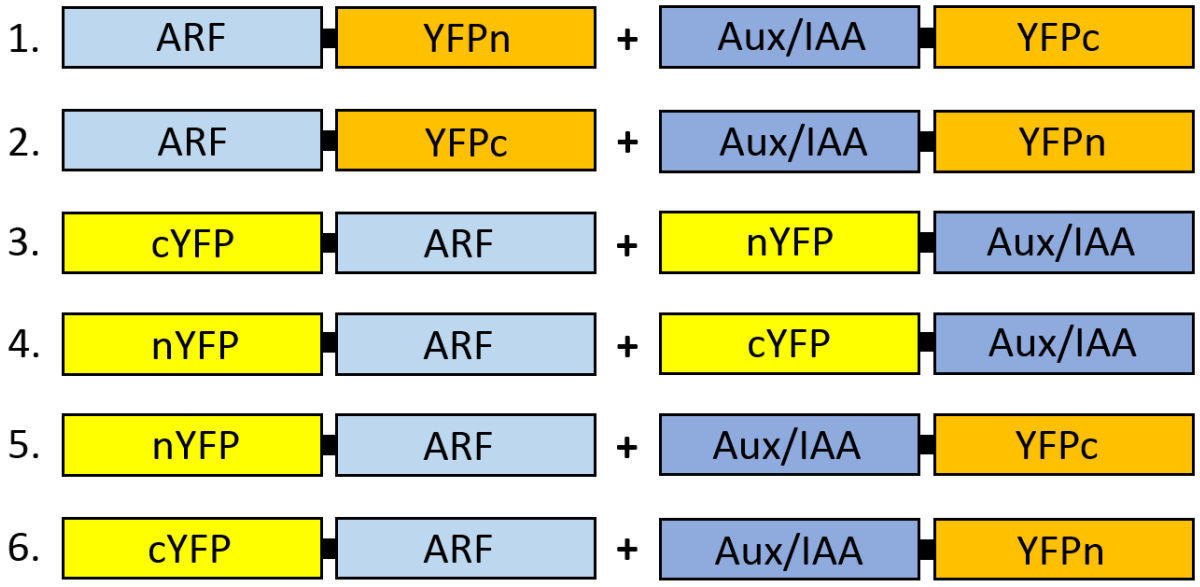


Figure 5.5 The six pSITE construct combinations of VviARF and VviIAA coding sequences with C- and N-terminal fusions of the C- and N-terminal halves of the yellow fluorescent proteins.

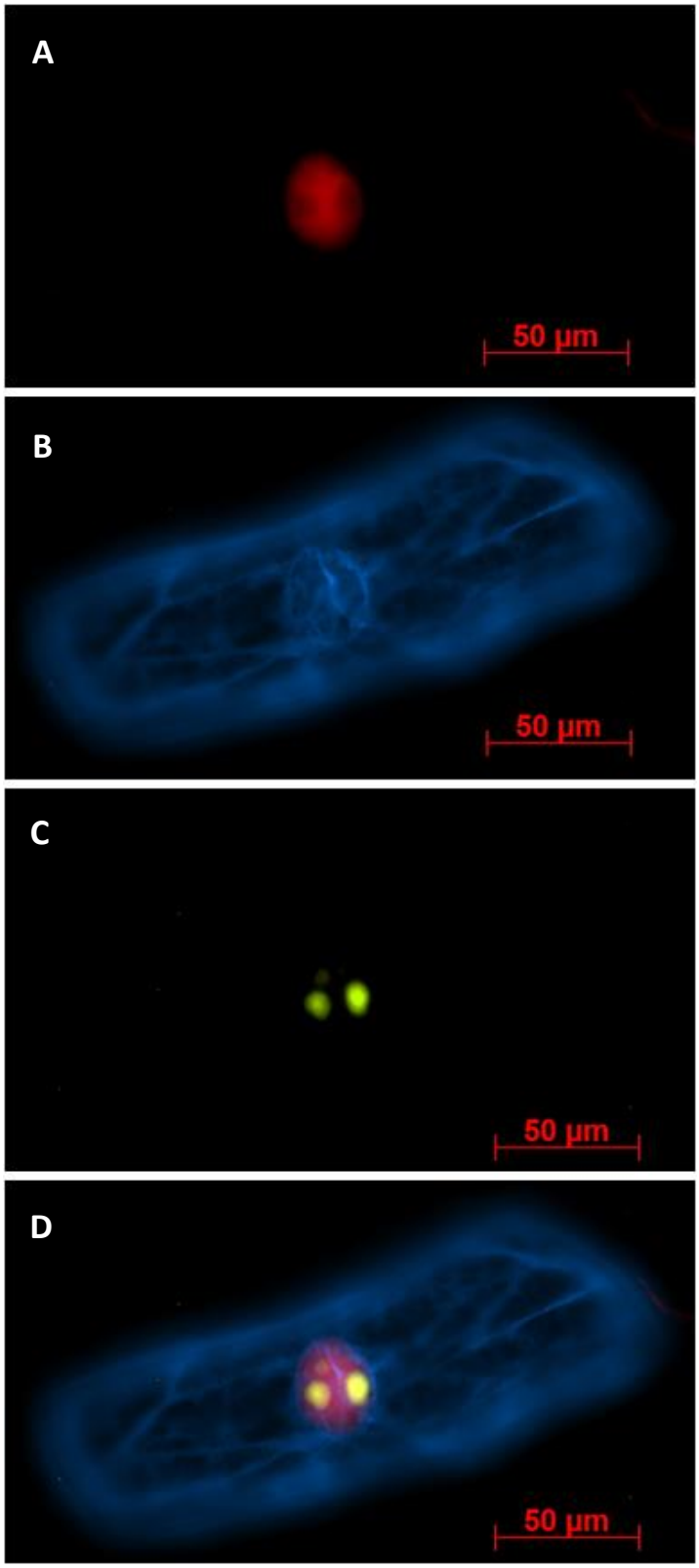


Figure 5.6 Onion cells photographed using three different channels during BiFC analysis and their overlay.

(A) DAPI stain – nucleus location (red), (B) pARF7-VviSNAP33-35S-CFP – transformation success reporter (cyan), and (C) N' terminal YFP fragment and C' terminal YFP fragment interaction – expression pattern of the genes of interest (yellow). (D) DAPI, YFP, CFP were then used to produce a multichannel overlay.

5.6.1 Testing the interacting partners from yeast library screens

In the yeast library screening two full-length coding sequences were isolated for *VviGRIP3* and *VviTrans-2,3-enoyl-CoA reductase-like*. To test the interaction between VviARF27 and these two proteins, the coding sequences were cloned into the pSITE vectors for BiFC using the six different combinations shown in Figure 5.5. Of the six construct combinations, #1 to #3 and #5 to #6 were trialled for VviGRIP3 and VviARF27, and none produced YFP expression consistent with the localisation of the GRIP proteins to the cell wall and ARF proteins to the nucleus. Combination #4 was not tested as VviGRIP3 was not successfully cloned into the pSITE cYFP-prey vector. Similarly, of the six construct combinations tested using VviTrans-2,3-enoyl-CoA reductase-like and VviARF27, none produced YFP expression.

5.6.2 Testing the ARF and Aux/IAA interacting partners

The N-terminal YFP construct combinations (Figure 5.5, 1 and 2) were the first to be tested for interactions between all VviARF and VviIAAs. No fluorescence was seen for any VviIAA + VviARF pair for either of these combinations. C-terminal YFP constructs were then cloned and the combinations between the N-terminal constructs and C-terminal constructs were tested (Figure 5.5, 3–6). The following results show the various interaction patterns and provide a representative example of the results of their bombardment into onion epidermal cells. No fluorescence was detected in bombardments containing full-length *VviIAA* sequences and a truncated *VviARF* sequence with the PB1 domains removed (Appendix F). In addition, no fluorescence was detected in bombardments containing *VviARF* or *VviIAA* sequences and pSITE-YFPn or pSITE-YFPc vectors containing no gene of interest (Appendix F).

5.6.2.1 VviARF4 + VviIAA19

The BiFC results confirmed that VviARF4 can interact with VviIAA19 and that these proteins are targeted to the nucleus (Table 5.4). With cYFP-VviARF4 + nYFP-VviIAA19, one of the four CFP positive cells had YFP expression; the YFP signal was visible as two large spots within the nucleus, potentially within the nucleoli (Figure 5.7_A). The majority of cells bombarded with nYFP-VviARF4 + cYFP-VviIAA19 and nYFP-VviARF4 and VviIAA19-YFPc had medium and strong nuclear YFP expression, respectively (Figure 5.7_B and C). The weakest expression was seen with cYFP-VviARF4 + VviIAA19-YFPn, with half of the cells photographed having faint to medium intensity nuclear YFP expression and the other half having no YFP expression (Figure 5.7_D).

Table 5.4 The pSITE VviARF4 and VviIAA19 bimolecular fluorescence results. The number of cells photographed and their expression patterns is described for the four construct combinations that produced fluorescence.

Construct combination	Cells photographed	Expression
cYFP-VviARF4 + nYFP-VviIAA19	4	1 with two large nuclear spots, 3 with no expression or weak nuclear background
nYFP-VviARF4 + cYFP-VviIAA19	6	5 medium nuclear, 1 none
nYFP-VviARF4 + VviIAA19-YFPc	8	6 strong nuclear, 2 none
cYFP-VviARF4 + VviIAA19-YFPn	14	3 faint nuclear, 4 medium nuclear, 7 background level nuclear/none

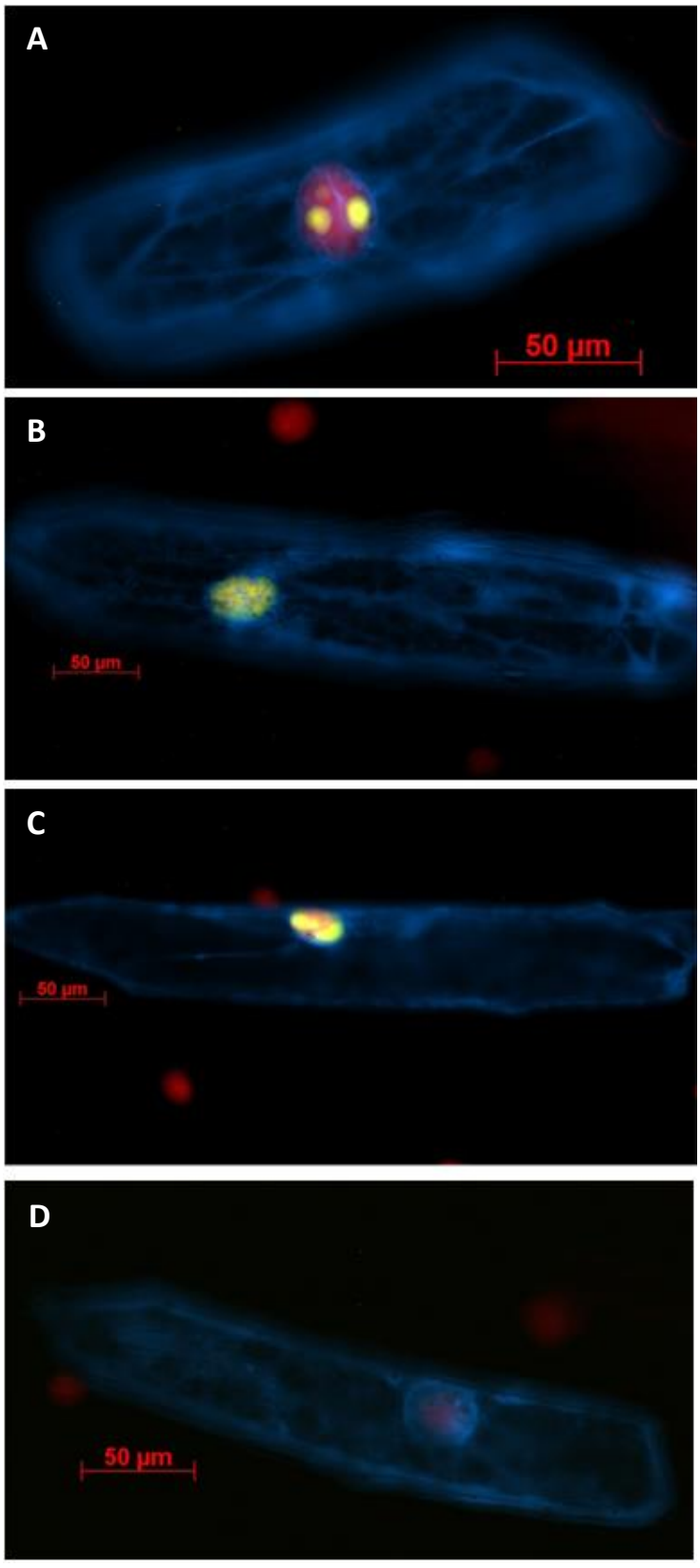


Figure 5.7 A representation of the fluorescence profiles seen between (A) cYFP-VviARF4 + nYFP-VviIAA19, (B) nYFP-VviARF4 + cYFP-VviIAA19, (C) nYFP-VviARF4 and VviIAA19-YFPc, (D) cYFP-VviARF4 + VviIAA19-YFPn. Channels as per Figure 5.6.

5.6.2.2 VviARF27 + VviIAA27

The BiFC results confirmed that VviARF27 can interact with VviIAA27 and that these proteins are targeted to the nucleus (Table 5.5). With cYFP-VviARF27 + nYFP-VviIAA27, nine of the 15 CFP positive cells had YFP expression; this YFP was present as large spots or speckles within the cells (Figure 5.8_A). The majority of cells bombarded with nYFP-VviARF27 + cYFP-VviIAA27 had a similar pattern of YFP expression with large spots or smaller speckles throughout the cells (Figure 5.8_B). The brightest YFP expression was present with nYFP-VviARF27 and VviIAA27-YFPc where the majority of cells had nuclear YFP expression with speckles or large spots present throughout the cell (Figure 5.8_C). The weakest expression was seen with cYFP-VviARF27 + VviIAA27-YFPn, with five of the six cells photographed having no YFP expression and one having large spots throughout the cell with faint nuclear expression (Figure 5.8_D).

Table 5.5 The pSITE VviARF27 and VviIAA27 BiFC results. The number of cells photographed and their expression patterns is described for the four construct combinations that produced fluorescence.

Construct combination	Cells photographed	Expression
cYFP-VviARF27 + nYFP-VviIAA27	15	9 with one or more spots or speckles, 6 with none
nYFP-VviARF27 + cYFP-VviIAA27	11	10 large spots/smaller speckles, 1 none
nYFP-VviARF27 + VviIAA27-YFPc	24	16 nuclear with speckles and/or large spots, 3 nuclear and weak expression through the cell, 2 nuclear, 2 faint nuclear, 1 none
cYFP-VviARF27 + VviIAA27-YFPn	6	5 none, 1 with large spots with faint nuclear expression

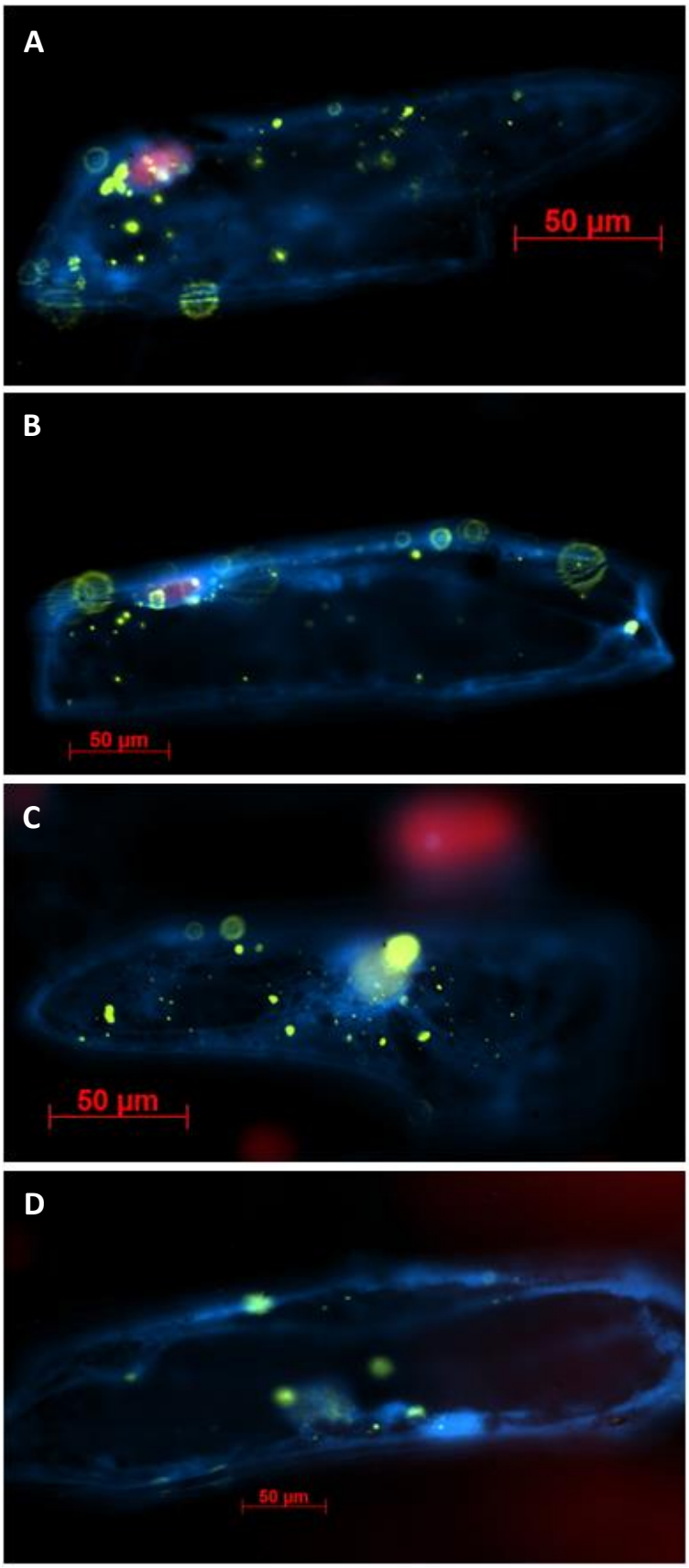


Figure 5.8 A representation of the expression seen between (A) cYFP-VviARF27+ nYFP-VviIAA27, (B) nYFP-VviARF27 + cYFP-VviIAA27, (C) nYFP-VviARF27 and VviIAA27-YFPc, (D) cYFP-VviARF27 + VviIAA27-YFPn. Channels as per Figure 5.6.

5.6.2.3 VviARF27 + VviIAA19

The BiFC results also confirmed that VviARF27 can interact with VviIAA19 and that these proteins are targeted to the nucleus. These results were the clearest of the three VviARF + VviIAA combinations (Table 5.6). All six cYFP-VviARF27 + nYFP-VviIAA19 CFP positive cells had YFP expression within the nucleus (Figure 5.9_A). The majority of cells bombarded with nYFP-VviARF27 + cYFP-VviIAA19 and nYFP-VviARF27 and VviIAA19-YFPc showed strong nuclear YFP expression (Figure 5.9_B, C). Once again the weakest expression was seen with cYFP-VviARF27 + VviIAA29-YFPn, with only one of four cells having faint nuclear YFP expression and the remaining three cells having no YFP expression (Figure 5.9_D).

Table 5.6 The pSITE VviARF27 and VviIAA19 BiFC results. The number of cells photographed and their expression patterns is described for the four construct combinations that produced fluorescence.

Construct combination	Cells photographed	Expression
cYFP-VviARF27 + nYFP-VviIAA19	6	All 6 nuclear
nYFP-VviARF27 + cYFP-VviIAA19	6	6 strong nuclear
nYFP-VviARF27 + VviIAA19-YFPc	13	12 strong nuclear, 1 medium nuclear
cYFP-VviARF27 + VviIAA19-YFPn	5	1 faint nuclear, 4 none

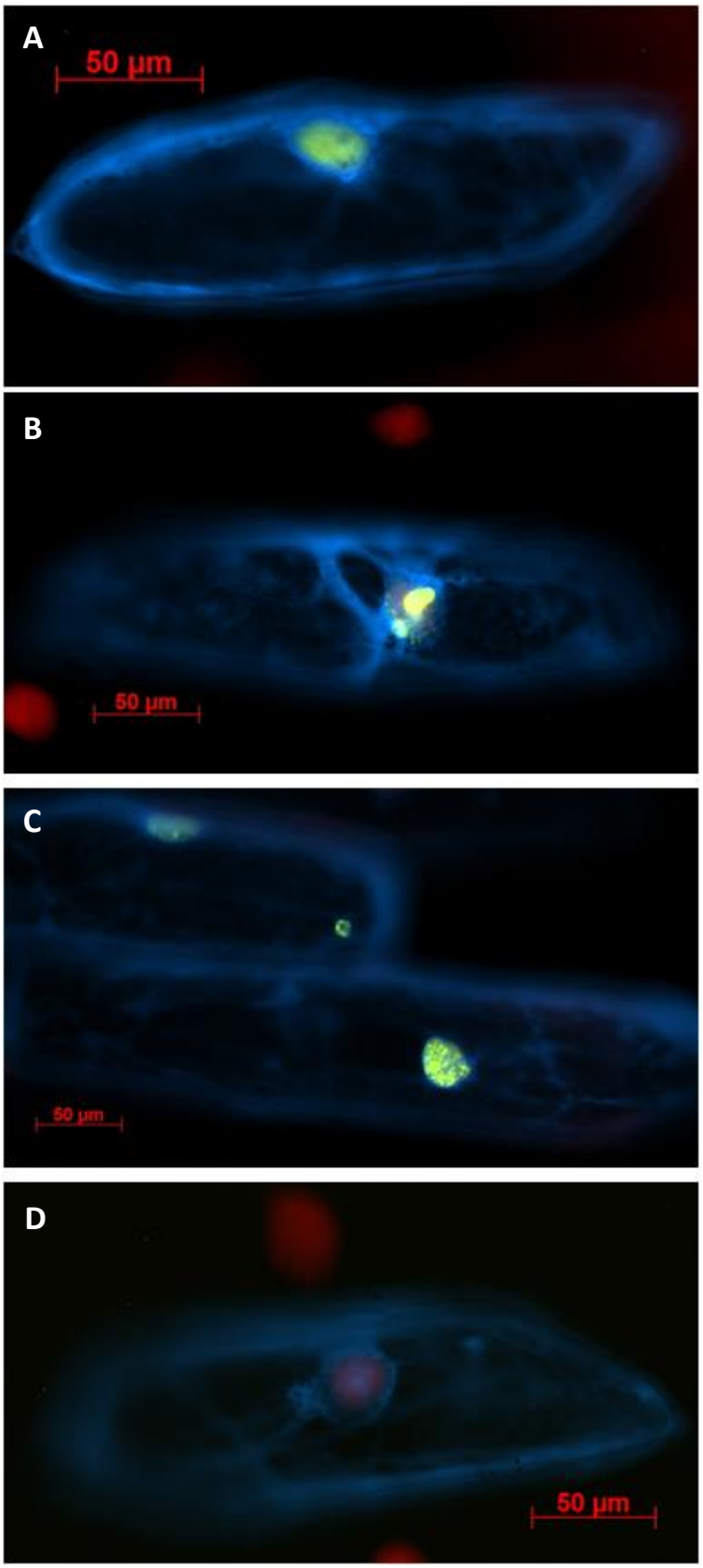


Figure 5.9 A representation of the expression seen between (A) cYFP-VviARF27 + nYFP-VviIAA19, (B) nYFP-VviARF27 + cYFP-VviIAA19, (C) nYFP-VviARF27 + VviIAA19-YFPc, (D) cYFP-VviARF27 + VviIAA19-YFPn. Channels as per Figure 5.6.

5.7 Discussion

5.7.1 Full-length sequences

In Chapter Three, coding sequences were identified through bioinformatic analysis for all *VviARF* and *VviIAA* family members. In this Chapter, coding sequences were cloned for three *VviARF* and three *VviIAA* family members. This revealed that two of the three *VviIAAs* differed from the sequence predictions and all three VviARF sequences differed from their predictions, however these differences did not appear to suggest any altered protein domains. The sequence differences suggest that the prediction of the splicing was incorrect in some cases, and the single nucleotide polymorphisms indicate that variety specific variation exists between the genome reference sequence (Pinot noir) and the cDNA, which was derived from Shiraz. In the case of VviARF27, the differences could be due to the polymerase or sequencing machinery having difficulty in amplifying the highly repetitive glutamine rich middle region or VviARF27 being amplified from Week 15 cDNA samples where the high sugar levels at this point may cause mRNA degradation or alternative splicing (Davis *et al.,* 2006). It is also possible that there are multiple isoforms of VviARF27. If this is the case, both carry a large insert in the middle. One has a simple insert that suggests the predicted sequence is incorrect as the inserted region matches the genomic sequence. The other isoform has a large insert but within this insert there are sequences that do not match the genomic sequence, potentially suggesting the presence of a transposon (Figure 5.2). The exact reason for the large number of differences present in VviARF27 is unclear and further analysis is required.

5.7.2 Yeast library screens

Yeast library screens were carried out using all three VviARF candidates as bait. Unfortunately, the yeast library screen with VviARF24 was unsuccessful in producing blue colonies, meaning that VviARF4 and VviARF27 became the focus of further analysis. The screens with both VviARF4 and VviARF27 yielded hundreds of yeast colonies that were blue in colour, suggesting potential interactions were occurring with prey from the Week 4 and Week 12 libraries, respectively. The colonies from these screens were streaked onto quadruple drop out plates and a number of blue colonies were maintained through to plasmid isolation as per the manufacturer's protocol. Of the interacting plasmids, 28 *VviARF4* prey and 11 *VviARF27* prey were sequenced. Very few full-length matches were identified and when combinations were retested in co-transformations not all interactions could be confirmed (Figure 5.3).

In the VviARF4 co-transformation with prey 3#29 (COP9-signalosome subunit) the yeast growth and blue colour intensity suggested that there was an interaction between the proteins. The COP9 signalosome has been found to play roles in plant development, including photomorphogenesis, auxin

response and flower development by regulating specific protein degradation (Serino and Deng, 2003). The isolated fragment was not the entire coding sequence, therefore the full-length COP9 gene would need to be isolated and tested to determine whether this interaction was maintained. The importance of protein degradation during auxin response suggests that COP9 may be a candidate worth following in future studies.

In the VviARF27 co-transformations determining the presence or absence of an interaction was complicated by the background auto-activation of VviARF27. It appeared that VviARF27 was potentially interacting with 16#3 (VvGRIP3) and 2#43 (VviTrans-2,3-enoyl-CoA reductase-like), both of which were full-length sequences and were subsequently investigated through BiFC interaction analysis. Robinson & Davies (2000) found that the grape ripening-induced (GRIP) transcripts were highly abundant in Shiraz berry cDNA libraries, with *VviGRIP3, 4, 13*, and *15* in early ripening (10 WPF) and *VviGRIP22, 28, 32, 51,* and *61* in later ripening (12 WPF). Based on their homology, VviGRIP3 and 4 were suggested to play a role in strengthening cell walls, potentially as a developmentally controlled preventative measure against pathogen attack. VviTrans-2,3-enoyl-CoA reductase-like is homologous to AtEnoyl-CoA, which is involved in the elongation of very long chain fatty acids that is required for cuticular wax, storage lipid and sphingolipid metabolism (TAIR, AT3G55360). There have been no studies to suggest the interaction of these two proteins with ARF proteins in the past, and the BiFC analysis failed to confirm *in planta* interaction. The predicted location of the VvGRIP3 protein within the cell wall and the VviTrans-2,3-enoyl-CoA reductase-like protein predicted to be located in the endoplasmic reticulum is also somewhat contradictory compared to the predicted nuclear localisation of VviARF27.

Previous studies used yeast library screens to isolate Aux/IAA proteins that interact with ARF transcription factors (Kim *et al.,* 1997) and it was hoped that this approach would yield similar protein interaction partners within this work. However, the yeast library screens within this work were relatively unsuccessful. This is consistent with a range of research articles that discuss the prevalence of false-positive interactions in yeast library screening (Koegl & Uetz, 2008; Brückner *et al.,* 2009). This lack of success may have been for a variety of reasons. Some problems were faced with the culture density when growing and mating the yeast such that the mating rates consistently produced lower than expected growth. Additionally, when trying to isolate single colonies, if a large amount of a yeast colony was streaked onto the quadruple drop out media the yeast may have grown on itself rather than the selection media. In addition, the large number of colonies and the streaking steps required to isolate single plasmids may have led to a dilution of the expected interacting partners. The removal of the DBD may have influenced the protein conformation and thus the ability of ARFs to bind with

their endogenous targets. However, previous work in rice with truncated ARF proteins indicated that most ARF activators without the DBD were still able to bind rice Aux/IAA proteins, whilst a single truncated ARF activator and two truncated ARF repressors were unable to interact with rice Aux/IAA proteins suggesting some activator ARF proteins retain functionality despite truncations whilst others do not (Shen *et al.,* 2010). Documented interactions between ARF repressors and Aux/IAA proteins are limited, hence it is unclear whether this lack of interaction is due to the absence of the domains or the ARF repressors themselves (Shen *et al.,* 2010). The libraries were prepared using a Clontech kit (Chapter 2) that was designed to be enriched with fragments larger than 600 bp, however, the majority of the fragments sequenced from prey plasmid isolation contained small fragments of genes less than 600 bp in length, rarely containing full protein sequences. Therefore, it is possible that there was non-specific binding of small gene fragments to the bait proteins. In addition, previous Y2H studies have used normalised Arabidopsis libraries, but this was not possible in this study (Causier & Davies, 2002). In the future, it would be ideal to use normalised grape libraries as this may reduce the prevalence of small non-specific fragments and the binding of the most highly expressed genes, such as the GRIP3 and GRIP4 proteins. Optimisation of the yeast library screen process would also be necessary to ensure a higher likelihood of the isolation of prey fragments. Auxin could be added to the media to test if this alters the number of true-positives isolated (Tiwari *et al.,* 2003). Additionally, with the 29 of 48 auxin signalling pathway members in grape having the highest transcript abundance at Week 1, a normalised Week 1 cDNA library should be generated to ensure the presence of interacting partners.

In addition to the standard yeast screening steps, yeast colony PCRs were tested to quickly identify VviIAA prey protein sequences. Degenerate primers were designed to amplify a number of the VviIAA proteins if they were present within the prey vector. Through this process three VviIAA sequences were identified, matching to VviIAA11, 19 and 41. As shown in Figure 5.1, VviARF4 and VviIAA41, and VviARF27 and VviIAA19 show the potential to interact based on their expression patterns in berry development. *VviARF27* and *VviIAA11* are both expressed pre- and post-veraison, decreasing towards veraison and increasing post-veraison, supporting the idea that they may interact (Figure 5.10). *VviARF27* and *VviIAA41* have opposing expression patterns post-veraison, however, they are both present pre-veraison and may interact during this period of berry development (Figure 5.10). This further supports the concept that the interacting partners were in fact present in the study but may have been lost through the multiple streaking steps involved in isolating single colonies.

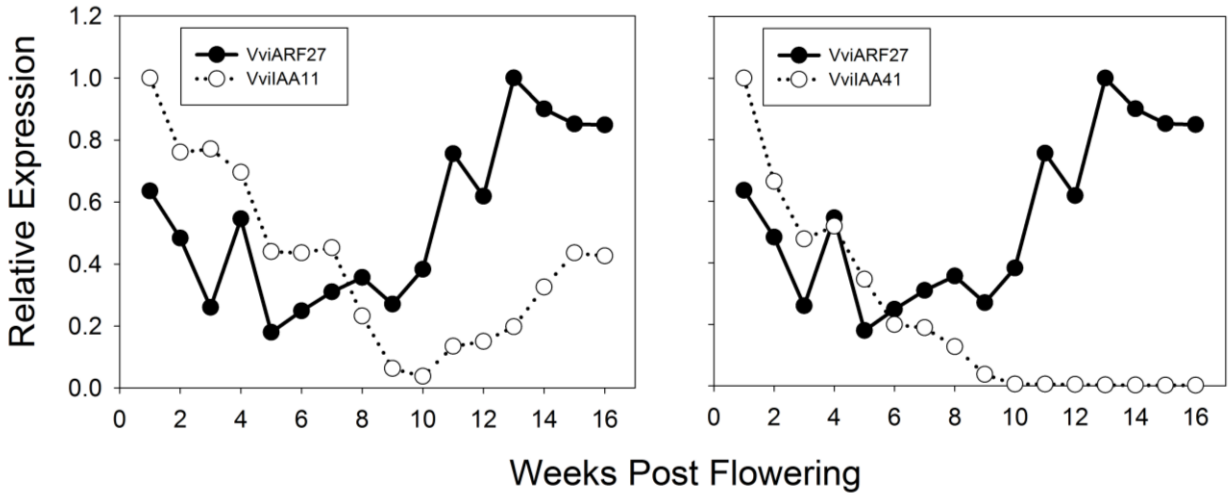


Figure 5.10 Expression patterns of the *VviARF27* candidates and the *VviIAA* candidates identified using PCR on yeast colonies.

Relative expression is used to compare the transcript patterns of the candidates during the 16 weeks of *V. vinifera* L cv. Shiraz berry development for each of the *VviARF-VviIAA* pairs.

5.7.3 Interaction analysis – yeast co-transformations and BiFC

This study was able to identify interactions between VviARF4+VviIAA19, and VviARF27+VviIAA19, and VviARF27+VviIAA27 using yeast 2-hybrid and BiFC. *VviARF27* and *VviIAA19* have similar expression profiles that peak after veraison towards the end of berry development, supporting a hypothesis that they may be interacting partners *in planta*. Contrasting this, *VviARF4* and *VviIAA19*, and *VviARF27* and *VviIAA27* have opposing expression patterns but clear interactions were still seen (Figure 5.11). These proteins may have the capacity to interact *in planta* although it appears unlikely that they may do so in berries. The proteins potentially interact in other organs within the plant where they are co-expressed, such as in flowers. Not all ARF-Aux/IAA combinations showed an interaction, suggesting some specificity.

Piya *et al.* (2014) conducted an in-depth interaction analysis between ARF-Aux/IAA in Arabidopsis, and showed that ARF activators interacted with the largest number of Aux/IAA proteins, which is consistent with the VviARF27 activator interacting with multiple VviIAA proteins in this work. In Piya *et al.* (2014) only six repressor ARFs were found to interact strongly with Aux/IAA proteins, none of which were as promiscuous as the ARF activators and this may be linked to the presence/absence of protein domains in the ARF and Aux/IAA proteins. Piya *et al.* (2014) identified that AtARF4, the closest homolog of VviARF4, interacted widely with AtIAA proteins. It may be that specific Aux/IAAs are 'stickier' and able to interact with multiple ARFs (Piya *et al.,* 2014) and the specificity and functional relevance of these interactions may depend on whether they have overlapping spatial and temporal expression *in planta*. Alternatively, the middle-region may not be the only factor determining whether an ARF protein is capable of functioning as a repressor protein. Interestingly, neither VviARF4 nor VviARF24 interacted with VviIAA27 which was selected due to the similarity of its berry expression pattern to those ARFs. This suggests that even if co-expression is occurring, this does not ensure an interaction between the proteins. One possibility is that as putative transcriptional repressor proteins, VviARF4 and VviARF24 may simply act alone in gene repression by binding directly to DNA and competing with ARF activators in promoter binding (Vert *et al.,* 2008). Piya *et al.* (2014) reported no auto-activation, which is inconsistent with the results of this study where VviARF27 had strong auto-activation when the DBD was present and minor auto-activation once the DBD was removed (Figure 5.3, Figure 5.4).

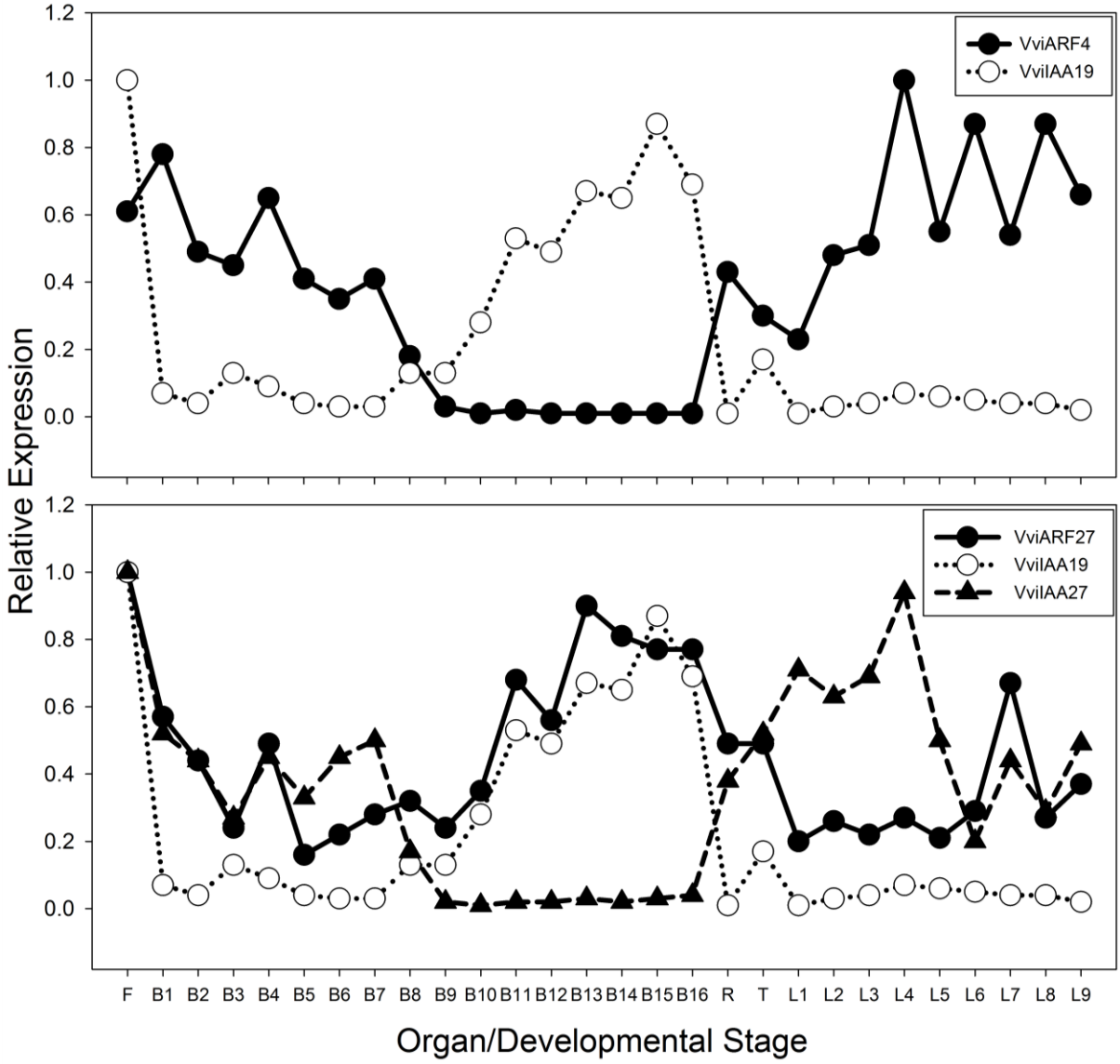


Figure 5.11 The expression patterns of the confirmed *VviARF* and *VviIAA* interacting partners confirmed using Yeast 2-Hybrid and bimolecular fluorescence analysis in all developmental series and organ types.

Relative expression is used to compare the transcript patterns of the candidates in flowers (F), 16 weeks of berry development (B1–B16), roots (R), tendrils (T), and nine stages of leaf development (L1–L9) in *V. vinifera* L. cv. Shiraz for *VviARF4* and *VviIAA19* and *VviARF27* and *VviIAA19* and *27*.

Interestingly, VviARF24 did not interact with either VviIAA19 or VviIAA27 in the co-transformation experiment and no interacting Aux/IAAs were identified in the yeast library screening. It is possible that these two issues are related and there may be no protein being produced, the protein may be produced or folded in an inactive form, or that the protein produced is toxic to the cells (Van Criekinge & Beyaert, 1999; Brückner *et al.,* 2009). Alternatively, it may be that VviARF24 is more similar to AtARF11 than AtARF18 and it not interacting with any IAA proteins *in planta* and is instead acting as a classical ARF repressor protein. To confirm protein production a protein pull-down experiment would be necessary to detect the presence of a protein. In addition to this, VviIAA41 could not be successfully transformed into yeast, possibly due to cell toxicity (Van Criekinge & Beyaert, 1999).

BiFC confirmed the interaction of VviARF4-VviIAA19 and VviARF27-VviIAA19 and showed that the proteins were located within the nucleus. Interestingly, there was a strong speckled pattern with VviARF27 and VviIAA27 which may suggest that there is protein aggregation. The position of the halves of YFP appears crucial to the ability of the proteins to interact, and also the intensity of the interaction. The strongest interactions were consistently seen with the nYFP-VviARF and VviIAA-YFPc vector combination (Figure 5.7, Figure 5.8 and Figure 5.9), as illustrated in the schematic in Figure 5.12. The next step would be to determine the functional relevance of these interactions through chromatin precipitation to identify the areas of DNA that the ARF proteins are binding to and controlling auxin mediated gene responses, and also through over-expression and knock-out mutant analysis in grape micro-vines (Chaïb *et al.*, 2010) and model species such as strawberry, tomato or Arabidopsis.

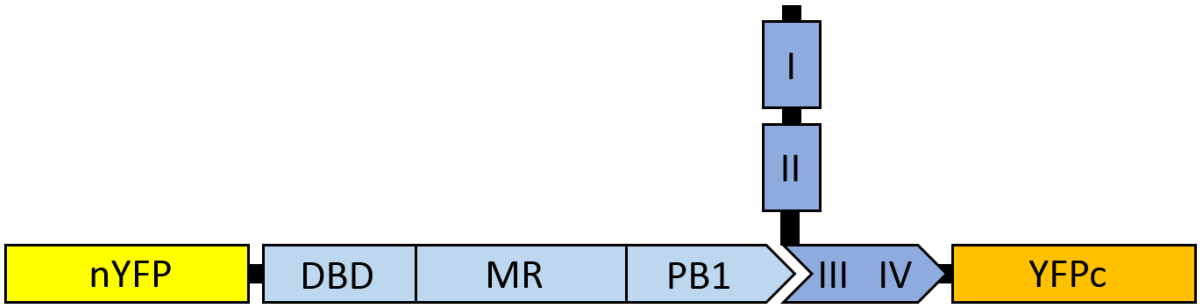


Figure 5.12 **A schematic representation of the strongest ARF and Aux/IAA interaction conformation in the BiFC analysis.**

The ARF protein is represented by the light blue coloured boxes and contains a DNA-binding domain (DBD), middle region (MR) and PB1 fold that forms a dimer with Domains III and IV in the Aux/IAA protein. The Aux/IAA protein is represented by the darker blue coloured boxes and contains Domains I - IV. The N terminal end of the YFP protein is fused at the C terminus to the ARF protein, whilst the C terminal end of the YFP protein is fused at the N terminus to the Aux/IAA protein. Based on a schematic in Hagen (2015).

The interaction of VviARF27 with VviGRIP3 and VviTrans-2, 3-enoyl-CoA reductase-like proteins detected in co-transformation in yeast was not confirmed by BiFC analysis, suggesting that they were not true interactions. The use of the 35S promoter driving gene expression in the same location may allow for the interaction of proteins that would not normally interact at their normal levels, may not co-localise, or be co-expressed in grape. Also, the yeast library screens and co-transformations used ARFs lacking their DBD, which may have impacted protein structure. However, in the BiFC analysis auto-activation is not a concern so full-length sequences could be used and the same negative results were seen. Whether or not this alters the interaction of the ARF and yeast prey proteins needs to be considered despite previous studies in rice showing truncated ARF proteins containing only the middle region + PB1 domain or the PB1 domain alone were capable of binding Aux/IAA proteins with only the intensity of the interaction in yeast being altered (Shen *et al.,* 2010).

This work describes the first yeast and BiFC interaction analysis with the ARF and Aux/IAA proteins from *V. vinifera*, however a comprehensive study, like that reported in Piya *et al.* (2014), would be informative and a good future step towards understanding the complexity of the ARF-Aux/IAA interaction network in *Vitis*. Protein pulldowns or genetic analysis would be required to determine the functional significance of these interactions.

5.7.4 Determining the phytohormone responsiveness of VviARF, VviIAA and VviAFB candidates will add to our understanding the auxin signalling pathway

VviARF and VviIAA proteins expressed with the same temporal and spatial expression patterns may play similar roles in fruit development in *Vitis*. However, the mechanisms behind how these proteins interact and what controls their expression is currently unknown. As phytohormones have been widely implicated in the control of fruit development it is possible that the presence or absence of hormones within the berries determines the patterns of expression of the members of the auxin signalling pathway and thus the capacity of different ARF and Aux/IAA proteins to interact. Since the levels of phytohormones present within the berry have been studied, we can relate gene expression in Chapter 4 to these. However, phytohormone treatment studies are required to determine whether the transcript levels of a gene are influenced by specific phytohormones. Some research has been done to understand which phytohormones stimulate the expression of these genes (Kohno *et al.,* 2012; Fujita *et al.,* 2012; Pilati *et al.,* 2017). Such a study in grape berries would enable a direct comparison to other studies that have used different tissues, and would add to the knowledge about the induction or repression of the family members that have not been functionally studied at this point.

Chapter 6 The responsiveness of auxin signalling pathway genes to phytohormones

6.1 Aim

The aim of this work was to understand how five phytohormones influence the expression of *VviARF*, *VviIAA* and *VviAFB* transcript levels pre- and post-veraison in *Vitis vinifera* L. cv. Shiraz berries and identify putative motifs present in the gene promoters that might be responsible for changes in transcript levels.

6.2 Introduction

Whole grape berry *ex planta* assays have been optimised and used previously with a range of phytohormones, including IAA, NAA, BTOA, Ethrel, ABA, and sucrose, to determine their effects on gene transcript levels (Böttcher *et al.,* 2010b, Gambetta *et al.,* 2010; Böttcher *et al.,* 2011). Gambetta *et al.* (2010) investigated ABA and sugar cross-talk within berries and found that berries grown on culture for 23 d increased in size when treated with 2 or 10% sucrose, by 21 and 8% respectively, whilst remaining green in colour. Different combinations of sucrose and ABA concentrations, however, led to smaller changes in berry weight but marked changes in anthocyanin accumulation, berry softening and expression of ripening-associated transcripts (Gambetta *et al.,* 2010). Comparing expression data between *ex planta* berry cultures with field-grown berries show that *ex planta* treatments mimic field-grown grapes, supporting the use of *ex planta* treatments as a reproducible and controlled method of determining the influence of phytohormones on transcript levels (Gambetta *et al.,* 2010).

To establish the effect of phytohormones on the auxin signalling pathway gene candidates at different stages of grape berry development, pre- and post-veraison *V. vinifera* L. cv. Shiraz berries were used for *ex planta* treatments. The transcripts had a range of accumulation patterns across berry development and may be playing important functional roles during this time. In pea epicotyl tissues the primary auxin response occurs within minutes of auxin exposure and is characterised by changes in the transcript levels of some *SAUR*, *GH3* and *Aux/IAA* genes, with some transcripts being 50–100 fold higher within 2 h of the phytohormone treatment (Theologis *et al.,* 1985; Abel & Theologis 1996; Chapman & Estelle, 2009). Other reports suggest a prolonged auxin induction, for example in *Capsicum chinense* L. fruit where *GH3* transcripts were upregulated from 30 min to 24 h (Liu *et al.,* 2005). The timing of the primary responses in grape is relatively unclear. The primary responses of *SAUR*, *GH3* and *Aux/IAA* genes begin a signalling cascade causing downstream transcriptional changes

in genes such as *ARFs*. *Ex planta* treated berries have a small area of tissue in their brush area exposed to the media and the time taken for the movement of the phytohormones into the whole berry is unknown. For these reasons sampling was conducted between 0 and 48 h to try to ensure the responses to the treatments were captured.

Phytohormone responsiveness is thought to be mediated through the presence of motif sequences within promoter regions and introns of phytohormone-responsive genes, which are responsible for protein-DNA interactions that regulate transcription (reviewed in Qiu *et al.,* 2016). The promoter region is divided into two parts; the core promoter, 50-100 bp upstream from the 5' UTR and start codon, which interacts with the transcriptional machinery, and the upstream regulatory region which contains sites or binding motifs for the binding of gene-specific regulators (Novina & Roy, 1996; Singh, 1998; Wu *et al.,* 2001; Dutt *et al.,* 2014). These motifs contribute to the complex expression profiles of genes, and the presence or absence of motifs within this regulatory region allows predictions to be made as to the phytohormones, biotic and abiotic factors that are regulating changes in transcription (Dutt *et al.,* 2014). Kumar *et al.* (2015) reported a correlation between the presence of phytohormone related cis-acting promoter elements and the differential expression of *Aux/IAA* and *ARF* genes in tomato and potato in response to specific phytohormone treatments, however, it was not strictly followed in the case of all genes. Specialised motifs have been identified for all phytohormones, including the AuxRE motif (Section 1.1.5), that is directly bound by ARF proteins in the upstream region of genes regulated by auxin (Liu *et al.,* 1994; Ulmasov *et al.,* 1995). Three motifs have been characterised as ABA-responsive elements; ACGT-containing abscisic acid response elements (ABRE), *cis*-regulatory elements (CRE) and coupling element 3 (CE3) (Hobo *et al.,* 1999; Gomez-Porras *et al.,* 2007). The ethylene responsive element (ERE) (Tapia *et al.,* 2005) is frequently present in ethylene-responsive genes. In this study promoter analysis was completed on the 5' UTR regions and 2 kb of the region upstream from the UTR to determine the presence of motifs and infer the factors regulating *VviARF* and *VviIAA* transcription. The *ex planta* phytohormone response data combined with the promoter analysis may therefore provide information on the integration and overlap of phytohormone signalling.

6.3 Results

6.3.1 Phytohormone application alters the expression of auxin signalling pathway family members in grape berry *ex planta* samples

Seven separate treatments were used, including the exogenous supply of the phytohormones; NAA, iP, epi-BL, ABA and Ethrel, and two types of control media. The first control included plates that

contained standard media with sucrose, as provided with each hormone treatment, to act as a direct comparison to determine phytohormone responsiveness. The second was a 'sucrose deficient' control, included to determine if the addition of sucrose in the media was influencing the transcript profiles when no phytohormones were present. The transcript levels of *VviARF*, *VviIAA* and *VviAFB* transcripts were then measured using RT-qPCR and the method described in Section 2.2.5.6. Week 6 pre-veraison berries and week 12 post-veraison berries were treated and samples collected at time 0, 3, 24 and 48 hours (Section 2.1.5.2, Figure 2.3). The arrangement of the berries on the media is shown in Figure 2.5, and this was replicated for the post-veraison berries. The fold-change difference in transcript level between each sample and the control was calculated, and the significance determined using Student's T-test, with P = 0.01 (Section 2.2.9.2). Only fold changes above 1.5 have been discussed here, using a criterion previously described by Morey *et al.* (2006). Tables of the results are in Appendix G. Pre-veraison, 47 gene candidates showed altered expression, compared to 36 post-veraison. A total of 21 of the 48 genes were unresponsive to all treatments pre-veraison; including one *VviAFB*, 11 *VviARFs* and nine *VviIAAs*. Post-veraison 23 of the 48 genes were unresponsive to all treatments; including five *VviAFBs*, nine *VviARFs* and nine *VviIAAs*. Thirteen transcripts were unresponsive to all treatments at both developmental stages; including *VviAFB10*, *VviARF1a, 1b, 2a, 26, 27, 29*, and *31*, and *VviIAA26, 34a, 34b, 43, 44,* and *45*.

The largest proportion of responses were down-regulation, with 74 examples of down-regulation and 23 examples of up-regulation (Figure 6.1, Appendix G). The general trend is that NAA causes up-regulation of transcripts, mainly *VviIAA* candidates, whilst ABA, iP, BL, Ethrel and sucrose deficient media cause the down-regulation of auxin signalling transcripts across all three gene families. The following 11 gene transcripts responded to a single treatment only; *VviAFB6, 7,* and *11*, *VvARF17*, and *32*, *VviIAA9*, *31, 33, 37, 41*, and *42* (Figure 6.1, Appendix G). No transcripts responded the same way to all treatments pre- and post-veraison, but seven gene transcripts were responsive to the same single treatment both pre- and post-veraison, including *VviIAA38* with NAA, *VviIAA15b, 19, 35* and *VviARF24* with Ethrel, and *VviARF2b*, and *25* with the sucrose deficient control, although the response times were not identical. Two gene transcripts had the opposite responses to the same treatment pre- vs. post-veraison; *VviARF25* with Ethrel treatment and *VviIAA15a* with sucrose deficient media. Pre-veraison the highest positive fold changes were *VviIAA19* (+2.7) and *VviIAA39* (+3.4) at 3 h, and *VviIAA36* (+2.8) at 24 h in NAA treated berries, and *VviIAA15a* (+3.1) at 3 h in berries treated with Ethrel. Post-veraison the highest positive fold changes were *VviIAA40* (+4.3), *VviIAA38* (+3.8), and *VviIAA15b* (+2.7) with NAA at 48h. The largest pre-veraison down-regulation fold changes were *VviARF4* (-2.5) at 24 h, *VviIAA15b* (-2.5) and *VviIAA19* (-2.7) at 48 h in the sucrose-deficient control. The post-veraison berries treated with Ethrel displayed the largest negative fold changes in the *ex*

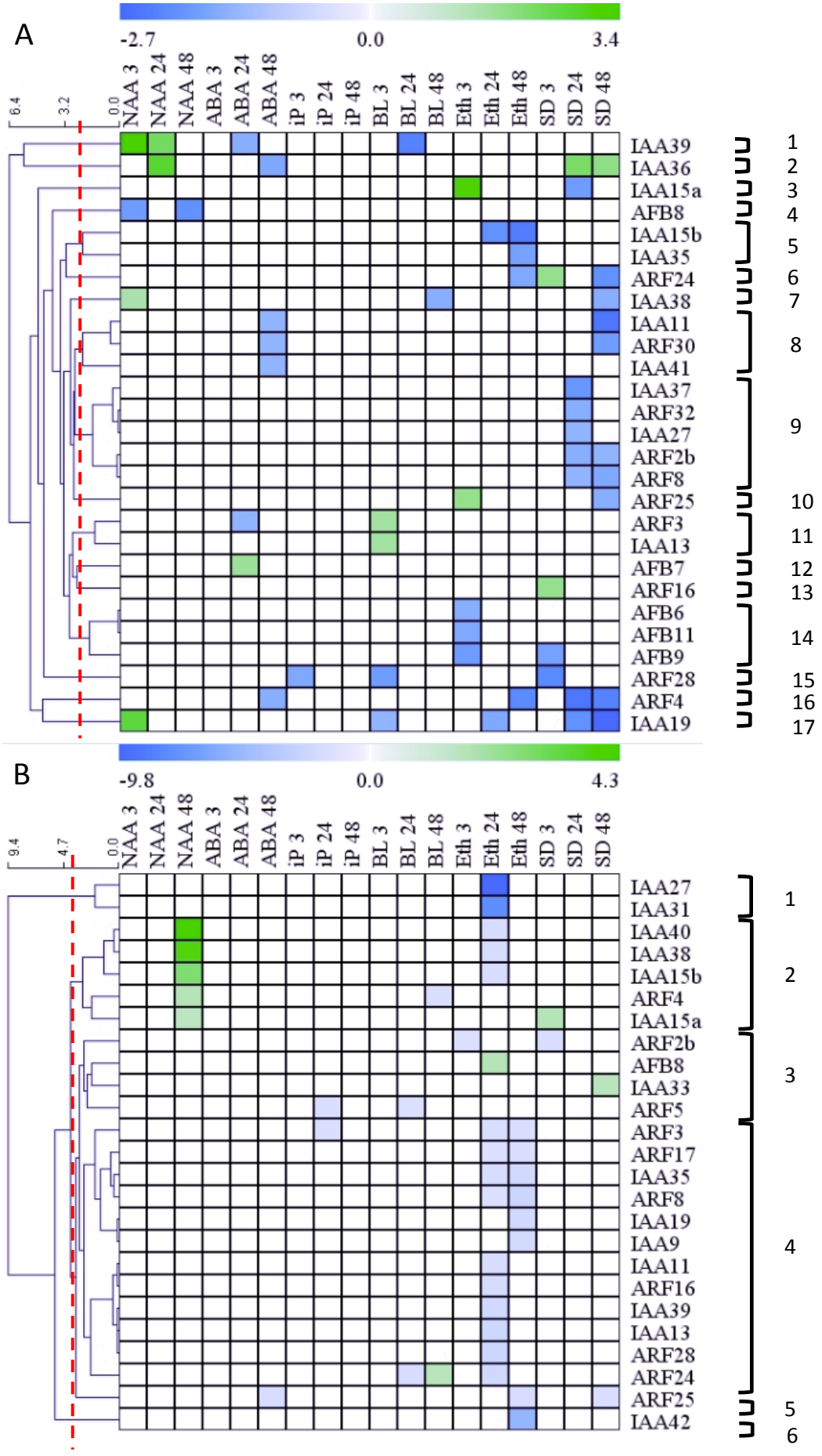
planta analysis, with *VviARF28* (-2.5, A), *VviIAA27* (-9.8), and *VviIAA31* (-7.7) at 24 h and *VviARF8* (-2.6, A) and *VviIAA42* (-5.3) at 48 h.

6.3.2 Hierarchical clustering of *ex planta* transcript changes identified pre-veraison and post-veraison clusters with shared phytohormone responses

Hierarchical cluster analysis was conducted on the statistically significant results from the pre- and post-veraison *ex planta* phytohormone treatments to identify genes that had similar response patterns to one or more of the six treatments (Figure 6.1). Pre-veraison there was a diverse range of patterns that did not cluster well, with a total of 17 clusters, 12 of which contained only single genes (Clusters 1–4, 6, 7, 10, 12, 13, 15–17) (Figure 6.1_A). General trends are discussed for the pre-veraison data with reference to the clusters where applicable. Upon NAA treatment pre-veraison, four *VviIAA* transcripts were up-regulated, *VviIAA19, 36, 38* and *39* (Clusters 1, 2, 7, 17), whilst a single *AFB* receptor, *VviAFB8* in Cluster 4, was down-regulated, most substantially at the 3 h time point (Figure 6.1_A). Between 24 – 48 h after ABA treatment, *VviARF3, 4,* and *30 (A),* and *VviIAA11, 36, 39*, and *41*, were all down-regulated, Cluster 8 contained three of these genes; *VviARF30* and *VviIAA11* and *41*. A single *AFB* receptor, *VviAFB7* in Cluster 12 was up-regulated. Only a single *ARF, VviARF28 (A)* was down-regulated by iP treatment at 3 h (Cluster 15). In Cluster 11 *VviARF3* and *VviIAA13* were up-regulated by BL treatment, whilst *VviARF28 (A), VviIAA19, 38* and *39* were down-regulated all at various time points. With Ethrel treatment, *VviARF25* and *VviIAA15a* were up-regulated, and *VviARF4* and *24*, *VviIAA15b, 19,* and *35*, and in Cluster 14 *VviAFB6, 9,* and *11* were all down-regulated. Finally, with the sucrose deficient media, 14 candidates showed down-regulation whilst only three showed up-regulation. *VviARF16* and *VviIAA36* were up-regulated, *VviARF24* was up-regulated at 3 h and down-regulated at 48 h. *VviARF2b, 4, 8 (A), 25, 28 (A), 30 (A),* and *32*, *VviIAA15a*, *15b, 19, 27, 37* and *18* and *VviAFB9* were all down-regulated. *VviARF2b, 8, 32, VviIAA27* and *37* form the largest pre-veraison cluster, Cluster 9, as all of these transcripts are down-regulated in the sucrose deficient media alone. Both Ethrel and sucrose deficient media led to transcriptional changes across all three time points. *VviARF30 (A)* was down-regulated at 48 h in both berries treated with ABA and with sucrose deficient treatment. Several genes appeared to be particularly responsive to treatment; *VviIAA36* and *39* were up-regulated by NAA treatment and down-regulated by ABA, supporting the opposing effects of these two phytohormones, additionally *VviIAA36* was up-regulated in the absence of sucrose, and *VviIAA39* was down-regulated with BL treatment (Clusters 1 and 2).

Six clusters were identified in the post-veraison experiment (Figure 6.1_B). The post-veraison Cluster 1 contained the most significantly down-regulated transcripts within these experiments with *VviIAA27* and *31* strongly down-regulated at 24 h in berries treated with Ethrel. Cluster 2 contained *VviARF4*,

and *VviIAA15a, 15b, 38* and *40* which were up-regulated at 48 h in berries treated with NAA. Additionally, *VviIAA15b, 38* and *40* were down-regulated at 24 h in Ethrel treated berries; VviARF4 was down-regulated at 48 h in berries treated with BL; and *VviIAA15a* was up-regulated at 3 h in the absence of sucrose. Cluster 3 has a less distinct cluster pattern: *VviARF2b* was down-regulated at 3 h in berries treated with both Ethrel and sucrose deficient media; *VviAFB8* was up-regulated at 24 h in berries treated with Ethrel; *VviIAA33* was up-regulated at 48 h in the absence of sucrose; and *VviARF5* was down-regulated at 24 h in berries treated with iP and BL. Cluster 4 was the largest cluster containing 11 transcripts with down-regulation in berries treated with Ethrel. The *VviIAA9* and *19* transcripts were down-regulated at 48 h, *VviARF3, 8* and *17*, and *VviIAA35* are down-regulated at 24 and 48 h, and *VviARF16, 24* and *28* and *VviIAA11, 13,* and *39* were down-regulated at 24 h. Additionally, *VviARF3* was down-regulated at 24 h in berries treated with iP, and *VviARF24* was down-regulated at 24 h and up-regulated at 48 h in berries treated with BL. Cluster 5 and 6 contained only single candidates, *VviARF25* and *VviIAA42*, respectively. *VviARF25* was down-regulated in ABA, Ethrel and sucrose deficient treatments, whilst *VviIAA42* was down-regulated at 48 h with Ethrel treatment. Interestingly, *VviIAA9* and *19* were also down-regulated at this time point with Ethrel treatment, these candidates may not cluster with *VviIAA42* due to the fold difference of their down-regulation. *VviIAA9* and *19* are down-regulated -2.1 and -2.2, respectively whilst *VviIAA42* was down-regulated by -5.3 fold (Appendix G).


A
-2.7
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IAA39
IAA36
IAA15a
AFB8
IAA15b
IAA35
ARF24
IAA38
IAA11
ARF30
IAA41
IAA37
ARF32
IAA27
ARF2b
ARF8
ARF25
ARF3
IAA13
AFB7
ARF16
AFB6
AFB11
AFB9
ARF28
ARF4
IAA19
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
B
-9.8
0.0
4.3
IAA27
IAA31
IAA40
IAA38
IAA15b
ARF4
IAA15a
ARF2b
AFB8
IAA33
ARF5
ARF3
ARF17
IAA35
ARF8
IAA19
IAA9
IAA11
ARF16
IAA39
IAA13
ARF28
ARF24
ARF25
IAA42
1
2
3
4
5
6

Figure 6.1 Heatmaps generated in MeV using HCL clustering of all the *VviARF*, *VviIAA* and *VviAFB* transcripts that were significantly up- or down-regulated in the *ex planta* treatments within the (A) pre- and (B) post-veraison experiments.

Blue indicates the level of down-regulation, green indicates the level of up-regulation. The red dashed line represents the cluster cut off point. Epi-brassinolide (BL), abscisic acid (ABA), 1-Naphthaleneacetic acid (NAA), cytokinin, isopentenyladenine (iP), sucrose deficient (SD). HCL clustering parameters used: *Gene tree selection* was used for tree selection, *optimise by gene leaf order* was used for ordering optimisation, *Euclidean distance* was used as the distance metric selection, and *average linkage clustering* was used as the linkage method selection.

6.3.3 Some auxin signalling genes respond to multiple phytohormones

Auxin signalling pathway transcripts were shown to have overlapping responses to different phytohormones at each time point, as illustrated in Figure 6.2 and Figure 6.3, with nine instances of overlap pre-veraison and six post-veraison including 11 and six genes respectively. In the pre-veraison experiment at 3 h, *VviIAA19* was shown to be up-regulated by treatment with NAA and down-regulated by BL and *VviARF28* was down-regulated by both iP and the sucrose-deficient media (Figure 6.2_A). Ethrel-regulated transcripts showed no overlap with the transcripts regulated by the other treatments. At 24 h *VviIAA19* was down-regulated by both Ethrel and the sucrose deficient media, *VviIAA36* was up-regulated by both NAA and the sucrose deficient media, *VviIAA39* was down-regulated by both epi-BL and ABA, and up-regulated by NAA (Figure 6.2_B). At 48 h *VviARF4* was down-regulated by both Ethrel and ABA, *VviARF24* and *VviIAA15b* were down-regulated by Ethrel and sucrose deficient media, *VviIAA38* was down-regulated by epi-BL and the sucrose deficient media, and *VviARF30* was down-regulated by both ABA and the sucrose deficient media, whilst *VviIAA36* was down-regulated by ABA and up-regulated by sucrose deficiency (Figure 6.2_C).

Within the post-veraison *ex planta* treatments fewer transcripts overlapped with their phytohormone responses (Figure 6.3). At 3 h, *VviARF2b* was down-regulated by both Ethrel and the sucrose-deficient media (Figure 6.3_A). At 24 h, although there were a large number of transcripts down-regulated by Ethrel, overlap was only seen with the down-regulation of *VviARF3* by Ethrel and iP, *VviARF5* by iP and epi-BL, and *VviARF24* by epi-BL and Ethrel (Figure 6.3_B). Only two transcripts had an overlap at 48 h in the post-veraison samples, these were the down-regulation of *VviARF25* by ABA, Ethrel and the sucrose-deficient media and *VviARF4* up-regulated by the treatment with NAA and down-regulated with the treatment of epi-BL (Figure 6.3_C). Three transcripts pre-veraison and one transcript post-veraison were up- or down-regulated by two or more phytohormones at the same time point, these included *VviIAA19* at 3 h, *VviIAA39* at 24 h, and *VviIAA36* at 48 h pre-veraison; and *VviARF4* at 48 h post-veraison.

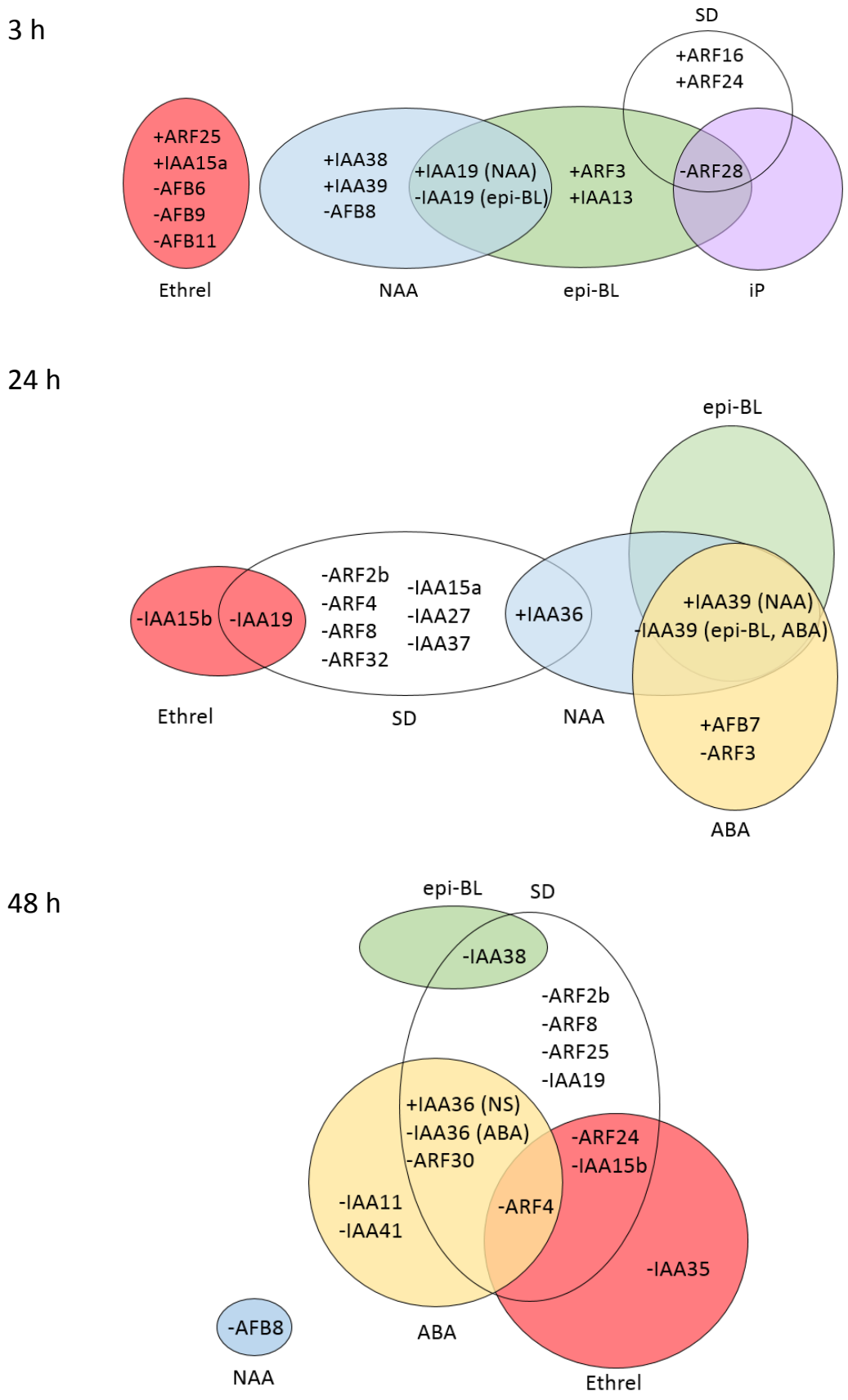

Figure 6.2 Venn diagrams of all *VviARF*, *VviIAA* and *VviAFB* transcripts that were significantly up- or down-regulated in the *ex planta* treatments pre-veraison.

The samples were collected at three hours post-treatment, 24 hours post-treatment, and 48 hours post-treatment. All with - indicating down-regulation and + as up-regulation. Epi-brassinolide (epi-BL), abscisic acid (ABA), 1-Naphthaleneacetic acid (NAA), cytokinin, isopentenyladenine (iP), sucrose deficient (SD). If the transcript levels of a gene candidate were altered in anyway, either up- or down-regulated, this was considered as overlap in the Venn diagrams and the different phytohormones inducing these changes were seen are marked in brackets within the Venn diagrams.

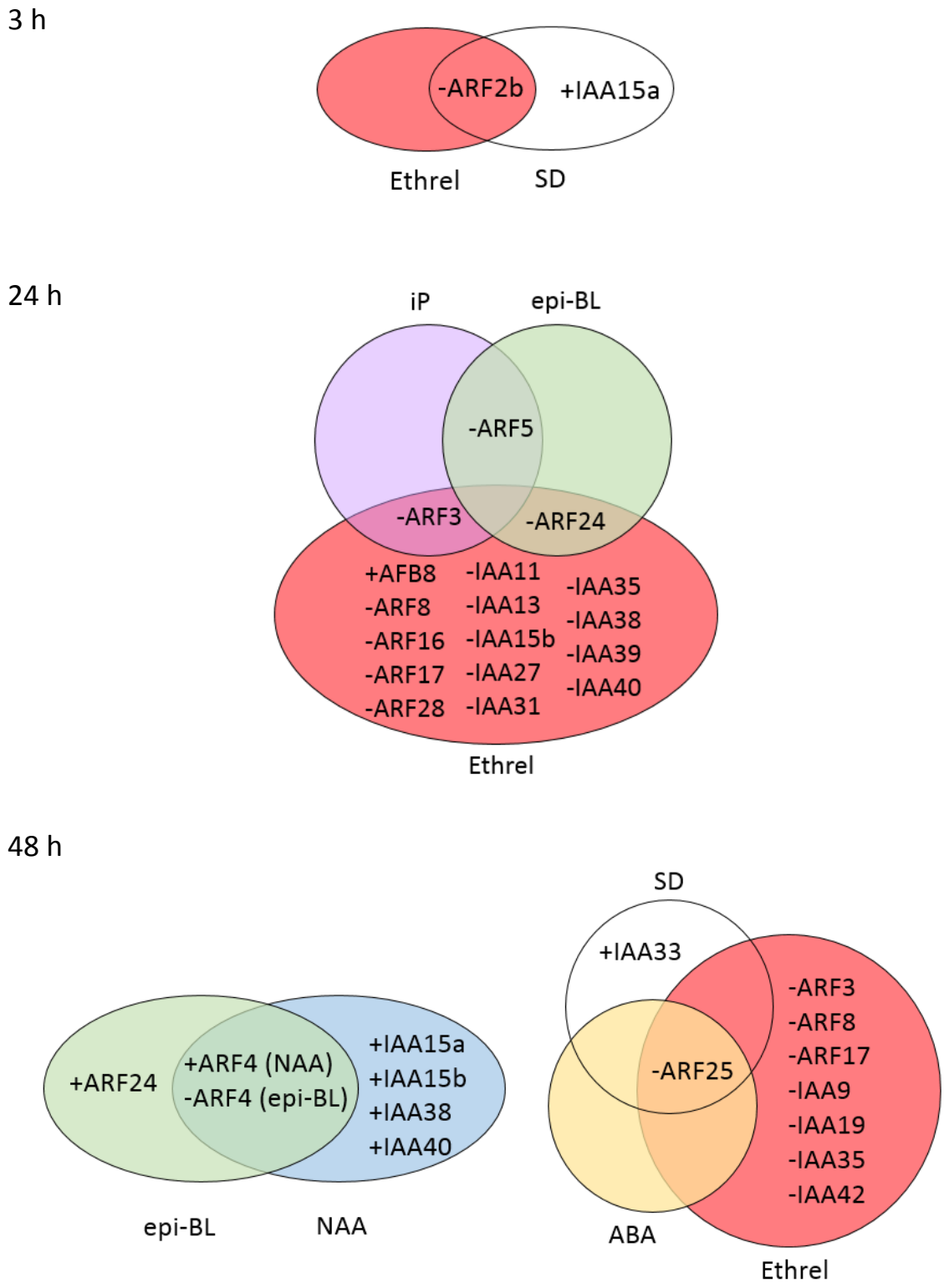

Figure 6.3 Venn diagrams of all *VviARF*, *VviIAA* and *VviAFB* transcripts that were significantly up- or down-regulated in the *ex planta* treatments post-veraison.

Refer to Figure 6.2 for figure details.

6.3.4 PlantPAN analysis identifies motif sequences in upstream regions of *VviARF* and *VviIAA* candidates

PlantPAN promoter analysis was completed on the 2 kb upstream of the 5' UTR of all *VviARF* and *VviIAA* candidates, in addition to the 5' UTR sequence. Due to the incomplete annotation and assembly of the *V. vinifera* genome the *VviAFB* promoters could not be analysed as many of the upstream regions were incomplete or unknown. The promoter analysis identified a large number of motifs that were related to different processes including plant phytohormone response, sugar response, development, and stress response. The phytohormone related motifs present included ABA, IAA, GA, cytokinin, and ethylene. The motifs were a minimum of four base pairs in size. Due to the nature of DNA and the large number of motifs present in the 2 kb fragments, a number of motifs may appear by numerical chance. For this reason an average value of the number of times a motif occurs in each promoter region was calculated across the *VviARFs* and *VviIAAs* plantPAN results (Appendix H), and a frequency of >2-fold was classified as significant. Additionally, if a motif occurred in less than half of the *VviARF* or *VviIAA* promoters the motif was considered significant.

Within the *VviARF* promoters, auxin response-related motif sequences were seen in all except *VviARF16*. Fifteen of the 19 *VviARFs* contained AuxRE binding motifs. ABA motifs were present in 13 of the 19 *VviARFs*, of these only *VviARF30 (A)* was seen to be ABA-responsive in this study (Table 6.1). Cytokinin motifs were present only in *VviARF24*, despite this the candidate did not appear to be iP responsive. Ethylene motifs were present in the selected upstream regions of *VviARF1a, 2a, 4, 25, 26* and *28*, and of these, *VviARF4, 25* and *28 (A)* were Ethrel-responsive, with *VviARF28 (A)* being up-regulated by Ethrel. Motifs involved in sugar and stress response were widely spread across the *VviARF* candidates. The *VviARF1b* promoter was the only promoter to contain a high number of the CArG motifs that are implicated in MADS box TF binding. These included the CCAAAAADGG motif present in *VviARF3* and eight promoter regions (data not shown) and the CArG box motif CYWWWWWWRG has been has been classified as a 'fruit development' motif. This motif was present in 12 of the 19 *VviARF* promoters.

As seen in the *VviARF* promoter analysis, the phytohormone motifs most prevalent across the *VviIAA* promoters were auxin and ABA motifs (Table 6.2). Auxin motifs were present in 17 of the 23 *VviIAA* candidates, excluding *VviIAA15b, 19, 26, 35, 42* and *44*, and as *VviIAA15b* and *19* were up-regulated by NAA this suggests that a key motif involved in auxin responsiveness may be missing within this promoter analysis or these are downstream changes. Only *VviIAA9* and *34a* contained AuxRE motifs. Cytokinin motifs were only present in *VviIAA15a* and *37*, despite neither of these candidates being iP-responsive in berries. Ethylene motifs were present in *VviIAA15a, 31, 33, 34a* and *40*, with *VviIAA15a*

being up-regulated by Ethrel, and *VviIAA31* and *40* being down-regulated by Ethrel. Motifs involved in sugar and stress responses were also widely spread across the *VviIAA* candidates. The 'fruit development' CArG box motif CYWWWWWWRG was present in 12 of the 23 *VviIAA* promoters.

Table 6.1 PlantPAN results for the 2 kb *VviARF* promoter fragments.

	***VviARF* Gene Name**																		
Type of motif	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Auxin	4*	10*	5*	1	14*	12*	6*	7*	-	12*	11	4*	5*	12	4*	4*	4*	6*	10*
Abscisic acid	1	-	11	3	2	10	12	-	-	-	18	39	12	3	27	-	3	-	1
Cytokinin	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-
Ethylene	5	-	2	-	-	2	-	-	-	-	-	4	2	-	4	-	-	-	-
Sugar	4	8	5	2	2	1	3	4	4	2	6	1	5	1	3	-	6	3	2
Stress	-	3	1	10	3	-	14	-	3	2	19	2	4	1	1	6	1	-	21
Fruit development[1]	-	3	1	-	-	1	-	3	1	-	1	2	1	1	-	3	-	3	1
Development	19	-	-	-	-	-	60	-	-	-	-	-	-	-	-	-	-	-	-

* - includes the AuxRE motif, [1] – CArG motifs

Table 6.2 PlantPAN results for the 2 kb *VviIAA* promoter fragments.

	***VviIAA* Gene Name**																						
Type of motif	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**
Auxin	22*	5	3	1	-	-	-	2	1	9	28*	3	-	1	2	2	2	1	1	-	2	-	8
Abscisic acid	1	1	2	19	-	3	8	13	3	2	1	2	3	2	3	-	1	1	2	1	3	-	-
Cytokinin	-	-	-	4	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-
Ethylene	-	-	-	13	-	-	-	-	2	4	4	-	-	-	-	-	-	2	-	-	-	-	-
Sugar	-	2	1	1	1	-	2	5	-	-	-	-	11	1	1	-	-	-	1	-	3	1	-
Stress	2	-	2	43	3	2	4	5	6	1	15	7	2	2	1	1	9	9	5	-	-	-	-
Fruit development[1]	-	1	3	2	1	1	3	-	2	-	1	1	-	-	-	1	-	-	-	-	3	2	-

* - includes the AuxRE motif, [1]- CArG motifs

6.4 Discussion

6.4.1 Results from *ex planta* phytohormone treatments indicate that the regulation of the auxin signalling pathway is complex

The phytohormone treatments highlight that the auxin signalling pathway is dynamic and highly responsive to a range of treatments. The primary response to auxin leads to effects on other phytohormones through signalling pathways, biosynthesis and catabolism which results in the proliferation of signals (Paponov *et al.,* 2008). The responses to treatments change between not only pre-veraison and post-veraison but also within the 48 h time period of sampling.

6.4.1.1 VviIAA candidates are up-regulated by NAA treatment

Arabidopsis *Aux/IAAs* have been shown to respond to exogenous IAA application with highly differential patterns dependent on dosage and timing of the applications (Abel *et al.*, 1994; 1995). NAA has an increased stability compared with IAA as GH3s are unable to conjugate NAA as efficiently as they conjugate IAA which leads to continued induction (Lee & Starratt 1992; Petrounia *et al.*, 1994; Böttcher *et al.*, 2010). Within this work, NAA treatment caused up-regulation of transcripts, mainly *VviIAA* candidates, seen both as an increase in *VviIAA* levels and indirectly as a decrease in the levels of the *VviAFB8* receptor which would reduce the levels of *VviIAA* proteins being degraded. The *VviAFB8* candidate is most closely related to *AtTIR1*, and it is the only candidate to be down-regulated by the NAA treatments, which is interesting as *AFB* gene transcription is not thought to be auxin responsive (Parry *et al.*, 2009; Figure 3.8, Figure 6.1_A). The reduction of *VviAFB8* transcript levels suggest that it is down-regulated in response to NAA within the pre-veraison period of grape development, allowing higher levels of Aux/IAA proteins to accumulate and repress ARF activators bound to the DNA or form functional hetero- or homodimers (Korasick *et al.,* 2014; Pierre-Jerome *et al.,* 2016; Prigge *et al.,* 2016). This suggests that the response may be a compensation for sustained exposure to high NAA concentrations, and is thus a homeostatic response trying to dampen the effects of increased NAA. Interestingly, *VviAFB8* transcript levels align closely with the free IAA concentration in berry development (Figure 4.1). In the climacteric fruit papaya, six of the 11 *ARF* candidates were down-regulated by IAA application to fruits (Liu *et al.,* 2015). Within this work only a single *VviARF* candidate was up-regulated by NAA application, suggesting a difference in responses to auxin application in climacteric vs. non-climacteric fruit.

VviIAA19, 36, 38 and *39* were up-regulated pre-veraison and *VviIAA15a, 15b, 38* and *40* were up-regulated post-veraison by the NAA treatments (Figure 6.1, Appendix G). The pre-veraison *VviIAA* transcripts are up-regulated within 24 h of the treatment. As auxin is present in untreated berries at

this stage it can be suggested that these four VviIAA might be involved in endogenous auxin signalling during the early berry development phase which includes cell division and expansion. Post-veraison the four *VviIAA* candidates and a single *VviARF4* candidate were all up-regulated at 48 h; the presence of the *VviARF* candidate and timing of this response suggests that this may be a secondary response (Appendix G). All post-veraison transcripts are minimally expressed during this period of berry development, although *VviIAA15b, 38* and *40* expression levels increase from 12 weeks onward (Figure 4.6). The functional role of these transcripts is difficult to infer from these results, VviARF4 and VviIAA15 may repress ripening, whilst VviIAA15b, 38 and 40 may be involved in berry expansion.

6.4.1.1.1 The hormone responsiveness of two VviIAA candidates compared with previous IAA treatments

In previous studies grape leaves were treated with auxin to determine the auxin responsiveness of two *VviIAA* candidates, *VviIAA9* and *VviIAA19* (Fujita *et al.,* 2012; Kohno *et al.,* 2012). In Fujita *et al.* (2012) leaf discs were treated with five different IAA concentrations at 6 h intervals and *VviIAA9* transcript levels were measured. Treatment with 1, 10 or 100 µM IAA increased *VviIAA9* expression in a dose-dependent manner, however, 10 nM and 100 nM did not significantly induce *VviIAA9* expression. Kohno *et al.* (2012) treated leaf discs with IAA using the same method as in Fujita *et al.* (2012) to test the auxin induction of *VviIAA19*. No response was seen with IAA application, however, a response was seen with BR application. These results are in direct opposition to what was seen within the *ex planta* experiment, where *VviIAA9* was not seen to have a response to auxin, however, *VviIAA19* did have an auxin response (Figure 6.1). In addition, *VviIAA19* appeared to be down-regulated by BR application. This suggests that different organs, such as berries and leaves, have different capacities to respond to the same hormone, and/or may indicate a difference in *VviIAA* candidate responses to IAA and NAA or that the responses were prior to the 3 h time point.

6.4.1.2 Ripening-associated phytohormones and the sucrose deficient media down-regulate the auxin signalling pathway

ABA, BR, cytokinin and ethylene have been associated with ripening in grapes, with ABA acting as a strong inducer of ripening-related physiological changes (Palejwala *et al.,* 1985; Ban *et al.,* 2003; Gény *et al.,* 2004; Jeong *et al.,* 2004; Symons *et al.,* 2006; Giribaldi *et al.,* 2010a; Böttcher *et al.,* 2015). ABA, iP, BL, Ethrel and sucrose deficient treatments cause the down-regulation of auxin signalling pathway candidates, with a few exceptions (Figure 6.1). This suggests that phytohormones, such as ABA, are having an opposing effect to the NAA treatment on some auxin signalling pathway members, most notably pre-verasion.

The largest number of transcriptional changes were seen with Ethrel application. Ethrel largely down-regulated transcript levels with the exception of *VviARF25* and *VviIAA15a* pre-veraison and *VviAFB8*

post-veraison (Appendix G). The 20 transcripts down-regulated in the post-veraison Ethrel treatment suggests that Ethrel is a strong negative regulator of the auxin signalling pathway during post-veraison berry development. Böttcher *et al.* (2013) found that the pre-veraison application of Ethrel activated the biosynthesis of auxin thus increasing auxin levels within grape berries. They observed a delay in ripening in Ethrel-treated fruit which may have been due to the increased auxin concentration in pre-ripening fruit and this is consistent with results in Davies *et al.* (1997). As ethylene application increases berry expansion and induces auxin biosynthesis, up-regulation of more *VviIAA* candidates might be expected. However, this was not observed, suggesting that although auxin and ethylene signalling pathways may be interacting, they also have independent effects on transcription of auxin signalling pathway candidates (Chervin *et al.,* 2008; Böttcher *et al.,* 2011). The down-regulation of three *VviAFB* receptors pre-veraison by Ethrel and one *VviAFB* receptor by NAA, and the up-regulation of one *VviAFB* receptor post-veraison by Ethrel indicates the timing of the application directly influences the response within the plant and supports the idea that Ethrel and NAA responses are linked. Less *VviAFB* receptor transcripts may indicate lower VviAFB protein levels present during the pre-veraison period. This decreased receptor level would reduce the VviAFB proteins binding auxin, and thus could potentially lead to less regulation of the VviIAA proteins. This is the opposite to the observed transcript pattern of high receptor levels during the pre-veraison period of untreated berries.

Eight of the nine transcripts whose transcript levels were altered upon ABA treatment were altered pre-veraison, with *VviARF3, 4, 30* and *VviIAA11, 36, 39, 41* all down-regulated pre-veraison and *VviAFB7* being up-regulated pre-veraison (Figure 6.1, Appendix G). Only *VviARF25* was down-regulated post-veraison. ABA is strongly associated with ripening, so the increase of *VviAFB7* suggests this receptor may play a role in mediating grape berry ripening. The copy number of *VviAFB7* is maintained at ~5000 from four WPF (Figure 4.2). All other transcripts were down-regulated in response to ABA suggesting that they may play roles in fruit maturation or repressing fruit ripening, and are therefore repressed by ABA as a promoter of ripening. This is complicated however, by the biphasic profile of endogenous ABA; as ABA levels are also high early in berry development (Wheeler *et al.*, 2009; Böttcher & Davies, 2012). It was speculated in Chapter 4 that six auxin signalling transcripts had expression patterns similar to the accumulation pattern of ABA and that they may have a relationship in berries, *ex planta* data only linked one of these six transcripts, *VviIAA11*, with ABA and it was down-regulated during the pre-veraison period. This indicates that comparing similar transcript and phytohormone accumulation patterns may not the best method of inferring relationships in grape development, however some relationships are supported. In Böttcher *et al.* (2013), the application of ABA and sucrose induced *VviGH3-1* transcript accumulation more than individual treatments of ABA,

sucrose or the ethylene-releasing compound Ethrel (ethephon), suggesting that phytohormones and sucrose act in tandem to regulate plant development. They additionally noted that the induction of *VviGH3-1* by ABA and sucrose was higher in treatments closer to veraison, when the endogenous auxin levels are low, supporting the idea that the responsiveness of auxin response genes is altered across development. ABA may be playing roles in fruit development other than in ripening and that the transcripts that are down-regulated pre-veraison may be repressed as a secondary response downstream of the ABA response in fruit maturation.

Increased levels of the cytokinin iP from veraison implicates iP in berry ripening (Böttcher *et al.,* 2015). Only three transcripts responded to iP treatment, all of which were down-regulated, suggesting that iP plays a minimal role in the auxin signalling pathway (Appendix G). In the berry developmental series the transcripts *VviARF5* and *28* clustered together in Cluster 6 (Figure 4.8), whilst *VviARF3* is in Cluster 7, all three transcripts have high expression levels pre-veraison and are minimally expressed post-veraison. The results suggest that these transcripts may be repressed downstream of iP post-veraison and may be involved in processes associated with cell division and expansion, however the role iP is playing in altering auxin signalling pathway transcription does not appear to be as crucial as NAA, ABA and Ethrel.

BR concentration is high at two WPF, decreases towards veraison and has a large peak post-veraison (Symons *et al.*, 2006). Both *VviARF3* and *VviIAA13* were up-regulated pre-veraison with the treatment of BL (Figure 6.1), suggesting that these transcripts may be involved in the BL response pre-veraison (Figure 4.8). *VviARF28*, and *VviIAA19, 38* and *39* were all down-regulated in response to BL in pre-veraison berries, whilst *VviARF4, 5* and *25* were down-regulated post-veraison. *VviARF24* was down-regulated at 24 h and up-regulated at 48 h in the post-veraison treatment highlighting the dynamic and rapidly changing phytohormone response. The relationship between auxin and BR is complex, the application of auxin can cause a decrease in BR levels, but alternatively auxin can also increase the plants sensitivity of BR and the transcription of BR biosynthetic genes (Caño-Delgado *et al.,* 2004; Turk *et al.,* 2005, Paponov *et al.*, 2008). For these reasons, the exact role each of these genes may be playing could be largely dependent on the timing of application and it is difficult to determine what role the up-regulation and down-regulation of *VviARF* and *VviIAA* candidates is playing in BR signalling and fruit development and further investigations would be required. As mentioned above for ABA, the BR accumulation pattern was linked in Chapter 4 to the transcript expression of six auxin signalling genes, only one of these transcripts, *VviIAA13*, was up-regulated pre-veraison by BL. Interestingly, the VviIAA candidates were both altered by phytohormones with similar accumulation patterns to the transcripts, however the VviAFB and ARF candidates were not.

Sucrose was provided in all of the *ex planta* media, with the exception of the sucrose deficient control, to mimic the sugar content in the berries at veraison and prevent osmotic stress (Gambetta *et al.,* 2010; Böttcher *et al.,* 2013a). Sucrose levels within *V. vinifera* berries throughout berry development are low, however sucrose may be transported into the berry cells and be rapidly converted to the hexose sugars, glucose and fructose, the levels of hexose sugars within the berry increase rapidly from veraison (Coombe, 1992; Davies & Robinson, 1996; Zhang *et al.,* 2006; Shiraishi *et al.,* 2010). It is unclear whether the sucrose in the media for the other treatments would be transported into the berry, possibly being actively converted by invertases into hexose sugars, and potentially causing changes in transcript levels due to the additional sugars. For this reason the sucrose deficient control was used to determine the effect of having no external sugars applied in the *ex planta* experiment, additionally, increased transcript response in the sucrose deficient media may suggest which of the transcripts are involved in osmotic stress (Gambetta *et al.,* 2010). Seventeen of the transcripts responded to the sucrose-deficient media pre-veraison, when the levels of sugar within the berry are low (Figure 2.1, Appendix G, Davies & Robinson, 1996), compared to only four transcripts post-veraison when the endogenous sugar levels have begun to increase (Figure 6.1). This response may be a stress response to the *ex planta* treatment that is enhanced by the sucrose deficiency, especially pre-veraison when berry sugar levels are low, or as an adjustment to the reduced sugar levels and osmotic stress (Gambetta *et al.,* 2010). The *VviARF16, 24* and *VviIAA36* transcripts increased pre-veraison and may be involved in stress response in damaged berries. Therefore, VviARF2b, 4, 8, 25, 28, 30, VviIAA15a, 15b, 19, 27, 37, 38, and VviAFB9 may be involved in fruit maturation, which is supported by NAA data for *VviIAA19, 36* and *38* where these transcripts are up-regulated by NAA (Figure 6.1). There is some consistency between the pre- and post-veraison results, with *VviARF2b* and *25* being down-regulated within both experiments, suggesting that even with the presence of endogenous sugars the same transcriptional responses are being triggered.

The overlap of responses of both *VviARF* and *VviIAA* candidates to multiple treatments within the same time period suggests that auxin signalling candidates are regulated by a range of phytohormones and factors, often dependent on the developmental stage (Figure 6.2_ A and B). The *VviAFB* receptors were regulated differently by all phytohormones and treatments, with the only overlap seen with Ethrel and the sucrose deficient media both down-regulating *VviAFB9* pre-veraison. A higher number of overlapping phytohormone responses were seen pre-veraison, correlating with the higher number of transcriptional changes occurring during this time. No clear patterns or relationships could be observed either pre- or post-veraison. Treatments of multiple phytohormones at the same time could be an interesting way to confirm if the overlap of these responses could be amplified by the presence

of more than one regulating phytohormone and may provide further information on the phytohormonal network regulating grape berry development.

6.4.2 Comparing the presence of motifs within gene promoters to *ex planta* phytohormone responses

Motif analysis has been used in a range of *ARF* and *Aux/IAA* publications to identify the presence of motif sequences that may indicate which phytohormones regulate the candidate genes. Wang *et al.* (2012) identified the presence of two motifs that are directly associated with auxin response and also ABRE elements in 1.5 kb *ARF* maize gene promoters. Audran-Delalande *et al.* (2012) found that none of the seven ethylene-regulated *Aux/IAAs* in tomato contained the conserved GCC-box motif which is often present in the promoter region of ethylene-responsive genes, however, five of the seven did contain the ERELEE4 motif, another ethylene-responsive motif. Within this work there was little consistency between phytohormone responses and the presence or absence of motifs in the 2 kb promoter sequences. Together these results support the idea that motif analysis is very speculative, and a more comprehensive analysis is needed (Section 6.3.4).

MADS-box transcription factor proteins have been found to play various roles in plant development and are known to bind CArG box motifs (West *et al.,* 1997; Becker & Theissen, 2003). The CArG box motif CYWWWWWWRG has been implicated in fruit development as a motif that acts as a binding site for the tomato MADS-box TF ripening-inhibitor (RIN), which is associated with fruit development and ripening (Vrebalov *et al.*, 2002; Giovannoni 2004). *SlRIN*, an *AGAMOUS-like 2 MADS-box* gene, has been implicated in ethylene biosynthesis, cell wall remodelling, and binding to the promoters of genes that control fruit maturation and pigment accumulation and has been found to bind the CYWWWWWWRG CArG box motif (Ito *et al.,* 2008; Fujisawa *et al.,* 2011; Martel *et al.,* 2011; Fujisawa *et al.,* 2012; *et al.,* 2013; Zhong *et al.,* 2013; Qiu *et al.,* 2016). These motifs have been classed as the 'fruit development' motif in Table 6.1 and Table 6.2. The CArG box motif CYWWWWWWRG, was present in 12 of the 19 VviARF and 12 of the 23 *VviIAA* promoters providing many potential RIN binding sites. The CCAAAAADGG motif, only present in *VviARF3* and *8* promoter regions, has been suggested to be linked with tomato fruit expansion through interaction with MADS-box transcription factors (Qiu *et al.,* 2016). This would be consistent with the *VviARF3* expression in berries, however, *VviARF8* expression would align more closely with a role in early berry development (Figure 4.4). Many of the VviARF and VviIAA transcripts contained multiple CArG box motifs that have been associated with flowering, including the 'CARGATCONSENSUS' motif and the 'CARGNCAT' motif (Shore & Sharrocks, 1995; Hepworth *et al.,* 2002). All of the *VviARF* candidates, except *VviARF26*, and many of the *VviIAA* have high expression in flowers. All of the *VviARF* candidates, except *VviARF32*, had high expression

in flowers (Figure 4.5). This suggests that a range of VviARF and VviIAA candidates may play roles in flower development.

6.4.3 Limitations and areas for future work

It is possible that transcriptional regulation patterns in this study were being masked by the presence of endogenous levels of ABA, BR, and IAA pre-veraison, and ABA, BR, and cytokinin post-veraison. The endogenous levels may dampen the results of phytohormone treatments as these transcripts will be high in the control treatment. Also, candidates may only be responsive at certain periods of development which were not sampled here. In addition, due to sampling restraints, the earliest time point for sample collection was three hours. It would be interesting to sample at a wider range of time points to capture a broader understanding of the timing of responses including 30 minutes and one hour. Calderon Villalobos *et al.* (2012) suggested that TIR1/AFB-Aux/IAA co-receptors have different affinities for different auxinic molecules. NAA was used in the experiments described here, and in future different auxinic molecules could be tested, including IAA. In addition to this, the TIR1/AFB-Aux/IAA co-receptor complex has different sensing and binding capacity for auxin, therefore different concentrations of auxinic molecules could be tested to ensure all responses are captured.

An in-depth analysis of two *SlARF2* candidates, sharing homology with *VviARF2a* and *2b*, was used to develop a model describing the role of these two ARFs in fruit development and highlights the importance of functional data in combination with similar analyses to those in this work in understanding modes of action (Hao *et al.,* 2015; Breitel *et al.,* 2016). SlARF2A and SlARF2B are transcriptional repressors that are up-regulated during ripening and are thought to act as positive regulators of tomato fruit ripening through targeting a negative regulator of the ripening process (Hao *et al.*, 2015; Brietel *et al.,* 2016). Down-regulation of either results in ripening defects, including a mottled ripening pattern, and silencing of both leads to severe ripening inhibition seen as reduced colour accumulation and enhanced firmness. Although the results suggest functional redundancy, *SlARF2A* had higher transcript levels in both vegetative and reproductive tissues and the down-regulation of *SlARF2A* is compensated for by an increase in *SlARF2B* but not the other way around. AuxRE and ERE (ethylene response elements) were identified in the 2 kb promoter regions of both genes. In mature green fruit *SlARF2A* was up-regulated by ethylene treatment while *SlARF2B* was not, and *SlARF2B* was up-regulated by auxin treatment (20 µM for 6 h) and *VviARF2a* within this study was not. SlARF2-down-regulated (RNA interference on both SlARF2A and B) plants produced less ethylene and showed down-regulation of MADS-box genes *RIN (RIPENING-INHIBITOR)* and *TOMATO AGAMOUS-LIKE 1 (TAGL1)*, a *SQUAMOSA promoter binding* protein *COLORLESS NON-RIPENING*, a *NAC-domain transcription factor NON-RIPENING*, and altered ethylene signalling and biosynthesis

gene expression, suggesting that it may also be worthwhile to check these transcripts in the grape data. Ethylene treatments were unable to recover the SlARF2 ripening inhibition. TAGL1 is reported to interact with RIN, and FRUITFUL (FUL1 and FUL2), forming higher order complexes that regulate tomato fruit ripening (Vrebalov *et al.,* 2009). Interestingly, both *VviARF2a* and *2b* are expressed post-veraison in Shiraz berries and *VviARF2a* was down-regulated by Ethrel at 3 hrs post-veraison in the *ex planta* analysis (Figure 6.1_A). The similarities seen between the studies may suggest some functional conservation. The gene silencing, gene overexpression and mutant transcriptome analysis experiments Hao *et al.* (2015) and Breitel *et al.* (2016) completed were required to develop possible modes of action, and suggest the types of further experiments that could be completed on the *Vitis* candidates to elucidate their roles within fruit development, possibly in grape microvines to get early fruit development (Chaïb *et al.,* 2010).

Although, Kumar *et al.* (2015) reported a good correlation between the presence of phytohormone-related motif sequences and the differential expression of *Aux/IAA* and *ARF* genes in two Solanaceae species in response to specific phytohormone treatments, this was not strictly followed in the case of all genes. This was also seen within this work, where a correlation was seen in some cases and not others leaving much of the expression data unexplained by motif analysis. This highlights the limitations of *in silico* promoter analysis and the complexity of the system. To address this in future work DNase I hypersensitive site (DHS) analysis could be used (Qiu *et al.,* 2016). DHS harnesses the DNase I hypersensitivity of actively transcribed regions by DNase I treating samples and using high-throughput sequencing to identify the regions of open chromatin. Qiu *et al.* (2016) used this method on two stages of fruit development to identify stage-specific active regulatory elements, however, it could be used on specific cell types and across a broader range of developmental stages. They found that 15% DHS were present within the region 1 kb upstream from the transcription start site, 20% of DHS were present within the region 2 kb upstream from the transcription start site, and nearly half were present in the 5’ UTR, exons, introns, 3’ UTR and the transcription termination site region (1 kb downstream of the transcription termination site). This method could be used to identify both active regulatory elements and transcripts that are being actively transcribed during specific stages of growth, identifying potentially interesting candidates for further research.

Chapter 7 Discussion, conclusions and future directions

Auxins are key regulators of plant development and have been implicated in the control of fruit development in a range of species (McAtee *et al.*, 2013; Kumar *et al.,* 2014). Auxins act through a complex signalling pathway that regulates the transcription of a large number of genes. Understanding the role of auxin signalling in grape berry and vegetative organ development may help to develop new viticultural management techniques through the manipulation of vegetative vigour, vine architecture and berry ripening. The primary aim of this study was to identify and characterise the *V. vinifera* auxin signalling pathway genes, focusing mainly on their roles in fruit development, through the analysis of transcript accumulation patterns, the interaction of select VviARF-VviIAA candidates and their responsiveness to different phytohormones. These results, taken together with functional data from *V. vinifera* and other species, has allowed a model to be developed that explains the role of auxin signalling pathway members in grape berry development (Figure 7.1).

Bioinformatic analysis identified six *VviAFB* receptors, 19 *VviARF* transcription factors and 23 *VviIAA* repressor sequences in *V. vinifera.* Many of the inconsistencies between the sequences identified from different databases and techniques, including NCBI, Phytozome and Genoscope, were likely to be genome misannotations or cultivar-dependent differences (Tables 3.4, 3.5, 3.6). Similar to previously characterised higher plants, the *VviAFB* sequences analysed in this work fall into four distinct clades and are located on different chromosomes (Figure 3.1, 3.8, Parry *et al.,* 2009). *VviARF* genes were also phylogenetically distant from each other and activator and repressor *ARF* candidates were distinguishable by their nucleotide and amino acid sequences (Figure 3.9). The *VviIAA* genes were the smallest in mRNA length, and the Baysian phylogeny based on the conserved regions of the sequences had some nodes with low posterior probability making putative 'pairs' hard to determine (Figure 3.10). Based on Figure 3.10, *VviIAA26-43, VviIAA27-42* and *VviIAA39-40* appeared to be genetically similar, sequence identity indicated that they were 47.5%, 38.9% and 74.5% identical, respectively (Appendix D, Figure D.9). *VviIAA26-43* and *VviIAA27-42* pairs had similar expression within berries, whilst *VviIAA39-40* were both highly expressed in flowers, however, they are located on different chromosomes (Figure 3.1). This supports the idea of neofunctionalisation of genetically similar genes (Papanov *et al.,* 2009) and suggests that the clear functional redundancy seen in other species, such as Arabidopsis, may not be present within the auxin signalling pathway in grape. The relatively large *VviARF* and *VviIAA* gene families and the differences between cultivars highlight that grape has evolved a complex auxin signalling pathway to mediate development and phytohormone responses.

The presence of distinct domains within the proteins of the auxin signalling family members may provide information about their functionality. All VviAFB proteins had an F-box motif and multiple LRRs, which in other species are necessary for their functionality (Worley *et al.*, 2000; Ramos *et al.*, 2001; Dharmasiri *et al.*, 2005b; Kepinski & Leyser, 2005; Tan *et al.*, 2007; Parry *et al.*, 2009; Lee *et al.*, 2009). The VviARF proteins all contain DBDs, suggesting that they all have the capacity to bind to AuxRE motifs in promoter sequences and act as transcriptional repressors or activators. However, three VviARF proteins (VviARF3, 8, 17) have no PB1 domains (Figure 3.6). The PB1 domains are purportedly essential for binding Aux/IAA repressor proteins, but some ARFs appear to lack this domain and instead interact with other protein families, such as bHLH (basic helix-loop-helix) proteins (Varaud *et al.,* 2011; Oh *et al.,* 2014; Simonini *et al.,* 2017). The absence or truncation of the PB1 domains in these VviARF proteins may mean that they are not regulated by Aux/IAA proteins, however, they may still be able to form hetero- or homodimers with other ARF proteins through the DD domain nearer the DBD (Pierre-Jerome *et al.,* 2016). Eleven of the 23 Aux/IAA proteins contained all four domains (I to IV) and three more had non-characteristic amino acids in Domain I but had all other domains intact (Figure 3.7). Domain I allows the interaction with TPL co-repressors, suggesting that the three VvIAA genes containing atypical amino acids and nine VvIAA genes lacking the domain completely may encode weaker repressors than the other VviIAA proteins containing it (Causier *et al.,* 2012). The four putative VviIAA proteins that lack the Domain II degron sequence may not be readily degraded, which may increase their half-lives and prevent a normal auxin response (Worley *et al.*, 2000; Ramos *et al.*, 2001; Zenser *et al.,* 2001; Tiwari *et al.,* 2004; Dreher *et al.,* 2006; Lee *et al.*, 2009). Independent of the variability in Domains I and II, 22 of the VviIAA proteins contain Domains III and IV that combine to create a similar domain to the PB1 domain in ARF proteins indicating the importance of this domain in VviIAA protein functionality (Pierre-Jerome *et al.,* 2016). The variation in the presence of the protein domains illustrates one of the many ways that control over the function of the auxin signalling pathway members has evolved within grape.

The transcript expression patterns across berry and leaf developmental series, flowers, roots and tendrils were analysed to determine when the auxin signalling pathway is most active and suggest what processes it might be associated with, in addition to identifying proteins that may interact with each other. This revealed eight clusters across berry development. Veraison appears to act as a key point of regulation, with most gene transcripts decreasing or increasing from this stage (Clusters 3, 6, and 7, Figure 4.9). The most common berry expression pattern is high expression pre-veraison and low expression post-veraison, with 39 of 46 genes having their highest transcript levels before week 8 (Clusters 4, 5, 7, and 8). This high pre-veraison expression is consistent with the IAA concentration, (Figure 4.1, Appendix E, Figure E.1) and correlates with the period of cell division, auxin-related cell

expansion and photosynthesis in berries. Based on homology and similar transcriptional profiles, VviARF28 (Cluster 6, Figure 4.9) may fulfil a similar role to SlARF7, which controls cell division in tomato (Vriezen *et al.,* 2008; de Jong *et al.,* 2009; 2011). SlARF4 (DR12) has been implicated in controlling the accumulation of chlorophyll in tomato fruit development (Jones *et al.,* 2002; Guillon *et al.,* 2008; Legland *et al.,* 2010; Sagar *et al.,* 2013) and like its homolog, *VviARF4* (Figure 4.4), *SlARF4* is expressed early in fruit development, its expression rapidly decreasing from the onset of ripening. This suggests that VviARF4 may also play roles in chlorophyll accumulation in grape berries. Interestingly, the expression of *VviAFB8*, the closest homolog to the main auxin F-box receptor in Arabidopsis *AtTIR1*, closely follows the pattern of IAA accumulation in berries but not in leaves emphasising the complexity of the auxin receptor regulation system. AtTIR1 has been identified as the most crucial receptor in Arabidopsis, its role being irreplaceable by any of the other AtAFB genes even when under the expression of the *AtTIR1* promoter (Parry *et al.,* 2009). With the close link between IAA levels and the *VviAFB8* transcript pattern in berries and high transcript levels in all tissues, it is possible that *VviAFB8* is the key auxin receptor in *V. vinifera* L. cv. Shiraz. Curiously, of the *VviIAA* genes, only *VviIAA27* and *42* showed similar expression patterns in the developmental series and organ data suggesting that they may play similar roles, such as a key role in pre-veraison berry and flower cell expansion.

In contrast to the majority of transcripts, the genes in Clusters 2, 3, and 8 (Figure 4.9), have high transcript levels during fruit ripening which correlate with the higher IAA-Asp and lower IAA concentrations (Figure 4.1, Appendix E, Figure E.1). These transcripts most likely play a role in berry ripening, potentially through an involvement in cell wall changes allowing softening or by controlling sugar or anthocyanin accumulation. Some of these changes may be due to interactions with other, process-specific, proteins such as bHLH transcription factors, reinforcing the concept that fruit ripening involves a well-orchestrated coordination of a wide range of regulatory genes (Varaud *et al.,* 2011; Nicolas *et al.,* 2013; Kumar *et al.,* 2014; Oh *et al.,* 2014; Simonini *et al.,* 2017). Interestingly, *SlARF2A* and *2B* are also highly expressed in ripening tomato fruit and are thought to be involved, through an interplay with several hormones, in fruit ripening by repressing a repressor of ripening thus allowing the up-regulation of ripening regulators and downstream ripening genes (Hao *et al.,* 2015; Breitel *et al.,* 2016). *SlARF2A* expression is responsive to ABA, ethylene and auxin at select stages of fruit development, dependent on the length of the treatment and the number of days post-treatment (Hao *et al.,* 2015; Breitel *et al.,* 2016). These findings indicate SlARF2A may be involved in competence to ripen, finely tuned through the interplay of the hormonal network (Hao *et al.,* 2015; Breitel *et al.,* 2016). The homolog *VviARF2b* is an ARF repressor and has a similar transcript accumulation pattern to SlARF2A and may be playing a similar role in grape berry ripening, however, direct comparisons of phytohormone responsiveness are difficult due to differences in experimental

design. Hao *et al.* (2015) exposed tomatoes to ethylene and auxin for 5 and 6 h, respectively, before sampling, and *SlARF2A* had no response to auxin and was up-regulated by ethylene. *VviARF2b* was also non-responsive to auxin but was down-regulated at 3 h post-veraison in response to Ethrel (Figure 6.1). A bHLH transcription factor, *Cell Elongation bHLH (VvCEB1)*, is responsive to auxin application and is thought to play a role in increasing cell size through cell expansion in grape, especially post-veraison (Nicolas *et al.,* 2013). *VvCEB1* accumulates mainly in berries, with a similar accumulation pattern to *VviIAA19, 31, 38* and *40* transcripts and has an overlapping pattern with IAA-Asp during ripening (Appendix E, Figure E.1). The overexpression of VvCEB1 in grape embryos alters the expression of several VviIAA proteins, including causing the up-regulation of *VviIAA19* transcription (Nicolas *et al.,* 2013). As both VviIAA19 and the ARF activator VviARF27 interact and their expression overlaps with that of *VvCEB1* during the ripening phase, these three proteins may interact and together play roles in post-veraison cell expansion.

Yeast 2-hybrid and BiFC analysis on a subset of *VviARF-VviIAA* transcripts with similar expression profiles determined that VviARF4ΔDBD and VviIAA19, and VviARF27ΔDBD with both VviIAA27 and VviIAA19 were able to interact and were localised to the nucleus (Figures 5.1, 5.4, 5.7, 5.8, and 5.9). Overlapping transcript accumulation patterns in particular organs indicate that these interactions are likely to occur *in planta*. Both *VviARF27* and *VviIAA19* have high transcript levels in flowers and in post-veraison berries, suggesting that they may interact in flowers and also play a role within berry ripening (Figure 5.11). VviARF27 and VviIAA27 interact in yeast two-hybrid and in BiFC analyses in onion cells, however, they have dissimilar expression patterns in both leaf and berry developmental series with the only overlapping patterns being high transcript levels within flowers suggesting that they may play a role in auxin signalling in flowers. *VviARF4* and *VviIAA19* have opposing expression patterns in berries and leaves. These proteins may have the capacity to interact *in planta* although it appears unlikely that they do so in berries or leaves, and may potentially interact in other tissues where they are co-expressed, such as in tendrils (Figures 4.5, 4.7). Alternatively, these proteins may interact only within a specific berry cell type, or their interaction may represent a false-positive interaction as discussed in Guilfoyle *et al.* (2015). Not all combinations showed an interaction, as both VviARF4ΔDBD and VviARF24ΔDBD did not interact with VviIAA27, which were selected due to the similarity of their berry transcript expression pattern with high transcript levels in pre-veraison berries. This suggests specificity of the interactions and may mean those that do interact may be functional *in planta*. VviARF27 is an ARF activator, and these are the only ARFs demonstrated to be regulated widely by Aux/IAAs (Guilfoyle, 2015). It is possible that VviARF4 and VviARF24, because they are ARF repressors, only interact with specific VviIAA proteins that are not present in the same berry transcript cluster.

In Chapter 6, NAA and Ethrel/ABA were shown to have antagonistic effects on the auxin signalling pathway, with NAA promoting the expression of the auxin signalling pathway members and Ethrel and ABA repressing them. Within the berry, endogenous auxin levels are high pre-veraison (Figure 4.1) and are thought to regulate cell division and the pre-veraison cell expansion, however, the auxin concentration must decrease to low levels for berry ripening to occur (Davies *et al.,* 1997). Ethylene and ABA are thought to be enhancers of fruit ripening, with concentrations of these phytohormones increasing early in fruit ripening (McAtee *et al.,* 2013). ABA appears to play an important role in ripening by switching off vegetative pathways, such as photosynthesis, and through the stimulation of the signalling cascade that causes the up-regulation of ripening-related transcription factors such as *VvNAC, VvMYB, VvERF* and *VvbZIP* (Palumbo *et al.,* 2014; Pilati *et al.,* 2017). Within this study, ethylene (applied as Ethrel) and ABA generally induced down-regulation of the auxin signalling pathway members, ABA most prevalently pre-veraison and Ethrel most prevalently post-veraison, suggesting the auxin signalling candidates predominantly play roles in the vegetative pathways that must be down-regulated to allow for ripening (Pilati *et al.,* 2017). The exception to this is seen with the *VviAFB* receptors, as NAA treatment repressed the *VviAFB8* (*AtTIR1* homolog) levels pre-veraison and ABA up-regulated *VviAFB7* pre-veraison, and Ethrel up-regulates *VviAFB8* post-veraison. Interestingly, Ethrel treatment pre-veraison, like NAA, down-regulated the transcript levels of the *VviAFB* receptors *VviAFB6, 9* and *11*. A decrease in *VviAFB* appears to be a homeostatic response to excess auxins and would be expected to reduce the berries sensitivity to auxins, whilst increases in *VviAFB* may lead to auxin hypersensitivity. The overexpression of plum TIR1 in tomato led to auxin hypersensitivity and fruit with enhanced fruit softening-associated ripening, supporting the concept that select auxin signalling genes play roles in fruit ripening, potentially through the up-regulation of cell-wall metabolism genes (El-Sharkawy *et al.,* 2016). These results highlight the complexity of fruit development, with a finely-tuned integration of phytohormone and gene regulation, with some auxin signalling members repressing ripening and others enhancing it, all dynamically controlled by phytohormones dependent on the stage of development. This is consistent with results in Böttcher *et al.* (2013b), who showed that the timing of the Ethrel application is important, with pre-veraison Ethrel application activating auxin biosynthesis and delaying fruit ripening. The down-regulation of VviAFB receptors levels pre-veraison may decrease the degradation of VviIAA proteins or act as a fine-tuning system in modulating the auxin response by reducing auxin sensitivity. *VviAFB7* up-regulation pre-veraison in response to ABA and *VviAFB8* up-regulation post-veraison in response to Ethrel suggests that the increase in the *VviAFB* receptor transcripts may increase the degradation of VviIAA proteins. As ethylene and ABA enhance ripening and auxin prevents it, the down-regulation of the auxin signalling pathway may help to enhance ripening. PlantPAN analysis identified that 15 of the 19 *VviARF*

genes had AuxRE motifs, whilst only two of the *VviIAA* genes did, suggesting that the transcription of *VviARFs* are widely regulated by *VviARF* proteins, but *VviIAA* genes are not (Tables 6.1, 6.2). The *VviARF* may regulate themselves in positive or negative feedback loops, or they may regulate each other to mediate auxin responses. It is thought that ARF repressors are likely to compete with ARF activators for AuxRE binding sites, providing an additional mechanism in facilitating the finely-tuned transcriptional regulation especially in concert with the formation of hetero- and homodimers (Vert *et al.,* 2008).

7.1 A proposed model of the role the auxin signalling pathway plays in fruit development and ripening in *V. vinifera* L. cv. Shiraz

The results of this study, together with the published literature, have allowed a model to be developed describing the mode of action of the auxin signalling pathway during *Vitis vinifera* L. cv. Shiraz berry development (Figure 7.1). The VviARF proteins bind to AuxRE motifs in the promoter region of auxin responsive genes via the DBD located at the N-terminus of the ARF proteins, often in complexes via the DD proximal to the DBD (Pierre-Jerome *et al.*, 2016). In the absence of auxin, VviARF activators and select VviARF repressors, such as VviARF4, will be actively repressed by VviIAA proteins and the TPL co-repressors by the interaction of the PB1 domain and Domains III and IV in the C-terminus of the ARF and Aux/IAA proteins, respectively (Mockaitis and Estelle, 2008). This prevents the transcriptional activation of the auxin responsive genes. In the presence of auxin, the Aux/IAA proteins are then targeted for proteasome-mediated degradation by the $SCF^{TIR1/AFB}$ complex. The ARF proteins then interact via the PB1 domain in the C-terminus of the protein and transcriptionally activate the auxin responsive genes, triggering a signalling cascade (Mockaitis and Estelle, 2008). In *V. vinifera* L. cv. Shiraz, the majority of the auxin signalling pathway members are highly expressed pre-veraison, when IAA levels are high. This is consistent with Aux/IAA proteins being rapidly induced by auxin, and downstream activation of auxin responsive genes by ARF proteins. As ARF and Aux/IAA candidates in other species have been shown to play roles in cell division, expansion and elongation and in chlorophyll accumulation in fruit it can be hypothesised that many of the 39 VviARF and VviIAA candidates that accumulate high levels of transcripts during the pre-veraison period may have similar functions (Jones *et al.,* 2002; Guillon *et al.,* 2008; Vriezen *et al.,* 2008; de Jong *et al.,* 2009; 2011; Legland *et al.,* 2010; Sagar *et al.,* 2013). IAA-Asp concentration increases rapidly during the ripening phase of berry development and there is some evidence that IAA-amino acid conjugates may themselves have biological activity (Staswick, 2009). Ethylene and abscisic acid concentrations also peak during this time, ethylene at the onset of ripening and abscisic acid early in ripening. It appears

that ethylene and ABA are key ripening regulators, and with select VviARF and VviIAA candidates they may regulate ripening during the post-veraison period, possibly through controlling cell expansion, softening, sugar and anthocyanin accumulation through repressing ripening repressors and/or the interaction with proteins, such as bHLH or MYB transcription factors (Nicolas *et al.,* 2013; Palumbo *et al.,* 2014; Hao *et al.,* 2015; Breitel *et al.,* 2016; Pilati *et al.,* 2017). Together, this suggests a finely-tuned network of proteins and phytohormones in which auxin and the majority of the auxin signalling pathway, mediate early berry growth and development pre-veraison, while ethylene, ABA and select VviARF and VviIAA proteins mediate berry ripening post-veraison. The network is complex and flexible, changing based on environmental cues and phytohormone exposure throughout berry development.

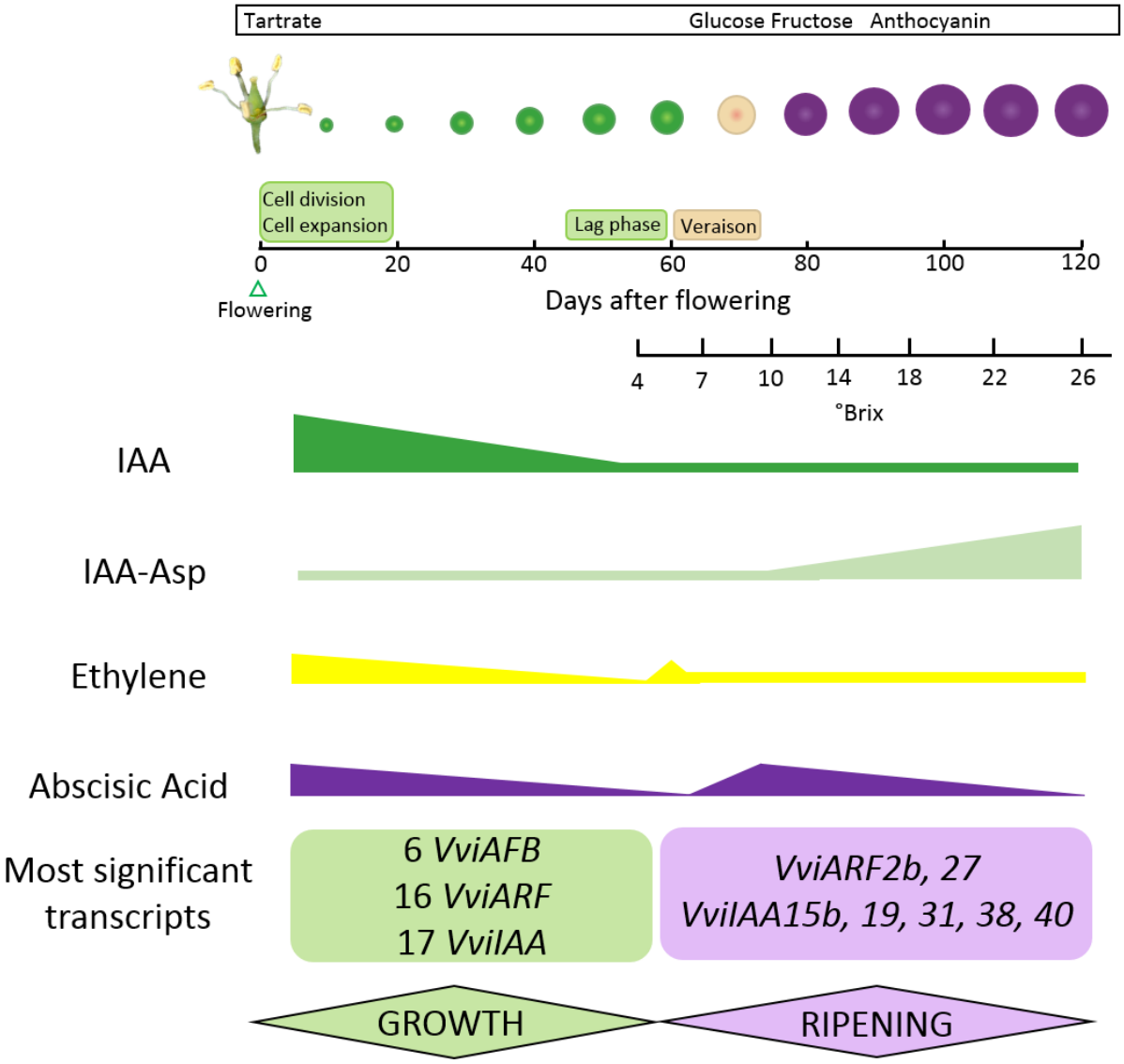

Figure 7.1 An integrated model of the auxin signalling pathway in grape berry development.

The stages of grape berry development are shown, starting with flowering and ending at ripe, harvestable berries. Indole-3-acetic acid (IAA) concentrations are high during the pre-veraison period during the period of berry growth. IAA-Aspartic acid (IAA-Asp) concentrations are high post-veraison, during the ripening phase of berry development, ethylene and abscisic acid levels also have peaks during this time, ethylene at the onset of ripening and ABA early in ripening. The majority of VviAFB, VviARF and VviIAA candidates may regulate pre-veraison growth and expansion while the IAA concentration is high. It appears that ethylene and ABA are key ripening regulators, and with select VviARF and VviIAA candidates they may regulate ripening during the post-veraison period. These hormones and auxin signalling genes form a finely tuned network regulating grape berry development (Adapted from Figure 4.1, Coombe, 1987; Coombe, 1995; Kennedy, 2002).

7.2 Significance to the discipline and future perspectives

A better understanding of the processes involved in berry development may aid in the decision making process regarding the choice and timing of existing viticultural treatments and the development of new strategies to enhance berry composition and production. Phytohormones are important controllers of reproductive and vegetative development and mediate responses to many environmental cues. This research represents the most in-depth analysis of the grape auxin signalling pathway components to date and has highlighted the pleiotropic roles the candidates play throughout plant development, with a focus on berry development. Auxin and the majority of auxin signalling pathway genes mediate early berry growth. ABA and ethylene appear necessary for fruit ripening, however, auxin signalling gene expression is also modulated during the ripening stage. Two key *VviARF* genes and four *VviIAA* candidates are implicated in fruit ripening, potentially in an auxin-independent manner. These results highlight the complexity and sophistication of the auxin signalling system within grape, with a flexible response system having evolved that controls gene expression in a wide range of tissues and organs based on environmental and phytohormonal cues. Evidence of this complexity is apparent within the large gene families with varied protein structures, different promoter sequences, diverse expression patterns and specific protein-protein interaction partners. This enhanced understanding of the role of phytohormones and the auxin signalling pathway may help to develop new viticultural approaches, such as timed phytohormone treatments, which may allow for more control over berry size and the ripening period. There is growing evidence that rising temperatures associated with climate change are altering the growing season of grapes (Davies *et al.,* 2015), hence the ability to manipulate grape development and control harvest dates would allow grapes to be harvested over a larger time period putting less pressure of harvesting machinery and processing facilities.

7.3 Future research directions

This study has identified many areas that would benefit from further experimentation to more comprehensively determine what roles the auxin signalling candidates are playing within plant development, and more specifically in grape berry development and ripening (Guilfoyle, 2015; Pilati *et al.,* 2017; Leyser, 2018). Tissue dissection could be completed on berry exocarp (skin), mesocarp (flesh) and endocarp/seeds to better resolve specifically narrow down the location of gene expression in the fruit throughout development and to avoid dilution issues which can cause underestimation of gene expression in localised areas. Completing a comprehensive yeast 2-hybrid screen like that done by Piya *et al.* (2014) with the VviARF and VviIAA candidates to identify more functional pairs, and complementing this with a more comprehensive BiFC analysis would expand our understanding of

interactions and create a map of interacting ARF-IAA candidates. Full-length clones for *VviIAA11* and *41* and the *V. vinifera COP9* genes could be isolated to determine if they interact in yeast 2-hybrid and BiFC analysis, in addition to isolating the *MYB* and *bHLH* factors thought to play roles in grape ripening to determine their capacity to interact with VviARF candidates (Nicolas *et al.,* 2013; Palumbo *et al.,* 2014; Hao *et al.,* 2015; Breitel *et al.,* 2016; Pilati *et al.,* 2017). As suggested in Guilfoyle (2015) it would be important to investigate interactions between ARF repressors and Aux/IAA proteins to determine the functional role that these play *in planta*, potentially also swapping the repressor and activator domains of the ARF proteins to determine if this completely alters their functionality. Investigating other protein-protein interaction mechanisms that do not have the auto-activation issues that were seen within this study could further enhance the analysis of protein-protein interactions and the isolation of novel interacting proteins. Chromatin immunoprecipitation analysis with VviARF proteins could be used to identify regions of DNA that they bind, allowing their target genes to be identified and allow the characterisation of what genes are involved in what processes, and which of the VviARF proteins mediate the different aspects of auxin responsive gene signalling. This will help to inform us of which processes we will influence when we alter auxin signalling. DNase I hypersensitivity (DHS) analysis could be performed on berry samples from different stages of development to identify regions of open chromatin and therefore the promoter regions being accessed by transcription factors (Qiu *et al.,* 2016). With the phytohormone treatments, methods could be developed to achieve faster treatment application and sampling to allow the initial primary responses to be captured.

Finally, the results presented here are in line with Kohno *et al.* (2012) and Fujita *et al.* (2012), indicating the need for functional data as a crucial puzzle-piece in characterising the grape auxin signalling pathway. Studies in heterologous systems are insufficient to fully understand functionality, supporting the need for transgenic grape to be made, potentially in grape microvines (Chaïb *et al.,* 2010). There are a range of experiments that could be completed in the grape microvine system, including determining the functional relevance of the VviARF4-VviIAA27, VviARF-VviIAA19/VviIAA27 interactions. Overexpression and/or gene-silencing in microvines may also provide information about the functionality of other VviAFB, VviARF and VviIAA candidates, providing clues as to what level of functional redundancy exists within the grape auxin signalling pathway. Ideally, as in tomato, there would be a lack of redundancy allowing mutant phenotypes to be detected and functionality inferred (Hao *et al.,* 2015).

Appendices

Appendix A Chemicals, enzymes, buffers, reagents, solutions and media

Table A.1 Antibiotics used in this work.

Name	Stock Solution Concentration (mg/mL)	Final Concentration (µg/mL)
Kanamycin	50	50
Ampicillin	100	100
Spectinomycin	50	50

Table A.2 Buffers and solutions.

Name	Components
1 kb Plus DNA Ladder	1 kb Plus DNA LadderTM (Invitrogen)
Agarose	Invitrogen UltraPure TM agarose ref 16500-500 (for gels)
+cis trans abscisic acid (ABA)	25 µM +cis trans abscisic acid (ABA)
4',6-diamidino-2-phenylindole	4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, Missouri, USA)
6-(γ,γ-Dimethyl-allylamino)-purine (iP)	25 µM 6-(γ,γ-Dimethyl-allylamino)-purine (iP) (Sigma-Aldrich) - Isopentenyladenine
24-epibrassinolide (BL)	25 µM 24-epibrassinolide (BL), Mikonik Technologies Ltd., New York, USA
Ethrel	150 µL/L Ethrel
6X Gel electrophoresis loading dye	15% Ficoll 0.25% bromophenol blue, 0.25% xylene cyanol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
10M Lithium Chloride	Filter sterilise and do not autoclave.
Luria-Bertani (LB) broth	1% Tryptone, 0.5% yeast extract, 1% NaCl, autoclaved at 121°C for 40mins
1-naphthaleneactic acid (NAA)	0.54 µM of 1-naphthaleneactic acid (NAA)
Methanol/water/acetic acid	Methanol/water/acetic acid (60:39:1, v/v/v)
10X PCR buffer	200 mM Tris HCl (pH 8) and 500 mM KCl (Invitrogen)
Phenol/chloroform/iso-amyl alcohol	Phenol/chloroform/iso-amyl alcohol (25:24:1) mixture, equilibrated to pH 8.0 with Tris 10 mM (store at 4°C). 0.1g 8-OH quinoline (0.1% in relation to phenol only)
Polyvinylpolypyrrolidone (insoluble PVPP)	Fluka analytical lot #BCBK1891V
RNA Loading Buffer	1 ml formamide (deionised), 350µl formaldehyde, 13.5µl 100x sterile TE, 86.5µl sterile MQ Water, bromophenol blue

Name	Components
20% SDS	Do not autoclave
3M Sodium acetate	3M sodium acetate (NaAc) pH 5.2, autoclaved
8M Sodium Perchlorate	Place 500g into 150ml of MQ water and stir. Once completely dissolved make up to 445ml with MQ water and place in a sterile Schott bottle. Do not autoclave.
0.2 M sodium hydroxide	0.2 M sodium hydroxide (NaOH),
10X TBE buffer	89 mM Tris, 89 mM Boric acid and 2 mM EDTA, adjusted to pH8, diluted 10-fold for 1X TBE buffer
TE	10mM Tris, 1mM EDTA (TE) pH 7.6, autoclaved
10mM Tris pH 8.0	1M Tris pH 8.0 stock, use 100 µl in 10 ml sterile MQ
1M Tris-Cl	1M Tris-Cl pH 8.0 (autoclaved)
2M Tris-Cl (for berries)	2M Tris-Cl, pH 8.3 (autoclaved)
2M Tris-Cl (for leaves and flowers)	2M Tris-Cl, pH 7.6 (autoclaved)
X-α-gal	5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside
X-gal (for blue/white selection)	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Table A.3 Media for bacterial growth.

Use for Media	Components/Contents/Recipe
Aureobasidin A	Aureobasidin A (AbA) (Clontech), made to concentration as instructed by manufacturer
LB agar with kanamycin	2.5 % LB, 1.5% select agar and 50 µg/mL kanamycin
LB agar with ampicillin	2.5 % LB, 1.5% select agar and 100 µg/mL ampicillin
Split YFP plates	2% Amresco Agar Bacteriological (lot 1474C349)
SD/–Leu broth	As supplied by Clontech, made to volume as instructed by manufacturer
SD/–Leu with agar	As above
SD/–Trp broth	As above
SD/–Trp with agar	As above
SD/–Leu/–Trp with agar (double drop out – DDO)	As above
SD/–Ade/–His/–Leu/–Trp with agar (quadruple drop out - QDO)	As above
SELECT Agar®, powder	As supplied by Invitrogen
Yeast peptone dextrose adenine (YPDA) broth	As supplied by Clontech, made to volume as instructed by manufacturer
YPDA with agar	As above

Appendix B Oligonucleotide primers

All primer sequences are listed in a 5’ to 3’ direction. All primer names are named with the original gene names based on Finet *et al*. (2012), Çakir *et al*. (2013) and Parry *et al*. (2009), and the updated Grimplet *et al*. (2014) names are listed beside them.

Table B.1 Primers used for standards and qPCR analysis.

Name of Primer	Grimplet name	Primer sequence
TIR1F	VviAFB8	CTTGGCCAATGCTGCAAAGCTGG
TIR1R	VviAFB8	TTCTGGCCTTGAATCTGGTCGCC
TIR1-LikeF	VviAFB7	TGTCGTCGGATGTCCAATGTGGC
TIR1-LikeR	VviAFB7	AGTCGGGACGGAAGCGTTCAATG
AFB23F	VviAFB9	AACGTGGCACTTCTGACGGACG
AFB23R	VviAFB9	CCGCAAGTACCTTGCAGCCTCC
AFB2-3.2-F*	VviAFB10	AAGATGCACCAGCTTCTGTTTG
AFB2-3.2-R*	VviAFB10	ACTCGGCAACTACCTTCAGAGC
AFB45F	VviAFB11	GAGATGCGGCTCTGCGATCTGG
AFB45R	VviAFB11	ACCACTAGACCAGGCATTGCTCG
AFB6F	VviAFB6	GATGCAGTGTGTGCTGAGTGGC
AFB6R	VviAFB6	TGCAGGCTGACATCCAGAGTGAC
ARF1.1-F 3	VviARF1a	ATGAATCAGCAACCAGGCCCCACC
ARF1.1-R 3	VviARF1a	ACACCGCTGAGGAAGTGAAGAG
ARF1.2-F	VviARF1b	ATGGCTGTTGGAAGGGCTGTGG
ARF1.2-R	VviARF1b	GTCCGCAGAGCTCACCTTTGATGTC
ARF2.1-F	VviARF2b	AAGAGTTGGAGACATCCCTCAGCAG
ARF2.1-R	VviARF2b	ATGCTCCATGGTCAATTCAGGCAGAC
ARF2.2-F	VviARF2a	ACCAGAGAGGAGGTGCAAAGGATG
ARF2.2-R	VviARF2a	ATGTCAAAGGAACCGGCTGACG
ARF2.3-F	VviARF26	GGAATTCCGGTCTATGGTGCAGAG
ARF2.3-R	VviARF26	AAGCAGTGATCATGAGGGTGTAGCTG
ARF3-F	VviARF3	AGCTGCAGGCTCTTTGGCTTTTCC
ARF3-R	VviARF3	AGGCTCTGAGTGCAACTGCTGG
ARF4 FWD1 New	VviARF4	ACTGCAGCTGGCTGTAAACTGTTCG
ARF4 REV1 New	VviARF4	TGGCTCGTCCCACTAAGTTGCC
ARF5-F	VviARF5	GTTTGTTGGCTGTGTCCGCTGC
ARF5-R	VviARF5	GCTGCATGCCCTCTTCACTCATC
ARF6-F	VviARF30	AGGAAGTGCAGCAGATGGGAAAACG

Name of Primer	Grimplet name	Primer sequence
ARF6-R	VviARF30	TTCTTGAGTCCTGCCGGCTTGC
ARF8.1-F	VviARF29	TCAGGTGGCAAGGGTGGCAAAG
ARF8.1-R	VviARF29	AGGCCAGGAAGCTTACTTTGAACAAG
ARF8.2-F	VviARF8	TCACCAGAGGATGTGCAGAAAATGGG
ARF8.2-R	VviARF8	TTCTCAGTACTCGAGCGACCCAGC
ARF9-F	VviARF25	TGGAAGGTGAAGGGACTACCATAAGC
ARF9-R	VviARF25	CGGCCAATTAGCCATCCTCTGC
ARF11-F	VviARF24	GCAAGGAGTAGCAGTTGGTCGTGC
ARF11-R	VviARF24	AGGGCAAAGCTCTCCTTTGATCTCG
ARF16.1-F	VviARF32	TCACCAGAATGGCCCACAGGAG
ARF16.1-R	VviARF32	ACCTTGCAGTGACCAGTCTCCAGG
ARF16.2-F	VviARF31	GCGGAATGCCGAAAATGGACTAGATTC
ARF16.2-R	VviARF32	CGTGCACTAAGTCCTTACTATCCATGC
ARF16.3-F	VviARF16	TGGATTCAAGCAGCGACAATGTAGGAG
ARF16.3-R	VviARF16	AAGGTCAGCATGGAACAAAGGATTTGG
ARF17-F	VviARF17	TCACCAGATAGCCAGGGCAGTG
ARF17-R	VviARF17	GCTGCAATTACCTTCAACAGGCTGC
ARF19.1-F	VviARF27	AGATCTGGGTCACGTGCCTGTC
ARF19.1-R	VviARF27	ATCCTCGTAGTGACCCTTCCATGC
ARF19.2-F	VviARF28	TGTGAACTGTGTACGCTGCATCAAG
ARF19.2-R	VviARF28	TGCCACCATCAGAACTACTACAGGC
Aux/IAA1-F	VviIAA37	GCACCTAGAGCCATGGAGAAATGC
Aux/IAA1-R	VviIAA37	ACATCCTGCTCAATCCATCTTGGC
Aux/IAA2-F	VviIAA27	AGGGCAATGGAGAAATGCAAGAGTCG
Aux/IAA2-R	VviIAA27	AAGGCACTCAAGGTTTGTAGCATTCAG
AuxIAA3 FWD NewACTUALLYREV	VviIAA36	GGTCTTGCACCAAGAGCAATGGAG
AuxIAA3 REV NewACTUALLYFWD	VviIAA36	TCAAAGCTTGAACACAGTGCTGC
Aux/IAA4-F 1	VviIAA9	TCCCAGGGCTGTGGAGAAATGC
Aux/IAA4-R 1	VviIAA9	CTGCTGGATGGATGGCAACAACC
AuxIAA4.1-F	VviIAA45	ACTAAAGAGCCCAATCCCAGTACATCC
AuxIAA4.1-R	VviIAA45	TCCACCCCACAAGACAAAATGTGG
Aux/IAA5-F	VviIAA35	TAGAGGCACCCATCCACAGTCTGC
Aux/IAA5-R	VviIAA35	AGCACATCCATCCATGATCCTCAGC
AuxIAA6 FWD2 New	VviIAA43	AGCTCAAAATGAATCATCTGCTGGCAC
AuxIAA6 REV2 New	VviIAA43	AATCCTGCCATGGTTTTTGCTTCCTC

Name of Primer	Grimplet name	Primer sequence
Aux/IAA7-F	VviIAA42	AGCCTCAGATTTCTGCTCCTGCTG
Aux/IAA7-R	VviIAA42	GGATCCTAACTTGCCTTCTGCACCC
Aux/IAA8-F	VviIAA15a	TGCAAGCGGGTACGGTTGATG
Aux/IAA8-R	VviIAA15a	AGTCGTGCTTGTGCAGGAAGG
Aux/IAA9-F similar	VviIAA34b	ACATTCATCGAGTCTGTGCAGCG
Aux/IAA9-R similar	VviIAA34b	AGCCTGCAAATCTGCCTGCACTTTC
Aux/IAA10NEWFWD 1	VviIAA26	AGGTTGCGCGTGTTGAAGAGC
Aux/IAA10NEWREV 1	VviIAA26	TGGTGCCTTTTCTTGCTTGCTGC
Aux/IAA11-F	VviIAA41	AAGGGTTCAGAGGCAATTGGGC
Aux/IAA11-R	VviIAA41	CCTCTTCTTTTCTCGGTCCCGCTG
AuxIAA11.1-F 1	VviIAA11	ATGGGAGACAAAGAAGCATGCGAATC
AuxIAA11.1-R 1	VviIAA11	TACGTTTCAAGCCATGGGTAGTTTTCC
Aux/IAA12NEWFWD 1	VviIAA15b	AGGACATGTTCTCCTGCTTCCCTATTC
Aux/IAA12NEWREV 1	VviIAA15b	GGTAGGCATGTAGTCGGATCCCTTC
Aux/IAA13 FWD New	VviIAA44	GCATCAAGCGTCTGAGAATTGTGCG
Aux/IAA13 REV New	VviIAA44	TGGGACTGGGCTGCCATTTTCATTTC
Aux/IAA22-F	VviIAA13	TGGACGGATTGCCTATTGGGAGG
Aux/IAA22-R	VviIAA13	TGGGAGGCATGGTTGTGTTGTGC
AuxIAA22.1-F	VviIAA39	TCATGAAGGGATCGGAATGAAACCCG
AuxIAA22.1-R	VviIAA39	AGGTGAAAACCGAGAGCCCAAGC
AuxIAA22.2-F	VviIAA40	AGACGCTAGAGGCTTGGGTTGTG
AuxIAA22.2-R	VviIAA40	TTTCCCTGGGTGAGAGACACGTCC
AuxIAA22.3-F	VviIAA38	ATGCTGGTGGGAGATGTTCCCTGG
AuxIAA22.3-R	VviIAA38	AGAAAGCAGCCCAAGCCTCTAGC
AuxIAA22.4-F	VviIAA19	TGAGGACAAAGATGGGGACTGGATGC
AuxIAA22.4-R	VviIAA19	ACTGCAGGCCGAAATTCTTGGTTTC
AuxIAA29-F	VviIAA34a	ATTCATTCAGTCGGTGGAGCGTC
AuxIAA29-R	VviIAA34a	ACCAGTCAATTTCTCTACCAGCCTTCC
AuxIAA31NewFWD 1	VviIAA31	ACCAACGTGTTGACTGGCCG
AuxIAA31NewREV 1	VviIAA31	AAGTTAGTGGCTTGGCGCTGG
AuxIAA33-F	VviIAA33	ACGAGCTCGATCTGTCCAACGC
AuxIAA33-R	VviIAA33	ATTCCTCTTCGCCGGCAAAATCCG

*- designed by Dr Christine Böttcher

Table B.2 Primers used for yeast library screens and yeast 2-hybrid analysis

Name of Primer	Grimplet name	Sequence	Use
VvARF4 FWD CDS	VviARF4	ATGGAAATTGATCTGAACCATG	To clone full-length ARF4 CDS
VvARF4 REV CDS	VviARF4	TCAGATTCTAATCACTGTTGGA	As above
VvARF11 FWD CDS	VviARF24	ATGGCGCATGGGAATAATATC	To clone full-length ARF11 CDS
VvARF11 REV CDS	VviARF24	CTATGGTTCAGTTCTTAACTCT	As above
VvARF19.1 FWD CDS	VviARF27	ATGAAAGCTCCACCAAATGGG	To clone full-length ARF19.1 CDS
VvARF19.1 REV CDS	VviARF27	TCATCGATTAAATGAGGCAGCT	As above
VvARF4 FWD BAIT	VviARF4	1CATGGAGGCCGAATTCATGGAAATTGATCTGAACCATGCA	Introduction of sites for cloning into the pGBKT7 bait plasmid
VvARF4 REV BAIT	VviARF4	GCAGGTCGACGGATCCTCAGATTCTAATCACTGTTGGAGA	As above
VvARF11 FWD BAIT	VviARF24	CATGGAGGCCGAATTCATGGCGCATGGGAATAATATCAGA	As above
VvARF11 REV BAIT	VviARF24	GCAGGTCGACGGATCCCTATGGTTCAGTTCTTAACTCTGA	As above
VvARF19.1 FWD BAIT	VviARF27	CATGGAGGCCGAATTCATGAAAGCTCCACCAAATGGGTTT	As above
VvARF19.1 REV BAIT	VviARF27	GCAGGTCGACGGATCCTCATCGATTAAATGAGGCAGCTGA	As above
VvARF4 FWD -DBD	VviARF4	ATGCCAAGAAATGGTCTTCCT	To clone ARF4 without the DBD
New VvARF4 REV CDS - DBD	VviARF4	TCAGATTCTAATCACTGTTGGAGA	As above
VvARF4 F -DBD BAIT	VviARF4	CATGGAGGCCGAATTCATGCCAAGAAATGGTCTTCCT	Introduction of the sites for cloning into the pGBKT7 bait plasmid without the DBD
VvARF11 FWD -DBD	VviARF24	ATGCCCTCATCTGTCATATC	To clone ARF11 without the DBD
New VvARF11 REV CDS - DBD	VviARF24	CTATGGTTCAGTTCTTAACTCTC	As above
VvARF11 F -DBD BAIT	VviARF24	CATGGAGGCCGAATTCATGCCCTCATCTGTCATATC	Introduction of the sites for cloning into the pGBKT7 bait plasmid without the DBD
VvARF19.1 FWD -DBD	VviARF27	ATGCAGCAGCCAGCTCTGTC	To clone ARF19.1 without the DBD

Name of Primer	Grimplet name	Sequence	Use
VvARF19.1 F -DBD BAIT	VviARF27	CATGGAGGCCGAATTCATGCAGCAGCCAGCTCTGTCTTCA	Introduction of the sites for cloning into the pGBKT7 bait plasmid without the DBD
VvARF4 FWD internal	VviARF4	AGGTCAGCCAAGGCGACATCTG	To sequence the full-length CDS
VvARF4 REV internal	VviARF4	AGATTTTCCAGCCAGGGATCTCAAAGG	As above
VvARF19.1 FWD internal	VviARF27	AGCAGCCAGCTCTGTCTTCATCAG	As above
VvARF19.1 REV internal	VviARF27	ACCCAGGCTTGAAAGACATGTTCG	As above
ARF11 int FWD2	VviARF24	TGTGGGTGATGATCCATGGC	As above
ARF11 int FWD	VviARF24	GAAGCTTCTATTGGCTGCCG	As above
ARF11 int FWD1	VviARF24	ACTATGGCTTTGCAGTTGGC	As above
ARF11 int REV1	VviARF24	TGCTGTCTTCTGTTAGGTCCTG	As above
NEW ARF19.1 intREV3	VviARF27	TTGATGCTCTGGTAGGTTCTGC	As above
NEW ARF19.1 intFWD3	VviARF27	AGGCTTGAATCTAGTCCAGTGG	As above
AuxIAA2 FWD short CDS	VviIAA27	ATGGAAAGCTCTGAGAAGCTCA	To clone the full-length Aux/IAA protein
AuxIAA2 FWD long CDS	VviIAA27	ATGTCTAAGCAACTGGAGCATG	As above
AuxIAA2 REV CDS	VviIAA27	CTAGTTGCGACTCTTGCATTTC	As above
AuxIAA11 FWD CDS	VviIAA41	ATGTCTATACCTCTAGAACATGATTAC	As above
AuxIAA11 REV CDS	VviIAA41	CTAGTTTCTGTTCTTGCATTTT	As above
AuxIAA22.4 FWD CDS	VviIAA19	ATGGCCCTAGGACTCGAGATC	As above
AuxIAA22.4 REV CDS	VviIAA19	CTAATCATTTATCTTCTGGAGTTCCTT	As above
AuxIAA2 F short CDS New	VviIAA27	ATGGAAAGCTCTGAGAAGCTCACC	As above
AuxIAA2 F long CDS New	VviIAA27	ATGTCTAAGCAACTGGAGCATGATTAC	As above
AuxIAA2 R CDS New	VviIAA27	CTAGTTGCGACTCTTGCATTTCTCC	As above
AuxIAA11 F CDS New	VviIAA41	ATGTCTATACCTCTAGAACATGATTACATAGGC	As above

Name of Primer	Grimplet name	Sequence	Use
AuxIAA11 R CDS New	VviIAA41	CTAGTTTCTGTTCTTGCATTTTTCCATG	As above
AuxIAA2 Prey FWD	VviIAA27	GGAGGCCAGTGAATTCATGTCTAAGCAACTG GAGCATGAT	Introduction of the sites for cloning into the pGADT7 prey plasmid
AuxIAA2 Prey REV	VviIAA27	CGAGCTCGATGGATCCCTAGTTGCGACTCTTG CATTTCTC	As above
AuxIAA11 Prey FWD	VviIAA41	GGAGGCCAGTGAATTCATGTCTATACCTCTAG AACATGAT	As above
AuxIAA11 Prey REV	VviIAA41	CGAGCTCGATGGATCCCTAGTTTCTGTTCTTGC ATTTTTC	As above
AuxIAA22.4 Prey FWD	VviIAA19	GGAGGCCAGTGAATTCATGGCCCTAGGACTC GAGATCACT	As above
AuxIAA22.4 Prey REV	VviIAA19	CGAGCTCGATGGATCCCTAATCATTTATCTTCT GGAGTTC	As above

1 – Bold sequences represent the overhang sequences introduced through PCR for cloning into the pGBKT7 bait or PGADT7 prey plasmids.

Table B.3 Primers used for bimolecular fluorescence analysis - Gateway cloning.

Name of Primer	Grimplet name	Sequence	Use
ARF4 attB1 FWD	VviARF4	1GGGGACAAGTTTGTACAAAAAAGCAGGCTT AATGGAAATTGATCTGAACCATGCA	The introduction of the attB1 cloning site and amplification of a full-length clone of ARF4
ARF4 attB2 REV	VviARF4	2GGGGACCACTTTGTACAAGAAAGCTGGGTA GATTCTAATCACTGTTGGAGAACT	As above, except an attB2 cloning site was introduced
ARF11 attB1 FWD	VviARF24	GGGGACAAGTTTGTACAAAAAAGCAGGCTTA ATGGCGCATGGGAATAATATCAGA	The introduction of the attB1 cloning site and amplification of a full-length clone of ARF11
ARF11 attB2 REV	VviARF24	GGGGACCACTTTGTACAAGAAAGCTGGGTAT GGTTCAGTTCTTAACTCTGAATC	As above, except an attB2 cloning site was introduced
ARF19.1 attB1 FWD	VviARF27	GGGGACAAGTTTGTACAAAAAAGCAGGCTTA ATGAAAGCTCCACCAAATGGGTTT	The introduction of the attB1 cloning site and amplification of a full-length clone of ARF19.1

Name of Primer	Grimplet name	Sequence	Use
ARF19.1 attB2 REV	VviARF27	GGGGACCACTTTGTACAAGAAAGCTGGGTATCGATTAAATGAGGCAGCTGAGGT	As above, except an attB2 cloning site was introduced
AuxIAA2 attB1 FWD	VviIAA27	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCTAAGCAACTGGAGCATGAT	The introduction of the attB1 cloning site and amplification of a full-length clone of Aux/IAA2
AuxIAA2 attB2 REV	VviIAA27	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTGCGACTCTTGCATTTCTCCAT	As above, except an attB2 cloning site was introduced
AuxIAA11 attB1 FWD	VviIAA41	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCTATACCTCTAGAACATGAT	The introduction of the attB1 cloning site and amplification of a full-length clone of Aux/IAA11
AuxIAA11 attB2 REV	VviIAA41	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTTCTGTTCTTGCATTTTTCCAT	As above, except an attB2 cloning site was introduced
AuxIAA22.4 attB1 FWD	VviIAA19	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCCTAGGACTCGAGATCACT	The introduction of the attB1 cloning site and amplification of a full-length clone of Aux/IAA22.4
AuxIAA22.4 attB2 REV	VviIAA19	GGGGACCACTTTGTACAAGAAAGCTGGGTAATCATTTATCTTCTGGAGTTCCTT	As above, except an attB2 cloning site was introduced
GRIP3 attB1 FWD	N/A	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCTTCTGCGTGCTCACTCGTG	The introduction of the attB1 cloning site and amplification of a full-length clone of GRIP3
GRIP3 attB2 REV	N/A	GGGGACCACTTTGTACAAGAAAGCTGGGTAATTGGCATGGATGGGTGGTGGAGC	As above, except an attB2 cloning site was introduced
Trans2_3enoylCoA attB1 F	N/A	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGGTCAAGGTGGTTACGCGG	The introduction of the attB1 cloning site and amplification of a full-length clone of Trans-2, 3 enoyl CoA
Trans2_3enoylCoA attB2 R	N/A	GGGGACCACTTTGTACAAGAAAGCTGGGTACAGGAATGGGGGTAGTATCACCCA	As above, except an attB2 cloning site was introduced
pAM REV3	N/A	TTCTGGAGAAAAATAGAGAGAGATAGA	Sequencing of the YFPn and YFPc vectors

Name of Primer	Grimplet name	Sequence	Use
ARF4_noIII_IV_RattB	VviARF4	GGGGACCACTTTGTACAAGAAAGCTGGGTACTTACCAGAATTTTGTGAGTTTGG	The introduction of the attB2 cloning site and generate a truncated ARF4 without domains III and IV
ARF19.1noIII_IV_RattB	VviARF27	GGGGACCACTTTGTACAAGAAAGCTGGGTACATACGCTGAGCCTGGTTTGTCCA	The introduction of the attB2 cloning site and generate a truncated ARF19.1 without domains III and IV
IAA2_noIII_IVRattB	VviIAA27	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGACACCCGGATCCTAACTTGCC	The introduction of the attB2 cloning site and generate a truncated Aux/IAA2 without domains III and IV
IAA22_4_noIII_IVRattB	VviIAA19	GGGGACCACTTTGTACAAGAAAGCTGGGTACATTTTGGTAGCTTCTGTTCGATC	The introduction of the attB2 cloning site and generate a truncated Aux/IAA22.4 without domains III and IV

1 – Bold sequences represent the attB1 overhang sequences introduced through PCR for cloning into the pDONR221 Gateway entry plasmid.
2 – Bold sequences represent the attB2 overhang sequences introduced through PCR for cloning into the pDONR221 Gateway entry plasmid.
3 – From the Dr Ian Dry lab group.

Table B.4 Controls.

Name of Primer	Grimplet name	Sequence	Use
Actin2 FWD*	VviActin	GCACCCTTCGCACGATATGA	qPCR analysis reference gene
Actin2 REV*	VviActin	TGACGCAAGGCAAGGACTGA	As above
M13 FWD	N/A	TGTAAAACGACGGCCAGT	Sequencing of inserts in plasmids
M13 REV	N/A	CAGGAAACAGCTATGACC	As above
T7 FWD	N/A	TAATACGACTCACTATAGGG	For sequencing BAIT and PREY vector
3' DNA-BD REV	N/A	TTTTCGTTTTAAAACCTAAGAGTC	For sequencing BAIT vector
3’ DNA-BD1	N/A	TAAGAGTCACTTTAAAATTTGTAT	As above
3' DNA-AD REV	N/A	AGATGGTGCACGATGCACAG	For sequencing PREY vector

*- provided by Dr Crista Burbidge, based on gene AM465189
1 – From Clontech

Appendix C Vectors

All vector names are named with the original gene names based on Finet *et al*. (2012), Çakir *et al*. (2013) and Parry *et al*. (2009), and the updated Grimplet *et al*. (2014) names are listed beside them.

Table C.1 Vectors generated in this work for the sequencing of standards for qPCR.

Plasmid Name	Grimplet name
pDRIVE-VvTIR1	VviAFB8
pDRIVE-VvTIR1-Like	VviAFB7
pDRIVE-VvAFB2/3	VviAFB9
pDRIVE-VvAFB2/3.2	VviAFB10
pDRIVE-VvAFB4/5	VviAFB11
pDRIVE-VvAFB6	VviAFB6
pDRIVE-VvARF1.1	VviARF1a
pDRIVE-VvARF1.2	VviARF1b
pDRIVE-VvARF2.1	VviARF2b
pDRIVE-VvARF2.2	VviARF2a
pDRIVE-VvARF2.3	VviARF26
pDRIVE-VvARF3	VviARF3
pDRIVE-VvARF4New	VviARF4
pDRIVE-VvARF5	VviARF5
pDRIVE-VvARF6	VviARF30
pDRIVE-VvARF8.1	VviARF29
pDRIVE-VvARF8.2	VviARF8
pDRIVE-VvARF9	VviARF25
pDRIVE-VvARF11	VviARF24
pDRIVE-VvARF16.1	VviARF32
pDRIVE-VvARF16.2	VviARF31
pDRIVE-VvARF16.3	VviARF16
pDRIVE-VvARF17	VviARF17
pDRIVE-VvARF19.1	VviARF27
pDRIVE-VvARF19.2	VviARF28
pDRIVE-VvAuxIAA1	VviIAA37
pDRIVE-VvAuxIAA2	VviIAA27
pDRIVE-VvAuxIAA3New	VviIAA36
pDRIVE-VvAuxIAA4	VviIAA9
pDRIVE-VvAuxIAA4.1	VviIAA45

Plasmid Name	Grimplet name
pDRIVE-VvAuxIAA5	VviIAA35
pDRIVE-VvAuxIAA6New	VviIAA43
pDRIVE-VvAuxIAA7	VviIAA42
pDRIVE-VvAuxIAA8	VviIAA15a
pDRIVE-VvAuxIAA9similar	VviIAA34b
pDRIVE-VvAuxIAA10	VviIAA26
pDRIVE-VvAuxIAA11	VviIAA41
pDRIVE-VvAuxIAA11.1	VviIAA11
pDRIVE-VvAuxIAA12New	VviIAA15b
pDRIVE-VvAuxIAA13New	VviIAA44
pDRIVE-VvAuxIAA22	VviIAA13
pDRIVE-VvAuxIAA22.1	VviIAA39
pDRIVE-VvAuxIAA22.2	VviIAA40
pDRIVE-VvAuxIAA22.3	VviIAA38
pDRIVE-VvAuxIAA22.4	VviIAA19
pDRIVE-VvAuxIAA29	VviIAA34a
pDRIVE-VvAuxIAA31New	VviIAA31
pDRIVE-VvAuxIAA33	VviIAA33

Table C.2 Vectors generated in this work for yeast library screening and yeast 2-hybrid analysis.

Plasmid Name	Grimplet name	Use
pCR-Blunt-VvARF4	VviARF4	Entry vector for yeast analysis
pCR-Blunt-VvARF11	VviARF24	As above
pCR-Blunt-VvARF19.1	VviARF27	As above
pCR-Blunt-VvAux/IAA2	VviIAA27	As above
pCR-Blunt-VvAux/IAA11	VviIAA41	As above
pCR-Blunt-VvAux/IAA22.4	VviIAA19	As above
pCR-Blunt-VvARF4ΔDBD	VviARF4	Entry vector for yeast analysis, without DBD
pCR-Blunt-VvARF11ΔDBD	VviARF24	As above
pCR-Blunt-VvARF19.1ΔDBD	VviARF27	As above
pGBKT7-VvARF4	VviARF4	Bait vector for yeast analysis
pGBKT7-VvARF11	VviARF24	As above
pGBKT7-VvARF19.1	VviARF27	As above
pGBKT7-VvARF4ΔDBD	VviIAA27	Bait vector for yeast analysis, without DBD

Plasmid Name	Grimplet name	Use
pGBKT7-VvARF11ΔDBD	VviIAA41	As above
pGBKT7-VvARF19.1ΔDBD	VviIAA19	As above
pGADT7-VvAux/IAA2	VviIAA27	Prey vector for yeast analysis
pGADT7-VvAux/IAA11	VviIAA41	As above
pGADT7-VvAux/IAA22.4	VviIAA19	As above

Table C.3 Vectors used and generated in this work for bimolecular fluorescence analysis.

Plasmid Name	Grimplet name	Use
pDONR 221-VvARF4	VviARF4	Entry vector for split-YFP analysis
pDONR 221-VvARF11	VviARF24	As above
pDONR 221-VvARF19.1	VviARF27	As above
pDONR 221-VvGRIP3	N/A	As above
pDONR 221-VvTrans2,3-enoyl-CoA	N/A	As above
pDONR 221-VvAux/IAA2	VviIAA27	As above
pDONR 221-VvAux/IAA11	VviIAA41	As above
pDONR 221-VvAux/IAA22.4	VviIAA19	As above
pDONR 221-VvARF4ΔDBD	VviIAA27	Entry vector for split-YFP analyais, without the DNA binding domain
pDONR 221-VvARF19.1ΔDBD	VviIAA19	As above
pDONR 221-VvAux/IAA2ΔIIIandIV	VviIAA27	For split-YFP analysis, without Domains III and IV
pDONR 221-VvAux/IAA22.4ΔIIIandIV	VviIAA19	As above
[1]pSITE-nEYFP-N1	N/A	For BiFC analysis negative control
pSITE-VviARF4-nEYFP (N1)	VviARF4	For BiFC analysis
pSITE-VviARF19.1-nEYFP (N1)	VviARF27	As above
pSITE-VviIAA2-nEYFP (N1)	VviIAA27	As above
pSITE-VviIAA22.4-nEYFP (N1)	VviIAA19	As above
pSITE-VviGRIP3-nEYFP (N1)	N/A	As above
pSITE-VviTrans-2,3-enoyl-CoA reductase-like-nEYFP (N1)	N/A	As above
pSITE-VviARF4ΔDBD -nEYFP (N1)	VviARF4	For BiFC analysis, without the DNA binding domain
pSITE-VviARF19.1ΔDBD-nEYFP (N1)	VviARF27	As above

Plasmid Name	Grimplet name	Use
pSITE-VviIAA22.4ΔDBD-nEYFP (N1)	VviIAA19	For BiFC analysis, without Domains III and IV
pSITE-cEYFP-N1	N/A	For BiFC analysis negative control
pSITE-VviARF4-cEYFP (N1)	VviARF4	For BiFC analysis
pSITE-VviARF19.1-cEYFP (N1)	VviARF27	As above
pSITE-VviIAA2-cEYFP (N1)	VviIAA27	As above
pSITE-VviIAA22.4-cEYFP (N1)	VviIAA19	As above
pSITE-VviGRIP3-cEYFP (N1)	N/A	As above
pSITE-VviTrans-2,3-enoyl-CoA reductase-like-cEYFP (N1)	N/A	As above
pSITE-VviARF4ΔDBD-cEYFP (N1)	VviARF4	For BiFC analysis, without the DNA binding domain
pSITE-VviARF19.1ΔDBD-cEYFP (N1)	VviARF27	As above
pSITE-VviIAA22.4ΔDBD-cEYFP (N1)	VviIAA19	For BiFC analysis, without Domains III and IV
pSITE-nEYFP-C1	N/A	For BiFC analysis negative control
pSITE-nEYFP-VviARF4 (C1)	VviARF4	For BiFC analysis
pSITE-nEYFP-VviARF19.1 (C1)	VviARF27	As above
pSITE-nEYFP-VviIAA2 (C1)	VviIAA27	As above
pSITE-nEYFP-VviIAA22.4 (C1)	VviIAA19	As above
pSITE-nEYFP-VviGRIP3 (C1)	N/A	As above
pSITE–nEYFP-VviTrans-2,3-enoyl-CoA reductase-like (C1)	N/A	As above
pSITE-nEYFP-VviARF4ΔDBD (C1)	VviARF4	For BiFC analysis, without the DNA binding domain
pSITE-nEYFP-VviARF19.1ΔDBD (C1)	VviARF27	As above
pSITE-nEYFP-VviIAA22.4ΔDBD (C1)	VviIAA19	For BiFC analysis, without Domains III and IV
pSITE-cEYFP-C1	N/A	For BiFC analysis negative control
pSITE-cEYFP-VviARF4 (C1)	VviARF4	For BiFC analysis
pSITE-cEYFP-VviARF19.1 (C1)	VviARF27	As above
pSITE-cEYFP-VviIAA2 (C1)	VviIAA27	As above
pSITE-cEYFP-VviIAA22.4 (C1)	VviIAA19	As above
pSITE-cEYFP-VviGRIP3 (C1)	N/A	As above
pSITE–cEYFP-VviTrans-2,3-enoyl-CoA reductase-like (C1)	N/A	As above

Plasmid Name	Grimplet name	Use
pSITE-cEYFP-VviARF4ΔDBD (C1)	VviARF4	For BiFC analysis, without the DNA binding domain
pSITE-cEYFP-VviARF19.1ΔDBD (C1)	VviARF27	As above
pSITE-cEYFP-VviIAA22.4ΔDBD (C1)	VviIAA19	For BiFC analysis, without Domains III and IV
[2]pART7-35S-VviSNAP33-CFP	N/A	For BiFC analysis positive control

1 – GenBank accession numbers: pSITE-cEYFP-C1 (GU734652), pSITE-cEYFP-N1 (GU734649), pSITE-nEYFP-C1 (GU734651) and pSITE-nEYFP-N1 (GU734648)
2 – from the Dr Ian Dry lab group
*Note – initially all combinations of pAM-35S-GWY-YFPn and pAM-35S-GWY-YFPc vectors were used to test the interaction of ARF and Aux/IAA candidates, however, some fluorescence was detected with the empty pAM-35S-GWY-YFPc when bombarded on its own suggesting false-positive results may occur. For this reason, the pSITE vectors were then used. The ARF-Aux/IAA combinations that were seen to positively interact in yeast were selected for analysis in pSITE vectors.
BiFC- bimolecular fluorescence
DBD- DNA binding domain

Appendix D Bioinformatic analysis

See the linked PDF documents or CD for the VviAFB, VviARF and VviIAA sequences in FASTA format and Figures D.1 – D.6.

Figure D.1 A MUSCLE protein alignment of the Aux/IAA sequences identified by Çakir *et al*. (2013) in grape with the AtARF1 and AtIAA1 protein sequences to highlight which sequences are ARF proteins and which are IAA proteins.

Figure D.1.pdf

Figure D.2 A MUSCLE protein alignment of the VvAFB sequences identified by Parry *et al*. (2009) in grape with the proteins identified in this work.

Figure D.2.pdf

Figure D.3 A MUSCLE protein alignment of the VvARF sequences identified by Wan *et al*. (2014) in grape with the proteins identified in this work.

Figure D.3.pdf

Figure D.4 A MUSCLE protein alignment of the Aux/IAA grape sequences from NCBI, Phytozome and Tablet to identify the VviIAA sequences used in this work. Once these sequences were refined down, all were compared to FGENESH+ RNAseq data to identify the correct intron/exon boundaries.

Figure D.4.pdf

Figure D.5 A MUSCLE protein alignment of the ARF grape sequences from NCBI, Phytozome and Tablet to identify the VviARF sequences used in this work. Once these sequences were refined down, all were compared to FGENESH+ RNAseq data to identify the correct intron/exon boundaries.

Figure D.5.pdf

Figure D.6 FASTA format of all VviAFB, VviARF and VviIAA coding sequences. The promoter sequences and the 5' untranscribed regions are in bold.

Figure D.6.pdf

Table D.1 The TIR1/AFB publication details and sequence identifier numbers.

Species	Gene name	Gene ID number	Publication and/or database
Arabidopsis	AtTIR1	AT3G62980	TAIR
	AtAFB1	AT4G03190	
	AtAFB2	AT3G26810	
	AtAFB3	AT1G12820	
	AtAFB4	AT4G24390	
Grape	VviTIR1 clade	Vitvi23591001	Parry *et al*., 2009,

Species	Gene name	Gene ID number	Publication and/or database
	VviTIR1 clade	Vitvi31072001	Vitisviniferapeptidev1, http://www.genoscope.cns.fr/spip/Vitis-vinifera-e.html
	VviAFB2 clade	Vitvi37929001	
	VviAFB2 clade	Vitvi6948001	
	VviAFB4 clade	Vitvi15144001	
	VviAFB6 clade	Vitvi9745001	
Poplar	PtTIR1 clade	Poptr572746	Parry *et al*., 2009, Populus trichocarpa (v.1.1, http://genome.jgi-psf.org/Poptr11)
	PtTIR1 clade	Poptr573509	
	PtAFB2 clade	Poptr549767	
	PtAFB2 clade	Poptr742822	
	PtAFB4 clade	Poptr410004	
	PtAFB4 clade	Poptr651225	
	PtAFB6 clade	Poptr568304	
	PtAFB6 clade	Poptr800382	
Apple	MdTIR1	MDP0000125975	Devoghalaere *et al*., 2012
	MdTIR101	MDP0000498419	
	MdAFB2	MDP0000268652	
	MdAFB102	MDP0000203334	
	MdAFB5	MDP0000809218	
	MdAFB105	MDP0000135966	
	MdAFB6	MDP0000305861	
	MdAFB106	MDP0000255696	
Tomato	SlAFB6	Solyc02g079190.2, SlyAC215365	Sol Genomics Network, Parry *et al*., 2009
	SlAFB4/5	Solyc04g074980.2, SlyCU928132	
	SlTIR1-like	Solyc06g008780.1	
	SlTIR1	Solyc09g074520.2, SlyAK320427	

ID – identification

Table D.2 The Aux/IAA publication details and sequence identifier numbers.

Species	Gene name	Gene ID number	Publication and/or database
Arabidopsis	AtIAA1	AT4G14560	TAIR
	AtIAA2	AT3G23030	
	AtIAA3	AT1G04240	
	AtIAA4	AT5G43700	
	AtIAA5	AT1G15580	

Species	Gene name	Gene ID number	Publication and/or database
	AtIAA6	AT1G52830	
	AtIAA7	AT3G23050	
	AtIAA8	AT2G22670	
	AtIAA9	AT5G65670	
	AtIAA10	AT1G04100	
	AtIAA11	AT4G28640	
	AtIAA12	AT1G04550	
	AtIAA13	AT2G33310	
	AtIAA14	AT4G14550	
	AtIAA15	AT1G80390	
	AtIAA16	AT3G04730	
	AtIAA17	AT1G04250	
	AtIAA18	AT1G51950	
	AtIAA19	AT3G15540	
	AtIAA20	AT2G46990	
	AtIAA26	AT3G16500	
	AtIAA27	AT4G29080	
	AtIAA28	AT5G25890	
	AtIAA29	AT4G32280	
	AtIAA30	AT3G62100	
	AtIAA31	AT3G17600	
	AtIAA32	AT2G01200	
	AtIAA33	AT5G57420	
	AtIAA34	AT1G15050	
Grape	VvAux/IAA1, (VviIAA37)	CBI17932.31, GSVIVT010007210012	Çakir *et al*., 2013
	VvAux/IAA4, (VviIAA9)	CBI19314.3, GSVIVT01009238001	
	VvAux/IAA8, (VviIAA15a)	CBI28126.3, GSVIVT01015449001	
	VvAux/IAA10*, (VviIAA26)	CBI36050.3, GSVIVT01016972001	
	VvAux/IAA11*, (VviIAA41)	CBI36106.3, GSVIVT01017046001	

Species	Gene name	Gene ID number	Publication and/or database
	VvAux/IAA12*, (VviIAA15b)	CBI36192.3, GSVIVT01017159001	
	VvAux/IAA22, (VviIAA13)	CBI23724.3, GSVIVT01027921001	
	VvAux/IAA25, (VviIAA34b)	CBI21052.3, GSVIVT01035866001	
Poplar	PoptrIAA3.1	eugene3.00700060	Kalluri *et al*., 2007, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html
	PoptrIAA3.2	estExt_fgenesh4_pg.C_LG_XIII0196	
	PoptrIAA3.3	fgenesh4_pm.C_LG_II000215	
	PoptrIAA3.4	eugene3.00051252	
	PoptrIAA3.5	eugene3.00081508	
	PoptrIAA3.6	estExt_Genewise1_v1.C_LG_X5603	
	PoptrIAA7.1	eugene3.00100709	
	PoptrIAA7.2	estExt_Genewise1_v1.C_LG_VIII2464	
	PoptrIAA9	estExt_fgenesh4_pm.C_LG_II0495	
	PoptrIAA11	estExt_Genewise1_v1.C_LG_II1635	
	PoptrIAA12.1	estExt_fgenesh4_pm.C_LG_X0141	
	PoptrIAA12.2	fgenesh4_pm.C_LG_VIII000731	
	PoptrIAA15	estExt_Genewise1_v1.C_LG_I9550	
	PoptrIAA16.1	estExt_fgenesh4_pg.C_700052	
	PoptrIAA16.2	estExt_fgenesh4_kg.C_LG_XIII0024	
	PoptrIAA16.3	grail3.0002049301	
	PoptrIAA16.4	grail3.0003037201	
	PoptrIAA19.1	gw1.I.9599.1	
	PoptrIAA19.2	estExt_fgenesh4_pm.C_LG_I0462	
	PoptrIAA19.3	estExt_fgenesh4_pm.C_LG_III0099	
	PoptrIAA20.1	grail3.0050017401	
	PoptrIAA20.2	grail3.0061005101	
	PoptrIAA26.1	estExt_fgenesh4_pg.C_LG_III0457	
	PoptrIAA26.2	gw1.I.413.1	
	PoptrIAA27.1	eugene3.01570047	
	PoptrIAA27.2	estExt_Genewise1_v1.C_LG_III0268	
	PoptrIAA27.3	estExt_fgenesh4_pm.C_LG_I0544	
	PoptrIAA28.1	gw1.XVIII.806.1	

Species	Gene name	Gene ID number	Publication and/or database
	PoptrIAA28.2	fgenesh4_pg.C_LG_VI001632	
	PoptrIAA29.1	eugene3.00061866	
	PoptrIAA29.2	eugene3.00181144	
	PoptrIAA29.3	fgenesh4_pg.C_LG_VI000485	
	PoptrIAA33.1	eugene3.00180818	
	PoptrIAA33.2	gw1.121.83.1	
	PoptrIAA34	gw1.X.53.1	
Apple	MdIAA1	MDP0000945260	Devoghalaere *et al*., 2012, Genome database for Rosaceae www.rosaceae.org
	MdIAA101	MDP0000295589	
	MdIAA4	MDP0000123816	
	MdIAA6	MDP0000324398	
	MdIAA106	MDP0000176753	
	MdIAA7	MDP0000010086	
	MdIAA107	MDP0000237499	
	MdIAA8	MDP0000580010	
	MdIAA13	MDP0000255223	
	MdIAA113	MDP0000164095	
	MdIAA14	MDP0000211848	
	MdIAA114	MDP0000262602	
	MdIAA16A	MDP0000663301	
	MdIAA16B	MDP0000363509	
	MdIAA18	MDP0000303142	
	MdIAA118	MDP0000208345	
	MdIAA19	MDP0000124810	
	MdIAA119	MDP0000213864	
	MdIAA20	MDP0000132805	
	MdIAA120	MDP0000250876	
	MdIAA220	MDP0000284467	
	MdIAA21	MDP0000270789	
	MdIAA121	MDP0000131759	
	MdIAA22	MDP0000753736	
	MdIAA122	MDP0000267601	
	MdIAA222	MDP0000130583	
	MdIAA24	MDP0000211934	
	MdIAA124	MDP0000246042	

Species	Gene name	Gene ID number	Publication and/or database
	MdIAA25	MDP0000296324	
	MdIAA27A	MDP0000090281	
	MdIAA127A	MDP0000456476	
	MdIAA27B	MDP0000174664	
	MdIAA127B	MDP0000801571	
	MdIAA28	MDP0000151877	
	MdIAA29	MDP0000157035	
	MdIAA129	MDP0000195460	
	MdIAA229	MDP0000543718	
	MdIAA32	MDP0000277775	
	MdIAA132	MDP0000299826	
	MdIAA33	MDP0000146848	
Tomato	SlIAA1	JN379431	Audran-Delalande *et al*., 2012, GenBank/EMBL data libraries
	SlIAA2	JN379432	
	SlIAA3	JN379433	
	SlIAA4	JN379434	
	SlIAA7	JN379435	
	SlIAA8	JN379436	
	SlIAA9	JN379437	
	SlIAA11	JN379438	
	SlIAA12	JN379439	
	SlIAA13	JN379440	
	SlIAA14	JN379441	
	SlIAA15	JN379442	
	SlIAA16	JN379443	
	SlIAA17	JN379444	
	SlIAA19	JN379445	
	SlIAA21	JN379446	
	SlIAA22	JN379447	
	SlIAA23	JN379448	
	SlIAA26	JN379449	
	SlIAA27	JN379450	
	SlIAA29	JN379451	
	SlIAA32	JN379452	
	SlIAA33	JN379453	

Species	Gene name	Gene ID number	Publication and/or database
	SlIAA35	JN379454	
	SlIAA36	JN379455	

ID – identification
1 – GenBank ID numbers
2 – Vitis Genoscope ID numbers
Names in brackets - Names used in this work
* - Similar sequences to the ones within this work but differences exist

Table D.3 The ARF publication details and sequence identifier numbers.

Species	Gene name	Gene ID number	Publication and/or database
Arabidopsis	AtARF1	AT1G59750	TAIR
	AtARF2	AT5G62000	
	AtARF3	AT2G33860	
	AtARF4	AT5G60450	
	AtARF5	AT1G19850	
	AtARF6	AT1G30330	
	AtARF7	AT5G20730	
	AtARF8	AT5G37020	
	AtARF9	AT4G23980	
	AtARF10	AT2G28350	
	AtARF11	AT2G46530	
	AtARF12	AT1G34310	
	AtARF13	AT1G34170	
	AtARF14	AT1G35540	
	AtARF15	AT1G35520	
	AtARF16	AT4G30080	
	AtARF17	AT1G77850	
	AtARF18	AT3G61830	
	AtARF19	AT1G19220	
	AtARF20	AT1G35240	
	AtARF21	AT1G34410	
	AtARF22	AT1G34390	
	AtARF23	AT1G43950	
Grape	VvARF1, (VviARF2b)	LOC100250592	Finet *et al*., 2012; Wan *et al*., 2014, NCBI gene locus tag numbers
	VvARF2, (VviARF25)	LOC100247833	

Species	Gene name	Gene ID number	Publication and/or database
	VvARF3, (VviARF8)	LOC100258129	
	VvARF4, (VviARF31)	LOC100256989	
	VvARF5, (VviARF4)	LOC100243320	
	VvARF6, (VviARF26)	LOC100246055	
	VvARF7, (VvARF16)	LOC100251645	
	VvARF8, (VviARF3)	LOC100245251	
	VvARF9, (VviARF30)	LOC100242923	
	VvARF10, (VviARF27)	LOC100257618	
	VvARF11, (VviARF28)	LOC100263801	
	VvARF12, (VviARF29)	LOC100260866	
	VvARF13, (VviARF1a)	LOC100264303	
	VvARF14, (VviARF32)	LOC100265118	
	VvARF15, (VviARF24)	LOC100265555	
	VvARF16, (VviARF2a)	LOC100268072	
	VvARF17, (VviARF17)	LOC100255673	
	VvARF18, (VvARF5)	LOC100254074	
	VvARF19, (VviARF1b)	LOC100263592	
Poplar	PoptrARF1.1	estExt_fgenesh4_pg.C_1500013	Kalluri *et al*., 2007, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html
	PoptrARF1.2	estExt_fgenesh4_pm.C_860029	
	PoptrARF2.1	estExt_fgenesh4_pm.C_LG_XII0386	
	PoptrARF2.2	eugene3.00150845	
	PoptrARF2.3	estExt_fgenesh4_pg.C_LG_I0563	
	PoptrARF2.4	eugene3.00031333	
	PoptrARF2.5	gw1.XIV.427.1	

Species	Gene name	Gene ID number	Publication and/or database
	PoptrARF2.6	fgenesh4_pg.C_LG_XIV000751	
	PoptrARF3.1	estExt_Genewise1_v1.C_LG_IV2935	
	PoptrARF3.2	fgenesh4_pg.C_scaffold_187000006	
	PoptrARF3.3	eugene3.08470003	
	PoptrARF4	gw1.IX.4827.1	
	PoptrARF5.1	estExt_fgenesh4_pg.C_LG_II0231	
	PoptrARF5.2	estExt_fgenesh4_pg.C_LG_V1503	
	PoptrARF6.1	fgenesh4_pg.C_LG_I002802	
	PoptrARF6.2	estExt_Genewise1_v1.C_LG_XI2869	
	PoptrARF6.3	fgenesh4_pg.C_scaffold_1006000001	
	PoptrARF6.4	eugene3.00020511	
	PoptrARF6.5	fgenesh4_pm.C_LG_V000490	
	PoptrARF7.1	fgenesh4_pm.C_LG_XVIII000014	
	PoptrARF7.2	fgenesh4_pm.C_scaffold_28000031	
	PoptrARF7.3	estExt_fgenesh4_pg.C_1640064	
	PoptrARF7.4	estExt_fgenesh4_pg.C_LG_VI0597	
	PoptrARF8.1	gw1.IV.3880.1	
	PoptrARF8.2	fgenesh4_pm.C_scaffold_44000056	
	PoptrARF9.1	estExt_fgenesh4_pm.C_LG_III0477	
	PoptrARF9.2	fgenesh4_pg.C_LG_I000784	
	PoptrARF9.3	fgenesh4_pm.C_LG_II000801	
	PoptrARF9.4	fgenesh4_pm.C_LG_XIV000195	
	PoptrARF10.1	eugene3.00660262	
	PoptrARF10.2	gw1.IX.4734.1	
	PoptrARF16.1	estExt_fgenesh4_pg.C_LG_X2014	
	PoptrARF16.2	eugene3.00080331	
	PoptrARF16.3	estExt_fgenesh4_pm.C_LG_XVI0323	
	PoptrARF16.4	gw1.28.631.1	
	PoptrARF16.5	gw1.28.632.1	

Species	Gene name	Gene ID number	Publication and/or database
	PoptrARF16.6	grail3.0050008401	
	PoptrARF17.1	eugene3.00020832	
	PoptrARF17.2	gw1.V.5081.1	
Apple	MdARF1	MDP0000194603, ADL36575	Devoghalaere *et al*., 2012, Genome database for Rosaceae www.rosaceae.org, GenBank sequences
	MdARF101	Not annotated	
	MdARF2	MDP0000232417, ADL36576	
	MdARF102	MDP0000268306	
	MdARF3	MDP0000179650, ADL36577	
	MdARF103	MDP0000173151	
	MdARF4	MDP0000134824	
	MdARF104	MDP0000185253	
	MdARF5	MDP0000886637	
	MdARF105	MDP0000876321	
	MdARF6	MDP0000256621	
	MdARF106	MDP0000232116	
	MdARF7	MDP0000221322	
	MdARF107	MDP0000274442	
	MdARF8	MDP0000310875	
	MdARF108	MDP0000258032	
	MdARF9	MDP0000153538	
	MdARF109	MDP0000319906	
	MdARF10	MDP0000190950	
	MdARF110	MDP0000156207	
	MdARF210	MDP0000319072	
	MdARF11	MDP0000139073	
	MdARF111	MDP0000259062	
	MdARF12	MDP0000138860	
	MdARF112	MDP0000123466	
	MdARF212	MDP0000138853	
	MdARF13	MDP0000412781	
	MdARF113	MDP0000225980	
	MdARF14	MDP0000929655	
	MdARF15	MDP0000211459	

Species	Gene name	Gene ID number	Publication and/or database
	MdARF115	MDP0000143749	
	MdARF16	MDP0000167246, ACI13681	
	MdARF116	MDP0000750392	
	MdARF216	MDP0000291384	
	MdARF17	MDP0000550049	
	MdARF117	MDP0000294251	
Tomato	SlARF1	Solyc01g103050.2	Zouine *et al*., 2014
	SlARF2A	Solyc03g118290.2	
	SlARF2B	Solyc12g042070.2	
	SlARF3	Solyc02g077560.2	
	SlARF4	Solyc11g069190.2	
	SlARF5	Solyc04g081240.2	
	SlARF6A	Solyc12g006340	
	SlARF6B	Solyc07g043620.3	
	SlARF7A	Solyc07g016180.2	
	SlARF7B	Solyc05g047460.3	
	SlARF8A	Solyc03g031970.3	
	SlARF8B	Solyc02g037530.3	
	SlARF9A	Solyc08g082630.3	
	SlARF9B	Solyc08g008380.3	
	SlARF10A	Solyc11g069500.2	
	SlARF10B	Solyc06g075150.3	
	SlARF16A	Solyc09g007810.3	
	SlARF16B	Solyc10g086130.1	
	SlARF17	Solyc11g013470.2	
	SlARF18	Solyc01g096070.3	
	SlARF19	Solyc07g042260.2	
	SlARF24	Solyc05g056040.2	

ID – identification
Names in brackets - Names used in this work

	VviAFB11	VviAFB6	VviAFB10	VviAFB9	VviAFB7	VviAFB8
VviAFB11		54.202%	50.586%	55.172%	53.598%	55.843%
VviAFB6	54.202%		53.187%	59.001%	58.348%	59.279%
VviAFB10	50.586%	53.187%		67.237%	55.271%	55.328%
VviAFB9	55.172%	59.001%	67.237%		62.369%	64.402%
VviAFB7	53.598%	58.348%	55.271%	62.369%		73.611%
VviAFB8	55.843%	59.279%	55.328%	64.402%	73.611%	

Figure D.7 The similarity matrix of the VviAFB coding sequences produced by a Geneious translational alignment.

	VviARF17	VviARF16	VviARF31	VviARF32	VviARF3	VviARF4	VviARF5	VviARF30	VviARF8	VviARF29	VviARF27	VviARF28	VviARF26	VviARF2a	VviARF2b	VviARF1a	VviARF1b	VviARF24	VviARF25
VviARF17		38.648%	40.806%	37.970%	30.669%	29.127%	25.391%	23.454%	32.155%	23.257%	25.882%	22.058%	30.081%	26.675%	27.941%	32.774%	32.255%	31.176%	30.690%
VviARF16	38.648%		65.084%	61.119%	32.554%	33.033%	29.970%	27.042%	34.364%	29.185%	31.109%	26.139%	37.063%	31.444%	33.733%	39.462%	39.508%	36.499%	37.209%
VviARF31	40.806%	65.084%		63.832%	32.899%	33.113%	29.478%	27.602%	35.096%	28.197%	30.324%	25.940%	37.384%	32.353%	34.294%	37.798%	37.176%	38.824%	37.566%
VviARF32	37.970%	61.119%	63.832%		30.442%	32.653%	29.531%	27.437%	31.463%	28.491%	30.344%	27.273%	34.047%	32.253%	34.146%	34.916%	35.066%	34.368%	33.740%
VviARF3	30.669%	32.554%	32.899%	30.442%		44.144%	32.114%	30.511%	32.593%	30.843%	32.304%	27.273%	39.299%	36.393%	38.089%	37.875%	35.064%	36.966%	38.196%
VviARF4	29.127%	33.033%	33.113%	32.653%	44.144%		35.437%	35.367%	32.405%	35.359%	37.523%	30.663%	42.345%	41.163%	43.667%	40.686%	39.138%	40.226%	41.601%
VviARF5	25.391%	29.970%	29.478%	29.531%	32.114%	35.437%		43.290%	33.297%	45.698%	48.415%	41.040%	34.575%	39.083%	38.301%	34.505%	33.994%	33.444%	34.624%
VviARF30	23.454%	27.042%	27.602%	27.437%	30.511%	35.367%	43.290%		35.969%	59.209%	46.414%	41.840%	33.835%	39.056%	36.562%	32.652%	32.849%	34.232%	34.973%
VviARF8	32.155%	34.364%	35.096%	31.463%	32.593%	32.405%	33.297%	35.969%		38.075%	35.787%	29.644%	35.603%	31.087%	34.114%	38.095%	41.297%	37.429%	37.331%
VviARF29	23.257%	29.185%	28.197%	28.491%	30.843%	35.359%	45.698%	59.209%	38.075%		46.911%	42.955%	34.520%	37.927%	37.112%	33.158%	33.157%	34.615%	34.452%
VviARF27	25.882%	31.109%	30.324%	30.344%	32.304%	37.523%	48.415%	46.414%	35.787%	46.911%		47.051%	35.568%	41.463%	39.733%	35.970%	35.525%	37.424%	37.403%
VviARF28	22.058%	26.139%	25.940%	27.273%	27.273%	30.663%	41.040%	41.840%	29.644%	42.955%	47.051%		30.165%	34.093%	34.276%	30.385%	29.472%	29.548%	30.241%
VviARF26	30.081%	37.063%	37.384%	34.047%	39.299%	42.345%	34.575%	33.835%	35.603%	34.520%	35.568%	30.165%		46.512%	48.880%	47.040%	45.967%	45.337%	47.028%
VviARF2a	26.675%	31.444%	32.353%	32.253%	36.393%	41.163%	39.083%	39.056%	31.087%	37.927%	41.463%	34.093%	46.512%		61.616%	43.614%	44.615%	44.497%	47.842%
VviARF2b	27.941%	33.733%	34.294%	34.146%	38.089%	43.667%	38.301%	36.562%	34.114%	37.112%	39.733%	34.276%	48.880%	61.616%		46.538%	46.131%	46.299%	48.789%
VviARF1a	32.774%	39.462%	37.798%	34.916%	37.875%	40.686%	34.505%	32.652%	38.095%	33.158%	35.970%	30.385%	47.040%	43.614%	46.538%		68.263%	50.605%	50.925%
VviARF1b	32.255%	39.508%	37.176%	35.066%	35.064%	39.138%	33.994%	32.849%	41.297%	33.157%	35.525%	29.472%	45.967%	44.615%	46.131%	68.263%		50.798%	51.315%
VviARF24	31.176%	36.499%	38.824%	34.368%	36.966%	40.226%	33.444%	34.232%	37.429%	34.615%	37.424%	29.548%	45.337%	44.497%	46.299%	50.605%	50.798%		69.927%
VviARF25	30.690%	37.209%	37.566%	33.740%	38.196%	41.601%	34.624%	34.973%	37.331%	34.452%	37.403%	30.241%	47.028%	47.842%	48.789%	50.925%	51.315%	69.927%	

Figure D.8 The similarity matrix of the VviARF coding sequences produced by a Geneious translational alignment.

	VviIAA33	VviIAA44	VviIAA34a	VviIAA34b	VviIAA26	VviIAA43	VviIAA31	VviIAA45	VviIAA11	VviIAA13	VviIAA42	VviIAA19	VviIAA15a	VviIAA15b	VviIAA40	VviIAA38	VviIAA39	VviIAA9	VviIAA36	VviIAA37	VviIAA35	VviIAA27	VviIAA41
VviIAA33		25.490%	23.519%	20.148%	20.833%	23.027%	30.254%	30.939%	25.535%	21.290%	18.833%	24.046%	28.019%	30.782%	26.036%	27.124%	26.736%	19.359%	25.074%	25.298%	28.715%	21.244%	20.358%
VviIAA44	25.490%		30.363%	28.696%	25.463%	29.464%	31.571%	32.343%	34.214%	28.810%	25.243%	29.412%	29.678%	32.873%	30.108%	33.028%	30.781%	26.011%	31.397%	34.034%	35.970%	27.365%	25.402%
VviIAA34a	23.519%	30.363%		40.296%	26.594%	30.244%	29.346%	33.668%	37.139%	29.319%	26.307%	34.506%	27.581%	28.636%	33.493%	34.123%	35.885%	26.159%	31.646%	31.339%	35.686%	25.256%	23.810%
VviIAA34b	20.148%	28.696%	40.296%		32.172%	26.971%	29.972%	27.704%	33.196%	30.869%	28.592%	33.183%	33.605%	32.787%	34.061%	34.493%	36.245%	29.666%	33.066%	33.592%	34.444%	30.263%	28.692%
VviIAA26	20.833%	25.463%	26.594%	32.172%		47.489%	25.676%	23.981%	31.236%	32.351%	24.422%	29.282%	29.942%	29.296%	30.081%	30.197%	29.964%	33.592%	35.480%	32.712%	39.583%	34.038%	31.781%
VviIAA43	23.027%	29.464%	30.244%	26.971%	47.489%		31.912%	30.303%	35.408%	30.527%	26.453%	29.787%	30.488%	32.222%	31.878%	30.603%	33.029%	26.582%	35.053%	32.396%	43.403%	27.904%	27.122%
VviIAA31	30.254%	31.571%	29.346%	29.972%	25.676%	31.912%		56.437%	36.377%	27.098%	27.982%	36.224%	32.621%	32.885%	38.409%	35.655%	38.579%	27.616%	32.751%	30.476%	41.667%	27.491%	26.793%
VviIAA45	30.939%	32.343%	33.668%	27.704%	23.981%	30.303%	56.437%		36.589%	27.961%	25.628%	35.965%	32.543%	33.821%	41.093%	38.632%	40.035%	26.644%	33.036%	32.735%	40.295%	27.305%	27.140%
VviIAA11	25.535%	34.214%	37.139%	33.196%	31.236%	35.408%	36.377%	36.589%		53.172%	28.287%	37.412%	36.450%	37.037%	40.789%	37.681%	39.545%	33.439%	39.394%	38.400%	43.915%	33.225%	32.919%
VviIAA13	21.290%	28.810%	29.319%	30.869%	32.351%	30.527%	27.098%	27.961%	53.172%		22.595%	29.770%	30.686%	31.028%	31.690%	30.643%	32.126%	32.580%	38.111%	36.181%	38.288%	32.655%	33.245%
VviIAA42	18.833%	25.243%	26.307%	28.592%	24.422%	26.453%	27.982%	25.628%	28.287%	22.595%		29.744%	29.063%	28.531%	34.983%	33.990%	34.323%	28.004%	34.061%	32.411%	37.708%	38.851%	32.308%
VviIAA19	24.046%	29.412%	34.506%	33.183%	29.282%	29.787%	36.224%	35.965%	37.412%	29.770%	29.744%		41.555%	39.545%	48.731%	47.291%	49.559%	33.110%	42.437%	42.259%	46.552%	34.932%	33.007%
VviIAA15a	28.019%	29.678%	27.581%	33.605%	29.942%	30.488%	32.621%	32.543%	36.450%	30.686%	29.063%	41.555%		54.962%	46.942%	45.814%	44.603%	34.652%	46.585%	44.689%	51.601%	37.879%	35.362%
VviIAA15b	30.782%	32.873%	28.636%	32.787%	29.296%	32.222%	32.885%	33.821%	37.037%	31.028%	28.531%	39.545%	54.962%		43.925%	44.651%	44.175%	33.611%	46.228%	43.990%	54.167%	36.265%	35.169%
VviIAA40	26.036%	30.108%	33.493%	34.061%	30.081%	31.878%	38.409%	41.093%	40.789%	31.690%	34.983%	48.731%	46.942%	43.925%		70.333%	74.510%	38.496%	46.552%	46.866%	52.965%	39.425%	36.075%
VviIAA38	27.124%	33.028%	34.123%	34.493%	30.197%	30.603%	35.655%	38.632%	37.681%	30.643%	33.990%	47.291%	45.814%	44.651%	70.333%		74.132%	35.690%	45.390%	45.570%	52.929%	38.851%	37.171%
VviIAA39	26.736%	30.781%	35.885%	36.245%	29.964%	33.029%	38.579%	40.035%	39.545%	32.126%	34.323%	49.559%	44.603%	44.175%	74.510%	74.132%		35.294%	45.536%	45.575%	54.409%	35.579%	35.248%
VviIAA9	19.359%	26.011%	26.159%	29.666%	33.592%	26.582%	27.616%	26.644%	33.439%	32.580%	28.004%	33.110%	34.652%	33.611%	38.496%	35.690%	35.294%		45.027%	42.242%	52.913%	48.202%	48.732%
VviIAA36	25.074%	31.397%	31.646%	33.066%	35.480%	35.053%	32.751%	33.036%	39.394%	38.111%	34.061%	42.437%	46.585%	46.228%	46.552%	45.390%	45.536%	45.027%		68.333%	80.312%	46.756%	45.192%
VviIAA37	25.298%	34.034%	31.339%	33.592%	32.712%	32.396%	30.476%	32.735%	38.400%	36.181%	32.411%	42.259%	44.689%	43.990%	46.866%	45.570%	45.575%	42.242%	68.333%		78.101%	44.408%	43.953%
VviIAA35	28.715%	35.970%	35.686%	34.444%	39.583%	43.403%	41.667%	40.295%	43.915%	38.288%	37.708%	46.552%	51.601%	54.167%	52.965%	52.929%	54.409%	52.913%	80.312%	78.101%		59.058%	60.526%
VviIAA27	21.244%	27.365%	25.256%	30.263%	34.038%	27.904%	27.491%	27.305%	33.225%	32.655%	38.851%	34.932%	37.879%	36.265%	39.425%	38.851%	35.579%	48.202%	46.756%	44.408%	59.058%		61.755%
VviIAA41	20.358%	25.402%	23.810%	28.692%	31.781%	27.122%	26.793%	27.140%	32.919%	33.245%	32.308%	33.007%	35.362%	35.169%	36.075%	37.171%	35.248%	48.732%	45.192%	43.953%	60.526%	61.755%	

Figure D.9 The similarity matrix of the VviIAA coding sequences produced by a Geneious translational alignment.

Appendix E Expression analysis

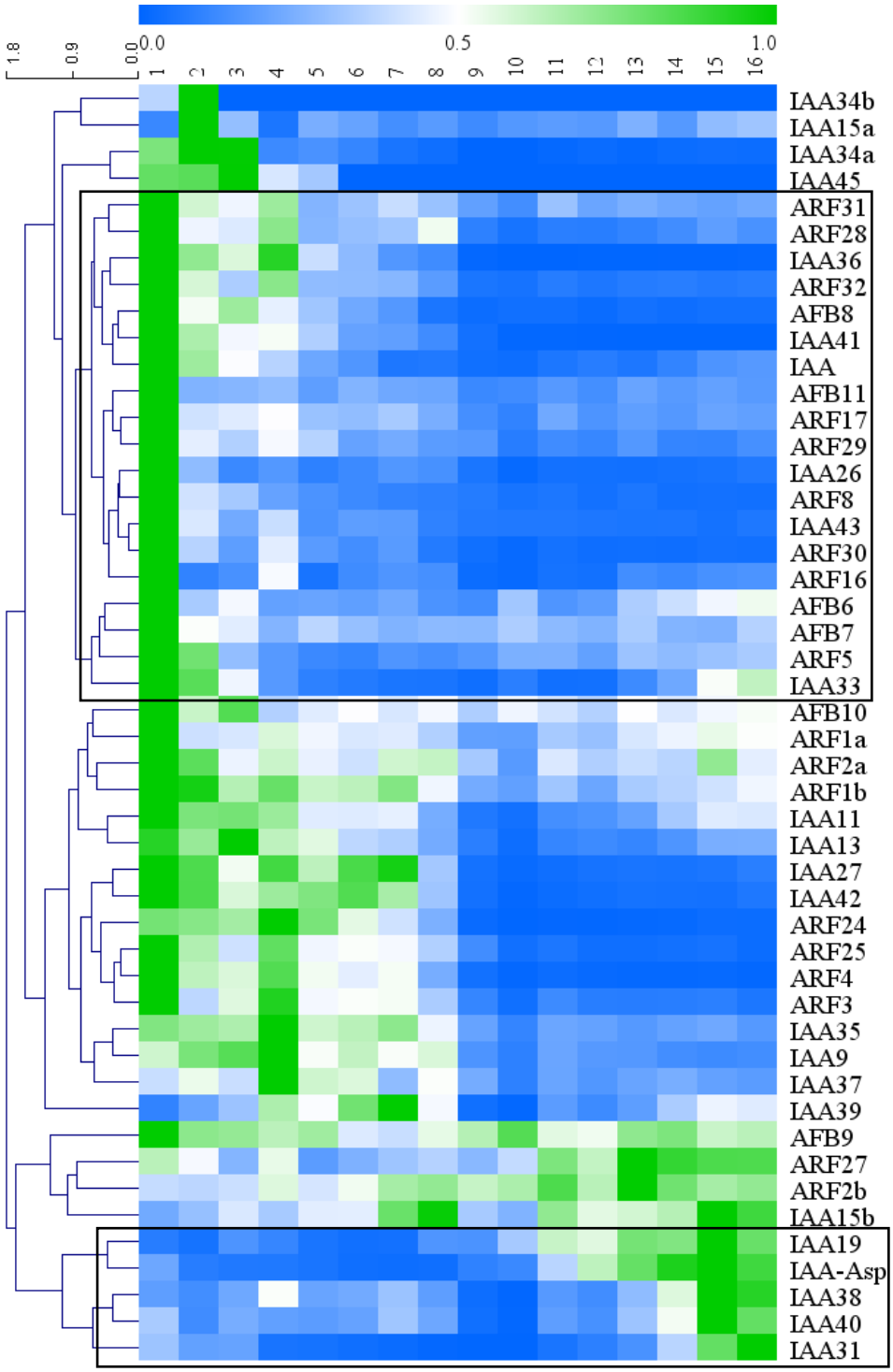

Figure E.1 Hierarchical clustering tree and heatmap of all VviAFB, VviARF, and VviIAA transcript profiles and IAA and IAA-Asp concentrations normalised between zero and one in *V. vinifera* L. cv. Shiraz berries across sixteen weeks post flowering.

The hierarchical clustering tree is shown on the left generated using MultiExperiment Viewer (Saeed *et al.*, 2003), using Gene tree selection for tree selection, optimise by gene leaf order for ordering optimisation, Euclidean distance was used as the distance metric selection, and average linkage clustering was used as the linkage method selection. The values above the tree indicate the distance between transcriptional profiles, computed as distance linkage. Clusters are determined as those that branch below a linkage distance of ~1, as indicated by the red

dashed line and are labelled on the far right. The colour scale labelled 0.0 to 1.0 represents the normalised transcript values, with blue indicating low levels and green high levels of relative expression.

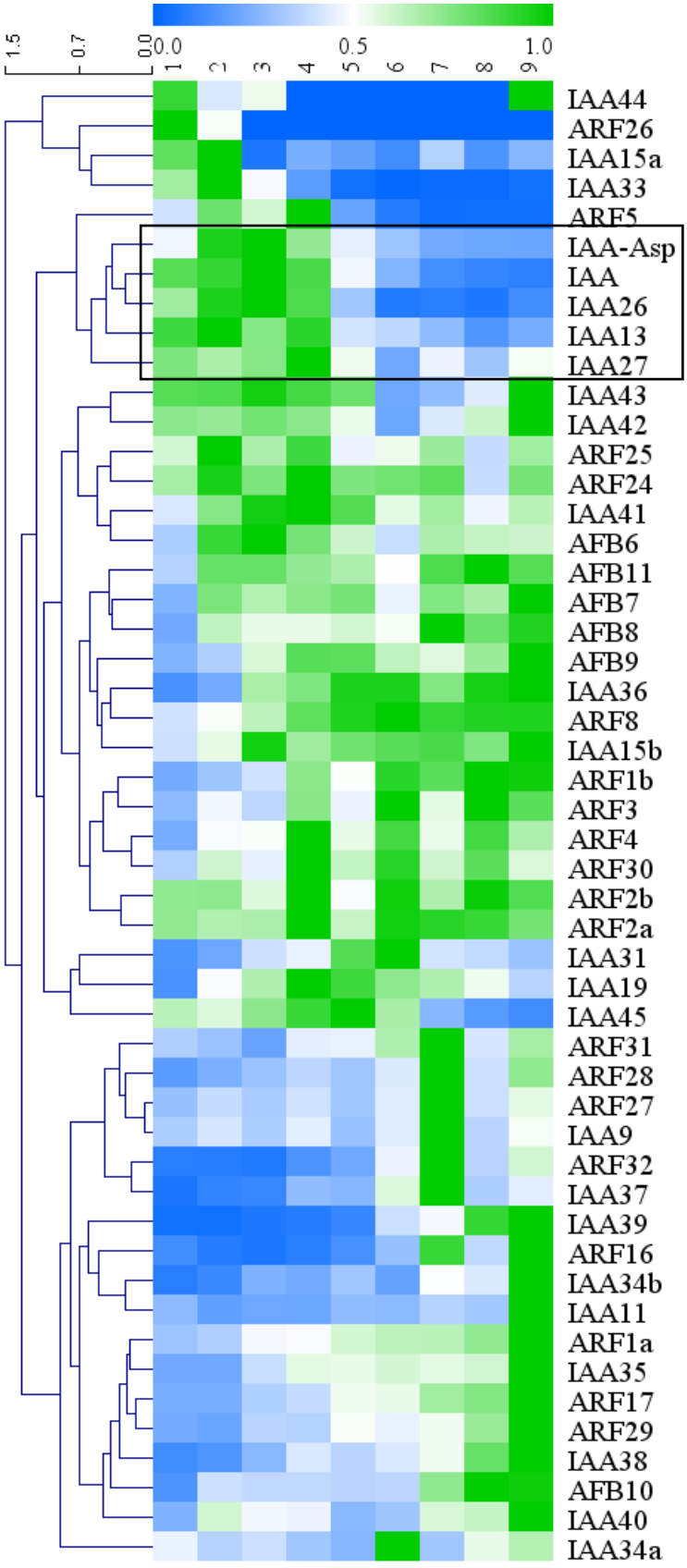

Figure E.2 Hierarchical clustering tree and heatmap of all VviAFB, VviARF, and VviIAA transcript profiles and IAA and IAA-Asp concentrations normalised between zero and one in V. vinifera L. cv. Shiraz leaves across nine leaf stages.

The hierarchical clustering tree is shown on the left generated using MultiExperiment Viewer (Saeed *et al*., 2003), using Gene tree selection for tree selection, optimise by gene leaf order for ordering optimisation, Euclidean distance was used as the distance metric selection, and average linkage clustering was used as the linkage method selection. The values above the tree indicate the distance between transcriptional profiles, computed as distance linkage. Clusters are determined as those that branch below a linkage distance of ~0.8, as indicated by the red dashed line and are labelled on the far right. The colour scale labelled 0.0 to 1.0 represents the normalised transcript values, with blue indicating low levels and green high levels of relative expression.

Appendix F Interaction analysis

Shown here are a selection of images of the VviARF and VviIAA negative controls.

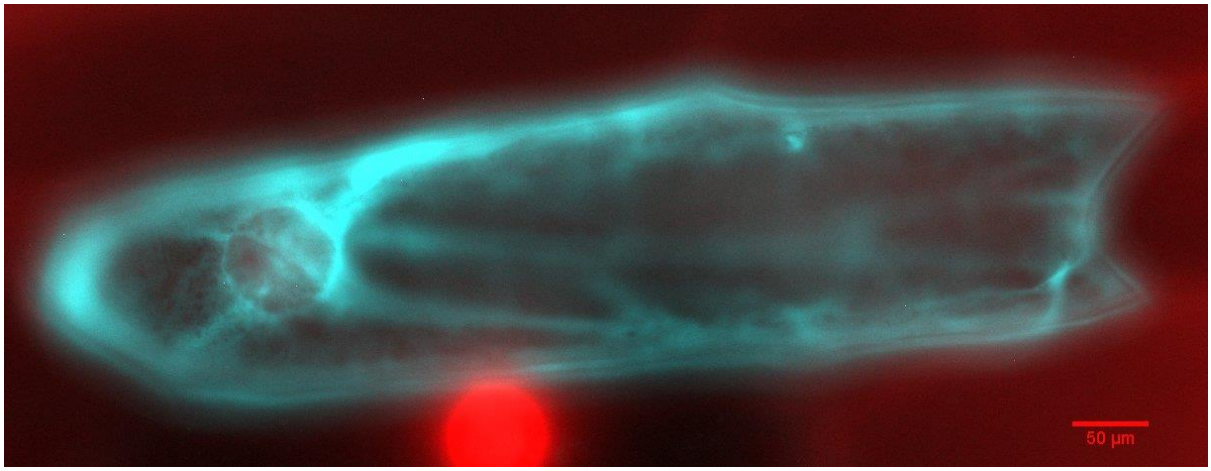

Figure F.1 A representation of the fluorescence profiles seen in VviARF27ΔPB1-cYFP + VviIAA19-YFPn bombarded onion cells. An overlay of DAPI, CFP and YFP channels. The PB1 domain has been removed from the VviARF protein.

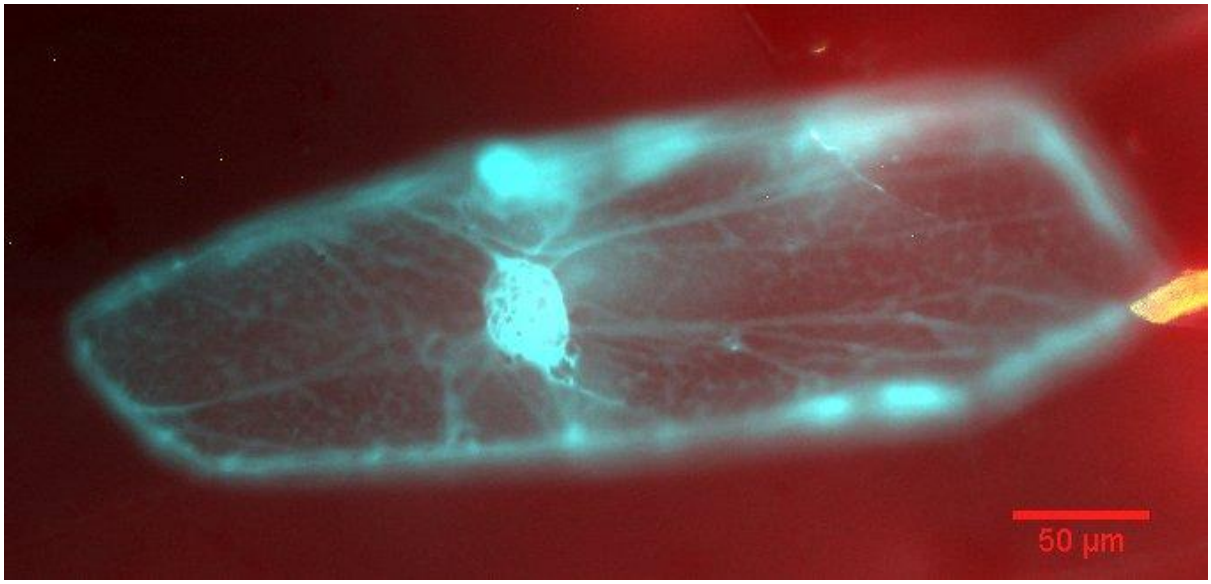

Figure F.2 A representation of the fluorescence profiles seen in VviARF4ΔPB1-YFPn + VviIAA19-YFPc bombarded onion cells. An overlay of DAPI, CFP and YFP channels.

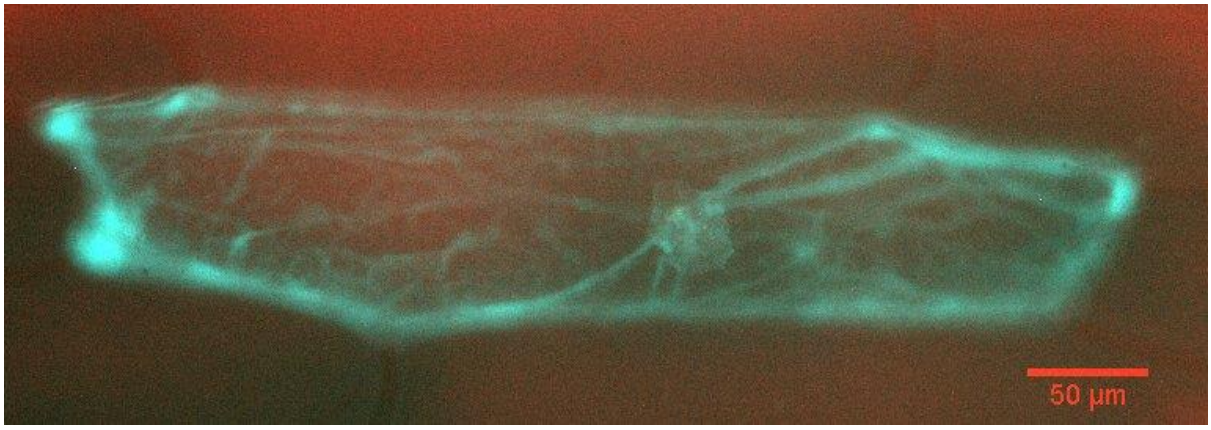

Figure F.3 A representation of the fluorescence profiles seen in VviARF27-YFPc + pSITE-nYFP bombarded onion cells. An overlay of DAPI, CFP and YFP channels.

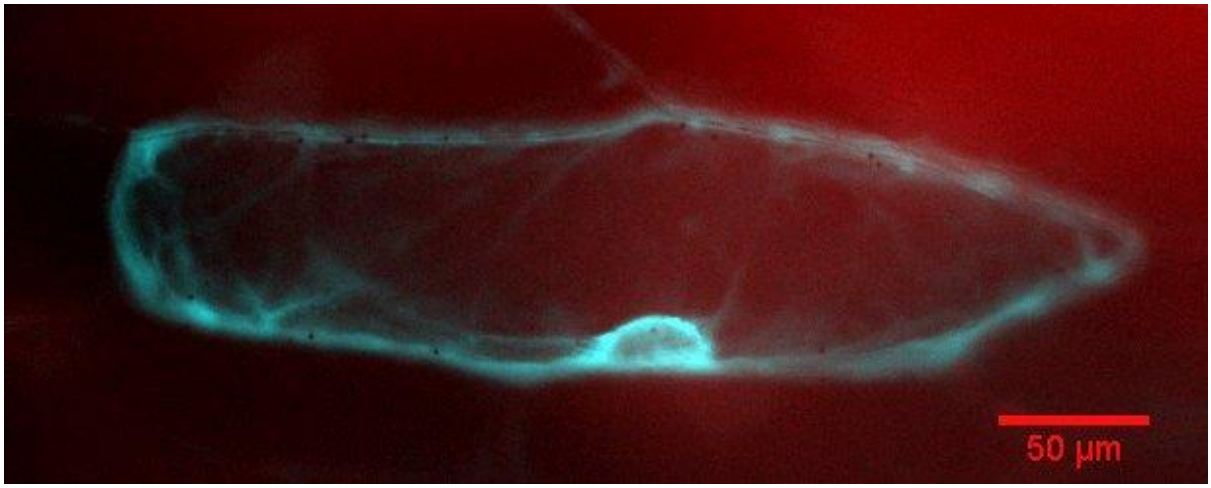

Figure F.4 A representation of the fluorescence profiles seen in VviARF27-YFPc + pSITE-YFPn bombarded onion cells. An overlay of DAPI, CFP and YFP channels.

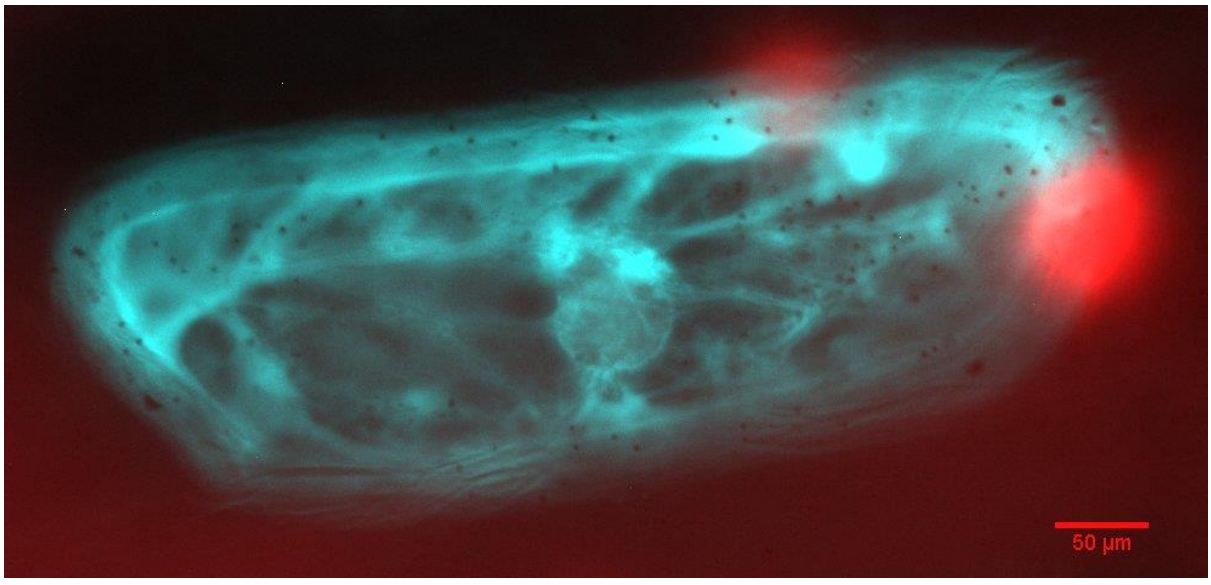

Figure F.5 A representation of the fluorescence profiles seen in VviIAA19-nYFP + pSITE-cYFP bombarded onion cells. An overlay of DAPI, CFP and YFP channels.

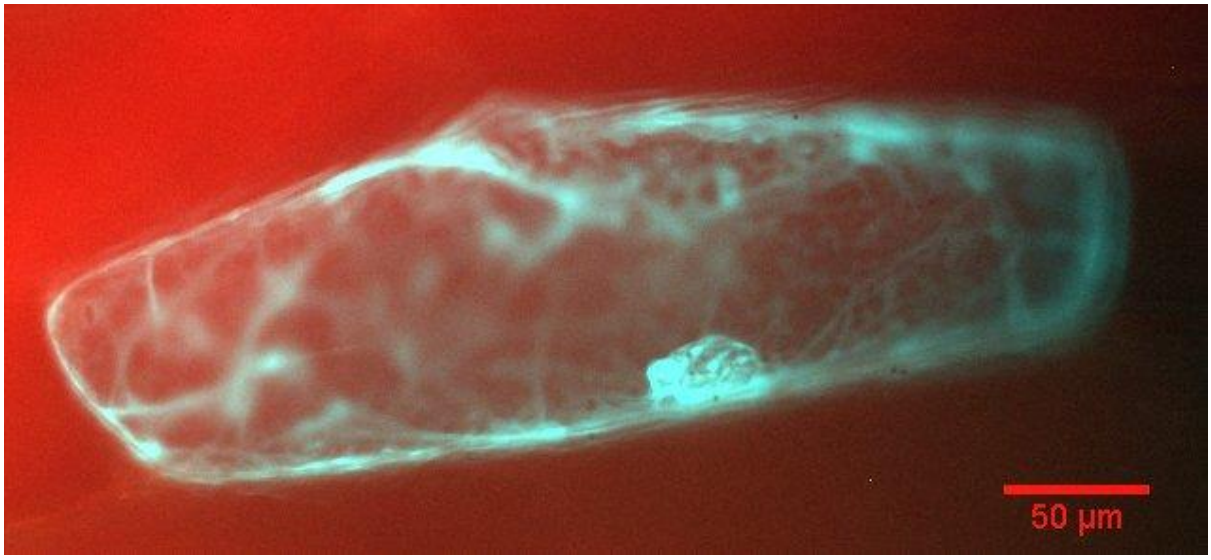

Figure F.6 A representation of the fluorescence profiles seen in VviIAA19-YFPc + pSITE-YFPn bombarded onion cells. An overlay of DAPI, CFP and YFP channels.

Appendix G Ex-planta berry treatments

Table G.1 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with 1-Naphthaleneacetic acid (NAA) pre-veraison. Significance P = 0.01 >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	NAA		
	3 h	24 h	48 h
VviIAA19	2.7		
VviIAA36		2.8	
VviIAA38	1.5		
VviIAA39	3.4	2.3	
VviAFB8	-1.9		-2.1

Table G.2 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with 1-Naphthaleneacetic acid (NAA) post-veraison. Significance P = 0.01 >1.5 fold change. Green boxes represent up-regulation.

Gene	NAA		
	3 h	24 h	48 h
VviARF4			1.7
VviIAA15a			1.5
VviIAA15b			2.7
VviIAA38			3.8
VviIAA40			4.3

Table G.3 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with abscisic acid (ABA) pre-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	ABA		
	3 h	24 h	48 h
VviARF3		-1.5	
VviARF4			-1.6
VviARF30			-1.5
VviIAA11			-1.5
VviIAA36			-1.7
VviIAA39		-1.6	
VviIAA41			-1.5
VviAFB7		1.7	

Table G.4 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with abscisic acid (ABA) post-veraison. Significance P = 0.01, >1.5 fold change. The blue box represent down-regulation.

Gene	ABA		
	3 h	24 h	48 h
VviARF25			-1.8

Table G.5 The VviARF transcripts that have significant changes in expression after treatment with cytokinin (iP) pre-veraison. Significance P = 0.01, >1.5 fold change. The blue box represents down-regulation.

Gene	iP		
	3 h	24 h	48 h
VviARF28	-1.7		

Table G.6 The VviARF transcripts that have significant changes in expression after treatment with cytokinin (iP) post-veraison. Significance P = 0.01, >1.5 fold change. The blue boxes represent down-regulation.

Gene	iP		
	3 h	24 h	48 h
VviARF3		-1.6	
VviARF5		-1.6	

Table G.7 The VviARF and VviIAA transcripts that have significant changes in expression after treatment with epi-brassinolide pre-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	BL		
	3 h	24 h	48 h
VviARF3	1.6		
VviARF28	-1.9		
VviIAA13	1.6		
VviIAA19	-1.5		
VviIAA38			-1.6
VviIAA39		-2.3	

Table G.8 The VviARF and VviIAA transcripts that have significant changes in expression after treatment with epi-brassinolide post-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	BL		
	3 h	24 h	48 h
VviARF4			-1.6
VviARF5		-1.5	
VviARF24		-1.8	1.6

Table G.9 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with Ethrel pre-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	Eth		
	3 h	24 h	48 h
VviARF4			-2.2
VviARF24			-1.7
VviARF25	1.8		
VviIAA15a	3.1		
VviIAA15b		-2.1	-2.4
VviIAA19		-1.7	
VviIAA35			-1.8
VviAFB6	-1.6		
VviAFB9	-1.9		
VviAFB11	-1.7		

Table G.10 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after treatment with Ethrel post-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	Eth		
	3 h	24 h	48 h
VviARF2b	-1.5		
VviARF3		-1.7	-1.7
VviARF8		-1.5	-2.6
VviARF16		-1.9	
VviARF17		-1.5	-1.7
VviARF24		-2.2	
VviARF25			-1.8
VviARF28		-2.5	

Gene	Eth		
	3 h	24 h	48 h
VviIAA9			-2.1
VviIAA11		-1.8	
VviIAA13		-2.4	
VviIAA15b		-1.8	
VviIAA19			-2.2
VviIAA27		-9.8	
VviIAA31		-7.7	
VviIAA35		-1.8	-2.2
VviIAA38		-1.9	
VviIAA39		-2.3	
VviIAA40		-1.9	
VviIAA42			-5.3
VviAFB8		1.7	

Table G.11 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after the ex-planta treatment with no sugar present in the media pre-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	-Sugar		
	3 h	24 h	48 h
VviARF2b		-1.6	-1.5
VviARF4		-2.5	-2.3
VviARF8		-1.5	-1.7
VviARF16	1.8		
VviARF24	1.8		-2.1
VviARF25			-1.6
VviARF28	-2.2		
VviARF30			-1.9
VviARF32		-1.6	
VviIAA15a		-1.9	
VviIAA15b			-2.5
VviIAA19		-2.1	-2.7
VviIAA27		-1.5	
VviIAA36		2.2	1.9
VviIAA37		-2.0	

Gene	-Sugar		
	3 h	**24 h**	**48 h**
VviIAA38			-1.6
VviAFB9	-1.8		

Table G.12 The VviARF, VviIAA and VviAFB transcripts that have significant changes in expression after the ex-planta treatment with no sugar present in the media post-veraison. Significance P = 0.01, >1.5 fold change. Green boxes represent up-regulation, blue boxes represent down-regulation.

Gene	-Sugar		
	3 h	**24 h**	**48 h**
VviARF2b	-1.7		
VviARF25			-1.6
VviIAA15a	1.7		
VviIAA33			1.6

Appendix H Promoter analysis

Table H.1 PlantPAN results for the 2 kb VviARF promoter fragments.

Includes the individual motifs, the totals that were used in Table 6.1, and the proposed function of the PlantPAN motifs.

		VviARF Gene Name																			
Type of motif	**Motif name**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Function from PlantPAN**
Auxin	ARF	1	2	1	-	3	3	1	1	-	3	-	1	1	-	1	1	1	1	2	AuxRE, TGTCTC
	ARFAT	3	6	3	-	9	9	3	3	-	9	-	3	3	-	3	3	3	3	6	ARF binding site found in the promoters of primary/early auxin response genes of Arabidopsis
	ASF1MOTIFCAMV	-	-	-	-	-	-	-	-	-	-	10	-	-	10	-	-	-	-	-	TGACG motifs are involved in transcriptional activation of several genes by auxin and/or salicylic acid
	AUXREPSIAA4	-	-	1	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	AuxRE (Auxin responsive element) of pea PS-IAA4/5 gene; Indoleacetic acid-inducible genes
	AUXRETGA2GMGH3	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	-	TGA-box #2 in putative auxin-responsive element (AuxRE) E1 of soybean GH3 promoter
	CACGCAATGMGH3	-	-	-	-	-	-	-	1	-	-	-	-	-	1	-	-	-	-	-	Sequence found in D4 element in Soybean GH3 gene promoter; Confers auxin inducibility
	CATATGGMSAUR	-	2	-	-	2	-	-	2	-	-	-	-	-	-	-	-	-	2	2	Sequence found in NDE element in soybean SAUR (Small Auxin-Up RNA) 15A gene promoter; Involved in auxin responsiveness
	GGTCCCATGMSAUR	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	Sequence found in NDE element in Soybean SAUR (Small Auxin-Up RNA) 15A gene promoter; Involved in auxin responsiveness
	SGBFGMGMAUX28	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	bZIP proteins SGBF-1 and SGBF-2 binding site in soybean GmAux28 gene promoter
	Total	4	10	5	1	14	12	6	7	0	12	11	4	5	12	4	4	4	6	10	
ABA	ABFs	-	-	-	-	-	-	2	-	-	-	1	1	1	-	3	-	-	-	-	A plant leucine zipper protein that recognizes an abscisic acid response element
	ABI4	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	AP2 family - Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes

		VviARF Gene Name																			
Type of motif	**Motif name**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Function from PlantPAN**
	ABREATCONSENSUS	-	-	-	-	-	-	2	-	-	-	1	1	1	-	3	-	-	-	-	ABA-responsive elements found in the promoter of ABA and/or stress-regulated genes, ABFs, a family of ABRE binding factors, ABF3 and ABF4 function in ABA signaling
	ABREAZMRAB28	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	ABA-responsive element found at -148 to -139 in maize rab28, ABA-inducible in embryos and vegetative tissues
	ABREBNNAPA	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	ABRE of napA storage-protein gene of Brassica napus, ABA responsive element
	ABREOSRGA1	-	-	-	-	-	-	-	-	-	-	2	6	-	-	2	-	-	-	-	ABRE (ABA responsive element) in rice RGA1 encoding a G protein alpha subunit;
	ABRETAEM	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	ABRE (ABA responsive element) found in wheat Em gene
	ABREZMRAB28	-	-	-	-	-	-	-	-	-	-	6	18	-	-	6	-	-	-	-	ABRE, ABA and water-stress responses found in maize rab28, ABA-inducible in embryos and vegetative tissues, responsible for the induction by ABA
	ACGTABREMOTIFA2OSEM	-	-	4	2	-	2	8	-	-	-	4	2	6	2	6	-	-	-	-	Experimentally determined sequence requirement of ACGT-core of motif A in ABRE of the rice gene, OSEM, DRE and ABRE are interdependent in the ABA-responsive expression of the rd29A in Arabidopsis
	AtMYB2	-	-	1	-	1	-	-	-	-	-	-	1	-	-	-	-	1	-	-	Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression
	CBF2	-	-	-	-	-	-	-	-	-	-	2	6	-	-	2	-	-	-	-	The cis-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene
	EMBP1TAEM	-	-	-	-	-	-	-	-	-	-	2	2	2	-	4	-	-	-	-	Binding site of trans-acting factor EMBP-1, wheat Em gene, binding site of ABFs (ABRE binding factors), expression ABFs is induced by ABA and various stress treatment, involved in ABA-mediated stress-signaling pathway;
	PROXBBNNAPA	-	-	-	1	1	-	-	-	-	-	-	1	-	1	1	-	2	-	1	Prox B (proximal portion of B-box) found in napA gene of Brassica napus, CA-rich sequence, required for seed specific expression and ABA responsiveness

		VviARF Gene Name																			
Type of motif	**Motif name**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Function from PlantPAN**
	RYREPEAT BNNAPA	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Dist B ABRE mediated transactivation by ABI3 and ABI3-dependent response to ABA
	SBOXATRBCS	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S-box conserved in several rbcS promoters in Arabidopsis, ABI4 binding site, Important for the sugar and ABA responsiveness of CMA5
	Total	1	0	11	3	2	10	10	0	0	0	17	38	11	3	24	0	3	0	1	
Cytokinin	CPBCSPOR	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	The sequence critical for Cytokinin-enhanced Protein Binding in vitro, found in the promoter of the cucumber NADPH-protochlorophyllide reductase gene
	Total	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	
Ethylene	ERELEE4	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ERE - ethylene responsive element of tomato E4 and carnation GST1 genes, related to senescence, ERE motifs mediate ethylene-induced activation of the U3 promoter region
	GCCCORE	-	-	2	-	-	2	-	-	-	-	-	4	2	-	4	-	-	-	-	Core of GCC-box found in many pathogen-responsive genes, has been shown to function as ethylene-responsive element
	Total	5	0	2	0	0	2	0	0	0	0	0	4	2	0	4	0	0	0	0	
Sugar	ACGTABOX	-	2	2	-	2	-	2	-	-	-	2	-	2	-	-	-	-	-	-	A-box according to the nomenclature of ACGT elements, responsible for sugar repression
	SREATMSD	1	1	-	-	-	-	1	1	-	1	1	1	1	1	1	-	2	1	1	Sugar-repressive element (SRE) found in 272 of the 1592 down-regulated genes after main stem decapitation in Arabidopsis
	SURE1STPAT21	1	2	-	1	-	1	-	1	1	1	-	-	-	-	1	-	1	1	1	Sucrose Responsive Element (SURE), a motif conserved among genes regulated by sucrose
	TATCCAOSAMY	1	2	3	1	-	-	-	1	2	-	2	-	1	-	1	-	2	1		TATCCA element found in alpha-amylase promoters of rice, binding sites of OsMYBS1, OsMYBS2 and OsMYBS3 which mediate sugar and hormone regulation of alpha-amylase gene expression
	TATCCAYMOTIF OSRAMY3D	1	1	-	-	-	-	-	1	1	-	1	-	1	-	-	-	1	-	-	TATCCAY motif found in rice, RAmy3D alpha-amylase gene promoter, responsible for sugar repression

		VviARF Gene Name																			
Type of motif	**Motif name**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Function from PlantPAN**
	Total	4	8	5	2	2	1	3	4	4	2	6	1	5	1	3	0	6	3	2	
Stress	ABREATRD22	-	-	-	-	-	-	1	-	-	-	1	-	1	-	-	-	-	-	-	ABRE (ABA responsive element) in Arabidopsis dehydration-responsive gene rd22
	ABRELATERD1	-	-	-	-	-	-	11	-	-	-	12	-	-	-	-	-	-	-	-	ABRE-like sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis
	ABRE-like	-	-	-	-	-	-		-	-	-	6	-	-	-	-	-	-	-	-	Molecular responses to dehydration and low temperature
	CBFHV	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Binding site of barley CBF1 and CBF2, CBFs are also known as dehydration-responsive element (DRE) binding proteins (DREBs)
	DRE1COREZMRAB17	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	DRE1 core found in maize rab17 gene promoter, is induced by ABA
	DRE2COREZMRAB17	-	1	-	1	1	-	1	-	1	-	-	-	1	-	-	1	-	-	2	DRE2 core found in maize rab17 gene promoter, rab17 is expressed during late embryogenesis, and is induced by ABA
	LTRE1HVBLT49	-	-	-	2	1	-	1	-	-	2	-	2	-	1	1	3	-	-	3	LTRE-1 (low-temperature-responsive element) in barley blt4.9 gene promoter
	LTREATLTI78	-	2	-	2	-	-	-	-	2	-	-	-	2	-	-	2		-	2	Putative low temperature responsive element (LTRE), found in Arabidopsis low-temperature-induced genes
	LTRECOREATCOR15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	Core of low temperature responsive element (LTRE) of cor15a gene in Arabidopsis, light signaling mediated by phytochrome is necessary for cold- or drought- induced gene expression through the C/DRE in Arabidopsis
	Total	0	3	1	10	3	0	14	0	3	2	19	2	4	1	1	6	1	0	21	
Fruit development	CArG	-	3	1	-	-	1	-	3	1	-	1	2	1	1	-	3	-	3	1	Recognised by RIN MADS box TF - RIN can directly bind to the promoters of ethylene biosynthesis genes, genes involved in cell wall remodeling and carotenoid biosynthesis, and genes involved in the control of fruit maturation and pigment accumulation, CYWWWWWWRG

		VviARF Gene Name																			
Type of motif	**Motif name**	**1a**	**1b**	**2a**	**2b**	**3**	**4**	**5**	**8**	**16**	**17**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**Function from PlantPAN**
	Total	0	3	1	0	0	1	0	3	1	0	1	2	1	1	0	3	0	3	1	
Development	Dof1	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Diversity and similarity among recognition sequences of Dof transcription factors
	Dof2	-	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	Diversity and similarity among recognition sequences of Dof transcription factors
	Dof2	-	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	ZN-FINGER, DOF
	Dof3	-	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	Diversity and similarity among recognition sequences of Dof transcription factors
	Total	19	0	0	0	0	0	60	0	0	0	0	0	0	0	0	0	0	0	0	

Table H.2 PlantPAN results for the 2 kb VviIAA promoter fragments.

Includes the individual motifs, the totals that were used in Table 6.2, and the proposed function of the PlantPAN motifs.

		VviIAA Gene Name																							
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
Auxin	ARF	5	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	AuxRE, TGTCTC
	ARFAT	15	-	-	-	-	-	-	-	-	-	18	-	-	-	-	-	-	-	-	-	-	-	-	ARF binding site found in the promoters of primary/early auxin response genes of Arabidopsis
	ASF1MOTIF CAMV	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	TGACG motifs are involved in transcriptional activation of several genes by auxin and/or salicylic acid
	AUXREPSIAA4	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	AuxRE (Auxin responsive element) of pea (P.s.) PS-IAA4/5 gene; Indoleacetic acid-inducible genes
	AUXRETGA2GMGH3	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	TGA-box #2 in putative auxin-responsive element (AuxRE) E1 of soybean GH3 promoter
	CACGCAATGMGH3	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	Sequence found in D4 element in Soybean GH3 gene promoter; Confers auxin inducibility
	CATATGGMSAUR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	Sequence found in NDE element in soybean SAUR (Small Auxin-Up RNA) 15A

		VvIAA Gene Name																						
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									gene promoter; Involved in auxin responsiveness
	CCTCGTGTCT CGMGH3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	-	-	Sequence found in D1 element in Soybean GH3 gene promoter, showed constitutive activity with TGTCTC element (AuxRE) Confers auxin inducibility
	GGTCCCATG MSAUR	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Sequence found in NDE element in Soybean SAUR (Small Auxin-Up RNA) 15A gene promoter; Involved in auxin responsiveness
	SEBFCONSS TPR10A	2	3	1	1	-	-	-	2	1	1	4	3	-	-	2	1	2	1	1	-	1	-	1	Binding site of the potato silencing element binding factor gene found in promoter of pathogenesis-related gene, similar to the auxin response element
	SGBFGMG MAUX28	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	bZIP proteins SGBF-1 and SGBF-2 binding site in soybean GmAux28 gene promoter
	Total	17	5	3	1	0	0	0	2	1	9	22	3	0	1	2	2	2	1	1	0	2	0	8	
Abscisic acid	ABFs	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A plant leucine zipper protein that recognizes an abscisic

		VvIAA Gene Name																							
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									acid response element
	ABI4	7	10	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-	AP2 family - Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes
	ABREATCONSENSUS	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ABA-responsive elements found in the promoter of ABA and/or stress-regulated genes, ABFs, a family of ABRE binding factors, ABF3 and ABF4 function in ABA signaling
	ABREATRD22	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	ABA responsive element in Arabidopsis dehydration-responsive gene rd22
	ABREMOTIFAOSOSEM	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Motif A ABRE-like sequence found in rice Osem gene promoter, important for regulation by ABA
	ABREOSRAB21	-	-	-	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-	ABA responsive element (ABRE) of wheat Em and rice rab21 genes
	ABREOSRGA1	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ABRE (ABA responsive element) in rice RGA1 encoding a G protein alpha subunit;
	ABREZMRAB28	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ABRE, ABA and water-stress responses found in maize rab28,

		VvIAA Gene Name																						
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									ABA-inducible in embryos and vegetative tissues, responsible for the induction by ABA
	ACGTABREMOTIFA2OSEM	-	-	-	5	-	-	4	-	-	2	-	-	-	2	2	-	-	-	-	-	2	-	-	Experimentally determined sequence requirement of ACGT-core of motif A in ABRE of the rice gene, OSEM, DRE and ABRE are interdependent in the ABA-responsive expression of the rd29A in Arabidopsis
	CBF2	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	The cis-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene
	DRE2COREZMRAB17	1	-	-	2	-	1	2	2	1	-	1	1	1	-	-	-	-	1	2	1	-	-	-	DRE2 core found in maize rab17 gene promoter, induced by ABA
	EMBP1TAEM	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Binding site of trans-acting factor EMBP-1, wheat Em gene, binding site of ABFs (ABRE binding factors), expression ABFs is induced by ABA and various stress treatment, involved in ABA-mediated stress-signaling pathway;

		VvIAA Gene Name																							
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
	PROXBBNNAPA	-	1	-	-	-	-	2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	Prox B (proximal portion of B-box) found in napA gene of Brassica napus, CA-rich sequence, required for seed specific expression and ABA responsiveness
	RYREPEATBNNAPA	-	-	-	6	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Dist B ABRE mediated transactivation by ABI3 and ABI3-dependent response to ABA
	SBOXATRBCS	-	-	2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	S-box conserved in several rbcS promoters in Arabidopsis, ABI4 binding site, Important for the sugar and ABA responsiveness of CMA5
	Total	8	11	2	19	0	3	8	21	3	2	1	2	3	2	3	0	1	1	2	1	10	0	0	
Cytokinin	CPBCSPOR	-	-	-	4	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	The sequence critical for Cytokinin-enhanced Protein Binding in vitro, found in the promoter of the cucumber NADPH-protochlorophyllide reductase gene
	Total	0	0	0	4	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	
Ethylene	ERELEE4	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	ERE - ethylene responsive element of tomato E4 and carnation GST1 genes,

		VvIAA Gene Name																							
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									related to senescence, ERE motifs mediate ethylene-induced activation of the U3 promoter region
	GCCCORE	-	-	-	-	-	-	-	-	2	-	4	-	-	-	-	-	-	2	-	-	-	-	-	Core of GCC-box found in many pathogen-responsive genes, has been shown to function as ethylene-responsive element
	TEIL	-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog
	Total	0	0	0	0	0	0	0	0	2	4	4	0	0	0	0	0	0	2	0	0	0	0	0	
Sugar	ACGTABOX	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A-box according to the nomenclature of ACGT elements, responsible for sugar repression
	AGMOTIFNTMYB2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	AG-motif found at -114 of the promoter of NtMyb2 gene, which are induced by various stress such as wounding or elicitor treatment
	SREATMSD	-	-	-	-	-	-	-	3	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	Sugar-repressive element (SRE) found in 272 of the 1592 down-regulated genes after main stem

		VvIAA Gene Name																						
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									decapitation in Arabidopsis
	SURE1STPAT21	-	-	-	1	-	-	-	-	-	-	-	-	2	-	-	-	-	-	1	-	-	-	-	Sucrose Responsive Element (SURE), a motif conserved among genes regulated by sucrose
	TATCCAOSAMY	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	TATCCA element found in alpha-amylase promoters of rice, binding sites of OsMYBS1, OsMYBS2 and OsMYBS3 which mediate sugar and hormone regulation of alpha-amylase gene expression
	TATCCAYMOTIFOSRAMY3D	-	2	1	-	-	-	-	2	-	-	-	-	2	1	1	-	-	-	-	-	2	1	-	TATCCAY motif found in rice, RAmy3D alpha-amylase gene promoter, responsible for sugar repression
	Total	0	2	1	1	1	0	2	5	0	0	0	0	11	1	1	0	0	0	1	0	3	1	0	
Stress	ABRE-like	-	-	2	2	-	1	2	2	-	1	-	1	-	2	1	-	-	-	-	-	-	-	-	Molecular responses to dehydration and low temperature
	ABRELATERD1	-	-	-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ABRE-like sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis
	ACGTATERD1	-	-	-	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ACGT sequence required for etiolation-induced expression of erd1

		VviIAA Gene Name																							
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									(early responsive to dehydration) in Arabidopsis
	AtMYB2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression
	AtMYC2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	6	-	-	-	-	-	Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression
	CRTDREHV CBF2	-	-	-	2	-	-	-	-	2	-	-	4	-	-	-	-	2	-	-	-	-	-	-	Preferred sequence for AP2 transcriptional activator HvCBF2 of barley, DNA binding is regulated by temperature
	LTRE1HVBL T49	2	-	-	1	3	1	-	1	2	-	1	-	-	-	-	1	-	1	-	-	-	-	-	LTRE-1 (low-temperature-responsive element) in barley blt4.9 gene promoter
	LTREATLTI78	-	-	-	-	-	-	2	2	2	-	-	2	2	-	-	-	-	2	4	-	-	-	-	Putative low temperature responsive element (LTRE), found in Arabidopsis low-temperature-induced genes
	LTRECOREAT COR15	-	-	-	-	-	-	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-	-	-	Core of low temperature responsive element of

		VvilAA Gene Name																						
Type of motif	**Motif name**	**9**	**11**	**13**	**15a**	**15b**	**19**	**26**	**27**	**31**	**33**	**34a**	**34b**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**Function from PlantPAN**
																									cor15a gene in Arabidopsis, light signaling mediated by phytochrome is necessary for cold- or drought- induced gene expression through the C/DRE in Arabidopsis
	Total	2	0	2	39	3	2	4	5	6	1	15	7	2	2	1	1	9	9	5	0	0	0	0	
Fruit develop-ment	CArG	-	1	3	2	1	1	3	-	2	-	1	1	-	-	-	1	-	-	-	-	3	2	-	Recognised by RIN MADS box TF - RIN can directly bind to the promoters of ethylene biosynthesis genes, genes involved in cell wall remodeling and carotenoid biosynthesis, and genes involved in the control of fruit maturation and pigment accumulation, CYWWWWWWRG
	Total	0	1	3	2	1	1	3	0	2	0	1	1	0	0	0	1	0	0	0	0	3	2	0	

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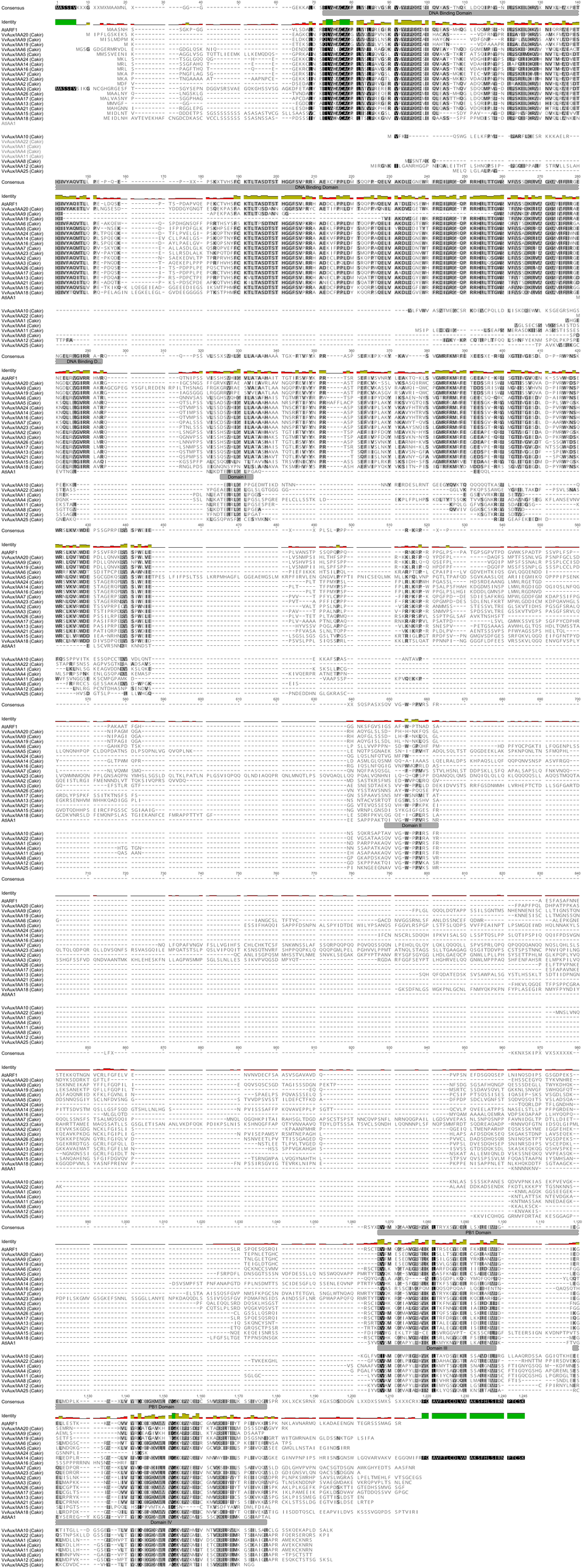


Consensus

F-Box Domain

Leucine Rich Repeat Region

Identity

VvAFB4 (Parry)
VviAFB11
VvAFB6 (Parry)
VviAFB6
VvAFB2B (Parry)
VviAFB10
VvAFB2A (Parry)
VviAFB9
VvTIR1A (Parry)
VviAFB7
VvTIR1B (Parry)
VviAFB8

Consensus

Leucine Rich Repeat Region

Identity

VvAFB4 (Parry)
VviAFB11
VvAFB6 (Parry)
VviAFB6
VvAFB2B (Parry)
VviAFB10
VvAFB2A (Parry)
VviAFB9
VvTIR1A (Parry)
VviAFB7
VvTIR1B (Parry)
VviAFB8

Consensus

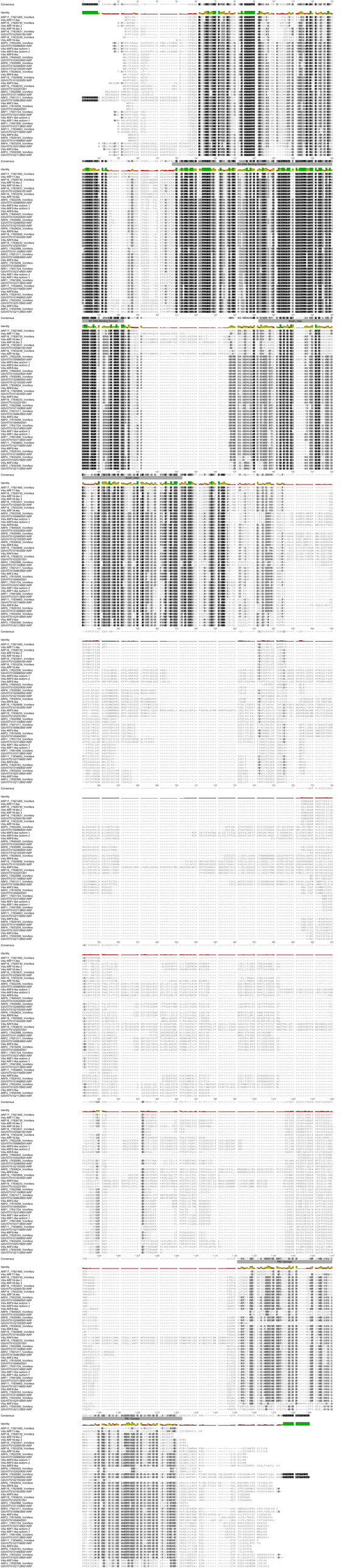
Leucine Rich Repe...

Leucine Rich Repeat Region

Identity

VvAFB4 (Parry)
VviAFB11
VvAFB6 (Parry)
VviAFB6
VvAFB2B (Parry)
VviAFB10
VvAFB2A (Parry)
VviAFB9
VvTIR1A (Parry)
VviAFB7
VvTIR1B (Parry)
VviAFB8


Consensus
Identity
B3 DNA Binding Domain
Auxin_resp
PB1 Domain



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