Identification and Partial Characterisation of Allene Oxide Synthase (EC 4.2.1.92) from *Vitis vinifera* L. *Sauvignon blanc*, a Key Enzyme in the Jasmonic Acid Biosynthetic Pathway, Whose Manipulation May Confer Increased Natural Resistance to *Botrytis cinerea* Infections

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Abstract

Identification and Partial Characterisation of Allene Oxide Synthase (EC 4.2.1.92) from *Vitis vinifera* L. *Sauvignon blanc*, a Key Enzyme in the Jasmonic Acid Biosynthetic Pathway, Whose Manipulation May Confer Increased Natural Resistance to *Botrytis cinerea* Infections

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Pathogen infection or plant disease cause major losses in crop production across many species. In grapevine, in particular, there is an ongoing need to decrease dependence on chemical agents as a method to control or manage pathogen infection. Therefore, new approaches need to be explored to provide effective methodologies or approaches to minimise the impacts of pathogen infections. Jasmonic acid is known to be an important compound in plants that orchestrates both wound and plant defence responsiveness against a range of plant herbivores and pathogens. Jasmonic acid, via complex signalling cascades, induces plant defence genes such as those encoding proteinase inhibitors (involved in the protection of plant from insect damage), defensins and thionin (involved in the production of antimicrobials), and a raft of biosynthetic genes that lead to the accumulation of antimicrobial secondary metabolite such as alkaloids, terpenoids, flavonoids, and glucosinolates. Furthermore, jasmonic acid also facilitates the interaction between other defence signalling pathways such as those mediated by salicylic acid and ethylene to acquire the most effective ways to combat herbivore and pathogen attacks. Allene oxide synthase is the first committed biosynthetic step in the formation of jasmonic acid. Previous studies indicate that genetic variation within allene oxide synthase that alter its biosynthetic capacity have the potential to confer to the host plant increased resistance to attack from fungal pathogens. Therefore characterisation of grapevine allene oxide synthase function and genetic variation is an important step in ascertaining the potential this enzyme to contribute to increased tolerance to a wide range of fungal pathogens.

Allene oxide synthase (hydroperoxide dehydratase; EC 4.2.1.92) is an enzyme belonging to the cytochrome P-450 (CYP74A) that known to catalyse the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides. A functional study of grapevine allene oxide synthase has not been previously reported. Therefore in this study we focused on the identification and functional characterization of the putative allene oxide synthase from *Vitis vinifera* L. *Sauvignon blanc* via complementation of an Arabidopsis allene oxide synthase null mutant. We investigated the

relationships between allene oxide synthase and the other members of the CYP74 family in grapevine, in terms of sequence similarities, subcellular localisations and transcriptional regulation, both spatially and in response to mechanical wounding. We also determined the range of genetic variation of the grapevine allene oxide synthase within a commercial grapevine population. Our findings clearly demonstrate that there is a single allene oxide synthase gene in grapevine and that this gene is able to function in a heterologous system (Arabidopsis) to compliment a null mutation in allene oxide synthase. We show that grapevine allene oxide synthase is localised within the chloroplast and likely associated with chloroplast membranes. In addition the remaining members of the grapevine CYP74 family are found to be localised in varying cellular locations, not necessarily those predicted by in silico sequence analysis. The members of the CYP74 family show differential spatial and developmental transcript accumulation in grapevine.

In order to assess the potential for increasing allene oxide synthase levels to increase biochemical flux through to jasmonic acid we overexpressed both the grapevine and Arabidopsis allene oxide synthases in a wild type Arabidopsis background. Our findings suggest that grapevine AOS might not be the only limitation in production of enhanced levels of jasmonic acid in response to wounding or pathogen attack. While we obtained increased levels of allene oxide synthase transcription, this did not result in a concomitant increase in jasmonic acid and consequently increases in the transcription of jasmonate regulated genes. However, while the alterations in jasmonate levels in the transgenic lines was below expectations, we did note that increased levels of jasmonate as a result of overexpression of allene oxide synthase did result in a limited and transient increase in tolerance to Botrytis infection. Investigation of the potential levels of genetic diversity of allene oxide synthase locus in grapevine indicated that this locus is highly conserved with no variation being evident among 100 vines in a commercial vineyard. While the levels of genetic variation strongly suggest that identification of suitable genetic variation in allene oxide synthase that would contribute to increased jasmonate accumulation from within existing grapevine populations is uneconomically practical or efficient. In conclusion our data suggests that to increase jasmonate mediated resistance against fungal disease in grapevine would likely require a coordinated alteration in allene oxide synthase as well as downstream genes in the biosynthetic pathway such as allene oxide cyclase and 12-oxophytodienoic acid reductase. To achieve such an alteration without resorting to transgenic approaches would require the use of a hybridization/breeding approach (which is currently unpalatable to industry) or identification of a suitable gain-of-function mutation from the native transposon mutation population that our group is currently producing.

Keywords: : Grapevine, jasmonic acid, Arabidopsis knock-out AOS, signalling, allene oxide synthase, genetic variation, qRT-PCR, binary vector.

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Chapter 1

Literature Review

1.1 Introduction

Worldwide, in 2014, approximately 7.4 million hectares were used to grow grapes, of which an estimated 287.7 million hectolitres of wine was produced (OIV, 2014). In New Zealand, a recent report by New Zealand Winegrower indicates that the grapevine planting area has grown to approximately 35,000 hectares, and with an estimated production of 320.0 million litres of wine in 2014 (New Zealand Winegrower, 2014). The New Zealand wine industry was reported to contribute approximately NZD$1.30 billion in export receipts to the New Zealand economy in the same year. In New Zealand, the *Sauvignon blanc* variety predominates, providing approximately 72% of wine production that signifying its outstanding contribution to the grapevine industry (New Zealand Winegrower, 2014). Due to this vital contribution to the industry, many researchers throughout New Zealand focus their research work and interest on the *Sauvignon blanc* variety.

In terms of sustainable production, wine vintage quality depends on the climate and seasonal weather conditions as well as the winemaker's skills and experience (Ashenfelter et al., 1995; Shanmuganathan et al., 2011). Besides this, disease infection provides the greatest challenge for the maintenance of fruit yield and quality while maintaining industry sustainability targets for production. In particular, destructive fungal diseases, such as *Botrytis cinerea* and mildews present a huge challenge for both viticulturists and winemakers. For example, it is estimated that *Botrytis cinerea* infections cause an annual loss of approximately USD$2 billion worldwide (Elmer and Michailides, 2007; Mundy et al., 2012). In New Zealand alone, *Botrytis cinerea* infections cost the wine industry approximately NZD$5000/ha for direct losses and an additional NZD$1500/ha to control this disease [Hoksbergen (2010) in (Mundy et al., 2012)]. Traditionally, disease control is achieved through application of chemical agents to control the infection. However, the use of chemical fungicides is becoming untenable due to increased public and regulatory concern over their application. Alternate strategies such as utilizing genetic improvement via hybridization/breeding are also limited due to industry concerns. The main form of genetic improvement that is traditionally acceptable, and extensively used by industry, has been limited to the identification of somaclonal mutants (bud sports) from within existing clonally-propagated grape populations.

However, current advances in functional genomics and the identification of the genetic basis for disease resistance offer an alternative method for identifying grapevines that, potentially, have more tolerance to disease infection while maintaining the quality of their fruit. Natural plant resistance to disease infection is based on a range of genetic determinants and the subsequent regulation of

specific biochemical pathways. Natural variation in plant resistance to a disease infection is as a result of genetic variation occurring in some plant cells as an adaptation the changing environmental conditions surrounding them (Meyers and Bull, 2002). In terms of grapevines, somatic mutations are the main source of genetic variation that is, subsequently, captured to form new clones in commercial vineyards (Carmona et al., 2008). However, natural variations of plant pathogen resistance appear to be quantitative and are often related to the biosynthesis of a signalling compound such as jasmonic acid (JA). Jasmonic acid is one of the signalling compounds responsible for the initiation of phytoalexins production in plants (Yamada et al., 1993; Nojiri et al., 1996). Phytoalexins are low molecular weight antimicrobial substances produced by plants as a response to a pathogen infection or stress, such as wounding or ultraviolet radiation (Kodama et al., 1988; Guest and Brown, 1997; Mert-Türk, 2002; Jeandet et al., 2013).

Allene oxide synthase (AOS), a CYP74 gene family member, plays a central role in jasmonate biosynthesis as this enzyme catalyses the first reaction in the pathway leading to JA production (Schaller and Stintzi, 2009; Gfeller et al., 2010). The AOS gene utilizes the products of lipoxygenase (LOX) activity as a precursor to produce JA (Gfeller et al., 2010). Lipoxygenase activities are not only an important element in the formation of JA but also in the formation of C_6 volatiles, which indicates the close functional relationship between the two compounds. Therefore, modulation of the JA level has a consequential impact on the signalling network of plant's responses to pathogen invasion or plant stress (Matsui et al., 2006; Wasternack, 2007). Evidence exists that genetic variation in AOS is able to contribute towards increased resistance to a pathogen infection, such as a Botrytis infection, in plants containing these variations (Pajerowska-Mukhtar et al., 2008). Moreover, initial work by Podolyan (2010) on six randomly-selected clones of *Sauvignon blanc* revealed a high number of putative SNPs within the coding sequence of the grapevine LOX gene. This provided an indication of a significant level of potentially valuable SNPs within the AOS sequence that might provide a source of variation within this gene that might contribute to improved responsiveness of grapevines to disease infection.

Given this background, this led us to an interest in whether field-grown *Sauvignon blanc* displays a similar genetic variation within the grapevine AOS gene as seen in LOX. If this were the case, then our main question is "Would it be possible to screen field-grown commercial vines for genetic variations that could lead to the identification of plants that could contribute to the production of an increased disease-resistant clone of *Sauvignon blanc*?" However, in order to address this question, the function of the AOS gene and its diversity in the *Sauvignon blanc* variety first needs to be identified and characterized. Therefore throughout this research project, we will answer questions about:

1. The function and character of the grapevine AOS gene isolated from the *Sauvignon blanc* grapevine genome.
2. The ability of grapevine AOS to increase a response against pathogen infection when it is overexpressed in a model plant system (*Arabidopsis thaliana*).

3. The degree of grapevine AOS gene diversity within a selected group population of *Sauvignon blanc* grapevines grown in a commercial vineyard.

1.2 Plant defences

Due to their immobility, plants become vulnerable to abiotic and biotic stresses, which can lead to large production crop losses in the agricultural industry. Examples of abiotic stresses include mechanical damage by wind, high salinity, high or low osmolarity, extreme temperatures, drought, ozone, reactive oxygen species and, even, UV light. Biotic stresses, however, are caused by living things, which include bacterial, viral or fungal infections (Wasternack and Hause, 2002; ten Hoopen et al., 2007). In order to combat these stresses, plants develop a wide range of defence mechanisms. These range from the presence of physical barriers to complex signalling networks leading to a host defence expression. Plant defence mechanisms not only occur in local tissues subjected to the stress but are also triggered in other healthy tissues as a systemic response. In environmental conditions that are suitable for pathogen development, the resistance or susceptibility of a plant to a particular pathogen is dependent upon two interrelated factors. These are the substrate requirements of the pathogen and the response of the plant to the pathogen (Guest and Brown, 1997). A plant's defence process is an action to induce defences mechanisms that prevent the pathogen from invading the plant cell and reproducing (Thatcher et al., 2005). Plant-pathogen interactions can be categorized into non-host and host resistance (Heath, 2000). Non-host resistance refers to where a plant is resistant to all races of a pathogen, whereas host resistance is where the plant is resistant to some, but not all, races of a pathogen. These two types of resistance have substantial overlaps but can be differentiated through the ability of the pathogen to overcome a series of obstacles (from the plants) to successfully infect a host plant (Thordal-Christensen, 2003)

Plants also employ the perception of non-specific elicitors, such as flagellin, the major protein component of the bacterial flagellum (Felix et al., 1999), and the possession of the corresponding resistance gene (*R*-gene), to initiate an active defence response, such as the hypersensitive response (HR), against all races of the pathogen (Thordal-Christensen, 2003). In contrast, in host resistance interactions, the pathogen is specific and this is where the plant develops the ability to recognize and trigger an effective defence mechanism against only some of the genotypes of the pathogens. Plant responses are commonly regulated by a single *R*-gene, the product of which participates either directly or indirectly in the perception of the avirulence (*avr*) gene product from the pathogen (Mysore and Ryu, 2004). In general, plant defence systems can be divided into two main classes based on their response, i.e. a passive or active defence response.

1.2.1 Passive defence responses

Plant defence responses are categorized as passive (also known as constitutive defence) when they are pre-existing in the plant, such as structural components or certain types of chemicals that are always present in the plant even in the absence of an abiotic and biotic stress. Plant cell walls are considered to be the major line of structural defence. Strong materials such as lignin (a highly impermeable substance for the pathogen and difficult for insects to chew), tough bark (the protective tissue of plant bodies), cuticle and wax (a fatty substance that is deposited on the surface of cell walls to prevent pathogens from penetrating plant cells) provide protection to the plant cell from pathogen invasion (Guest and Brown, 1997) as well as give strength and rigidity. Besides physical protection, cell wall also incorporates with a wide variety of chemical defences that can be rapidly activated when the presence of a pathogen is detected (Guest and Brown, 1997). For example, cell walls contain protein and enzymes that are not only involved in induced plant defence mechanisms but also actively strengthen the wall during cell growth (Bradley et al., 1992). When a plant detects the presence of a potential pathogen, an enzyme catalyses an oxidative burst that produces highly reactive oxygen molecules that not only are capable of damaging the cell of invading organisms (Lamb and Dixon, 1997; Montillet et al., 2005) but also help strengthen the cell walls by catalysing cross-linkages between cell wall polymers and serving as signals to neighbouring cells about the attack (Bradley et al., 1992; Lamb and Dixon, 1997).

A plant's chemical defences arise from its main secondary metabolic routes, such as the phenylpropanoid, isoprenoid and alkaloid pathways (Iriti and Faoro, 2009). Some of these secondary metabolites may have antibacterial, antimicrobial and insecticidal properties that can inhibit pathogen development or growth. These defensive compounds of the plant can be either constitutive, stored in an inactive form, or induced in response to the insect or microbial attacks (War et al., 2012). In the chemical constitutive defence mechanism, these compounds, also known as phytoanticipins, are excreted into the external environment, accumulate in the dead cells or are stored in the vacuole in an inactive form (Guest and Brown, 1997). Some plant peptides also inhibit the development of fungi, bacteria, viruses and insects. They act as proteinase and polygalacturonase-inhibitors, as ribosome inhibitors, or lectins. These inhibitors interfere with the pathogens' nutrition and retard their development (Guest and Brown, 1997). Unlike the simple chemicals derived from secondary metabolites, peptides are simply produced by transcription and translation of a single gene, which means they can be delivered relatively rapidly after infection with limited inputs of energy and biomass (Aerts et al., 2008). However, this innate immune response of plants has been regarded as a primordial defence system because they constitutively express or accumulate in all plant organs during normal development (Guest and Brown, 1997; Stotz et al., 2013).

1.2.2 Active defence responses

Passive plant defence mechanisms, such as structural barriers and constitutive antimicrobial compounds, are designed for non-specific protection against the colonization of a wide range of pathogens infection. However, some pathogens that manage to overcome these obstacles will be faced with induced responses by the plants. These responses rely upon pathogen recognition to trigger a series of signalling cascades that, eventually, activate numerous plant defence pathways (Thatcher et al., 2005). Plant-induced defences are described as active defence mechanisms because they are response to an invading pathogen and require host mechanisms to function (Hutcheson, 1998). Active defence responses can be activated by different types of pathogens, including viruses, bacteria, fungi, nematodes or abiotic stresses, as previously described in section 1.2 (León et al., 2001). Plant-induced defence responses are generally based on the interaction of pathogen elicitors with plant receptors, and the subsequent transduction of this interaction triggers defence response in the plant (Thatcher et al., 2005). In general, there are three separate classes of active plant defence responses that can be identified, based on their eliciting signal: primary responses, secondary responses and a systematically acquired response.

Primary responses are localized in the cells and only activated when in contact with, or infected by pathogens. Primary responses involve the recognition of specific signal molecules which belong to, or are displayed by, the pathogens. These signal molecules are often a critical component in the cell activities of a pathogen (Hutcheson, 1998; Mur et al., 2008) and, as a result, frequently trigger programmed cell death such as a HR-response (Hutcheson, 1998) at the infected site. Hyper sensitive response can be described as the rapid death of plant cells associated with disease resistance (Goodman and Novacky, 1994). The HR-response is a multitude of biochemical processes that includes: a rapid oxidative burst, an increase in cytoplasmic streaming, cytoplasmic aggregation followed by granulation, membrane disruption, cellular decompartmentalization and browning at the infected site. The cumulative effect of this process is the death of the infected cell as well as those adjacent to it, and that creates an unfavourable environment for disease development and prevents further damage by pathogen colonization (Guest and Brown, 1997; Heath, 1998). The HR-response is triggered by the presence of an *avr*-gene in the pathogen that produces a direct or indirect product that is recognized by the plant via a corresponding *R*-gene. If the interaction between the two genes product, *avr*- and *R*-gene is compatible, disease will develop and plant will be infected by the pathogen. However, if the interaction is incompatible, the plant will develop resistance to the particular pathogen (Morel and Dangl, 1997).

Induction of a secondary response occurs in the cells adjacent to the initial infection site as a response to diffusible signal molecules, known as elicitors, produced by the primary response (Hutcheson, 1998; Thatcher et al., 2005). This response relies on pathogen recognition to trigger a series of signalling cascades that activate numerous defence pathways (Thatcher et al., 2005; Kachroo

and Kachroo, 2009). Following pathogen attack, the early defence responses are often amplified through the generation of secondary response compounds, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which activate plant defence mechanisms both locally (at the site of infection) and systematically (through non-infected tissues). Secondary compound SA, JA and ET are signalling molecules that activate components of the signal transduction cascade and that lead to the expression of plant defences or protectant genes, such as pathogenesis related proteins (PR-protein), glutathione *S*-transferases (GST), proteinase inhibitors and the production of antimicrobial secondary metabolites, such as phytoalexins (Guest and Brown, 1997; Kunkel and Brooks, 2002; Thatcher et al., 2005; Koornneef and Pieterse, 2008).

The third category of active defence response is associated with systemically acquired resistance induced by the production of hormones throughout the entire plant (Guest and Brown, 1997; Vallad and Goodman, 2004). Systemic resistance confers long-lasting protection against a broad range of pathogens (Durrant and Dong, 2004). Systemic resistance involves the *de novo* production of PR-protein, such as chitinases and glucanases, or the synthesis of antimicrobial compounds, such as phytoalexins (Heil and Bostock, 2002; Zhang et al., 2013). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two forms of plant-induced resistance in this category. Both SAR and ISR are preconditioned and activated prior to an infection that results in resistance against a pathogen challenge or mechanical stresses (Vallad and Goodman, 2004). SAR and ISR resistance can be differentiated via the nature of the elicitor and the regulatory pathway involved (Knoester et al., 1999; Maleck et al., 2000; Schenk et al., 2000; van Wees et al., 2000; Yan et al., 2002). For example, SAR can be induced by the exposure of roots or foliage to biotic or biotic elicitors and is also dependent on the phytohormone, SA, and the accumulation of PR-protein. In comparison, ISR can be induced by the exposure of the roots to specific strains of plant growth-promoting rhizobacteria. ISR is dependent on the phytohormones, ET and JA, but independent of SA, and is also not associated with the accumulation of PR-proteins (Heil and Bostock, 2002; Vallad and Goodman, 2004; Thatcher et al., 2005).

1.3 Plant signalling

Plants have to evolve their defence strategies to protect them from pathogen attacks and threats by herbivores. Some defences are pre-formed while others are induced upon attack. These strategies are likely employed due to the deleterious or high energy costs needed to maintain them continuously (Baldwin, 1998). Pathogen-associated molecular patterns (PAMPs) are pathogen-derived molecules that are conserved throughout various classes of microbes and contribute to general microbial fitness. Whereas, effectors are species, race or strain-specific and contribute to pathogen virulence (Thomma et al., 2011). Defence signalling pathways are generally induced when plants recognize PAMPs, or effector molecules, produced by the pathogen. Recognition of these molecules is

designated as PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Kachroo and Kachroo, 2009; Thomma et al., 2011). PTI is induced when pattern recognition receptors (PRRs) in the plant recognize PAMPs, whereas, ETI is induced when a strain-specific *avr*-protein from the pathogens interacts directly or indirectly with the corresponding plant *R*-protein (Kachroo and Kachroo, 2009).

Pathogens secrete a series of chemical compounds to establish an interaction with the plant host and facilitate colonization (Castro and Fontes, 2005). The main chemical compounds secreted by the pathogen are hydrolytic enzymes that have the ability to degrade the cell wall components which allow the pathogen to invade plant tissues (Collmer and Keen, 1986; Walton, 1994). Other compounds include toxins that interfere with the host's metabolic functions. These toxins have many effects, including altering cell membrane permeability, inactivating enzymes leading to interruption of essential metabolic pathways (Quigley and Gross, 1994; Scholz-Schroeder et al., 2001), the interruption of growth regulators leading to an imbalance in hormones causing a disruption to normal plant development (Mengiste et al., 2003; Suckstorff and Berg, 2003) and causing polysaccharides to block the water translocation mechanisms in the vascular system (Leigh and Coplin, 1992; de Pinto et al., 2003).

Plant-induced responses are activated only after contact with the pathogen or mechanical stresses (Castro and Fontes, 2005). Pathogen-derived elicitors, such as PAMPs, wounding, glycan and systemin, all cause a rapid depolarisation in the electric potential of the plasma membrane (Ryan, 2000; de Bruxelles and Roberts, 2001; ten Hoopen, 2002). This depolarisation is associated with an efflux of K^+ and Cl^- ions and an influx of Ca^{2+} and H^+ ions through controlled protein phosphorylation and dephosphorylation events across the plasma membrane (Nurnberger and Scheel, 2001; ten Hoopen, 2002; Thatcher et al., 2005). These events signal the production of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and reactive nitrogen such as nitric oxide (NO) (McDowell and Dangl, 2000; Hancock et al., 2002). As a plant defence, H_2O_2 stimulates a direct microcidal effect and strengthens the plant cell wall by stimulating the lignification process and cross-linking around the plant cell walls (Thatcher et al., 2005). H_2O_2 and NO, together, induce the expression of defence-associated genes, such as phenylalanine ammonia-lyase (PAL), pathogenesis-related (PR) and glutathionine S-transferase (GST) (Bi et al., 1995; Delledonne et al., 1998; Desikan et al., 1998; Durner et al., 1998).

Transient changes in the ion permeability across the plasma membrane are consequence of, and cause, the rapid death of host cells at the infected site (Guest and Brown, 1997; Thatcher et al., 2005). A ring of cells surrounding the dead cells or necrotic lesions become refractory to subsequent infections (Fritig et al., 1998). This phenomenon, also known as localized acquired resistance, often triggers non-specific resistance throughout the plant, providing durable protection against future infection by a broad range of pathogens (Sticher et al., 1997). Metabolic alternation in acquired

resistance triggered plants to induce responses that includes defence regulators, such as SA, ET and lipid-deprive metabolites (Fritig et al., 1998). In addition, it also triggers the induction of phospolipases (PLPs), which act on lipid-bound unsaturated fatty acids within the membrane and result in the release of a signal compound known as JA (Wang, 2001). Evidence also shows that JA, SA and ET play pivotal roles in the signal pathway leading to the up-regulation of pathogen defence-related genes in plants (Koornneef and Pieterse, 2008).

Following pathogen attacks or mechanical stresses, the early defence signalling events are often amplified through the generation of secondary signalling molecules. This may lead to defence activation, both locally at the infection site and systemically in non-infected tissues (Thatcher et al., 2005). As described earlier in this section, the earliest known events after the attack or stimuli include ion fluxes across the plasma membrane, changes in cytoplasmic calcium concentration, the generation of ROS and changes in protein phosphorylation patterns, which appear to be associated with intercellular signal generation by the plant's defence system (de Bruxelles and Roberts, 2001). However, these early events are unlikely to all be directly responsible for inducing defence gene expression. Instead, they act as a mass of data to initiate the production of signal molecules, which then mediate the induction of the defence gene expression (de Bruxelles and Roberts, 2001). Signalling molecules are low molecular mass regulators that are capable of inducing plant defence mechanisms and depend on the pathogen or stimuli elicitor (de Bruxelles and Roberts, 2001; Garcia-Brugger et al., 2006). In plants, despite several elicitors being involved in regulating the plant defence response, the three main plant specific phytohormones involved in triggering plant defences are SA, JA and ET (Rojo et al., 2003). These molecules do not function independently but influence each other through a complex network of regulatory interactions (Kunkel and Brooks, 2002; Thatcher et al., 2005). In the plant kingdom, oxylipin is known as one of the most important signalling molecules related to plant stress responses and innate immunity (Eckardt, 2008)

1.4 Plant oxylipins

Oxylipins are signalling molecules involved in various stages of plant development, growth regulation and responses to environmental stimuli (Savchenko et al., 2014). In response to plant stresses, oxylipins are involved in signal transduction, which induces the expression of target genes and also interacts with other signalling molecules, such as ET and SA (Rojo et al., 2003). These form a complex signalling network pathway to fine tune the induction of plant defences. Therefore, oxylipins represent one of the main defence signalling mechanisms employed by plants against pathogens. In response to a pathogen attack, the phospholipase A superfamily of proteins catalyses the hydrolysis of phospholipids to generate the corresponding free fatty acid (also known as polyunsaturated fatty acids or PUFAs) (Shah, 2005). Oxylipin biosynthesis begins with the oxygenation of PUFAs by LOX

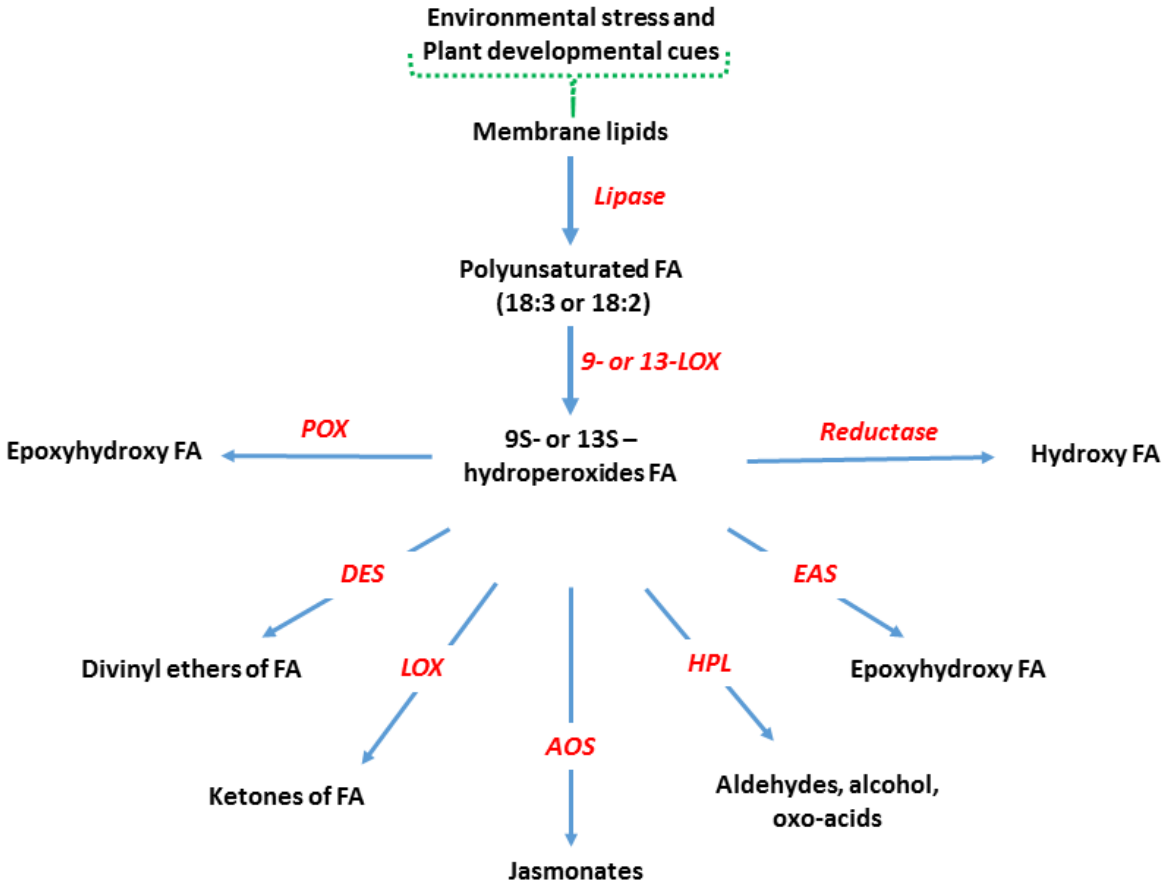

Figure 1.1 Oxylipin biosynthesis pathway

Oxylipin is a collective term for oxygenated metabolites derived from polyunsaturated fatty acids (PUFAs). Biosynthesis of various oxylipin, regulated by plant developmental or environment signals via lipase, mediated the release of PUFAs from membrane lipid, additional oxygen molecules were catalysed by LOXs to form hydroxyl PUFAs that were, subsequently, metabolised by various enzyme systems to produce an array of hydroperoxy fatty acids. The oxylipin biosynthesis pathway is also known as the lipoxygenase biosynthesis pathway. FA, fatty acid; LOX, lipoxygenase; AOS, allene oxide synthase; POX, peroxygenase; DES, divinyl ether synthase; HPL, hydroperoxide lyase; EAS, epoxy alcohol synthase. Figure adapted from Howe and Schmiller (2002), Feussner and Wasternack (2002) and Savchenko et al. (2014)

to form a fatty acid hydroperoxide (Stumpe and Feussner, 2006; Schneider et al., 2007). Lipoxygenase oxygenate from most common PUFAs available as as linoleic and linolenic by inserting an oxygen molecule at the specific position to produce 9- or 13-hydroperoxide substratet in plant (Hughes et al., 2009). PUFA hydroperoxide substrate can be further metabolised by a group of enzymes, known as CYP74 family, to produce an array of different oxylipins, such as jasmonates, aldehydes, ketols, epoxides and divinyl ethers (Shah, 2005) as illustrated on the figure 1.1. The level of each oxylipin compound will increase dramatically in response to environmental stimuli or from a development input (Hughes et al., 2009)

1.5 Cross-talk signalling

Despite the importance of plant hormones for the regulation of plant growth, development, reproduction and survival, hormones are also essential as primary signals in regulating plant defence mechanisms (Pieterse et al., 2009). When challenged with a pathogen or mechanical stress, plants produce complex responses that activate different signalling cascades, which lead to the activation of local and systemic defence systems; for example, antimicrobial defence systems (Rojo et al., 2003). These different signalling pathways form a complex network that influence each other, through positive and negative interactions, to equip the plant with a powerful regulatory capacity to finely tune the immune response. In addition, they also help the plant to minimize energy costs to induce plant responses (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Pieterse et al., 2009). Despite a number of plant hormones being involved in communicating and triggering plant defence mechanisms (Spoel and Dong, 2008), plant hormones SA, JA and ET are recognized as the three major, endogenous, defence hormones (Kunkel and Brooks, 2002; Bari and Jones, 2009; Pieterse et al., 2012). Pathogens that require a living host (biotrophs) are commonly more sensitive to a SA-mediated defence response, whereas, pathogens that kill the host and feed on the contents (necrotrophs) or herbivorous insects are generally affected more by a JA/ET-mediated defence (Glazebrook, 2005; Howe and Jander, 2008). Generally SA, JA and ET signalling cascades do not activate defences independently but, rather, establish complex interactions that determine the response to the attack (Kunkel and Brooks, 2002; Rojo et al., 2003). Crosstalk between SA, JA and ET signalling pathways has not only emerged as an important regulatory mechanism but also serves as a backbone to induce defence signalling networks where other hormone pathways, such as abscisic acid (ABA), auxin, gibberellin (GA), cytokinin (CK) and brassinosteroids, feed into it (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011).

In plant crosstalk, interactions between SA and JA signalling generally appear to be antagonistic, whereas SA and it derivatives block the JA biosynthesis pathway by preventing the release of the JA precursor from the chloroplast (Rojo et al., 2003). The reverse is also true for the effect of SA on JA signalling. It is also reported that JA accumulation negatively regulates SA signalling (Rojo et al., 2003; Thatcher et al., 2005). In contrast to the SA and JA interaction, JA/ET signalling shows positive or

synergistic interactions where both signalling pathways can be activated by the same precursor, e.g. methyl jasmonic (MeJA) (Schenk et al., 2000). Both JA and ET signalling are also required for the expression of the defence-related genes in response to a pathogen infection (Ellis et al., 2002; Kunkel and Brooks, 2002; Thatcher et al., 2005). Similar to the SA and JA interactions, SA and ET also portray a negative interaction (Rojo et al., 2003) where SA blocks the activity of the transcription factor expression that is induced by ET (Gu et al., 2000). Clustering of genes reveals that more genes are activated by JA/ET and inhibited by SA, than show the reverse pattern when activated by SA but inhibited by JA/ET (Thatcher et al., 2005).

1.6 CYP74 enzyme family

As mentioned previously, oxylipins play a crucial role in plant cell signalling and defence mechanisms (Feussner and Wasternack, 2002; Stumpe and Feussner, 2006). The diversity of oxylipin compounds is created by a unique group of enzymes called the CYP74 family, a non-classical cytochrome belonging to the P450 superfamily group (Gogolev et al., 2012; Toporkova et al., 2013). In contrast to most P450 family members, CYP74 enzymes family do not use oxygen molecules as their catalyst. Instead, they catalyse the isomerisation or dehydration of fatty acid hydroperoxides as both substrate and oxygen donors (Mosblech et al., 2009; Gogolev et al., 2012). Another feature of the CYP74 enzymes that differentiates them from the other P450 superfamily members is their low affinity to carbon monoxide (CO) (Matsui, 1998; Froehlich et al., 2001). Three well known family members of CYP74 enzymes are two dehydrases [allene oxide synthase (AOS) and divinyl ether synthase (DES)] and one isomerase [hydroperoxide lyase (HPL)] (Toporkova et al., 2013).

The AOS branch pathway is seen to be the most dominant among all the oxylipin biosynthesis pathways competing for the hydroperoxide substrate (Stumpe et al., 2006). This is because jasmonates, the end product of this pathway, have high biological activity and regulate vitally important processes in plants (Savchenko et al., 2014), such as plant growth and development, flower formation, gene expression, fertility and photosynthesis (Creelman and Mullet, 1997; Chen et al., 2011; Goetz et al., 2012; Wasternack, 2014). The AOS enzyme transforms the fatty acid hydroperoxide substrate into an unstable allene oxide, which is then converted into 12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (AOC) (Schaller and Stintzi, 2008). Compound OPDA is then further converted to jasmonic acid by a few cycles of β-oxidation (Yan et al., 2013).

The HPL branch pathway is probably the main competitor of AOS for hydroperoxide substrate consumption (Figure 1.1). The HPL catalyses the oxidative cleavage of the hydrocarbon backbone of fatty acid hydroperoxides (Zhu et al., 2012) and this leads to the formation of short chain C_6 aldehydes and ω-oxo acid fatty acids or C_{12} aldehydes from 13-hydroperoxide, whereas 9-hydroperoxide of fatty acid forms a C_9 aldehyde compound (Savchenko et al., 2014). Volatile aldehydes and their derivatives are the most studied of the HPL branch metabolites. These metabolites, collectively named Green Leaf

Volatiles (GLV), are a major aroma component in fruit and green leaves. These compounds have been reported to be directly and indirectly involved in plant protection against insects and pathogens (Shiojiri et al., 2006). It has also been reported that these compounds could be toxic against various bacterial and fungal pathogens (Arroyo et al., 2007; Kishimoto et al., 2008) or emitted to attract predators to attack pest herbivores (Scala et al., 2013).

Divinyl ether synthase (DES) catalyses the conversion of hydroperoxides into the fatty acid, divinyl ether. The function of divinyl ether in biological systems remains largely unknown (Itoh and Howe, 2001). However, recent reports provide evidence that colneleic acid (CA) and colnelenic acid (CnA) are divinyl ether compounds that play important roles in plant defences against pathogenic fungi (Shah, 2005; Fammartino et al., 2007)

1.6.1 Substrate specificity of CYP74 family enzymes

Oxygenation of fatty acids proceeds through distinct enzyme activities. This activity gives rise to a series of diverging metabolic pathways that, eventually, yield a large array of different oxygenated and non-oxygenated derivatives (Blee, 1998; Vancanneyt et al., 2001). In the lipoxygenase pathway, lipoxygenase enzymes introduce molecular oxygen to unsaturated fatty acids, such as linoleic and linolenic acid, to yield either 9- or 13-hydroperoxides that later become the substrates for CYP74 enzymes. The CYP74 enzyme subfamilies are distinguished by enzymatic identity and substrate specificity. The AOS gene from flax was the first member of the CYP74 family to be isolated (Song et al., 1993); hence, AOS was sub-grouped as CYP74A. Within this subfamily, all enzymes are known to use specific 13-hydroperoxide as a substrate and, thus, are called 13-AOS (Stumpe and Feussner, 2006). This, however, is not the case for all the known AOS in plants. There are two other types of AOS with different substrate specificities. The AOS isolated from barley show no substrate specificity for either 9- or 13-hydroperoxides and are, therefore, called 9/13-AOS (Maucher et al., 2000; Stumpe and Feussner, 2006). An AOS from tomato and potato have a specificity to 9-hydroperoxides as a substrate, so are categorized as 9-AOS (Itoh et al., 2002; Stumpe et al., 2006). Due to their high sequence similarity, 9- and 9/13-AOS are grouped into a separate sub-group known as CYP74C. It was also reported that some HPLs also accept either 9- or 13-hydroperoxides as a substrate, as reported in cucumber (Matsui et al., 2000), or are specific to 9-hydroperoxides as a substrate, as reported in almond (Mita et al., 2005). Therefore, due to the substrate specificity, 9- and 9/13-HPL are included in the CYP74C sub-group (Matsui, 2006). HPL enzymes with a preference to 13-hydroperoxidases as a substrate are sub-grouped into CYP74B families. Group 13-HPLs are known to be widespread throughout the plant kingdom and are present in almost every plant that has been examined so far (Matsui et al., 2006). Furthermore, they are also reported to be involved in plant defence responses to pathogen attacks (Vancanneyt et al., 2001; Arimura et al., 2005). In contrast to 13-HPL, 9/13-HPL (CYP74C sub-group members) activity could not be detected in every plant, but is known to be involved

in flavour compounds and the production of C_6-aldehyde, which has antibacterial properties (Matsui et al., 2006). DES enzymes are grouped into the CYP74D sub-group. DESs are known to differ in substrate specificity: DES from tomato, tobacco and potato use 9-hydroperoxydes (Itoh et al., 2002) as a substrate; whereas DES from garlic uses 13-hydroperoxydes as a substrate (Stumpe et al., 2008).

1.6.2 Intracellular localization of CYP74

As reported previously, CYP74 enzyme activity often occurs in chloroplasts, therefore it is predicted to be localized in chloroplasts (Stumpe and Feussner, 2006). This localization seems likely because CYP74 polypeptide sequences encoded a protein region that characterized chloroplastic transit peptide sequence that targeted to chloroplast membrane (Bruce, 2000; Howe et al., 2000). However, most CYP74 enzymes in plants have been found localized in various organs and tissues and are often localized with LOXs (Froehlich et al., 2001; Hughes et al., 2009). Product from 13-LOX metabolism provide 13-hydroperoxides fatty acid as substrates to 13-AOS (CYP74A) where their activity takes place in the plastid to yield jasmonates as an end product of this pathway. Another route for the 13-hydroperoxide substrate is through 13-HPL (CYP74B), which shows the same plastidal localization as the CYP74A subfamily members but in different compartments (Hughes et al., 2009). According to Froehlich and a co-worker, there are different localizations inside the chloroplast for tomato AOS and HPL, whereas AOS is localized in the inner membrane and HPL in the outer membrane (Froehlich et al., 2001). This would indicate the possible compartmentalization of the substrate and/or CYP74 enzymes within the plastids.

Unlike the CYP74A and CYP74B sub-groups, there is a little information on the subcellular localization of the CYP74C and CYP74D sub-groups (which use the 9-hydroperoxide substrate from 9-LOX metabolisms) (Hughes et al., 2009). Despite the fact that 9-LOXs localize in the cytosol, different localizations of CYP74C and CYP74D enzymes have been reported. Fammartino et al. (2007) reported that DES in tobacco localizes in the cytosol, whereas, Stumpe and Feussner (2006) found that potato 9-AOS was detected in the amyloplasts and leucoplasts. However, in *Petunia inflate*, 9-AOS was reported to be localized in the tonoplast (Xu et al., 2006). Another CYP74 enzyme, 9-HPL (enzyme associated with the CYP74C sub family) from almond, was shown to be localized in the microsomes and was also associated with lipid bodies (Mita et al., 2005). Moreover, 9/13-HPL from *Medicgo truncatula* was shown to be associated with lipid bodies together with distribution in the cytosol.

1.6.3 CYP74 as a plant defence system

Although these enzymes play important roles in plant defences, there has been little research undertaken on CYP74 enzymes but with the exception of AOS. The research has focused on AOS due to the biosynthetic end product, jasmonate, a signal molecule that regulates the plant's responses to biotic and abiotic stresses (Turner et al., 2002). Reports indicate that AOS in tomatoes, barley,

Arabidopsis and potatoes was activated in response to wounding (Maucher et al., 2000; Sivasankar et al., 2000; Park et al., 2002). Meanwhile, HPLs are associated with the production of GLVs, important compounds that contribute to aroma and flavour in plants. Although it has been reported that HPLs contribute to plant defence mechanisms (Noordermeer et al., 2001), the majority of studies have focussed on their relationship to aroma and flavour compounds. While, to date, the DES enzyme has not had much work undertaken to elucidate its specific relationship to plant defence mechanisms.

As mentioned previously, CYP74 enzymes have been phylogenetically classified into CYP74A, CYP74B, CYP74C and CYP74D and, with some exceptions, plant AOS enzymes belong to CYP74A (Stumpe and Feussner, 2006). The most well-known AOS enzyme used 9- or 13-hydroperoxide as a substrate in respect to 9- or 13-AOS. To date, only the AOS enzymes from barley (Maucher et al., 2000) and rice (Ha et al., 2002; Agrawal et al., 2004) are known to use both 9- and 13-hydroperoxide as substrates. In JA biosynthesis, only the 13-AOS enzyme was known to be involved in JA production (Yan et al., 2013). Aside from an AOS enzyme isolated from guayule (Pan et al., 1995) and barley (Maucher et al., 2000), all known AOS enzymes protein sequences encodes chloroplast transit peptides region that are associated with membrane-bound proteins. This shows that during JA biosynthesis, AOS enzymes are localized in the chloroplast plastid membranes. Interestingly, although the AOS enzyme in barley lacks a chloroplast-transit peptide, it was found localized in plastids (Maucher et al., 2000). Different plants or species carry different copy numbers of the AOS gene. For example, in Arabidopsis, only one copy number of the AOS gene has been reported, whereas, in rice, four AOS genes have been reported (Laudert et al., 1996; Agrawal et al., 2004). As mentioned previously, AOS is the first enzyme to initiate the reaction of a branch pathway leading to the production of JA (Laudert and Weiler, 1998). This is a clear indication that AOS activity is crucial to controlling the influx of the 13-hydroperoxide substrate into the JA biosynthesis pathway. Interestingly, overexpression of AOS in different plants or species exhibits different results. Overexpression of flax AOS in transgenic potato increased the basal JA level 6 to 12-fold (Harms et al., 1995) but overexpression of the Arabidopsis AOS enzyme (AtAOS) in either Arabidopsis or tobacco did not alter the basal level of JA (Laudert et al., 2000). Different basal expression levels of AOS from different plants or species may be an indication that AOS could be the bottle neck (or not) for JA production in the respective plants (Yan et al., 2013).

AOS gene expression in plants are also stimulated by mechanical wounding and also by its own biosynthetic pathway end product, such as JA or MeJA, as well as its own reactant, OPDA, in many plant species (Harms et al., 1995; Laudert and Weiler, 1998). Another strong indication of AOS enzymes as crucial components in plant defence mechanisms is when the AOS function in Arabidopsis is disrupted or knocked out. In these studies plants show a male-sterile phenotype and JA induction does not respond to wounding treatment (Park et al., 2002).

1.6.4 Allene oxide synthase (AOS) plays an important role in the production of jasmonate signalling compounds

Jasmonate (JAs) are biologically active intermediates in the pathway for JA biosynthesis, as well as the biologically active derivatives of JA that interact with other signalling plant hormones to form a complex signalling network (Turner et al., 2002; Wu et al., 2008) in response to plant stimuli, such as wounding and pathogen infection. When a plant is challenged with this stimulus, OPDA levels rise dramatically. In previous work on Arabidopsis, when plants were challenged by an appropriate stimulus, such as wounding, AOS transcript (Laudert et al., 1996), as well as its polypeptide levels increased at the plant damage site (Laudert and Weiler, 1998). Furthermore, the AOS gene transcript is induced by its own reaction products, OPDA and JA, as well as ET and SA (Kubigsteltig et al., 1999). This attribute indicates that regulation of the expression the AOS protein plays a major role in controlling JA production and signalling (Laudert and Weiler, 1998).

To assess further role of the AOS gene in wound signalling transduction in plants, Park et al (2002) knocked-out the AOS gene function in Arabidopsis (Arabidopsis *aos* mutant) by the insertion of T-DNA within the exon region of the AOS gene sequence. Transcript levels of wound-inducible genes, an Arabidopsis lipoxygenase *2* (AtLOX2) and a vegetative storage protein 2 (AtVSP2) (Laudert and Weiler, 1998; Utsugi et al., 1998), were measured after wounding as an indication of AOS gene response. The results showed that after wound treatment, the transcripts of AtLOX2 and AtVSP2 were not induced and remained at the untreated level. This attribute indicated that wound signal transduction to AtLOX2 and AtVSP2 was not generated due to the lack of AOS gene function to activate the production of endogenous JAs (Park et al., 2002). Mutations in AOS function also led to JAs' insensitive responses, which increased their disease susceptibility to the infecting pathogen. Furthermore, the AOS enzyme is particularly important in JA biosynthesis because it is the first enzyme that catalyses the reaction that leads to the production of these signalling compounds (Laudert and Weiler, 1998; Turner et al., 2002)

1.7 Jasmonic acid

Jasmonic acid is a member of jasmonates, a growing class of signalling molecules and plant hormones that are derived from polyunsaturated acids (PUFAs) via the octadenoid pathway (also known as the oxylipin or lipoxygenase biosynthetic pathway) (Schaller and Stintzi, 2008). These compounds are widely distributed in plants and are affected by a variety of processes, including fruit ripening, production of viable pollen, root growth, tendril coiling, seed germination, and plant growth and development (Turner et al., 2002). Besides that, JA also plays an important role as a signalling molecule in plant defences, particularly defence against insects, herbivores and necrotrophic pathogens. Jasmonic acid has been viewed as the end product of the pathway and as a bioactive compound. However, current findings show that this biological activity is not limited to JA only but also

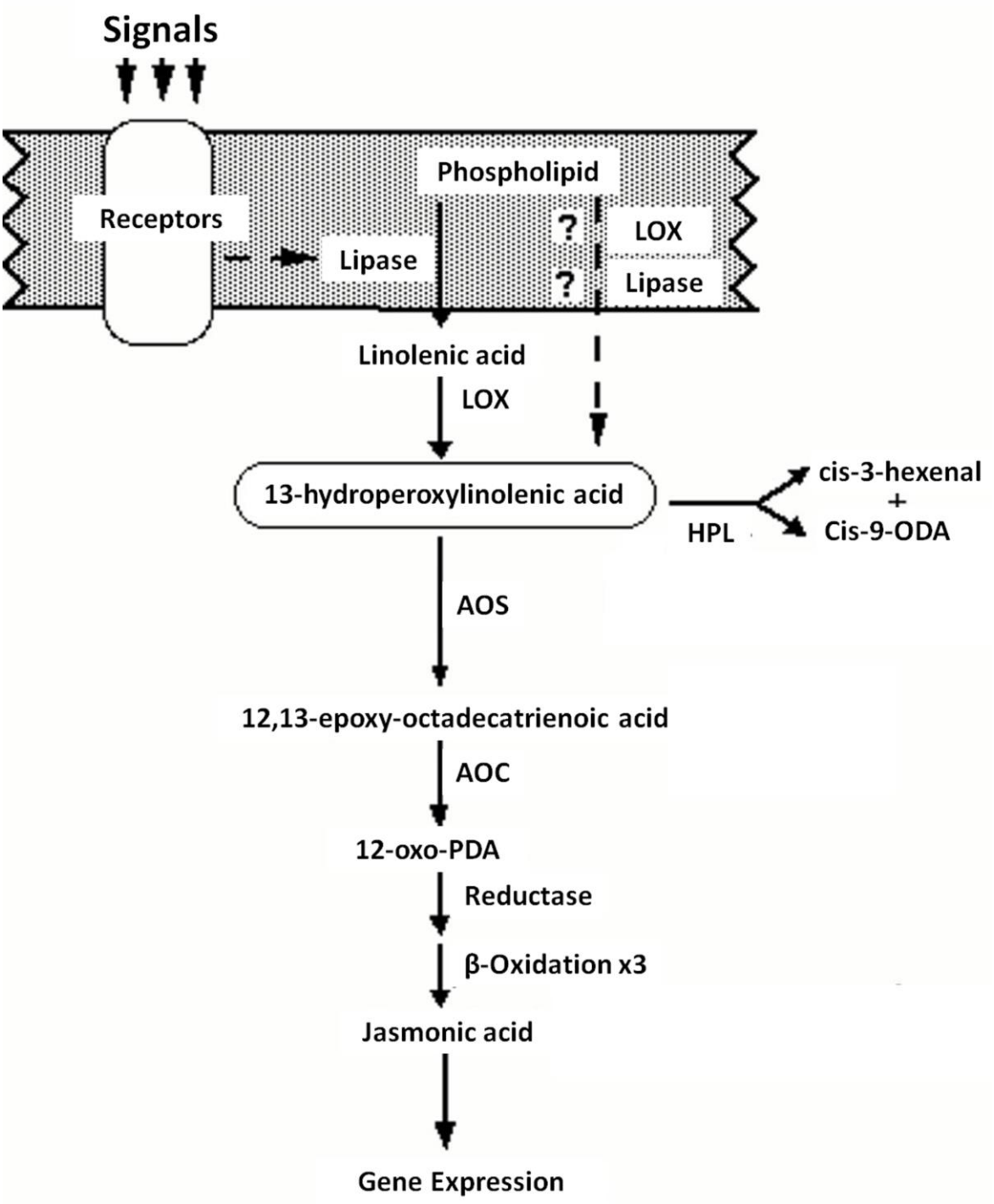

Figure 1.2 Jasmonic acid biosynthesis pathway
Biosynthetic pathway of jasmonic acid. AOS is the first enzyme catalysed in the conversion of 13-hydperoxides substrate to produce jasmonic acid. Jasmonic acid and it derivatives facilitate the signalling mechanism to regulate gene expression related to plant defences. Figure adapted from Creelman and Mullet (1997)

extends to many metabolites and conjugates, as well as its biosynthetic precursors (Schaller and Stintzi, 2008).

Jasmonic acid is a natural hormone regulator that induces proteinase inhibitor proteins in response to pathogen attack and development (Gfeller et al., 2010). Jasmonic acid is synthesized by converting α-linolenic acid into OPDA by a series of enzymatic reactions in the chloroplasts (Kazan and Manners, 2008). OPDA is then transported to the peroxisome where it undergoes a series of β-oxidations to generate JA (Wasternack, 2007; Kazan and Manners, 2008), as illustrated by figure 1.2. Pathogen attack and wounding utilize their own unique receptors (dependent upon pathogen type) in order to trigger the JA-mediated response (Antico et al., 2012). Some elicitors, such as ion influxes across the plasma membrane, changes in cytoplasmic calcium concentration, generation of ROS and changes in protein phosphorylation, are the earliest events that can lead to the activation of production of JA. Other important elicitors include cell wall glycans, such as oligogalacturonidase, and the peptide hormone, systemin (de Bruxelles and Roberts, 2001).

Systemin systematically regulates the activation of over 20 defence genes, including JA signalling, as a response to attacks by herbivores and pathogens (Ryan, 2000; Sun et al., 2011). Once JA is generated, it diffuses from the peroxisome into the cytosol where it can undergo subsequent reactions to, or from, various JA derivatives (Acosta and Farmer, 2010; Antico et al., 2012). Upon infection, necrotrophic fungal attack on plant seems to benefit from the host cell death as a source of nutrients instead of preventing the spread of infection (Glazebrook, 2005). Therefore, plants have had to evolve an alternate mechanism of defence that is mediated by JA. The JA dependent signalling pathway causes increased JA synthesis and initiates the expression of defence effector genes to produce antimicrobial peptides, such as defensin (PDF1.2), thionins (thi2.1) and the anti-insect vegetative storage protein (VSP) (Glazebrook, 2005)

1.8 Regulation of the biosynthesis of jasmonates

The main research interest for oxylipins has largely focused on JAs and their roles as regulators of plant defence-related responses and developmental processes (Creelman and Mullet, 1997). Therefore, many of the physical roles for JAs signalling compounds are well understood and genes encoding for all the biosynthetic enzymes have been cloned from a range of plant species (Itoh and Howe, 2001). Jasmonic acid biosynthesis and signalling are interlinked by a positive feedback loop where the synthesis of JAs are stimulated by their own products, as illustrated in figure 1.3 (Laudert and Weiler, 1998; Sasaki et al., 2001). Genes that encode enzymes involved in the biosynthesis of JAs are inducible by JA (Wasternack et al., 2006) and analysis of the AOS promoter also shows that AOS gene activity increases upon methyl jasmonic acid (JA derivative) treatment (Kubigsteltig et al., 1999).

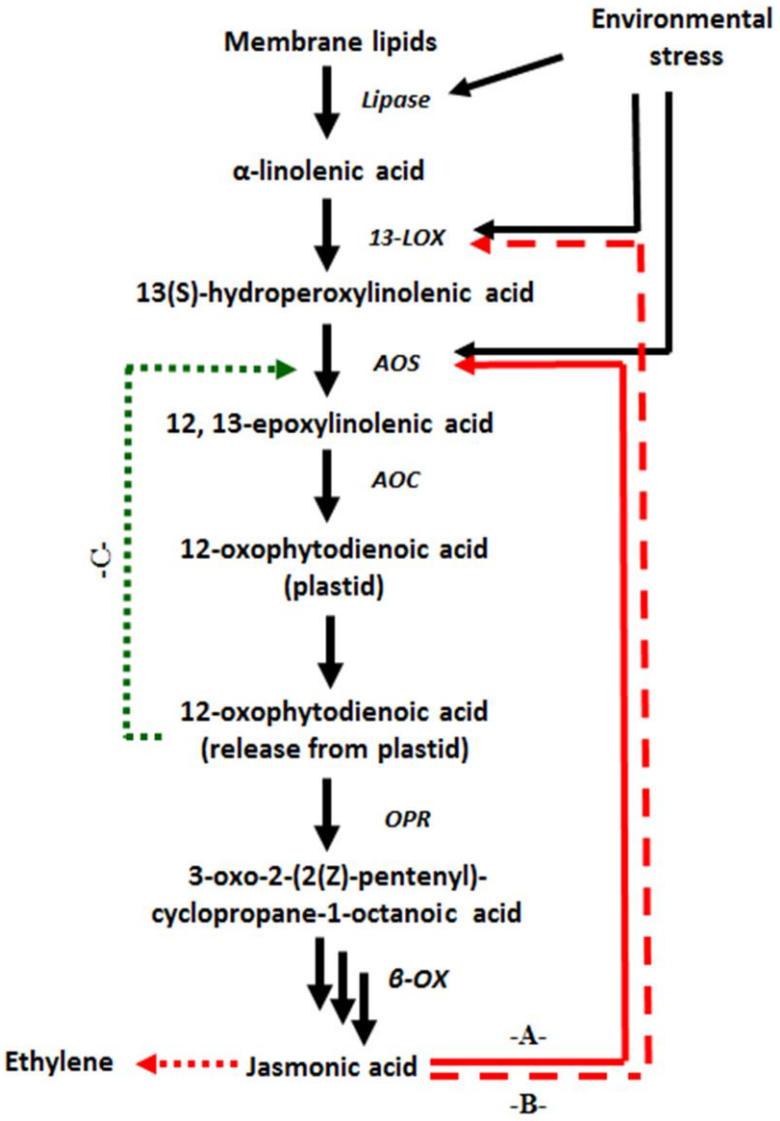

Figure 1.3 Jasmonic acid biosynthesis regulatory process
Regulatory processes of how Jasmonic acid production was regulated by its own products and was also regulated by substrate availability. LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase β-Ox, β-oxidation. Alphabet **-A-** (red arrow), **-B-** (dash red arrow) and **-C-** (dash green arrow) indicate positive feedback induction by its own biosynthetic products. Figure adapted from Laudert and Weiler (1998).

Regulation by its own end product indicates that JAs biosynthesis involves a positive feedback mechanism (Browse, 2005; Wasternack, 2007). Experimental evidence supports this observation, as shown by mutants with constitutively elevated JA levels, such as *cev1* (gene mutation that caused constitutive expression of VSP1), displaying a phenotype attributed to JA treatment (Ellis et al., 2002) and showing regulation of AOC expression (Wasternack, 2007). The Arabidopsis defective in cellulose synthase3 (*cev1* mutant), not only shown elevated levels of JA and OPDA but constitutive JA responses, such as expression of VSP1 (Ellis and Turner, 2001; Ellis et al., 2002). Alteration of the JA capacity form in this mutants seems to be caused or at least partially, by a defect in the positive feedback regulation of JA biosynthesis (Delker et al., 2006). Furthermore JAs deficient mutants such as 12-oxophytodienoate reductase 3 (OPR3) or coronatine insensitive (COI1), show decreased AOC levels of transcription abundance (Stenzel et al., 2003) and caused an increased susceptibility to insect and pathogen attack. It has also been shown that JAs formation takes place only upon external stimuli, such as wounding or pathogen infection (Harms et al., 1995; Park et al., 2002; Wasternack, 2007). Wounding causes a rise in the level of JAs production but this is only transiently expressed and appears before the expression of LOX, AOS or AOC (Howe et al., 2000; Stenzel et al., 2003). However, in plants over-expressing AOS or AOC constitutively, no elevated JAs levels before wounding or other stimuli have been detected (Laudert et al., 2000). Therefore, this observation suggests that JA biosynthesis is regulated by substrate availability (Wasternack, 2007). It was also reported that plant JA and MeJA possess transferable properties from the leaves to the roots or to other tissues (Thorpe et al., 2007). In fact, JA and MeJA are considered as long distance signalling compounds. These signalling compounds can be transported to distal plant sites via air and vascular processes to perform their functions as long distance signals (Heil and Ton, 2008). Later, the transported JAs move into receiver tissues and are converted into the active form of JAs, jasmonoyl-isoleucine (JA-Ile), which eventually activates JA-inducible gene expression (Tamogami et al., 2008).

1.9 Jasmonates as signalling compounds

According to Acosta and Farmer (2010), there are five main steps in JAs synthesis and signalling. The first step is the initiation of JAs synthesis in the plastid. Through the action of LOX, AOS and AOC enzymes, PUFAs was converted to OPDA and dinor-OPDA substrate compounds for JA production. Dinor-OPDA is a product of the parallel pathway for JA biosynthesis where LOX and AOS enzymes used hexadecatrionoic (16:3) compound as a precursor instead of linolenic acid (18:3). The second step is the completion of JA synthesis in the peroxisomes. OPDA and dinor-OPDA substrates are converted to JA by the action of OPR3 enzymes and a beta-oxidation process. JA is then exported by a yet to be determined mechanisms to the cytosol. The third major step occurs in the cytosol. Many derivative active compounds, such as jasmonoyl-isoleucine (JA-Ile) and jasmonoyl-L-tryptophan (JA-Trp), are created in the cytosol (ten Hoopen et al., 2007; Acosta and Farmer, 2010). At this stage

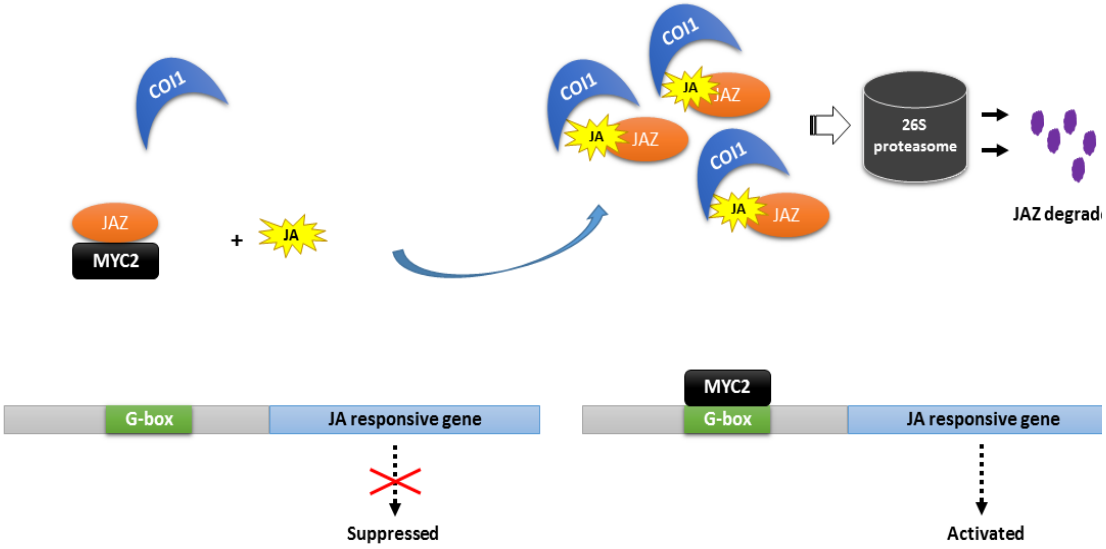

Figure 1.4 Jasmonate signalling mechanisms
JAZ proteins are normally bound to transcription factors and inhibit their activity. In response to attack, JA derivatives (jasmonoyl–isoleucine, JA–Ile, marked with a star) stabilize the interaction between COI1 and JAZ. The JAZ protein is probably then modified by ubiquitin (U), so marking it for destruction. **d)** JAZ is destroyed, liberating the transcription factors; **e)** this allows transcription of genes that produce proteins involved in defence and development, as well as of JAZ genes to restrain the jasmonate response. (The COI1 component is a complex of the SCF-COI1 enzyme which is only shown as "COI1" on the diagram.) Figure adapted from Farmer (2007) and Hou et al. (2010)

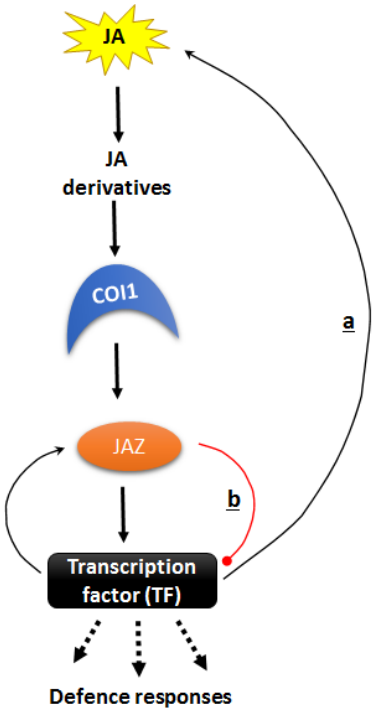

Figure 1.5 Primary regulatory cycle in jasmonate signalling
Synthesis of jasmonic acid (JA) is a self-promoting (feed-back positive loop-**a**) regulation. Newly discovered negative feedback (loop-**b**) regulation involving JAZ proteins and transcription factors (TF), such as MYC2. These two regulatory cycles may be interlocked, but we can expect to find further complexity in the mechanism if, for example, some JAZ proteins bind to transcriptional repressors. Indeed, evidence for secondary regulatory loops already exists. In a regulatory circuit that is not shown here, MYC2 can repress the synthesis of its own transcript. Figure adapted from Farmer (2007).

biochemical diversification occurs. Jasmonic acid is a starting point for the synthesis of many other compounds involved in signalling. For example, JA-Ile and JA-Trp are made in the cytosol where JA-Ile conjugate play an important role in jasmonate signalling pathway and JA-Trp inhibitor act as an inhibitor to auxin response. Step four is where JAs act as signalling compounds. This process takes place in the nucleus where the COI1 receptor binds to JA-Ile, the major active form of JA, to form part of an SCF ubiquitine E3 ligase complex or a SCF-COI1 complex (Yan et al., 2009). The SCF-COI complex is a multi-subunit machine that specifies and mediates protein ubiquitination for the targeted degradation of ZIM-domain (JAZ) proteins by the 26S proteasome. COI1 binds to JAZ proteins, which eventually target the protein complex for degradation by the 26S proteasome (Chini et al., 2007). JAZ proteins are known as negative regulators of transcription of JAs-responsive genes. Interaction of JAZ proteins with the transcription factor, MYC2, (Chini et al., 2007; Chung et al., 2009) suggest that this protein complex controls JAs-related gene expression by preventing the function of the transcriptional activator (Acosta and Farmer, 2010). However, how the exact mechanism works is not yet well understood. Thereafter, destruction of JAZ proteins via the SCF ubiquitine E3 ligase complex liberates transcription factors associated with JAZ complexes and allows for gene expression of the target genes, as illustrated by figure 1.4. The final step of JAs synthesis and signalling is the production and targeting of JAs synthesis enzymes. Gene encoded LOX, AOS, AOC, OPR3 (oxo-phytodienoic acid reductase 3) are activated.

As mentioned above, JAs signalling compounds control diverse aspects of plant growth and defence mechanisms. However, they have also been shown to inhibit plant growth, but the mechanisms are still not well understood (Zhang and Turner, 2008; Noir et al., 2013). Zhang (2008) reported that JAs suppress plant growth by delaying the mitosis process in cells. Further study by Noir et al. (2013) shows that JAs control leaf growth by repressing the proliferation of plant cells and the onset of endoreduplication. Therefore, it is important for the plant to control JAs signalling after the initial activation to avoid an out of control response. One way to control this signalling is to metabolize its bioactive form in order to inactivate it (Chung et al., 2009). This inactivation is known as a negative regulatory feedback loop in JA signalling. During the negative feedback process, the plant cell produces a new JAZ protein variant that, again, represses the corresponding transcription factor. This new JAZ protein variant does not contain a JAs motif (JAs is an active site for COI1 binding) and, as a consequence, this new JAZ variant is not recognized by COI1 (Figure 1.5). Therefore, they are not subjected to JAs-induced proteasome degradation (Chung et al., 2009).

1.10 *Arabidopsis thaliana* as a model plant

Arabidopsis thaliana (Arabidopsis) is a small dicotyledonous species belonging to the *Brassicaceae* or mustard family (Smyth, 1990). Arabidopsis is closely related economically important

crops plants such as cabbage and broccoli. Instead, Arabidopsis has been used for genetic, biochemical and physiological studies as a result of several traits that make it desirable for laboratory study. Arabidopsis is suitable as a plant model because of its usefulness in genetically modified experiments. Arabidopsis is a simple plant that needs only light, air, water and a few minerals to complete its life cycle and this makes it suitable to grow in controlled environments, such as greenhouses, growth chambers or plant growth rooms. Other important features of Arabidopsis include its short life cycle, meaning many plants can be grown in a short period of time to gain a result especially to investigate the function of genetic modification. It is also small in size meaning that it requires only a small space for growth. Arabidopsis also produces plenty of seeds through self-pollination and this is another advantage of using it as a plant model (Koornneef and Meinke, 2010). The Arabidopsis genome is relatively small (125 Mb) and has been completely sequenced (Initiative, 2000) making it easier for gene organization, such as manipulation and cloning, to be performed. Furthermore, these plants have a small number of chromosomes (5) and this simplifies genetic mapping. This is a big advantage for the analysis and understanding the effect of newly introduced genes.

However, the major advantage of using Arabidopsis as a model plant is because it has been widely used in research activities and its features have been well-studied. An important breakthrough for Arabidopsis research was the establishment of an efficient transformation protocols using *Agrobacterium tumefaciens*. Researchers use these protocols to introduce genes of interest back into Arabidopsis for subsequent analysis and the production of plant mutants through the random disruption of endogenous genes. Transformation techniques in Arabidopsis are now well-studied and its genotype is independent, allowing, if needed, the generation of a large number of transformants for each generation. Therefore, this allows the generation of plants that not only express (or overexpress) the gene of interest but also means the study of the localization and quantification of expression patterns in specific tissues is possible. Efficient techniques to screen for mutant plants in Arabidopsis have also been established. Development of random T-DNA mutagenesis procedures, followed by the establishment of transformation, enabled researchers to use genetic enhancers and suppressors of specific mutant phenotypes to screen mutant plants; for example, by using genes resistant to antibiotics or herbicides.

As has been repeatedly reported, JA not only plays an important role in plant defence mechanisms but is also a crucial component in plant development, such as in pollen maturation and dehiscence (Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). A knock-out of AOS gene function in Arabidopsis was achieved using a T-DNA insertion into the AOS nucleotide sequence that completely blocks the JA biosynthetic pathway that results in a male sterile phenotype in Arabidopsis *aos* mutant plants. Furthermore, this Arabidopsis *aos* mutant did not respond to wound treatment (Park et al., 2002). These two attributes enable researchers to study the biosynthesis mechanisms and wound transduction of essential plant hormones.

1.11 *Nicotiana benthamiana* as a model plant

Nicotiana benthamiana (*N. benthamiana*) is a unique species belonging to the *Solanaceace* family that is endemic to Australia (Goodin et al., 2008). Although several species belonging to *Solanaceace*, such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*) or tobacco (*Nicotiana tobaccum*) are economically important, *N. benthamiana* is not. However, *N. benthamiana* has become an increasingly important subject as a model plant to study host-pathogen interactions, particularly those involved in plant virus interactions (Goodin et al., 2008). In terms of chromosomes, *N. benthamiana* comprises 19 pairs of chromosomes. The haploid genome was estimated to be ≈ 3136 Mbp, approximately 20-fold larger than the size of the *Arabidopsis thaliana* genome (157 Mbp) (Burbidge, 1960; Bennett and Leitch, 1995; Bennett et al., 2003; Chase et al., 2003; Bennett and Leitch, 2005).

In the plant research community, *N. benthamiana* has been widely adopted as a plant model due to breakthroughs in three major technical advances, as discussed by Goodin et al. (2008). The first technical advance was the ability to express foreign genes from a plant virus vector. Using this technology, a researcher can trace viral movement within living plant cells and define the protein targeted to them. Besides that, it also provides new insights into fundamental aspects of plant biology, such as the opening of plasmodesmata and macromolecule movement within the living cells (Chapman et al., 1992; Cruz et al., 1996; Escobar et al., 2003; Lucas, 2006). The second technical advance was based on the invention of the virus-induced gene silencing (VIGS) technique (Kumagai et al., 1995; Thomas et al., 2001). Using the VIGS technique, researchers can directly down-regulate any gene-of-interest in the plant which, therefore, transforms *N. benthamiana* into a powerful reverse-genetic system (Ratcliff et al., 2001; Liu et al., 2002; Burch-Smith et al., 2004; Fu et al., 2005; Dong et al., 2007). Moreover, the VIGS technique is able to reduce the issues from the genetic redundancy effect if the cDNA used for the silencing gene is homologous to more than a single member of a multiple gene family (Goodin et al., 2008). The third technical advance was the utilization of the agro-infiltration technique. Using the agro-infiltration technique, researchers are able to observe the expression of the protein of interest when fused with an auto-fluorescence protein and expressed transiently in plant cells (Voinnet et al., 2003; Goodin et al., 2008). Although methodologically, agro-infiltration seems too simple to be useful, in reality, this technique is the most facile means for transiently expressing proteins; it is straightforward and well suited for high-throughput studies in plant cells. These three major technologies for manipulating protein and gene expression in plant cell are best suited to *N. benthamiana* as a plant model system.

1.12 Green fluorescent protein as a gene expression marker

Green fluorescent protein (GFP) is a chemiluminescent protein isolated from jellyfish, *Aequorea victoria* (Chalfie et al., 1994). When calcium binds to the photoprotein, aequorin, it produces

blue light (Chalfie et al., 1994; Baubet et al., 2000). This blue light excites the aequorin companion protein, the GFP receptor fluorophore that emits green light (Tsien, 1998; Baubet et al., 2000). Purified GFP is made from 238 amino acid residues and absorbs blue light between 395 to 470 nm and emits light green light at 509 nm with a shoulder at 540 nm (Morin and Hastings, 1971; Chalfie et al., 1994). The GFP chromophore is a *p*-hydroxybenzylideneimidazolinone, which is derived from the primary amino acid sequence through the crystallization of ser-tyr-gly within the hexapeptide structure (Cody et al., 1993; Tsien, 1998).

The GFP is one of the most widely studied and exploited protein as a result of its ability to be used as a marker for gene expression and protein targeting in intact cells or organisms. The GFP's ability to generate a highly visible and efficiently emitting internal fluorophore; as well as being a very stable protein fluorescence, and with the feasibility of fusing with other proteins without affecting them, makes it a favourite choice as a gene expression marker among researchers (Chalfie et al., 1994; Tsien, 1998; Creemers et al., 2000; Zimmer, 2002). This GFP is also known to be very stable in plant cells and shows only a little photobleaching (Sheen et al., 1995). Using GFP as a gene expression marker is considered to have several advantages over other visual marker genes. First, the fluorescence emission by GFP does not require an exogenous substrate and the procedure does not affect the tissues examined (Pang et al., 1996; Maor et al., 1998). In comparison, β-Glucuronidase (*GUS*) expression is cytotoxic and firefly luciferase (*Luc*) requires luciferin (exogenous substrate) for detection, whereas plant anthocyanins are generally useful only in mature, differentiated cells (Ow et al., 1986; Jefferson et al., 1987; Klein et al., 1989; Lloyd et al., 1992; Millar et al., 1995; Twyman et al., 2002). Secondly, GFP polypeptide size is relatively small (26.9 kDa); therefore, it can tolerate both N- and C-terminal protein fusion making it suitable for protein localization and intracellular protein trafficking studies (Wang and Hazelrigg, 1994; Davis et al., 1995; Kaether and Gerdes, 1995). Thirdly, GFP mutants with shifted wave-lengths for absorption and emission have been isolated, which allows simultaneous use and detection of multiple reporter genes (Heim et al., 1994; Delagrave et al., 1995; Heim et al., 1995). Depending on the experimental layout, quantification of GFP expression can be measured using a range of different methods, such as conventional hand-held UV lamps, anti-GFP antibodies for immunological assays, or confocal laser scanning microscopes (Harper et al., 1999; Richards et al., 2003; Stewart et al., 2005; Stephan et al., 2011). In plant cells, detection and quantification of GFP using an imaging device is often disrupted by auto-fluorescence from plant tissues caused mainly by chlorophyll. However, this interference can be reduced or eliminated by using specific optical filters (Chiu et al., 1996).

1.13 *Botrytis cinerea*

Botrytis cinerea (Botrytis), the causal agent of grey mould, is a haploid *Euascomycetes* belonging to the class of *Leotiomycates* (http://www.genoscope.cns.fr/spip/-Botrytis-cinerea-.html, accessed in

2015). Botrytis is an airborne pathogen with a necrotrophic lifestyle that is a problem to at least 235 plant species (Williamson et al., 2007; ten Have et al., 2010). As a result of the ability of Botrytis to indiscriminately infect different plant tissues and species of plants, it is a major pre- and post-harvest problem for important economic crops around the world (Williamson et al., 2007). The very wide range of infection symptoms may indicate that Botrytis might use an arsenal of weapons to attack host plants (Williamson et al., 2007; El Oirdi et al., 2010).

The infection process of Botrytis begins with the attachment of conidia to the surface of the host, followed by penetration through physical pressure or secretion of enzymes to breach the plant's surface defences (van Kan, 2003; van Kan, 2006). During the penetration stage, Botrytis synthesizes extracellular enzymes that degrade pectin, the major component and most complex polysaccharide in the plant cell wall, which allows its growth inside the plant (Cabanne and Doneche, 2002; Soulie et al., 2003; Kars et al., 2005; El Oirdi et al., 2010). Botrytis kills the host cells before invading them with hyphae to form a primary lesion (van Kan, 2006; El Oirdi et al., 2010). Finally, the plant tissues are macerated and nutrients are converted into a fungal biomass before sporulation. Invasion of plant tissue from Botrytis triggers multiple plant defence responses, including a PR-protein (Hammerschmidt, 1999; Van Loon and Van Strien, 1999). It has been reported that Botrytis infections in tomato and Arabidopsis induce expression of multiple genes encoding defence-related proteins, such as the SA, ET and JA pathways (Thomma et al., 2001; Diaz et al., 2002; El Oirdi and Bouarab, 2007). The SA, ET and JA pathways are widely known as markers of defence pathways in plants.

1.14 Research prospect

The literature review highlighted the important role of AOS in JA biosynthesis pathway and its vital function in orchestrating plant defence mechanisms against pathogens. Therefore, we have decided to focus our study on AOS gene in grapevine due to its role in JA production and as part of research project to answer the questions that we have outlined in section 1.1. In order to answer these questions, first we will identify and partially characterise the putative grapevine AOS via complementation of a null mutation in AOS in Arabidopsis. We also will look at the sub-cellular localization of the CYP74 family of proteins, responsiveness to wound treatment as well as their spatial and developmental (berries) transcript accumulation. This will allow contextualisation of AOS within the CYP74 clade of enzymes. In order to determine whether increased activity of AOS might contribute to increase JA mediated responses to pathogen infection both the grape and Arabidopsis AOS genes will be overexpressed in Arabidopsis under the control of constitutive CaMV 35S promoter. Our prediction is that overexpression of grapevine AOS will provide higher amount of enzymes that should therefore increase biochemical flux through to JA and consequently an improvement in resistance to pathogen infection. Finally, in order to investigate whether sufficient natural genetic variation exists within grapevine we will estimate grapevine AOS genetic diversity within selected population of

commercial grapevine. This will provide insight into the possibility of using AOS gene as a target to screen individual grapevines, searching for potential bud sports that might contribute to plants with increased resistance to fungal infections.

Chapter 2

Partial characterization of CYP74 family members

2.1 Introduction

Due to their immobility, plants are constantly exposed to a variety of biotic and abiotic threats. In order to overcome these threats, plants have developed defence systems that rely on pre-formed and induced responses. The pre-formed defence system as such continues the production of antimicrobial compounds or structural barriers providing a wide range protection to plants. However, pre-formed defences provide non-specific protection to the plants. They becomes surperfolous once these plant defences are defeated by a threat, such as attack by an herbivore or pathogen. Therefore, in order to overcome this problem, the plant has developed another defence system, which relies on the recognition of the threat, leading to the activation of a specific suite of genes. Activation of these specific targeted genes leads to the activation of a complex signalling cascade of defences in plants (Chinnusamy et al., 2004). When plants are exposed to stresses (abiotic or biotic stress), specific ion channels and kinase cascades are activated that lead to the accumulation of ROS, phytohormones, SA, JA and ET (Thatcher et al., 2005; Rejeb et al., 2014). As a consequence, plants re-programme their genetic machinery to activate an adequate defence response in order to minimize the biological damage caused by the stress (Fujita et al., 2006; Rejeb et al., 2014). Among important plant biochemical compounds that plants release as part of their defence mechanisms are the oxylipins. Oxylipins are one of the most important and well-studied signal molecule families in plant defence mechanisms. Formation of oxylipins in plants is mostly synthesised by enzymes that belonging to CYP74 family members (Wasternack and Feussner, 2008).

Grapevines (*Vitis vinifera)* are economically important not only in the wine industry but also in the production of juice, dried fruit and table grapes (Ferreira et al., 2004; Fiori et al., 2009). However, grapevines, especially the *Vitis vinifera* species, are highly susceptible to an array of diseases that cause significant economic losses to the wine industry. Traditionally, disease control can be achieved through application of chemical agents to control the infections. Nevertheless, the use of chemical fungicides is becoming untenable due to increased public and regulatory concerns over their application. Alternate strategies utilizing genetic improvement via hybridization/breeding are limited due to industry concerns. The main form of genetic improvement traditionally acceptable, and extensively used by industry, has been limited to the identification of somaclonal mutants (bud sports) within existing clonally-propagated grape populations. Current advances in functional genomics and identification of the genetic basis for disease resistance has opened up a number of opportunities. Natural variation in plant pathogen resistance appears to be often quantitative and usually related to

the biosynthesis of signalling molecules that are mostly associated to oxylipin compounds (Pajerowska-Mukhtar et al., 2008). The CYP74 family enzymes are known to be involved in synthesizing and diversifying oxylipin compound production in plants (Eckardt, 2008), including grapevines.

2.1.1 CYP74 enzymes in grapevines

There has been little work undertaken on the identification and characterization of CYP74 enzymes family members in grapevines. Most of the work on CYP74 enzymes family members in grapevines has focused on the CYP74B/C sub-group of the family, which comprises both 13-HPL and 9/13-HPLs. These enzymes are involved in the production of C_6 and C_9 aldehyde compounds, respectively, and represent a major fraction of the volatile profiles that contribute to the typical "green" aroma in grape juice and wine (Zhu et al., 2012). As mentioned by Podolyan (2010), VvHPL catalyses the cleavage of fatty acid hydroperoxides to produce C_6 and C_9 aldehyde compounds, which are crucial to grape flavour. His report also indicates that HPLA (a 13-HPL group) responds to both wounding and pathogen attack in berries by increasing transcription abundance up to 3-fold six hours after wounding (Podolyan, 2010). This is consistent with VvHPL's proposed function as an important compound in response to plant stresses, as reported in previous research in other plants (Shiojiri et al., 2006).

Up until now, a fully characterized AOS gene (CYP74A sub-family enzyme) from *Vitis vinifera* has not been reported. Another prevalent CYP74 gene member, DES, has also not been reported or investigated in *Vitis vinifera*. Therefore, in this experiment to characterize CYP74 family members from grapevines, we isolated a putative VvAOS using a gene homologue approach to a previously characterized AOS gene from *Arabidopsis thaliana*. However, due to limited information about function of the DES in plant systems and our main interest in investigating AOS gene variation as a key factor to increasing grapevine tolerance to Botrytis, we did not include DES as part of our objective to characterize CYP74 gene family members in *Vitis vinifera*. In this chapter, CYP74 family members of *Vitis vinifera* L. cv *Sauvignon blanc* were characterized via polypeptide sequence similarity, sub-cellular localization, gene transcription responses to abiotic stress and their gene expression distribution within grapevine tissues.

2.2 Materials and methods

2.2.1 Gene Identification of CYP74 gene family members in grapevines

Identification of the grapevine AOS gene homologue

In order to identify a putative AOS gene homologue in grapevines, the previously identified AOS gene (accession: At5g42650) in *Arabidopsis thaliana* (AtAOS) was used as a reference to search for its homologues across the grapevine genome available in Genoscope (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) and The National Centre for Biotechnology Information (NCBI), USA databases (Altschul et al., 1990). Using the nucleotide BLAST (Altschul et al., 1990) tool to search for AtAOS gene homologues, the "Choose Search Set" section was set to "reference RNA sequences (refseq_rna)" and limited to "*Vitis vinifera* (taxid:29760)". The "Program Selection" section was set to the "Somewhat similar sequences (blastn)" option. A nucleotide sequence that had high identiy to AOS in grapevines was selected. The full sequence of the AOS grapevine gene candidate was obtained and used to BLAT search (BLAST-Like alignment tool, http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat) in the Grape Genome Browser bioinformatics tool by Genoscope. The complete sequence retrieved from the Genoscope database was used as a reference to clone the AOS gene from the grapevine cv *'Sauvignon blanc'*.

Identification of grapevine HPL genes

Nucleotide sequences of previously identified HPL genes from other plant species were used in BLAST and BLAT alignments via the Genoscope grapevine database (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/ blast.pl) in order to identify HPL gene homologues in the grapevine genome. The identified HPL gene homologue sequences were analysed using the web-based GenScan software (http://genes.mit.edu/ GENSCAN.html), to identify intron-exon regions within the coding sequence. Identification of the HPL gene homologue in grapevines was established and carried out by Andriy Podolyan and Jackie White, a PhD student and staff researcher from The Chris Winefield Research Group (2010). The full method for identifying these genes can be referred to in Podolyan's thesis (Podolyan, 2010).

2.2.2 Primer design

Primer pairs were designed to isolate the putative VvAOS gene from grapevines based on the sequence data retrieved from the Genoscope database. VvAOS primers were developed using the Primer3 Plus program (Untergasser et al., 2012) with the addition of an extra "CACC" nucleotide residue at the 5' end of the forward primer to accommodate direct cloning into the pENTR/D-TOPO (Life Technologies, NZ) plasmid. The Primer3 Plus program was used in its default settings except for primer size (Min: 20, Opt: 22: Max 26) and primer Tm (Min: 60, Opt: 63, Max: 65). Primer pairs selected

by Primer3 Plus were then tested for specificity using the NCBI Primer-BLAST algorithm against the Refseq mRNA database, and limited to *Vitis vinifera* (Altschul et al., 1990). Primer synthesis was carried out by Integrated DNA Technologies, Inc. (Custom Science Ltd, NZ). Primer sequences for the amplification of a putative VvAOS gene are shown in table 2.1. Grapevine HPL (VvHPLs) primers were designed based on the sequences previously identified using the Genoscope database by Andriy Podolyan and Jackie White (Podolyan, 2010). Primer sequences for VvHPLs gene amplification also shown in table 2.1.

Gene ID	Forward primer (5' – 3')	Reverse primer (5' – 3')	Amplicon (bp)
VvAOS	*CACC*ATGGCGTCCCCTTCTCTAACTTTC	**TCA**AAAACTGGCTCGCTTTA	1563
VvHPLA	*CACC*ATGTTGTCTTCCACGGTCATG	**TCA**GTTAGCTTTCTCAACGGCGG	1461
VvHPLB	*CACC*ATGTCATCCTTGTCTTCTTCTTC	**TCA**AGTGTAACTGGACTTGGTCA	1452
VvHPLC	*CACC*ATGTCATCCTCGTCTTCTTCTTC	A**TCA**AGTGTAACTGGTCTTGGTCA	1452
VvHPLD	*CACC*ATGTCCTCTTCTTCTTCCTCTCTTC	**CTA**CGATACGTGCGTAATTGACTT	1497
VvHPLE	*CACC*ATGTCCTCTTCTTCTTCCTCTCTTC	**CTA**CGATACGTGCTTAATTGACTTG	1497
VvHPLF	*CACC*ATGTCTTCATCTTCTGATAAAAACGA	**CTA**ACTGTCCGTGGCCTTG	1464

Table 2.1 Oligonucleotide PCR primers used to amplify predicted grapevine HPLs
For protein localization purposes, VvCYP74 gene members were amplified using forward primers in which stop codon (bold) was removed.

2.2.3 Plant DNA Extraction

Genomic DNA (gDNA) was extracted from young leaves of *Vitis vinifera* L. cv *Sauvignon blanc* and used as a template for PCR amplification. Young leaves (approximately 2.5-3 cm long) were collected from the Lincoln University research vineyard (Lincoln University, NZ). Tissue material was snap frozen in liquid nitrogen (N_2), ground, and stored at -80°C until used for gDNA extraction. Approximately 100 mg of ground leaf tissue was use as starting material for grapevine genomic (gDNA) extraction using a NucleoSpin Plant II kit by Macherey-Nagel (Norrie Biotech, NZ). The protocol for gDNA extraction followed the manufacturer's instructions. Plant genomic DNA was eluted into a 1.7 mL micro-centrifuge tube and was quantified using a Qubit fluorometer (Life Technologies, NZ).

2.2.4 Grapevine CYP74 genes amplification and cloning

Amplification of the grapevine allene oxide synthase gene

The grapevine AOS gene candidate (putative VvAOS) was amplified via the PCR method using 50 ng of *Vitis vinifera* L. cv *Sauvignon blanc* gDNA as a template. High-fidelity PrimeSTAR HS DNA polymerase by Takara Bio (Norrie Biotech, NZ) was used to amplify the putative VvAOS gene fragment. The PCR reaction was performed according to the standard method suggested by the manufacturer. Amplification was carried out using a 50 µL PCR reaction in a GenePro thermocycler (Bioer Tech, NZ) under the following conditions: initial denaturation: 98°C for 10 s, followed by 35 cycle of

denaturation: 98°C for 15 s, annealing: 60°C for 30 s, extension: 72°C for 1.5 minutes and final extension: 72°C for five minutes. PCR products were separated through electrophoreses using 1% agarose gel in 1xTBE buffer [TBE buffer was prepared according to the protocol described by Sambrook and Russell (2001)] and run under 90 V of power for 40 minutes. The expected size of the putative VvAOS amplicon, approximately 1.5 kb, was excised from the agarose gel and purified using the AxyPrep DNA Gel Extraction kit (RayLab, NZ) following the protocol provided by the manufacturer. The purified PCR product was quantified using a Qubit fluorometer (Life Technologies, NZ) according to instructions supplied by the manufacturer. Amplicons of putative VvAOS were cloned into pENTR/D-TOPO (Life Technologies, NZ) following the standard protocol provided by the manufacturer for TOPO-based cloning.

The newly ligated products were transformed into chemically competent *Escherichia coli* (*E.coli*) dH5α cells according to the method described by Sambrook and Russell (2001). Approximately 100 µL of a mixture of newly transformed dH5α competent cells were grown on a Luria Bertani (LB) agar plate (solid media containing 1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, 1.5% bacto-agar, pH 7.5) containing 50 µg/mL of kanamycin as an antibiotic selection marker and incubated at 37^{o}C for 16-18 hours. Single transformed colonies were inoculated in 3 mL of LB broth (1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, pH 7.5) containing 50 µg/mL of kanamycin and incubated at 37^{o}C in the rotary shaker for 16-18 hours at 250 rpm. Cells were harvested by centrifugation at 5000xg for five minutes and a mini-plasmid extraction was carried out using standard protocols, as described by Sambrook and Russel (2001). Purified pENTR/TOPO-D carrying the VvAOS gene (pENTR:VvAOS) plasmid were sequenced using universal M13 forward/reverse primers, (ReadyMade primers by Integrated DNA Technologies, Singapore) which bound to specific sites on the flanking region of the DNA insert. Approximately 200 ng of pENTR:VvAOS plasmid and 5 pmol of primer in a 7.5 µL final volume was used per sequencing reaction. Sequencing reactions were carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Big-Dye chemistry and with subsequent separation and detection on an ABI Prism 3130*xl* Genetic Analyser (Life Technologies Ltd, NZ) by the Department of Bio-Protection, Lincoln University, New Zealand. Sequencing results were analysed with Lasergene SeqMan Molecular Biology software by DNASTAR Inc (DNASTAR inc, Madison, USA).

Grapevine hydroperoxide lyase genes

Grapevine HPL (VvHPL) was amplified using the primer pairs listed in table 2.1. Complementary DNA (cDNA) was used as a template for HPL amplification via the PCR method and was prepared by pooling cDNA from berries of eight developmental stages. Taq DNA polymerase by Qiagen (Bio-Strategy Ltd, NZ) was used with the final concentration of reagents in a 50 µL PCR reaction: 1× CoralLoad PCR Buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.2 µM of each primer, 2.5 units of Taq DNA Polymerase and 2 µL cDNA. The PCR reactions were carried out using iCycler thermocycler

(BioRad, NZ) with the following PCR parameters: 1 cycle at 94°C for 3 min, 40 cycles (94°C for 15 s, 50-58°C for 15 s, 72°C for 30-180 s) and final extension at 72°C for 5 min. The PCR products were then separated on 1% agarose electrophoresis gel. The expected sized DNA fragments were excised, gel-purified, sequenced and cloned into pENTR/D-TOPO (pENTR:VvHPLs). In this experiment, gene identification, cloning and construction of pENTR:VvHPLs plasmid vector were prepared by Jackie White, a staff researcher from The Chris Winefield Research Group (2010).

2.2.5 Phylogenetic tree development

Multiple alignments and cladograms of CYP74 family members were generated using the software program "Multiple Alignment using Fast Fourier Transform" or MAFFT version 7 (Katoh and Standley, 2013) software program. The predicted amino acid sequence of VvAOS was generated from the putative VvAOS DNA sequence using the Seqbuilder program within the Lasergene molecular biology software suite (DNASTAR Inc, Madison, USA). The sequences for other CYP74 plant species were identified based on previously published results (Howe and Schilmiller, 2002; Mei et al., 2006; Stumpe and Feussner, 2006; Kongrit et al., 2007; Pajerowska-Mukhtar et al., 2008; Stumpe et al., 2008; Wu et al., 2008; Podolyan, 2010; Podolyan et al., 2010; Zhu et al., 2012) their amino acid sequences were retrieved from the NCBI database (Table 3.3).

2.2.6 Karyotype development

The putative VvAOS location within the grapevine chromosome was predicted using the Ensembl Plants *Vitis vinifera* karyotype software application available on their website (plants.ensembl.org/Vitis_vinifera/Location/Genome, assessed in 2014). A putative VvAOS nucleotide sequence was converted to a BED data file format using only three of the required columns (chromosome number, start position, stop position) before being uploaded into the Ensembl Plants *Vitis vinifera* karyotype website. The output from the Ensembl website was then saved in the PNG format for ease of viewing.

2.2.7 Gene localization via green fluorescent protein (GFP) fusion in *Nicotiana benthamiana*

Binary vector construction for the localization of grapevine CYP74 gene family members

The binary vector, pB7FWG2 (Karimi et al., 2002), carrying CaMV 35S as a gene promoter and containing an N-terminal GFP fusion protein as a gene marker was used to uncover the sub-cellular localization of the VvCYP74 protein in *Nicotiana benthamiana.* For VvCYP74 protein localization purposes, due to the pB7FWG2 system carrying an N-terminal GFP fusion, all VvCYP74 gene members were amplified with a reverse primer minus a stop codon (refer to table 2.1, but stop codon was removed) and cloned into pENTR/TOPO-D (Life Technologies, NZ). Approximately 150 ng of pENTR

plasmid carrying VvCYP74 gene members (pENTR:VvCYP74) was added to 300 ng of the pB7FWG2 binary vector to perform an LR recombination reaction. A Gateway LR Clonase Enzyme Mix kit by Invitrogen Inc. (Life Technologies, NZ) was used to complete the recombination, following the protocol of the standard reaction as suggested by the manufacturer. Approximately 2 µL of the completed LR reaction mixture was added to 100 µL of chemically competent cells for plasmid transformation into *E.coli* (DH5α). The method for plasmid transformation into *E.coli* and the preparation of competent cells followed the standard protocol, as described by Sambrook and Russell (2001). Newly transformed *E.coli* cells with the binary vector carrying the putative VvCYP74 genes was selected using LB plates (Section 2.2.4) containing 50 µg/mL spectinomycin as an antibiotic selection marker. A mini-plasmid preparation for the newly constructed plasmid was carried out following to the standard protocol, as described by Sambrook and Russell (2001). Integration of the VvAOS gene into the pB7FWG2 binary vector (pB7FWG2:VvCYP74) was assessed via the PCR method using a pART 35S primer (forward primer located at 137 bp from 3'end of CaMV 35S promoter sequence) and the respective VvCYP74 gene reverse primer.

Transient agrobacterium mediated transformation of Nicotiana benthamiana leaves

Approximately 2 µL (10-50 ng) of the pB7FWG2:VvCYP74 vector plasmid was added to 100 µL of an *Agrobacterium tumefaciens* (Agrobacterium) cell culture and incubated on ice for five minutes. The mixture of Agrobacterium cells and pB7FWG2:VvCYP74 vector plasmid was transferred to a sterile ice-cold electroporation cuvette for transformation. Agrobacterium cell preparation for transformation and electroporation transformation of Agrobacterium followed the standard protocol described in the Agrobacterium protocol (Wise et al., 2006). Transformed Agrobacterium with pB7FWG2:VvCYP74 was assessed via a colony PCR method using combination pART 35S (forward) and the respective VvCYP74 gene reverse primer.

A fresh, single colony of Agrobacterium, transformed with pB7FWG2:VvCYP74 (mediated-Agrobcaterium), was inoculated in 3 mL LB media containing 50 µg/mL spectinomycin as an antibiotic selection marker and incubated in a shaking incubator at 28°C, 250 rpm for two days. About 100 µL of the fresh Agrobacterium culture was used to inoculate 50 mL LB media containing 50 µg/mL of spectinomycin in a 500 mL flask and incubation was continued in a shaking incubator at 200 rpm, 28°C until the cells grew to an OD600 of between 0.8 - 1.0. Agrobacterium cells were harvested by centrifugation at 5000xg for 20 min at room temperature and re-suspended to OD600 = 0.2 in re-suspension medium (Full strength MS medium with vitamins, 3% (w/v) sucrose and 150 µM acetosyringone, pH 5.8) and the culture incubated under the same conditions for an additional two hours.

Using a 1 mL syringe, approximately 100 µL of the mediated-Agrobacterium culture was infiltrated into the underside of an attached young *N. benthamiana* leaf (approximately 3 cm x 1.5 cm

leaf size). The control leaf was infiltrated with a wild type Agrobacterium culture. Inoculated plants were grown for an additional three days at 25°C with 16 hours light/day. Three days after agro-infiltration, the leaf was removed from the plant and kept in a covered petri dish containing moist filter paper to maintain the fresh leaves. The infiltrated leaves were sectioned by hand, set-up on microscope slides and observed under a confocal microscope (Leica TCS SP5 system by Leica Macrosystems, Bio-strategy Ltd, NZ) for transient VvCYP74 protein sub-cellular localization in plant cell compartments. Localization of GFP fluorescence was observed under 501 to 551 nm (green colour) wavelengths, whereas chlorophyll autofluorescence was detected at 621 to 701 nm (red colour) wavelengths. Images shown on the confocal microscope were documented and analysed via the Leica Application Suite Advanced Fluorescence lite version 2.6 software (Leica Microsystem, Germany).

2.2.8 Trial plant conditions

All grapevine cv *Sauvignon blanc* materials were sourced from three different locations: Booker vineyard (Brancott Estate, Marlborough, NZ), Lincoln University research vineyard (Lincoln University, Canterbury, NZ) and a wounding experiment grown in the glasshouse at Lincoln University, in September 2013.

2.2.9 Expression of CYP74 genes in grapevine tissues

Samples from a range of grapevine tissues (i.e. roots, young leaves, tendrils, inflorescences, seeds, pulp and skins) were collected, snap frozen in liquid N_2 and stored at -80°C before RNA extraction, as described in Section 2.2.11

2.2.10 Grapevine leaf wounding

In order to examine grapevine responses to threats, we used young grape leaves as tissue samples and wound treatment to stimulate plant responses. Wound treatment of leaves tissues represents a critical stress to which a plant is commonly exposed in nature (Brilli et al., 2011). Furthermore, mechanical wounding clearly induces expression of many genes that function in defence against threats (de Bruxelles and Roberts, 2001). Wound treatment in plants causes changes in gene expression that contribute to tissue defence and repair (Reymond et al., 2000). Plant respond to wounding by initiating an array of different defences regulated by complex networks of inter- and intracellular signalling pathways (de Bruxelles and Roberts, 2001). As a consequence, wounding is often used as an experimental procedure to investigate plant defence response to their threats (de Bruxelles and Roberts, 2001).

Experimental design of wounding trial

Fruiting cuttings were used as a source of plant material for the wounding experiment. Hardwood cuttings six nodes long were collected from dormant canes and stored at 4°C until use. The canes were obtained from *Vitis vinifera* cv. *Sauvignon blanc* located at the Lincoln University research vineyard, Canterbury, NZ. Fruitful cuttings were grown according to the method described in Mullins and Rajasekaran (1981). In order to initiate the root growth from the hardwood cutting, each cane was cut transversally just below the level of the lowest bud and placed in a tray containing pumice (1-4 mm grade) approximately 8-10 cm into the substrate. Trays were then placed in the shade house with an electric hot-pad to maintain the 'soil' temperature at between 24-26 ^{o}C. Air temperature was maintained between 4 to 10 ^{o}C. Under these conditions, root growth was promoted but bud formation

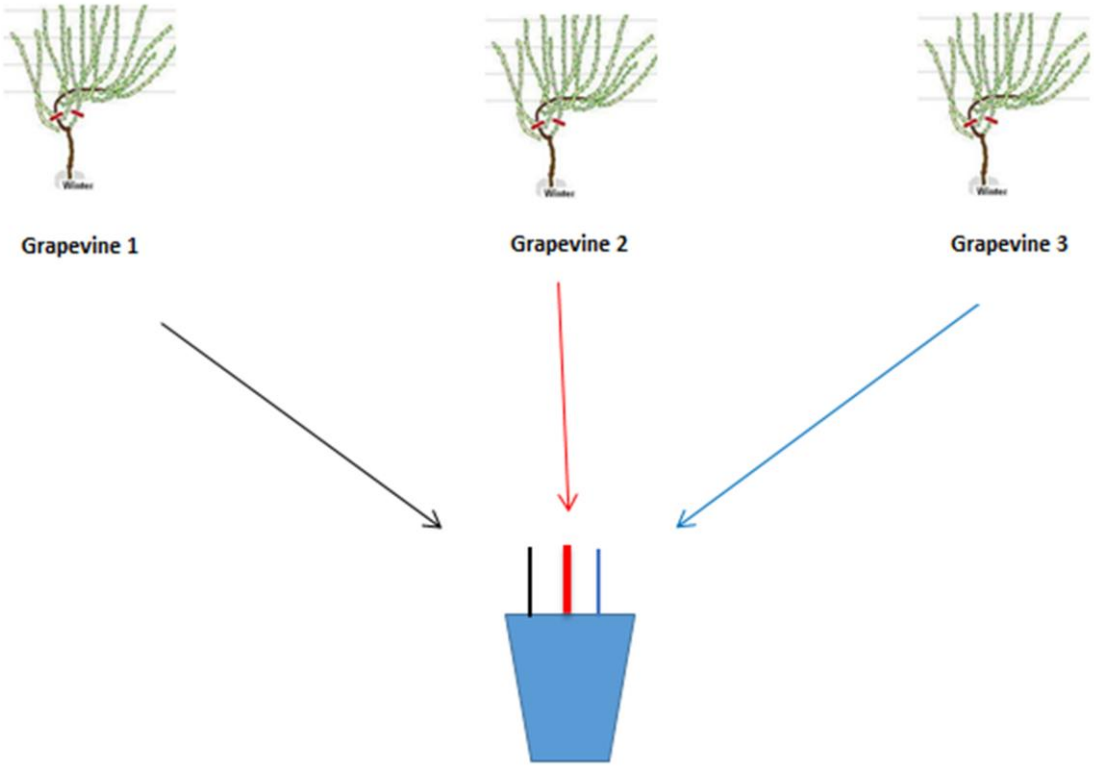

Figure 2.1 Mullins vine combination plants
Combination of grapevines in one pot diagram illustrates that each pot comprises three shoots taken from three different sources of mother plant. Hardwood cuttings were grown based on the standard method by Mullin (Mullins and Rajasekaran, 1981)

was suppressed. Trays were regularly watered and monitored for approximately four week before transferred into plastic pots (PB8 plastic pot by Egmont Commercial, NZ) filled with potting mix in the Lincoln University plant nursery (Lincoln University, NZ). Nursery temperature environment was at 25 ^{o}C during the day and 18 ^{o}C during the night. Potting mix used contained with 800 L of composted bark and 2 L pumice (supplemented with 2 kg of fertilizer (Osmocote: 16-3.9-10 NPK), 1 kg agricultural lime, 1 kg hydraflo (Scott Australia Pty Ltd, Aus.). The experiment was set up so that each 4 L pot contained three canes originating from three different mother-plants, as illustrated in figure 2.1. Approximately

42 pots were prepared for this experiment to accommodate the number of wounded and control replicates needed.

Wound treatment of leaves

Without being separated from the plant, young leaves (approximately 2.5 – 3 cm long) were subjected to mechanical wounding by pressing a pair of small pliers against the leaf to induce a wound response. The wounded leaves were then collected at different time intervals - 0 (pre-wound), 0.5, 1, 3, 6, 12 and 24 hours after wounding. For each time point collection, leaves were collected from all three shoots in a pot and immediately snap-frozen in liquid N_2 before being stored at -80°C. Pre-wounded leaves also were collected prior to wounding as an untreated experimental control (labelled as zero hour). Three sets of wounded leaf samples were collected for each time point over the following three days to serve as experimental biological replicates. In order to minimize the volatile organic compound (VOC) effect released from wounded plants to their neighbours over the three-day experiment, each group of wounded plants (biological replicates) were separated approximately 10 metres away from the non-wounded plants. Each set of biological replicate were separated for 12 hours in order to minimize the effect of VOC response occur in plants through the atmosphere.

2.2.11 Total RNA extraction and cDNA synthesis

Frozen leaves were ground to a fine powder in the presence of liquid N_2 with a cold mortar and pestle for RNA extraction. Approximately 100 mg of each sample was used for total RNA extraction using the Spectrum Plant Total RNA kit (Sigma-Aldrich, NZ) following the instructions provided by the manufacturer. Total RNA DNase I treatment was performed on column following to instruction described by the Spectrum Plant Total RNA kit manufacturer (Sigma-Aldrich, NZ). The extracted RNA concentration was measured using a Qubit® fluorometer with the Qubit® RNA buffer and dye (Life Technologies Ltd, NZ), after calibration using the standards supplied by the manufacturer. RNA integrity was verified by running total RNA samples on a 1.5% denaturing agarose gel, as described by Sambrook and Russell (2001), and visualized by UV excitation of ethidium bromide on a BioRad GelDoc XR System (BioRad Laboratory Pty Ltd, NZ). Total RNA quality was assessed using a DeNovix DS-11 spectrophotometer (DeNovix Inc, Wilmington, USA) where both the spectrum (λ=200nm-300 nm) and individual absorbances were recorded (λ=230 nm, 260 nm and 280 nm). The 206/280 nm and 230/260 nm ratio were used to estimate total RNA purity. Ratio 260/280 nm was used to indicate protein contamination (a value lower than 1.8 indicated significant protein contamination) whereas, 230/260 ratio value ≈ 2.1 indicated a good quality of total RNA. Complementary DNA (cDNA) was synthesized from total RNA using a PrimeScript Reverse Transcriptase kit by Takara Bio Inc (Norrie Biotech, NZ) following the protocol provided by the manufacturer. Each total RNA sample was assessed for gDNA contamination via the PCR method using actin as a target gene. Approximately 500 ng of total RNA was used in a 10 µL reaction to synthesize cDNA and this was, subsequently, diluted 20-fold with

UltraPure Dnase/Rnase-Free Distilled Water (Life Technologies, NZ). Newly synthesized cDNA was assessed for gDNA contamination using a PCR approach with actin as the target gene (primer sequences used to assess for gDNA contamination are listed in Appendix A.1). For contamination assessment of both total RNA and cDNA synthesis, actin qPCR primers were used and the PCR program was followed according to the parameters described in section 2.2.4 except for the extension time was shorten to 30 s. Actin qPCR primers (target actin gene in grapevines) were designed across an intron, so that any genomic contamination would show as a larger DNA fragment (≈ 166 bp) appearing from the expected 82 bp amplified from the cDNA template.

2.2.12 Quantitative Real Time PCR

Quantitative Real Time PCR (qRT-PCR) was performed on cv. *Sauvignon blanc* cDNA synthesized from selected grapevine tissues and wounded leaves. Relative expression assays of VvAOS and VvHPL transcripts were carried out using an Eco™ Real-Time PCR System by Illumina (Illumina, dnature Ltd, NZ) with Eco software version 5.0.16.0. An Eppendorf epMotion 5070 liquid handling robot (Eppendorf, NZ) was used to aliquot the cDNA template and master mix into the Eco qRT-PCR plates (Illumina, dnature, NZ). In the qRT-PCR assessment, all cDNA samples used were synthesized from 500 ng of total RNA of each samples (Section 2.2.11). Primer pairs (qRT-PCR primers) were used to assess transcript abundance of each target gene (as shown in Appendix A.1). A 10 µL volume for the qRT-PCR reaction consisted of 4 µL of cDNA samples, 6 µL of master mix [0.2 µL of 10 µM of each forward and reverse primers, 5 µL of 2x SYBR Premix Ex Taq™ II (Tli RNase H Plus)] by Takara Bio Inc. (Norrie Biotech, NZ) and 0.6 µL of UltraPure DNase/RNase-Free Distilled Water (Life Technologies, NZ)]. UltraPure DNase/RNase-Free Distilled Water (Life Technologies, NZ) replaced the cDNA template as a non-template control. Real-time PCR was carried out, as follows: polymerase activation: 95°C for 60 s followed by 35 cycles of 2-step PCR, denaturation at 95°C for 15 s and annealing at 62°C for 30 s, melting curve denaturation at 95°C for 15 s, annealing at 55°C for 15 s, denaturation again at 95°C for 15 s and final step was an incubation at 40°C for 30 s. Since qRT-PCR was preformed using 2-step PCR, the fluorescent signal was measured at the end of the annealing step (at 62°C for 30 s) of each PCR cycle to determine the relative change in amplified products.

For each assay, a standard curve was prepared using serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) of plasmid containing a clone of the gene target. The standard curve was created based on eight points of a 10-fold dilution starting from 1 ng/µL of plasmid concentration. Relative analysis was carried out using Actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference genes (Reid et al., 2006). Each gene assay (including reference genes) was performed in triplicate. However, for consistency purposes for qRT-PCR analysis in grapevine tissue types, threshold quantification cycle (Cq) values over 30 were not included in quantification calculation analysis but just indicated as detected but not-quantified (NQ). Samples with did not produce Cq value after 35 cycles

were labelled as not-detected (ND). The list of primers used for the qRT-PCR quantification assay is shown on Appendix A.1. Whereas results data were presented as an amount of gene copy numbers. Concentration of DNA amplicons in nanogram per microliter (ng/µL) which were derived from standard curve were converted to copy number detected followed to the formula shown below:

$$\text{Number of copies (molecules)} = \frac{X\ (ng) * 6.0221 \times 10^{23}\ \text{molecules/mole}}{(N * 660\ \text{g/mole}) * 1 \times 10^{9}\ \text{ng/g}}$$

where X = amount of amplicon (ng), N = length of dsDNA amplicon and average mass of 1 bp dsDNA used = 660 g/mole. Conversion formula was adapted from Integrated DNA Technologies Inc. (http://sg.idtdna.com/site). Statistical analysis for significant differences among the two sets of data, wound treated and control samples, were analysed via a simple T-Test by MS Excel 2013 (Microsoft Office 2013, USA). Simple T-Test analysis was carried out based on these attributes: wounded data, control data, two tails with assumption of unequal variance and critical value = 0.05 (P value = 0.05).

2.3 Results

2.3.1 Characterization of a putative allene oxide synthase gene in grapevines

Using published AOS sequence data and interrogation of resources at the National Center for Biotechnology Information (NCBI) and Vitis genome resources held at Genoscope (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/, assessed in 2010), a pair of VvAOS primers were designed to amplify a putative allene oxide synthase gene from grapevines. This primer pair was used to amplify AOS from grapevine genomic DNA. Given the lack of available data referring to AOS transcription and because the genomic copy of AOS appeared to be intron-less, genomic DNA was chosen as a template rather than RNA/cDNA. However, a putative VvAOS gene from *Vitis vinifera* was confirmed as intron-less via PCR amplification using cDNA as a template and the amplicon was sequenced. Putative VvAOS polynucleotide sequences amplified from gDNA and cDNA templates displayed as 100% identical (Appendix C.1).

A single amplicon of approximately 1.5 kb was amplified (Figure 2.2), cloned into pENTR/D-TOPO and sequenced for analysis. The full sequence of the putative VvAOS obtained consisted of a single open reading frame of 1563 bp, encoding a polypeptide of 520 amino acids, and with a molecular mass of 58.13 kDa. BLAST (Altschul et al., 1990) and BLAT (BLAST-like alignment tool, http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis, accessed in 2014) search tools were used to determine the similarity of the clone sequence amongst other AOS genes. A BLAST search on the NCBI database (attribute, database: Refseq_rna, organism: *Vitis vinifera*, optimize for highly similar sequences) showed that the nucleotide sequence of the putative VvAOS gene was 99% identical to the predicted *Vitis vinifera* AOS gene (accession: XM_002283744). Whereas, a BLAT search on the grape genome browser [Database: Grape genome (12X), accessed in 2014] showed the putative VvAOS gene was 99.9% identical to an intron-less partial region of the grapevine genome at chromosome 18 (9911314:: 9912876), as illustrated in the assembled karyotype (Figure 2.3). The putative VvAOS gene sequence did not match 100% with sequences available in the NCBI and Genoscope databases, probably due to the different source of the grapevine variety used. The putative VvAOS was sequenced from *Sauvignon blanc* but the sequences available in both databases were sequenced from *Pinot noir* variety.

On the polypeptide level, the deduced polypeptide sequence of putative VvAOS was 72% identical to *Hevea brasiliensis* (Norton et al., 2007); 73% to *Lonicera japonicaI*, (Jiang et al., 2009); 69% to *Glycine max* (Wu et al., 2008); and 68% to *Solanum tuberosum*, (Pajerowska-Mukhtar et al., 2008). Further interrogation indicated that a putative VvAOS polypeptide sequence was incorporated with approximately 47 amino acid residues of predicted chloroplast transit peptide (cTP) regions, as prediction analysis was carried out via ChloroP1.1 (Emanuelsson et al., 1999), TargetP1.1 (Emanuelsson et al., 2000) and iPSORT (Bannai et al., 2002), a heme-binding site conserved domain (xPxxxNKQCxGKD)

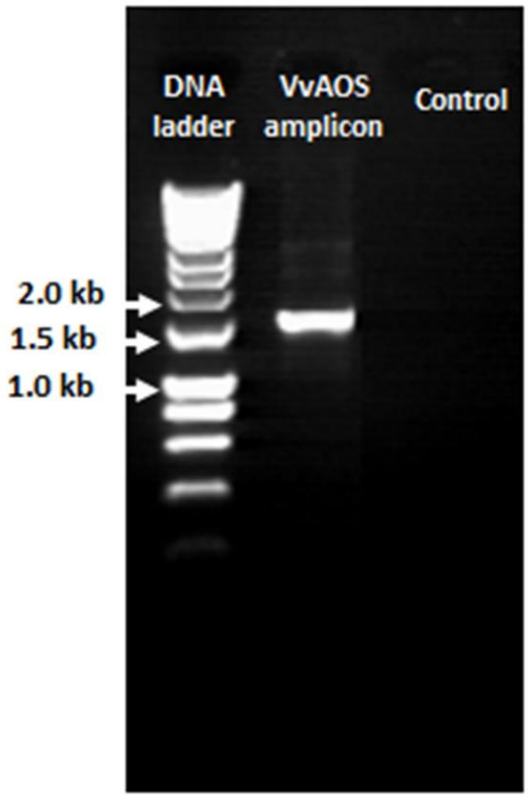

Figure 2.2 PCR amplification of a putative VvAOS gene from the grapevine genome
Putative VvAOS gene amplified from *Sauvignon blanc* genomic DNA as a template. The DNA ladder used to measure amplicon size was HyperLadder I (1 kb) from Bioline (Total Lab System, NZ).

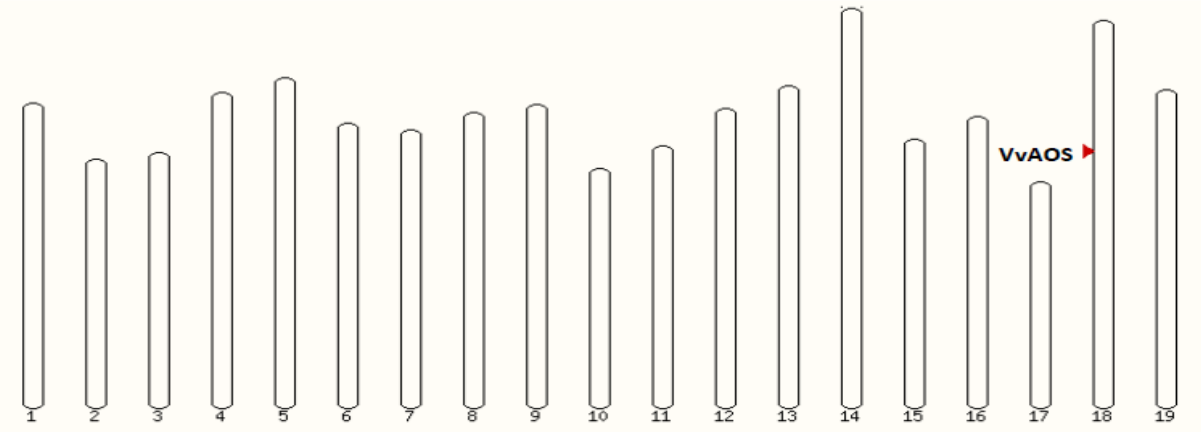

Figure 2.3 Location of VvAOS gene
Karyotype was generated on Ensemble website using the sequence location collected from the 0NCBI database.

```
        M  A  S  P  S  L  T  F  P  S  L  Q  L  Q  F  P  T  H  T  K
1       atggcgtccccttctctaactttcccttccctgcaactacaattcccaacacacacaaaa   60
        S  S  K  P  S  K  H  K  L  I  V  R  P  I  F  A  S  V  S  E
61      tcatctaagccatccaagcataagctcattgttcgcccgatatttgcctctgtttctgag  120
        K  P  S  V  P  V  S  Q  S  Q  V  T  P  P  G  P  I  R  K  I
121     aaaccatcggtaccggtttctcagtctcaggtgacgccccccggtccaatcaggaaaatt  180
        P  G  D  Y  G  L  P  F  I  G  P  I  K  D  R  L  D  Y  F  Y
181     cccggagattatggtctccctttcatcggtcccataaaagatcgtcttgattatttctat  240
        N  Q  G  R  E  E  F  F  R  S  R  A  Q  K  H  Q  S  T  V  F
241     aatcaaggcagagaagagttcttcaggtccagagcccagaaacaccagtcaaccgtgttc  300
        R  S  N  M  P  P  G  P  F  I  S  S  N  S  K  V  I  V  L  L
301     cggtccaacatgccacccggccctttcatctcctccaactccaaagtcatcgttttactg  360
        D  G  K  S  F  P  V  L  F  D  V  S  K  V  E  K  K  D  V  F
361     gatggaaagagttttcctgtactctttgacgtttccaaagttgaaaaaaaggacgttttc  420
        T  G  T  F  M  P  S  T  E  F  T  G  G  F  R  V  L  S  Y  L
421     accggaactttcatgccctccaccgaattcaccggcggattcagagttctttcctatctc  480
        D  P  S  E  P  D  H  T  K  L  K  R  L  L  F  F  L  L  Q  S
481     gatccatccgagcccgatcacaccaaactcaagcgcctcctcttcttcctcctccagtcc  540
        S  R  D  R  I  I  P  E  F  H  S  C  F  S  E  L  S  E  T  L
541     agccgcgacaggatcatcccagagttccattcctgcttctccgagctctccgagaccctt  600
        E  S  E  L  A  A  K  G  K  A  S  F  A  D  P  N  D  Q  A  S
601     gaaagcgaactcgcagcaaaaggcaaagccagtttcgccgaccctaacgatcaggcatcc  660
        F  N  F  L  A  R  A  L  Y  G  T  K  P  A  D  T  K  L  G  T
661     ttcaactttcttgctcgcgctctctacggcaccaagccggctgataccaaactgggtact  720
        D  G  P  G  L  I  T  T  W  V  V  F  Q  L  S  P  I  L  T  L
721     gacgggcctggcttaatcacgacatgggttgtcttccagttgagtcccatcctcactcta  780
        G  L  P  K  F  I  E  E  P  L  I  H  T  F  P  L  P  A  F  L
781     ggcctacccaagtttatagaagaacccctatccacacttttccactcccggcatttctg   840
        A  K  S  S  Y  Q  K  L  Y  D  F  F  Y  D  A  S  T  H  V  L
841     gctaaatcaagttaccagaagctctatgacttcttctacgacgcgtcaactcatgttctg  900
        D  E  G  E  K  M  G  I  S  R  E  E  A  C  H  N  L  L  F  A
901     gacgaaggtgagaagatggggatatcaagagaggaagcttgccacaacctccttttcgcc  960
        T  C  F  N  S  F  G  G  M  K  I  I  F  P  T  I  L  K  W  V
961     acgtgctttaattccttcggagggatgaaaatcatctttccaacaattctcaaatgggtc  1020
        G  R  G  G  V  K  L  H  T  Q  L  A  Q  E  I  R  S  V  V  K
1021    ggtcgaggaggagtgaaactgcacacccaattagcccaggagattagatctgtcgtcaaa  1080
        S  N  G  G  K  V  T  M  A  S  M  E  Q  M  P  L  M  K  S  T
1081    tccaacggcggaaaagtgaccatggcgtccatggagcagatgccgctgatgaagtctact  1140
        V  Y  E  A  F  R  I  E  P  P  V  A  L  Q  Y  G  K  A  K  Q
1141    gtatacgaagccttccggatcgaacccccctgtcgcattgcagtacggcaaggcgaagcag  1200
        D  L  V  I  E  S  H  D  S  V  F  E  V  K  E  G  E  M  L  F
1201    gatctggtgatcgaaagccacgactctgtttttgaagtcaaagaaggtgaaatgttgttc  1260
        G  Y  Q  P  F  A  T  K  D  P  K  I  F  E  R  S  E  E  F  V
1261    gggtaccaaccgttcgccaccaaagacccgaaaatcttcgaacgatccgaagagttcgtg  1320
        P  D  R  F  V  G  E  G  E  K  L  L  K  H  V  L  W  S  N  G
1321    ccggatcggttcgtgggtgagggtgagaagctgctgaagcacgtgctctggtcaaacgga  1380
        P  E  T  E  N  P  T  L  G  N  K  Q  C  A  G  K  D  F  V  V
1381    cctgaaaccgagaatccaaccttggggaataagcagtgcgcaggtaaagacttcgtggtg  1440
        L  A  A  R  L  F  V  V  E  L  F  L  R  Y  D  S  F  D  I  E
1441    ctggccgccaggctatttgtggtggagctgttcctgcgttacgattcctttgacatcgag  1500
        V  G  T  S  L  L  G  S  A  I  N  L  T  S  L  K  R  A  S  F
1501    gttggcacgtcgctgttgggttcagccatcaatctaacctccctaaagcgagccagtttt  1560

1561    tga                                                          1563
```

Figure 2.4 Full sequence of VvAOS nucleotides and amino acids

Full sequence of allene oxide synthase gene (ORF) together with the deduced amino acid sequence amplified from *Vitis vinifera* L. Cv *Sauvignon blanc* genomic DNA. Predicted chloroplast transit peptides are underlined in red. The heme-binding domain (PxxxNKQCxGKD) is highlighted in yellow and FNxxGGxKxxxP, a highly conserved motif (helix-I) in CYP74A enzymes, is highlighted in light blue. Methionine (M in blue) indicates a possible starting codon across nucleotide sequences during translation.

(Maucher et al., 2000) and a helix-I domain (FNxxGGxKxxxP), a highly conserved motif among the CYP74A enzyme sub-group (Chapple, 1998; Pajerowska-Mukhtar et al., 2008). The presence of these conserved domains was a further indication of possible AOS gene function in grapevines (Figure 2.4).

2.3.2 Phylogenetic relationship of CYP74 family members

As mentioned in the previous chapter (Section 1.5), CYP74 family members are classified based on which specific substrate (9-, 13- or 9/13-hydroperoxide) they utilized. In order to predict the biochemical function of the putative AOS gene from grapevines (referred to as putative VvAOS from now on), a multiple sequence alignment analysis at polypeptide level between VvAOS and AOS amino acid sequences previously identified from other plant species (Table 2.3) was carried out and interrogated. Similarity data presented on the cladogram diagram, indicated polypeptide sequence interrelationships and predicted the putative VvAOS sub-family group. Characterized amino acid sequences of CYP74 family members from other plant species (Table 2.3) were retrieved from the NCBI database based on published data sequences (Howe and Schilmiller, 2002; Mei et al., 2006; Stumpe and Feussner, 2006; Kongrit et al., 2007; Pajerowska-Mukhtar et al., 2008; Stumpe et al., 2008; Wu et al., 2008; Podolyan, 2010; Zhu et al., 2012).

Current research in our group (the Winefield Research Group, Lincoln University, NZ) has identified another gene in *Sauvignon blanc* that can be categorized as a CYP74 gene family member. This gene was identified as nucleotide sequence encoding hydroperoxide lyase (HPL) enzyme, an enzyme known to catalyse the formation of C_6 and C_9 aldehyde compounds in plants (Matsui et al., 2000; Wan et al., 2013). Therefore, as part of the partial gene identification and classification of grapevine CYP74 gene family members, all six grapevine HPLs (grapevine HPL and it variations) polypeptide sequences identified in our research group were included in this phylogenetic analysis to gain a wider understanding of CYP74 enzyme relationships in grapevines. All amino acid sequences identity of CYP74 from grapevines are shown in table 2.2.

ID	NCBI protein accession	Predicted amino acid length	Predicted Sub-family group
VvHPL-A	XP_002272991	487	CYP74B
VvHPL-B	XP_002281159	483	CYP74C
VvHPL-C	XP_002281190	483	CYP74C
VvHPL-D	XP_002281201	499	CYP74C
VvHPL-E	XP_002281213	499	CYP74C
VvHPL-F	XP_002281226	487	CYP74C
VvAOS		520	CYP74A

Table 2.2 Identify CYP74 enzymes from grapevine
Amino acid sequences of grapevine CYP74 family members previously identified and cloned within our research group.

Species	ID	NCBI amino acid accession
Arabidopsis thaliana	AtHPL	AAC 69871
	AtAOS	CAA 63266
Artemisia annua	AaAOS	ADZ 24000
Capsicum annuum	CaHPL	AAA 97465
	CaDES	ABH 03632
Cucumis melo	CmAOS	AAM 66138
	CmHPL	AAK 54282
Cucumis sativus	CsHPL1	AA F64041
Glycine max	GmAOS	NP 001236445
	GmAOS	NP 001236432
Hordeum vulgare	HvHPL	CAC 82980
	HvAOS1	CAB 86384
	HvAOS2	CAB 86383
Ipomoea nil	InAOS	BAK 52267
Linum usitatissimum	LuAOS	AAA 03353
Lycopersicon esculentum	LeHPL	AAF 67142
	LeDES	AAG 42261
	LeAOS1	CAB 88032
	LeAOS2	AAF 67141
	LeAOS3	AAN 76867
Medicago sativa	MsHPL1	CAB 54847.
	MsHPL2	CAB 54848
	MsHPL3	CAB 54849
Medicago truncatula	MtAOS	CAC 86897
	MtHPL1	CAC 86898
	MtHPL2	CAC 86899
Musa accuminata	MaHPL	CAB 39331
Nicotiana attenuata	NaHPL	CAC 91565
	NaAOS	CAC 82911
Nicotiana tabacum	NtDES	AAL 40900
Oryza sativa	OsAOS-1	AAL 17675
	OsAOS-2	AAL 38184
	OsAOS-3	AAP 75620
Psidium guajava	PgHPL	AAK 15070
Parthenium argentatum	PaAOS1	CAA 55025
Solanum tuberosum	StHPL	CAC 44040
	StDES	CAC 28152
	StAOS1	CAD 29735
	StAOS2	CAD 29736
	StAOS3	CAI 30876
	StAOS	AAN 37417
Vitis vinifera	VvHPL1	ADP 88810
	VvHPL2	ADP 88811
	VvHPL	ACZ 17394
Zea mays	ZmHPL	AAS 47027

Table 2.3 Characterized plant CYP74 family members
CYP74 enzymes retrieved from the NCBI database based on published articles by Stumpe et al (2006 and 2008), Howe and Schilmiller (2002), Podolyan (2010), Zhu et al (2012), Mei et al. (2006), Kongrit et al. (2007), Wu et al. (2008) and Pajerowska-Mukhtar et al. (2008).

Multiple alignments sequences and cladogram diagrams of CYP74 polypeptide sequences were generated using the software program "Multiple Alignment using Fast Fourier Transform" or MAFFT version 7 (Katoh and Standley, 2013). This software is available online at Computational Biology Research Consortium website (http://mafft.cbrc.jp/alignment/software/, accessed in 2015). The alignment results were used to construct a CYP74 family member cladogram using a bootstrapped neighbour-joining method algorithm with 1000 replicates. Sub-family groupings shown from the cladogram diagram were consistent with results reported in Howe et al. (2002), Stumpe and Feussner (2006) and Stumpe et al. (2008). The chloroplast transit peptide (cTP) was not as highly constrained as the functional component of the enzyme so, as a consequence, it could cause an inaccurate relationship during the production of the cladogram diagram. Therefore, the multiple alignment sequences and construction of the cladogram diagram were repeated with the predicted cTP region removed. Prediction of a cTP region to each CYP74 family member polypeptide sequence was identified using ChloroP 1.1 software (Emanuelsson et al., 1999). Results indicated that both cladogram gave similar outcomes.

The cladogram diagram, derived from the putative grapevine and previously characterized CYP74 amino acid sequences from other plant species (Table 2.3), is shown in figure 2.5. The cladograms suggested that the putative VvAOS polypeptide sequence was closely related to the CYP74A sub-family group, which also suggest that these enzymes utilized 13-hydroperoxide as a substrate. Moreoever, the interrelationships of the entire CYP74 family in grapevine clearly showed a strong separation between their sub-families, CYP74A, CYP74B, CYP74C and CYP74D. Grapevine HPL (referred to as VvHPL from now on) enzymes were segregated into two separate groups based on their proposed substrate used 9-, 13- or 9-/13-hydroperoxide. Among grapevine HPLs, only VvHPLA was grouped as a CYP74B sub-family and predicted to utilize 13-hydroperoxide as a substrate; whereas, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF were grouped in the CYP74C sub-family, which utilized either 9- or 13-hydroperoxide as a substrate.

In plants, AOS enzymes are known to be the enzyme that uses 13-hydroperoxide as a substrate to produce JA as an end product (Feussner and Wasternack, 2002; Yan et al., 2013). Therefore, we predicted that the putative VvAOS protein sequence identified might be involved in plant defence mechanisms in grapevine due to its polypeptide sequence being highly homologous to other AOS from other plant species. Furthermore, VvAOS protein was classified within 13-AOS group that the only known to be involved in JA biosynthesis. Further interrogation of the interrelationships among AOS enzymes (published sequences only) from other plant species and grapevines indicated that the putative VvAOS was closely related to flax (LuAOS), guayule (PaAOS) and sweet wormwood (AaAOS), as shown in figure 2.6.

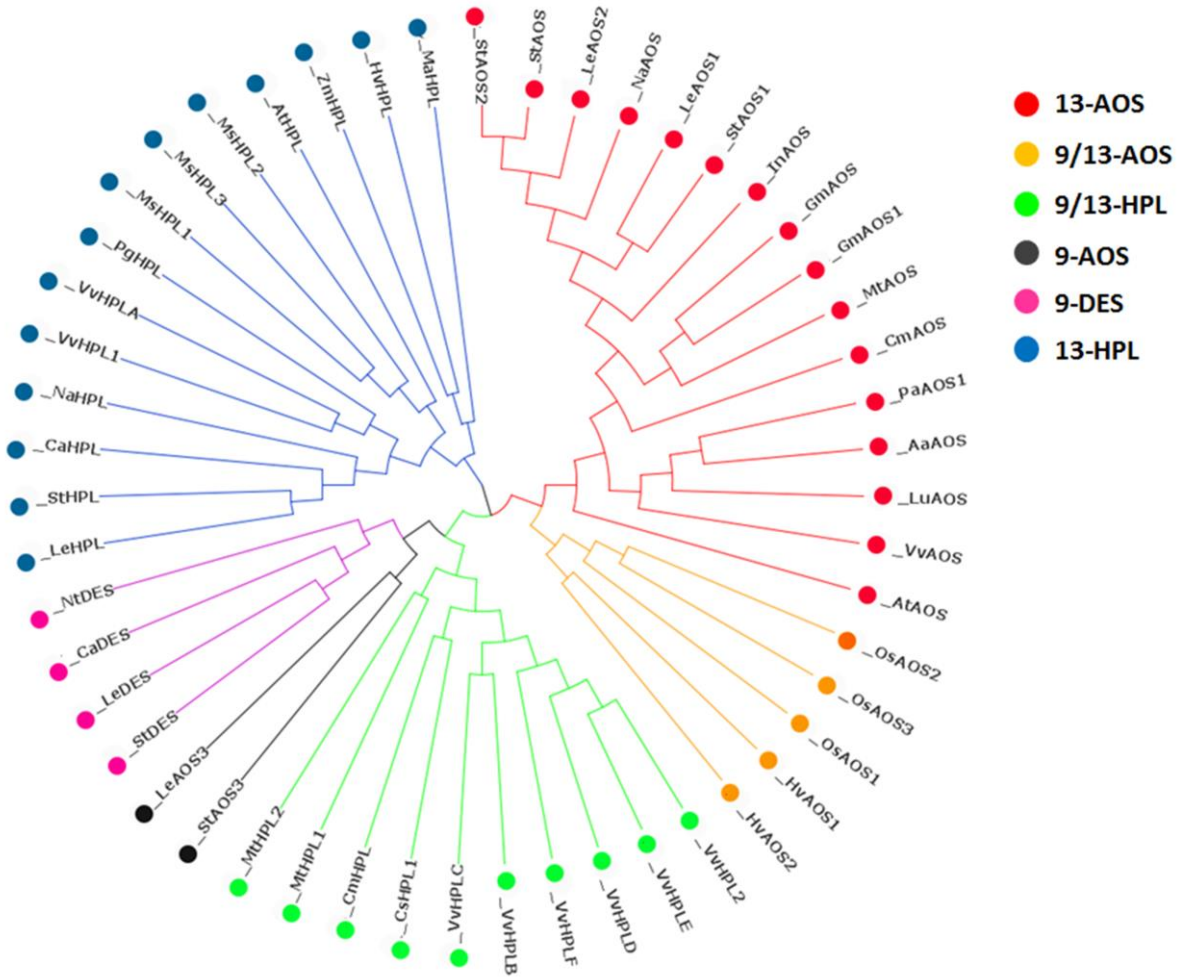

Figure 2.5 Cladogram diagram of CYP74 family members

The cladogram diagram was drawn using a bootstrapped neighbouring-joining method via MAFFT software version 7 (Katoh and Standley, 2013). CYP74 sub-family members are discriminated by node colours. Sub-family CYP74A (13-AOS) is in red, CYP74B (13-HPL) in blue, CYP74C (9/13-AOS, 9/13-HPL and 9-AOS) in yellow, green and black. The last sub-family, CYP74D (9-DES) is in magenta. The sub-family classification was adapted from Stumpe and Feussner (2006) and Stump et al. (2008).

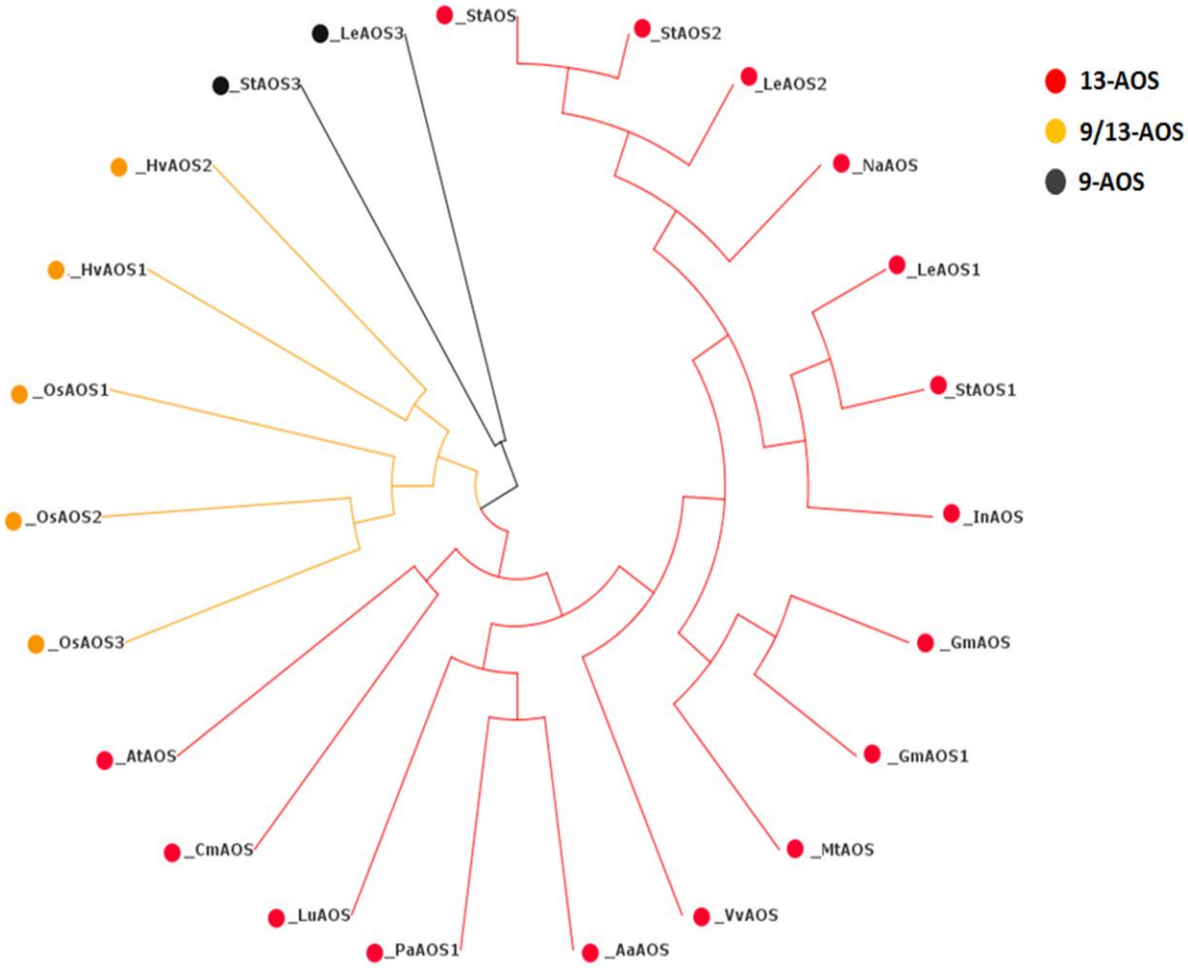

Figure 2.6 Cladogram of AOS enzymes

The cladogram diagram of AOS was drawn using a bootstrapped neighbouring-joining method via MAFFT software version 7 (Katoh and Standley, 2013). Allene oxide synthase groups are discriminated by node colours. Enzymes using 13-hydroperoxide substrate (13-AOS) is in red, 9/13-hydroperoxide substrate (9/13-AOS) in yellow and 9-hydroperoxide substrate (9-AOS) in black. Grapevine AOS (VvAOS) was grouped among enzymes using 13-hydroperoxide as a substrate (13-AOS). Classification was adapted from Stumpe and Feussner (2006) and Stump et al. (2008).

2.3.3 Subcellular localization of transiently expressed of grapevine CYP74 family members in *Nicotiana benthamiana* leaves

In order to determine the sub-cellular localization of grapevine CYP74 gene expression, a green fluorescent protein (GFP) fusion system was used to localize proteins of interest to specific plant cell compartments. Green fluorescent protein is widely used as a biological marker due to its stability and it generally does not interfere with the function of the protein of interest (Zimmer, 2002). Furthermore, it was easy to visualize GFP expression under a confocal microscope. Several reports have described AOS and HPL protein localization as being in the chloroplasts (Maucher et al., 2000; Froehlich et al., 2001; Mita et al., 2005; Pajerowska-Mukhtar et al., 2008). The precise localization of grapevine CYP74 enzymes is critical to understanding the biochemical role that they may play in a cell's system. In order to demonstrate CYP74 family protein localization in grapevines, full length putative VvAOS and VvHPLs genes lacking the stopping codon at the 3' end were fused in-frame to the coding sequence of GFP and placed under the regulation of a CaMV 35S promoter in the pB7FWG2 binary vector (Karimi et al., 2002). Genetic maps for the plant binary vector, pB7FWG2 (Appendix B.4), transformed with VvCYP74 genes are shown on the Appendix B.5, where transformation of each VvCYP74 i.e. VvHPLA, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF genes member into Agrobacterium is shown in figure 2.7.

Introduction of VvCYP74-GFP constructs into *N. benthamiana* leaf was performed, as described in section 2.2.7. Three days after agro-infiltration, leaves were observed under a confocal microscope. Imaging of GFP expression and chlorophyll autoflourescence was observed under 30% laser power. Sub-cellular localization of both VvAOS and VvHPLs fusion proteins are shown in figures 2.8 and 2.9. Sub-cellular localization of grapevine AOS (CYP74A) showed that the protein was clearly incorporated within the chloroplasts or plastids. This was shown by the small punctate green or were detected embedded within the chloroplast plastids on the confocal images. Furthermore, polypeptide sequence prediction software indicates that putative VvAOS contains a chloroplast transit peptide sequence, corroborates that this protein is produced in chloroplasts (Bruce, 2000). This result is consistent with the other 13-AOS sub-cellular localizations identified from other plants (Froehlich et al., 2001; Pajerowska-Mukhtar et al., 2008)

Despite the expectation that VvHPL proteins would be localized within chloroplast outer membranes (Farmaki et al., 2007; Bak et al., 2011), results showed that, except for the VvHPLE protein that appeared to be localized within the chloroplast membrane, most VvHPLs protein not only localize within the chloroplast or plastid but also were observed to be co-localized within the cytoplasm. Localization of the VvHPL protein group, which were associated with 9/13-HPL family members, showed similar patterns of localization with 9/13-HPL identified in *Medicago truncatula,* almond, and cucumber. It was reported that HPL protein grouped within 9/13-HPL family member in *Medicago truncatula,* almond, and cucumber were found to be associated with lipid bodies together with cytosolic distribution and also detected in the endoplasmic reticulum (ER) fraction (Mita et al., 2005;

De Domenico et al., 2007; Hughes et al., 2009). However, to date, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF that identify (Chris Winfield research group) within grapevine (*Sauvignon blanc*) have not been functionally characterized. Furthermore, there was little information available on sub-cellular localization within CYP74 enzymes that used 9/13-hydroperoxides as a substrate. Therefore, further work is needed to elucidate further relationships between protein localization, substrate and their functions.

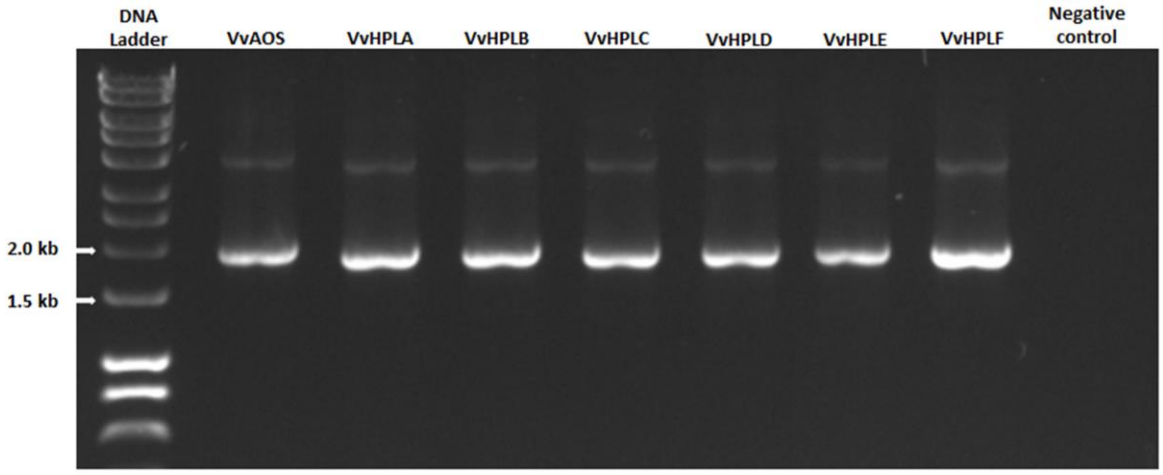

Figure 2.7 Colony PCR of transformed Agrobacterium with respective pB7FWG2:VvCYP74 binary vector
Colony PCR assessment of Agrobacterium transformed with respective pB7FWG2:VvCYP74 binary vector using combination primers of pART 53S (forward) and respective VvCYP74 reverse. VvCYP74 genes are VvAOS, VvHPLA, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF. Additional 0.273 bp derived from junction sequence between CaMV 35S promoter and gene of interest (GOI).

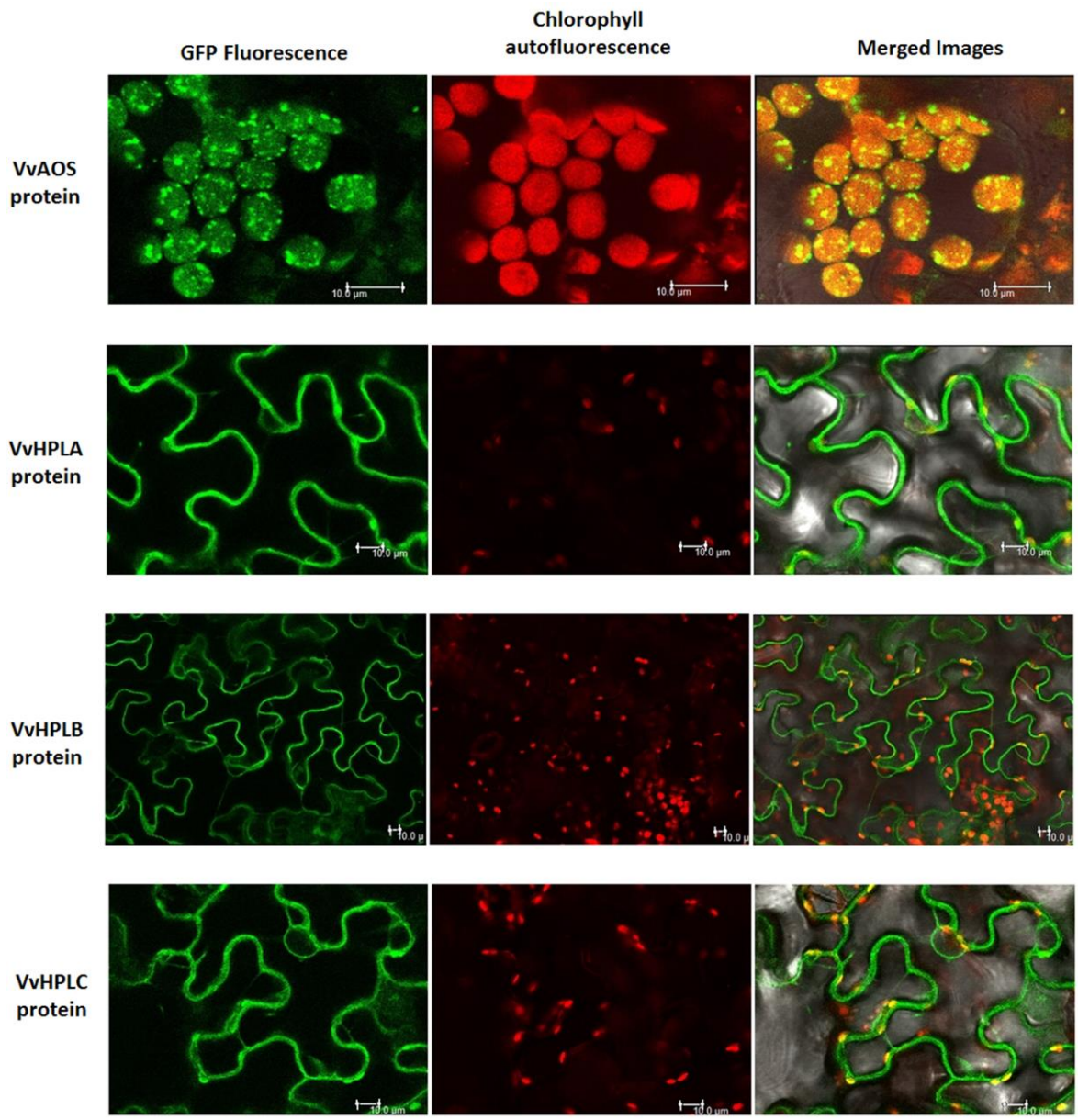

Figure 2.8 Subcellular localization of the VvCYP74 gene fused with GFP in *Nicotiana benthamiana* leaves (continue)

Sub-cellular localization of VvAOS, VvHPLA, VvHPLB and VvHPLC proteins within plant cell compartments in *Nicotiana benthamiana* leaves and visualized by confocal microscopy. Negative control are shown on the next figure.

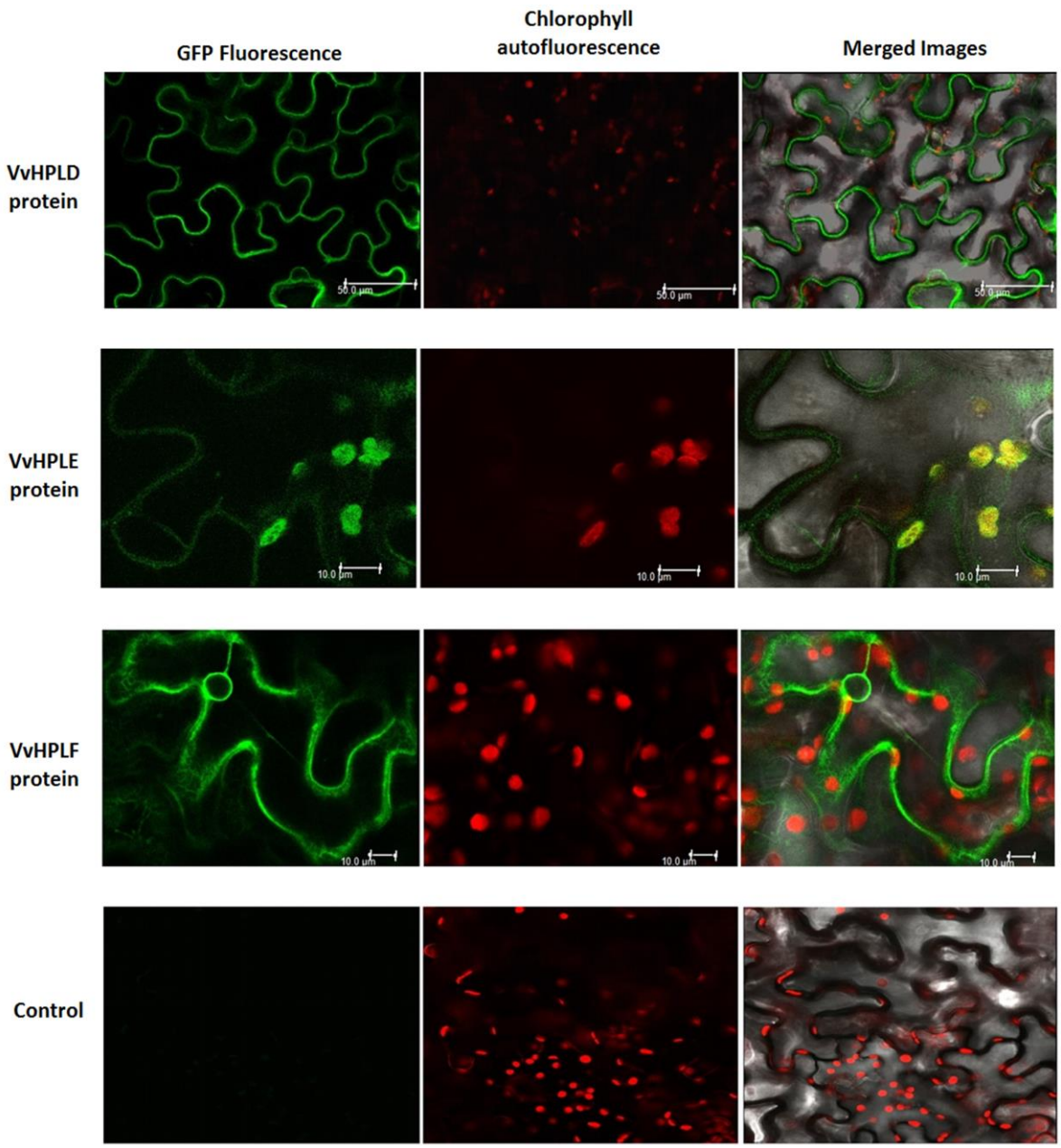

Figure 2.9 Subcellular localization of the CYP74 gene fused with GFP in *Nicotiana benthamiana* leaves (continued)

Sub-cellular localization of VvHPLD, VvHPLE, and VvHPLF protein within plant cell compartment in *Nicotiana benthamiana* leaves and visualized by confocal microscopy. Negative control leaf was infiltrated with Agrobacterium wild type.

2.3.4 Distribution of CYP74 genes expression in grapevine tissues

In order to understand the physiological role of the CYP74 family of enzymes in grapevines, their transcriptional expression was examined in different tissue types. The transcript abundance of each VvCYP74 gene was assessed to give an indication of their specific role in grapevines. Grapevine tissues were collected from the roots, young leaves, tendrils, inflorescences, seeds, pulp and skins. Quantitative Real Time PCR was used to measure transcript abundance of putative VvAOS, VvHPLA, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF in the collected tissues. Due to the high similarity of nucleotide sequences among VvHPL genes (see Appendix C.2 for VvHPL genes alignment sequences), qRT-PCR primers of VvHPLB, VvHPLC, VvHPLD and VvHPLE were designed to include the 3'UTR region in their sequence. This strategy was used ensure the qRT-PCR assay was able to clearly discriminate each of the VvHPL genes identified in grapevines. Therefore, in order to ensure that qRT-PCR primer pairs have a specific target (gene target), were able to effectively amplify gene targets without primer dimers, and had no cross amplification among the target genes (VvHPLs or VvAOS), each qRT-PCR primer was assessed via the endpoint PCR method. For this assessment, a mixture of several grapevine cDNA tissues (leaves, whole berries and inflorescences) was used as a template for qRT-PCR reactions (as described in the material and methods, section 2.2.12) and Eco™ Real-Time PCR System by Illumina (Illumina, dnature Ltd, NZ) as a thermocycler machine (same thermocycler used to run the qRT-PCR assay). Results for a single amplicon amplified from each qRT-PCR primer pairs are shown in Appendix A.3 (Reference genes, VvActin and VvGAPDH, are also included).

Results from qRT-PCR analysis indicated that the level of the VvCYP74 family gene expression varied in the different grapevine tissues types (Figure 2.10), and this outcome was in agreement with the end point PCR amplification results (Figure 2.11). Transcript abundance of the VvAOS gene in all tissues tested was generally high. Among the VvHPL enzymes, VvHPL, the qRT-PCR results showed that only the VvHPLA gene was highly expressed in all tissue samples. Interestingly, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF (using 9/13-HPL as a substrate) genes showed mixed expression patterns across grapevine tissues (Figure 2.10). Transcript abundance showed that the VvHPLB gene was expressed in roots, inflorescences and seeds but not quantified (NQ) in the leaves, tendrils, pulp and skins. Whereas the VvHPLC gene was expressed in the roots and leaves but not detected (ND) in any other tissue tested. Of the other VvHPLs classified as CYP74C family members, the VvHPLD gene was expressed in roots and inflorescences but NQ in leaves, tendrils and seeds whereas it was ND in pulp and skins while the VvHPLE gene was expressed in inflorescences and seeds but NQ in roots, tendrils and leaves, whereas ND in pulp and skins. Lastly, the VvHPLF gene was expressed in tendrils, inflorescences and seeds but NQ in roots, leaves, pulp and skins. In summary, the VvHPL gene falls within the CYP74C gene family and members show different patterns of expression in different tissues and these might be an indication of their specific functions in respect to tissue types in grapevines. However, further assessment was needed to elucidate their clear function in grapevines.

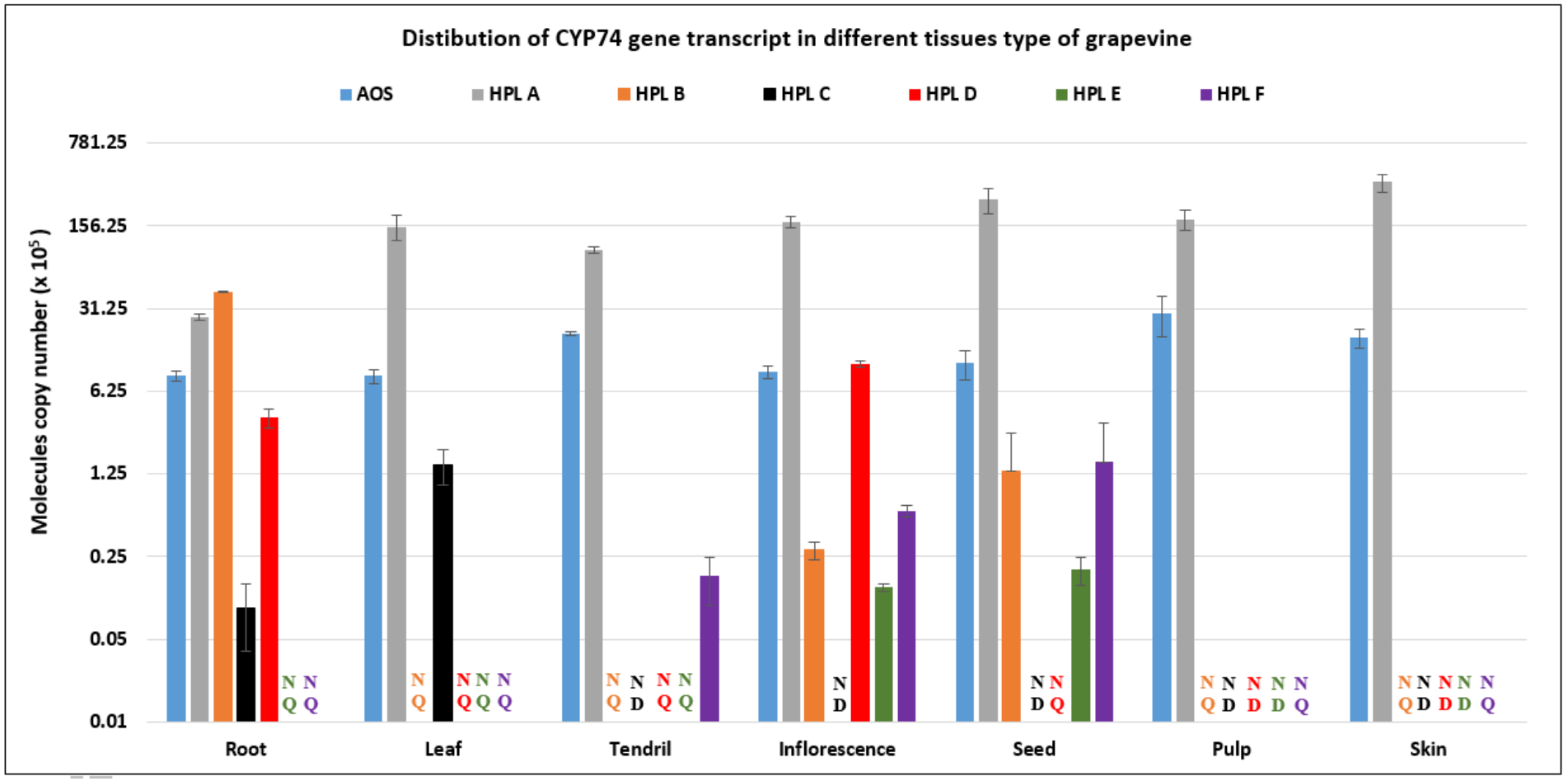

Figure 2.10 qRT-PCR analysis of CYP74 gene expression in grapevine

Distribution of CYP74 enzyme activity in different tissue, i.e. roots leaves, tendrils, inflorescences, seeds, pulp and skins in grapevine are shown on the graph above (log graph base 2). For each respective tissue sample, 500 ng of total RNA was used as a starting material for cDNA synthesis. Not quantified (NQ) and not detected (ND) gene transcripts were labelled as a "single and double star", respectively. NQ defined as Cq values above 30 and ND as zero amplification after 35 cycles.

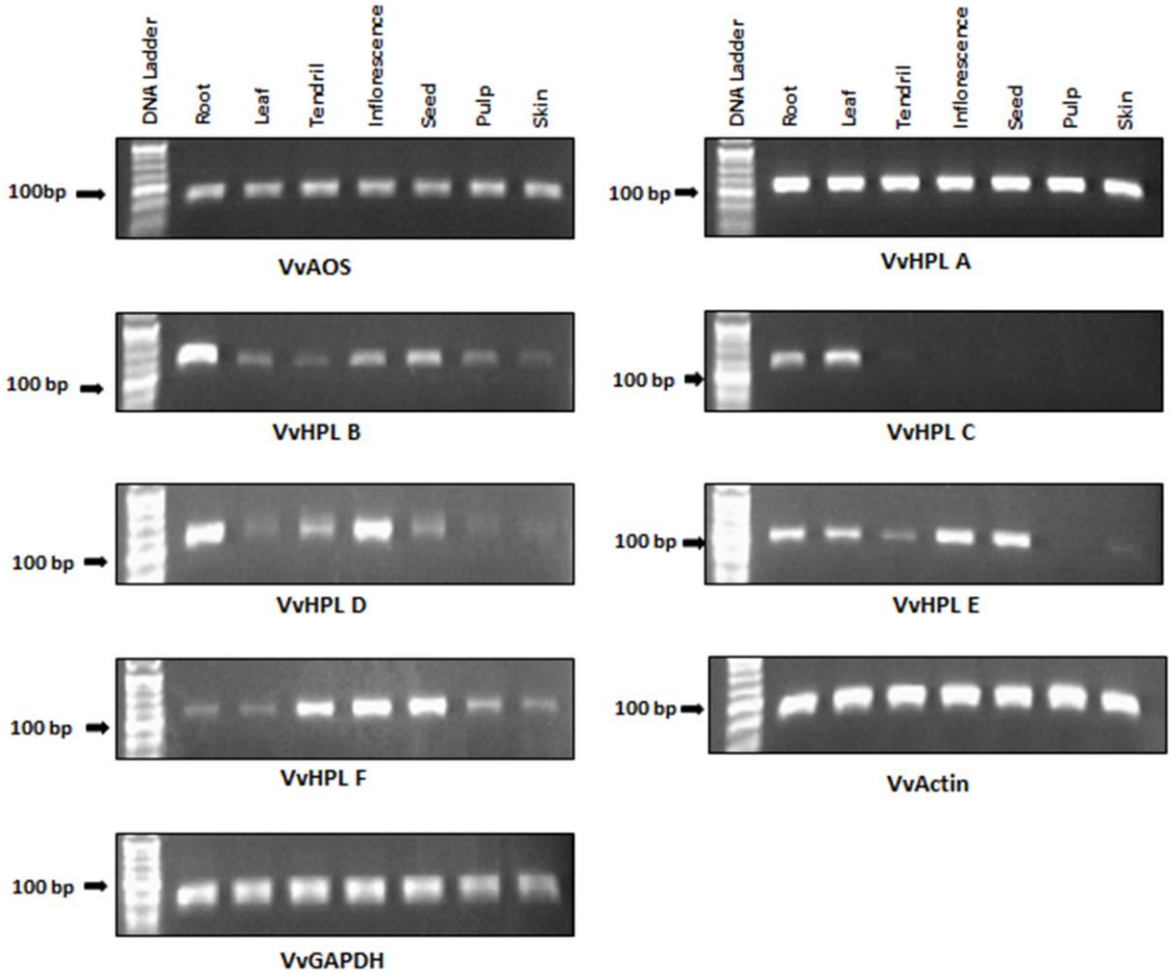

Figure 2.11 End-Point PCR of CYP74 gene amplification using cDNA template
Gel photos shown above are PCR end point products to indicate CYP74 expression gene in roots leaves, tendrils, inflorescences, seeds, pulp and berry skins. HyperLadder V (25bp) from Bioline (Totallab, NZ) was used to measure DNA fragment size.

2.3.5 Responses of CYP74 family to wound treatment in grapevine leaves

Young leaves (approximately 2.5 – 3 cm long) from grapevine Mullins vines were subjected to mechanical wounding (Figure 2.12) and collected at different time points, as described in section 2.2.10. In order to quantify transcript abundance, the relative expression of VvCYP74 genes were assayed via the qRT-PCR method, as described in the material and methods (Section 2.2.12). Transcript abundance of each VvCYP74 gene family members, VvCYP74A (VvAOS), VvCYP74B (VvHPLA) and VvCYP74C (VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF), were examined. Results from the qRT-PCR analysis in each samples were presented as total gene copy numbers detected in 4 µL of cDNA synthesized from 500 ng of total RNA samples. Interrogation of preliminary qRT-PCR data (Appendix A.5) showed that VvHPLF generally displayed threshold quantitation cycle (Cq) values above 30 cycles on wound treated samples and the majority were not detected on the control (non-wound treated) samples even at 35 cycles. Therefore, due to the incomplete Cq data, the VvHPLF gene was not included in further analysis in order to avoid making incorrect conclusions related to the response of this gene to wound treatment.

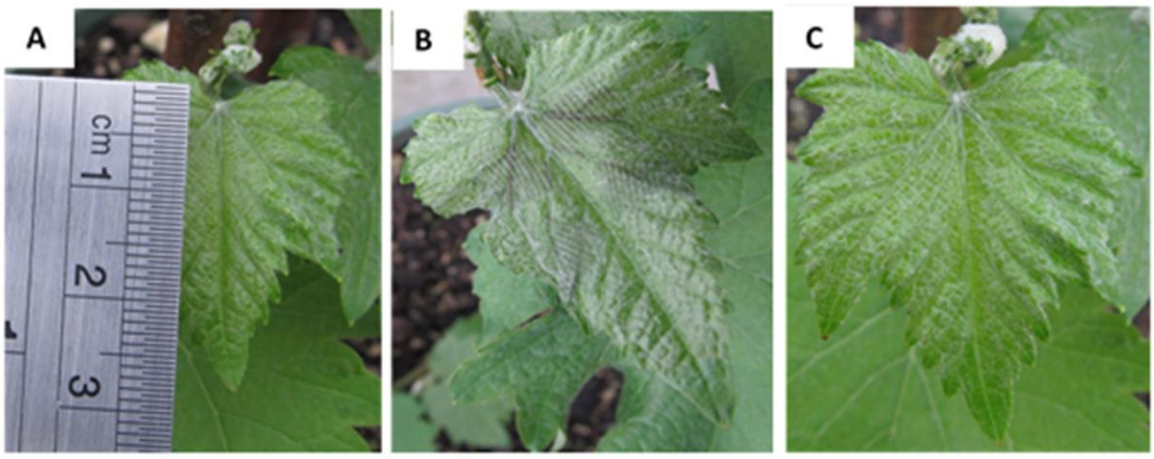

Figure 2.12 Grapevine young leaves with wound treatment
Average size and wound damage of young leaves collected for CYP74 family enzymes response to plant abiotic stress in grapevines. A) Approximately 2.5 to 3 cm long; B) Average leaf conditions six hours after wound treatment; and C) Average non-wounded (control) leaves.

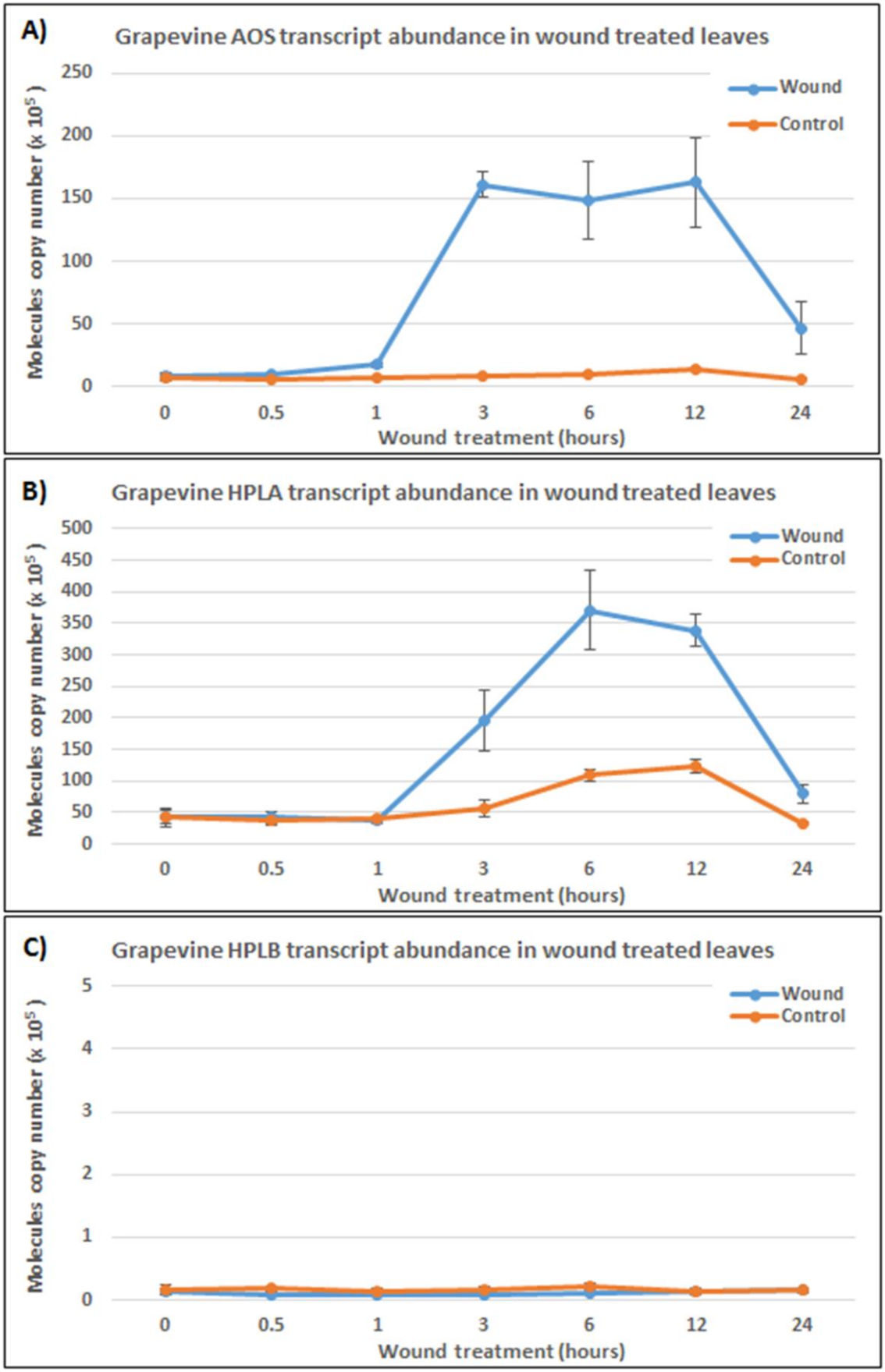

Figure 2.13 VvCYP74 gene expression as a response to wound treatment (continued)
Grapevine AOS (A), HPLA (B) and HPLB (C) transcript abundance in wounded leaves. Bars represent standard deviation of mean from three biological replicate samples.

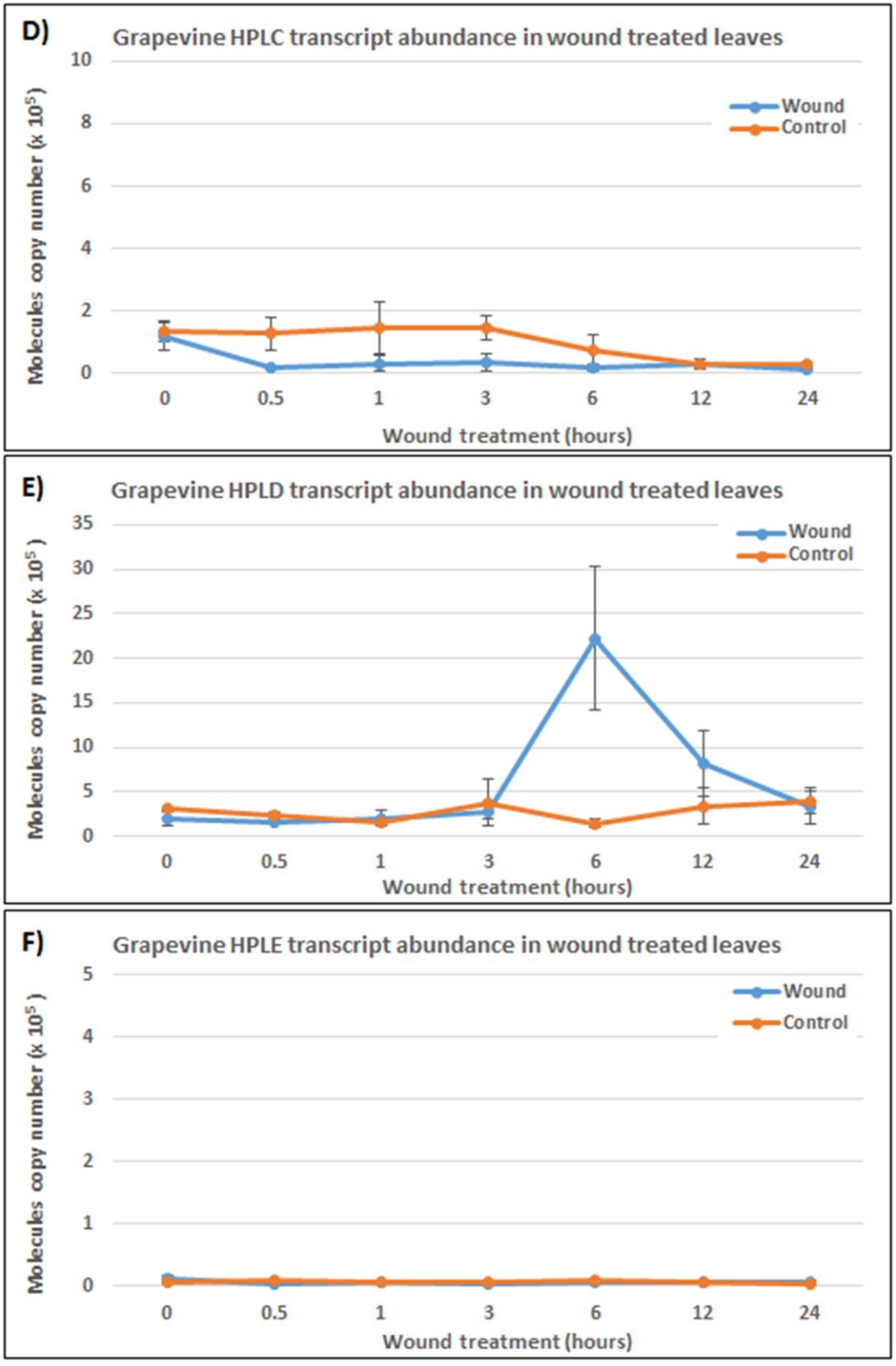

Figure 2.13 CYP74 genes expression as a response to wound treatment (continued)
Grapevine HPLC (D), HPLD (E) and HPLE (C) transcript abundance in wounded leaves. Samples were collected over the course of 24 hours of wound treatment and transcript abundances were quantified and compared to control sample. Control samples at each collection time point were included to accommodate the possibility of circadian effect on GOI. Bars represent standard deviation of mean from three biological replicate samples .

Results from qRT-PCR analysis of each CYP74 gene member in wounded grape leaves and control samples collected at different time points over the course of 24 hours wound treatment are shown in figure 2.13. The results clearly showed that the putative VvAOS response to wounding, as expected, in which transcript abundance increased significantly at 3, 6 and 12 hours (simple T-Test, $P < 0.05$) after wounding compared to the control samples (Figure 2.13A). Comparison analysis indicated that transcript abundance in wound treated samples peaked at three hours (16-fold higher compared to the control at a similar time point) and gradually declined to a near normal level at 24 hours after wounding. Likewise, VvHPLA and VvHPLD showed a significant increase (simple T-Test, $P < 0.05$) as a response to wounding but this differed in terms of the pattern of transcript accumulation compared to the VvAOS gene (Figure 2.13B and 2.13E). Both these VvHPLs peaked at six hours which were three hours later compare to VvAOS expression before returning to near normal level after 24 hours wounding. However, in terms of gene induction magnitude at the expression level relative to control samples, the VvAOS gene registered a larger (approximately 20-fold) value followed by VvHPLD (14-fold) and then VvHPLA (4-fold). On the other hand, VvHPLB, VvHPLC and VvHPLE did not show a significant response (simple T-Test, $P > 0.05$) to wound treatment at any time point relative to their control samples (Figure 2.13C, 2.13D and 2.13F) which indicated that these particular genes were not elevated by wounding in leaves within 24 hours.

2.4 Discussion

In this chapter, we identified one putative grapevine AOS gene sequence that displayed highly similar characteristics to AOS genes from other plants, such as the appearance of highly conserved motifs, predicted to carried a cTP region, high sequence similarities (both at polypeptide and nucleotide levels) and was grouped by a NCBI database search as P450 superfamily members. Together with another six VvHPLs previously identified within our research group, we carried out partial characterization of CYP74 family members in grapevines. Throughout this characterization experiment, we first localized grapevine CYP74 family enzymes within plant cell compartments via GFP fusion technique in *N. benthamiana* leaves, followed by study of their possible physiological role in grapevine systems by examining their transcript level in different tissue types and, finally, their response to wound treatment, as an indication to their possible involvement in plant defence mechanisms.

Protein sub-cellular localization is important because knowing the protein environment will help to elucidate protein function in which the protein of interest operate (Scott et al., 2005). Protein location in plant cell compartments can influence their function by controlling access or their availability to interact with other molecular interaction partners (Scott et al., 2005). For CYP74 enzyme family members, numerous reports describe their activity in green tissues as often occurring in chloroplasts (Feussner and Wasternack, 2002; Stumpe and Feussner, 2006; Hughes et al., 2009). This localization seems to be likely because production of the CYP74 substrates, 9- and 13-hydroperoxide,

takes place in plastids (Hughes et al., 2009). Furthermore, many of the polypeptide sequences of CYP74 enzymes comprise cTP regions, which are associated with membrane-bound proteins. However, the sub-cellular localization of plant enzymes ascribed to the CYP74 family is still largely unclear (Mita et al., 2005). Some CYP74 family members targeted chloroplasts (Pajerowska-Mukhtar et al., 2008) but with different localizations. For example, in tomato, AOS targeted the inner, but HPL targeted the outer, chloroplast membrane (Froehlich et al., 2001). Others were reported to target the cytosol, microsomes and lipid bodies, as shown by HPL enzymes (9-HPL) in almond (Mita et al., 2005) and (9/13-HPL) in *Medicago truncatula* (De Domenico et al., 2007). CYP74 enzyme in grapevines, putative VvAOS and VvHPLs, seem to be targeted within chloroplast and cytosol compartments in plant cells.

The putative VvAOS, as expected, was clearly localized within the chloroplast membrane. However, an interesting result from this work was the localization of VvHPL family members, i.e. VvHPLA and VvHPLD, which were predicted to contain a cTP region but did not localize within the chloroplast membrane. Although, in general, HPLs enzymes were initially thought to be localized in the plastids due to HPL research in Arabidopsis, in which the proteins appeared to be membrane-bound (Farmaki et al., 2007) and the existence of potential signal peptides within the HPL amino acid sequence, which ought to target plastids or mitochondria (Bak et al., 2011), but all grapevine HPLs, except the VvHPLE result was opposite to what was expected. This result is quite interesting since 13-LOX is responsible for providing the substrate for both 13-AOS and 13-HPL within the chloroplasts (Feussner and Wasternack, 2002; Hughes et al., 2009). In contrast, VvHPLA (predicted to be 13-HPL) did not localize within chloroplasts. This indicated a more complicated picture for the formation of C_6 volatiles in grapevines where there was likely a specific transfer of LOX products out of the chloroplast to the location of the 13-HPL for cleavage to form C_6 aldehyde. Up to date, other VvHPL's i.e. VvHPLB, VvHPLC, VvHPLD and VvHPLF that identify (in our research group) within grapevines have not had their function characterized. However, these VvHPLs, which were predicted to be associated with the 9/13-HPL groups were shown to be localized within cytoplasmic or plasma membranes. In *Medicago truncatula,* almond, and cucumber, these proteins (9/13-HPL enzymes) are also found to be specifically associated with lipid bodies together with cytosolic distribution and are also detected in the ER fraction (Mita et al., 2005; De Domenico et al., 2007; Hughes et al., 2009). However, there is a little information on sub-cellular localization within the CYP74 enzyme family that use 9/13-hydroperoxides as a substrate. Therefore, further work is needed to elucidate further relationships between protein localization, substrate and function of VvCYP74B and VvCYP74C in grapevines.

Despite the known poor overall correlation between mRNAs and their protein products, it was reported that some studies have noted that certain classes of gene have higher correlation with protein expression (Koussounadis et al., 2015). These classes of genes often have tight synchrony regulation with their respective protein products. For example regulation of secreted protein which would only require transcription when needed or cell cycle genes which are time-dependent

(Koussounadis et al., 2015). In the case of AOS gene, it was shown that the increase of its transcript abundance lead to increase of JA level in Flax (Harms et al., 1995), Nicotiana (Laudert et al., 2000) and Arabidopsis (Park et al., 2002) whereas the expression of two HPL in grapevine were reported have a significant correlation with the accumulation of their volatile products. Therefore, in this experiment, in order to understanding the physical or specific role of grapevine CYP74 enzymes, we used gene expression as a proxy to estimate their activity within different tissue types of grapevine. First, as widely reported, AOS plays an important role in the production of jasmonates, a signalling compound heavily involved in a plant's response to stress and development (Sivasankar et al., 2000; Park et al., 2002; Santino et al., 2013). Therefore, it was not surprising that VvAOS was significantly expressed in all tissues tested. Furthermore, this observation may imply that, in general, CYP74A enzyme family members play a vital role in the whole grapevine plant system.

Second, hydroperoxide lyases (HPL) are enzymes associated with production of C_6 and C_9 aldehyde volatile compounds, important contributors to the characteristic flavour of fruit, green leaves and plant defences (Noordermeer et al., 2001; Taurino et al., 2013). Enzyme HPLs are categorized based on their substrate specificity: i) 13-HPL catalyses the 13-hydroperoxide substrate to produce C_6 and C_{12} aldehydes; ii) 9-HPL uses 9-hydroperoxides as a substrate to form C_9 aldehyde; and iii) 9/13-HPL is able to consume both 9- and 13-hydroperoxides as a substrate to produce both C_6 and C_9 aldehyde compounds (Zhu et al., 2012). Among the VvHPL genes assessed, it was interesting to see that only VvHPLA was highly expressed in all tissues, specifically, especially in skins (most abundant transcript). This result was consistent with the observations reported by Zhu et al. (2012) where 13-HPL transcripts were abundant in berries, leaves, tendrils and stems of grapevines. This result was interesting because Zhu et al. (2012) also mentioned that, C_6 aldehyde content was far higher than the content of C_9 aldehyde in grape berries throughout the ripening stages. Since VvHPLA is classified as the 13-HPL type, which was responsible for the formation of C_{12} and C_6 compounds (Creelman and Mulpuri, 2002; Matsui, 2006; Pinot and Beisson, 2011; Scala et al., 2013), this result may suggest that the degradation of fatty acids in grape berries occurred mainly with assistance from 13-LOX and 13-HPL rather than 9-LOXs and 9-HPL. Therefore, this observation may also explain why VvHPLA gene expression was relatively high in berries.

Gene expression of other VvHPLs i.e. VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF was assessed. Those grouped as 9/13-HPL (VvCYP74C) type displayed different patterns of expression compared to the 13-HPL (VvCYP74B) type. The results showed that VvCYP74C gene members display mixed expression pattern across grapevine tissues. This may indicate that each VvCYP74C gene member exhibited different physical functions across grapevine tissues. Again, these outcomes are consistent with the results reported by Zhu et al. (2012). Another interesting result was the expression of grapevine CYP74B compared to VvCYP74C in skins and pulp. The grapevine CYP74B enzyme used 13-HPL as a substrate (VvHPLA) showed high levels of gene expression in skins and pulp, whereas

VvCYP74C, an enzymes used 9/13-HPL as a substrate display gene expression either low (NQ) or not detected (ND) in the similar tissues. Low gene expression of VvCYP74C gene members in skin and pulp tissues supported our previous suggestion about 13-LOX's vital role in the formation of aromatic compounds in grape berries. Besides that, expression of VvCYP74B and VvCYP74C genes also indicated locations for C_6 and C_9 aldehyde production and their function not only in the production of aromatic compound but as signalling molecules. However, interestingly, that most of the VvCYP74C gene members displayed high expression in seeds and inflorescences might indicate their involvement in early developmental stages of fruits and floral organ in grapes (Zhu et al., 2012). Nevertheless, VvCYP74C gene functions and mechanisms are still not fully understood. Therefore, further evidence was needed to understand their function and mechanisms.

2.5 Conclusions and future prospects

The CYP74 family is a group of enzymes that belong to the superfamily of cytochrome P450, which generates oxygenated metabolites or oxylipin compounds derived from polyunsaturated fatty acids (Stumpe and Feussner, 2006). Oxylipin plays a diverse role in plants, including plant defence systems, plant development, fruit ripening and flavour. CYP74 family enzymes were grouped into three sub-families based on their enzyme activities, as described previously. In this chapter, seven enzymes belonging to the CYP74 family in grapevine were examined. Phylogenetic analysis categorized VvAOS and VvHPLA as belonging to the CYP74A and CYP74B sub-families, respectively, whereas VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF were grouped in the CYP74C sub-family. Similar to results reported in other plant species, putative VvAOS was localized within the chloroplast membrane, but unlike other results, VvHPLA was localized within the plant cell plasma membrane and cytoplasm. Most of the VvHPL proteins grouped into the CYP74C sub-family were localized within the plant cell plasma membrane and cytoplasm. However, interestingly, the VvHPLE protein appeared to be generally localized within chloroplasts.

The distribution of the CYP74 gene family expressed within grapevines were varied in different tissue types. Generally, transcript abundance of VvAOS and VvHPLA genes (which were classified within the CYP74A and CYP74B gene families, respectively) showed strong expression in all tissues examined (root, leaf, inflorescence, seed, pulp and skin), whereas gene expression of VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF (which were classified within the CYP74C gene family) were expressed differently in the different tissue types examined. Interestingly, the transcript level of all VvHPL grouped among CYP74C family (enzymes used 9/13-HPL as a substrate) displayed low expression (either unquantified or undetectable) in the pulp and skins, which may indicate a lesser role in the formation of aromatic compounds in berries but the high expression of CYP74B indicated the opposite role. Therefore, this may suggest that VvHPLA (member of CYP74B gene family) should be the focus for further research for gaining a better insight into the relationship between VvHPL enzymes group

and aroma/flavour in grape berries. Despite the report of CYP74 enzymes playing an important role in plant abiotic stimuli (Hughes et al., 2009), our results showed that not all CYP74 enzyme members were stimulated by abiotic stress or wounding in grapevines. Among CYP74 enzymes member examined only VvAOS (CYP74A), VvHPLA (CYP74B) and VvHPLC (CYP74C) responded to wounding treatment. The strongest response (based on transcript abundance) was displayed by VvAOS gene with a relative expression increase of approximately 16-fold three hours after wound treatment. Whereas, while VvHPLA and VvHPLC gene responded to mechanical wounding, it but not as strong as in VvAOS. The expression of both genes only reached peak levels after six and 12 hours with an increment of approximately 4- and 5- fold, respectively.

Among grapevine CYP74 gene family members, VvAOS, grouped within the CYP74A family members exhibited the most interesting features towards plant defence systems, such as, its enzyme localization site in the chloroplast, high gene expression occurred in the whole plant and it responded strongly to mechanical stimuli. Therefore, the VvAOS gene was selected for further analysis to gain a wider insight about the role of CYP74s plant defence systems. However, in order to use this gene for further work to understand its role, the VvAOS gene needed to be verified for its biochemical function in grapevines. Therefore, the next chapter focuses on elucidating VvAOS's biochemical function via complementation of the knock-out AOS gene function in Arabidopsis (Park et al., 2002)

Chapter 3

Characterization of Allene oxide synthase in grapevine

3.1 Introduction

The oxidation products of polyunsaturated fatty acids (PUFAs), collectively known as oxylipins, are a highly diverse group of substances that play important roles at various stages in the developmental processes and stress responses in plants (Andersson et al., 2006; Acosta and Farmer, 2010). Despite recent progress in understanding the function of some oxylipins, the roles of the vast majority of plant oxylipins still remain unclear (Yan, Borrego et al. 2013). Among plant oxylipins, jasmonates (JAs) are one example that has been extensively studied. JAs are a collective group of compounds (Figure 3.1), but only jasmonoyl isoleucine (JA-Ile), jasmonyl-L-tryptophan (JA-Trp) and OPDA are considered as major bioactive compounds in this group that initiate signalling processes involved in plant defences or plant development (Fonseca et al., 2009; Acosta and Farmer, 2010; Gfeller et al., 2010; Yan et al., 2013). In the pathway that leads to the production of JA, AOS is the enzyme that catalyses the first committed reaction of the JA biosynthesis pathway (Laudert and Weiler, 1998; Turner et al., 2002). An interesting feature of AOS is that it can be induced by its own reaction product, OPDA, as well as the pathway's end product, JA, suggesting that regulation of the AOS gene is a focal point for the control of the JA biosynthetic pathway (Laudert and Weiler, 1998; Kubigsteltig et al., 1999).

3.1.1 Allene oxide synthase of grapevines

Up until now, no research undertaken on allene oxide synthase in grapevines (*Vitis vinifera* L. cv *Sauvignon blanc*) has been reported. However, from our work in the previous chapter (Chapter 2), we identified and cloned a putative AOS gene from *Vitis vinifera* L. cv *Sauvignon blanc*. The grapevine AOS that we identified had characteristics and likely a function that resembled functional AOS identified from other plant species. For example, grapevine putative AOS displayed high similarity to other known AOS sequences (both at the nucleotide and amino acid level), comprised all the conserved motifs that characterized an AOS protein sequence, protein localization occur within the chloroplast plastid membrane and also responded to wound treatment (Harms et al., 1995; Laudert et al., 2000; Park et al., 2002; Pajerowska-Mukhtar et al., 2008).

Therefore, in this chapter, our objective is to characterize the functionality of the putative AOS gene isolated from *Vitis vinifera* L. cv *Sauvignon blanc* via complementation of the AOS gene function in Arabidopsis *aos* mutant plants. An Arabidopsis *aos* mutant is a plant in which its endogenous AOS gene function has been knocked out and, as a consequence, it exhibits a male sterile phenotype due

to the dysfunctional AOS gene in their system (Park et al., 2002). Recovery from the male sterile phenotype in an Arabidopsis *aos* mutant plant after the introduction of the putative AOS gene from grapevines will verify its functionality as an AOS gene in *Vitis vinifera* L. cv *Sauvignon blanc.*

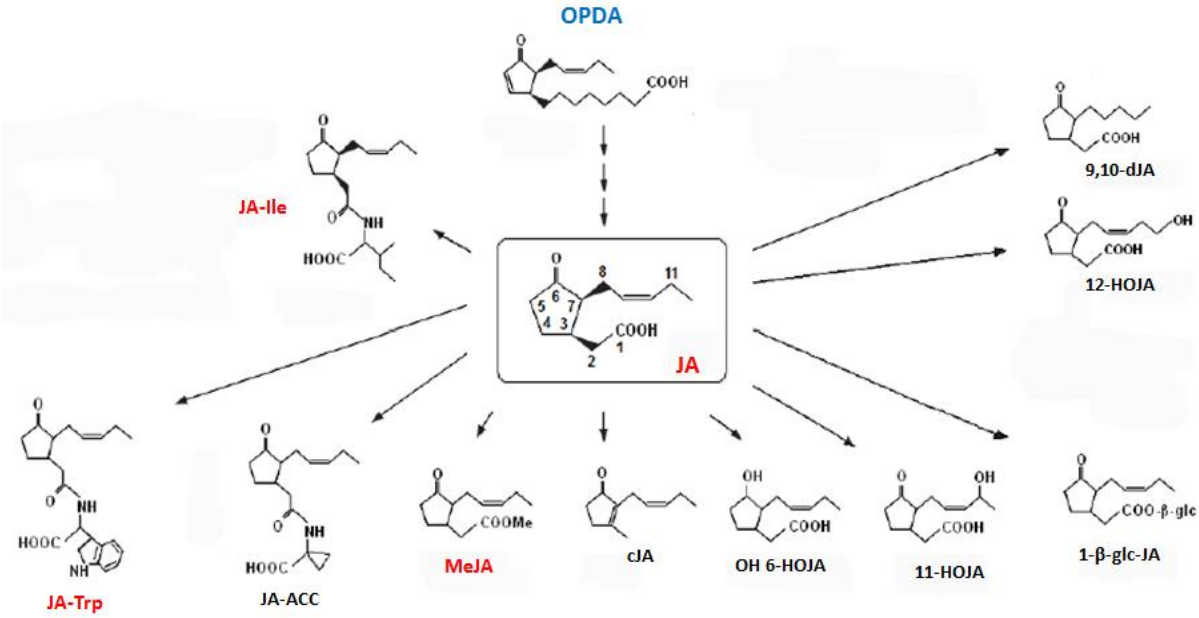

Figure 3.1 Metabolites derived from JA in plants
Jasmonic acid and it derivatives. Most active compounds are highlighted in red and, OPDA, as a precursor to JA, production is highlighted in blue. Adapted from Gfeller et al, 2010

3.2 Materials and methods

3.2.1 Plant material for *Arabidopsis thaliana aos* mutant

Heterozygous seeds of the Arabidopsis *aos* mutant were obtained from the Arabidopsis Biological Resource Centre (ABRC), Ohio State University via The Arabidopsis Information Resource database (TAIR). In order to confirm its genotype, five to six Arabidopsis *aos* mutant seeds were planted directly in a 7 cm long x 7 cm wide and x 8 cm tall plastic tray containing seed sowing mix soil prepared by the Lincoln University plant nursery. The seed sowing mix soil contained a mixture of peat and sterilized pumice at a ratio of 3:1, respectively, with additional fertiliser of Osmocote exact mini, i.e. NPK (2 g/L), dolomite (4 g/L) and hydraflo (1 g/L). Trays containing Arabidopsis *aos* mutant seeds were stratified at 4°C for two days before being transferred to the Lincoln University plant growth room facility. Plant growth room conditions were as follows: 16 hours light/day, temperature at 20°C, 60–70% humidity and light intensity of approximately 100 µmol/m^2sec provided by cool white fluorescent tubes. The genotype of each Arabidopsis *aos* mutant plant on the tray was confirmed via PCR, as described below.

Leaf tissue harvested from approximately three to four week-old plants was used directly as samples for PCR analysis using the KAPA3G Plant PCR Kit as per the manufacturer's instructions (Custom Science, NZ). Primers for PCR amplification were obtained from the work reported by Park et al. (2002). A combination two of these primers, TJRB (Fwd): 5' – CGGGCCTAACTTTTGGTGTGATGATGCT – 3', CYP74AF (Fwd): 5' – AACATATGCTCAAGGGATGGAGCTAAAAG - 3' and CYP74AR (Rev): 5' – CGAACATGTAGAGCAGCAACTGATTATACA - 3' were used to assess the genotypes of each Arabidopsis seedling. Samples that produced both DNA amplicons (2.5 kb and 400 bp) for primer combinations CYP74AF-CYP74AR and TJRB-CYP74AR, respectively, confirmed the Arabidopsis seedlings as containing a heterozygous T-DNA insertion in the Arabidopsis AOS gene.

3.2.2 Amplification of the Arabidopsis plant promoter

The Arabidopsis AOS promoter (promAOS) was amplified from genomic DNA isolated from wild type *Arabidopsis thaliana* ecotype *Columbia* leaves. Approximately 50 ng of Arabidopsis gDNA was used as a template for promAOS amplification. A 2.2 kb of DNA upstream of the predicted initiating ATG of the AtAOS gene (Accession: At5g42650) encompassing the AtAOS promoter, was cloned. The 3' primer (AtPRT-Rev: 5'- CTGAAT*ATCG*ATCTATTCGAAACAGTG -3') was designed to bind to the AtAOS locus immediately next to, but excluding, the translation initiation codon. The 5' primer (AtPRT-Fwd: 5'- TAGCCCTTACGAAAGAAAACACTATTTG-3') was positioned 2.2 kb upstream of the cognate translation initiation codon. According to the previous report (Benhamed et al., 2008) promoter sequences of up to 2 kb from the translation initiation codon are sufficient to recapitulate genuine transcription patterns. The reverse primer was designed to have a restriction site, *Cla I,* (underlined

site of reverse primer AtPRT-Rev) to accommodate cloning of the fragment gene into the pARTBGW binary vector. The promAOS was amplified using High-fidelity PrimeSTAR HS DNA polymerase from Takara Bio Inc. (Norrie Biotech, NZ), to minimize any PCR amplification errors and to produce a blunt ended DNA amplicon. Amplification was carried out in a 50 µL reaction volume using GenePro thermocycler by Bioer Technology (Ngaio Diagnostic, NZ) with the following reaction conditions: Initial denaturation - 98°C for 10 s, followed by 35 cycles of denaturation: 98°C for 15 s, annealing: 60°C for 30 s, extension: 72°C for two minutes with a final extension of: 72°C for five minutes. PCR amplification products were separated using agarose gel electrophoresis, as described in chapter 2 (Section 2.2.4). PCR products of the desired size were then excised from the gel using a razor blade and purified using an AxyPrep DNA gel Extraction kit (RayLab, NZ) according to the manufacturer's instructions.

3.2.3 Cloning of AtAOS promoter into pARTBGW binary vector

The plant binary vector, pARTB-GW-egfpER (pARTBGW), 16.41 kb in size, was obtained from Plant and Food Research Institute, Canterbury, New Zealand, which used a cauliflower mosaic virus 35S promoter (CaMV 35S prompter) to drive the transcription of transgenes and possessed the phosphinothricin-N-acetyltransferase resistance gene to confer resistance to the herbicide glufosinate, a non-selective herbicide (commercially known as BASTA) as a selection marker. In order to accurately test the ability of the VvAOS gene to complement the Arabidopsis *aos* mutation it was necessary to replace the CaMV 35S prompter in this vector with the promAOS. Approximately 500 ng of the binary vector, pARTBGW, was double digested with *PmlI* and *Cla I*, restriction enzyme (New England Biolabs, Generesearch Ltd, NZ), as per the manufacturer's instructions, to separate the pARTBGW backbone from the CaMV 35S promoter. The digested plasmid was separated on 1% agarose gel to facilitate the separation of the pARTBGW backbone (approximately 15.0 kb) from the unwanted CaMV 35S promoter fragment (approximately 1.3 kb). The pARTBGW backbone was excised and gel purified using the AxyPrep DNA gel Extraction kit (RayLab, NZ) as per the manufacturer's instructions. The purified fragment was quantified using a Qubit® Fluorometer (Life Technology, NZ) before ligation.

In order to facilitate directional cloning of the promAOS, the 3'-primer was designed to contain a unique *Cla I* restriction enzyme site. Approximately 500 ng of purified promAOS amplicon was treated with *Cla I* (New England Biolabs, Generesearch Ltd, NZ) to create a 3' overhang on the 3' end of the amplicon. Restriction enzymes digests were carried out in a 50 µL reaction, as suggested in the protocol provided by the manufacturer. After a one hour incubation at 37°C, the reaction was inactivated by incubation of the restriction digestion mixture at 65°C for 20 minutes. The digested AtAOS promoter amplicon was purified using AxygPrep PCR Clean-Up kit (RayLab, NZ). The purified amplicon was then quantified using a Qubit® Fluorometer (Life Technologies, NZ) before ligation with a prepared pARTBGW binary vector, which had the CaMV-35S cassette removed.

Incorporation of promAOS into the pARTBGW binary vector was carried out as follows: 45 ng of the digested promAOS amplicon was added to 100 ng of digested pARTBGW in a 10 µL volume ligation reaction using the Mighty Mix DNA Ligation Kit (Takara Inc, Norrie Biotech, NZ) as per the manufacturer's instructions. The entire ligation reaction was used to transform 100 µL of *Escherichia coli* (strain DB3.1) competent cells via the heat shock transformation method, as described by Sambrook and Russell (2001), and was plated on the LB agar media (1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, 1.5% bacto-agar, pH 7.5) containing 50 µg/mL of spectinomycin as the antibiotic selection agent. The transformed bacteria were identified through colony PCR (see Appendix D.3 for the method). Positively identified transformed colonies were used to inoculate 3 mL of LB broth (1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride, pH 7.5) containing 50 µg/mL of spectinomycin and then grown in the shaking incubator (250 rpm) at 37°C overnight. After inoculation, the plasmid was purified using a standard plasmid extraction method, as described by Sambrook and Russell (2001). Prior to sequencing the purified DNA to confirm that the construction of the new binary vector was correct, the DNA preparation was further purified using AxygPrep PCR Clean-Up kit (RayLab, NZ).

3.2.4 Construction of a putative VvAOS binary vector for plant transformation

Approximately 150 ng of pENTR/D-TOPO carrying the putative VvAOS gene (pENTR:D-TOPO:VvAOS; see section 2.2.4) was added to 300 ng of the newly constructed binary vector pARTBGW:promAOS (as described above) to perform an LR recombination reaction with the Gateway Clonase Enzyme Mix kit as per the manufacturer's instruction (Invitrogen Inc., Life Technologies, NZ). After recombination, 2 µL of the LR reaction mixture was used to transform 100 µL of DH5α competent cells, as described previously. Incorporation of the VvAOS gene into the newly transformed pARTBGW binary vector containing the AtAOS promoter in place of the original CaMV 35S promoter, was confirmed via PCR using the following PCR primers: Int2pAOS (Fwd): 5'-CCACTAAATTCACTATTTTCATTCACA-3' and VvAOS-Rev: 5'- TCAAAAACTGGCTCGCTTTA-3'. Int2pAOS primer binding site was located at 153 bp from the 3' end of AtAOS promoter sequence. Positively identified constructs from the PCR analysis were purified using AxygPrep PCR Clean-Up kit (RayLab, NZ) before being subjected to sequencing for further confirmation of the correct construction of the binary vector. Binary vector pARTBGW comprised the Arabidopsis AtAOS promoter and incorporated with VvAOS gene was labelled as pARTBGW:promAOS:VvAOS.

3.2.5 Arabidopsis *aos* mutant dip floral transformation with binary vector pARTBGW:promAOS:VvAOS

Transformation of pARTBGW:promAOS:VvAOS into *Agrobacterium tumefaciens* was carried out via an electroporation method, as described in chapter 2 (Section 2.2.7). Preparation for floral dip

transformation of the Arabidopsis *aos* mutant using the transformed Agrobacterium was carried out according to the protocol described in the previous chapter (Section 2.2.7). After the final incubation, 50 mL of mediated-Agrobacterium cell culture was added to 950 mL of infiltration media [3% sucrose (w/v) and 150 µM acetosyringone] with 0.05% (v/v) of Pulse (Nufarm, NZ) in a 1 L beaker. After several unsuccessful attempts to isolate and transform the homozygous Arabidopsis *aos* mutant, it was decided to transform a heterozygous line of this mutant. Healthy heterozygous Arabidopsis *aos* mutant plants were grown, as described above (Section 3.2.1). In order to obtain more floral buds per plant, inflorescences were clipped after most plants had formed primary bolts to relieve apical dominance and encourage synchronized emergence of multiple secondary bolts. These plants were subjected to floral dip transformation when most secondary inflorescences were about 1 - 10 cm tall (approximately four to eight days after clipping) using a standard protocol (Bent, 2006). Transformed Arabidopsis *aos* mutant plants were grown in the plant growth room under the conditions described above (Section 3.2.1). Mature siliques were harvested following to the standard protocol, as described by Bent (2006). Seeds harvested from these plants were labelled as T_0-seeds.

3.2.6 Screening plant transformants

Primary plant transformants

All T_0 – seeds (resulting from the transformation) were germinated directly on seed sowing mix (soil composition as in section 3.2.1) in a seedling tray (37 cm long x 23 cm wide x 6 cm deep). Seedling preparation and plant growth conditions were described in section 3.2.1. The T_0-seed was a mixture of transformed and un-transformed heterozygote and homozygote Arabidopsis *aos* mutants and wild type plants. Transformed plants (T_1-generation) containing the promAOS:VvAOS construct were selected by spraying 1.7 % (v/v) commercial BASTA herbicide (Agpro, NZ) when germinated T_0-seeds produced the first pairs of true leaves. Transformed plants resistant to BASTA herbicide were labelled as T_1-plants and grown under the same conditions until siliques were ready for harvesting. While these plants were maturing, individual T_1-plants were assessed for the presence of the VvAOS gene and a homozygous Arabidopsis *aos* mutant genotype using the PCR method, as described in section 3.2.1. Primer combinations used for this assessment were CYP74AF – CYP74AR, TJRB (Fwd) – CYP74R and VvAOS (Fwd-Rev) (Table 3.1). Homozygous Arabidopsis *aos* mutants transformed with promAOS:VvAOS were consequently identified based on the scoring of PCR amplicons, as shown in table 3.1, below. Seed was only collected and retained from plants that contained both the promAOS:VvAOS transgene and the homozygous *aos* allele. Each of the homozygous *aos* mutant genotype T_1-plants positive for the promAOS:VvAOS gene was treated as an independently transformed plant line. Seeds collected from each T_1-plant were labelled as T_1-seeds.

Primer pairs	Homozygous promAOS:VvAOS	Heterozygous promAOS:VvAOS	Wild type plant
CYP74 F and R (2.5 kb)	Negative	Positive	Positive
TJRB and CYP74 R (0.4 kb)	Positive	Positive	Negative
VvAOS(Fwd) and (Rev) (1.5 kb)	Positive	Positive	Negative

Table 3.1 Scoring table to identify homozygous *aos* mutant transformed with promAOS:VvAOS gene
Homozygous promAOS:VvAOS plants were identified via aPCR method using a combination of CYP74AF, CYP74R, TJRB, VvAOS(Fwd) and VvAOS(Rev) primers. Indication, "Positive" – PCR product amplified and "Negative" – No PCR product amplified

Generating homozygous T_3-plants

Approximately 100 to 150 seeds from each plant line were transferred into 2 mL Eppendorf tubes and sterilized using a vapour phase sterilization method. Seeds in open tubes were placed on a rack in a desiccator jar containing a beaker with 100 mL of commercial strength bleach. Prior to sealing the desiccator jar, 5 mL of concentrated hydrochloric acid (HCl) was carefully added to the bleach in a fume hood to generate chlorine gas. The vessel was immediately sealed and left for approximately four hours to sterilize the seeds. After sterilization, seeds were scattered evenly on BASTA selection plates [one-half-strength Murashige and Skoog medium (MS) with vitamins, 3% (w/v) sucrose, 0.8% (w/v) phyto agar and 10 mg/mL BASTA]. Seeds on the BASTA selection agar plates were stratified by placing the plates in 4°C for two days and then subsequently transferred to a tissue culture room for two to three weeks. The tissue culture room conditions were maintained at 16 hours of light per day, with a light intensity of approximately 100 µmol/m^2sec provided by cool white fluorescent tubes, and at a constant temperature of 20°C. Individual lines were scored for their segregation ratio of BASTA resistance to susceptibility. Only plant lines that exhibited a germination segregation ratio of approximately 3:1 were selected for further analysis. Between eight and ten plants from each of the selected lines were eventually transferred to soil and grown in the growth room, as described above. Seed was harvested from each individual plant and plant line and labelled as T_2-seeds.

T_2 - seeds were sterilized and planted out, as described above. The resulting T_3-plantlets, which gave 100% germination on the BASTA selection plates, indicated a homozygous line transformed with the VvAOS gene. These plant lines were selected as the source material for further analysis of VvAOS function in the Arabidopsis *aos* mutant. Seeds collected from the homozygous plant transformed with VvAOS gene were labelled as T_3 – seeds. Phenotypic examination was carried out via naked eye and under a light microscope (Olympus SZX16 [Olympus Ltd, NZ) to differentiate between homozygous Arabidopsis *aos* mutants, transformed homozygous Arabidopsis *aos* mutants and wild type plants. Examinations were based on phenotypic differences in the leaves, flowers, number of siliques and their size, floral organ developmental stages, siliques and inflorescence formation and average of seeds viability in each silique. For viability seed counting, all siliques involved in viable seed counting were at

approximately similar sizes from both transformed homozygous Arabidopsis *aos* mutants and wild type plants.

3.2.7 Analysis of homozygous Arabidopsis *aos* mutant transformed with VvAOS gene response to wound treatment

Homozygous Arabidopsis *aos* mutants transformed with the VvAOS gene were grown in the plant growth room under the conditions described above (Section 3.2.1) with the exception of the light conditions were altered to 12 hour light/day to induce the production of more and larger leaves on the plants. Each pot contained five to six individual plants. In each pot, only three to four of these individual plants were subjected to wounding. On the wounded plant, between three or four leaves of similar sizes were subjected to wounding. Wounding was achieved by using a small pair of pliers to crush the leaves in a manner that generated maximum mechanical injury for each leaf in order to induce consistent wounding. For standardization, each leaf was subjected to only one mechanical injury with approximately equal wounding pressures.

For the wound treatment experiment, young leaf tissues approximately 1-2 cm in size from eight week-old plants were treated with mechanical damage and samples were harvested in a time course as follows: 0 (pre-wound), 0.5, 1, 2, 4, and 6 hours. Due to the time and research fund constraints, unwounded control from each time course sample was not collected. However, all Arabidopsis plants (homozygous *aos* mutant, heterozygous *aos* mutant and wild type) were grown in the control environment (plant growth room). Therefore the possibility of diurnal expression of AOS was minimum. Samples were collected from three biological replicates and each biological replicate was a collection of four to five different individual plants in one pot. The time periods for sample collection were based on information from Park et al. (2002). In order to minimize crosstalk among plants via volatile organic compounds (VOC), all transformed plants involved for wound treatment experiment were transferred from the plant growth room to a Westinghouse Biological Safety Cabinet Class II (MedLab Instrument, New Zealand) and the wounding experiment was carried inside. The Westinghouse Biological Safety Cabinet Class II (MedLab Instrument, New Zealand) provided constant air flow to avoid VOC accumulating in the atmosphere around the plants. The environmental conditions within the cabinet were as follows; constant air velocity – 0.44 m/s and light - 20 $\mu mol/m^2sec$. The plants remained in the cabinet post wounding until leaf samples for those plant were collected. For sample collection at each time period, wounded leaves from respective transformed plants in the biosafety cabinet were collected and immediately snap frozen into liquid N_2. After sampling, plants were removed from the biosafety cabinet and returned to the growth room.

Frozen leaves samples were stored at -80°C before grinding for total RNA extraction. Leaf samples were ground manually to a fine powder in the presence of liquid N_2 using a mortar and pestle. Approximately 50 mg of fine ground leaves were used as a starting material for total RNA extraction.

Total RNA extraction, quantification, validation and cDNA synthesis were carried out following the methods described in sections 2.2.11. Transcript abundance analysis was carried out via the qRT-PCR technique, as described in Section 2.2.12. Reference genes, Arabidopsis F-BOX (accession: At5g15710) and elongation factor 1-alpha (accession: At5g60390) were used to normalize the transcript expression of the genes of interest in the transgenic plant leaf samples. In this study, beside quantifying the VvAOS and AtAOS gene transcripts, wound-induced genes (also known as JA-responsive genes) *Arabidopsis thaliana* vegetative storage protein 2 (AtVSP2) and lipoxygenase 2 (AtLOX2) were included to investigate the immediate effect of transgenic VvAOS overexpression in the transformed plants (Utsugi et al., 1998; Creelman and Mulpuri, 2002; Park et al., 2002). Primer pairs for qRT-PCR analysis used in this experiment are shown in the Appendix A.2. Statistical analysis to determine significant differences among the multiple data collected from individual transformed plant lines was carried out using one-way ANOVA (no blocking) by GenStat version 16 (VSN International Ltd, UK) with Fisher's Protected LSD multiple comparisons ($P < 0.05$). Data results from qRT-PCR analysis were presented as the number of gene copies detected in 4 µL of cDNA sample synthesized from 500 ng of the total RNA.

3.2.8 Vapour phase method for jasmonic acid extraction

Extraction and analysis of jasmonic acid (JA) content in transgenic leaf samples was carried out essentially according to the protocol, as described by Schmelz et al. (2004) and Mishina and Zeier (2006). Leaf samples from transformed Arabidopsis promAOS:VvAOS transgenic lines were ground to a fine powder with a mortar and pestle in the presence of liquid N_2. Approximately 100 mg of each plant line was used to extract jasmonic acid. In a 2 mL tube, 600 µL of pre-heated (70°C) extraction buffer (water:1-propanol:HCl = 1:2:0.005) was added to each sample and vortexed. An internal standard consisting of 20 µL of 20 µg/mL dihydrojasmonic acid (TCI Chemical Industry Co. Ltd, Tokyo, Japan) in methanol was added to each sample followed by subsequent addition of 1 mL of dichloromethane (DCM). The mixture was centrifuged for 1 min at 10,000 x g to separate the DCM and extraction buffer layers. The organic phase at the bottom of the tube was transferred to a 4 mL vial. Sodium sulphate (Na_2SO_4) was added to remove traces of water from the organic solvent solution.

In order to make the polar compound, jasmonic acid, more volatile and, thus, amenable to GC/MS analysis, the carboxylic group was esterified to yield methyl jasmonate. Derivatisation was carried out by adding 2 µL of 2 M trimethylsilyldiazomethane (TMS) in hexane (Sigma-Aldrich New Zealand Ltd, NZ) to each sample followed by incubation of the mixture for 5 min at room temperature. The derivatisation reaction was stopped by adding 2 µL of 2 M acetic acid in hexane. The derivatized extract was then transferred to a 4 mL glass vial with a hole in the cap and a PTFE/silicone septum. An 18 gauge syringe needle and a volatile collection trap containing 30 mg Super-Q (VCT-1/4X3-SPQ; Analytical Research Systems, Inc., FL, USA) Super Q absorbent filter (Altech, USA) was inserted through the septum to collect volatile compounds from the organic extract (see figure 3.2a for the experimental

setup). Super-Q is a highly stable divinylbenzene polymer absorbent that is tolerant to water vapour and temperatures up to 300°C. A gentle steam of N_2 gas (flow rate 0.8 L/min) was introduced into the vial through the 18 gauge needle. The assembled vial setup was then placed in a dry block at 70°C to accelerate the evaporation of the derivatized volatile components in the organic extract. After the organic solvent was completely evaporated (approximately three to five minutes), the 4 mL vial containing the sample was transferred to a second heating block set at 200°C and samples were incubated for two minutes. This step is required to collect compounds with low volatility. After the vapour phase extraction was completed, the volatile compounds trapped in the filter were eluted with 150 µL DCM into a 1.5 mL GC vial and immediately stored at -80°C until needed for analysis by gas chromatography/mass spectrometry (GC/MS). An aliquot of 40 µL was used for GC/MS analysis which was carried out using Lincoln University facilities (Lincoln University, NZ).

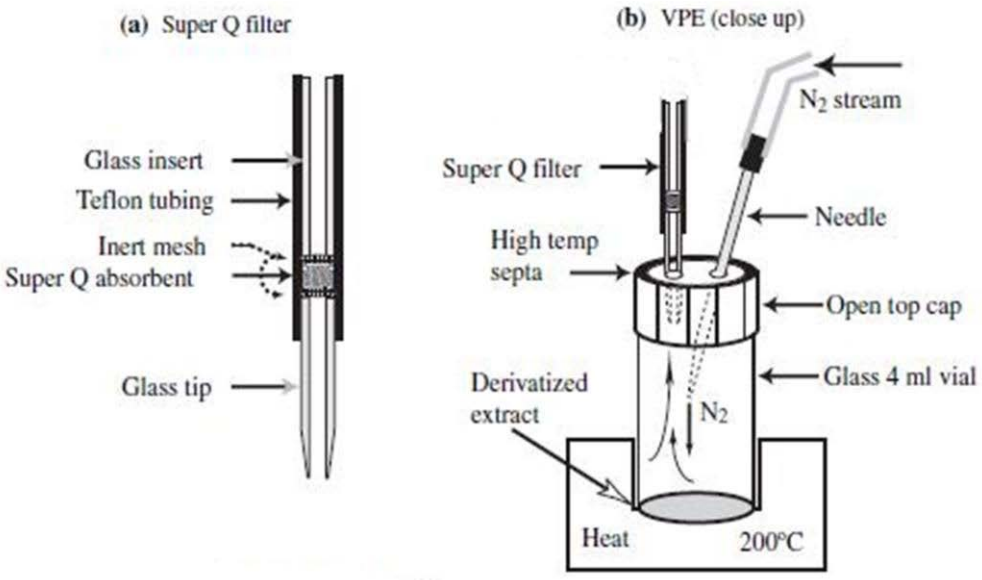

Figure 3.2 Vapour phase extraction layout
Layout for vapour phase extraction set up to collect plant volatile compounds, including derivatized JA (methyl jasmonate). Adapted from Schmelz et al. (2004)

3.2.9 GC-MS analysis of jasmonic acid

Samples of derivitized jasmonic acid (methyl jasmonate) for GC-MS analysis were eluted in dichloromethane (DCM) solvent. Eluted samples were analysed using a Shimadzu GCMS-QP2010 (Shimadzu, Japan) fitted with a Restek Rxi-1ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte, PA, USA). A CTC-Combi PAL auto-sampler (Shimadzu AOC-5000) was used to inject a 3 µL of sample into the GC injection port, operating in a high pressure injection splitless mode at 220°C and 168 kPa for 40 seconds. After injection, the column oven was held at 50°C for three minutes, then heated to 320°C at 8°C min^{-1}, and held at this temperature for eight minutes. Helium was used as

a carrier gas with the constant linear velocity set at 44.4 cm/sec in split mode (1.5 mL/min). The mass spectrometer (MS) was operated in the single ion monitoring mode with selected masses used to identify methyl jasmonate (target ion m/z 151, confirming ions – m/z 193 and m/z 224) and the internal standard, dihydro-methyl-jasmonate (target ion m/z 156, confirming ions – m/z 153 and m/z 195). The temperature of the capillary interface was 320°C, with the MS source temperature set at 230°C. Initial confirmation of retention times was performed for the compound of interest by injecting the individual standards and matching their mass spectra with the spectra of reference compounds in the NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST05). The quantitative determination of methyl jasmonate in a sample was performed using the GC Solution software provided by the GC-MS instrument manufacturer (Shimadzu Scientific Instruments, NZ). The methyl jasmonate peak was integrated and the area was related to the area of the internal standard peak. JA content was calculated using the following formula, where 3.5 is a correction factor:

$$JA\left(ng \cdot g^{-1}FW\right) = \frac{area_{m/z}\left(JA\right) \cdot 3.5 \cdot 200}{area_{m/z}\left(dihydroJA\right) \cdot FW}$$

3.3 Results

3.3.1 Development of the plant binary vector system

In order to accurately determine the ability of the cloned VvAOS gene to complement the AtAOS mutant, it was decided to generate transformed lines of the mutant with VvAOS driven by the native Arabidopsis AOS promoter (promAOS). To achieve this, the pARTBGW binary vector obtained from Plant and Food Research Institute, Canterbury, New Zealand, which possessed a CaMV35S promoter to drive transgene transcription, was replaced with an approximately 2.2 kb amplicon that contained the promAOS region. The initial amplification product for the promAOS fragment from genomic DNA of Arabidopsis WT is shown in figure 3.3A. In order to confirm the incorporation of promAOS and VvAOS genes into pARTBGW binary vector (pARTBGW:promAOS:VvAOS), PCR amplification was carried out using the newly constructed vector as a template and a primer combination of Int2pAOS(Fwd) and VvAOS-Rev (Figure 3.3B).

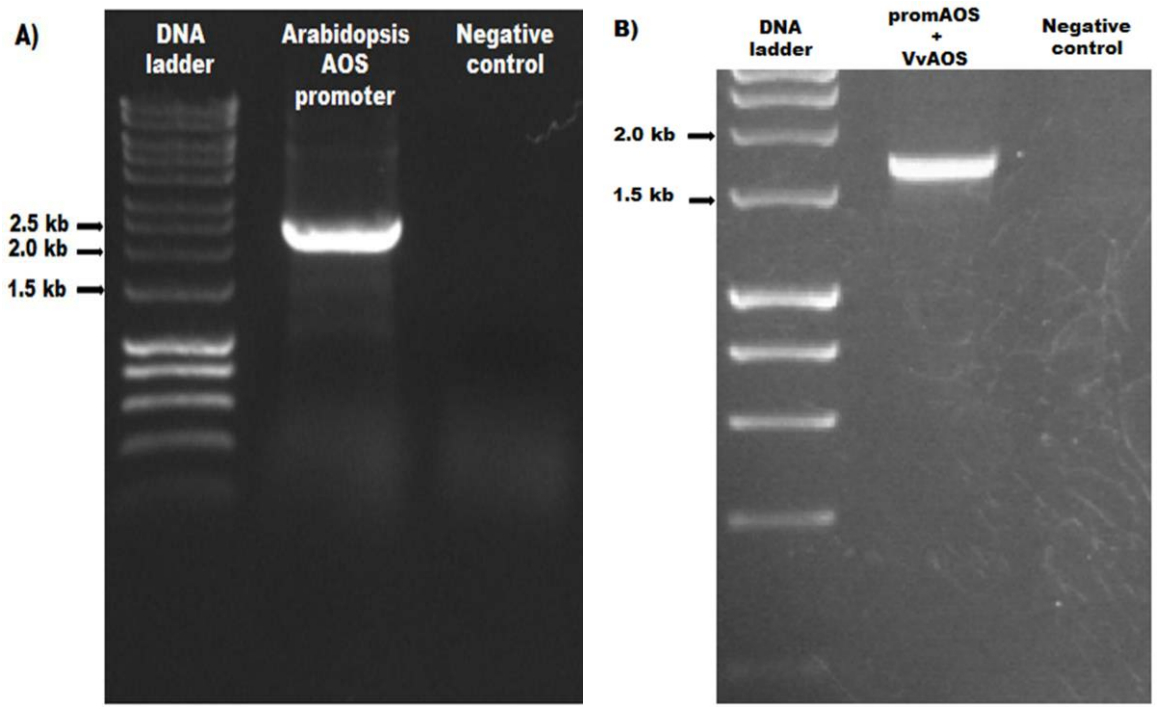

Figure 3.3 Arabidopsis AOS promoter and pARTBGW:promAOS:VvAOS binary vector
Gene regulator CaMV 35S promoter in original pARTBGW vector was replaced with native promAOS. A) DNA amplification of promAOS region approximately 2.2 kb in size whereas B) is a confirmation of the incorporation of promAOS:VvAOS into pARTBGW via the PCR method. Expected amplicon size approximately 1.7 kb. DNA ladder used was Hyperladder (1 kb) a from Bioline (Total Lab system Ltd, NZ)

3.3.2 Complementation of the AOS function in Arabidopsis *aos* mutant with VvAOS

Transformation, screening and identification of homozygous Arabidopis aos mutant complemented with VvAOS gene

As described by Park et al. (2002), the Arabidopsis *aos* mutant was a complete male sterile due to the lack of jasmonic acid (JA) production. In Arabidopsis, there is only one copy of AOS gene present on chromosome 5 and alternative enzymes have not been reported to catalyse this step in the synthesis of JA and it derivatives (Laudert et al., 1996). As illustrated in figure 3.4, insertion of T-DNA located 98 bp upstream of the invariant cysteine residue within heme-binding domain completely removed the AOS enzyme's function in Arabidopsis (Park et al., 2002).

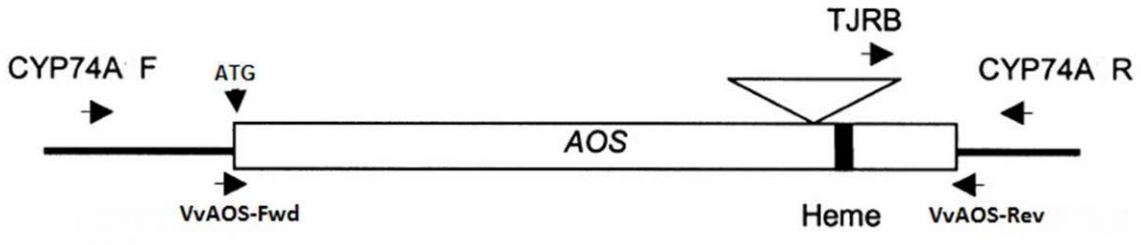

Figure 3.4 Schematic map of knock-out AtAOS gene
The open triangle depicts the location of the T-DNA insertion position within AtAOS gene. Black close box indicates the conserved heme-binding domain (Heme). Arrows show the locations and direction of primers used to screen for Arabidopsis *aos* mutant (CYP74A F, CYP74A R and TJRB). Adapted from Park et al. (2002)

The standard approach for complementation of the Arabidopsis *aos* mutant required using plants that were homozygous for the AtAOS mutation alleles. Given that the homozygous lines were male sterile, fertility was reported to be restored via application of exogenous JA (Park et al., 2002). However, in our hands, this approach proved to be very difficult and an alternative approach based on the transformation of plants that were heterozygous for the mutation was adopted. Floral dip transformation of the heterozygous Arabidopsis *aos* mutant was redirected to produce segregants with respect to the mutated AOS gene in a ratio of 1:2:1 (wild-type:heterozygous mutant:homozygous mutant) based on simple Mendelian genetics inheritance. In this experiment, heterozygote Arabidopsis *aos* mutant plants were prepared for transformation, as described in section 3.2.1. Subsequently, T_1-transformants plants were PCR screened to identify plants that were both homozygous for the AtAOS mutation (Section 3.2.5) and that were transformed with the complementation construct by virtue of BASTA resistance. However, heterozygote Arabidopsis *aos* mutants and homozygous Arabidopsis *aos* mutants carrying the complementation construct were predicted to be indistinguishable based solely on the recovery of male fertility (the main recessive phenotype conferred by the AOS mutation). Consequently, subsequent generations were also PCR screened and assessed for progeny segregation via BASTA plate selection to identify plants that were homozygous both for the AtAOS mutation and a single insertional locus for the VvAOS

complementation constructs (Section 3.2.6). Primary transformants of Arabidopsis *aos* mutant carrying the promAOS:VvAOS gene were screened using BASTA herbicide plates yielded 39 individual plant T_1-plant lines. Among the 39 primary transformants, eight were identified as homozygous for the Arabidopsis AOS mutation and also contained the promAOS:VvAOS transgene (from here on this on will be referred as *aos*:promAOS:VvAOS plant lines), 22 plants were found to be heterozygous for the Arabidopsis AOS mutation and nine plants were found to not contain the AOS mutation. The segregation ratio for the transformed lines was found to be 1:2.275:0.89, which is in agreement with a simple 1:2:1 Mendelian segregation ratio and chi-square test, as discussed above. However, two of the eight *aos*:promAOS:VvAOS plants did not survive, therefore, only six plants were used to produce T_3-plant lines that were used as source material for further analysis. These six *aos*:promAOS:VvAOS plant lines were L5, L28, L29, L30, L31 and L32. PCR analysis results for the six individual *aos*:promAOS:VvAOS plants using combination CYP74AF, CYP74AR, TJRB, VvAOS-Fwd and VvAOS-Rev are shown in figure 3.5 below.

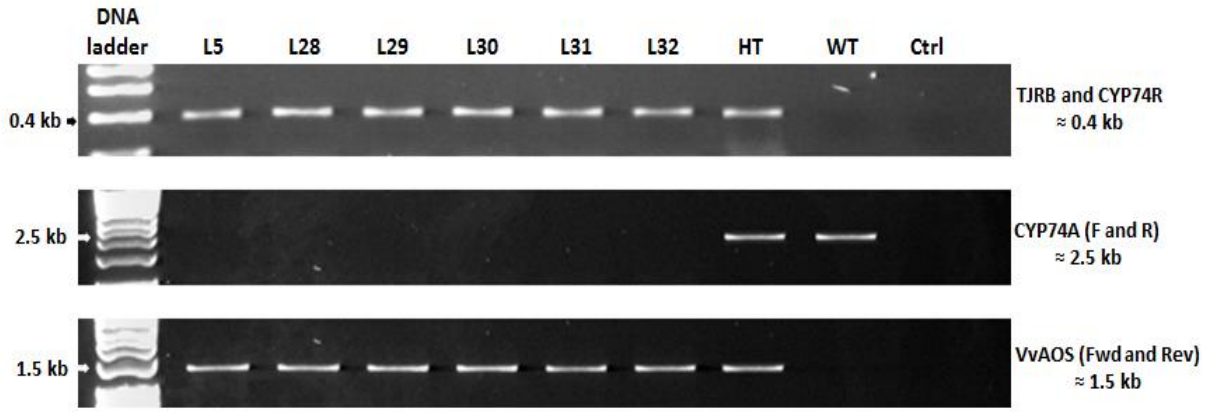

Figure 3.5 Results showign *aos*:promAOS:VvAOS plants via the PCR amplification method
Result show six *aos*:promAOS:VvAOS plants – L5, L28, L29, L30, L31 and L32 plants had their genotype confirmed via the PCR amplification method. HT-(Heterozygous promAOS:VvAOS) and WT- (wild type) were included as a plant control and Ctrl (control) as a PCR reaction control. DNA ladder was HyperLadder I (1kb ladder) from BioLine (Total Lab System, NZ) was used to measure DNA fragment size.

Putative VvAOS gene expression complements male sterile phenotype of homozygous Arabidopsis aos mutant

Homozygous T_3-plant lines for Arabidopsis *aos:*promAOS:VvAOS transgenics were used as source material for plant phenotypic analysis. As in qualitative phenotypic analysis, plant growth development, siliques and inflorescence development, silique formation and floral organ formation was taken from the average of six plant lines of *aos:*promAOS:VvAOS. For the analysis to compare the complemented phenotype in *aos:*promAOS:VvAOS plant lines, Arabidopsis lines homozygous for the *aos* mutation and WT plants were grown together under similar conditions, as described in the material and methods (Section 3.2.1). Results showed that at a developmental level the Arabidopsis

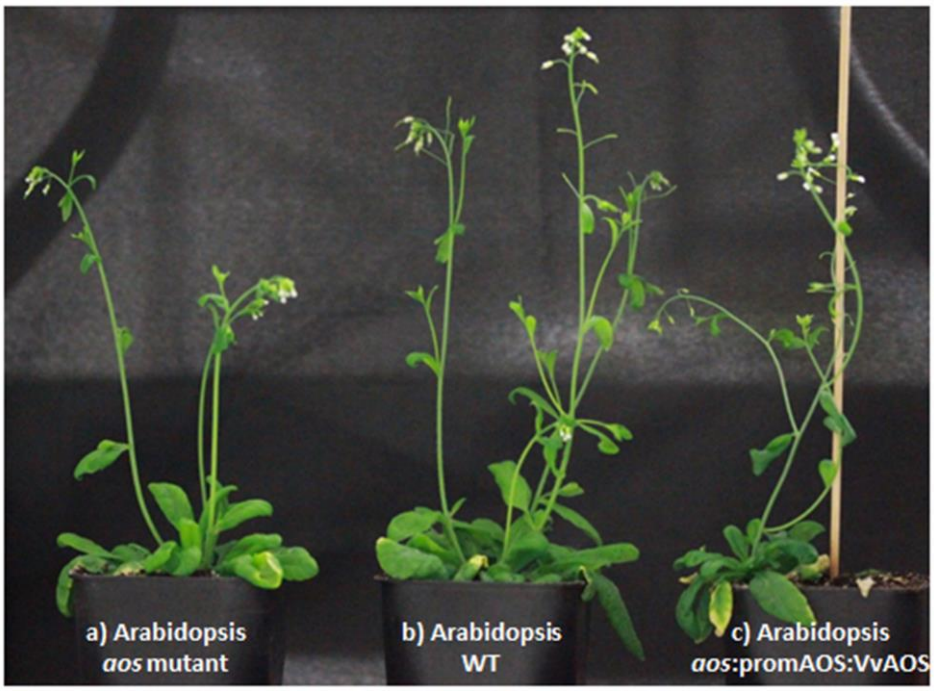

Figure 3.6 Growth and development of *aos* mutant, WT and *aos*:promAOS:VvAOS plants
Plants were approximately six weeks-old. No apparent phenotype defects between plants except for silique development in Arabidopsis *aos* mutant plants, which indicated that disruption or recovery of AOS gene function displayed a minimal impact on growth and development in *Arabidopsis thaliana*

Figure 3.7 Silique and Inflorescence development
Inflorescences show normal development in all plants. However, siliques failed to elongate in Arabidopsis *aos* mutant plants (a) Siliques elongate normally in *aos*:promAOS:VvAOS (b) and WT (c) plants and produce seeds.

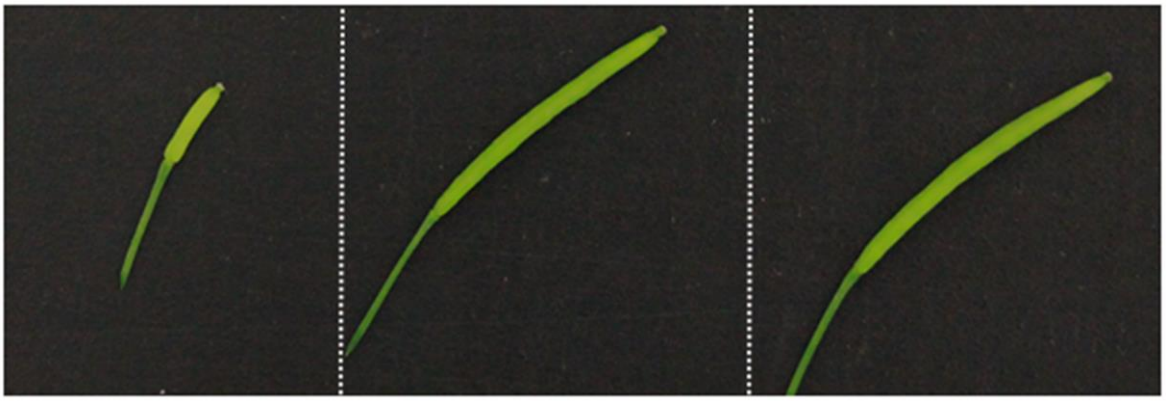

Figure 3.8 Silique formation
Development of siliques in *aos*:promAOS:VvOAS plant (b) show similar sizes with WT (c). As a comparison, siliques did not develop in Arabidopsis *aos* mutant plants (a).

Figure 3.9 Floral organ formation
Anthers did not fully develop in Arabidopsis *aos* mutant plants (a), therefore, they failed to pollinate stigmatic papillae and, as a consequence, no seeds were produced. In contrast, anthers developed fully in both *aos*:promAOS:VvAOS and WT plants and pollinates the stigmatic papillae. Both plants produced healthy silique with approximately similar numbers of seeds. White arrow indicates an anther position whereas red arrow indicates the location of stigma.

aos mutant, *aos:*promAOS:VvAOS and WT plants showed no apparent defects except for aberrant silique formation in the Arabidopsis *aos* mutant plant (Figure 3.6). In the early stages of development, all plants displayed normal inflorescence formation but siliques failed to elongate in the Arabidopsis *aos* mutant as compared to the normal elongation of siliques in both the *aos:*promAOS:VvAOS and WT plants (Figure 3.7). Further investigation showed that silique development in *aos:*promAOS:VvAOS was normal and displayed a similar size to that found in the WT plants (Figure 3.8). In addition, the average number of viable seeds counted from WT plants (Table 3.2) indicated that male sterile phenotype had completely recovered. For viable seed counting, siliques were collected from L28 and L30 which, subsequently, were the plant lines used for wound response assessment. Due to the failure of the Arabidopsis *aos* mutant siliques to elongate in a manner consistent with WT, floral organs just prior to opening were examined. Results showed that at this stage, as expected, anthers did not fully develop in the Arabidopsis *aos* mutant and, consequently, pollen was not released onto the stigmatic papillae and fertilization did not occur (Figure 3.9a). In comparison, in the *aos:*promAOS:VvAOS and WT plants, the anthers did fully develop and fertilisation was able to take place normally (Figure 3.9b and 3.9c). Clearly, the male sterile phenotype was recovered by the introduction of VvAOS gene in Arabidopsis *aos* mutant plant. However, a question that still remained was whether the introduction of the VvAOS gene in the transgenic *aos:*promAOS:VvAOS lines was able to complement the *aos* mutation in terms of the plants' responses to environmental stimuli, such as abiotic stresses. To investigate this matter, we used a mechanical wounding approach to examine *aos:*promAOS:VvAOS transgenic line responses to wounding.

Plant lines	*aos* mutant plant (Average per silique)	*aos*:promAOS:VvAOS plant (Average per silique)	WT plant (Average per silique)
Plant 1-L28	0	42.7 (± 2.6)	45.8 (± 7.5)
Plant 2-L30	0	40.5 (± 4.6)	43.7 (± 4.7)

Table 3.2 Viable seeds from complemented Arabidopsis and Wild type
Average of viable seeds counted from six individual silique from each plant. For consistency, all siliques involved were at the approximate similar sized from both *aos*:promAOS:VvAOS and WT plants.

3.3.3 Complemented Arabidopsis *aos* mutant plant response to wound treatment

As mentioned in the material and methods (Section 3.2.7), in order to investigate transgenic *aos*:promAOS:VvAOS plant line responses to mechanical wounding, transcript abundance of VvAOS, AtAOS, AtLOX2 and AtVSP2 genes were measured. These data were compared to the same data generated from the WT and Arabidopsis *aos* mutant lines. Validation of total RNA integrity, and assays for RNA and cDNA synthesis contamination from genomic DNA were carried out following the protocol, as described in chapter 2 (Sections 2.2.11). In this experiment, the qRT-PCR primer pairs used for the qRT-PCR analysis are shown in the Appendix A.2. As mentioned previously, six *aos*:promAOSVvAOS plant lines were identified. However, due to limitations in both time and research funding, we were

not able to perform preliminary VvAOS transcript abundance analysis on all transformed plant lines. Our attempt to select the best performing transgenic plant lines was based on the phenotypes observed, such as average size of siliques, average number viable seeds per silique and average number of siliques produced. However, as there was little to discriminate the lines in terms of physical phenotypic differences *aos*:promAOS:VvAOS plant lines L28 and L30 were selected randomly for analysis of their responses to wounding. The results indicated that transcript abundance of the VvAOS gene in L28 and L30 increased upon wound treatment (Figure 3.10a), which was consistent with the fact that the transgene was being driven by the AtAOS promoter. Over the course of the six hour wound treatment, VvAOS transcript abundance in L28 peaked at one hour but for line L30 the peak of transcript abundance was observed at two hours after wounding. As expected, the VvAOS gene was able to complement wound response of homozygous Arabidopsis *aos* mutant, as shown in both L28 and L30. However, it was surprising that the transcript abundance of VvAOS in complemented Arabidopsis *aos* mutants was very high (approximately 59-fold higher in both L28 and L30) than in their respective samples in WT at two hours after wounding. Another surprise result from this experiment was the knock-out endogenous AtAOS gene response to wound treatment in both the *aos*:promAOS:VvAOS lines and Arabidopsis *aos* mutant plant lines. Results showed that transcript abundance of endogenous AOS (knock-out gene) significantly increased after wound treatment and peaked at one hour before declining to near normal level four hours after wounding in both L28 and L30. Whereas, although it was a small increment of endogenous AtAOS transcript in Arabidopsis *aos* mutant but it was significant at one hour after wounding compared to samples at different time points (ANOVA, $P < 0.001$). As a comparison, AtAOS transcript abundance peak at two hours and declined to near normal level six hours after wounding in the WT.

As shown by the results above, the VvAOS transgene clearly complemented the *aos* mutation response to wound treatment but how about their signal transduction pathway? To answer this simple question, we measured the expression of AtLOX and AtVSP2, two JA responsive genes in *aos*:promAOS:VvAOS plants. The results showed that transcript abundance of AtLOX2 gene was found to increase to approximately the same level in both L28 and L30 but to only two thirds of the expression level observed for wild type plants at peak level two hours after wounding (Figure 3.11a). In contrast to the WT plant and *aos*:promAOS:VvAOS lines, AtLOX2 transcript abundance in Arabidopsis *aos* mutant plants did not respond to wound treatment. Statistical analysis for significant differences showed that no significant differences (ANOVA, $P=0.21 > 0.05$) among Arabidopsis *aos* mutant samples collected over the course of the six hour wound treatment. Another JA-responsive gene, AtVSP2, also responded to wound treatment at the approximate level in both L28 and L30 but, again, only to two thirds of the expression shown in WT plants at the peak level, eight hours after wounding. Again, the AtVSP2 gene in Arabidopsis *aos* mutants did not respond at any point of wound treatment.

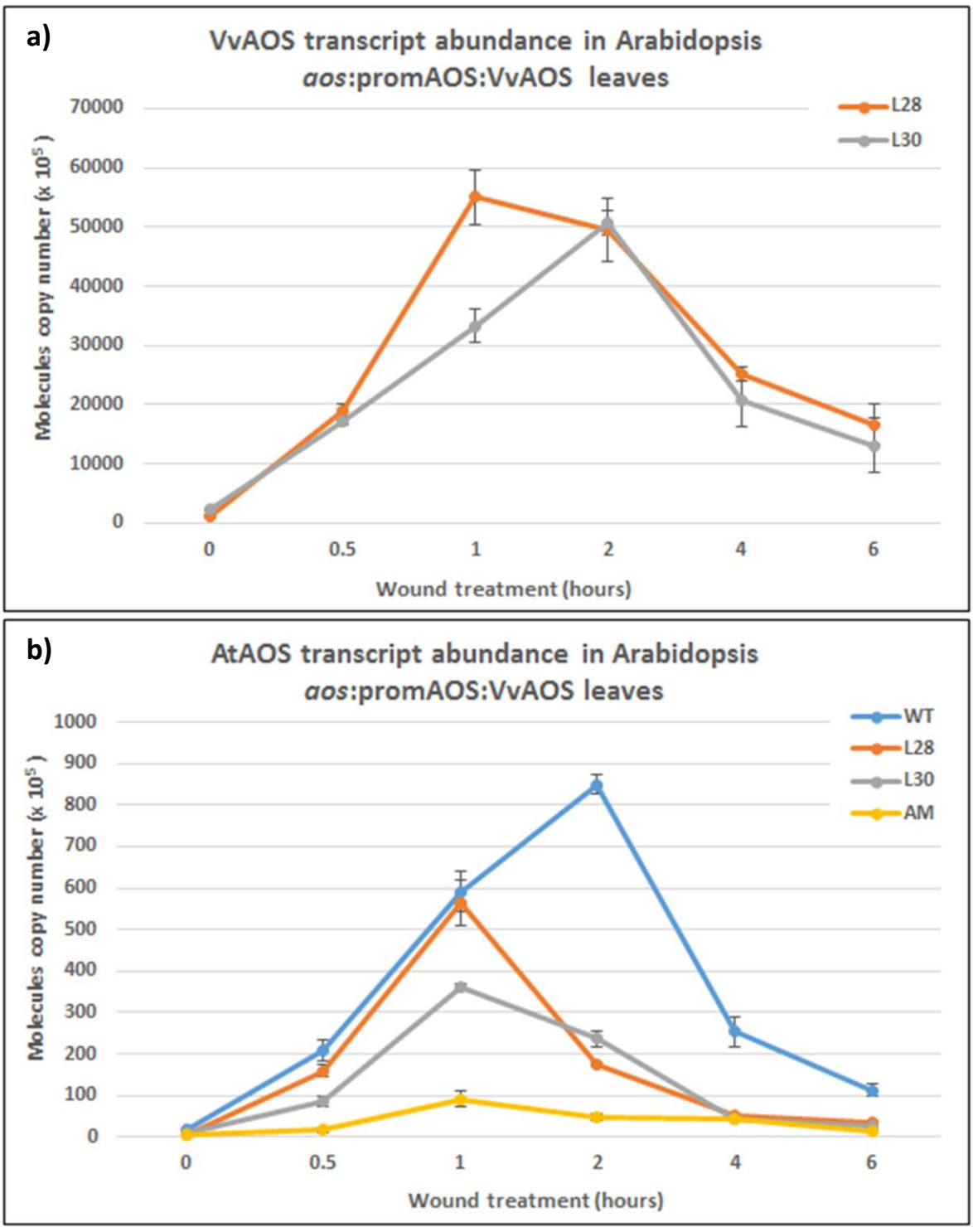

Figure 3.10 Transcript abundance of VvAOS and AtAOS genes in Arabidopsis *aos*:promAOS:VvAOS plant lines

Transcript abundance of VvAOS and AtAOS genes were measured from *aos*:promAOS:VvAOS plant L28 and L30. As a comparison, similar transcript genes were also measured in WT and Arabidopsis *aos* mutant plants. Graph a) transcript abundance of VvAOS gene and b) transcript abundance of AtAOS gene. L28 and L30 – two complemented Arabidopsis *aos* mutant lines, WT – Arabidopsis wild type, AM – homozygous Arabidopsis *aos* mutant and bar represent standard deviation of the mean from three biological replicates of samples

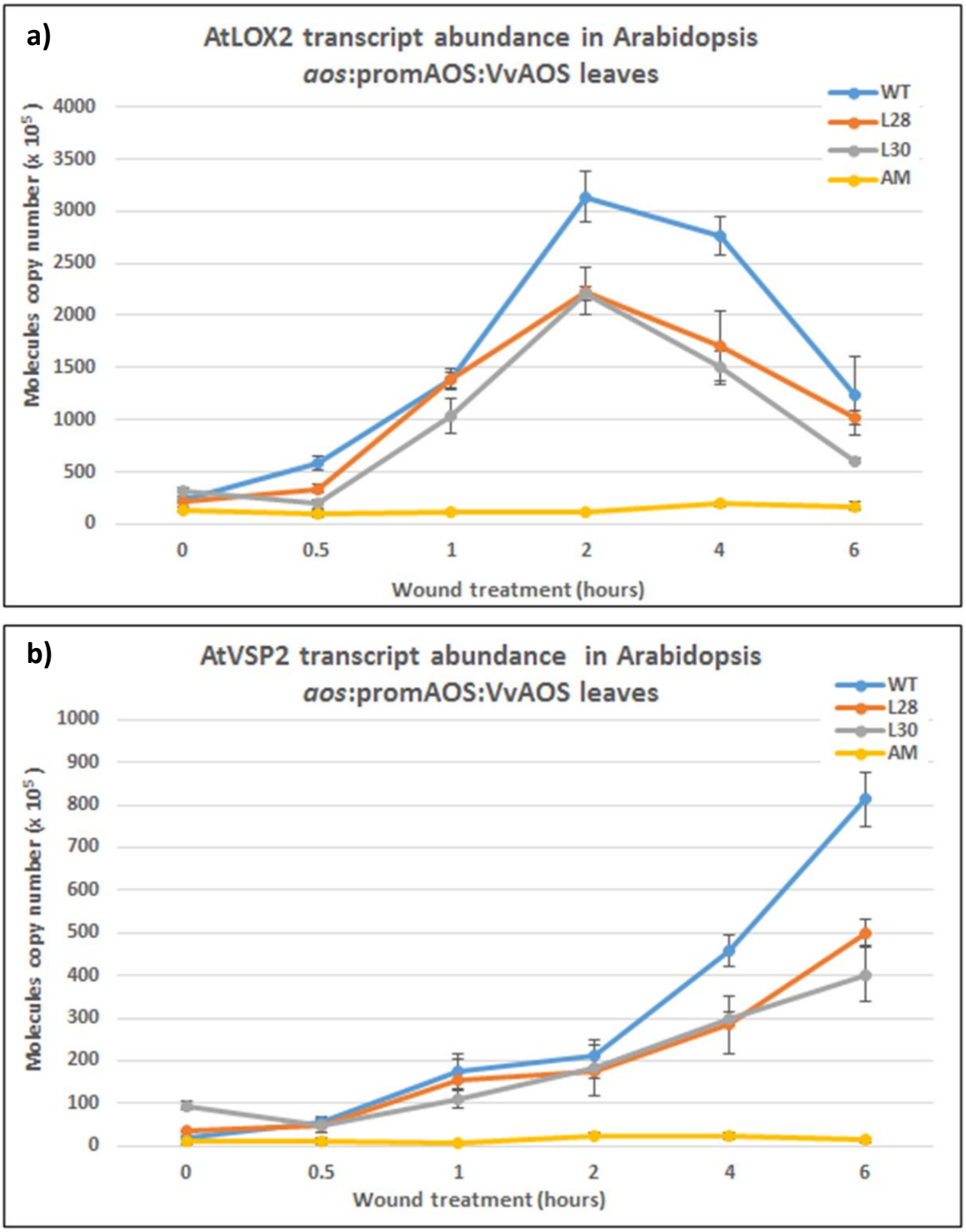

Figure 3.11 Transcript abundance of AtLOX2 and AtVSP2 genes in Arabidopsis *aos*:promAOS:VvAOS plant lines

Transcript abundance of JA-responsive genes, AtLOX2and AtVSP2 genes were also measured from *aos*:promAOS:VvAOS plants, L28 and L30. As a comparison, similar transcript genes were measured in WT and *aos* mutant plants. Graph a) transcript abundance of AtLOX2 gene and b) transcript abundance of AtVSP2 gene. L28 and L30 – two complemented Arabidopsis *aos* mutant lines, WT – Arabidopsis wild type, AM – homozygous Arabidopsis *aos* mutant and bar represent standard deviation of mean from three biological replicate of samples

No significant differences were shown by the statistical analysis for significant difference values (ANOVA, P = 0.48 > 0.05) among samples collected from Arabidopsis *aos* mutants over the course of the six hour wound treatment. Generally, in this experiment, results indicated that signal transduction to regulate JA biosynthesis production was recovered by the introduction of VvAOS gene in the homozygous Arabidopsis *aos* mutant. However, for further confirmation, JA content in the *aos*:promAOS:VvAOS transgenic lines needed to be quantified.

3.3.4 Recovery of jasmonic acid biosynthesis

As shown in the previous experiments, complementation of the Arabidopsis *aos* mutant with the VvAOS gene, driven by the promAOS, exhibited a recovery in the transcription of two JA responsive genes and a recovery in male fertility of the mutant lines. However, a question that remained was that while the presence of VvAOS resulted in a recovery of mutant phenotypes as compared to wild type plants, the accumulation of transcripts of JA responsive genes (AtLOX2 and AtVSP2) was lower in the transgenic complementation lines than the wild type, suggesting that levels of jasmonic acid accumulating were likely to be lower in the transgenic lines as compared to wild type plants. This question was of particular importance due to the observations above, where the levels of VvAOS transcription in the transgenic lines were much higher than AtAOS in wild type plants and included a recovery of transcription of the mutated AtAOS in the transgenic lines in response to wounding. Using a published GC-MS method (Schmelz et al., 2004; Mishina and Zeier, 2006) we measured the levels of total JA at 0 (pre-wound), 1 and 6 hours after wound treatment in the samples. In this experiment, due to resource constraints, we could only measure JA compounds from L30 with WT and Arabidopsis *aos* mutant plants as comparison controls. Selection of L30 as a complemented sample was due to VvAOS transgene expression closely resembling the expression of endogenous AOS gene in WT sample.

Results indicated that in a pre-wound condition JA accumulation in WT and L30 plants was approximately at the similar level. After a one hour wound treatment, JA accumulation occurred in both plants (WT and L30) but the level of JA in L30 was only 61 percent compared to the amount accumulated in WT (Figure 3.12). Moreover, in Arabidopsis *aos* mutant plants using the same method JA accumulation was not detected in any of the 0, 1 and 6 hour samples. This confirmed the ability of VvAOS transgene to recover JA biosynthesis pathway, which had been thwarted by the knock-out endogenous AtAOS gene.

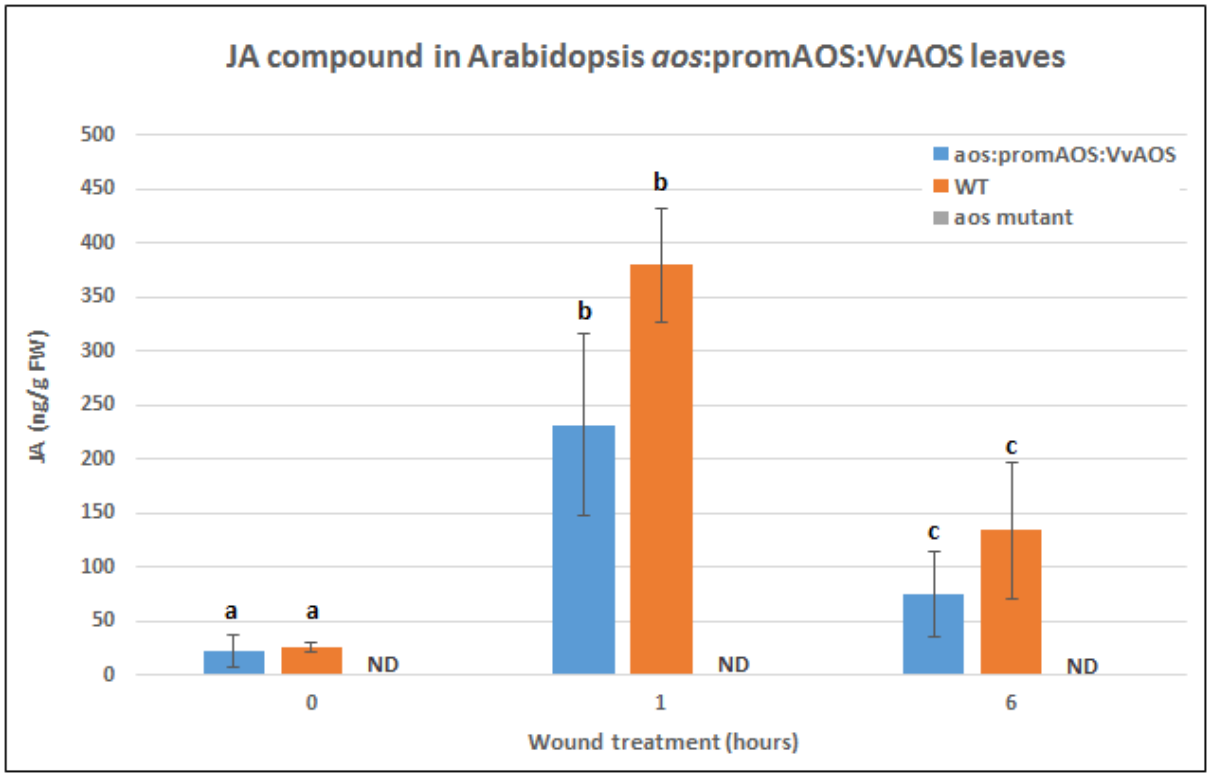

Figure 3.12 Jasmonic acid accumulation in wound treated Arabidopsis *aos*:promAOS:VvAOS leaves
JA accumulation was measured from L30 of wound treated Arabdiopsis *aos*:promAOS:VvAOS leaves at 0, 1 and 6 hours via GCMS method, as described by Schmelz et al. (2004) and Mishina and Zeier (2006) . As a comparison, JA was also measured from WT and Arabidopsis *aos* mutant plants. However, JA accumulation was not detected in any of Arabidopsis *aos* mutant plant samples. Bar represent standard deviation of the mean from three biological replicate samples. Alphabet (a, b and c) re-present no significant different between JA accumulation in WT and L30 at the respective time samples whereas ND indicate that no JA accumulation detected.

3.4 Discussion

In order to confirm that the cloned VvAOS was, in fact, an AOS gene we decided to investigate whether the grape orthologue was capable of complementing the Arabidopsis *aos* mutant. The mutation in the AOS gene was caused by a T-DNA insertion at 98 bp upstream of the invariant cysteine residue within the heme binding domain (Figure 3.4). The presence of the T-DNA has been reported to lead to complete removal of JA production and the presentation of a male sterile phenotype due to the blocking of JA biosynthesis (Park et al., 2002). Arabidopsis was known to have only a single copy of AOS gene in its genome (Laudert et al., 1996); therefore, using this complementation approach would have allowed us to confirm the biochemical and physiological activity of the VvAOS gene in an Arabidopsis background system. In order to provide a more realistic assessment of the ability of VvAOS to complement the AtAOS mutation we decided not to use the strong constitutive CaMV35S promoter, opting rather to use a construct in which the expression of the VvAOS transgene would be driven by a copy of the AtAOS promoter. Using promAOS as a gene regulator for the VvAOS transgene will replicate the natural regulation of endogenous AtAOS in the Arabidopsis system compared to the constitutive CaMV 35S promoter.

In addition to their functions in plant defence mechanisms, JAs also play crucial roles in plant development, such as in pollen maturation and dehiscence. Loss of AOS function disrupted a JA signalling component that caused defects in stamen development by blocking the elongation of anthers filaments formation, delayed anther dehiscence and production of non-viable pollen at floral stage 13 due to the anther failing to pollinated the stigma (Browse, 2009; Reeves et al., 2012; Song et al., 2013). The introduction of VvAOS gene in Arabidopsis *aos* mutant system (referred to as *aos*:promAOS:VvAOS) recovered the male sterility phenotype was a major indication that the cloned VvAOS encoded a functional AOS, which likely functioned in a similar manner in grapevines. Development of normal silique size, recovery of siliques and viable seed production, and fully developed stamens in *aos*:promAOS:VvAOS plant compared to WT indicated that the VvAOS gene was able to recover the lost function of the JA biosynthetic pathway in Arabidopsis. Furthermore, transcript accumulation of JA-responsive genes, AtLOX2 and AtVSP2, in L28 and L30 also indicated that JA signalling and its component network had been partially recovered.

As part of this experiment, we quantified VvAOS and JA-responsive genes transcripts (AtLOX2 and AtVSP2) as an indication for recovery of JA transduction signal in a complemented Arabidopsis *aos* mutant (Park et al., 2002). As expected, the introduction of transgene VvAOS enabled the recovery of the JA signalling pathway in Arabidopsis *aos* mutants but some of the results were beyond our expectation. First, although endogenous AOS in homozygous Arabidopsis *aos* mutant was completely knocked out, and we expected it will not respond to wound treatment but eventually its transcript abundance increased significantly within one hour after wounding. Second, transgene VvAOS transcript abundance in Arabidopsis *aos*:promAOS:VvAOS was exceptionally high compared to the

endogenous AOS gene transcript abundance needed in WT to activate a plant's response to wound treatment. Third, JA accumulation and transcript abundance of both JA-responsive genes (AtLOX2 and AtVSP2) in Arabidopsis *aos*:promAOS:VvAOS lines was only two thirds compared to WT considering VvAOS transcript gene was 59-fold higher than the AtAOS transcript.

Although there was no solid evidence from data presented in this experiment, it was tempting to speculate that the observation mentioned above could be a critical feature in understanding JA biosynthesis mechanisms in Arabidopsis *aos*:promAOS:VvAOS plants. Therefore, we proposed several possible reasons behind these three unexpected results. First, the endogenous AtAOS gene in the homozygous Arabidopsis *aos* mutant response to wound treatment indicated its gene regulator was still active and involved in AtAOS gene regulation. However, at this point endogenous AtAOS was not regulated by its own biosynthetic product but was activated by the early signal transduction mechanism created upon mechanical wounding. Early events associated with wound signalling included a rapid increase in the levels of cytosolic Ca^{2+}, membrane depolarization, inhibition of a proton ATPse in the plasma membrane and the activation of MAPK activity (Maffei et al., 2007; Hu et al., 2009; Arimura et al., 2011; Zebelo and Maffei, 2015) followed by the release of linolenic acid from membrane phospholipids and the subsequent activation of the production of the hydorperoxide substrate by lipoxygenase (Bonaventure and Baldwin, 2010). Substrate availability activated promAOS to produce AOS enzymes for the conversion to 12-oxophytodienoic acid (OPDA) but this did not materialize due to their knock-out function. Therefore, this explained the small increment of endogenous AtAOS gene transcript that occurred in Arabidopsis *aos* mutant plants. It was a small increment because no JA had been produced to stimulate a positive feedback loop to enhance a large signal transduction (see Figure 2.3). A similar phenomenon was also observed in Arabidopsis *aos*:promAOS:VvAOS (complemented Arabidopsis *aos* function). Due to the AOS function recovery, JA production in *aos*:promAOS:VvAOS plant was continuously activating the production of the hydroperoxide substrate via a positive feedback loop (Sasaki et al., 2001; Turner et al., 2002). As a consequence, endogenous AtAOS transcription was continuously produced up to one hour after wounding. However, this plant system shut down production in order to prevent further damage due to the accumulation of this useless transcript RNA (Schubert et al., 2004; Dalakouras et al., 2011). Interestingly, the activation of endogenous AtAOS was also a strong indication that the JA signalling pathway in Arabidopsis *aos*:promAOS:VvAOS was recovered.

Another unexpected result was the high transcript abundance of VvAOS genes in *aos*:promAOS:VvAOS lines compared to WT and yet the transcript abundance of both JA responsive genes (AtLOX2 and AtVSP2) and JA accumulation in *aos*:promAOS:VvAOS, were only approximately two thirds of these amount compared to the WT samples. These results provide a conundrum phenomenon because high level of VvAOS transcript did not translated efficiently into the production of JA compound. The use of the AtAOS promoter to drive VvAOS transcription in *aos* mutant plant

should have delivered similar levels of transcription as the wild type AOS. Although it was reported in cereal that having multiple copies insertion of transgene can enhance the expression level in proportion to gene copy number (Stoger et al., 1998; Gahakwa et al., 2000; Kohli et al., 2010), the high levels of VvAOS transcript accumulation observed are unlikely to arise solely due to the location of transgene insertion(s) because two independent transgenic lines tested were exhibited similarly high levels of transcript accumulation. There was also a possibility that by taking only 2.2 kb of the promoter region of AtAOS, it may have not captured all of the CIS-regulatory elements characteristic of the endogenous AtAOS gene promoter and have inadvertently removed CIS elements controlling levels of transcription of the wild type AtAOS gene. This in turn may lead to an apparent increase in 'strength' of this promoter that produce high transcript of transgene. Besides that, position effect of transgene VvAOS might be influenced by AtAOS local regulatory element. Integration of transgene can be influenced by a local enhancer nearby that could change the expression profile (Kohli et al., 2010). According to this report, the enhancer interacts with the regulatory element in the transformation construct to control transcription where the final expression pattern reflected the combined influence of both regulatory elements. Transgene VvAOS was designed to be driven by a similar regulator with local AtAOS. Therefore, high transcript abundance of VvAOS gene might also be influenced by local regulators.

However, the most puzzling result was the disconnection between the observed levels of VvAOS transcription and the relatively low levels of JA accumulation. Two possibilities may explain these observations. The first is that the VvAOS enzyme does not perform well in the Arabidopsis background, leading to poor production of JA despite the high levels of VvAOS protein that might be assumed to accumulate given the very high levels of transcription of the transgene. Secondly there is a possibility that there is an interaction between the re-activation of transcription of the mutant allele and the VvAOS transgenic allele. Recent reports have shown that T-DNA containing mutant alleles are often silenced via a siRNA-mediated chromatin silencing mechanism (Gao and Zhao, 2013). The T-DNA construct that is present in the AtAOS mutated allele derives from the pD991-AP3 vector. This T-DNA contains the Arabidopsis APETALA3 promoter fused to β-glucuronidase (GUS) and a nopaline synthase (NOS) promoter fused to the neomycin phosphotransferase (NPTII) gene, used to select primary transgenic lines. T-DNA mutants that have resulted from the insertion of the pD991-AP3 T-DNA have been shown to epigenetically silence the AP3 allele in mutant lines and that the degree of epigenetic regulation is dependent on the genomic location of the T-DNA (Hayakawa et al., 2015). The structure of the *aos* mutant flowers do not indicate that the T-DNA is epigenetically regulating AP3 in this line. However transcriptional reactivation of the T-DNA containing AOS allele may well stimulate silencing activity at this locus, leading to silencing of the VvAOs transgenic allele. It is not clear, given the level of sequence identify shared between the AtAOS and VvAOs gene sequences (Figure 3.13), whether sufficient sequence similarity exists to drive silencing of the VvAOS. However in a recent report

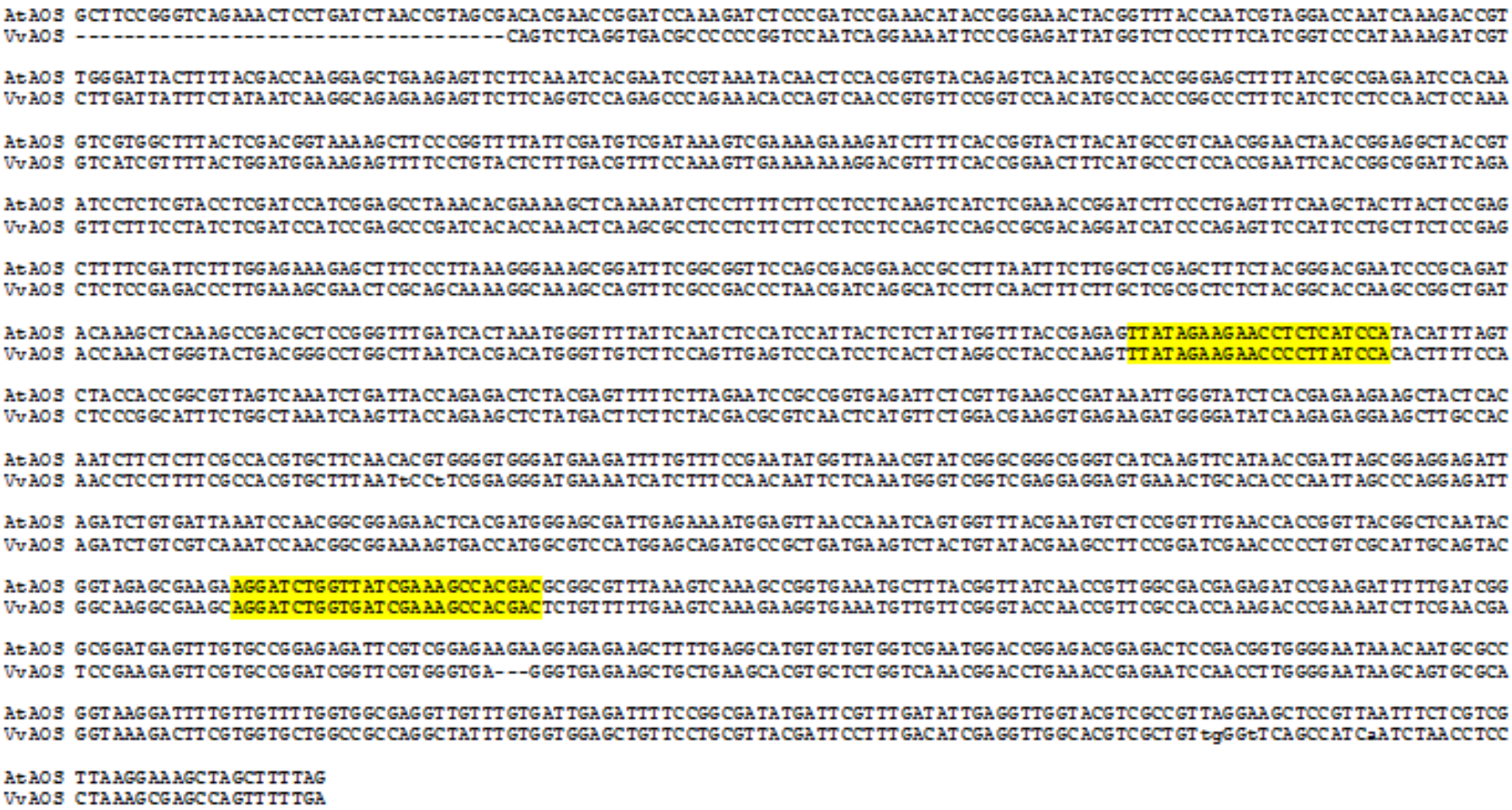

Figure 3.13 Nucleotide sequence alignment between VvAOS and AtAOS gene

Nucleotide sequence alignment between VvAOS and AtAOS genes with chloroplast transit peptide region removed. Yellow shaded colour indicated a possible small RNA fragment (siRNA) that initiated PTGS within AtAOS and VvAOS transcripts

where a VvAOS-RNAi construct was used to transiently transform strawberry fruit, it was shown that the VvAOS construct was able to silence the endogenous strawberry AOS gene leading to a decrease in JA accumulating in the transformed fruit (Jia et al., 2016). It is therefore possible that we are observing a similar phenomenon in our Arabidopsis complementation lines and activation of the mutant AtAOS allele is epigenetically impacting VvAOS transcription.

If in fact transgene VvAOS was silence by the mechanisms mention above, the apparent high levels of VvAOS transcript observed may be an artefact of the qPCR design the targeted sequence for the qRT-PCR assay may not report accurate levels of transcript. This phenomena has been tested in Human cell systems where it was shown that selection of inappropriate target regions of the gene for qRT-PCR analyses may result in an inappropriate assessment of the level of knockdown contributed by siRNA derived gene-silencing (Holmes et al., 2010). However further work to investigate the relative kinetic capacity of the VvAOS and AtAOS enzymes, presence of siRNAs targeting the VvAOS transgene, levels of methylation on the AtAOS and VvAOS alleles and testing a range of different qRT-PCR primer sets to probe the levels of VvAOS transcription are required to confirm these hypotheses

3.5 Conclusions and future prospects

It was clear from the experiments undertaken that the VvAOS encoded a functional AOS as evidenced by its ability to complement the Arabidopsis *aos* mutant phenotypes. However, in using the AtAOS promoter to drive the expression of the VvAOS transgene we observed an unexpectedly high level of VvAOS transcription compared to what was present in wild type plants. However, both levels of JA and transcription of JA responsive genes were found to be lower than in wild type Arabidopsis. At this point it was unclear what the precise mechanism behind this unexpected observation was but we have speculated a several possible reasons for this phenomenon, as discussed above. However, this unexpected observation could be a critical feature to understanding further involvement of AOS enzymes in JA biosynthesis mechanisms in Arabidopsis *aos*:promAOS:VvAOS plants. Therefore, in order to understand this mechanism, further assessment needed to be carried out to unravel the true reason. In this assessment, provision of a functional copy of VvAOS led not only to the recovery of the physical phenotype but also recovery of the wound responsiveness of the mutant AOS locus in Arabidopsis *aos* mutants.

As mentioned previously, a number of AOS genes isolated from different plant species were overexpressed in several model plants. Overexpression of Arabidopsis AOS in *Arabidopsis thaliana* and *Nicotiana tabacum* plants did not alter the basal level of JA but displayed an earlier JA peak and higher levels of JA compared to the control plants (Laudert et al., 2000). Whereas overexpression of an AOS-like gene from soybean enhanced tolerance to insect attack in transgenic tobacco (Wu et al., 2008) and overexpression of flax AOS induced accumulation of JA in transgenic tobacco (Wang et al., 1999). Furthermore, overexpression of rice AOS in transgenic rice increased the endogenous JA level, PR gene

expression and resistance to fungal infection (Mei et al., 2006). Therefore, for further work, we look forward to overexpressing this VvAOS gene in *Arabidopsis thaliana* under the control of the constitutive CaMV 35S gene regulator.

Chapter 4

Over expression of grapevine allene oxide synthase in Arabidopsis wild type background

4.1 Introduction

In plants, induced defence mechanisms are one of the strategies that plants employ to defend themselves from herbivore or pathogen attacks. Induced defence mechanisms are based on the interaction between a pathogen elicitor and plant receptors. The subsequent gene transduction from this interaction triggers a series of signalling cascades that activate several plant defence pathways (Anderson et al., 2005; Thatcher et al., 2005; Mithofer and Boland, 2012). Activation of this signalling cascade leads to the expression of plant defence genes, such as the pathogenesis-related (PR) proteins, glutathione S-transferases (GST), peroxidases, proteinase inhibitor (PIN 2) and the production of phytoalexic secondary metabolites (Feussner and Wasternack, 2002; Thatcher et al., 2005). One of several important signalling compounds involved in mediating plant induced defence responses is jasmonic acid (JA). In this study, we investigated the possibility of enhancing plants' resistance to pathogen attack by increasing JA production using overexpression of the AOS gene in Arabidopsis plants as a model system.

In the wine industry, *Botrytis cinerea* (Botrytis) infections in grapevines are one of the major issues for crop management and wine making (Diguta et al., 2010; Saito et al., 2013; New Zealand Winegrower, 2014). Botrytis infections are characterized by the rapid destruction of grapevine leaf or berry tissues as the pathogen proceeds to colonize the plant. In grapevines, a Botrytis infection causes yield losses and reduces the quality of fruit for winemaking (Saito et al., 2013). Although Botrytis is also responsible for the production of well-known sweet white wines (Nobel wines), Botrytis infections most commonly cause undesirable effects, including the degradation of aroma compounds and the production of “mouldy” and “earthy” off-flavours and aromas that are generally not appreciated by consumers (Bell and Henschke, 2005; Sarrazin et al., 2007; Lee et al., 2009; Hong et al., 2011). Management of Botrytis infections commonly makes use of synthetic or organic fungicides that eventually increase production costs and also present producers with significant environmental and consumer concerns over the excessive use of these fungicides. Currently, there is no natural resistance to Botrytis or other fungal infections in commercially-relevant wine grape varietals, so identification of natural genetic variants that exhibit increased activity of natural defence mechanisms needs to be explored as an alternative and new approach to improve control of pathogen infections in grapevines. One of the most important signalling pathways that responds to pathogen attack is the JA biosynthetic pathway. Jasmonic acid is a key signalling compound in plants' responses to biotic and abiotic stresses

as well as in their development (Wasternack, 2014). As previously reported, JA formation is thought to be largely controlled by fluxes through AOS (Laudert and Weiler, 1998). However, other points of regulation have been postulated, such as AOC (Schaller and Stintzi, 2009; Stenzel et al., 2012) and OPR (Schaller et al., 2000; Schaller and Stintzi, 2009). Nevertheless, due to the literature surrounding the role of AOS in the regulation of JA, the AOS gene is a suitable candidate to be explored as a new approach to combating grapevine disease infections.

4.2 Overexpression of allene oxide synthase gene from grapevines in the Arabidopsis system

In the previous chapter (Chapter 3), we identified and characterized the sole AOS gene in the grapevine genome (VvAOS) via complementation of the AOS mutant in *Arabidopsis thaliana*. Following the introduction of the VvAOS gene, the male sterile phenotype that characterized the Arabidopsis *aos* mutant completely recovered and this verified the identity of the grapevine gene as a functional AOS. In order to gain further insights into the role of the VvAOS gene in the grapevine defence system, we overexpressed this newly characterized gene in the wild type *Arabidopsis thaliana* cv *Columbia* (Arabidopsis WT) in order to investigate the possibility of additional VvAOS gene copies increasing plant performance against pathogen infections and, therefore, to raise the possibility of producing grapevines less susceptible to Botrytis or necrotrophic pathogen infections via increasing JA production.

4.3 Materials and Methods

4.3.1 Construction of VvAOS and AtAOS binary vectors for plant transformation

Approximately 150 ng of Entry clone plasmid (Life Technology, NZ) carrying the VvAOS gene (pENTR/D-TOPO/VvAOS) was added to 300 ng of the binary vector, pARTBGW, to perform an LR recombination reaction using Gateway Clonase Enzyme Mix kit as per the manufacturer's instruction (Invitrogen Inc., Life Technologies, NZ). The resulting plant binary (35S:VvAOS) was purified using the AxygPrep PCR Clean-Up kit (RayLab, NZ) before being sequenced, as described previously in section 2.2.4, to confirm the identity of the insert. A binary vector containing the Arabidopsis AOS gene (35S:AtAOS) was also developed, as described above, to provide a comparative control to the 35S:VvAOS construct. The Arabidopsis AOS gene was amplified from Arabidopsis wild type cDNA, essentially according to the protocol described in section 2.2.4. Amplification was based on the AtAOS sequence, as reported by Park et al. (2002) and Pajerowska-Mukhtar et al. (2008) (accession: At5g42650). The primer pair used to amplify the AtAOS gene was as follows: AtAOS-Fwd*: 5'-CACC*ATGGCTTCTATTTCAACCCCTTTTC-3' and AtAOS-Rev*:* 5'-CTAAAAGCTAGCTTTCCTTAACGACGA-3'. An additional "CACC" was added at the 5' end of the AtAOS forward primer to accommodate the directional cloning of the AtAOS gene into the pENTR/D-TOPO plasmid according to the manufacturer's instructions (Life Technologies, NZ). In order to confirm the insertion of VvAOS or AtAOS genes into the pARTBGW binary vector, the forward primer, pART35S (Fwd) 5'-GACGTTCCAACCACGTCTTCAAAGCAA-3' (located at 137 bp from 3' end of CaMV 35S promoter gene sequence) was designed for PCR and sequencing verification. Verification via the PCR method was carried out using pART35S (Fwd) and the respective reverse gene primer (VvAOS-Rev or AtAOS-Rev primer) with pARTBGW:35S:VvAOS (or AtAOS) plasmid as the template. For the nucleotide sequencing verification, position of the pART35S primer for sequencing were illustrated on the diagram below (Figure 4.1).

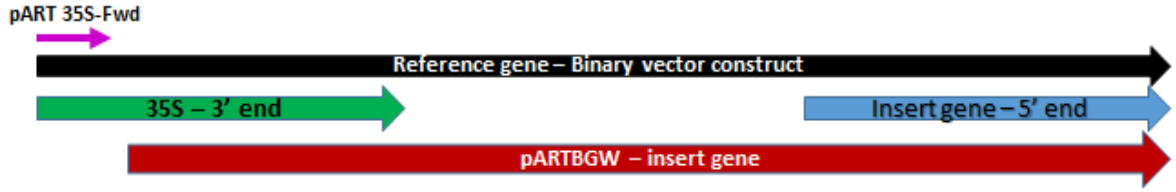

Figure 4.1 Graphic illustration of binary vector construct junction sequencing between 35S promoter and the inserted gene
Sequencing results to confirm the insertion of VvAOS or AtAOS genes into the pARTBGW vector. The forward primer, pART 35S, was used to sequence the junction between the 3' end of the 35S promoter and the 5' end of the insert gene (VvAOS or AtAOS). The reference gene, pARTBGW/35S/VvAOS (or AtAOS), was constructed via the LR recombination method using Lasergene software (DNASTAR Inc, Madison, USA).

4.3.2 Transformation of the 35S:VvAOS and 35S:AtAOS gene constructs into *Arabidopsis thaliana* cv *columbia*

Arabidopsis thaliana cv *Columbia* (Arabidopsis) plants were grown, essentially according to the growth conditions described for the Arabidopsis *aos* mutant plants (Section 3.2.1). Transformation of the AtAOS gene into Arabidopsis WT also was carried as a comparative experiment to Arabidopsis transformed with the VvAOS gene. Healthy Arabidopsis WT were subjected to floral dip transformation using Agrobacterium GV3101 transformed with either 35S:VvAOS or 35S:AtAOS constructs, essentially as described in section 3.2.5. Twenty primary transformants from 35S:VvAOS, and 24 from 35S:AtAOS constructs, were selected and taken through the process, as described in section 3.2.6, to produce eight and six independently transformed lines of homozygous 35S:VvAOS and 35S:AtAOS plants, respectively. Integration of VvAOS or AtAOS within the Arabidopsis WT genome was validated via the PCR method using a combination of VvAOS-Fwd/-Rev or pART35S/AtAOS-Rev primers respectively and genomic DNA from transformed Arabidopsis as a template. DNA extraction and PCR amplification conditions essentially modified from those described in sections 2.2.3 and 2.2.4 respectively. Transformant plant lines were screened for the homozygous allele of VvAOS or AtAOS gene in Arabidopsis transgenic (35S:VvAOS or 35S:AtAOS) using BASTA selection media followed to the methodology described in section 3.2.6. Homozygous Arabidopsis (35S:VvAOS or *35*:AtAOS) transgenic plants were produced at the T_3- generation and these plants were used as source material for further assessment to quantify transcript abundance, quantification of JA and use in plant disease assays.

4.3.3 Assessment of Arabidopsis homozygous 35S:VvAOS and 35S:AtAOS transgenic plants

In order to determine the impact of overexpressing transgenic AOS (VvAOS or AtAOS) in Arabidopsis, it was decided to measure changes in the accumulation of jasmonic acid in the transformed and control plants. Wounding the plants prior to the measurement and observation of the rates of accumulation of JA over time was considered to be important, as described in previous reports (Wang et al., 1999; Pajerowska-Mukhtar et al., 2008; Wu et al., 2008).

Individual transgenic plants lines assessment for wound response

Both homozygous transgenic 35S:VvAOS and 35S:AtAOs were assessed for their responses to wound treatment. Eight transgenic lines from 35S:VvAOS plants, five from 35S:AtAOS plants and a wild type (control plant) were selected for these assessments. All plants involved were grown in pots under controlled environmental conditions, as described in section 3.2.1. Each pot contained five to six individual plants. For wound treatment experiment, six-week-old transgenic and WT (plant control) plants were used. From the five to six plants in each pot only three to four individual plants were subjected to wounding. On each individual plant subjected to wounding, between three to four leaves

of approximately similar size were subjected to wounding. Wounding was achieved by using a small pair of pliers to crush the leaves in order to induce wound signalling in a manner that generated maximum mechanical injury for each leaf. For standardization, each leaf was subjected to only one mechanical injury of an approximately equal pressure. Leaves that were subjected to wound treatment were left attached to the plant for a period of six hours before being harvested and immediately snap frozen using liquid N_2. Frozen leaf samples were stored at -80°C before being used to extract total RNA. Approximately 50 mg of ground leaves were used as a starting material for total RNA extraction. Total RNA extraction, quantification, validation and cDNA synthesis were carried out following the methods described in sections 2.2.11. Transcript abundance analysis was carried out via the qRT-PCR technique as described in Section 2.2.12. Arabidopsis F-BOX (accession: At5g15710) and elongation factor 1-alpha (accession: At5g60390) were used as reference genes to normalize the transcript expression genes of interest in both transgenic plants. The selection of Arabidopsis F-BOX (AtF-Box) and elongation factor 1-alpha (AtEF1α) genes as a reference gene were based on the report by Lilly et al. (2011). Statistical analysis for significant differences among the multiple data collected from the individual transgenic plant lines were analysed using one-way ANOVA (no blocking) by GenStat version 16 (VSN International Ltd, UK) with Fisher's Protected LSD multiple comparison ($P < 0.05$). Data results from qRT-PCR were presented as the number of gene copies detected in 4 µL of cDNA synthesized from 500 ng total RNA samples.

Analysis of Arabidopsis transgenic plants' responses to wound treatment

Three lines from each transgenic plant (both transgenic 35S:VvAOS and 35S:AtAOS plants) were selected for wound treatment analysis. All transgenic plants involved were grown and organized, as described in the section above, with the exception of the light condition, which was altered to 12 hour light/day to induce the formation of more and larger leaves. For the wound treatment experiment, leaves of eight-week-old transgenic plants were harvested at various time points as follows: 0 (pre-wound), 0.5, 1, 3 and 6 hours. Samples were collected from three biological replicates with each biological replicate representing a collection of four to five different individual plants harvested from a single pot. Time periods for the collection of samples were selected, based on experiments and information published for other plant species, with respect to AOS and their subsequent JA responses to wounding or plant stress. In order to minimize crosstalk among plants via volatile organic compounds (VOC), all transgenic plants involved for wound treatment experiment were carried out in the Westinghouse Biological Safety Cabinet Class II (MedLab Instrument, New Zealand) as describe in section 3.2.7. Leaf samples were stored at -80^{o}C and total RNA was subsequently extracted, as described above. In this study, wound-induced genes (and also known as JA-responsive genes) were included to investigate the immediate effect of overexpression of transgenic VvAOS and AtAOS. Transcript abundance analysis was carried out using qRT-PCR, reference genes, statistical significant analysis and qRT-PCR data presentation analysis, as described above.

4.3.4 Quantification of jasmonic acid compounds from Arabidopsis transgenic 35S:VvAOS plant

Two selected transgenic 35S:VvAOS lines and a WT were selected for jasmonic acid (JA) quantification via the vapour phase extraction (VPE) technique, as described earlier (Section 3.2.8). Briefly, approximately 100 mg of ground leaves from each selected samples was used as starting material for this extraction. In order to quantify JA and its various conjugates, samples or, more specifically, jasmonic acid need to be derivatized to methyl jasmonic acid. Derivatization and quantification of JA via gas chromatography mass spectrometry (GC-MS) was carried out based on the method described in the previous chapter (Sections 3.2.8 and 3.2.9). The results were presented as weight of JA per gram of fresh weight (ng/g FW) based on the formula described in the previous chapter (Section 3.2.9).

4.3.5 *Botrytis cinerea* detached leaf disease assay

A detached leaf system assay was carried out to determine the correlation between induced gene expression in transgenic plants and their responses to a Botrytis infection. The rate of lesion formation on the infected transgenic detached leaves was designed to demonstrate that the transgenic plants were resistant to Botrytis infection. Slower rates of lesion formation will indicate that the transgenic plant was less susceptible to pathogen attack.

Botrytis cinerea inoculation and spore suspension

The isolate of Botrytis used in this study was kindly donated by Assoc Prof. Marlene Jaspers (Lincoln University, NZ) and maintained on table grape berries held at 23°C. Spores formed on the inoculated fruit were then used to inoculate PDA agar plates [24 g/L Potato Dextrose Broth by Duchefa Biocheme (Total Lab Systems Ltd, NZ), 15 g/L Agar by Sigma-Aldrich (Sigma-Aldrich, NZ)]. Plates inoculated with Botrytis were incubated at 23°C in 16 hours light/day for three weeks. Botrytis spores were harvested by flooding the PDA agar plate containing the Botrytis inoculant with approximately 10-15 mL of sterile water containing Tween 80 at a final concentration of 0.05% v/v. Conidia were scraped from the PDA agar surface using a sterile L-shaped spreader and the conidial suspension was filtered through a homemade sterile glass wool filter [in 5 mL disposable pipette tips (Global Science, NZ)] and the filtrate collected in sterile 50 mL Falcon tubes. Conidial suspensions were pelleted by centrifugation at 1200 x g for two minutes. The conidial pellet was washed twice with 15 mL of sterile deionized water before being collected by centrifugation at 1200 x g for two minutes (Walter et al., 2006). After the final wash step, Botrytis spores were re-suspended into 1 mL of 25% table grape juice (25% of juice filtered through a 0.22 µm filter to eliminate foreign bacteria or fungi) (Denby et al., 2004). In order to estimate spore concentration in this suspension, 94.5 µL of the spore suspension was aliquoted into a fresh 1.5 mL Eppendorf tube and mixed with 5.5 µL of 0.4% trypan blue solution

(Thermo Fisher Scientific Ltd, NZ) for 60 minutes (Govrin and Levine, 2000). Numbers of Botrytis spores per mL in the suspension were estimated using a haemocytometer.

Detached leaf assay

Arabidopsis transgenic plants were grown under following conditions, as described in the previous section (Section 3.2.1). Leaves from transgenic plants, approximately eight weeks old were used as a material source for the detached leaf assay. Eight-week-old plants were selected to match the plant age used in the wound treatment experiment. Detached leaves were placed on plates containing filter paper moistened with sterile water placed. The Botrytis spore suspension with a concentration of 682 spores/µL, were germinated in 25% of grape juice for two hours before being inoculated on the leaf surface. On each leaf, 5 µL (682 spores/µL) of the Botrytis spore suspension was inoculated on the adaxial surface of the detached leaf. As an experimental control, 5 µL of the 25% grape juice solution was placed on the adaxial surface of the control leaves. All the inoculated leaves were placed in a covered container, which was sealed with parafilm to maintain high humidity for Botrytis growth. The detached leaf assay incubation conditions were as follows: temperature 20°C, light intensity of approximately 100 µmoL/m^2/sec provided by cool white fluorescent tubes and a light cycle of 16 hours per day. Lesion formations were scored 96 hours after inoculation as an indication of Botrytis infection, where the size of a lesion formation will be documented as a general indication of the plant's resistance to pathogens (Liu et al., 2007).

4.4 Results

4.4.1 Construction of the Binary system

As mentioned in chapter 3 (Section 3.2.3), the plant binary vector, pARTB-GW-egfpER (pARTBGW) was obtained from Plant & Food Research Ltd, Canterbury, New Zealand; the binary vector selection marker and gene regulator genetic map described in the Appendix B.1. The transgenes (VvAOS or AtAOS) were integrated into the pARTBGW binary vector system (to produce constructs, henceforth named 35S:VvAOS and 35S:AtAOS) and transformed into *Agrobacterium tumefaciens* (mediated-Agrobacterium) in order to facilitate the transformation of these gene into *Arabidopsis thaliana* cv. *colombia*. Gene insertion of VvAOS or AtAOS genes in the pARTBGW binary vector was confirmed via the PCR method (Figure 4.2) and through gene sequencing analysis (Appendix C.6).

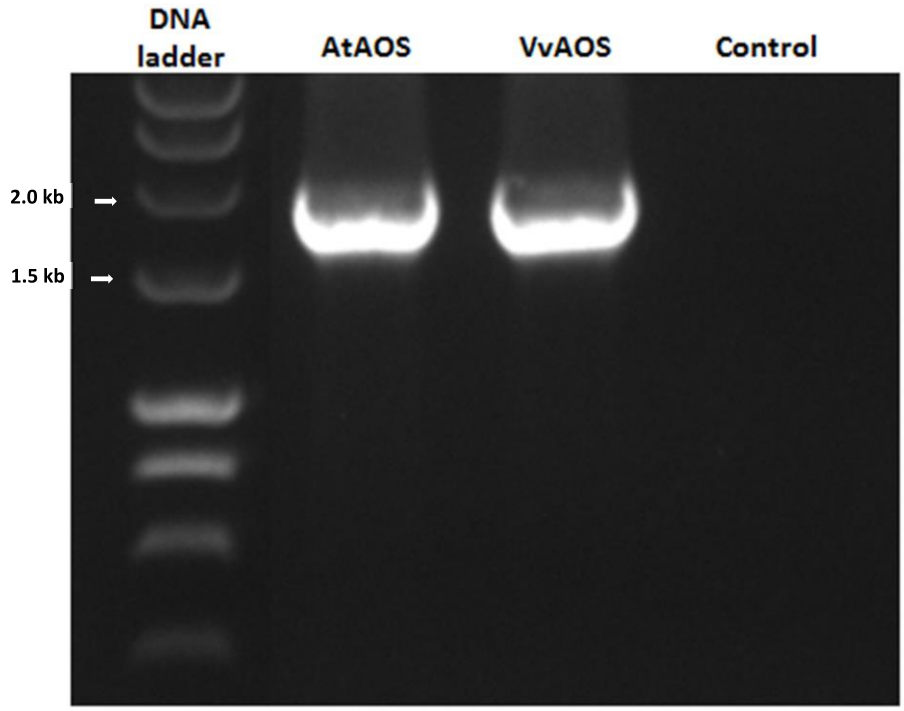

Figure 4.2 PCR approach to assess AtAOS and VvAOS incorporation in pARTBGW binary vector
The single PCR product of approximately 1.8 kb indicated the AtAOS or VvAOS insertion into the pARTBGW binary vector. An additional, approximately 0.3 kb, gene insert was generated from an extra sequence from the 3' end of 35S promoter sequence. A HyperLadder I (1 kb ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as a DNA ladder to measure the DNA fragment sizes.

4.4.2 Integration of VvAOS and AtAOS genes into *Arabidopsis thaliana*

Prior to analysis of the impacts of AOS overexpression in Arabidopsis, lines that were homozygous for the transgene were required. Subsequently, T_1-transformant plants were PCR-screened to identify plants that carried the AOS transgene that had been transformed with the pARTBGW binary construct by virtue of BASTA resistance. Subsequent generations were also PCR screened for the presence of the transgene and assessed for the segregation of their progeny via BASTA plate selection to identify plants that were homozygous for the AOS transgene alleles. Primary transformants of Arabidopsis carrying 35S:VvAOS genes were screened via the PCR method (described in section 4.3.2) and BASTA herbicide plates yielding 20 individual plants (T_1-plant lines). Among the 20 primary transformants, only 18 were identified as plants carrying the 35S:VvAOS transgene. Among the 18 transformed lines, the first eight plants that were found to have their progeny segregated in a ratio of 3:1 (recovery over dead) were used to produce T_3-plants as a plant source material for further analysis. These eight plants were labelled as Arabidopsis 35S:VvAOS transgenic plants, VvAOS-6, VvAOS-7, VvAOS-8, VvAOS-9, VvAOS-11, VvAOS-12 VvAOS-14 and VvAOS-15. PCR screening to identify VvAOS insertion on eight selected transgenic plants are shown in figure 4.3. The primary transformants of Arabidopsis carrying the 35S:AtAOS gene were also screened using the PCR method (described in section 4.3.2) and the BASTA herbicide plates yielded 24 individual plants (T_1-plant lines). Among the 24 transformed lines, the first six plants that were found to have their progeny segregated in a ratio of 3:1 (recovery over dead) were used to produce T_3-plants as plant source materials for further analysis. These six plants were labelled as Arabidopsis 35S:AtAOS transgenic plants AtAOS-11, AtAOS-13, AtAOS-14, AtAOS-16 and L AtAOS-18. PCR screening to identify AtAOS insertion on six selected transgenic plants are shown in figure 4.4.

4.4.3 Preliminary screening for Arabidopsis 35S:VvAOS and 35S:AtAOS transgenic plants' responses to wound treatment

In section 4.3.2, we identified eight Arabidopsis 35S:VvAOS transgenic and five Arabidopsis 35S:AtAOS transgenic plants. In this experiment we assessed all transgenic plant for their response to mechanical wounding. Each transgenic plant was subjected to a six-hour wound treatment before their AOS gene transcripts were measured. First, in Arabidopsis 35S:VvAOS transgenic lines and, secondly, in Arabidopsis 35S:AtAOS transgenic lines.

Arabidopsis 35S:VvAOS transgenic lines

The eight homozygous Arabidopsis 35S:VvAOS transgenic lines were identified as follows: VvAOS-6, VvAOS-7, VvAOS-8, VvAOS-9, VvAOS-11, VvAOS-12, VvAOS-14 and VvAOS-15. All eight transgenic lines were assessed for their response to wounding based of a six-hour wound-treatment. To facilitate the preliminary analysis of the transgenic lines to identify lines for further study (which exhibited differing levels of transgene expression and responsiveness) a single time point at six hours

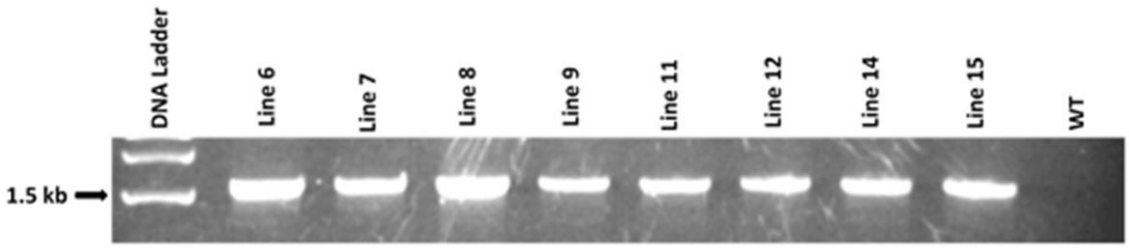

Figure 4.3 Validation of VvAOS gene integrated into the Arabidopsis *35S:*VvAOS genome
Validation of eight Arabidopsis transgenic *35S:*VvAOS using the PCR method. Amplification of the 1.5 kb DNA fragment indicated an integration of transgene VvAOS gene into the Arabidopsis genome. The hyperladder 1 kb (DNA Ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as a DNA ladder.

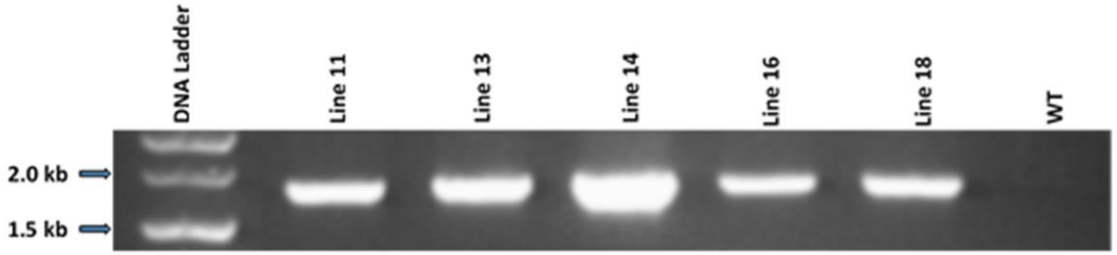

Figure 4.4 Validation of additional copy of AtAOS integrated into Arabidopsis *35S:*AtAOS genome
Validation of five Arabidopsis transgenic *35S:*AtAOS using end point PCR approach. Amplification of 1.8 kb DNA fragment indicated the integration of transgene AtAOS gene into the Arabidopsis genome. Transgene and endogenous AtAOS were differentiated by using pART 35S(Fwd) primer located at the 3' end of CaMV 35S promoter region pair with AtAOS(Rev) primer. Therefore, an additional 0.23 kb emerged from usage of some part of the 35S promoter region. The hyperladder™ 1 kb (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used a as DNA ladder.

was chosen. Numerous reports have stated that it was important to study the impact following a wound treatment to ensure full activation of the pathway (Reymond et al., 2000; de Bruxelles and Roberts, 2001; León et al., 2001; Schilmiller and Howe, 2005). Therefore, we employ a wound treatment approach to study the impact of overexpress AOS gene JA biosynthetic pathway in Arabidopsis system. A six-hour post-wound treatment was selected based on previous reports that indicated that in wounded leaves, a transient increase of AOS mRNA reached a peak level at around six hours (Harms et al., 1995; Laudert and Weiler, 1998; Siqueira-Júnior et al., 2008). After six hours, samples of leaves were collected and transcript abundance of transgene VvAOS and endogenous AtAOS were quantified. Results indicated that after a six-hour wound treatment, VvAOS gene expression was generally higher compared to the expression of their endogenous AtAOS gene in the same 35S:VvAOS transgenic plants (Figure 4.7). Whereas, endogenous AtAOS gene expression in 35S:VvAOS transgenic plants were comparable to AtAOS expression in WT plant. In general, VvAOS transcript abundance in transgenic plants can be clustered into three groups, i.e. low: VvAOS-7, VvAOS-8, VvAOS-9; medium: VvAOS-11, VvAOS-12, VvAOS-14; and high: VvAOS-6, VvAOS-15. Whereas, in AtAOS gene expression, most transgenic plants exhibited a comparable gene expression in which VvAOS-9 had the lowest level and VvAOS-6 had the highest level. In order to investigate the effect of the VvAOS gene overexpressed in Arabidopsis to JA biosynthesis regulation further, three transgenic lines were selected based on this primary response result. The three transgenic lines are shown below:

1. VvAOS-6 – due to the highest expression of both the endogenous and transgene the among transgenic lines
2. VvAOS-9 – the lowest expression of both the endogenous and transgene among the transgenic lines.
3. VvAOS-15 - exhibited a stable expression of both the endogenous and transgene among transgenic lines (when VvAOS-6 and VvAOS-9 excluded). This was shown by the small standard deviation (SD) value among three biological replicates used.

Arabidopsis 35S:AtAOS transgenic lines

As a comparison, five Arabidopsis transgenic 35S:AtAOS were also assessed for their responses to wound treatment. The five transgenic lines were identified as AtAOS-11, AtAOS-13, AtAOS-14, AtAOS-16 and AtAOS-18. The assessment was carried out under similar treatments and conditions as for the transgenic 35S:VvAOS plants. However, for this assessment, qRT-PCR primers were not able to be designed to differentiate between the endogenous and transgene AtAOS. This experiment was designed to investigate the effect of increasing overall AOS transcripts and, therefore, presumably AOS activity in JA the biosynthetic pathway. Transcript abundance of the AtAOS gene in six Arabidopsis 35S:AtAOS transgenic lines is shown in figure 4.6. The results indicated that AtAOS transcript abundance in 35S:AtAOS transgenic plants were exceptionally low compared to the total copy number

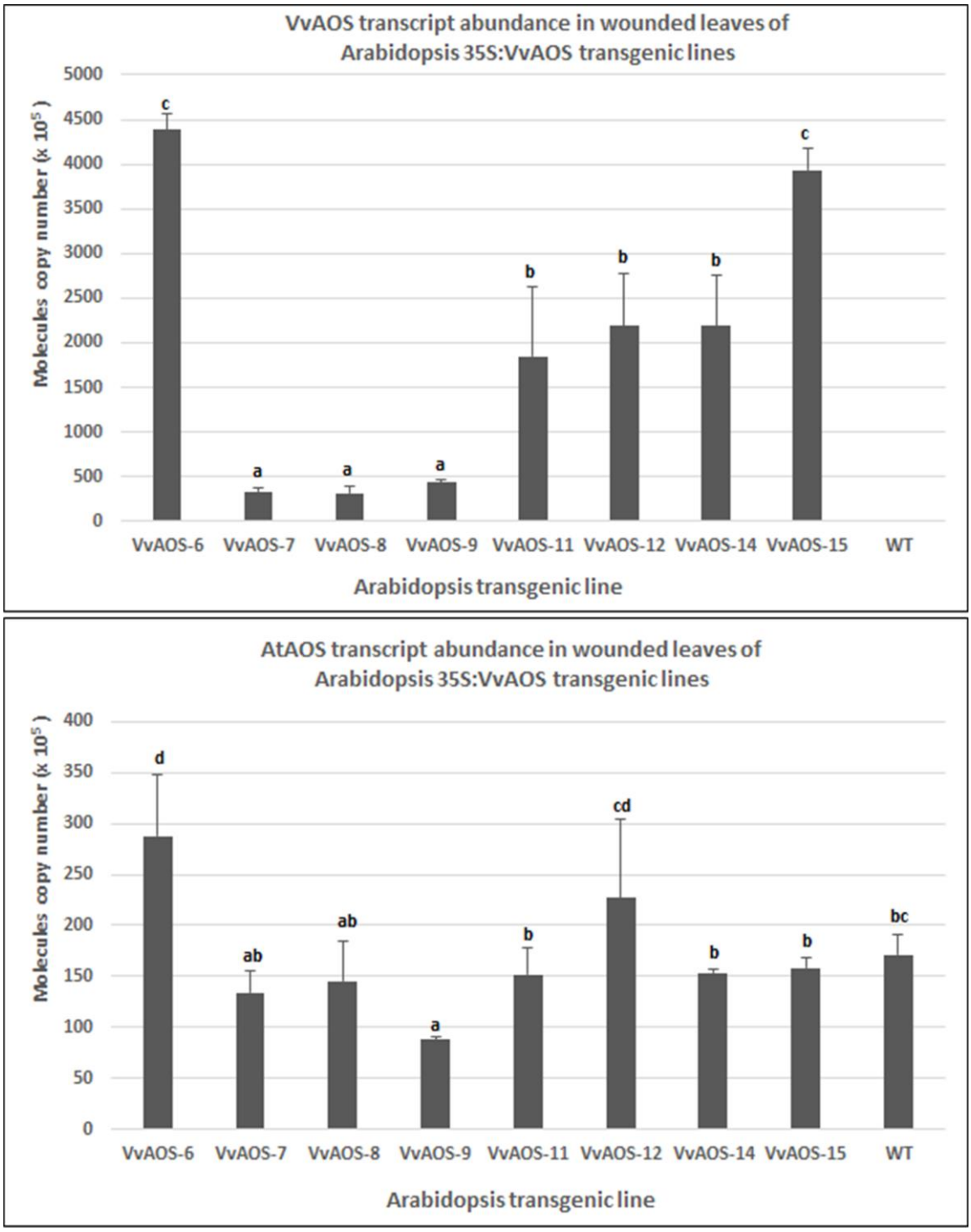

Figure 4.5 Transcript abundance of VvAOS and AtAOS genes in Arabidopsis 35S*:*VvAOS transgenic lines
Eight homozygous Arabidopsis *35S:*VvAOS transgenic lines, i.e. VvAOS-6, VvAOS-7, VvAOS-8, VvAOS-9, VvAOS-11, VvAOS-12, VvAOS-14 and VvAOS-15, were screened for their responses to mechanical wounding. Gene transcripts of VvAOS and AtAOS genes were quantified at six hours after wounding. A gene transcript of AtAOS from a wild type was used as a comparison. Bars represent standard deviation of the mean from the three biological replicate samples collected. Letters represent statistical significant differences among the multiple data collected.

of AOS transcripts in the 35S:VvAOS transgenic plants. The vast differences in the total level of AOS transcript abundance between 35S:VvAOS transgenic plants (Figure 4.5) and 35S:AtAOS transgenic plants (Figure 4.6) was quite a surprising result considering that both transgenes were regulated by the same gene promoter. Generally, AtAOS gene expression of transgenic 35S:AtAOS lines can be categorized into three groups, i.e. low – AtAOS-13; medium – AtAOS-16, AtAOS-18; and high – AtAOS-11, AtAOS-14. For further investigation of the effect of AtAOS gene overexpression in the Arabidopsis JA biosynthesis pathway, three transgenic lines were selected based on this primary response result;

1. AtAOS-11 – Highest AtAOS gene expression among the transgenic 35S:AtAOS lines
2. AtAOS-13 – Lowest and most stable AtAOS gene expression among the five transgenic 35S:AtAOS lines
3. AtAOS-18 – Medium and showing more stable gene expression among three biological replicates compared to AtAOS-16 (low SD value)

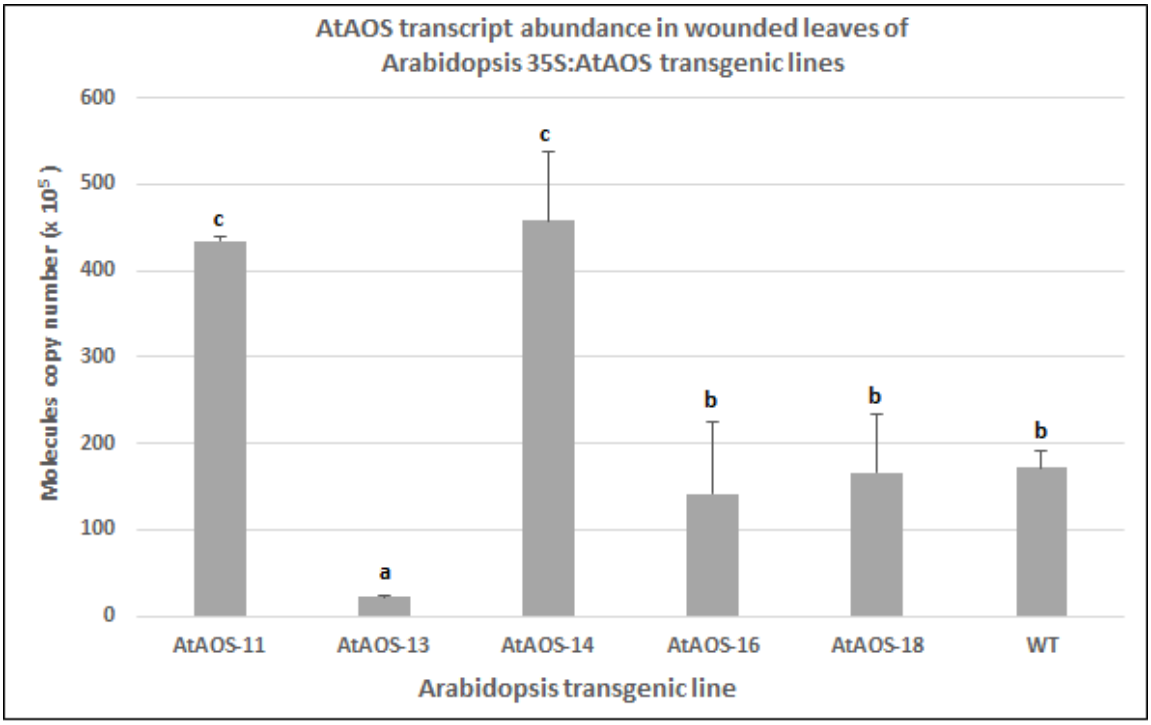

Figure 4.6 Transcript abundance of AtAOS genes in Arabidopsis *35S:*AtAOS transgenic lines
Five homozygous Arabidopsis *35S:*AtAOS transgenic lines, i.e. AtAOS-11, AtAOS-13, AtAOS-15, AtAOS-16 and AtAOS-18 and a WT were screened for their responses to wound treatment. AtAOS gene expressions were quantified at six hours after wounding. Bars represent standard deviation of the mean from three biological replicate samples. Letters represent statistical significant differences among the multiple data collected

4.4.4 Arabidopsis transgenic plants response to wound treatment

In order to determine the impact of the ectopic expression of either VvAOS or AtAOS in Arabidopsis in response to wounding, three transgenic lines from each Arabidopsis transgenic plant (Section 4.4.3) were subjected to a six-hour time course wound treatment. Over the course of six hours, samples were collected at 0 (pre-wound), 0.5, 1, 3, and 6 hours after wounding. Time course and time point sample collections were selected based on the previous experiment and information from other plant species with respect to AOS gene and, subsequent, JA responses to wound or plant stresses (Harms et al., 1995; Laudert et al., 2000; Sivasankar et al., 2000; Park et al., 2002; Pajerowska-Mukhtar et al., 2008; Wu et al., 2008). To investigate transgenic plants' responses to wound treatment, two wound-induced/JA-responsive genes in Arabidopsis, AtVSP2 and AtLOX2, were also measured at each time point of wound treatment (Utsugi et al., 1998; Creelman and Mulpuri, 2002; Park et al., 2002). First, we assessed three Arabidopsis 35S:VvAOS transgenic lines followed by another three transgenic lines from Arabidopsis 35S:AtAOS plants. Assessments were carried out separately.

Arabidopsis transgenic 35S:VvAOS plants' responses to wound treatment

In this experiment, 35S:VvAOS transgenic lines, VvAOS-6, VvAOS-9 and VvAOS-15, were selected. Samples were collected at 0 (pre-wound), 0.5, 1, 3 and 6 hours after wounding, as described in section 4.3.3. Transcript abundance of transgene AOS (VvAOS), endogenous AOS (AtAOS), AtLOX2 and AtVSP2 genes were quantified via qRT-PCR at each time point. Transcript abundance results are shown in figures 4.7 and 4.8. Transcription of VvAOS genes indicated that in the pre-wound condition, all transgenic lines assessed exhibited a high basal level of gene expression of VvAOS, as expected (Figure 4.7). Over the course of the six-hour wound treatment, it was evident that VvAOS-6 and VvAOS-15 exhibited a stable level of expression of between 41 and 45 x 10^7 copies in 4 µL of cDNA samples synthesized from 500 ng total RNA, respectively. However, a slight drop from 41 to 13 x 10^7 and 45 to 22 x 10^7 was noted in VvAOS-6 and VvAOS-15, respectively, at three to six hours after wound treatment. Whereas in VvAOS-9, which exhibited much higher levels of basal expression, it was found to exhibit very variable levels of expression as evidenced by the large standard deviation within the biological replicates. Interestingly, this line exhibited a decline in transcription from 41 to 3 x 10^7 copies in 4 µL of cDNA samples synthesized from 500 ng total RNA over the course of the six-hour wound treatment. Statistical multiple comparison analysis using ANOVA (GeneStat Version 16, International Ltd, UK) showed that VvAOS gene expression between VvAOS-6 and VvAOS-15 showed no significant differences ($P > 0.05$) over the course of the six-hour wound treatment and this might be an indication of the true pattern for VvAOS gene expression in homozygous transgenic plants. On the other hand, AtAOS gene expression in all transgenic lines (Figure 4.9) were much lower compared to the VvAOS gene.

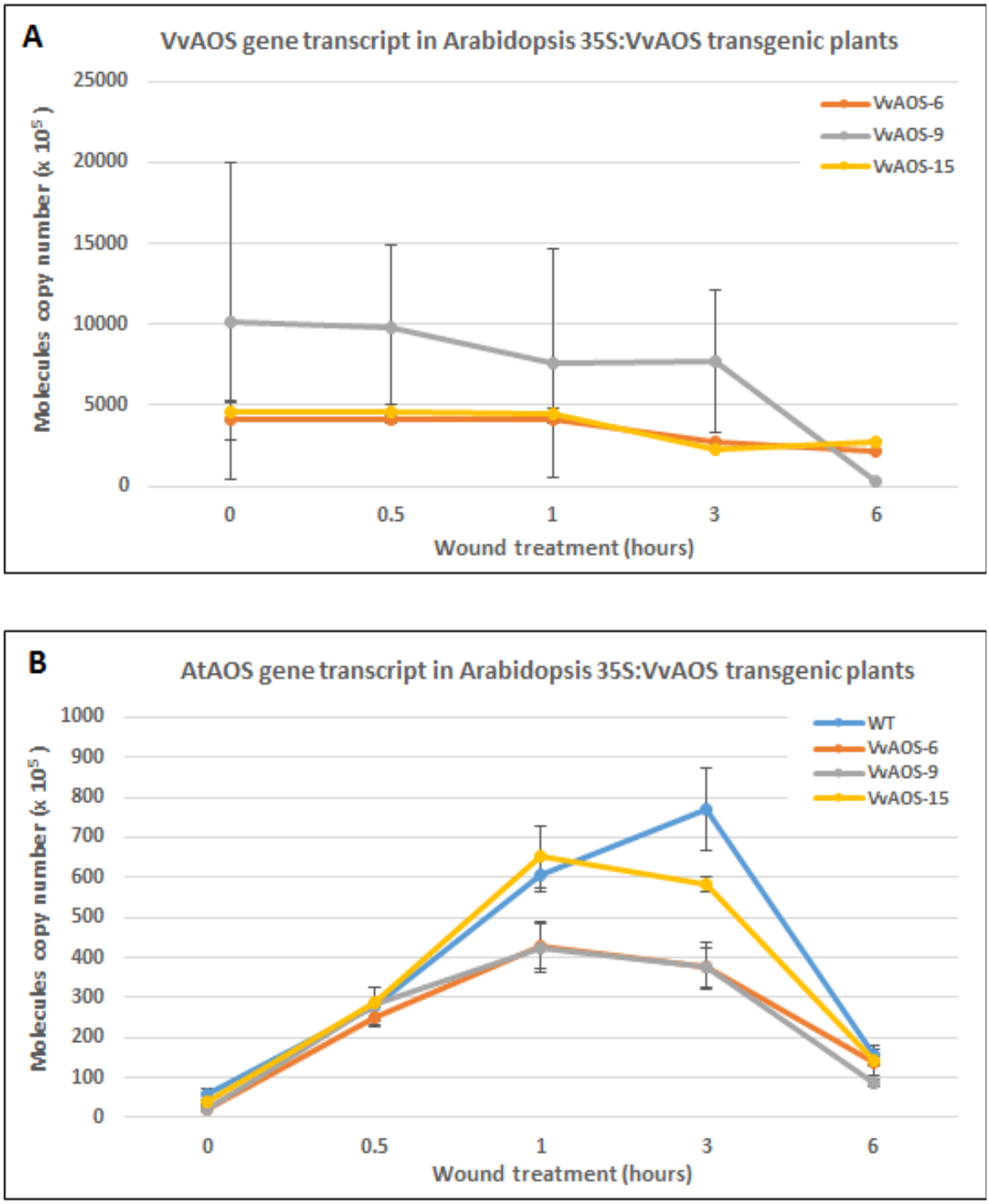

Figure 4.7 Transcript abundance of transgene VvAOS and endogenous AtAOS in three lines of Arabidopsis transgenic *35S:*VvAOS plants

Transcript abundance of VvAOS and AtAOS genes were quantified from three selected transgenic *35S:*VvAOS plants (VvAOS-6, VvAOS-9 and VvAOS-15) after a six-hour time course of wound treatment via a qRT-PCR approach. Bars represent the standard deviation of the mean from three biological replicate samples.

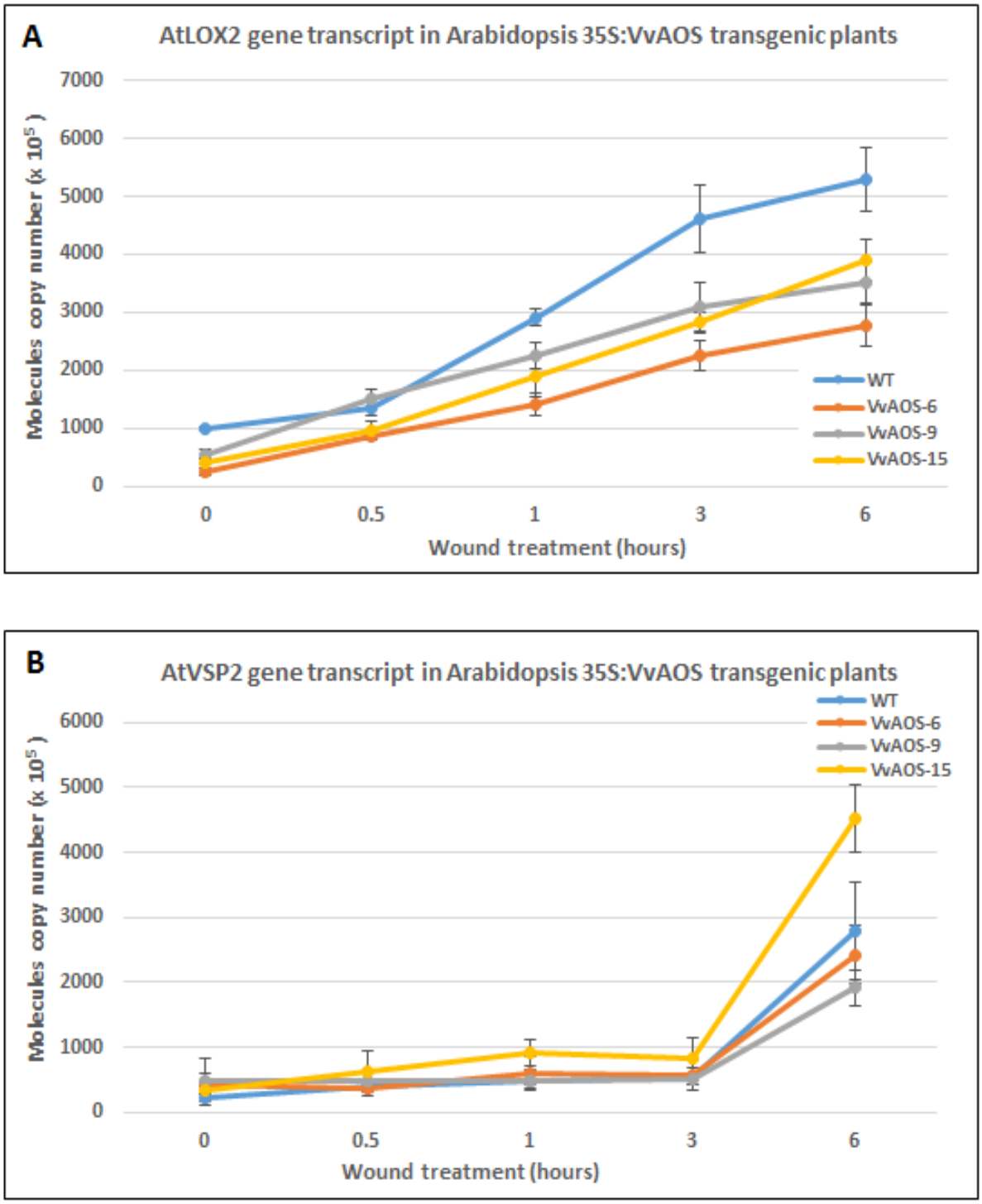

Figure 4.8 Transcript abundance of AtLOX2 and AtVSP2 in three lines of Arabidopsis transgenic *35S:*VvAOS plants

Transcript abundance of AtLOX2 and AtVSP2 genes were quantified from three selected transgenic *35S:*VvAOS plants (VvAOS-6, VvAOS-9 and VvAOS-15) after a six-hour time course of wound treatment via a qRT-PCR approach. Bars represent the standard deviation of the mean from three biological replicate samples.

By comparison, expression of the AtAOS gene in each transgenic line pre-wounding was at a comparable level to AtAOS expression in the Arabidopsis wild type (WT). After wound treatment, AtAOS expression in all transgenic lines increased and peaked at one hour but exhibited different expression magnitudes before declining to near pre-wound condition levels six hours after wounding. AtAOS gene transcripts in transgenic VvAOS-6 increased from 0.2 to 4.3 x 10^7, from 0.2 to 4.3 x 10^7 in VvAOS-9 and 0.4 to 6.5 x 10^7 in VvAOS-15 copies in 4 µL of cDNA samples synthesized from 500 ng total RNA. In comparison, AtAOS expression in the WT increased and peaked at three hours, during which gene transcripts increased from 0.7 to 7.6 x 10^7 copies in 4 µL of cDNA samples synthesized from 500 ng total RNA before declining to near pre-wound conditions six hours after wounding. An interesting observation worth mentioning from this comparison was that the time of AtAOS expression in 35S:VvAOS transgenic plants declined two hours earlier compared to AtAOS expression in WT might suggest an early suppression on endogenous AOS (Figure 3.7B).

Both JA-responsive genes, AtLOX2 and AtVSP2 in all three transgenic lines showed increased gene expression as a response to wound treatment (Figure 4.8). Interestingly, at the pre-wound condition, both JA-responsive genes in 35S:VvAOS transgenic lines exhibited approximately similar levels to their respective genes' expression in the WT (Figures 4.8A and 4.8B). This was a clear indication that high levels of VvAOS gene expression in pre-wound conditions did not elevate the transcription of JA-responsive genes. Generally, over the course of the six-hour wound treatment, AtLOX2 gene expression exhibited in all transgenic 35S:VvAOS lines were below to the transcript level in WT. This was a surprising result because we expected that AtLOX2 gene expression in the transgenic lines will be higher than the expression in WT because of the high turnover of overall AOS gene transcripts in transgenic lines. Whereas, in AtVSP2, the transcript level only showed an increase six hours after wounding and exhibited the highest expression level of VvAOS-15. Again, our expectation was AtVSP2 gene expression will be much higher in the transgenic lines compared to WT, as reported by Park et al (2002). Although AtVSP2 transcript abundance in transgenic VvAOS-15 was higher compared to the WT (only a 0.5-fold difference), this result alone was not strong enough to conclude that AtVSP2 expression in transgenic plant was significantly high compared to the gene expression in WT. Furthermore, AtVSP2 transcript abundances of VvAOS-6 and VvAOS-9 were below the transcript abundance in WT.

Arabidopsis transgenic 35S:AtAOS plants' responses to wound treatment

As a comparison to the transgenic 35S:VvAOS plants, the transcript abundance of AtAOS, AtLOX2 and AtVSP2 genes in three selected lines of transgenic 35S:AtAOS plants were also quantified. The three transgenic lines were AtAOS-11, AtAOS-13 and AtAOS-18, as mentioned in section 4.4.3. As already mentioned in the materials and methods section 4.3.4, transcript abundance of the endogenous and transgene AOS were not discriminated for quantification purpose. Transcript

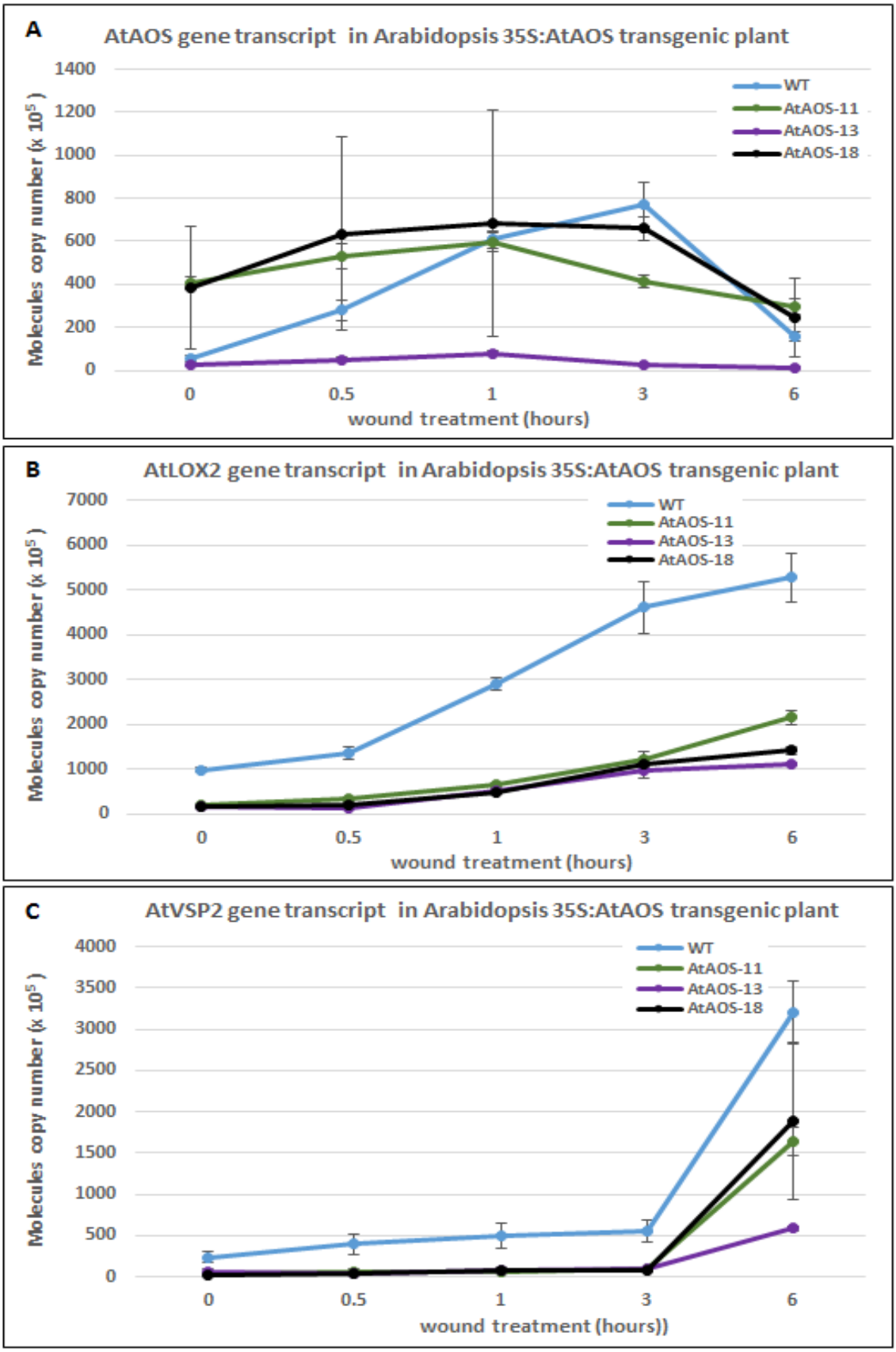

Figure 4.9 Transcript abundance of AtAOS, AtLOX2 and AtVSP2 in three lines of Arabidopsis transgenic *35S:*AtAOS plants.

Transcript abundance of AtAOS, AtLOX2 and AtVSP2 genes were quantified from three selected transgenic *35S:*AtAOS plants (AtAOS-11, AtAOS-13 and AtAOS-18) after a six-hour time course wound treatment via a qRT-PCR approach. Bars represent standard deviation of the mean from three biological replicate samples.

abundance of each transgenic 35S:AtAOS line is shown in figure 4.9A. In the pre-wound condition, transgenic lines exhibited a different level of AtAOS gene expression. Transgenic AtAOS-11 exhibited approximately 40 x 10^6 transcript copies in 4 µL of cDNA synthesized from 500 ng of total RNA sample whereas, in AtAOS-13, there were only 2 x 10^6 transcript copies. Transgenic AtAOS-18, on the other hand, exhibited a similar expression level as with AtAOS-11 but was found to exhibit a very variable level of expression, as evidenced by the large standard deviation value within the biological replicates collected. In comparison, transcript copies quantified in WT were 6 x 10^6 in 4 µL of cDNA synthesized from 500 ng of total RNA sample. This indicated that, in general, transgenic AtAOS-11 and AtAOS-18 exhibited high basal levels of AtAOS gene expression compared to the AtAOS-13 and WT samples. Interestingly, AtAOS expression in transgenic 35S:AtAOS showed a similar pattern to the endogenous AtAOS expression in transgenic 35S:VvAOS. AtAOS gene expression in all transgenic lines peaked one hour after wound treatment but at a different magnitude. Transgenic AtAOS-11 transcripts increased from 40 to 60 x 10^6, from 39 to 68 x 10^6 in AtAOS-18 but only from 2 to 8 x 10^6 in AtAOS-13 copies in 4 µL of cDNA synthesized from 500 ng of total RNA sample. In comparison, AtAOS expression in WT peaked at three hours when the gene transcripts had increased from 6 to 77 x 10^6 copies of cDNA synthesized from 500 ng of total RNA sample before declining to a near pre-wound condition level. An interesting observation in this comparison was the AtAOS expression magnitude in AtAOS-13. Transgenic AtAOS-13 exhibited an exceptionally low expression of the AtAOS gene over the course of the six-hour wound treatment compared to the other transgenic lines. However, despite this low expression a noticeable AtAOS transcript abundance increased at one hour (from 2 to 8 x 10^6 copies) after wound treatment.

Although both JA-responsive genes, AtLOX2 and AtVSP2, in all transgenic 35S:AtAOS responded to the six-hour time course treatment, despite the high basal level of AtAOS gene transcripts in transgenic lines (except for AtAOS-13), their JA-responsive expressions were much lower compared to the expression of similar genes in WT (Figures 4.9B and 4.9C). Again, this was a clear indication that a high basal level in AOS transcripts did not alter the transcription regulation of JA-responsive genes in the transgenic lines. Our comparisons observed between Arabidopsis transgenic 35S:VvAOS and 35S:AtAOS plants showed that:

1. Despite both transgenes being regulated by similar promoters the transcript abundance of the VvAOS gene (transgenic 35S:VvAOS) was exceptionally high compared to the AtAOS gene (transgenic 35S:AtAOS)
2. The patterns of AtAOS gene in transgenics, 35S:VvAOS and 35S:AtAOS, were similar but with different magnitudes.
3. In reference to transcript abundance in WT samples, it clearly shown on the graph that generally the transcript abundance of both JA responsive genes in transgenic 35S:VvAOS (Figure 3.8A and

4.8B) were higher compared in respective genes in transgenic 35S:AtAOS (Figure 4.9B and 4.9C) plants.

4.4.5 Botrytis disease assay on transgenic Arabidopsis 35S:VvAOS and 35S:AtAOS detached leaves

In order to demonstrate correlations between AOS gene expression and plant defences against botrytis infections in both sets of Arabidopsis transgenic lines, we infected eight-week-old detached leaves with Botrytis spores re-suspended in 25% of grape juice. In this experiment, we expected that plants that had exhibited a high level of expression of JA-responsive genes will produce smaller Botrytis lesions on infected leaves compared to wild type plants. For this assay, we selected transgenic lines that exhibited stable AOS gene expression over the six-hour wound treatment. Therefore, in transgenic 35S:VvAOS, we selected VvAOS-6 and VvAOS-15, and in transgenic 35S:AtAOS, we selected AtAOS-13 and AtAOS-18. As a comparison, the WT was also included. The results for the Botrytis infection assay are shown in figures 4.10 and 4.11. Botrytis infections (shown by lesion formation) were documented by photographs at 48, 72 and 96 hours after inoculation. Results indicated that at 96 hours after Botrytis inoculation, there were no apparent differences in lesion size formation among all detached leaves infected except for the AtAOS-13 line. Lesion size formation between VvAOS-6, VvAOS-15, AtAOS-18 and WT were similar and inconclusive (transgenic 35S:VvAOS) in showing which plants were less susceptible to pathogen infection. On the other hand, the size of lesion formation in AtAOS-13 was a noticeably larger than in the rest of the samples, which gave a clear indication that this particular transgenic line was more susceptible to pathogen infection compared to the rest of the samples.

4.4.6 Molecular modeling of VvAOS and AtAOS protein sequences

We were puzzled by the counter intuitive levels of VvAOS transcript accumulation observed in the complementation lines which were approximately 150 times higher than wild type AOS expression and the relatively low levels of jasmonic acid and jasmonic acid responsive gene transcription observed. A number of scenarios might explain this phenomenon, one of which was that the grapevine AOS might be compromised in its catalytic activity due to some alteration in tertiary structure. We therefore decided to model the grapevine AOS sequence and compare this to the Arabidopsis model. Protein sequences of both VvAOS and AtAOS were used as query to identify suitable modelling template. Using Swiss-modelling interactive tools (http://swissmodel.expasy.org/interactive) we identify 3dsi.2.A crystal structure (http://swissmodel.expasy.org/templates/3dsi.2) as most suitable template to build a 3D protein structural model for both VvAOS and AtAOS sequences. We chose this 3dsi.2.A crystal structure (Lee et al., 2008) as a template because it share a highest similarity with VvAOS (68%) and 100% identical to AtAOS protein sequence. In order to search for gross differences

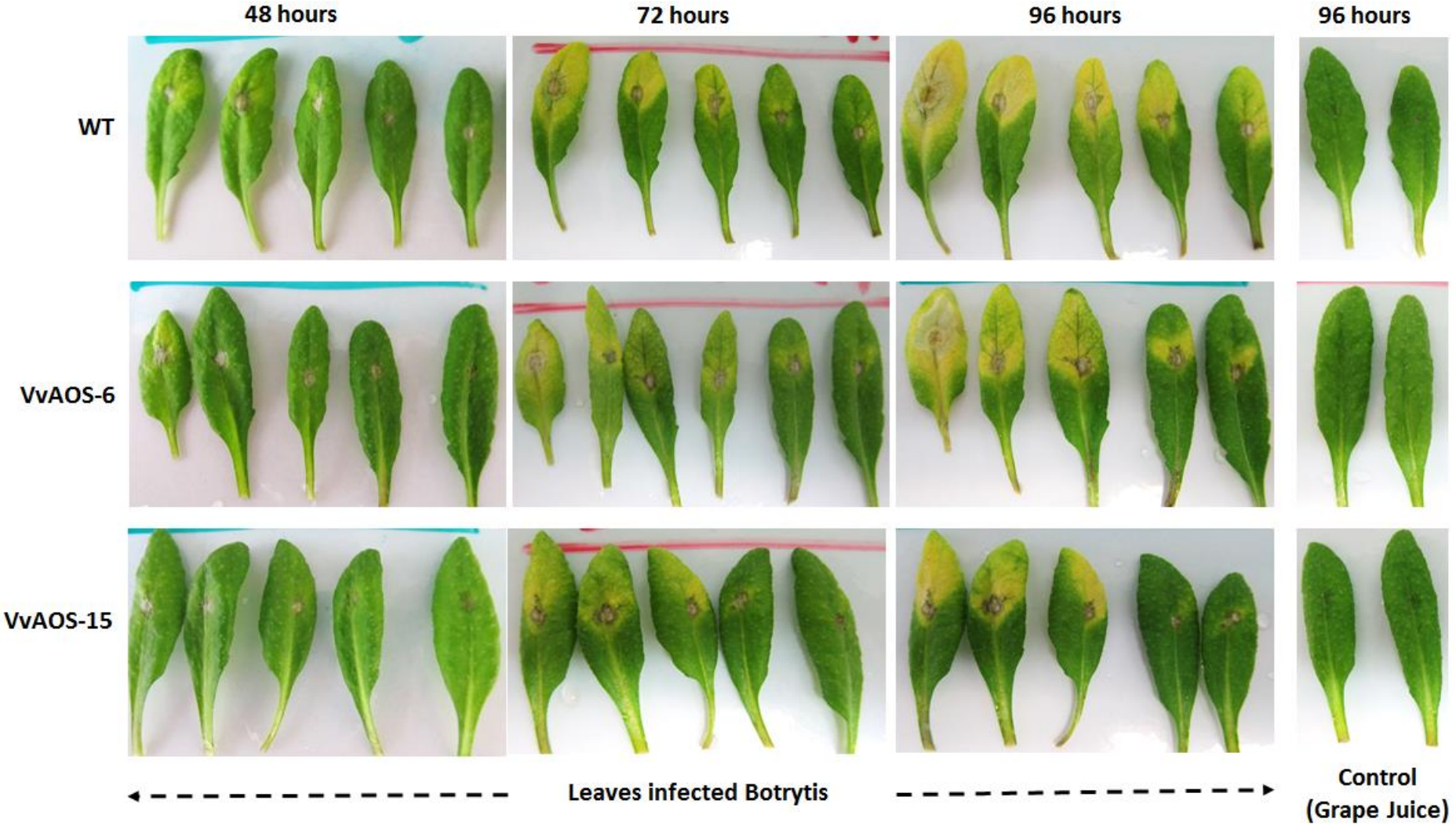

Figure 4.10 Botrytis disease assay on Arabidopsis transgenic 35S:VvAOS plants.
In this 35S:VvAOS line, out of six leaves infected with Botrytis, two most severely infected leaves are shown on the figure above. Transgenic VvAOS-6 shows a similar infection response to WT based on the lesion infection size, transgenic VvAOS-15 shows a stronger response to infection with smaller sized infection lesions compared to VvAOS-6 and WT – plant control.

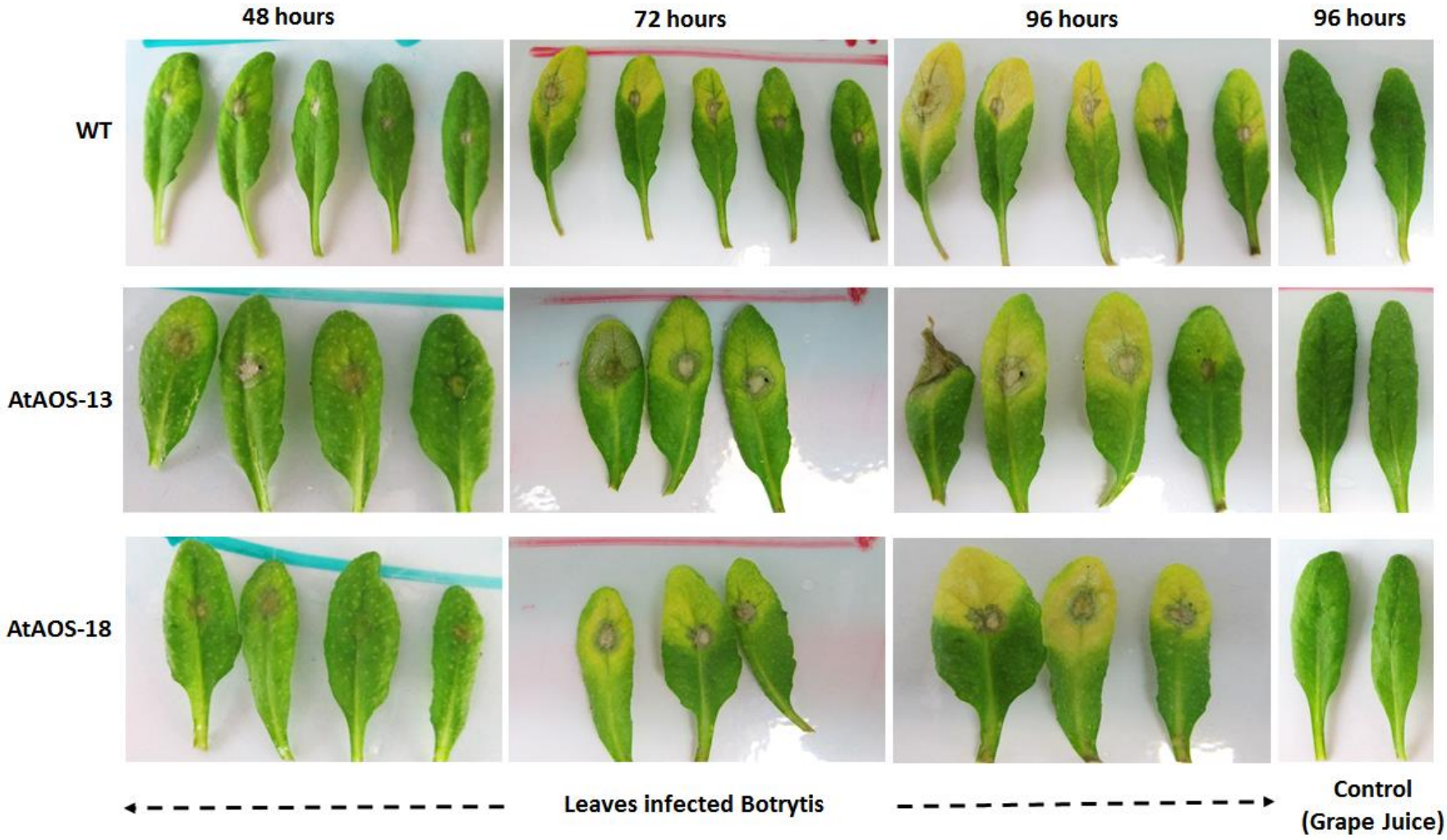

Figure 4.11 Botrytis disease assay on Arabidopsis transgenic 35S:AtAOS plants

In this 35S:AtAOS line, out of six leaves infected with Botrytis, two most severely infected leaves are shown on the figure above. Transgenic AtAOS-13 – AtAOS expression was strongly supressed and showed the biggest area of lesion infection, transgenic AtAOS-18 - highest AtAOS expression of transgenic 35S:AtAOS line showed smaller area of lesion infection compared to transgenic AtAOS-13 and WT – plant control.

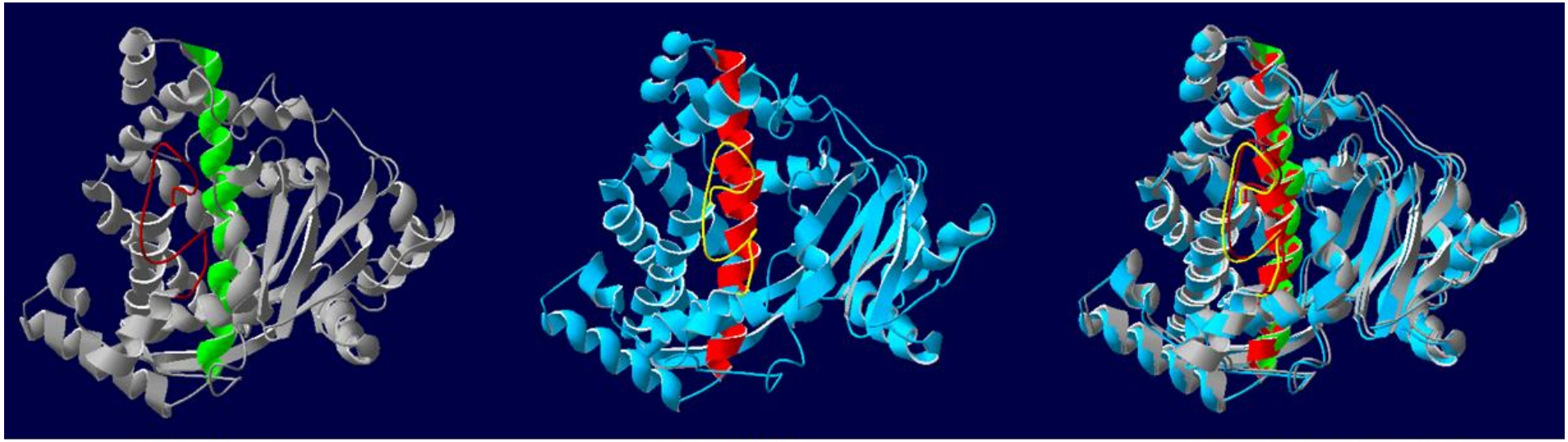

Figure 4.12 Prediction of allene oxide synthase crystal structure in Arabidopsis and Grapevine
Predicted AOS enzymes structures between AtAOS and VvAOS were overlapped to see any apparent differences between the two enzymes.Helix-I motif region was highlighted in green and red, whereas heme-binding site in brown and yellow for AtAOS and VvAOS 3D-protein structure respectively.

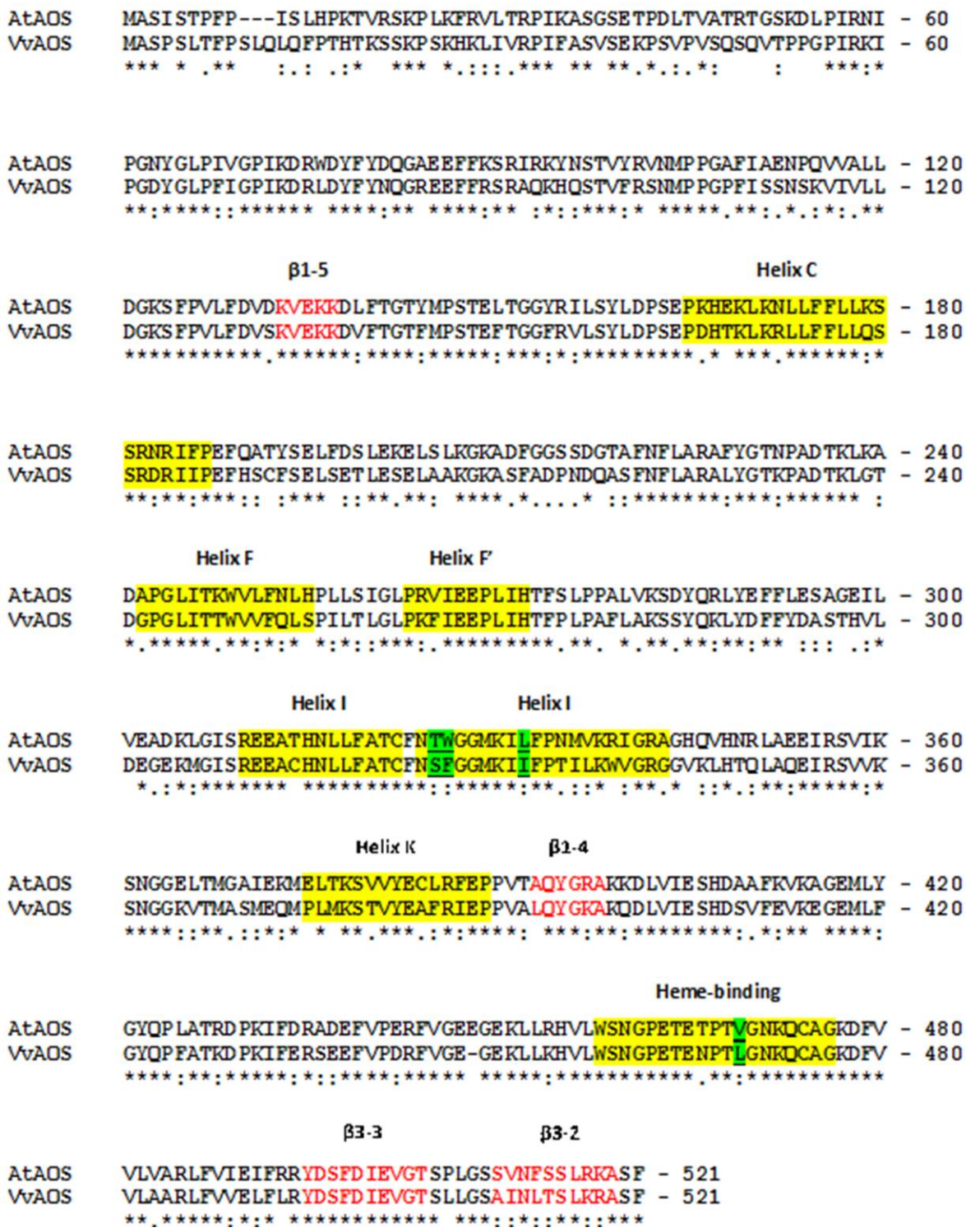

Figure 4.13 Alignment sequence of AtAOS and VvAOS protein

Alignment sequence between AtAOS and VvAOS protein sequences to identify amino acid substitutions within important motifs in VvAOS enzymes protein sequences relative to AtAOS. Amino acid substitution within helix-I and heme-binding sites were identified in green letters whereas hydrophobic residues within AOS active sites were identified in blue boxes. Important helix motif and β-sheet region were identified in yellow and red letters whereas the heme-binding site is in a red box.

between VvAOS and AtAOS protein structure, their predicted 3D-protein structural model were compared by superimposing their predicted model on each other using Swiss-PDB Viewer (Guex and Peitsch, 1997; Johansson et al., 2012) that was acquired from Swiss-Pdb Viewer website (http://spdbv.vital-it.ch/). In order to analyse amino acid differences between VvAOS relative AtAOS protein sequence at their active sites, both protein sequences were aligned using CLUSTALW (Thompson et al., 1994) and residues differences were identified within Helix-I and Heme-binding motifs region. Helix-I and Heme-binding site conserved regions were identified based on the AOS crystal structure carried out in guayule (Li et al., 2008). Superimposition of the predicted structural models of VvAOS compared to AtAOS showed that there are no obvious differences between the two proteins at the structural level (Figure 4.12 and 4.13). However, close examination on the predicted enzymes active sites show that there are two amino acid residues differences present within helix-I motif (FNxxGGxKxxxP) and one in heme-binding site (PxxxNKQCxGKD) in VvAOS protein sequence relative to AtAOS protein. The amino acid substitutions in these regions corresponded to a serine to threonine, phenylalanine to tryptophan and isoleucine to leucine (F-N-[**S/T**]-[**F/W**]-G-G-M-K-I-[**I/L**]-L-P). In addition at the heme-binding site, there was an amino acid residue substitution of a leucine to Valine (P-T-[**L/V**]-G-N-K-Q-C-A-G-K-D).

4.4.7 Quantification of Jasmonic acid compound in Arabidopsis transgenic 35S:VvAOS plant via Gas Chromatography-Mass Spectrometry analysis.

In order to confirm a correlation between VvAOS gene expression and JA regulation in transgenic 35S:VvAOS plants, JA was quantified via derivatization of JA conjugates into methyl jasmonic acid using trimethylsilyldiazomethane as a methylation agent. Using this method, the methylation agent converts carboxylic acid to methyl esters to increase the volatility all compounds including JA to enable gas chromatography to separate of them (Schmelz et al., 2004). Jasmonic acid was quantified from the 35S:VvAOS transgenic lines via GC-MS with dihydrojasmonic acid (dhJA) as an internal standard for the quantification calculation, as described in the materials and methods (Section 4.3.4). As a control, the JA content in WT samples was also quantified. However, due the time constraints and limitations of research funding, we only quantified JA from two transgenic 35S:VvAOS plant lines with WT as a control. In addition, we were only able to quantify JA from 0 (pre-wound), 0.5, 1 and 6 hours post-wounding. The sampling periods for wound treated samples were selected based on previous reports by Laudert et al. (2000) and Park et al. (2002) which indicated that JA accumulation peaked at one hour after wounding in Arabidopsis leaves. Transgenic VvAOS-6 and VvAOS-15 lines were selected due to their consistency in VvAOS and AtAOS gene expression at the transcription level. The results indicated that, between the two selected transgenic plant lines, VvAOS-15 accumulated JA to the highest level ($P < 0.05$), whereas VvAOS-6 and WT accumulated JA to similar levels but lower than those observed for VvAOS-15 (Figure 4.14). Interestingly, this result was in accordance with the

result of AtVSP2 (JA- responsive) gene expression at a transcription level (Section 4.4.4). Besides that, results also indicated that JA was not elevated in the pre wound condition which, therefore, clearly indicated that JA compounds were not elevated by the high levels of transgene VvAOS (or AtAOS) transcript abundance at the basal level.

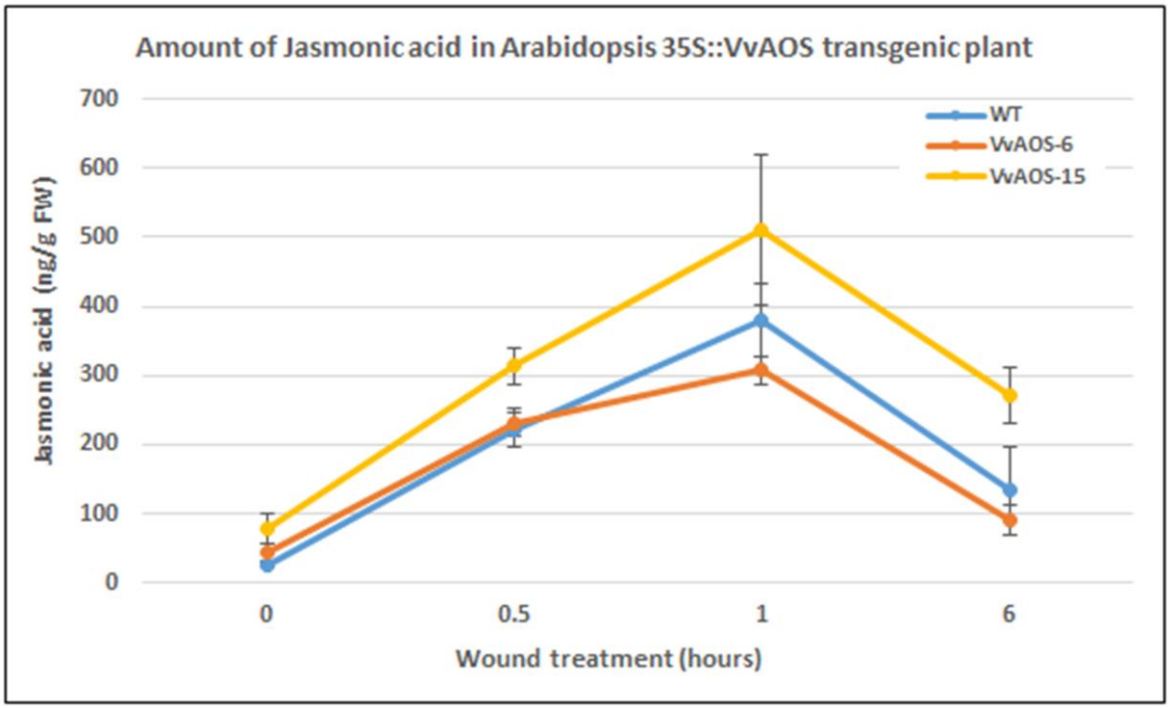

Figure 4.14 Regulation of jasmonic acid biosynthesis in selected Arabidopsis *35S:*VvAOS transgenic plants
Amount of jasmonic acid quantified from 100 mg of leaves samples using GC-MS. Bars represent standard deviation of the mean from three biological replicates

4.5 Discussion

It discussed previously (Literature Review - Section 1.6.4) jasmonates play an important role in regulating plant development and AOS was an enzyme that catalysed the first step in the biosynthesis pathway which may indicate its vital role as a focal point for JA production. Therefore, in order to examine the effect of the VvAOS gene, which may lead to increased JA regulation in grapevines, we overexpressed VvAOS gene in *Arabidopsis thaliana* cv *Columbia* (Arabidopsis). In this chapter, our objective was to investigate further functions of the VvAOS gene regulation effect on JA production when overexpressed under the control of the constitutive CaMV 35S promoter. Our question is "Would grapevine AOS be able to increase the plant's ability to defend itself against pathogen attack when AOS overexpressed in Arabidopsis?" Previous reports indicated that overexpression of AOS in a model plant system can confer enhanced tolerance to insect attack through an increased endogenous JA level, PR gene expression, and host resistance to fungal infections (Wang et al., 1999; Laudert et al., 2000; Mei et al., 2006). Based on these reports we tested the capability of VvAOS to improve plant defence mechanisms in Arabidopsis as a model for the possible impact of increasing AOS levels in grapevines. As part of this experiment, in order to assess immediate effect of VvAOS gene up-regulation in wound treated leaves, transcripts of the known wound-induced and JA-responsive genes, AtLOX2 and AtVSP2, also were measured. These experiments were compared with data generated from a comparable set of transgenic lines that overexpressed Arabidopsis AOS (AtAOS) as a control.

Based on previous reports (Harms et al., 1995; Wang et al., 1999; Laudert et al., 2000; Park et al., 2002; Mei et al., 2006; Pajerowska-Mukhtar et al., 2008; Wu et al., 2008) we developed a number of hypotheses for this overexpression experiment. First, AOS gene overexpression meant the addition of at least one copy of a similar gene function into a model plant that could confer increased JA production. However, in light of the previous work (chapter 4) on complementation of the AOS knock-out function in Arabidopsis *aos* mutant, the VvAOS transgene under the control of AtAOS promoter produced massive amount of VvAOS transcripts that we speculated could be as a result of crosstalk between the transgene promoter (promAOS:VvAOS) and endogenous AOS (promAOS:Knock-out *aos* function). As a consequence, this might cause immediate PTGS mechanisms in overexpress plant (OE) upon mechanical stimulation. Therefore, we employed a constitutive CaMV 35S promoter as a gene regulator instead of promAOS. We expected that the use of CaMV 35S promoter would deliver a high level of AOS transcripts but the transcription mechanism uncoupled from the endogenous promAOS response that might have had a more profound impact than just adding extra copies of transcripts. Therefore, our second hypothesis, was using the constitutive CaMV 35S promoter to deliver very high levels of AOS transcripts to achieve a higher level of JA in OE-plants. In OE-plants, although the literature suggested that JA production was limited by substrate availability (Wasternack, 2007), we expected that high levels of VvAOS transcripts regulated by CaMV 35S promoter will provide high

amount of AOS enzymes available within the JA biosynthetic pathway to compete with other enzymes (enzymes that used the same substrate, such as hydroperoxide lyase and divinyl ether synthase) when high levels of substrate available after wound stimulation and this will confer a high level of JA production. Increased levels of JA will confer more resistance on OE-plants to pathogen attack (Ellis et al., 2002; Chen et al., 2006). Lastly, by investigating transgenic lines that shared similar levels of transgene expression we may be able to assess the comparative efficiencies of VvAOS and AtAOS in an Arabidopsis plant's background.

Based on these hypothesis, and also information from the previous report mentioned above, we developed expectations that overexpression of the grapevine AOS gene under the regulation of CaMV 35S promoter in Arabidopsis will deliver high accumulation of AOS transcripts that will help to regulate higher production of JA compounds in OE-plants compared to WT. It has previously been reported that high JA levels will confer more tolerance on OE-plants to pathogen attack (Wasternack, 2007). However, despite the excellent theoretical prospects from the literature (Harms et al., 1995; Wang et al., 1999; Laudert et al., 2000; Park et al., 2002; Mei et al., 2006; Pajerowska-Mukhtar et al., 2008; Wu et al., 2008), our results did not support the expectation mentioned above. Generally, our results indicated that overexpression of grapevine AOS did not significantly increase JA production in Arabidopsis transgenic or their ability to respond significantly to pathogen attack or mechanical wounding. This result was not only unexpected but was also irregular with the previous report in respect to AOS gene overexpression from other plant species. Therefore, in order to comprehend the possible mechanism behind these unexpected results in Arabidopsis 35S:VvAOS transgenic plants, we interrogated further details in our data to rationalize the reasons behind it.

We began with the unexpectedly high level of VvAOS gene transcripts in transgenic 35S:VvAOS plants. The results showed that high levels of overall AOS transcript abundance in transgenic 35S:VvAOS (at the basal level and after wound treatment) did not significantly contribute to the increase in JA production or transcription levels of both JA-responsive genes compared to WT. Based on this result, our next question is “Why does high transcript level of AOS did not confer high JA production in OE-plants?” The transgenic plants comprised transgene and endogenous AOS that was regulated by two different promoters, i.e. the constitutive CaMV 35S promoter and promAOS. The constitutive CaMV 35S promoter has the ability to regulate gene expression independently and continuously which, as a result, confers a high level of gene transcription; whereas, the promAOS was an inducible promoter where its performance was modulated by environmental conditions and external stimuli, including abiotic factors such as wounding. The presence of the CaMV 35S promoter explained why the transgene VvAOS transcript was maintained at a high basal level and with unchanged transcript levels across the six-hour wound treatment in transgenic VvAOS-6 and VvAOS-15 lines (Figure 4.7). However, a previous report by Schaller (2001) suggested that the constitutive and higher basal levels of JA might not be an effective way to induce signal transduction as the relative

increase of JA over a short period of time was more critical for the induction of JA production or transcription of JA-responsive genes. Based on this suggestion, a study by Mei et al. (2006) was able to demonstrate that a relative increase of *Oryza sativa* AOS (OsAOS) transcripts over a short period of time not only increased JA production but also resulted in the activation of many JA-responsive genes. Therefore, this explained the result that the high level of VvAOS transcripts did not elevate the transcription of JA-responsive genes or JA production but that the relative increase of endogenous AtAOS transcripts in a short period of time did. Therefore, this experiment result is consistent with previous report (Mei et al., 2006) indicate that, JA induction or activation of JA-responsive genes might not be regulated by the availability of AOS enzymes but by the sudden increase of substrate availability within the JA biosynthesis pathway.

Nevertheless, JA induction mechanisms via relative increases in AOS transcripts over a short period of time did not explain why the high transcript abundance of VvAOS did not confer high levels of JA production compared to the much lower transcript abundance of AtAOS transcripts in WT. We speculated that the mechanism behind this issue could be due to the different affinity between VvAOS and AtAOS enzymes towards the local hydroperoxide substrate available within the Arabidopsis system in transgenic 35S:VvAOS plants. Pajerowska-Mukhtar (2008) suggest that amino acid substitution close to StAOS2 in the *Solanum tuberosum* substrate binding site could possibly change the enzymes' substrate affinity and other kinetic properties that might influence the quantitative defence responses. At the nucleotide level, VvAOS and AtAOS sequences were only 68% identical and were 66% identical at the amino acid level. If the amino acid differences occurred within active sites of VvAOS enzyme protein sequences, this might affect their substrate affinity towards local substrates available in the Arabidopsis system compared to AtAOS enzyme proteins. In order to comprehend this issue further, we began by comparing predicted protein structures between the VvAOS and AtAOS enzyme to search for the apparent differences between the structures of the two proteins. At the protein structural level, there were no apparent differences detected between the two enzymes' protein conformational structures, as illustrated in figure 4.12. For further analysis, we interrogated amino acid differences within important conserved motifs in VvAOS protein sequences relative to the AtAOS protein sequence, such as helices motifs and heme-binding sites. According to Li et al. (2008), the crystal structure of guayule AOS had a very narrow and deep pocket on the distal side of the heme motif. The substrate binding pocket was formed by helices F and I, and loops between helix C and β1-5, between helices F and F', between helix K and β1-4, and between β3-2 and β3-3, and consisted mainly of hydrophobic residues, such as Phe-92, Phe-275 and Phe-278, and the presence of a polar residue (Asn-276) in the active site that was very close to the heme conserved motif (Li et al., 2008). Based on the crystal structure reported by Li et al. (2008), we interrogated amino acid substitution in the VvAOS protein relative to the AtAOS protein sequence within their active sites.

Among the important conserved motifs that formed active sites with the AOS enzyme, we drew our attention to amino acid differences occurring within helix-I and heme-binding site in VvAOS protein sequences relative to AtAOS protein sequences. We decided to focus our interrogation on the helix-I and heme-binding conserved motifs because of the unique interactions between these two motifs within the active site (Li et al., 2008). It was previously reported that the amino acid sequence within the helix-I conserved motif was very important in determining these enzyme activities (Toporkova et al., 2013). Toporkova et al. (2013) reported that a single amino acid substitution (located at 292) occurred within the helix-I conserved motif in flax divinyl ether synthase (LuDES) can convert its function to allene oxide synthase (LuAOS) enzyme activity. Whereas a substitution of two amino acid residue occurring within the helix-I conserved motifs (located at 295 and 297) in the tomato AOS enzyme can convert their function to produce tomato HPL products (Toporkova et al., 2008). Considering the importance of helix-I and heme-binding conserved motifs in determining AOS enzyme activities, we interrogated amino acid substitution occurring within these motifs in the VvAOS protein sequence relative to the AtAOS protein sequence. Our interrogation showed that there were two amino acid differences detected within the helix-I conserved motif (FNxxGGxKxxxP) and the heme-binding site (xxPxxxNKQCxGKD), as illustrated in figure 4.13. Amino acid substitution within the helix-I and heme-binding sites in the VvAOS enzyme protein structure relative to the AtAOS enzyme protein structure might not enough convert their function (proven by VvAOS gene complementing AOS gene function in Arabidopsis *aos* mutants) but we speculated that these substitutions could alter their specific affinity significantly towards local substrates in the Arabidopsis system. As a comparison, transgenic 35S:AtAOS plant lines that did not encompass two different AOS genes exhibited exceptionally low transcript abundance compared to transgenic 35S:VvAOS plants and were at a comparable level compared to WT.

However, the possibility of low substrate affinity of VvAOS protein enzyme mechanisms toward local substrates in Arabidopsis did not explain why endogenous AtAOS gene transcripts in transgenic 35S:VvAOS declined two hours earlier compared to WT and that might cause a significant reduction in JA production. In order to comprehend this issue, we proposed a gene silencing mechanism effect, i.e. either or both transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Stam et al., 1997; Schubert et al., 2004; Kohli et al., 2010; Sohn et al., 2011) that act as plant defence mechanisms against foreign genes occurring in transgenic 35S:VvAOS plants as an explanation. In general, unexpected gene silencing mechanisms in Arabidopsis transgenic plants were not unique. Several reports have indicated that unexpected gene silencing occurred in certain Arabidopsis transgenic plant lines. For example, Dixanger et.al (2008) reported an unexpected silencing effect of T-DNA tags occurring in SALK, FLAG and GABI transgenic Arabidopsis collections. According to this report, effects from commonly used T-DNA tagging lines resulted in the silencing of a variety of diverse constructs, using the CaMV 35S promoter, caused by unintended homology-dependent

transcriptional gene silencing caused by T-DNA insertion. The potential factor that can trigger the gene silencing mechanism was the presence of promoter sequences that were common to both the T-DNA insertion and the unlinked transgene that was silenced by the T-DNA (Mlotshwa et al., 2010). For example, SALK, GABI and FLAG mutant lines all carry a copy of the CaMV 35S promoter and a study of SALK and GABI lines found that high proportion of those lines induced transcriptional silencing of transgenes expressed from the CaMV 35S promoter (Daxinger et al., 2008). Mlotshwa et.al (2010) also explained that the propensity of individual T-DNA insertion lines to trigger CaMV 35S promoter homology-dependent transcriptional silencing in *dcl3-1* line (SALK_005512) was probably due to complex integration patterns of T-DNA which promoted production of dsRNA from the CaMV 35S promoter sequence on the T-DNA and, consequently, gave rise to CaMV 35S promoter siRNAs. In Arabidopsis transgenic lines, the production of siRNA could trigger both TGS and PTGS through methylation of homologous sequences in *cis*- and *trans*- forms of the 35S promoter (Matzke et al., 2004; Bhullar et al., 2007; Matzke et al., 2009). In addition, it was reported that siRNA also appeared to target direct or indirect specific mRNA sequences to trigger PTGS mechanisms (Dalakouras et al., 2011). Furthermore, gene silencing primarily evolved as a plant defence mechanism to protect plants against foreign nucleic acids, including viruses and active transposable elements (Baulcombe, 2005; Wang and Metzlaff, 2005; Dalakouras et al., 2011). In plant defence mechanisms, the introduction of exogenous transgenes containing genetic elements that plants probably recognised as a foreign genes (Salinas et al., 1988) and detected as a threat, were eventually silenced by plant defence mechanisms (Dalakouras et al., 2011). Therefore, the Arabidopsis natural defence mechanisms could also play a significant role in the unexpected result shown.

Although several factors can trigger gene silencing mechanisms in our transgenic plants (transgenic 35S:VvAOS and transgenic 35S:AtAOS) but, in this particular experiment, we suspected that the introduction of additional constitutive CaMV 35S promoter with T-DNA insertion and insertion of transgene AOS (either VvAOS or AtAOS) into the Arabidopsis genome were the main factors. Generally, our results indicated that all transgenic 35S:VvAOS plants selected exhibited a high level of VvAOS transcript abundance pre- and post-wound treatment; whereas, the AtAOS gene transcript only increased post-wound treatment. At post-wound treatment, both genes transcripts (VvAOS and AtAOS) showed a decline one hour after wounding but at different rates (Figure 4.7). Based on this observation, this result, first, indicated that the gene silencing mechanisms were only triggered after concentration of AOS transcript abundance surpassed their threshold points (Schubert et al., 2004) and, secondly, this silencing mechanism did not completely silence the AOS gene function but reduced their gene expression at either the TGS or PTGS levels (Tang et al., 2007; Carthew and Sontheimer, 2009; Fan et al., 2011). Transgene VvAOS was under the control of a strong promoter that was consistently active which, as a result, conferred a high basal level of VvAOS gene transcripts in transgenic 35S:VvAOS plants. When stimulated by wounding, promAOS activated and initiated

transcription mechanisms in the endogenous AtAOS gene. The addition of AtAOS transcript as a result of the activation of promAOS lead to the accumulation of more aberrant transcripts within the transgenic 35S:VvAOS plant system that, when they exceeded a tolerable level, activated plant defence mechanisms via either TGS or PTGS or both mechanisms (Schubert et al., 2004; Wassenegger and Krczal, 2006; Mourrain et al., 2007; Dalakouras et al., 2011). As a result, regulation of both AOS genes in transgenic 35S:VvAOS started to reduce just one hour after wounding.

Similar mechanisms were also observed in transgenic 35S:AtAOS plants. Due to the effect of the constitutive CaMV 35S promoter on transgene AtAOS, transgenic 35S:AtAOS plants exhibited a high basal level of AtAOS transcripts but did not trigger gene silencing mechanisms. However, after wounding, transcription mechanisms of endogenous AOS were activated and the additional AOS transcripts led to the activation of gene silencing after the overall levels of AOS transcripts in the transgenic 35S:AtAOs plant systems exceeded a tolerable level. As a consequence, AOS transcription was reduced one hour after wounding. In comparison, AtAOS gene transcripts in WT increased but decreased at only three hours after wounding, a delay of two hours compared to both transgenic plants. Early reduction of overall AOS gene transcripts might explain why transcript abundance of JA-responsive genes, AtLOX2 and AtVSP2, were lower in transgenic plants compared to WT. As a consequence of the early reduction of AOS gene transcription, transduction signals generated to regulate JA-responsive genes were much weaker in both transgenic plants compared to WT. This suggestion supported the result shown in figures 4.8 and 4.9 where transcript abundance of JA-responsive genes in WT were exceptionally high compared to both transgenic plants.

Interestingly, among the transgenic plants, selected transgenic 35S:AtAOS line AtAOS-13 showed a strong suppression of AtAOS transcripts. Recent reports have suggested that spontaneous transgene silencing occurring in transgenic *N. benthamiana* was related to the number of transgenes incorporated into their genome (Sohn et al., 2011). If more than one transgene insertion located on two different chromosomes were detected and incorporated into their genome, it will cause spontaneous gene silencing through TGS and PTGS mechanisms (Sohn et al., 2011). Transgenes incorporating more than one copy into their genomes were often facilitated by the ectopic expression of CaMV 35S promoter (Harper and Stewart, 2000), multiple copies, or more than two T-DNA inserted in the same chromosome (Tang et al., 2007) and direct (DR) or inverted (IR) transgene repeats (Schubert et al., 2004). The initiation of gene silencing was recognized as involving the formation of complementary dsRNA which, subsequently, triggered gene silencing (Mourrain et al., 2007). This spontaneous gene silencing effect explained the results shown in transgenic AtAOS-13 (Transgenic 35S:AtAOS). In AtAOS-13, AtAOS gene transcript abundance was below the transcript level of similar genes in WT, which indicated that AtAOS genes had already undergone spontaneous suppression in pre-wound conditions. As a comparison, other transgenic plants (transgenic VvAOS-6, VvAOS-9, VvAOS-15, AtAOS-11 and AtAOS-18) comprised high accumulations of overall AOS genes transcripts

under pre-wound conditions and suppression only begun at one hour after wounding. Previous reports also mentioned that all plants containing more than a single T-DNA insertion were methylated on the promoter and its activity was reduced, with amount of methylation and reduction of promoter activity correlated with the number of T-DNA copies (Chalfun-Junior et al., 2003). Furthermore, it was also hypothesized that the expression of the two T-DNA copies remained below the threshold but when the threshold was exceeded the sum of the four T-DNA (homozygous) copies likely initiated the silencing process (Weinhold et al., 2013). Therefore, incorporation more than one copy number of the transgene 35S:AtAOS line, AtAOS-13, may explain why the results shown in this transgenic line were distinct from the other selected lines.

In order to appraise further transgenic plants (35S:VvAOS and 35S:AtAOS transgenic) response to different type stimuli, detached leaves of eight-week-old plants were infected with Botrytis spores re-suspended them in 25% grapevine juice media. Leaves for these assays were selected from transgenic lines, VvAOS-6 and VvAOS-15, from transgenic 35S:VvAOS and AtAOS-13 and AtAOS-18, from transgenic 35S:AtAOS, whereas WT was included as a control. Generally, the Botrytis disease assay outcomes were in agreement with the JA-responsive gene especially with AtVSP2 transcript abundance results. The results indicated that among all transgenic plants selected (include transgenic 35S:VvAOS and transgenic 35S:AtAOS), the transgenic line AtAOS-13 showed as most susceptible to Botrytis infection, as shown by the largest size of lesion formation from Botrytis infection on their detached leaves. This observation was as expected because among all the transgenic lines selected, transgenic AtAOS-13 exhibited the lowest JA-responsive gene transcript abundance. However, Botrytis disease assay results for transgenic VvAOS-6, VvAOS-15, AtAOS-18 and WT (control) were inconclusive. The sizes of a lesion formed as a result of Botrytis infection on the respective detached leaves of transgenic VvAOS-6, VvAOS-15, AtAOS-18 and WT cannot be differentiated. However, this result was supported by the AtVSP2 transcript (JA-responsive gene) level in each of the respective transgenic lines. Transcript abundance level of AtVSP2 in VvAOS-6, VvAOS-15, AtAOS-18 and WT were not exceptionally different from each other. As an example, the largest difference of AtVSP2 gene transcripts shown between VvAOS-15 and AtAOS-18 was only 2-fold (Figures 4.8 and 4.9). Therefore, it was, apparently, acceptable that transgenic plant responses to Botrytis infection within these selected transgenic lines cannot be differentiated via lesion formation on their detached leaves. Furthermore, plant defence mechanisms toward Botrytis infection were known to be influenced by several phyto-hormones that interacted negatively or positively on them (Windram et al., 2012; Wang et al., 2013). This complex phyto-hormone interaction sometimes caused contradictory results about the influence of individual hormone on assay either susceptible or resistance to pathogen infection (Windram et al., 2012).

Nevertheless, the progression of Botrytis growth within 96 hours (photographed at every 24 hours) shows a noticeable slower at 48 hours in transgenic VvAOS-15 compare to VvAOS-6, AtAOS-18

and WT. According to Windram et al. (2012), the relative timing of different plant hormones in response to Botrytis infection are diverse. The sequential involvement of plant signalling molecules in response to Botrytis infection data indicates that most of the JA-responsive genes were start expressed around 16 hours followed by ABA at 20 hours and SA at 22 hours after inoculation (Windram et al., 2012). This observation suggest that genes expression of JA-responsive gene only occur in a short time period (between 16 – 20 hours after inoculation) before ABA-responsive genes been upregulated. ABA and JA/ET signalling known to have an antagonistic interaction (Anderson et al., 2004; Windram et al., 2012). Previous reports reveal that ABA appeared to have a negative effect on defence against Botrytis in both tomato (Audenaert et al., 2002) and Arabidopsis (Adie et al., 2007). These observations are consistent with our result. Expression of JA-responsive genes in transgenic VvAOS-15 are the highest among transgenic lines selected. Therefore, progression of Botrytis colony growth on transgenic VvAOS-15 detached leaf is much slower during this small time window (between 16 – 20 hours after inoculation) compare to other transgenic plant lines and WT. This explained a noticeable Botrytis growth progression different in the early stage of the infection rather than at 96 hours after inoculation. This explanation also apply to transgenic AtAOS-13 as a transgenic less resistance to Botrytis infection since this particular line exhibit the lowest JA-responsive regulation as a response to wounding among transgenic line selected. Nevertheless, even at the early stage of Botrytis infection, transgenic VvAOS-6, AtAOS-18 and WT still not be able to discriminate distinctively for their response to Botrytis infection. Unlike response to mechanical wounding which is robust and rapid, plant response to pathogen infection based on the plant recognition to pathogen effector that eventually trigger plant defence mechanisms (Boyd et al., 2013). Defence mechanisms triggered by this plant-pathogen recognition interaction initiate sequential activation of diverse plant hormones such as salicylic acid, ethylene, abscisic acid and jasmonic acid as a strategies to overcome pathogen invasion (Windram et al., 2012; Denance et al., 2013). Therefore this might suggests that the influx of AOS enzyme alone within plant defence system did not enough to confer high resistance to pathogen attack but crosstalk among defence signalling network balance the immune response to acquire the best way to prevent penetration or pathogen infection. Therefore this might provide some explanation as to why some of the transgenic plants high levels of JA-responsive gene transcript but did not clearly show improved response to pathogen attack compared to the other transgenic plant lines especially at 96 hours after Botrytis inoculation.

As mentioned previously, due to limited funding and time constraints we only managed to quantify JA from two selected transgenic 35S:VvAOS plant lines, i.e. VvAOS-6 and VvAOS-15 and with WT as a control. As expected, regulation of JA biosynthesis from selected transgenic 35S:VvAOS lines was in agreement with the results shown by AtVSP2 (JA-responsive gene) transcript abundance. Generally, JA levels in transgenic VvAOS-6 and VvAOS-15 lines were comparable with the levels of JA expressed in WT. Although JA regulation in transgenic VvAOS-15 was statistically significantly higher

compared to VvAOS-6 and WT, the amount of the increment of JA was not significant enough to conclude that introduction of the VvAOS gene regulated by the CaMV 35S promoter significantly increased the ability of Arabidopsis plants to shield themselves from abiotic and biotic stresses compared to WT. However, Kohli *et al.* (2010) mentioned that there were many factors that influenced transgene stability which led to high variable expressions of the transgene. One of the most important factors was the position of the transgene insertion effect, which reflected the influence of genomic DNA surrounding the site of transgene integration (Wilson et al., 1990; Matzke and Matzke, 1998). Furthermore, Schubert *et al.* (2004) also reported that position effect of transgene expression in the Arabidopsis genome can be up to 2-fold under the regulation of CaMV 35S promoters. Variation of JA accumulation shown between the two selected transgenic 35S:VvAOS lines were in agreement with the report by Park (2002). Therefore, we believed that the transgene position effect was one of the main reasons to explain the variation of JA accumulation in transgenic 35S:VvAOS plants.

Another observation worth mentioning was the magnitude of the sudden increase (a shorter time to reach peak expression) of AtAOS gene expression in transgenic plants, as described in the Results section (Section 4.4.4). The magnitude of the sudden increase of AtAOS transcription in all samples selected (two transgenic plant lines and WT) was agreement with the JA level in plants. For example, in VvAOS-15, the sudden increase of AtAOS gene expression was highest and also displayed the highest levels of JA production. Whereas both WT and VvAOS-6 showed no significant differences in the sudden increase of AtAOS expression and also displayed no significant differences in the levels of JA production. In relation to magnitude of the sudden increase, similar correlations can be observed within the transgenic 35S:AtAOS line. Due to high basal levels of AtAOS transcripts in AtAOS-13 and AtAOS-18, the magnitude of the sudden increase of AtAOS transcript in these respective transgenic lines was small and, as a consequence, lower transcript abundance of the JA-responsive gene was produced (Figure 4.11). These observations might be an indication that not only was the sudden increase of AtAOS gene expression critical to regulate JA production (Schaller, 2001; Mei et al., 2006) but also that the magnitude of the sudden increase will shape the level of JA production in Arabidopsis transgenic plants.

Besides the internal factors discussed above, several external factors could also have had a major influence on our results. Throughout this study, we have been working with the JA biosynthetic pathway that was not only stimulated by mechanical wounds but was also sensitive to diverse environmental stimuli, such as UV light, temperature or humidity (Ramakrishna and Ravishankar, 2011). Besides that, it was widely known that plants also have the ability to communicate with their plant neighbours via VOC which, eventually, can activate the JA biosynthetic pathway (Scala et al., 2013). Moreover, plant responses to stimuli were orchestrated by the combination or crosstalk between three major signalling pathways, i.e. salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) and their activation was known to be mutually antagonistic or synergistic based on the stimuli type

(Koornneef and Pieterse, 2008). Therefore, different stimuli types will influence the activation of different signalling pathways either by regulation or suppression. As our experiment progressed to the transgenic plant wound treatment assay, we encountered several issues that could influence the regulation of the JA biosynthesis pathway even at the transcription level. The first was technical problems with the experimental facility. Due to it being a new facility, the progress of the experiments was interrupted with technical "teething" problems, such as inconsistences in temperature, humidity and light intensity. As mentioned above, our subject plants might have responded to these inconsistent changes. Secondly, the plant growth facilities were also shared with other researchers who were conducting a diverse range of experiments. While growing our plants for the experiment, other plants species populations, such as grapevines (*Vitis vinifera*), tobacco (*Nicotiana tocaccum*), garlic (*Allium sativum*) and *N. benthamiana* were also being grown for different experiments. Due to stresses applied from the different experimental methods and techniques on these plant populations, the neighbouring plants could interact or crosstalk with our experimental plants via VOCs that eventually activated our subject plants' defence mechanisms even before the wound treatment assay started, which we use as a pre-wound sample. Thirdly, insect infestation and fungal infections. While growing our plant populations, we also encountered with minor insect and fungal problems, which influenced our plant responses to the wound treatment assay, especially in the Botrytis infection detached leaf assay. Fourthly, the method of sample collection for wound-treated leaves. For each time point collection, the samples were collected from different soil pots. This meant these plants could have already been influenced by different levels of environmental effects, such as insect infestation in the soil before the wound treatment assay started.

As potential reasons causing the unexpected results have been dissected, we suggested modifications to certain aspects of the materials and methods in order to achieve more informative and accurate results for future investigation of overexpression in the VvAOS gene. First, employ an inducible promoter instead of a constitutive promoter to overexpress the AOS gene. As discussed above, constitutive and higher basal level of JA did not effectively establish signal transduction pathway to regulate the expression of JA-responsive genes but the relative increase of JA in short period of time is more critical for the induction. Furthermore, transgene VvAOS with induced promoter influence on the JA biosynthesis pathway can be assayed based on the induction time period for gene expression to reach maximum level rather than their high basal availability. Secondly, screen a larger population of homozygous transgenic plants to acquire a more stable VvAOS gene expression among individual transgenic plants. Thirdly, since AOS was involved in the biosynthesis pathway that was easily stimulated by environmental conditions, homozygous transgenic plants should be grown in separate plant growth rooms (or containment) away from other plants to avoid possible crosstalk influences between plant neighbours. More importantly, grow transgenic plants free from any insect

infestation or pathogen problems. Lastly, if possible, sources of leaf materials should be collected only from same group of plants source for the whole time course wound treatment of sampling.

4.6 Conclusion and future prospects

In this chapter we overexpressed and characterized the VvAOS gene into Arabidopsis plants to answer our primary question "Would grapevine AOS be able to increase plant ability to response against pathogen when overexpress in Arabidopsis?" In order to answer this question we established a homozygous transgenic 35S:VvAOS and transgenic 35S:AtAOS (as a comparison control) plant population as source material for these studies. A constitutive CaMV 35S promoter was used as gene regulator due to its ability to regulate gene expression independently and continuously which, therefore, provided the appropriate amounts of additional AOS enzymes continuously in the Arabidopsis JA biosynthesis pathway. However, research findings showed that overexpression of grapevine AOS did not significantly increase the transgenic ability of Arabidopsis transgenic as a response to pathogen attack or mechanical wounding. However further analysis of the results indicated that the unexpected result may have been influenced by other factors, such as:

1. Plant gene silencing mechanisms in transgenic Arabidopsis was triggered early due to the presence of T-DNA, CaMV 35S promoter and insertion of transgene AOS, which caused weak signal transduction to induce JA biosynthesis.
2. Sudden increased AOS transcript abundance was more critical to regulate JA biosynthesis pathway than their availability. Therefore, employing an inducible promoter to regulate transgene in Arabidopsis transgenic plant will be more suitable than a constitutive promoter.
3. Quantitative assessment for transgene performance to regulate JA biosynthesis was less relevant due to the low affinity of the VvAOS enzyme toward substrate availability in the Arabidopsis system.
4. Un-optimized Arabidopsis transgenic growth conditions, such as temperature, humidity and light intensity due to the brand new plant growth room facility.
5. Plant crosstalk between Arabidopsis transgenic plants with other plant species that were undergoing different experimental approaches were grown in the same closed containment plant room.

Chapter 5

Identification of natural genetic variation of allene oxide synthase in 100 individual grapevines

5.1 Introduction

Traditionally disease control is achieved through application of chemical agents to control infection. Alternate strategies utilizing genetic improvement via hybridization/breeding are limited in grapevine due to industry concerns. Current advances in functional genomics and identification of the genetic basis for disease resistance open up a number of industry opportunities. Natural variation of plant pathogen resistance appear to be quantitative and often related to the biosynthesis of a signalling compound, jasmonates (JAs) (Pajerowska-Mukhtar et al., 2008). JAs are responsible for signalling the production of phytoalexins, a wide group of compounds that are responsible for a wide range of plant defence mechanisms (Yamada et al., 1993; Zhou et al., 1999; De Geyter et al., 2012). Allene oxide synthase (AOS) plays a central role in JAs biosynthesis as this enzyme catalyses the first reaction in the branch pathway leading to JA production. Evidence exists that genetic variation of AOS in *Solanum tuberosum* able to contribute to increased resistance to pathogen attack in plants containing these variation (Pajerowska-Mukhtar et al., 2008; Pajerowska-Mukhtar et al., 2009). As often such genetic variations do not produce visible changes in morphology, somatic mutations that alter AOS function may never be recognized especially in commercial crops such as grapevine populations.

5.2 Genetic variation of allene oxide synthase

Study by Pajerowska et al. (2008) on potato (*Solanum tuberosum* L.), the function of a endogenous allene oxide synthase 2 (StAOS2) allelic variation was investigated through a complementation approach in the same Arabidopsis *aos* mutant (Park et al., 2002) that we have utilised in our study as complementation AOS function result describe in chapter 3. Their study showed that StAOS2 variations have varying degrees of phenotype complementation as well as differing effects on JA and OPDA levels when each allele was individually expressed in Arabidopsis *aos* mutant. To correlate the differential JA and OPDA expression to the levels of pathogen resistance, they infected Arabidopsis StAOS2 complemented line with pathogen. The results of this experiment show that StAOS2 sequence variation in potato resulted in plants expressing different levels of JA and consequently have differing levels of resistance to key necrotrophic pathogens. This study suggests that genetic variation within AOS and varying levels of JA, play a key role in disease resistance in plants. Based on this and other reports indicating the significance of variation within AOS gene in disease

responses, we decided to investigate the levels of genetic variation that exist in AOS in a small population of field grown vines.

5.2.1 Significant genetic variation of AOS exist in field planted grapevine

In commercially grown wine grapes, varieties and clones are developed almost exclusively through somatic mutations derived from clonally propagated plant material which represent the main source of genetic variation used to improve the quality of fruit and viticulture (Carmona et al., 2008). However, the levels of genetic variation and most importantly the rate of mutation is unknown in the field due to; first, they are clonally propagated so the level should be low to none and second, studies have shown that between clones there is substantial sequence and structural differences. Nevertheless little is known about the levels of genetic variation between individual plants in a vineyard. Mutations occurring in cells of the shoot apical meristem tissue as well as transposable elements represent an important source of somaclonal variation in most plant include grape vine (Larkin and Scowcroft, 1981; Lizamore, 2013). However in grapevine, typically only those mutations which produce significant changes in growth and development, or morphological changes such as different pigmentation, alternations to leaf morphology are identified. Mutations that result in non-visible changes such as response to disease are often never recognized and many somatic mutations are presumably lost during pruning on a seasonal basis. Therefore, selection for useful somaclonal variants within elite grape vine cultivars for important viticulture traits such as disease resistance is relatively inefficient. In order to select useful somatic mutation in the field, more effective method needed to identify this valuable variation within existing grapevine population in the commercial vineyard. Previous work by Pajerowska et al. (2008) indicate that molecular genetic can be utilized as a tool to identified useful gene mutation via genetic variation diversity occur within key genes that may lead to the improvement of desirable phenotype. Information gather from the genetic variation can be subsequently used to develop efficient screening tools to identify useful gene mutation presence in the individual grapevine. In this experiment, we interested in the individual grapevine that contain useful AOS gene variation. AOS gene known to have the capability to increase plant resistance to stress, pathogen infection, or wounding (Beale and Ward, 1998; Chung et al., 2008; Kazan and Manners, 2008). Previous report also indicate that specific genetic variations of the AOS gene, has been shown to result in varying in sensitivity to pathogen infection (Pajerowska-Mukhtar et al., 2008). In the previous chapter, we successfully identified and clone AOS from grapevine that can be used as a VvAOS nucleotide sequences reference.

Initial work by Podolyan (Podolyan, 2010) on six randomly selected clone of *Sauvignon blanc* revealed a number of putative single nucleotide polymorphisms (SNP's) found within coding sequence of VvLOXA genes. This interesting finding leads us to question whether the AOS gene contains a diversity of SNP's between individual plants. If it does, can these gene variations lead to an

improvement of grapevine's response to stress or pathogen infection? The AOS enzyme catalyst the first reaction in the production of JA (Kubigsteltig et al., 1999). Natural variation of potato AOS2 causes varying levels of JA and pathogen resistance in complemented Arabidopsis *aos* mutant (Pajerowska-Mukhtar et al., 2008) clearly indicates its role in plant pathogen response. Therefore, the open question is "Does AOS gene variation and its function in grapevine effect the plant response to stresses such as wounding and pathogen attack?" will be interesting area to be address. Therefore in this chapter, based on the initial finding by Podolyan (2010), the VvLOX enzyme located upstream of the JA biosynthesis pathway contains several SNP's, the research objective is to determine the range of VvAOS gene variation in a small *Sauvignon blanc* population.

5.3 Materials and Methods

5.3.1 Plant material

Leaf apical material was collected from 100 individual of *Vitis vinifera* L. cv *Sauvignon blanc* vines located on the 786 row of Booker vineyard (Brancott Estate, Malborough, New Zealand), southern part of Wairau Valley near Blenheim. Samples were keep in the ice-dry during transportation to Lincoln university laboratory. Upon arriving, samples were swiftly transferred to minus 80^{o}C freezer prior to use.

5.3.2 Plant DNA extraction

Leaf apical tissues from 100 individual *Vitis vinifera* L. cv *Sauvignon blanc* vines along a single row was used as the source of grapevine genomic DNA (gDNA) for PCR amplification. Tissue material was snap frozen in liquid N_2 and ground to fine powder for gDNA extraction. Apical leaf tissues samples were grounded to fine powder in the present of liquid N_2 with cold mortar and pestle. Approximately 100 mg of grounded samples as a starting for the genomic extraction. Genomic extraction were carried out following to the methodology describe from the previous section 2.2.3. Each sample than was aliquot at final concentration 10 ng/µL.

5.3.3 A Targeting-Induced Local Lesion IN Genomes (TILLING)

Grapevine AOS (VvAOS) was amplified using VvAOS (Fwd and Rev) primers as shown in table 2.1. Genomic DNA of the 100 individual grapevine were pooled into 10 group where each group was a pooled of 10 individual gDNA. Genomic samples were group a as follow: A (1-10), B(11-20), C (21-30), D (31-40), E (41-50), F (51-60), G (61-70), H (71-80), I (81-90) and J (91-100). Each genomic pooled was a mixture of 10 ng/µL (approximately 1 µL from the genomic solution) from each of the ten individual gDNA. As for VvAOS gene amplification, approximately 50 ng (or 5 µL) of plant genomic mixture from the each genomic pool were used as a template for PCR reaction. PCR amplification was followed to the method describe in section 2.2.4. Ex-Taq DNA polymerase from Takara Inc (Norrie Biotech, NZ) was used for the amplification. In order to separate VvAOS gene fragment from any trace of DNA, PCR product was separated through electrophoreses using a 1% agarose gel as describe in section 2.2.4. Expected sized of DNA fragment, 1.5 kb were excised and gel purify as describe in 2.2.4. Each VvAOS DNA fragments from the genomic pool was quantified using a Qubit fluorometer (Life Technologies, NZ) according to instructions supplied by the manufacturer

Approximately 600 ng of VvAOS amplicons were used for heteroduplex formation and endonucleases digestion. Heteroduplex formation was achieved as follows: denaturation - 98°C for 2 min, Annealing – 95 to 85 °C (-2°C/s) and 85 to 25°C (-0.1 °C/s) and hold at 4°C. Heteroduplex of VvAOS amplicons were treated with CEL I nuclease from Surveyor mutation detection kit (Transgenomic, Inc).

Heteroduplex amplicons digestion mixture was followed to the standard protocol provided by the manufacturer. Digested DNA amplicons were separated into 2.5% Agarose gel. Separation of digested VvAOS amplicon was visualized by UV excitation of ethidium bromide on a BioRad GelDoc apparatus (Bio Rad Laboratories Pty Ltd, NZ).

5.3.4 Direct Sanger sequencing of VvAOS amplicons

Direct Sanger sequencing of AOS amplicons was carried out using Lincoln University sequencing facility. For Sanger sequencing, 100 individual grapevine genomic were pooled into 20 groups of 5 individual genomic DNA samples. Genomic samples were group a as follow: : A (1-5), B (6-10), C (11-15), D (16-20), E (21-25), F (26-30), G (31-35), H (36-40), I (41-45), J (46-50), K (51-55), L (56-60), M (61-65), N (66-70), O (71-75), P (76-80), Q (81—85), R (86-90), S (91-95) and T (96-100). VvAOS gene was amplified and amplicons were purified as describe in the section 5.3.3. Purified VvAOS amplicons were sequence using three primers combination as given as follow: VvAOS Fwd (Forward primer): 5'-ATGGCGTCCCCTTCTCTAAC-3', IntVvAOS Fwd (Internal Forward primer): 5'-ACCCGGCCCTTTCATCTCCTC-3' and VvAOS Rev (Reverse primer): 5'-TCAAAAACTGGCTCGCTTTA-3'. VvAOS gene amplicon was sent for sequencing to the Lincoln University sequencing facility and sequencing results were analysed with Lasergene SeqMan Molecular Biology software by DNASTAR Inc (DNASTAR inc, Madison, USA). Position and direction of each primer used for sequencing are shown on the figure 5.1

Figure 5.1 Location and direction of primers used for VvAOS gene sequence
Three set of primers were used to obtained complete coverage of VvAOS full sequence.

5.4 Result and Discussion

5.4.1 Genetic variation of VvAOS gene in 100 individual grapevine

As reported by Pajerowska-Mukhtar et al. (2008), StAOS2 variation causes varying levels of JA and range of pathogen response in complemented Arabidopsis *aos* mutant. Based on this report we speculate that single nucleotide polymorphisms (SNP) occur within VvAOS gene sequence could be an important variation that can confer grapevine to less susceptible to pathogen attack. However in order to study this SNPs, diversity of VvAOS gene variation within grapevine population need to be estimated. In this experiment, we investigate the diversity of VvAOS gene variation from 100 individual *Sauvignon blanc* planted within 786 row at Booker vineyard (Brancott Estate, Marlborough, NZ). To characterize any variation present, we employ TiLLING and Direct Sanger Sequencing approaches.

5.4.2 Targeting Induced Local Lesion in Genomes (TiLLING) for AOS variation

Targeting-induced Local Lesion in Genomes or TiLLING technique is describe as general reverse genetic strategy that used to locate a series of induce point mutation in the gene of interest and was first reported by Claire McCallum and colleagues at the Fred Hutchinson Cancer Research Centre in Seattle in 1990's (McCallum et al., 2000). In this assay, TiLLING approach was used to detect single point mutation in heteroduplex VvAOS gene amplicons by harnessing the capability of CEL-I endonuclease that able to recognize and digest single nucleotide base pair mismatch. Genomic of 100 individual *Sauvignon blanc* were organized into 10 groups and VvAOS gene were amplified and DNA amplicons were digested as describe in the material and method (Section 5.3.3). Result of VvAOS amplicon digested by CEL-I shown on the figure 5.2. Digested heteroduplex VvAOS amplicons show an identical pattern in all group which indicate that there was a little if any difference between any of the 10 pools of amplicon obtained. Multiple bands shown on the gel electrophoresis indicate a mismatch present at a single position but similar band size appeared across in all 10 group suggest that this particular SNP present at a similar position within VvAOS amplicon signify heterozygosity of VvAOS allele in grapevine genome. Also on some amplicons groups such as B, F, H I and J show intense band at the top of the gel corresponds to the intact VvAOS (1.5 kb) indicate that most of the amplicons did not digested by CEl-I endonuclease which might suggest that VvAOS amplicon did not carry high diversity of mismatch or variation within their sequence. Generally, TiLLING approach did not successfully detect any significant DNA variation present within VvAOS amplicons within the population of 100 individual *Sauvignon blanc.* Therefore to investigate this, we employed a more sensitive approach to detect the presence of VvAOS SNPs i.e. direct Sanger sequencing method.

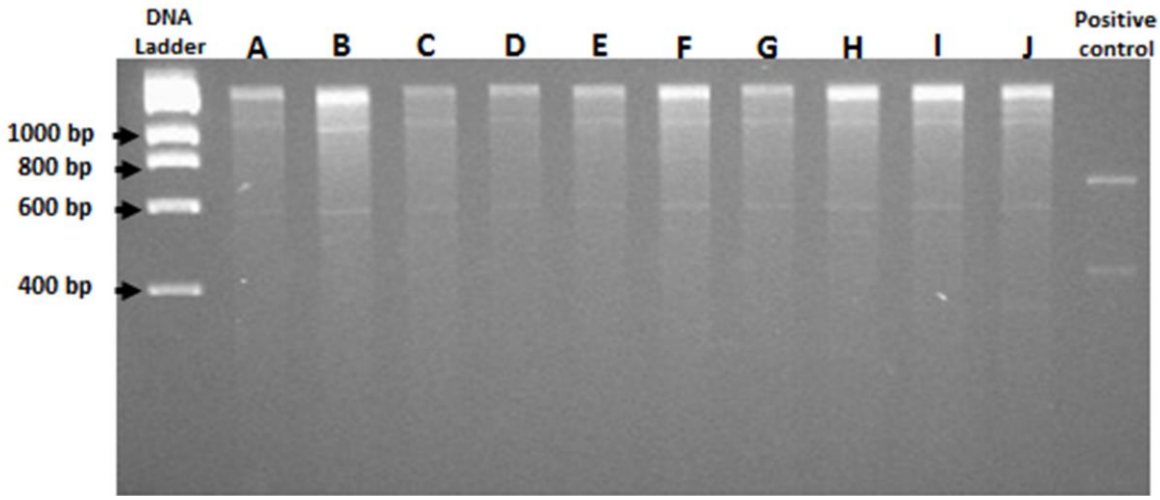

Figure 5.2 Restriction enzymes digestion of heteroduplex VvAOS gene amplicon using CEL-I
Genomic DNA (gDNA) of 100 individual grapevine were pooled into 10 group (each pool comprise 10 individual gDNA). Grapevine samples were given numbers label from 1 to 100 in apprehend samples that comprise unique SNP's. Pool of gDNA were labelled as follow: A (1-10), B(11-20), C (21-30), D (31-40), E (41-50), F (51-60), G (61-70), H (71-80), I (81-90) and J (91-100). Positive samples was provided by manufacturer as a control for endonuclease activity to be able to digest single nucleotide mutation within the heteroduplex of DNA amplicons. DNA ladder used was HyperLadder I (1 kb) from Bioline (Totallab, NZ) to extimate DNA fragments size after CEL-I enzymes digestion.

5.4.3 Direct Sanger sequencing

Direct Sanger derived sequence is more sensitive method used to detect SNP's within VvAOS gene sequence. In order to increase the SNP's detection, 100 individual genomic DNA of grapevine were pooled into 20 groups instead to 10 groups compare to TiLLING method as describe in section 5.3.3. VvAOS amplicons from each genomic DNA pool were purified and sequence using primers mention and methodology as describe in material and method section. Location and direction for each sequencing primer is illustrated on the figure 5.1. Result shows that DNA sequences obtained from the 20 samples shows only three sequencing variations i.e. 1) in pool C - location 553, 2) pool B - location 1489 and 3) Pool A, C and M – location 1504 as illustrated on the figure 5.3. Initial result direct Sanger sequencing show that at location 553 bp, variation indicate a base pair substitution from G to A. Whereas, at the locations of 1489 and 1504 bp indicate an insertion and deletion respectively. However, further interrogation of the raw sequence data indicate that only 1 of these variation represent a clear demonstrable SNPs i.e. at the location 553. Closer examination of the sequence showed that due to the even peak height of the two bases, A and G, at this position clearly shows a heterozygote allele substitution and furthermore all plant contained the exact same set of sequences (Figure 5.4). This result also confirmed our previous finding via TiLLING method demonstrated by two DNA fragment (approximately 1 and 0.5 kb in size) appeared on the agarose gel (Figure 5.2). Therefore, this is a strong indication of VvAOS gene exist as a heterozygous allele within grapevine.

Figure 5.3 Location of genetic variation occur within VvAOS gene sequence
Direct Sanger sequencing result shows three potential SNP's detected at the location 1) 533, 2) 1489 and 3) 1504. Blue arrows 1, 2 and 3 indicate an approximation location of each SNPs.

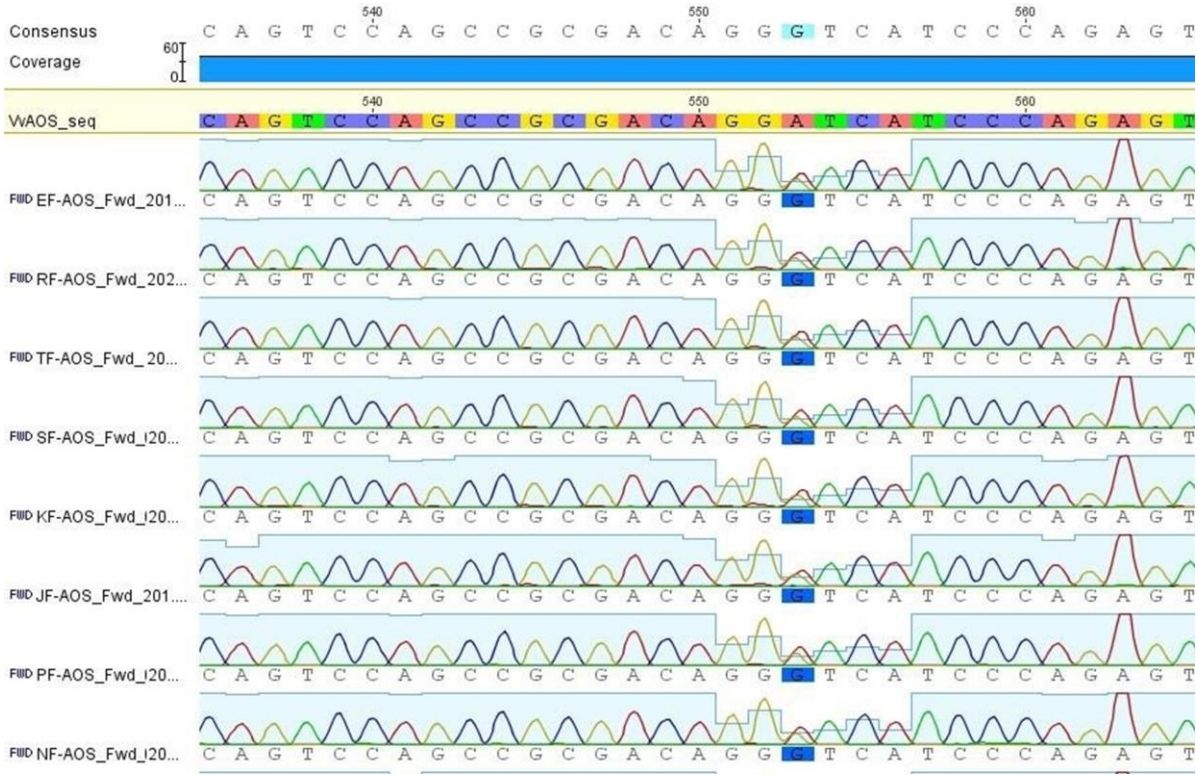

Figure 5.4 Grapevine AOS genes sequencing excerpt from assembly contiq.
Excerpt from the contig assembly of VvAOS Sanger-sequencing run as produced by Geneious Version 7 (Biomatters, NZ). Bases in blue in individual sequences highlight at the presence of an A and A G occurring at equal amounts in each pooled DNA sample, indicating the presence of 2 allele of AOS

5.5 Discussion

In this chapter, our assessment is based on the two previous reports. First, reported by Pajerowska-Mukhtar et al. (2008) indicate that variation occur within AOS2 in *Solanum tuberosum* exhibit different level of JA and range of pathogen response in complemented Arabidopsis *aos* mutant. Based on this report, we interested to investigate the possibility of VvAOS variation that caused grapevine less susceptible to pathogen attack. Second, previous report by Andriy Podolyan (2010) as part of his PhD thesis indicate that there was a significant number of SNP present within Lipoxygenase A (LOXA) coding sequence in 10 individual of grapevine. Therefore, this interest us to investigate whether VvAOS gene also contain a similar diversity of SNPs. High diversity of VvAOS gene variation among small population of grapevine is a vital feature to develop a tool or method to screen an important gene variation hidden within large population of vine in commercial vineyard. In this assessment, we start with population of 100 individual grapevine from the commercial vineyard. It is a small population but 10 times larger than the population reported by Andry Podolyan (2010). First we screen the 100 individual grapevine for VvAOS gene diversity via TiLLING approach. The TiLLING result clearly indicate that gene variation of VvAOS within the population of 100 grapevine is very low and at this point result is inconclusive. SNP's detected is represent the heterozygosity of VvAOS allele in grapevine genome and not a true variation. However, there are a few issues that make TiLLING method not suitable to detect gene variation in VvAOS gene amplicons. First, resolution of 2.5% agarose gel is not suitable for viewing smaller DNA fragments. VvAOS amplicons only 1.5kb in sized, if SNP located closed to each other or at the end of the fragment (either end's -5' or 3'), it will be difficult to view the DNA fragment on the 2.5% of the gel agarose. Excitation signal by ethidium bromide is difficult to detect in small and low quantities DNA fragments whereas large DNA fragments will be closed to original sized of VvAOS, therefore again it difficult to aim and conclude the variation result based on DNA size different. Second, CEL-I endonuclease base pair mismatch substrate preference is C/C ≥ C/A ~ C/T ≥ G/G > A/C ~ A/A ~ T/C > T/G ~ G/T ~ G/A ~ A/G ≥ T/T (Oleykowski et al., 1998). Substrate preference could be the factors to determine endonuclease digestion efficiency among base pair mismatch present in the heteroduplex amplicons. Third, false positive or false negative due to the endonuclease enzyme digestion reaction efficiency or human error. Previous report also indicate that approximately 5% and 4% report give false negative and false positive respectively in TiLLING or EcoTiLLING methods (Barkley and Wang, 2008). Taken all together the disadvantage of using TiLLING method as discussed above, it is clear that more sensitive method needed to measure the approximate frequency of SNP's occur within VvAOS gene. Therefore, Direct Sanger Sequencing a more sensitive method were used as an alternative method to detect SNP's within a pool of VvAOS amplicons.

In order to increase the possibility of SNPs detection within VvAOS gene among 100 individual grapevine, we employed a more sensitive i.e. direct Sanger sequencing method. Via this method, we only manage to identify one credible SNP located at 533 bp from the 5' end which indicate the

heterozygosity of VvAOS allele in grapevine genome. However, this result confirm our previous result from the TiLLING method. Low variation of VvAOS gene among 100 individual grapevine quite surprising considering high variation of VvLOXA, an upstream enzymes involved in JA biosynthesis pathway but understandable. It is understandable because from our previous discussion (discussion section in chapter 4) indicate that a single amino acid substitution occur especially within active site of AOS protein sequence can change an entire function of AOS enzymes (Toporkova et al., 2008). Therefore it is understandable that VvAOS gene sequence within grapevine genome a highly conserved to preserve it originality and function efficiency.

However the diversity difference between VvLOXA and VvAOS could be due to the several factors. First, due to the size of respective gene sequence i.e VvLOXA is almost twice larger at cDNA level and four time larger at the genomic level compare to VvAOS gene size. Second, is due to the nature of LOX gene function redundancy (Vellosillo et al., 2007) expose to the gene duplication (Cooke et al., 1997; Zhang, 2012). Third, is due to the critical function of VvAOS in JA biosynthesis regulation which an important component in plant development and regulation (Laudert et al., 2000). During plant development JA mediated the following processed; male and female organ development, embryo development, sex determination, seed germination, seedling development, root growth, gravitropism, trichome formation, tuber formation, leaf movement and leaf senescence. He and Zhang (2006) hypothesized that less important gene have higher rates of successful duplication where important gene is measured by the fitness reduction caused by the deletion of the gene. One of the character of important gene drawn was genetic stability, particularly the stability of central cellular and development processes which may be essential for the survival of organisms (He and Zhang, 2006). Duplication of important gene could cause genetic perturbation by doubling gene dosage, is therefore one expect that important gene tend to have reduce duplicability. Therefore, VvAOS gene sequence is highly conserved in grapevine. However, although we haven't seen significant sequence variation within the grapevine AOS locus, this does not mean that there isn't genetic diversity within the population. Result analysis using Next Generation Sequencing methods from Pinot noir clones show that there are surprisingly high level of genetic diversity (Chris Winefield, Pers. Comms.). However this diversity appears to mostly be associated with transposable elements (TEs) rather than SNPs (This et al., 2006; Carrier et al., 2012). Given the apparently high levels of both sequence and structural diversity among clones, it is reasonable to assume that there is an unknown yet possibly high rate of somatic mutation occurring in field grown grapevines (from which these clones have been identified and isolated). However what is currently unclear is the rates of mutation. Consequently while there is a low level of divergence in the small population we have screened there may still be important variation accumulating in the field populations. Considering the expenses and time consume during screening, it is unlikely that using this approach will prove efficient or economically practical.

5.6 Conclusion and future prospect

In the previous chapter (Chapter 4), we manage to identify and characterized AOS gene function in grapevine. One of our primary question is "What is the estimation of grapevine AOS genetic variation diversity within the population of grapevine grown in the commercial vineyard?" By estimating grapevine AOS variation diversity, we will be able to estimate number of individual and cost involved to identify high quality of variation that confer grapevine less susceptible to pathogen attack. In this experiment we use 100 individual of grapevine as a population samples. From this analysis, it is clear that there is no detectable genetic variation present within 100 individual *Sauvignon blanc* vines.

Chapter 6

Conclusion and future prospects

6.1 Research project summary

We started this research project with a simple question, “How to identify a grapevine variety less susceptible to disease infection but maintain it wine quality?” If we can address this question, we will be able to reduce our dependent usage to chemical agent to control disease problem. In order to address this question, we look to a several potential solutions including genetic improvement or genetic modification (GM). However due to industry and public concerns, genetic improvement in grapevine has been limited only to identification and utilisation of soma clonal mutants whereas the use of GM grapevines is not an option due to strict New Zealand government policy. Therefore, we shifted our focus to study the natural somatic genetic variation in particular genetic loci in grapevine that may contribute to increased tolerance to pathogen infection. In this research project, we identified that the plant cytochrome CYP74 enzyme family are a potential target due to their special role in the metabolism of hydroperoxides and oxylipin which is one of the main defence mechanisms employed by plants. Among CYP74 enzymes members, we interrogated the natural variation occurring at the allene oxide synthase (AOS) allele as a key focal point due to its role as the first committed gene in the jasmonic acid biosynthetic pathway. Based on a previous report by Pajerowska-Mukhtar (2008) which indicated that sequence variation within AOS gene in *Solanum tuberosum* apparently leads to differing accumulation of JA in potato. Jasmonic acid and its derivatives are endogenous signalling compounds that are heavily involved in regulating plant defence mechanisms. Until up to this thesis written, alternative biosynthetic pathway lead to the production of JA has not been reported in plant other than the one with AOS branch pathway. Throughout this research project we concentrated on the identification and partial characterisation of the CYP74 gene family members in grapevine, with particular focus on the characterization and elucidation of VvAOS gene function. Allene oxide synthase represents a potential target for the improvement of grapevine tolerance to disease, particularly necrotrophic fungi such as Botrytis. In order to determine the possibility of utilising naturally occurring genetic variation at the AOS locus we determined the naturally occurring genetic variation within a small population of field grown vines.

6.2 Characterization of grapevine CYP74 gene family member

As part of our research objectives, we identified and partially characterised grapevine AOS alongside six HPLs gene which comprise the entire grapevine CYP74 family. Phylogenetic analysis confirmed that the putative grapevine AOS gene (VvAOS) belongs to the CYP74A family uses 13-

hydroperoxide as a substrate (13-AOS group) and is proposed to be physically associated with the chloroplastic membrane. Investigation of the transcript abundance of VvAOS showed consistent levels of transcript across all tissues types tested i.e. leaf, tendril, root, inflorescence, skin, pulp and seed indicating that this gene may play a vital role in different parts and stages of grapevine growth and development. This is not surprising due to the critical role that AOS enzymes play in JA biosynthesis and that JA plays such an important role across a diverse range of plant functions (Wasternack et al., 2013; Wasternack and Hause, 2013). The six grapevine HPLs (VvHPLs) genes can be divided into 2 groups. Grapevine HPLA belongs to the CYP74B family which uses 13-hydroperoxides as a substrate (13-HPL group). However while possessing a putative chloroplast localisation peptide at the N-terminus of the predicted peptide we found that a VvHPLA:GFP fusion peptide did not localise to the chloroplast in transient expression experiments in tobacco leaves (Figure 2.8). Interestingly, VvHPLA expression was found to be consistent across in all tissues types tested which indicate that it may have a ubiquitous role in grapevine. However given the unique localisation pattern suggests its mode of action may differ from other species in which 13-HPLs have been characterised. 13-Hydroperoxide lyases are known to catalyse the production of C_6 and C_{12} aldehydes which collectively are important compounds that are both involved in flavour/aroma production as well as in plant defence (Taurino et al., 2013). Five other VvHPLs (VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF) were predicted to belong to the CYP74C gene family which have been reported to use either, or both, 9- and 13-hydroperoxides as a substrates and form the 9/13-HPL group. Grapevine HPLs belonging to the CYP74C family show variation in their sub-cellular localization and are found either within the cytoplasm or associated with cell membrane. The transcription patterns of this group was found to be quite diverse both spatially and in quantity. The range of 9/13 HPLs present in grapevine raises a number of questions given the breadth of subcellular localisations and gene expression patterns. With the ability of these enzymes to potentially utilise either 9 or 13-hydroperoxides these enzymes may play a previously unreported role in both the formation of green leaf volatiles, and C9 aldehyde derived phytoalexins within grapevine. The presence of 9-lipoxygenases (9-LOXs) strongly suggests that under certain conditions and developmental stages grapevine is capable of the production of C9 aldehydes (Podolyan, 2010). However the duality of catalytic behaviour of the 9/13-HPLs also implicates these enzymes in a range of potential roles for further production of 13-hydroperoxides under conditions other than those that have been previously reported (Zhu et al., 2012). Consequently to fully understand the involvement of the cyp74 family in aldehyde and phytoalexin formation will require further in depth analysis of both their individual biochemistry and cellular functions.

6.3 Characterization of allene oxide synthase in grapevine

Due to our interest in jasmonic acid(s) as a vital component in mediating plant defence responses, we focused our attention on VvAOS gene as a potential biosynthetic bottle neck that could

regulate biochemical flux to JA formation. However, to date, in depth characterisation of AOS in grapevine has not been carried out. Therefore, as part of our research objectives, we first identified and isolated the sole putative grapevine AOS orthologue predicted in the grapevine reference genome sequence, using the previously characterized Arabidopsis AOS gene sequence in Arabidopsis to assist in an indepth interrogation of the annotated genome seuqence. The putative grapevine AOS identified and cloned from Sauvignon blanc was proven to be a functional AOS via complementation of the Arabidopsis AOS mutant.

6.4 Over expression of grapevine allene oxide synthase in Arabidopsis wild type

Increasing levels of JA has been shown to contribute to increased tolerance of plants to necrotrophic pathogens such as Botrytis (Rowe et al., 2010). Increasing biochemical flux into the JA biosynthetic pathway via alteration of AOS levels has been shown to alter both JA levels and JA responsiveness in downstream pathways (Park et al., 2002; Pajerowska-Mukhtar et al., 2008). As we were unable to directly alter levels of AOS in grapevine we decided to investigate the impact of altering levels of AOS in Arabidopsis on JA production and alterations in expression patterns of JA responsive genes by ectopically expressing both the Arabidopsis AOS and the grapevine AOS gene. Having shown that the Introduction of putative AOS gene isolated from grapevine recovered the male-sterile and JA signalling transduction pathway phenotypes of the *aos* mutant we confirmed the it functionality as an AOS gene in grapevine

As our findings suggest that overexpression of VvAOS gene in Arabidopsis did not confer significantly increased resistance to disease infection such as Botrytis, we provide possible explanation to comprehend these issues. First, while there was high abundance of transgenic VvAOS transcript in OE-lines, these levels of expression did not confer increased responsiveness in plant defence mechanisms. This suggests that other downstream enzymes such as AOC or OPR3 within JA biosynthesis pathway are equally important in regulating JA production. Therefore influx of biochemical substrate alone by increasing AOS enzymes activity is not necessarily the most effective way to increase JA production and as a consequence did not confer increased resistance to pathogen infection. Second, our analysis of the grape AOS sequence indicated the presence of substitution of amino acid residues located within important motifs, namely helix-I and the heme-binding binding site within active site. Previous reports (Toporkova et al., 2008; Toporkova et al., 2013) indicate that amino acid substitutions that occur within this highly preserved motif can alter the function of AOS, such as substrate affinities and other kinetic properties (Pajerowska-Mukhtar et al., 2008; Toporkova et al., 2008; Toporkova et al., 2013). Two amino acid substitutions at the helix-I motif and one in heme-binding site within VvAOS protein sequence (relative to AtAOS protein sequence) could possibly alter the enzymes kinetic properties toward substrates present in Arabidopsis and therefore differing

activities compared to the endogenous Arabidopsis enzyme. Third, combination of early initiation of gene silencing and JA induction mechanisms. Regulation of JA production is induced by the sudden increase of AOS transcript rather than their availability (Mei et al., 2006). Employing CaMV 35S promoter as a regulator to VvAOS gene in Arabidopsis transgenic plant provide a high basal of overall AOS transcript abundance but prevent from creating high magnitude of sudden increase of AOS transcript in transgenic plant where high basal level of AOS transcript caused an early activation of gene silencing mechanisms that also initiate an early repression to AOS transcription. Therefore as a consequence, Arabidopsis transgenic plant fail to produce strong signal to upregulate JA production.

6.5 Identification of natural genetic variation of allene oxide synthase in small population of grapevine

With tentative evidence that alterations in AOS activity may impact positively levels of JA production and responsiveness to JA we wanted to explore the potential levels in sequence divergence is grapevine AOS. In this assessment, we focused on exploring levels of grapevine AOS sequence variation within 100 individual grapevine collected from commercial vineyard. Our aim was to estimate VvAOS genetic variation diversity within the population of commercial grapevine grown in the field. By estimating levels of VvAOS variation, we would be able to estimate number of individuals required to be screened and the cost involved to identify suitable forms of variation in AOS that might confer grapevines that are less susceptible to pathogen attack. However, surprisingly in light of earlier data our results are clear indicate that genetic variation diversity of VvAOS gene present within 100 individual grapevine plants are very low. In light of the central role of this pathway to reproductive development and stress responsiveness this finding is not necessarily surprising. However in light of our own groups studies and reports of high levels of sequence divergence among clones of Pinot noir (Carrier et al., 2012), the finding is somewhat puzzling. Further reduced representation genome sequencing of individual vines will be required to provide a clear indication of the levels and rates of formation of both SNP and other structural variation accumulating in clonally propagated grapevines.

6.6 Future prospect

This project was initiated to investigate the possibility to develop suitable molecular tools to identify novel genetic variation that might confer disease resistance to individual grapevines. We chose grapevine AOS as a gene target due to its critical role in regulating the wound and pathogen signalling molecule, JA. Having proved that the putative AOS in grapevine encodes a functional AOS gene capable of complementing an AOS mutant in Arabidopsis, we showed that simply increasing levels of AOS transgenically was insufficient to significantly impact Arabidopsis' responses to Botrytis infection. These results raise a number of important questions, some of a technical nature and some biological. Technically it is clear that ectopic expression of AOS in Arabidopsis as model plant leads to a range of

potential issues, many typical of over-expression experiments, such as gene silencing due to the introduction of the T-DNA (Mlotshwa et al., 2010) or environmental issues. Production of Arabidopsis transgenic plant either via constitutive CaMV35S or endogenous AtAOS promoter as a gene regulator lead to unpredictable levels of gene expression. Although it's been reported that increase of biochemical influx into JA biosynthesis pathway could alter JA-responsive expression, our result suggest that increase influx of biochemical substrate alone by increasing enzymes activity via overexpression is not the only limitation factor for JA regulation. Arabidopsis transgenic plant that exhibited high level of VvAOS gene expression did not lead to increase JA level suggest that other point of regulation are also involved.

The other points of JA regulation that involved and could be an interesting area to be explored for future prospect are; first, phosphorylation/dephosphorylation (Krebs and Beavo, 1979) of AOS enzyme mechanisms to activate/deactivate protein activity as a response to high concentration of this enzymes available within the overexpressed Arabidopsis plant system. However, phosphorylation /dephosphorylation of AOS enzymes mechanisms as far as we know have never been reported yet. Therefore this mechanisms could be an interesting area to be explore. Second, the ability of Arabidopsis as a transgene host plant to recognized and control (upregulate or downregulate) transgene VvAOS transcription mechanisms. As we have been speculated previously (section 4.4), unlike endogenous AOS, Arabidopsis system might not be able to recognised transgene VvAOS transcription and overturned their mechanisms when required. Therefore, high transcript abundance of transgene VvAOS might triggered early plant defence mechanisms in order to repress their further transcription to avoid damage (Schubert et al., 2004; Dalakouras et al., 2011) or alter mRNA steady-state condition (Prelich, 2012). Further understanding on Arabidopsis as a transgene host plant model to perceive foreign gene will provide a deeper insight not only to elucidate VvAOS gene regulation mechanisms but also to understand how to assess other genes overexpression mechanisms in Arabidopsis at large. Third, the possibility of other enzymes located downstream within JA biosynthetic pathway, AOC and OPR3 are also equally important in determining JA regulation. The AOC enzymes catalyse a crucial step in JA biosynthetic pathway because only this enantiomeric form is the substrate for the naturally occurring (+)-7-*iso*-JA which lead to the formation of (+)-7-*iso*-JA-Ile, the most bioactive compound among jasmonate and it derivative's (Schaller et al., 2000; Stenzel et al., 2003; Staswick and Tiryaki, 2004; Fonseca et al., 2009; Stenzel et al., 2012). Furthermore, AOC also catalysed the production of first bioactive compound, OPDA within JA biosynthetic pathway (Schaller et al., 2008) and AOC gene itself is an OPDA responsive gene (Stenzel et al., 2003). Therefore, this suggest that AOC play an important role in regulating JA production. Whereas, OPDA alone is a bioactive compound that play an important role in plant defence mechanisms and also the precursor to the JA production (Stintzi et al., 2001; Scalschi et al., 2015). The first step in conversion of OPDA to JA is catalyse by OPR3 where this enzymes determine OPDA availability for JA biosynthesis (Scalschi et al., 2015). Since OPDA and JA

appear to be different signalling molecules (Stintzi et al., 2001; Scalschi et al., 2015), OPR3 also seem to play a key role in controlling the pool of OPDA and JA to respond to stresses (Díaz et al., 2012). Therefore, in order to effectively increase JA production, targeting transcription factors that co-ordinately regulate all three enzymes, namely AOS, AOC and OPR3 may be a better target instead of target each enzymes individually

Considering that AOS, AOC and OPR3 enzymes are equally important in JA regulation, identification of a simple SNP among these enzymes gene sequence might be insufficient to identify or regulate useful disease resistance material within grapevine. Therefore, instead of focusing on these three enzymes, mutations within transcription factor that co-ordinately regulate AOS, AOC and OPR3 might be more useful. According to Alves et al (2014), transcription factors (TF) a main regulators for gene expression at the transcription level. Alternation of TF activity will alter the transcriptome that leading to metabolic and phenotypic changes in plant response to stress (Alves et al., 2014). In plants, there six major family of TF in defence mechanisms i.e. basic leucine zipper containing domain proteins (bZIP), amino-acid sequence WRKYGQK (WRKY), myelocytomatosis related proteins (MYC), myeloblastosis related proteins (MYB), apetala2/ethylene-responsive element binding factors (AP2/EREBP) and no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), and cup-shaped cotyledon (CUC) (NAC) (Singh et al., 2002; Van Verk et al., 2009; Alves et al., 2014). All of these TFs family could be a potential target to be screen to identify useful disease resistance material in grapevine.

Besides SNPs, other potential mechanisms that can spontaneously upregulate gene expression is via the mobility of transposable elements (TEs). Transposable elements is a small DNA fragment that are competent to integrate into new position in the genome and known to produce a wide variety of change in plant gene expression and function (Lisch, 2013; Makarevitch et al., 2015). Several known TEs also tend to transpose into 5’ end of plant genes mean that the promoter and enhancer elements within their TEs potential alter gene expression (Lisch, 2013). Therefore, insertion of TEs either in AOS, AOC, OPR3 or TFs gene sequences might coordinate increase upregulation of their gene expression and eventually confer high resistance to disease. Currently, our group is specifically producing “TEs induced grapevine population”. Mutations (via TEs insertion) within TFs that coordinate increase upregulation of AOS, AOC and OPR3 might be present in this “TEs induced grapevine population” and this can be used as a tool to screen natural gene variation in the vineyard.

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Appendix A

Real-Time PCR primers used

A.1 qRT-PCR primers used to quantify transcript abundance in grapevine

Gene ID	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)	Accession	Location
VvHPLA-qPCR	CTGAAACGGAGTTCCAGCAT	GCTTGATGTAACTGGCGCTGT	112	XM_002272955	CDS region
VvHPLB-qPCR	TCCGGTGTGCTTTGTATGTT	AGATACATAGTAAATTCATGTTTCATCC	119	XM_002281123	3'-UTR region
VvHPLC-qPCR	CGGTGTGCTTTGTATGTTTT	TCGATGTAATAGTAAATTCATGTTT	119	XM_002281154	3'-UTR region
VvHPLD-qPCR	TTCAACATTGAGTGCGGAAC	TCGTGCCTTTCTACGATACGTG	115	XM_002281165	3'-UTR region
VvHPLE-qPCR	GGTTTAACATTTAAAGCTTATCATTCC	GGGCATTCTATTTCCTTTTTCAC	108	XM_002281177	3'-UTR region
VvHPLF-qPCR	AGAGAAGCTACACCGCGAAC	CATATACCACTGACTTGGTCAAACT	113	XM_002281190	CDS region
VvAOS-qPCR	CTTCGGAGGGATGAAAATCA	GGCTAATTGGGTGTGCAGTT	82	Our sequence	CDS region
VvActin-qPCR	CTTGCATCCCTCAGCACCTT	TCCTGTGGACAATGGATGGA	82	EC969944	CDS region
VvGAPDH-qPCR	TTCTCGTTGAGGGCTATTCCA	CCACAGACTTCATCGGTGACA	70	Multiple	CDS region

Table A.1 qRT-PCR primers used to quantify transcript abundance in grapevine.
qRT-PCR primers pair and gene target used in transcriptional profiling CYP74 gene family member in grapevine. Housekeeping gene VvActin and VvGAPDH were synthesize based on Reid et al. (2006) report.

A.2 qRT-PCR primers used to quantify transcript abundance in Arabidopsis

Gene ID	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)	Accession
AtAOS-qPCR	GATGGGAGCGATTGAGAAAATGG	CCTTCTTCGCTCTACCGTATTGA	101	AT5G42650
AtLOX2-qPCR	TCTTCCTCAGCGATGATAGCAC	ATGACGTAGCATCATAGCCTGG	117	AT3G45140
AtVSP2-qPCR	GTACTGGTTGTGGTTAGGGAC	AACTTCCAACGGTCACTGAG	120	AT5G24770
AtFBOX-qPCR	GGCTGAGAGGTTCGAGTGTT	GGCTGTTGCATGACTGAAGA	108	ATG515710
AtEF1α-qPCR	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	76	AT5G60390

Table A.2 qRT-PCR primers used to quantify transcript abundance in Arabidopsis
Shows qRT-PCR primers used in semi-quantification of target gene in complemented AOS gene function in Arabidopsis *aos* mutant. Housekeeping gene AtF-Box and AtEF1α were synthesize based on the primers reported in Lilly et al. (Lilly et al., 2011) and Czechowski et al. (Czechowski et al., 2005)

A.3 Amplification of a single product for each qRT-Primers used

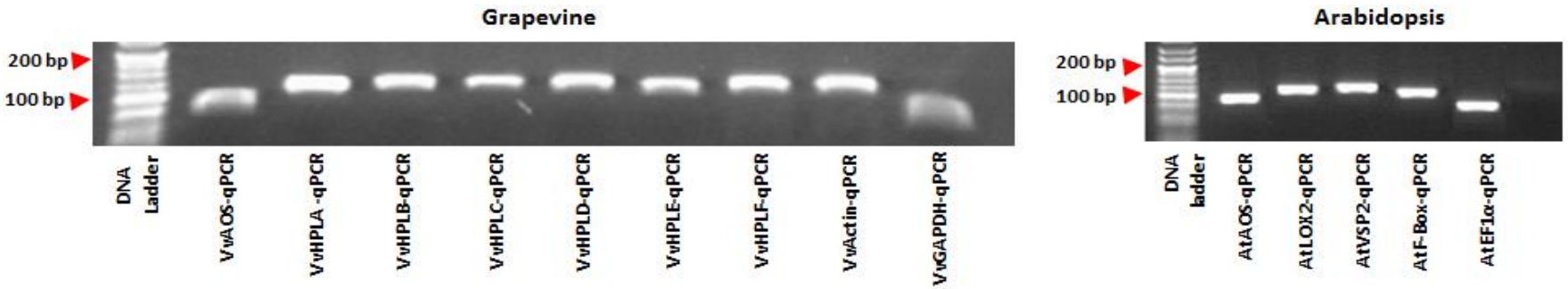
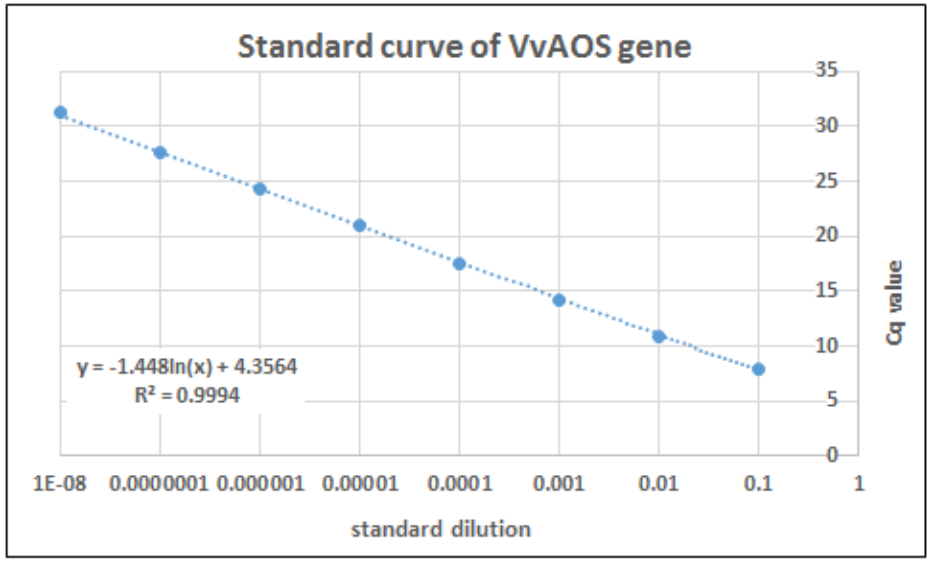
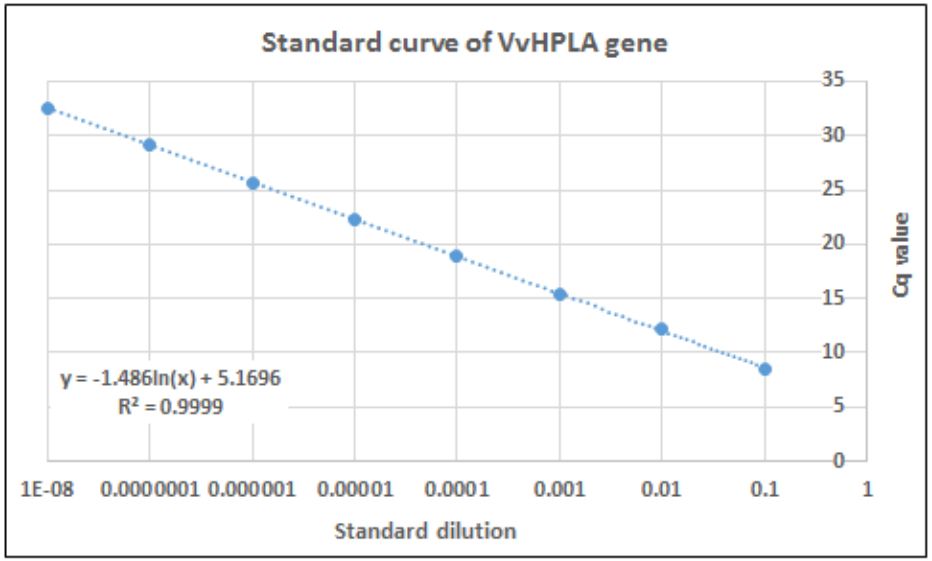
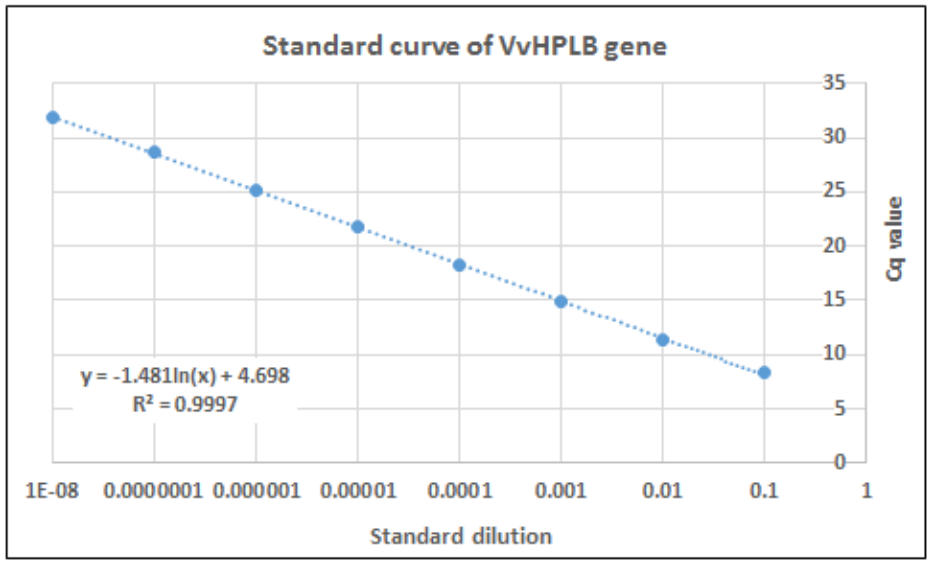


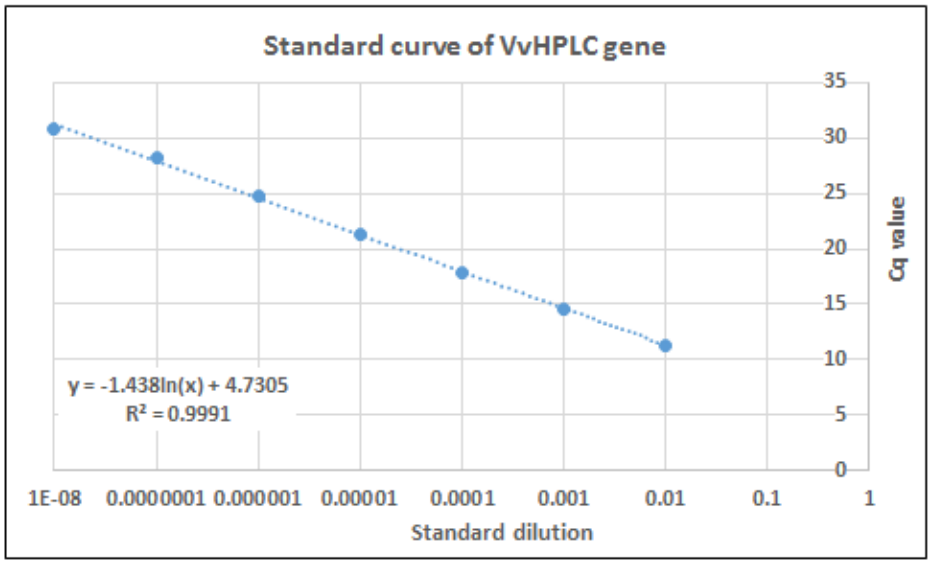
Figure A.1 Amplification of a single product for each qRT-Primers
Amplification of a single product for each qRT-Primers used Each qRT-PCR primers pair was verified for their specific target by producing a single amplicon product. As for PCR amplification template, a mixture of cDNA samples from leaf, whole berries and inflorescence were used in grapevine and cDNA from leaf in Arabidopsis qRT-PCR primers assay. The DNA ladder used was HyperLadder V (25bp ladder) from BioLine (Total Lab System, NZ) to measure DNA amplicons size.

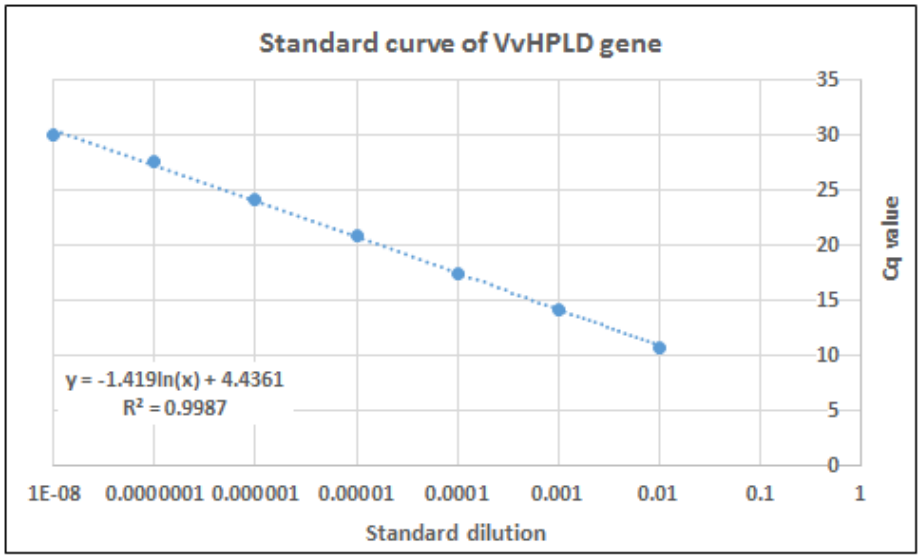
A.4 Standard curve for all qRT-PCR primers pair used in this research project

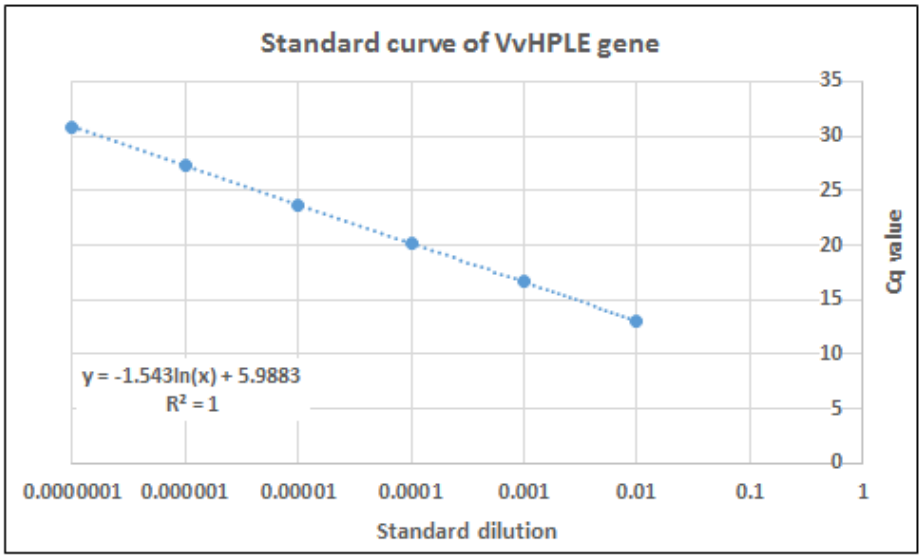









Standard curve of VvHPLD gene
y = -1.419ln(x) + 4.4361
R² = 0.9987
Cq value
Standard dilution
1E-08
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
35
30
25
20
15
10
5
0


Standard curve of VvHPLE gene
y = -1.543ln(x) + 5.9883
R² = 1
Cq value
Standard dilution
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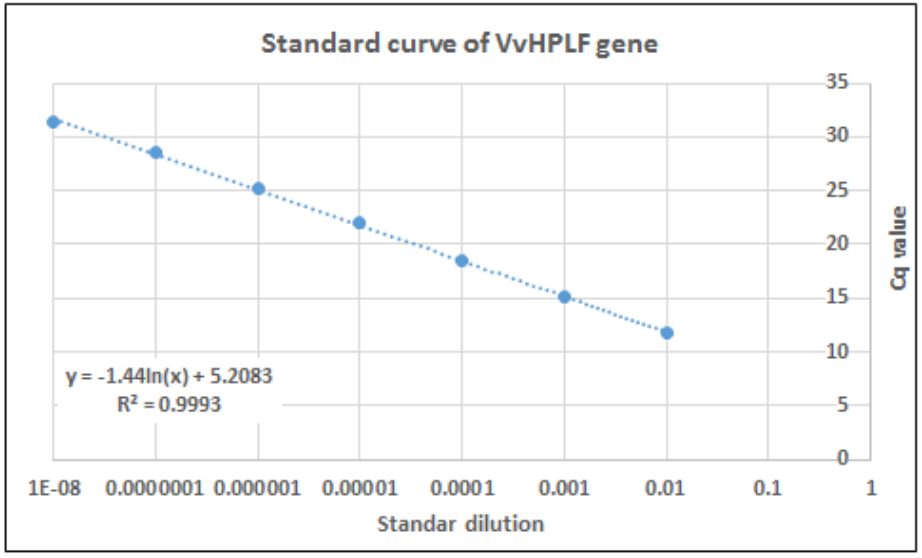
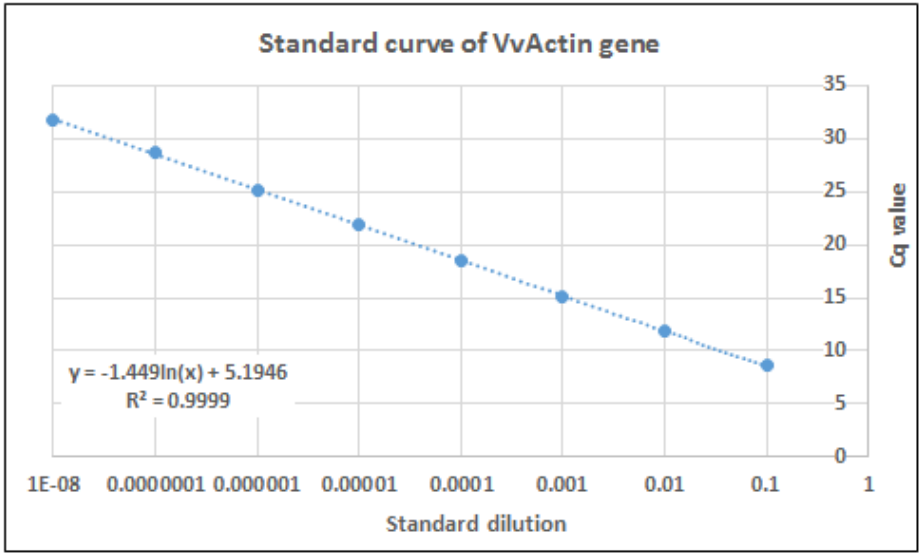
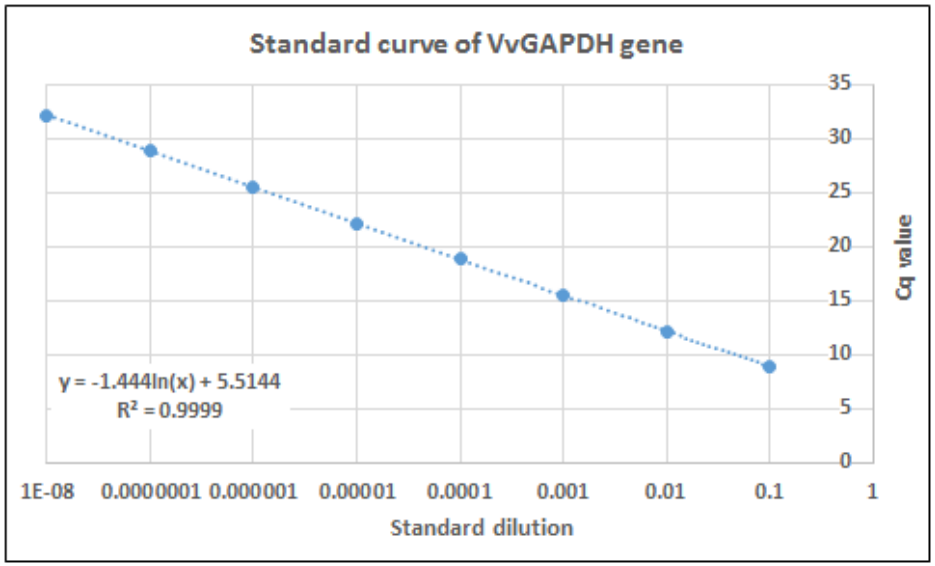
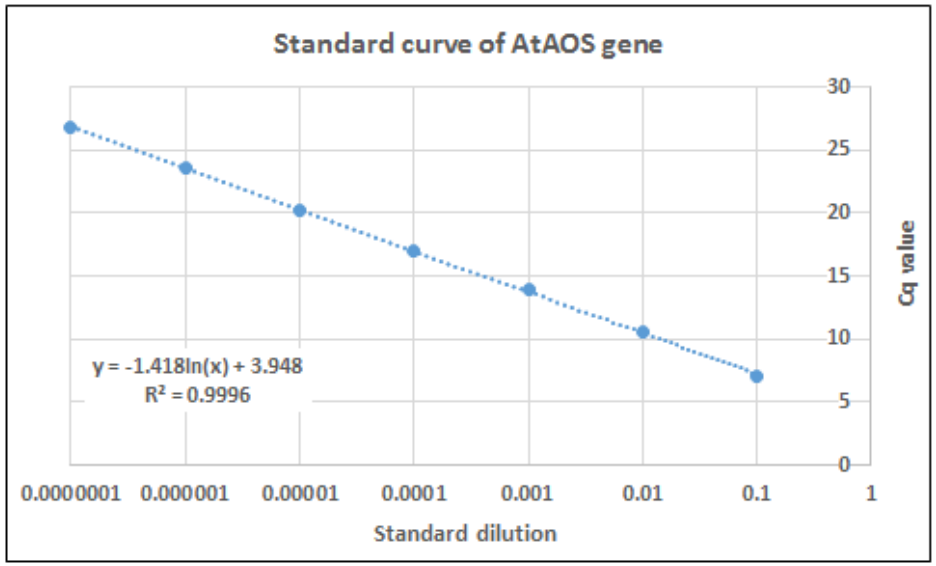
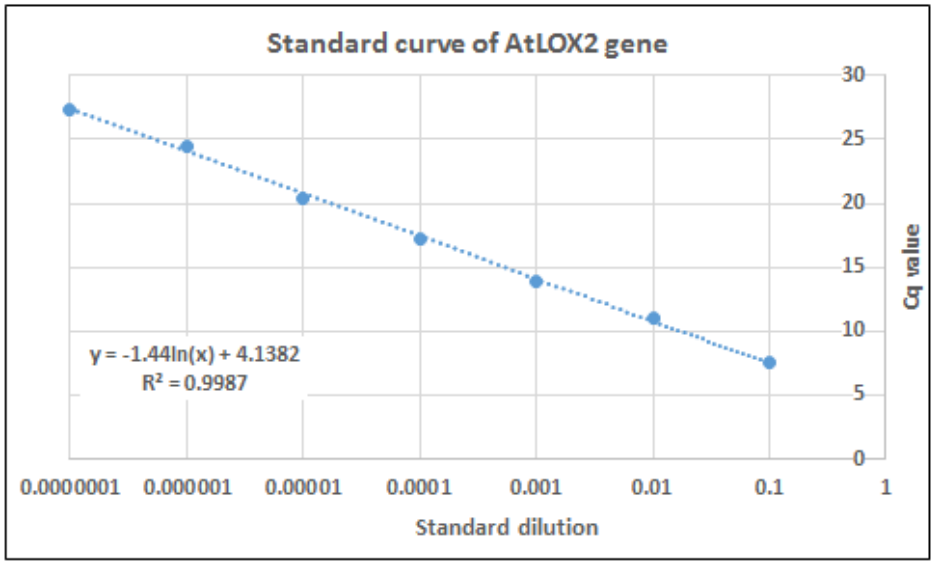
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
35
30
25
20
15
10
5
0

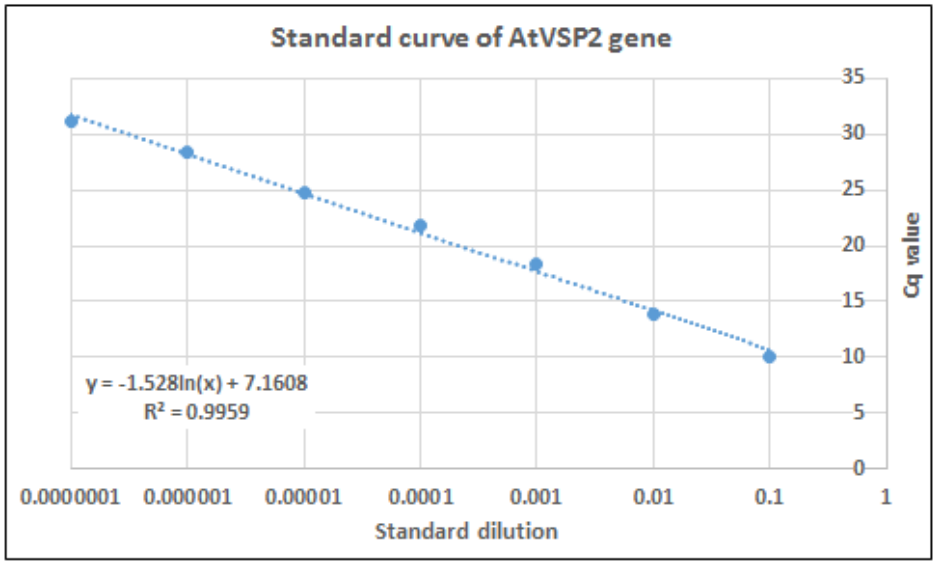

Standard curve of VvHPLF gene
y = -1.44ln(x) + 5.2083
R² = 0.9993
Cq value
Standar dilution
1E-08
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
35
30
25
20
15
10
5
0


Standard curve of VvActin gene
y = -1.449ln(x) + 5.1946
R² = 0.9999
Cq value
Standard dilution
1E-08
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
35
30
25
20
15
10
5
0


Standard curve of VvGAPDH gene
y = -1.444ln(x) + 5.5144
R² = 0.9999
Cq value
Standard dilution
1E-08
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
0
5
10
15
20
25
30
35


Standard curve of AtAOS gene
y = -1.418ln(x) + 3.948
R² = 0.9996
Cq value
Standard dilution
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
0
5
10
15
20
25
30


Standard curve of AtLOX2 gene
y = -1.44ln(x) + 4.1382
R² = 0.9987
Cq value
Standard dilution
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
0
5
10
15
20
25
30


Standard curve of AtVSP2 gene
y = -1.528ln(x) + 7.1608
R² = 0.9959
Cq value
Standard dilution
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
0
5
10
15
20
25
30
35

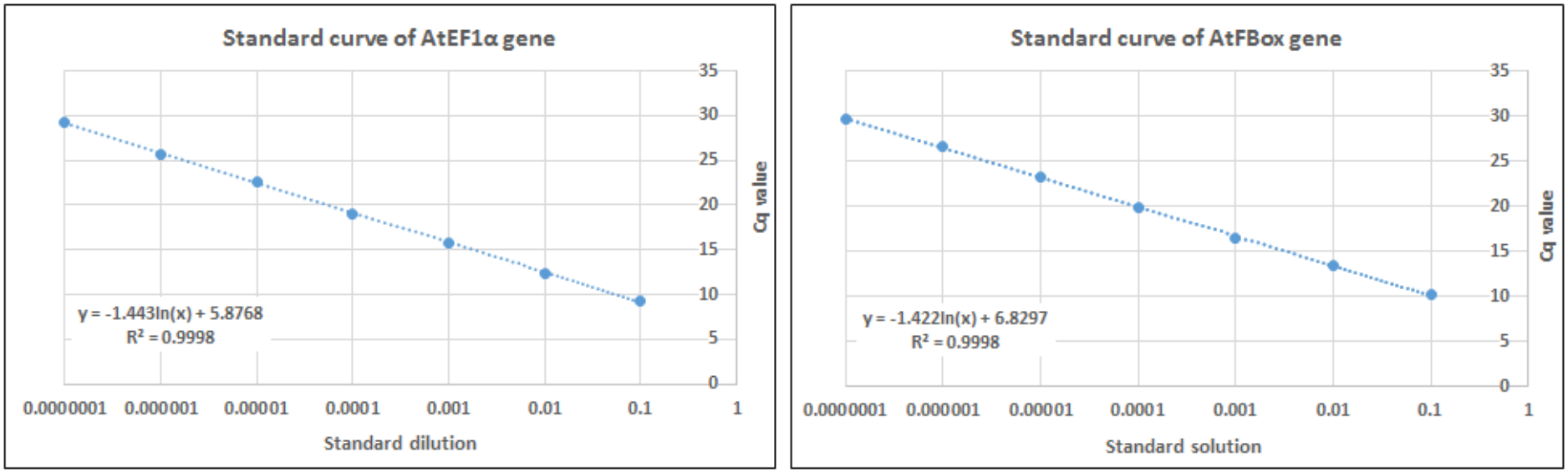


Figure A.2 Standard curve of all genes target

Standard curve for each gene target from grapevine and Arabidopsis (including reference genes) was generated using serial dilution from 1 ng/µL concentration of linearized plasmid (plasmid carried target gene). Amplification was carried out as describe in respective Material and Method.

A.5 qRT-PCR Cq value of VvCYP74 genes family members transcript quantified from wound treated and control samples in grapevine leaves.

	Wound Treatment (Hours)	VvAOS (Cq)		VvHPLA (Cq)		VvHPLB (Cq)		VvHPLC (Cq)		VvHPLD (Cq)		VvHPLE (Cq)		VvHPLF (Cq)		VvACTIN (Cq)		VvGAPDH (Cq)	
		WL	CL	WL	CL	WL	CL	WL	CL	WL	CL	WL	CL	WL	CL	WL	CL	WL	CL
Biological replicate 1	0	25.15	24.16	23.72	22.94	30.70	31.69	27.71	26.61	31.91	31.67	29.39	31.46	N/A	N/A	17.66	17.67	18.27	18.11
	0.5	23.47	25.16	22.69	23.13	31.64	30.19	29.46	26.24	32.01	31.89	31.43	30.13	34.26	N/A	17.20	17.38	17.40	17.74
	1	22.97	24.86	22.63	23.07	31.96	31.52	29.38	27.32	30.71	33.05	31.03	30.71	31.43	N/A	17.56	17.83	17.59	18.25
	3	20.00	24.61	22.50	22.46	31.62	30.42	27.27	27.05	30.29	32.45	31.14	30.82	31.53	N/A	17.45	18.11	17.82	18.29
	6	20.76	24.34	20.09	21.75	32.01	30.60	30.16	27.45	29.11	32.67	31.48	30.54	30.47	N/A	18.18	17.88	18.92	18.00
	12	18.72	23.40	20.18	21.07	30.97	31.17	29.37	29.37	28.90	31.65	31.16	30.68	27.96	N/A	18.04	17.53	18.29	17.46
	24	22.33	24.31	21.90	22.98	30.37	30.83	30.06	28.38	32.52	31.04	30.45	31.66	30.30	N/A	17.66	17.42	18.21	17.61
Biological replicate 2	0	24.00	25.33	22.44	23.13	31.65	30.48	29.64	27.29	32.44	32.12	31.16	30.87	N/A	N/A	17.42	18.16	17.78	18.68
	0.5	24.16	24.78	22.65	22.96	31.41	30.86	29.64	26.94	30.63	32.10	31.15	30.58	32.82	32.91	17.75	17.89	18.06	18.20
	1	23.01	25.00	22.60	23.42	30.77	31.18	29.86	26.38	31.50	32.47	30.64	30.82	32.04	N/A	17.45	17.98	17.75	18.50
	3	19.92	24.35	20.16	22.46	30.82	30.29	29.99	26.35	31.85	31.82	30.58	30.41	32.36	N/A	17.49	17.73	17.84	17.75
	6	20.10	24.39	19.76	21.46	31.26	30.17	30.95	27.26	29.79	32.29	30.94	29.98	31.19	N/A	17.49	17.80	18.23	18.03
	12	20.27	23.62	19.63	21.15	31.04	30.26	29.74	29.82	30.61	31.93	31.00	30.56	29.86	N/A	17.60	17.59	17.96	17.95
	24	21.09	24.61	21.49	22.99	30.65	30.11	30.49	29.25	31.40	31.50	30.75	30.80	29.84	N/A	17.48	17.52	17.76	17.75
Biological replicate 3	0	24.58	24.93	23.05	23.12	30.88	30.33	30.73	27.13	31.40	31.40	30.27	30.87	N/A	N/A	18.25	17.52	18.45	17.87
	0.5	24.51	24.99	23.08	23.14	31.02	30.19	30.19	27.96	32.70	31.84	31.56	30.77	34.45	N/A	17.82	17.74	18.45	18.18
	1	23.70	24.66	23.45	23.01	31.20	30.19	28.16	27.39	32.79	32.50	30.69	30.84	32.22	N/A	17.75	17.56	18.32	17.97
	3	20.04	24.55	20.87	22.61	31.26	31.10	30.92	26.94	31.37	30.33	31.52	31.60	31.66	N/A	17.50	17.77	18.20	17.66
	6	21.04	24.44	20.25	21.84	30.96	30.22	29.55	30.16	29.56	33.59	30.78	31.08	31.89	32.64	17.85	18.18	18.65	18.33
	12	20.41	23.81	19.58	21.25	31.32	30.41	29.44	28.43	30.27	30.71	31.03	30.54	29.40	33.06	18.30	17.71	18.44	18.03
	24	22.43	25.00	22.26	23.23	30.39	30.28	30.13	29.07	30.78	30.61	30.53	31.17	29.80	31.84	17.64	17.59	18.03	17.93

Table A.3 qRT-PCR Cq value of VvCYP74 genes family

Appendix B

Vector construct genetic maps

All vector genetic maps were constructed via Lasergene molecular biology software suite (DNASTAR Inc, Madison, USA).

B.1 Plant binary vector - pARTBGW

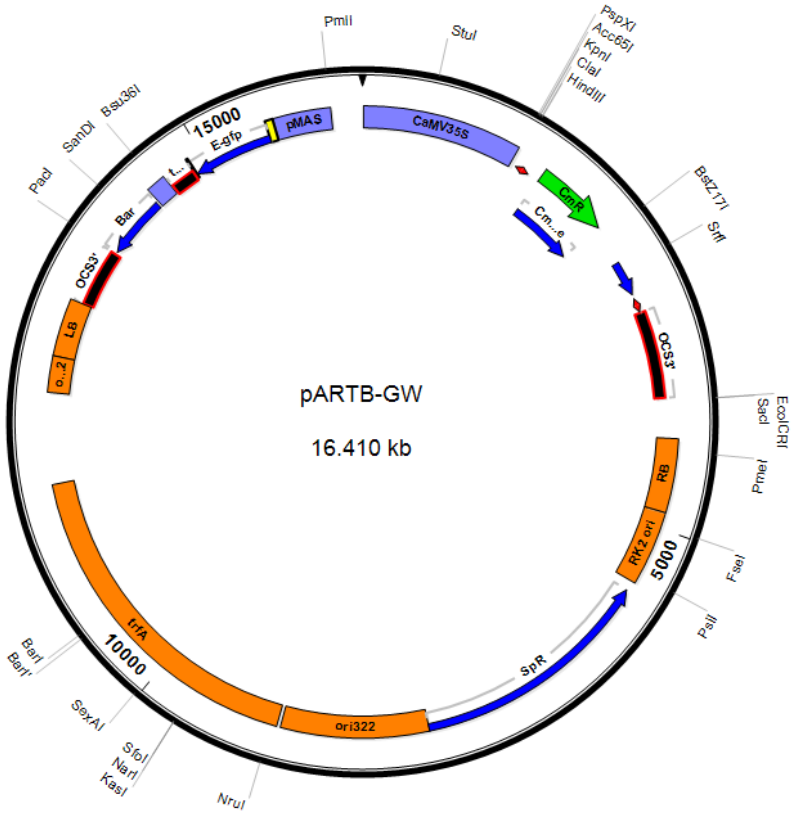


Figure B.1 Binary vector pARTBGW
This plant binary vector was obtained from Plant and Food Research Institute, Canterbury, New Zealand, which incorporated with cauliflower mosaic virus 35S promoter (CaMV 35S prompter) as a gene regulator to desired gene and possessed the phosphinothricin-N-acetyltransferase resistance gene to confer resistance to the herbicide glufosinate, a non-selective herbicide (commercially known as BASTA) as a selection marker

B.2 Plant binary vector - pARTBGW:promAOS:VvAOS

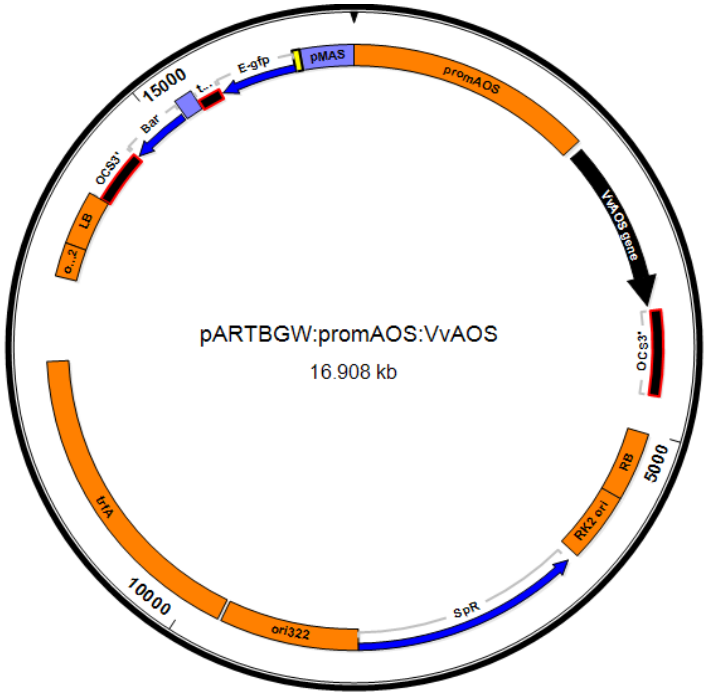
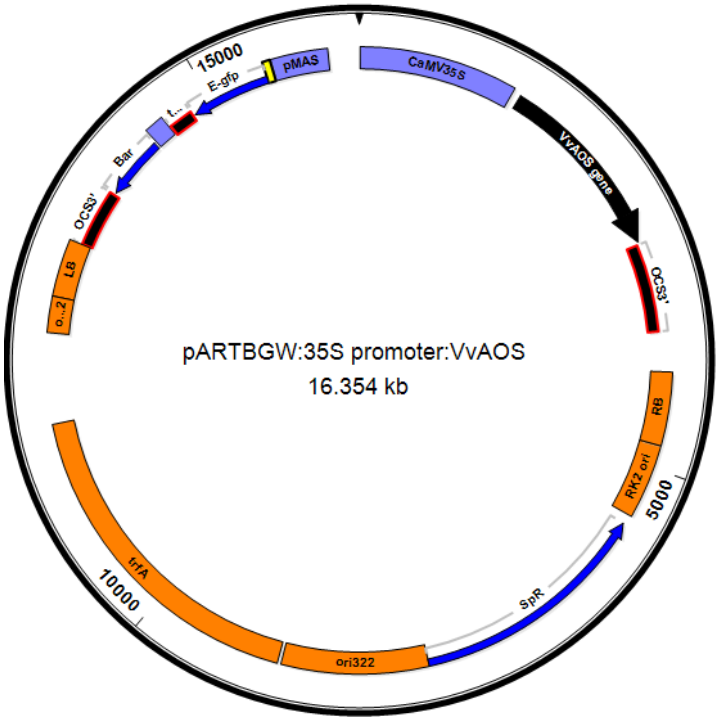


Figure B.2 Binary vector pARTBGW:promAOS:VvAOS
This plant binary vector, pARTBGW incorporated with VvAOS gene and used plant promoter isolated from Arabidopsis (promAOS) as a gene regulator for the desired gene. This vector was used to transform homozygous Arabidopsis *aos* mutant to characterized VvAOS gene via male-sterile complementation phenotype.

B.3 Plant binary vector – pARTBGW:35S promoter:VvAOS or AtAOS



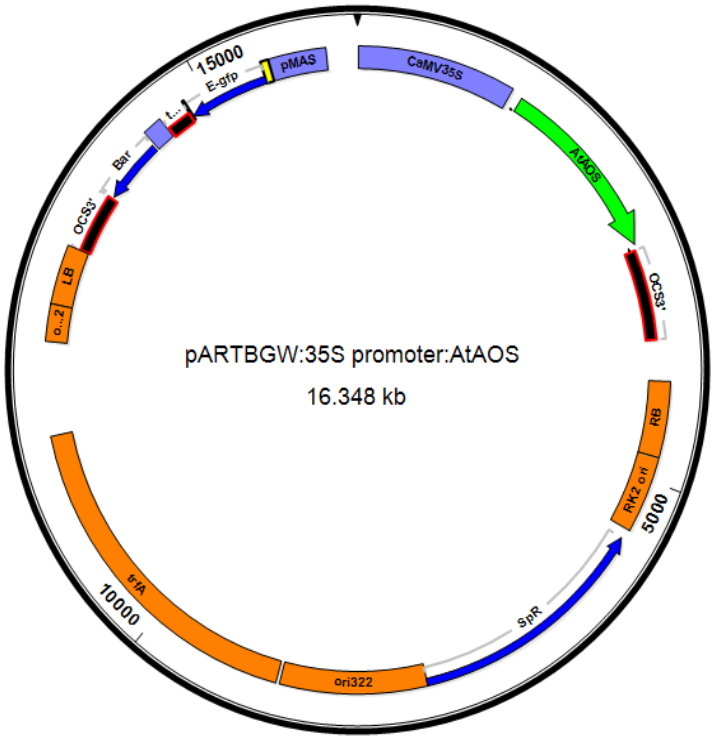


Figure B.3 Binary vector pARTBGW:35S promoter:VvAOS or AtAOS
This plant binary vector, pARTBGW incorporated with VvAOS or AtAOS gene and used constitutive CaMV 35S promoter (35S promoter) as a regulator to desired gene. This vector was used to transformed *Arabidopsis thaliana* with VvAOS or AtAOS genes to investigate their overexpression in Arabidopsis background

B.4 Plant binary vector – pB7FWG2

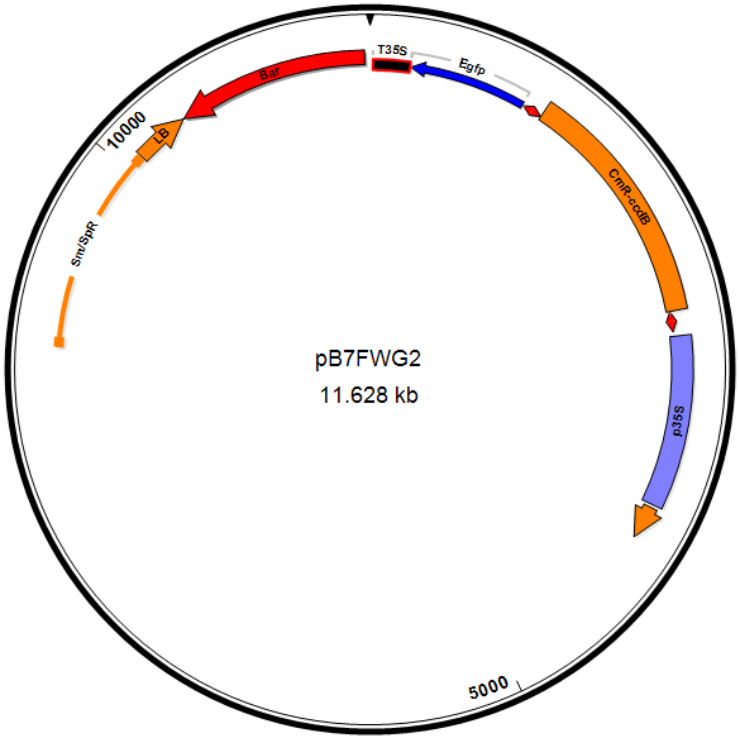


Figure B.4 Binary vector pB7FWG2
The binary vector, pB7FWG2 (Karimi et al., 2002), used CaMV 35S promoter as a regulator to desired gene and containing an N-terminal GFP fusion protein as a gene marker to uncover the sub-cellular localization of the VvCYP74 protein in *Nicotiana benthamiana*

B.5 Plant binary vector – pB7FWG2:VvCYP74

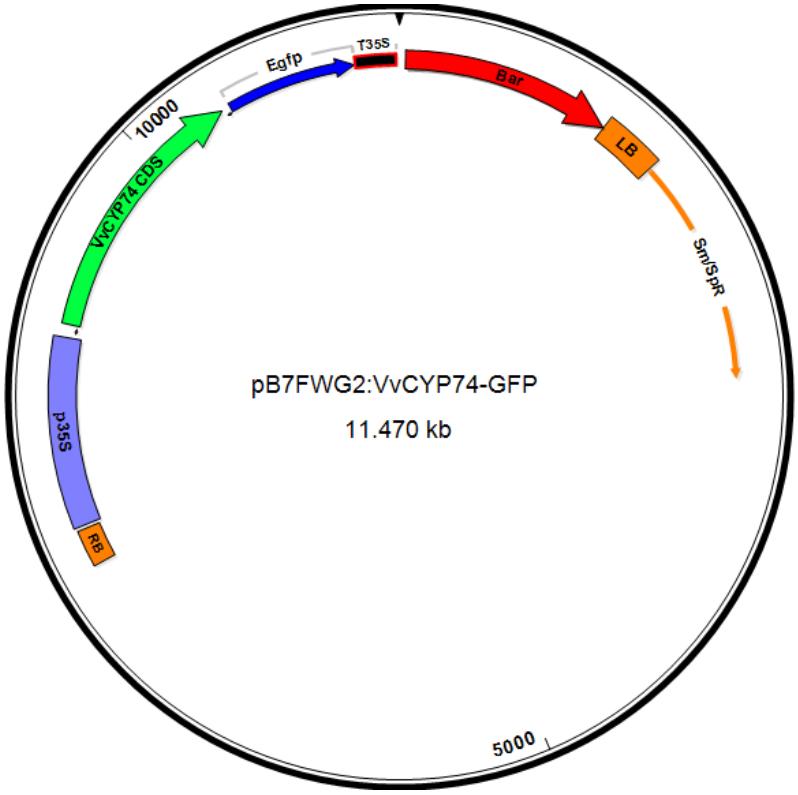
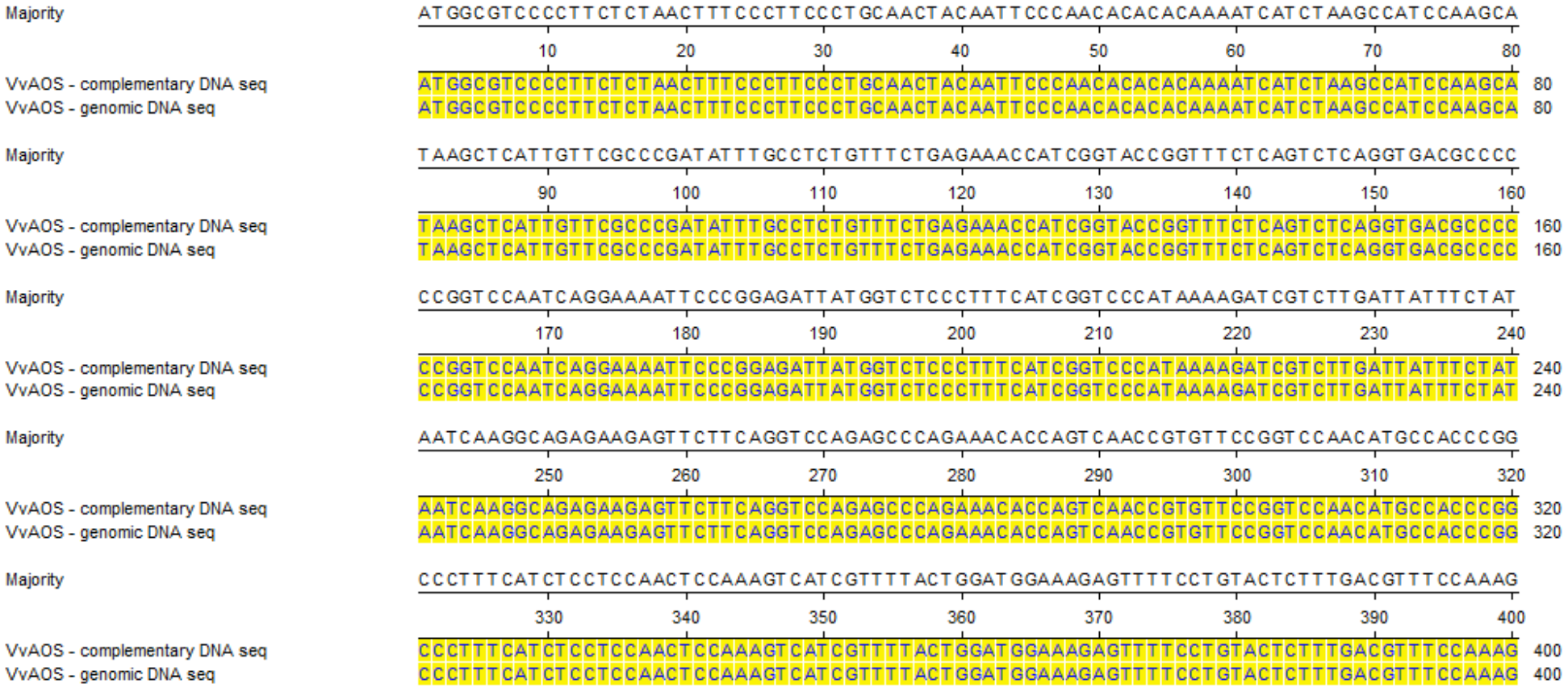


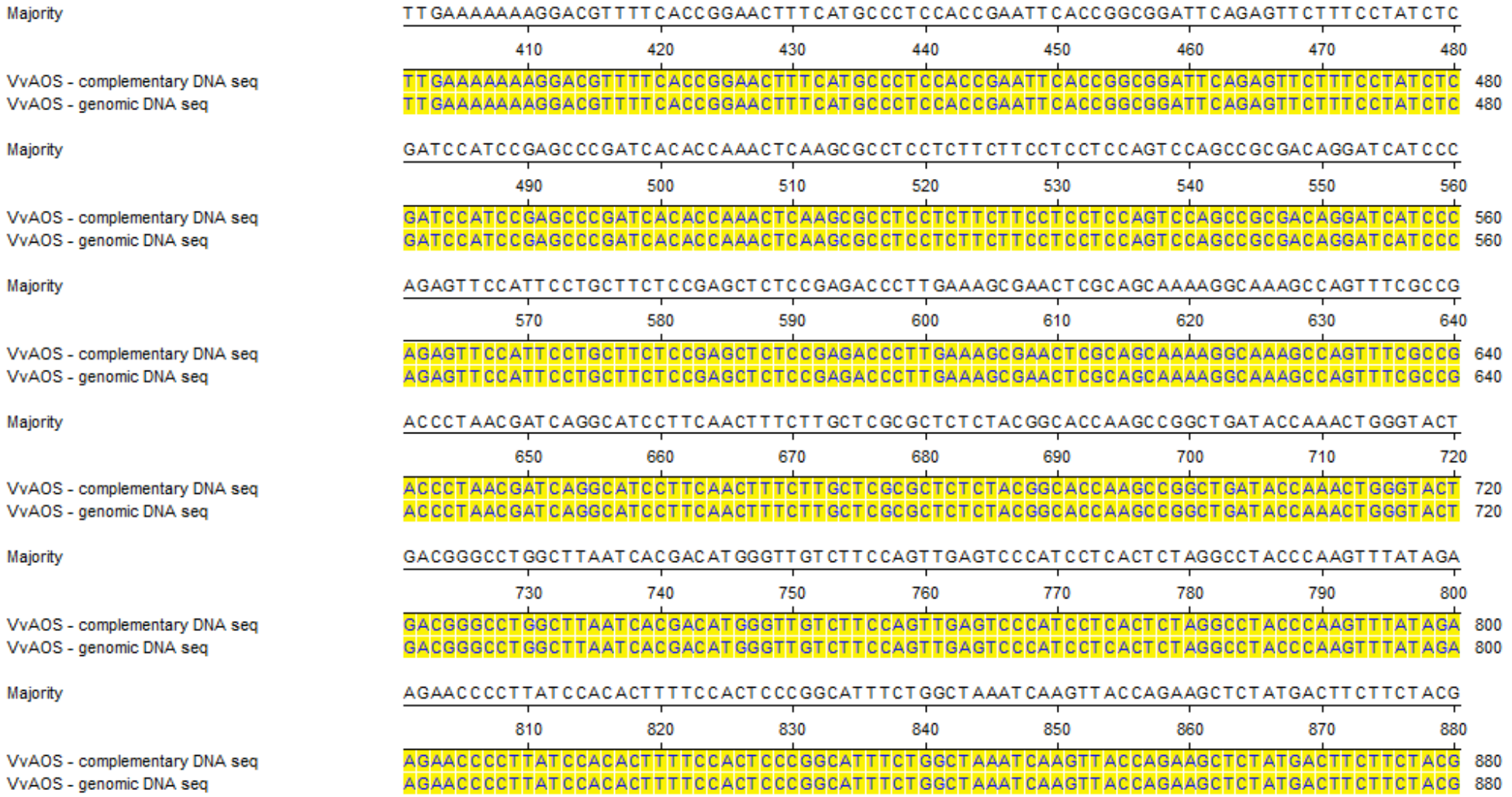
Figure B.5 Binary vector pB7FWG2:VvCYP74
This plant binary vector, pB7FWG2:VvCYP74 were used to transformed young leaf of *Nicotiana benthamiana* in order to arrest the sub-cellular localization of grapevine CYP74 gene expression. Each of the grapevine CYP74 genes member was fused green fluorescent protein (GFP) fusion in order to localize their transient expression into specific plant cell compartments. Grapevine CYP74 (VvCYP74) genes used were; VvAOS, VvHPLA, VvHPLB, VvHPLC, VvHPLD, VvHPLE and VvHPLF

Appendix C

Sequence alignments

C.1 Alignment sequences of grapevine AOS gene between complementary and genomic DNA template




Majority
TTGAAAAAAAGGACGTTTTCACCGGAACTTTCATGCCCTCCACCGAATTCACCGGCGGATTCAGAGTTCTTTCCTATCTC
410 420 430 440 450 460 470 480
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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TTGAAAAAAAGGACGTTTTCACCGGAACTTTCATGCCCTCCACCGAATTCACCGGCGGATTCAGAGTTCTTTCCTATCTC 480
Majority
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490 500 510 520 530 540 550 560
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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GATCCATCCGAGCCCGATCACACCAAACTCAAGCGCCTCCTCTTCTTCCTCCTCCAGTCCAGCCGCGACAGGATCATCCC 560
Majority
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570 580 590 600 610 620 630 640
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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AGAGTTCCATTCCTGCTTCTCCGAGCTCTCCGAGACCCTTGAAAGCGAACTCGCAGCAAAAGGCAAAGCCAGTTTCGCCG 640
Majority
ACCCTAACGATCAGGCATCCTTCAACTTTCTTGCTCGCGCTCTCTACGGCACCAAGCCGGCTGATACCAAACTGGGTACT
650 660 670 680 690 700 710 720
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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ACCCTAACGATCAGGCATCCTTCAACTTTCTTGCTCGCGCTCTCTACGGCACCAAGCCGGCTGATACCAAACTGGGTACT 720
Majority
GACGGGCCTGGCTTAATCACGACATGGGTTGTCTTCCAGTTGAGTCCCATCCTCACTCTAGGCCTACCCAAGTTTATAGA
730 740 750 760 770 780 790 800
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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GACGGGCCTGGCTTAATCACGACATGGGTTGTCTTCCAGTTGAGTCCCATCCTCACTCTAGGCCTACCCAAGTTTATAGA 800
Majority
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810 820 830 840 850 860 870 880
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
AGAACCCCTTATCCACACTTTTCCACTCCCGGCATTTCTGGCTAAATCAAGTTACCAGAAGCTCTATGACTTCTTCTACG 880
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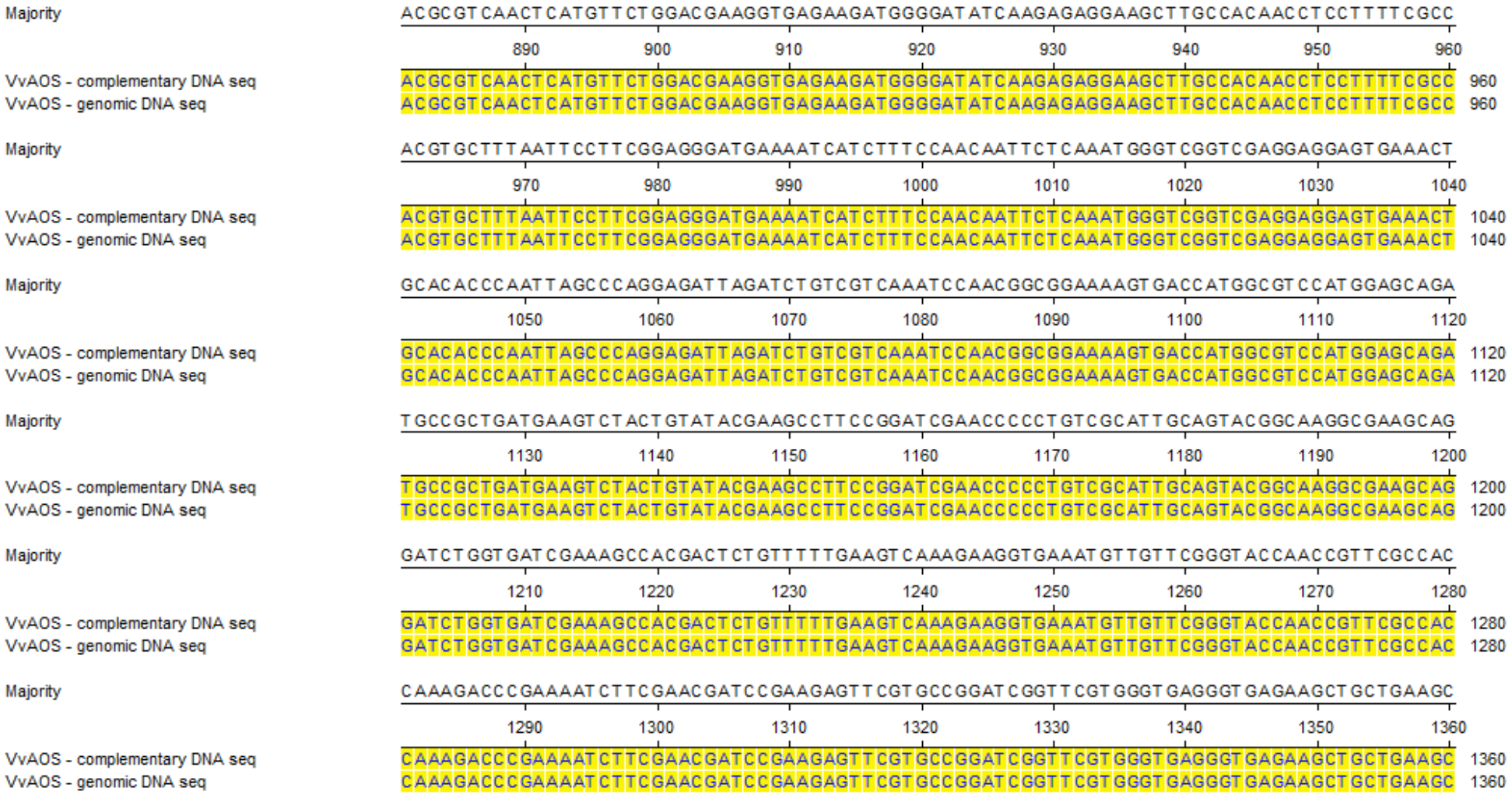
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VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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Majority
ACGTGCTTTAATTCCTTCGGAGGGATGAAAATCATCTTTCCAACAATTCTCAAATGGGTCGGTCGAGGAGGAGTGAAACT
970 980 990 1000 1010 1020 1030 1040
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
ACGTGCTTTAATTCCTTCGGAGGGATGAAAATCATCTTTCCAACAATTCTCAAATGGGTCGGTCGAGGAGGAGTGAAACT 1040
Majority
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1050 1060 1070 1080 1090 1100 1110 1120
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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Majority
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1130 1140 1150 1160 1170 1180 1190 1200
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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Majority
GATCTGGTGATCGAAAGCCACGACTCTGTTTTTGAAGTCAAAGAAGGTGAAATGTTGTTCGGGTACCAACCGTTCGCCAC
1210 1220 1230 1240 1250 1260 1270 1280
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
GATCTGGTGATCGAAAGCCACGACTCTGTTTTTGAAGTCAAAGAAGGTGAAATGTTGTTCGGGTACCAACCGTTCGCCAC 1280
Majority
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1290 1300 1310 1320 1330 1340 1350 1360
VvAOS - complementary DNA seq
VvAOS - genomic DNA seq
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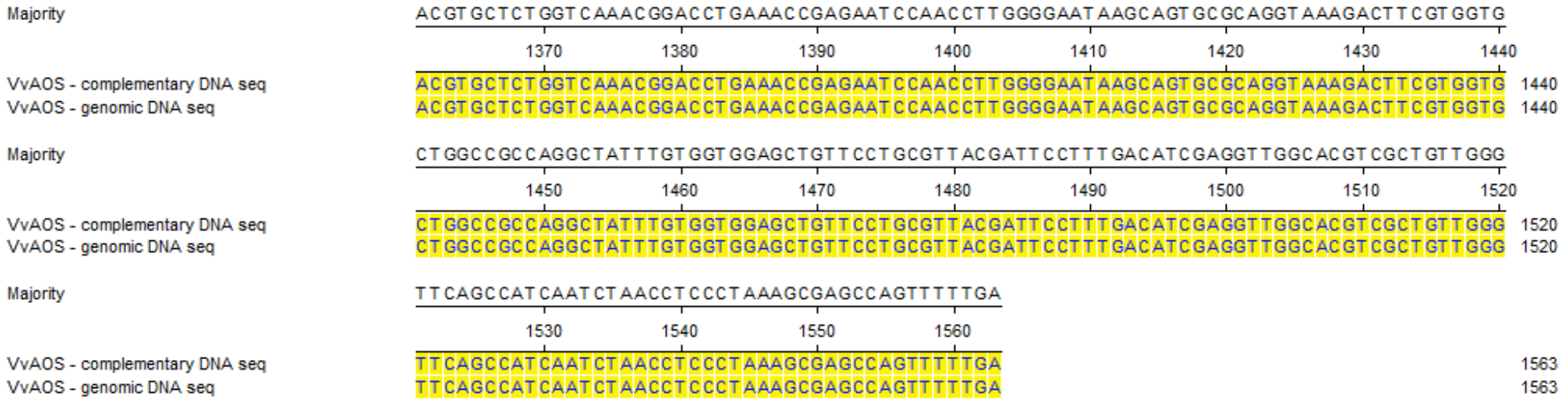
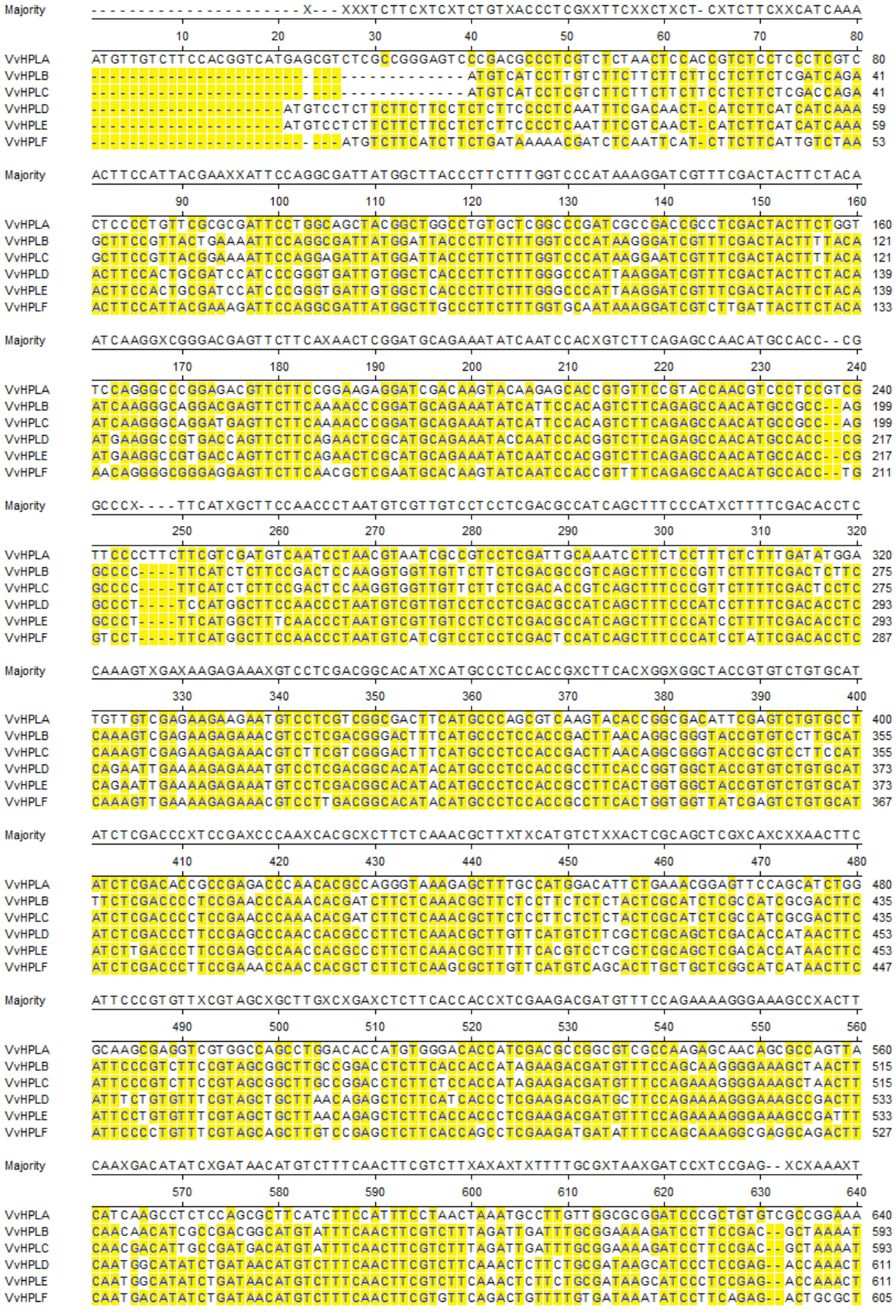
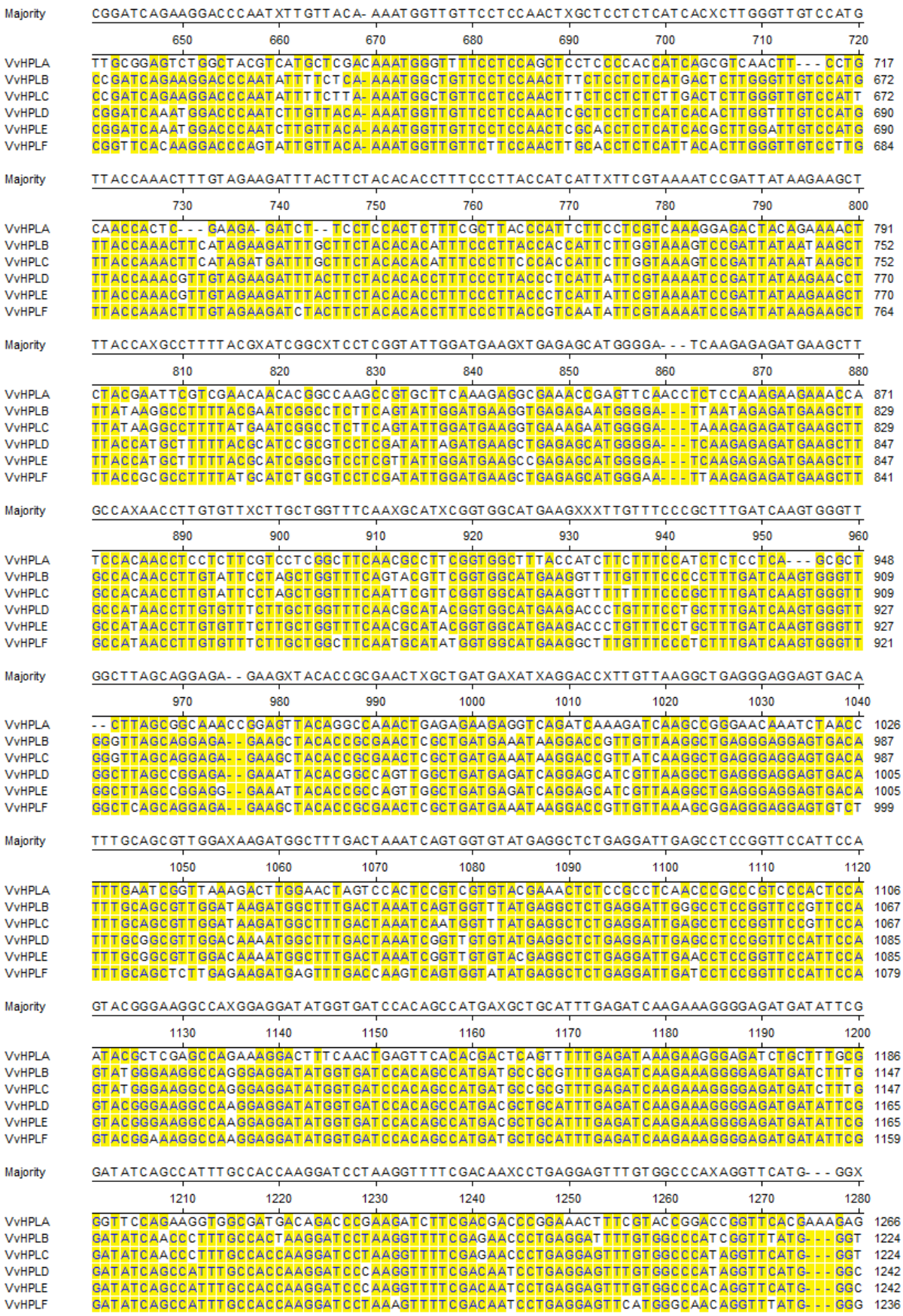


Figure C.1 Alignment sequences of VvAOS nucleotide sequence between cDNA and gDNA template

Grapevine AOS nucleotide sequences amplified from complementary DNA (cDNA) and genomic DNA (gDNA) were align to identify their intron region. Alignemnt sequence was carried out using MegAlign program within the Lasergene molecular biology software suite (DNASTAR Inc, Madison, USA). Yellow shade indicate nucleotide match among grapevine HPL sequences

C.2 Alignment sequences of grapevine HPLs nucleotide sequences

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VvHPLB CCGATCAGAAGGACCCAATATTTTCTCA-AAATGGCTGTTCCTCCAACTTTCTCCTCTCATGACTCTTGGGTTGTCCATG 672
VvHPLC CCGATCAGAAGGACCCAATATTTTCTTA-AAATGGCTGTTCCTCCAACTTTCTCCTCTCTTGACTCTTGGGTTGTCCATT 672
VvHPLD CGGATCAAATGGACCCAATCTTGTTACA-AAATGGTTGTTCCTCCAACTCGCTCCTCTCATCACACTTGGTTTGTCCATG 690
VvHPLE CGGATCAAATGGACCCAATCTTGTTACA-AAATGGTTGTTCCTCCAACTCGCACCTCTCATCACGCTTGGATTGTCCATG 690
VvHPLF CGGTTCACAAGGACCCAGTATTGTTACA-AAATGGTTGTTCTTCCAACTTGCACCTCTCATTACACTTGGGTTGTCCTTG 684
Majority TTACCAAACTTTGTAGAAGATTTACTTCTACACACCTTTCCCTTACCATCATTXTTCGTAAAATCCGATTATAAGAAGCT
730 740 750 760 770 780 790 800
VvHPLA CAACCACTC---GAAGA-GATCT--TCCTCCACTCTTTCGCTTACCCATTCTTCCTCGTCAAAGGAGACTACAGAAAACT 791
VvHPLB TTACCAAACTTCATAGAAGATTTGCTTCTACACACATTTCCCTTACCACCATTCTTGGTAAAGTCCGATTATAATAAGCT 752
VvHPLC TTACCAAACTTCATAGATGATTTGCTTCTACACACATTTCCCTTCCCACCATTCTTGGTAAAGTCCGATTATAATAAGCT 752
VvHPLD TTACCAAACGTTGTAGAAGATTTACTTCTACACACCTTTCCCTTACCCTCATTATTCGTAAAATCCGATTATAAGAACCT 770
VvHPLE TTACCAAACGTTGTAGAAGATTTACTTCTACACACCTTTCCCTTACCCTCATTATTCGTAAAATCCGATTATAAGAAGCT 770
VvHPLF TTACCAAACTTTGTAGAAGATCTACTTCTACACACCTTTCCCTTACCGTCAATATTCGTAAAATCCGATTATAAGAAGCT 764
Majority TTACCAXGCCTTTTACGXATCGGCXTCCTCGGTATTGGATGAAGXTGAGAGCATGGGGA---TCAAGAGAGATGAAGCTT
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VvHPLA CTACGAATTCGTCGAACAACACGGCCAAGCCGTGCTTCAAAGAGGCGAAACCGAGTTCAACCTCTCCAAAGAAGAAACCA 871
VvHPLB TTATAAGGCCTTTTACGAATCGGCCTCTTCAGTATTGGATGAAGGTGAGAGAATGGGGA---TTAATAGAGATGAAGCTT 829
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VvHPLF TTACCGCGCCTTTTATGCATCTGCGTCCTCGATATTGGATGAAGCTGAGAGCATGGGAA---TTAAGAGAGATGAAGCTT 841
Majority GCCAXAACCTTGTGTTXCTTGCTGGTTTCAAXGCATXCGGTGGCATGAAGXXXTTGTTTCCCGCTTTGATCAAGTGGGTT
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VvHPLA TCCACAACCTCCTCTTCGTCCTCGGCTTCAACGCCTTCGGTGGCTTTACCATCTTCTTTCCATCTCTCCTCA---GCGCT 948
VvHPLB GCCACAACCTTGTATTCCTAGCTGGTTTCAGTACGTTCGGTGGCATGAAGGTTTTGTTTCCCCCTTTGATCAAGTGGGTT 909
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VvHPLE GCCATAACCTTGTGTTTCTTGCTGGTTTCAACGCATACGGTGGCATGAAGACCCTGTTTCCTGCTTTGATCAAGTGGGTT 927
VvHPLF GCCATAACCTTGTGTTTCTTGCTGGCTTCAATGCATATGGTGGCATGAAGGCTTTGTTTCCCTCTTTGATCAAGTGGGTT 921
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970 980 990 1000 1010 1020 1030 1040
VvHPLA --CTTAGCGGCAAACCGGAGTTACAGGCCAAACTGAGAGAAGAGGTCAGATCAAAGATCAAGCCGGGAACAAATCTAACC 1026
VvHPLB GGGTTAGCAGGAGA--GAAGCTACACCGCGAACTCGCTGATGAAATAAGGACCGTTGTTAAGGCTGAGGGAGGAGTGACA 987
VvHPLC GGGTTAGCAGGAGA--GAAGCTACACCGCGAACTCGCTGATGAAATAAGGACCGTTATCAAGGCTGAGGGAGGAGTGACA 987
VvHPLD GGCTTAGCCGGAGA--GAAATTACACGGCCAGTTGGCTGATGAGATCAGGAGCATCGTTAAGGCTGAGGGAGGAGTGACA 1005
VvHPLE GGCTTAGCCGGAGG--GAAATTACACCGCCAGTTGGCTGATGAGATCAGGAGCATCGTTAAGGCTGAGGGAGGAGTGACA 1005
VvHPLF GGCTCAGCAGGAGA--GAAGCTACACCGCGAACTCGCTGATGAAATAAGGACCGTTGTTAAAGCGGAGGGAGGAGTGTCT 999
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1050 1060 1070 1080 1090 1100 1110 1120
VvHPLA TTTGAATCGGTTAAAGACTTGGAACTAGTCCACTCCGTCGTGTACGAAACTCTCCGCCTCAACCCGCCGTCCCACTCCA 1106
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VvHPLC TTTGCAGCGTTGGATAAGATGGCTTTGACTAAATCAATGGTTTATGAGGCTCTGAGGATTGAGCCTCCGGTTCCGTTCCA 1067
VvHPLD TTTGCGGCGTTGGACAAAATGGCTTTGACTAAATCGGTTGTGTATGAGGCTCTGAGGATTGAGCCTCCGGTTCCATTCCA 1085
VvHPLE TTTGCGGCGTTGGACAAAATGGCTTTGACTAAATCGGTTGTGTACGAGGCTCTGAGGATTGAACCTCCGGTTCCATTCCA 1085
VvHPLF TTTGCAGCTCTTGAGAAGATGAGTTTGACCAAGTCAGTGGTATATGAGGCTCTGAGGATTGATCCTCCGGTTCCATTCCA 1079
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1130 1140 1150 1160 1170 1180 1190 1200
VvHPLA ATACGCTCGAGCCAGAAAGGACTTTCAACTGAGTTCACACGACTCAGTTTTTGAGATAAAGAAGGGAGATCTGCTTTGCG 1186
VvHPLB GTATGGGAAGGCCAGGGAGGATATGGTGATCCACAGCCATGATGCCGCGTTTGAGATCAAGAAAGGGGAGATGATCTTTG 1147
VvHPLC GTATGGGAAGGCCAGGAGGATATGGTGATCCACAGCCATGATGCCGCGTTTGAGATCAAGAAAGGGGAGATGATCTTTG 1147
VvHPLD GTACGGGAAGGCCAAGGAGGATATGGTGATCCACAGCCATGACGCTGCATTTGAGATCAAGAAAGGGGAGATGATATTCG 1165
VvHPLE GTACGGGAAGGCCAAGGAGGATATGGTGATCCACAGCCATGACGCTGCATTTGAGATCAAGAAAGGGGAGATGATATTCG 1165
VvHPLF GTACGGAAAGGCCAAGGAGGATATGGTGATCCACAGCCATGATGCTGCATTTGAGATCAAGAAAGGGGAGATGATATTCG 1159
Majority GATATCAGCCATTTGCCACCAAGGATCCTAAGGTTTTCGACAAXCCTGAGGAGTTTGTGGCCCAXAGGTTCATG---GGX
1210 1220 1230 1240 1250 1260 1270 1280
VvHPLA GGTTCCAGAAGGTGGCGATGACAGACCCGAAGATCTTCGACGACCCGGAAACTTTCGTACCGGACCGGTTCACGAAAGAG 1266
VvHPLB GATATCAACCCTTTGCCACTAAGGATCCTAAGGTTTTCGAGAACCCTGAGGATTTTGTGGCCCATCGGTTTATG---GGT 1224
VvHPLC GATATCAACCCTTTGCCACCAAGGATCCTAAGGTTTTCGAGAACCCTGAGGAGTTTGTGGCCCATAGGTTCATG---GGT 1224
VvHPLD GATATCAGCCATTTGCCACCAAGGATCCCAAGGTTTTCGACAATCCTGAGGAGTTTGTGGCCCATAGGTTCATG---GGC 1242
VvHPLE GATATCAGCCATTTGCCACCAAGGATCCCAAGGTTTTCGACAATCCTGAGGAGTTTGTGGCCCACAGGTTCATG---GGC 1242
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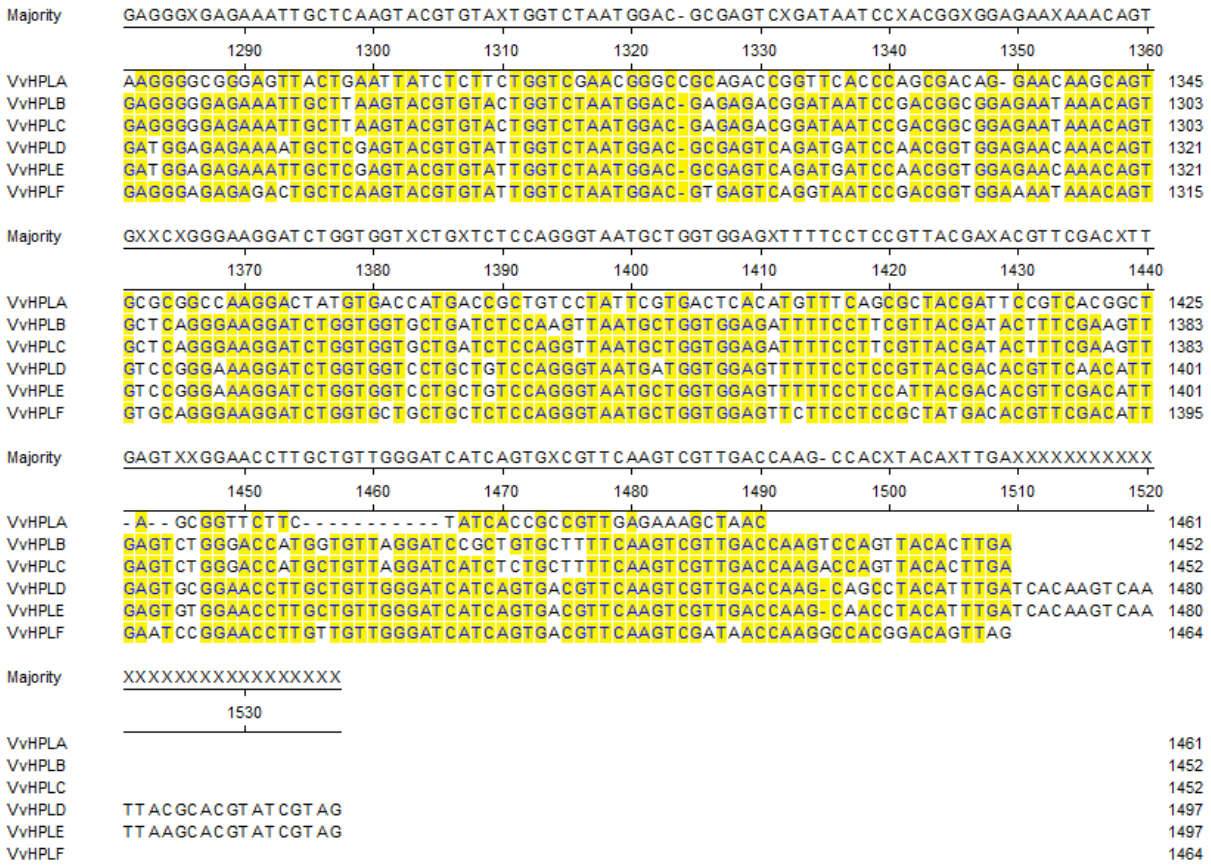

Figure C.2 Alignment sequences of grapevine HPLs nucleotide sequences

Grapevine HPLs nucleotide sequences i.e. HLPA. HPLB, HPLC, HPLD, HPLE and HPLF were align in order to identify suitable site for qRT-PCR primers. Alignment result shows that grapevine HPLs sequences are highly identical within their coding sequence region. Alignment sequence was carried out using MegAlign program within the Lasergene molecular biology software suite (DNASTAR Inc, Madison, USA). Yellow shade indicate nucleotide match among grapevine HPL sequences

C.3 Multiple alignment of CYP74 enzymes polypeptide sequences

```
PaAOS1          ---------------------------------------MDPSSKPLREIPGSYGIPFF 20
AaAOS           ----------------------FSATSPDTTTTTTTTGSNTDNKNLPIRPIPGSYGIPFY 38
AtAOS           --------------------------ASGSETPDLTVATRTGSKDLPIRNIPGNYGLPIV 34
LeAOS1          ------------------------------------------------AQKVPGDYALPLV 13
StAOS1          --------------------ASVSERPPYISSPSPSPSPPVKQAKLPTRKVPGDYGLPLV 40
InAOS           -------------------SATVSDTPP---SVSLSPVP----EKLPKRKIPGDYGLPLI 34
StAOS2          --------------------------------LSEKPTIVVTQPTKLPTRTIPGDYGLPGI 29
StAOS           --------------------------------LSEKPTIVVTQPTKLPTRTIPGDYGLPGI 29
LeAOS2          MALTLSFSLPLPSLHQKIPSKYSTFRPIIVSLSDKSTIEITQPIKLSTRTIPGDYGLPGI 60
NaAOS           -----------------------------------AVTQSSEFTKLPIRTIPGDYGLPLI 25
GmAOS           --------------------ASVSEKPPLPAVSVTSPEP----SKLPIRKIPGDCGFPVI 36
GmAOS1          --------------------ASVSEKPPLPAVSVTSPEP----SKLPIRKIPGDCGFPVI 36
MtAOS           --------------------SSVSEKPPF-QVSISQPQT----TKLPIRKIPGDYGIPFI 35
CmAOS           --------------------SSSSSSLQVPQRIVSPPEP----TKLPLRKVPGDYGPPMF 36
LuAOS           -------------------ASLFGDSPIKIPGITSQPPPSSDETTLPIRQIPGDYGLPGI 41
VvAOS           ---------------------------------QSQVTPPG-----PIRKIPGDYGLPFI 22
HvAOS1          ---------------------------------MNQSAIG-----SLVPRQAPGSYGLPFV 23
HvAOS2          ---------------------------------MNQSGMARSDEGSLVPREVPGSYGLPFV 28
OsAOS2          ----------------------------------MELGVP------LPRRPVPGSYGVPFV 21
OsAOS3          ----------------------------------MELGVP------LPRRPVPGSYGVPFV 21
OsAOS1          ----------------------------------MELGVP------LPRRPVPGSYGVPFV 21
CsHPL1          -----------------------------------MAS---SSPELPLKPIPGGYGFPFL 22
CmHPL           -----------------------------------MATPSSSSPELPLKPIPGGYGFPFL 25
VvHPL2          ------------------------------------------------------------
VvHPLE          ------------------------------------------------------------
VvHPLD          ------------------------------------------------------------
VvHPLF          ----------------------------MSSSSDKNDLNSSSSLSKLPLRKIPGDYGLPFF 33
VvHPLB          ---------------------------------MSSLSSSSSSSRSELPLLKIPGDYGLPFF 29
VvHPLC          ---------------------------------MSSSSSSSSSSRPELPLRKIPGDYGLPFF 29
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MtHPL2          ---------------------------------MASSKQEQSSTNKELPLKQIPGSYGLPFI 29
StDES           -----------------------------------MSSYSELSN-LPIREIPGDYGFPII 24
LeDES           -----------------------------------MSSYSELSN-LPIREIPGDYGFPII 24
CaDES           -----------------------------------MSSYSESPK-LPVREIPGDYGFPII 24
NtDES           -----------------------------------MSSFLVSSNNLPEREIPGDYGFPII 25
StAOS3          ----------------------MANTKDSYHIITMDTKESSIPNLPMKEIPGDYGVPFL 37
LeAOS3          ----------------------MANTKDSYHIITMDTKESSIPSLPMKEIPGDYGVPFF 37
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MsHPL2          ----------------------MSLPPPIPPPSLTTPPKARPTELPIRQIPGSYGWPLL 37
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LeHPL           ---------------------------------MNSAPLSTPAPVTLPVRSIPGSYGLPLV 28
StHPL           ------------------------------MIPIMSSAPLSTPAPVTLPVRTIPGSYGLPLL 32
CaHPL           ------------------------------MIPIMSSAPLSTATPISLPVRKIPGSYGFPLL 32
NaHPL           ------------------------------------------------GGYGWPLL 8
VvHPL1          ------------------------------------------------AIPGSYGWPVL 11
VvHPLA          ------------------------------------------------------------
PgHPL           ------------------------------------------------GSYGWPLL 8
HvHPL           ----------------------------------------AAMAPPPPKPIPGGYGAPVL 20
ZmHPL           ------------------------------------------------------------
MaHPL           ------------------------------------------------------------


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AtAOS           GPIKDRWDYFYDQG-AEEFFKSRIRKYNSTVYRVNMPPGA--FIAENPQVVALLDGKSFP 91
LeAOS1          GPWKDRLDYFYNQG-KNEFFKSRIQKHQSTVFRTNMPPGP--FISFNPNVVVLLDGKSFP 70
StAOS1          GPWKDRLDYFYNQG-KNEFFKSRIQKHQSTVFRTNMPPGP--FISFNPNVVVLLDGKSFP 97
InAOS           GPWKDRLDYFYNQG-REEFFRSRVQKYGSTVFRTNMPPGP--FISFSPNVVVLLDGKSFP 91
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GmAOS1          GPLKDRQDYFYKQG-RDEFFKSRIQKYQSTVFRTNMPPGP--FLAPNPNVVVLLDAKTFP 93
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VvHPL2         ---------------------------------ANMPPGP--FMALNPNVVVLLDAISFP 25
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VvHPLD         ---------------------------------ANMPPGP--SMASNPNVVVLLDAISFP 25
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VvHPLC         GPIRNRFDYFYNQG-QDEFFKTRMQKYHSTVFRANMPPGP--FISSDSKVVVLLDTVSFP 86
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MtHPL2         GPIFDRHDYFYNQG-RDKFFSTRIQKYNSTIFRTNMPPGP--FISSNPRVIALLDAASFP 86
StDES          SAIKDRYDYFYNQG-EDAWFHNKAEKYKSTVVKINMAPGP--FTSNDYKLVAFLDANSFV 81
LeDES          SAIKDRYDYFYNQG-EDAWFHNKAEKYKSTVVKINMAPGP--FTSNDYKLVAFLDANSFV 81
CaDES          SAIKDRYDYFYNQG-EDAWFHGKAEKYKSTVVKINMAPGP--FTSNDYKLVAFLDATSFV 81
NtDES          SAIKDRYDYFYKQG-EDVWFHSKAEKYNSTVVKINMAPGP--FTSNDYKLVAFLDANSFV 82
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LeAOS3         GAIKDRYDFHYNQG-ADEFFRSRMKKYDSTVFRTNVPPGP--FNARNSKVVVLVDAVSYP 94
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StHPL          GPIADRLDYFWFQK-PENFFTKRMEKHKSTVFRTNVPPCFPFVGSVNPNVVAVLDVKSFS 91
CaHPL          GPLWDRLDYNWFQK-LPDFFSKRVEKYNSTVFRTNVPPCFPFFLGVNPNVVAVLDVKSFA 91
NaHPL          GPISDRLDYNWFQG-PNTFFTKRIEKHKSTVFRTNVPPCFPFFLGVNPNVVAVLDVKSFS 67
VvHPL1         GPIADRLDYFWFQG-PETFFRKRIDKYKSTVFRTNVPPSFPFFVGVNPNVIAVLDCKSFS 70
VvHPLA         ----------------------IDKYKSTVFRTNVPPSFPFFVDVNPNVIAVLDCKSFS 37
PgHPL          GPISDRLDYFWFQG-PETFFRKRIEKYKSTVFRANVPPCFPFFSNVNPNVVVVLDCESFA 67
HvHPL          GPLRDRLDYFWFQG-PEEFFRRRAAQHRSTVFRANIPPTFPFFVGINPRVIAIVDTAAFT 79
ZmHPL          ----------------------AAAHRSTVFRTNIPPTFPFFVGVDPRVVAIVDAAAFT 37
MaHPL          ------------------------------------------------------------


PaAOS1         ILFDVSKVEKKDLFTGTYMPSTKLTGGYRVLSYLDPSEPRHAQLKNLLFFMLKNSSNRVI 138
AaAOS          TLFDVTKVEKKDLFTGTYMPSTELTGGHRVLSYLDPSEPKYAPLKNMVFFMLKNSIKKII 156
AtAOS          VLFDVDKVEKKDLFTGTYMPSTELTGGYRILSYLDPSEPKHEKLKNLLFFLLKSSRNRIF 151
LeAOS1         VLFDVSKVEKKDLFTGTFMPSTDLTGGYRVLSYLDPSEPNHAKLKKLMFYLLSSRRNEVI 130
StAOS1         ILFDVSKVEKKDLFTGTFMPSTDLTGGYRVLSYLDPSEPNHAKLKKLMFYLLSSRRNEVI 157
InAOS          TLFDPGKVEKRDLFTGTFMPSTELTGGYRILSYLDPSEPKHAQLKQLMFFLLSSRRGHVI 151
StAOS2         VLFDVSKVEKKDLFTGTYMPSTELTGGYRVLSYLDPSEPNHEKLKKLMFFLLSSRRDHVI 146
StAOS          VLFDVSKVEKKDLFTGTYMPSTELTGGYRVLSYLDPSEPNHEKLKKLMFFLLSSRRDHVI 146
LeAOS2         VLFDASKVEKKDLFTGTFVPSTELTGGYRILSYLDPSEPNHEKLKKLMFFLLSSRRDHVI 177
NaAOS          TLFDVSKVEKKDLFTGTFMPSTELTGGYRVLSYLDPSEPTHEKLKKLLFFLLSSRRDYII 142
GmAOS          VLFDNSKVEKKDVFTGTFMPSTELTGGYRVLSYLDPSEPKHALLKQLMFFLLKSRRAHVI 153
GmAOS1         ILFDNSKVDKRDVFTGTFMPSTQLTGGYRVLSYLDPSEPKHSLLKQLMFFLLKSRRAHVI 153
MtAOS          VLFDASKIDKTDVFTGTYTPSTELTGGYRVLSYLDPSEPKHEQLKKLMFFLLKSRSRHVI 152
CmAOS          VLFDHSKVEKKDLFTGTYMPVTELTGGYRVLSYIDPSEPDHAKLKQLIFFLLKHRRDKIM 153
LuAOS          VLFDMSKVEKKDLFTGTYMPSTELTGGYRILSYLDPSEPNHTKLKQLLFNLIKNRRDYVI 158
VvAOS          VLFDVSKVEKKDVFTGTFMPSTEFTGGFRVLSYLDPSEPDHTKLKRLLFFLLQSSRDRII 139
HvAOS1         VLFDVDKVEKKNLFTGTYMPSTSLTGGFRVCAYLDPSEPTHTKVKQLLFSLLASRKDAVI 140
HvAOS2         VLFDVTKVEKKNLFTGTYMPSTSLTGGFPVCSYLDPSEPTHTKVKQLLFSLLASRKDAFI 145
OsAOS2         VLFDVAKVEKRDVFTGTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFV 138
OsAOS3         VLFDVAKVEKRDVFTGTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFV 138
OsAOS1         VLFDVAKVEKRDVFTGTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFV 138
CsHPL1         ILFDTTKVEKRNILDGTYMPSLSFTGGIRTCAYLDPSETEHTVLKRLFLSFLASHHDRFI 139
CmHPL          ILFDTAKVEKRNILDGTYMPSLSFTGNIRTCAYLDPSETEHSVLKRLFLSFLASRHDRFI 142
VvHPL2         ILFDTSRIEKRNVLDGTYMPSTAFTGGYRVCAYLDPSEPNHALLKRLFTSSLAARHHNFI 85
VvHPLE         ILFDTSRIEKRNVLDGTYMPSTAFTGGYRVCAYLDPSEPNHALLKRFFTSSLAARHHNFI 85
VvHPLD         ILFDTSRIEKRNVLDGTYMPSTAFTGGYRVCAYLDPSEPNHALLKRLFMSSLAARHHNFI 85
VvHPLF         ILFDTSKVEKRNVLDGTYMPSTAFTGGYRVCAYLDPSETNHALLKRLFMSALAARHHNFI 150
VvHPLB         VLFDSSKVEKRNVLDGTFMPSTDLTGGYRVLAFLDPSEPKHDLLKRFSFSLLASRHRDFI 146
VvHPLC         VLFDSSKVEKRNVFVGTFMPSTDLTGGYRVLPYLDPSEPKHDLLKRFSFSLLASRHRDFI 146
MtHPL1         ILFDNAKVEKRDVLDGTFMPSTDFFGGYRTCAFQDTAEPSHSLLKRFIFHILSSKHDTFI 143
MtHPL2         ILFDNKKVEKLNVLDGTFMPSTKFTGGYRVCAYLDTTEPNHALIKGFYLNTLLLRKDTFI 146
StDES          CMFDNSLIDKTDTLGGTFKPGKEYYSGYRPVAFIDTKDPNHAALKGYILSAFAKRHNLFI 141
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LeDES           CMFDNSLIDKTDTLGGTFKPGKEYYGGYRPVAFIDTKDPNHAALKGYILSSFAKRHNLFI 141
CaDES           YMFDNTLIDKTDTLGGTFKPGKEYYGGYRPVAFVDTKDPNHAALKGYILSSFAKRHNLFI 141
NtDES           YMFDNSLIDKTDTLGGTFKPGKEYYGGYRPVAFVDTSDPNHAALKNYILTSFAKRHNLFI 142
StAOS3          ILFDNSQVDKENYFEGTFMSSPSFNGGYKVCGFLGTTDPKHTTLKGLFLSTLTRLHDKFI 154
LeAOS3          ILFDNSQVDKENYFEGTFMSSPSFNGGYKVCGFLGTSDPKHTTLKGLFLSTLTRLHDKFI 154
MsHPL1          HLFDMDLVDKRDVLVGDFVPSVEFTGNIRVGVYQDVSEPQHAKAKNFSMNILKQSSSIWV 156
MsHPL3          HLFDMDLVDKRDVLVGDFVPSVEFTGNIRVGVYQDVSEPQHAKAKNFSMNILKQSSSIWV 156
MsHPL2          HLFDMDLVDKRDVLVGDFVPSVEFTGNIRVGVYQDVSEPQHAKAKNFSMNILKQSSSIWV 156
AtHPL           HLFDMDLVDKRDVLIGDFRPSLGFYGGVCVGVNLDTTEPKHAKIKGFAMETLKRSSKVWL 130
LeHPL           HLFDMEIVEKANVLVGDFMPSVVYTGDMRVCAYLDTSEPKHAQIKNFSQDILKRGSKTWV 147
StHPL           HLFDMEIVEKANVLVGDFMPSEVYTGDMRVCAYLDTSEPKHAQIKNFSLDILKRSSKTWV 151
CaHPL           HLFDMEIVEKANVLVGDFMPSVVYTGDMRVCAYLDTSEPKHTQIKNFSLDILKRSSKTWV 151
NaHPL           HLFDMEIVEKANVLVGDFMPSVKYTGDMRVCAYLDTSEPKHTQIKNFSLDILKRSSKTWV 127
VvHPL1          FLFDMDVVEKKNVLVGDFMPSVKYTGDIRVCAYLDTAETQHARVKSFAMDILKRSSSIWA 130
VvHPLA          FLFDMDVVEKKNVLVGDFMPSVKYTGDIRVCAYLDTAETQHARVKSFAMDILKRSSSIWA 97
PgHPL           HLFDMEIVEKSNVLVGDFMPSVKYTGNIRVCAYLDTSEPQHAQVKNFAMDILKRSSKVWE 127
HvHPL           ALFDPELVDKRDCLIGPYNPSDSFTGGTRVGVYLDTEEPEHERTKAFAMDLLRRSSRVWA 139
ZmHPL           ALFDPDLVDKRDILIGPYNPGAGFTGGTRVGVYLDTQEEEHARVKTFAMDLLHRSARTWS 97
MaHPL           ------VVEKKNILIGDYMPSLSFTGDTRVVVYLDPSEPDHARVKSFCLELLRRGAKTWV 54


PaAOS1          PQFETTYT-ELFEGLEAELAKNG------KAAFNDVGEQAAFRFLGRAYFNSNPEET-KL 190
AaAOS           PEFQKTYN-ELFDELEAELSNKG------KAFFNDVGEQTAFRFLGRAYLNTNPEET-KI 208
AtAOS           PEFQATYS-ELFDSLEKELSLKG------KADFGGSSDGTAFNFLARAFYGTNPADT-KL 203
LeAOS1          PEFHNSYS-ELFETLENELSTKG------KAGLNAANDQAAVNFLARSLYGINPQDT-EL 182
StAOS1          PEFHNSYS-ELFETLENELSTKG------KARLNAANDQAAFNFLARSLYGINPQDT-KL 209
InAOS           PEFHRSFT-EMFEGLEKEVASKG------KVGLNAANDQAAFNFLARSWFGVDPAGT-KI 203
StAOS2          PKFHETYT-EFFETLDKEMAEKG------TAGLNSGNDQAAFNFLARSLFGVNPVET-KL 198
StAOS           PKFHETYT-EFFETLDKEMAEKG------TAGLNSGNDQAAFNFLARSLFGVNPVET-KL 198
LeAOS2          PEFHETYT-ELFETLDKEMEEKG------TVGFNSGSDQAAFNFLARSLFGVNPVET-KL 229
NaAOS           PQFHESYT-ELFKTLEKEMEKNG------KADLNSANDQAAFNFLARSLYGANPVET-KL 194
GmAOS           SEFHASYK-ELFHALEANLAEAG------KASFGDANDQAAFNFLSRSLFNSNPADT-KL 205
GmAOS1          SEFHASYK-DLFHELEANLAEAG------KASFGDANDQAAFNFLARSLFNSNPADT-KL 205
MtAOS           PEFQSCYR-EFFNALENQLAENG------HASFADNNDQAAFNFLNRALFGVNPVDT-EL 204
CmAOS           PEFHSTFS-ELFETLEKDLAAAG------RAEYNASGEQAAFNFLARSLFGADPVDS-KL 205
LuAOS           PEFSSSFT-DLCEVVEYDLATKG------KAAFNDPAEQAAFNFLSRAFFGVKPIDT-PL 210
VvAOS           PEFHSCFS-ELSETLESELAAKG------KASFADPNDQASFNFLARALYGTKPADT-KL 191
HvAOS1          PAFRSHFS-SLLATVESQLVLSG------KSNFNTLNDFTSFEFIADTYFGVLPSAS-DL 192
HvAOS2          PAFRSHFS-SLLATVESQLLLSG------KSNFNTLNDATSFEFIGDGYFGVLPSAS-DL 197
OsAOS2          PVFRSNFG-ALLDTVESQLASGGG-----KSDFTALNDATSFEFIGEAYFGVRPSASSSL 192
OsAOS3          PVFRSNFG-ALLDTVESQLASGGG-----KSDFTALNDATSFEFIGEAYFGVRPSASSSL 192
OsAOS1          PVFRSNFG-ALLDTVQSQLASGGG-----KSDFTALNDATSFEFIGKAYFGVRPSASSSL 192
CsHPL1          PLFRSSLS-EMFVKLEDKLADKNK-----IADFNSISDAVSFDYVFRLFSD-GTP-DSTL 191
CmHPL           PLFRSSLS-EMFVKLEDKLSEKKK-----IADFNSISDSMSFDYVFRLLSD-GTP-DSKL 194
VvHPL2          PVFRSCLT-ELFTTLEDDVSRKGK------ADFNGISDNMSFNFVFKLFCD-KHPSETKL 137
VvHPLE          PVFRSCLT-ELFTTLEDDVSRKGK------ADFNGISDNMSFNFVFKLFCD-KHPSETKL 137
VvHPLD          SVFRSCLT-ELFITLEDDASRKGK------ADFNGISDNMSFNFVFKLFCD-KHPSETKL 137
VvHPLF          PLFRSSLS-ELFTSLEDDISSKGE------ADFNDISDNMSFNFVFRLFCD-KYPSETAL 202
VvHPLB          PVFRSGLP-DLFTTIEDDVSSKGK------ANFNNIADGMYFNFVFRLICG-KDPSDAKI 198
VvHPLC          PVFRSGLP-DLFSTIEDDVSRKGK------ANFNDIADDMYFNFVFRLICG-KDPSDAKI 198
MtHPL1          PLFQTNLT-EHFTDLEKELAGKHQ-----KASFNTSIGGITFNFLFKLITD-KNPSETKI 196
MtHPL2          PLFKTILS-DGFNEIEDGLSSKSG-----KADFNSMVSVASFNFMFKLFCDDKNPSETIL 200
StDES           PLFRNSLSDHLFNNLEKQVTEQGK------SDFNALLPTMTFNFIFRLLCDQTNPSDTVL 195
LeDES           PLFRNTLSDHLFNNLEKQVTEQGK------ADFNALLPTMTFDFIFRLLCDQKNPSDTVL 195
CaDES           PLFRNSLSDHLFNDLEKQVSEQGK------SDFNALLPNMTFGFIFRLLCDQTNPSDTVL 195
NtDES           PLFRNSVSDHLFQNLEKQVSDQGK------SDFNALLPNMTFGFIFRLLCDQTNPSDTVL 196
StAOS3          PIFTTSIT-QMFTSLEKELSEKGT------SYFNPMSDNLSFEFLFRLFCEGKNPVDTSV 207
LeAOS3          PIFTTSIT-SMFTSLEKELSEKGT------SYFNPIGDNLSFEFLFRLFCEGKNPIDTSV 207
MsHPL1          PELISNLD-IFLDQIEATLSNS------SSASYFSPLQKFLFTFLSKVLARADPSLDPKI 209
MsHPL3          PELISNLD-IFLDQIEATLSNS------SSASYFSPLQKFLFTFLSKVLARADPSLDPKI 209
MsHPL2          PELISNLD-IFLDQIEATLSKS------SSASYFSPLQQFLFTFLSKVLARADPSLDSKI 209
AtHPL           QELRSNLN-IFWGTIESEISKN------GAASYIFPLQRCIFSFLCASLAGVDASVSPDI 183
LeHPL           PTLLKELD-TMFTTFEADLSKS------NTASLLPALQKFLFNFFSLTILGADSSVSPEI 200
StHPL           PTLLKELD-TMFTTFEADLSKS------KEASLLPALQKFLFNFFSLTLLGADPSVSPEI 204
CaHPL           PTLVKELD-TLFGTFESDLSKS------KSASLLPALQKFLFNFFSLTFLGADPSASPEI 204
NaHPL           PTLVNELN-SMFETFESDISKS------NSASLLPTMQKFLFNFFSLSLLGANPSASPEI 180
VvHPL1          SEVVASLD-TMWDTIDAGVAKS------NSASYIKPLQRFIFHFLTKCLVGADPAVSPEI 183
VvHPLA          SEVVASLD-TMWDTIDAGVAKS------NSASYIKPLQRFIFHFLTKCLVGADPAVSPEI 150
PgHPL           SEVISNLD-TMWDTIESSLAKD------GNASVIFPLQKFLFNFLSKSIIGADPAASPQV 180
HvHPL           PEFLEGVD-GMLAAIESDLAAG----KEGGASFLVPLQRCIFRFLCRSVASADPAAEGLV 194
ZmHPL           ADFRASVG-AMLDAVDAEFGKDDGSDKKPSASYLVPLQQCIFRFLCKAFVGADPSADWLV 156
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MaHPL          SSFLSNLD-VMLATIEQGIAKD------GSAGLFGPLQKCIFAFLCKSIIGADPSVSPDV 107


PaAOS1         GTSAPTLISSWVLFNLAPTLDLGLP---WFLQEPLLHTFRLPAFLIKSTYNKLYDYFQSV 247
AaAOS          GKDGPKLIGTWVLFNLGPLLRLGLP---WFVEEPLLHTFRLPAALVKKNYNKLYDFFESC 265
AtAOS          KADAPGLITKWVLFNLHPLLSIGLP---RVIEEPLIHTFSLPPALVKSDYQRLYEFFLES 260
LeAOS1         GTDGPKLIGKWVLFQLHPLLILGLP---KVLEDLVMHTFRLPPALVKKDYQRLYNFFYEN 239
StAOS1         GTDGPKLIGKWVLFQLHPLLILGLP---KVLEDLVMHTFRLPPALVKKDYQRLYNFFYEN 266
InAOS          GNDGPNLVGKWVVFNLHPLLVLGLP---KGLEEALLHTFRLPAALVKKDYQRLYEFFYAN 260
StAOS2         GTDGPTLIGKWVLLQLHPVLTLGLP---KFLDDLILHTFRLPPFLVKKDYQRLYDFFYTN 255
StAOS          GTDGPTLIGKWVLLQLHPVLTLGLP---KFLDDLILHTFRLPPFLVKKDYQRLYDFFYTN 255
LeAOS2         GTDGPALIGKWILLQLHPVITLGLP---KFLDDVLLHTFRLPPILVKKDYQRLYDFFYTN 286
NaAOS          GTDGPTLIGKWVLFQLHPLLTLGLP---KVLDDFLLHNFRLPPALVKKDYQRLYDFFYES 251
GmAOS          GLDGPKIVQKWVLFQIGPILRLGLP---QFLEESTIRTFRLPFSLIKKDYQRLYDFFYES 262
GmAOS1         GRDGPKIVQKWVLFQLGPILRLGLP---QFLEESTIRSFRLPFSLIQKDYQRLYDFFYQS 262
MtAOS          GLDGPKMVQKWVLFQLGPVLKLGLP---KFVEDSMIHNFRLPFRLIKKDYQRLYDFFYAS 261
CmAOS          GRDAPKLIAKWVLFQLGPVLSLGLP---KVVEELLLRTVRLPPALIKADYRRLYDFFYKS 262
LuAOS          GKDAPSLISKWVLFNLAPILSVGLP---KEVEEATLHSVRLPPLLVQNDYHRLYEFFTSA 267
VvAOS          GTDGPGLITTWVVFQLSPILTLGLP---KFIEEPLIHTFPLPAFLAKSSYQKLYDFFYDA 248
HvAOS1         GTTGPAKAAKWLIFQLHPLVTFGLP---MILEEPLLHTVLLPPIFVSGDYKALYKYFYAA 249
HvAOS2         GTTGPAKAAKWLIFQLHPLVTLGLP---MILEEPLLHTVHLPPFLVSGDYKALYKYFFAA 254
OsAOS2         GTGGPTKAALWLLWQLAPLTTLGLP---MIIEDPLLHTLPLPPFLISSDYKALYAYFAAA 249
OsAOS3         GTGGPTKAALWLLWQLAPLTTLGLP---MIIEDPLLHTLPLPPFLISSDYKALYAYFAAA 249
OsAOS1         GTGGLDQGRLWLLWQLAPLTTLGLP---MIIEDPLLHTLPLPPFLISSDYKALYAYFAAA 249
CsHPL1         AADGPGMFDLWLGLQLAPLASIGLPKIFSVFEDLIIHTIPLPFFPVKSRYRKLYKAFYSS 251
CmHPL          AAEGPGMFDLWLVFQLAPLASIGLPKIFSVFEDLVIHTIPLPFFPVKSGYRKLYEAFYSS 254
VvHPL2         GSNGPNLVTKWLFLQLAPFITLGLSMLPNVVEDLLLHTFPLPSLFVKSDYKKLYHAFYAS 197
VvHPLE         GSNGPNLVTKWLFLQLAPLITLGLSMLPNVVEDLLLHTFPLPSLFVKSDYKKLYHAFYAS 197
VvHPLD         GSNGPNLVTKWLFLQLAPLITLGLSMLPNVVEDLLLHTFPLPSLFVKSDYKNLYHAFYAS 197
VvHPLF         GSQGPSIVTKWLFFQLAPLITLGLSLLPNFVEDLLLHTFPLPSIFVKSDYKKLYRAFYAS 262
VvHPLB         RSEGPNIFSKWLFLQLSPLMTLGLSMLPNFIEDLLLHTFPLPPFLVKSDYNKLYKAFYES 258
VvHPLC         RSEGPNIFLKWLFLQLSPLLTLGLSILPNFIDDLLLHTFPFPPFLVKSDYNKLYKAFYES 258
MtHPL1         GDSGPTLVQTWLAAQLAPLATAGLPKIFNYLEDVLIRTIPIPAWTVKSSYNKLYEGLMEA 256
MtHPL2         GDQGPKMFDTWLLFQLAPLATLGPPKIFNYLEDILLRTVPFPACLTRSSYKKLYEAFSTS 260
StDES          GAQGPEHLRKWLFPQLIP--SLSAKKLPNIIEDTLFHNFLIPFGFIKSDYNKLVDAFSKS 253
LeDES          GAQGPEHLRKWLFPQLIP--SLSAKKLPNIIEDMLFHNFLIPFGFIKSDYNKLVDAFSKS 253
CaDES          GAQGPEHLRKWLFPQLIP--SLSARKLPSFIEDLLFHNFLIPFGFVKSDYQKLVDAFSKS 253
NtDES          GAQGPEHLRKWLFPQLIP--SLSARKLPSFIEDLLFHNFLIPFGLVKSDYNKLVDAFSKN 254
StAOS3         GTNGPKIVDKWVFLQLAPLISLGLKFVPNFLEDLVLHTFPLPYFLVKGDHQKLYNAFYNS 267
LeAOS3         GPNGPKIVDKWVFLQLAPLISLGLKFVPNFLEDLVLHTFPLPYILVKRDHQKLYNAFYNS 267
MsHPL1         AESGSSMLNKWLAVQLLPTVSVGTI---QPLEEIFLHSFSYPYALVSGDYKNLYNFIKQH 266
MsHPL3         AESGSSMLNKWLAVQLLPTVSVGTI---QPLEEIFLHSFSYPYALVSGDYKNLYNFIKQH 266
MsHPL2         AESGSSMLNKWLAVQLLPTVSVGTI---QPLEEIFLHSFSYPYALVSGDYNNLYNFIKQH 266
AtHPL          AENGWKTINTWLALQVIPTAKLGVVP--QPLEEILLHTWPYPSLLIAGNYKKLYNFIDEN 241
LeHPL          ANSGYIFLDSWLAIQLAPTVSIGVL---QPLEEILVHSFAYPFFLVKGNYEKLVQFVKNE 257
StHPL          ANSGYIFLDSWLAIQLAPTVSIGVL---QPLEEILVHSFAYPFFLVKGNYEKLVQFVKNE 261
CaHPL          ANSGFAYLDAWLAIQLAPTVSIGVL---QPLEEIFVHSFSYPYFLVRGGYEKLIKFVKSE 261
NaHPL          ANSGYVMLDTWLAIQLAPTVSIGLL---QPLEEIFVHSFNYPFFLVKGSYEKLIQFVKNE 237
VvHPL1         AESGYVMLDKWVFLQLLPTISVNFL---QPLEEIFLHSFAYPFFLVKGDYRKLYDFVEQH 240
VvHPLA         AESGYVMLDKWVFLQLLPTISVNFL---QPLEEIFLHSFAYPFFLVKGDYRKLYEFVEQH 207
PgHPL          AKSGYAMLDRWLALQLLPTINIGVL---QPLVEIFLHSWAYPFALVSGDYNKLYQFIEKE 237
HvHPL          DRYGLFILDVWLGLQLLPTQKVGAIX--QPLEELLLHSFPFPSILAKPGYDLLYRFVAKH 252
ZmHPL          DNFGFTILDIWLALQILPTQKIGLV---QPLEELLIHSFPLPSFLIWPGYYVLYRFIEKH 213
MaHPL          GENGFVMLDKWLALQLLPTVKVGAIP--QPLEEILLHSFPLPFFLVSRDYRKLYEFVEKQ 165


PaAOS1         ATPVMEQA-EKLGVPKDEAVHNILFAVCFNTFGGVKILFPNTLKWIGLAG-ENLHTQLAE 305
AaAOS          SGEIIEHA-KSLGLEKDEAVHNILFTLCFNTFGGIKILFPNTLKWLGRAG-TNLHTQLAE 323
AtAOS          AGEILVEA-DKLGISREEATHNLLFATCFNTWGGMKILFPNMVKRIGRAG-HQVHNRLAE 318
LeAOS1         STSVLDEA-EKIGISREEACHNLLFATCFNSFGGIKIFFPNMLKWIGRAG-AKLHSQLAQ 297
StAOS1         STSVLDEA-EKIGISREEACHNLLFATCFNSFGGIKIFFPNMLKWIGRAG-AKLHSQLAQ 324
InAOS          STEILDEA-ENLGLSREEACHNLLFATCFNSFGGMKIFFPNMIKWIGRGG-AKLHAQLAR 318
StAOS2         SASLFAEA-EKLGISKEEACHNLLFATCFNSFGGMKIFFPNMLKSIAKAG-VEVHTRLAN 313
StAOS          SASLFAEA-EKLGISKEEACHNLLFATCFNSFGGMKIFFPNMLKSIAKAG-VEVHTRLAN 313
LeAOS2         SANLFIEA-EKLGISKDEACHNLLFATCFNSFGGMKIFFPNMLKSIAKAG-VEIHTRLAN 344
NaAOS          STAVLNEA-GNFGISRDEACHNLLFATCFNSFGGMKIFFPNMLKWIARAG-VELHIRLAN 309
GmAOS          SGLVLDEA-ERLGITRDEACHNLLFATCFNSFGGMKLFFPNVLKWIGRAG-VKLHARLAE 320
GmAOS1         SGSVLDEA-ERLGITRDEACHNLLFATCFNSFGGMKLFFPNVLKWIGRAG-VKLHARLAE 320
MtAOS          SGFALEEA-ERLDVSKEEACHNLLFATCFNSFGGMKLFFPNLMKWIGRGG-VRLHTKLAT 319
CmAOS          SEAVFEEA-DRLGISREEACHNLLFTTCFNSFGGMKIFFPNMIKWIGRAG-VNLHTRLAR 320
LuAOS          AGSVLDEA-EQSGISRDEACHNILFAVCFNSWGGFKILFPSLMKWIGRAG-LELHTKLAQ 325
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VvAOS          STHVLDEG-EKMGISREEACHNLLFATCFNSFGGMKIIFPTILKWVGRGG-VKLHTQLAQ 306
HvAOS1         ATKALDMA-ESLGLNRDEACHNLLFATVFNSYGGLKVMLPGFLGRIAEAG-EKFHQRLAA 307
HvAOS2         ATKALDTA-EGLGLKRDEACHNLLFATVFNSYGGLKVLLPGILARIADSG-EKFHKKLVT 312
OsAOS2         ASQALDAA-EGLGLSREEACHNLLFATVFNSYGGFKLLLPQILSRVAQAG-EKLHERLAA 307
OsAOS3         ASQALDAA-EGLGLSREEACHNLLFATVFNSYGGFKLLLPQILSRVAQAG-EKLHERLAA 307
OsAOS1         ASQALDAA-EGLGLSREEACHNLLFATVFNSYGGFKLLLPQILSRVAQAG-EKLHERLAA 307
CsHPL1         SGSFLDEA-EKQGIDREKACHNLVFLAGFNAYGGMKVLFPTILKWVGTGG-EDLHRKLAE 309
CmHPL          SGSFLDEA-EKQGIDREKACHNLVFLAGFNAYGGMKVLFPTLLKWVGTAG-EDLHRKLAE 312
VvHPL2         ASSILDEA-ESMGIKRDEACHNLVFLAGFNACGGMKTLFPALIKWVGLAG-EKLHRQLAD 255
VvHPLE         ASSLLDEA-ESMGIKRDEACHNLVFLAGFNAYGGMKTLFPALIKWVGLAG-GKLHRQLAD 255
VvHPLD         ASSILDEA-ESMGIKRDEACHNLVFLAGFNAYGGMKTLFPALIKWVGLAG-EKLHGQLAD 255
VvHPLF         ASSILDEA-ESMGIKRDEACHNLVFLAGFNAYGGMKALFPSLIKWVGSAG-EKLHRELAD 320
VvHPLB         ASSVLDEG-ERMGINRDEACHNLVFLAGFSTFGGMKVLFPPLIKWVGLAG-EKLHRELAD 316
VvHPLC         ASSVLDEG-ERMGIKRDEACHNLVFLAGFNSFGGMKVFFPALIKWVGLAG-EKLHRELAD 316
MtHPL1         GTTVLDEA-EKMGIKREEACHNLVFTLGFNAFGGLTNQFPILIKWVGLAG-ADLHKKLAD 314
MtHPL2         ATTMLNEA-EKAGLKRSEALHNIIFTAGFNAYGGLKNQFPILFKWLGSSG-EELHKELAN 318
StDES          AVSILDEA-EKLGIKREEAVQNILFLVGINMFAGLNAFSPHLFRFVGEAG-ASLHTQLAK 311
LeDES          AVSMLDEA-EKLGIKREEAVQNILFLVGINMFAGLNAFFPHLFRFVGEAG-ASLHTQLAK 311
CaDES          AVSMLDEA-EKLGIKREEAVHNMLFLVGINMFAGLNAFFPHLIRFVGEAG-PNLHTRLAN 311
NtDES          AGSMLDEA-EKLGIKREEAVHNILFLVGINMFAGLNAFFPHLIRFVGEAG-PTLHARLAK 312
StAOS3         MKDILDEA-EKLGVKREEACHNFIFLAGFNSYGGMKVFFPSLIKWIGTSG-PTLHTRLVK 325
LeAOS3         MKDILDEA-EKLGVKRDEACHNFVFLAGFNSYGGLKVFFPSLIKWIGTSG-PSLHARLVK 325
MsHPL1         GKEVIKNG-TEFGLSEDEAIHNLLFVLGFNSYGGFSIFLPKLIESITNGP-TGLQEKLRK 324
MsHPL3         GKEVIKSG-TEFGLSEDEAIHNLLFVLGFNSYGGFSIFLPKLIESIANGP-TGLQEKLRK 324
MsHPL2         GKEVIKSG-TEFGLSEDEAIHNLLFVLGFNSYGGFSIFLPKLIESIANGP-TGLQEKLRK 324
AtHPL          AGDCLRLGQEEFRLTRDEAIQNLLFVLGFNAYGGFSVFLPSLIGRITGDN-SGLQERIRT 300
LeHPL          AKEVLSRAQTEFQLTEQEAIHNLLFILGFNAFGGFSIFLPTLLGNLGDEKNADMQEKLRK 317
StHPL          AKEVLNRAQTEFQLTEQEAIHNLLFILGFNAFGGFTIFLPTLLGNLGDEKNAEMQEKLRK 321
CaHPL          AKEVLTRAQTDFQLTEQEAIHNLLFILGFNAFGGFTIFLPTLLGNLGDEKNAEMQEKLRK 321
NaHPL          AKEVLNRGKSEFGLTEQEAIHNLLFILGFNAFGGFSIFLPTLLGNLGDEKNAELQEKLRN 297
VvHPL1         GQAVLQRGETEFNLSKEETTHNLLFVLGFNAFGGFTIFFPSLLS-ALSGK-PELQAKLRE 298
VvHPLA         GQAVLQRGETEFNLSKEETIHNLLFVLGFNAFGGFTIFFPSLLS-ALSGK-PELQAKLRE 265
PgHPL          GREAVERAKAEFGLTHQEAIHNLLFILGFNAFGGFSIFLPTLLSNILSDT-TGLQDRLRK 296
HvHPL          GAESVAVGVTNHGMSEKDAINNILFLLGFNAFGGFSVFLPFLILQIG-KD-AALRARLRD 310
ZmHPL          GAEAVAYAEAQHGIGKKDAINNILFVLGFNAFGGFSVFLPFLVAKVG-GA-PALRERLRD 271
MaHPL          GQEVVRRAETEHGLSKHDAINNILFVLGFNAFGGFSVFFPTLLTTIGRDK-TGLREKLKD 224


PaAOS1         EIRGAIKSYGD-GNVTLEAIEQ-MPLTKSVVYESLRIEPPVPPQYGKAKSNFTIESHD-A 362
AaAOS          EIRNAIKVHGG-GKVTMAAMEQ-MPLMKSVVYESLRIEPPVALQYGKAKKDMTIESHD-A 380
AtAOS          EIRSVIKSNG--GELTMGAIEK-MELTKSVVYECLRFEPPVTAQYGRAKKDLVIESHD-A 374
LeAOS1         EIRSVISSNSG--KVTMAAMEK-MPLMKSVVYESLRIEPPVASQYGRAKHDMVIESHD-A 353
StAOS1         EIRSVISSNSG--KVTMAAMEK-MPLMKSVVYESLRIEPPVASQYGRAKHDMVIESHD-A 380
InAOS          EIRSVVKSNGG--KVTMAGMEQ-MPLMKSVVYEALRIEPPVPAQYGRAKRDFVVESHD-A 374
StAOS2         EIRSEVKSAGG--KITMSAMEK-MPLMKSVVYEALRVDPPVASQYGRAKQDLKIESHD-A 369
StAOS          EIRSEVKSAGG--KITMSAMEK-MPLMKSVVYEALRVDPPVASQYGRAKQDLKIESHD-A 369
LeAOS2         EIRSEVKSAGG--KITMSAMEK-MPLMKSVVYEALRVDPPVASQYGRAKQDLKIESHD-A 400
NaAOS          EIRSAVKSAGG--KITMSAMEK-MPVMKSVVYEALRIDPPVASQYGRAKRDLMIESHD-G 365
GmAOS          EIRSAVRSGGG--EISMAAMEK-MPLMKSVVYEAFRIDPPVALQFGRAKRDLIIESHD-H 376
GmAOS1         EIRSAVRGAGG--EITMAAMEN-MPLMKSVVYEAFRIDPPVPLQFGRAKRDLIIESHD-H 376
MtAOS          EIREAVRSAGG--EITMAAMEN-MPLMKSVVYEAFRIDPPVPLQFGRAKRDMVIENHE-N 375
CmAOS          EIRTAVKANGG--KITMGAMEQ-MPLMKSVVYEALRIEPPVPVQYGRAKKDLVVESHD-A 376
LuAOS          EIRSAIQSTGG-GKVTMAAMEQ-MPLMKSVVYETLRIEPPVALQYGKAKKDFILESHE-A 382
VvAOS          EIRSVVKSNGG--KVTMASMEQ-MPLMKSTVYEAFRIEPPVALQYGKAKQDLVIESHD-S 362
HvAOS1         EVRTAVADAGG--KVTIEALEK-MELTKSAVWEALRLEPPVKFQYGRAKVDMNIESHD-A 363
HvAOS2         EIRAAVAEAGG--KVTIEALEK-MELTKSAVWEALRLDPAVKFQYGRAKADMNIESHD-A 368
OsAOS2         EIRSAVADAGG--NVTLAALEK-MELTRSVVWEALRLDPPVRFQYGRAKADLEIESHD-A 363
OsAOS3         EIRSAVADAGG--NVTLAALEK-MELTTSVVWEALRLDPPVRFQYGRAKADLEIESHD-A 363
OsAOS1         EIRSAVADAGG--NVTLAALEK-MELTRSVVWEALRLDPPVRFQYGRAKADLEIESHD-A 363
CsHPL1         EVRTTVKEEGG---LTFSALEK-MSLLKSVVYEALRIEPPVPFQYGKAKEDIVIQSHD-S 364
CmHPL          EVRTTVKEEGG---LTFSALEK-MSLLKSVVYEALRIEPPVPFQYGKAKEDIVIQSHD-S 367
VvHPL2         EIRSIVKAEGG---VTFAALDK-MALTKSVVYEALRIEPPVPFQYGKAKEDMVIHSHD-A 310
VvHPLE         EIRSIVKAEGG---VTFAALDK-MALTKSVVYEALRIEPPVPFQYGKAKEDMVIHSHD-A 310
VvHPLD         EIRSIVKAEGG---VTFAALDK-MALTKSVVYEALRIEPPVPFQYGKAKEDMVIHSHD-A 310
VvHPLF         EIRTVVKAEGG---VSFAALEK-MSLTKSVVYEALRIDPPVPFQYGKAKEDMVIHSHD-A 375
VvHPLB         EIRTVVKAEGG---VTFAALDK-MALTKSVVYEALRIGPPVPFQYGKAREDMVIHSHD-A 371
VvHPLC         EIRTVIKAEGG---VTFAALDK-MALTKSMVYEALRIEPPVPFQYGKAREDMVIHSHD-A 371
MtHPL1         EIRAIVREEGG---VNLYALDK-MTLTKSTVYEALRIEPAVPYQYAKAREDLVVQSHD-A 369
MtHPL2         EIRTVVKQEGG---VTIQSLEK-MPLVKSVVYEAMRIEPAVPYQYAKAREDLIVKSHD-A 373
StDES          EIRTVIKEEGGA--ITLSAINK-MSLVKSVVYETLRLRPPVPLQYGKAKKDFMVQSHD-A 367
LeDES          EIRSVIKEEGGA--ITLSAINK-MSLVKSVVYETLRLRPPVPLQYGKAKKEFMVQSHD-A 367
```

```
CaDES           EIRTAIKEEGGA--ITLSAINK-MSLVKSVVYETLRLRPPVPLQYGKAKKDFMVQSHD-A 367
NtDES           EIRTAIKEEGGA--VTLSAINK-MSLVESIVYETLRLRPPVPLQYGKAKKDFMVQSHD-A 368
StAOS3          EIRTAVKEAGG---VTLSAIDK-MPLVKSVVYETLRMDPPVPFQTVKARKNIIVSNHE-A 380
LeAOS3          EIRTAVKEAGG---VTLSAIDK-MPLVKSVVYETLRMDPPVPFQTVKARKNIIITNHE-S 380
MsHPL1          EAREKGGS-----TLGFDSLKE-LELINSVVYETLRMNPPVPLQFGRARKDFQLSSYD-S 377
MsHPL3          EAREKGGS-----TLGFDSLKE-LELINSVVYETLRMNPPVPLQFGRARKDFQLSSYD-F 377
MsHPL2          EAREKGGS-----TLGFDSLKE-LELINSVVYETLRMNPPVPLQFGRARKDFQLSSYD-S 377
AtHPL           EVRRVCGSG-S--DLNFKTVNE-MELVKSVVYETLRFNPPVPLQFARARKDFQISSHD-A 355
LeHPL           EVRDKVGVN-PE-NLSFESVKE-MELVQSFVYETLRLSPPVPSQYARARKDFKLSSHD-S 373
StHPL           EVRDKVGVN-PE-NLSFESVKE-MELVQSFVYETLRLTPPVPSQYARARKDFKLSSHD-S 377
CaHPL           EVREKVGTN-QE-NLSFESVKE-MELVQSFVYESLRLSPPVPSQYARARKDFMLSSHD-S 377
NaHPL           EVREKVGLK-TE-NLSFESVKE-MELVQSFVYETLRLSPPVPSQYARARKDFKLSSHD-S 353
VvHPL1          EVRSKIKPG-T--NLTFESVKD-LELVHSVVYETLRLNPPVPLQYARARKDFQLSSHD-S 353
VvHPLA          EVRSKIKPG-T--NLTFESVKD-LELVHSVVYETLRLNPPVPLQYARARKDFQLSSHD-S 320
PgHPL           EVRAKGGP-----ALSFASVKE-MELVKSVVYETLRLNPPVPFQYARARKDFQLKSHD-S 349
HvHPL           EVRAALDQH-DG-EVGFASVKG-MPLVRSTVYEVLRMNPPVPLQFGRARRDFVLRSHGGE 367
ZmHPL           EVRRAMVGK-DG-EFGFATVREGMPLVRSTVYEMLRMQPPVPLQFGRARRDFVLRSHGGA 329
MaHPL           EVRRVMKSRGEK-RPSFETVRE-MELVRSTVYEVLRLNPPVPLQYGRARTDFTLNSHD-A 281


PaAOS1          TFEVKKGEMLFGYQPFATKDPKVFD-RPEEYVPDRFVGD-GEALLKYVWWSNGPETESPT 420
AaAOS           VFKVKEGEMLFGYQPFATKDPKIFD-RPEESVPDRFVGE-GEKLLKYVTWSNGPETETPT 438
AtAOS           AFKVKAGEMLYGYQPLATRDPKIFD-RADEFVPERFVGEEGEKLLRHVLWSNGPETETPT 433
LeAOS1          SFEIKEGELLYGYQPFATKDPKIFD-RSEEFVADRFKGEEGEKLLKHVLWSNGSETENAS 412
StAOS1          SFEIKEGELLYGFQPFATKDPKIFD-RSEEFVADRFIGEEGEKLLKHVLWSNGSETENPS 439
InAOS           VFEVKEGEMLFGFQPFATKDPKIFD-RAEEFVPDRFTGENANELLSHVLWSNGPETESPT 433
StAOS2          VFEVKKGEMLFGYQPFATKDPKIFD-RPEEFVADRFVGE-GEKLLKYVLWSNGPETESPT 427
StAOS           VFEVKKGEMLFGYQPFATKDPKIFD-RPEEFVADRFVGEEGEKLLKYVLWSNGPETESPT 428
LeAOS2          VFEVKKGEILFGYQPFATKDPKIFD-RPGEFVADRFVGEEGEKLLKHVLWSNGPETESPT 459
NaAOS           VFEVKKGEMLFGYQPFATRDPKIFD-RPDEFVPDRFVGEEGEKLLKHVLWSNGPETESPT 424
GmAOS           AFQVKEGEMLFGYQPFATKDPRIFE-RAEEFVGDRFVGEEGEKLLKHVLWSNGPETESPT 435
GmAOS1          AFQVKEGEMLFGYQPFATKDPRIFE-RAEEFVGDRFVGEEGEKLLKHVLWSNGPETESPT 435
MtAOS           GFLVKKGELLLGYQPFATKDPKIFE-RAEEFVADRFVGDEGEKLLKHVLWSNGPESQSPT 434
CmAOS           AFEIKEGEVICGYQPFATRDPKIFD-RADELVPDRFTGE-GEELLKHVIWSNGPETQSPS 434
LuAOS           AYQVKEGEMLFGYQPFATKDPKIFD-RPEEFVADRFVGE-GVKLMEYVMWSNGPETETPS 440
VvAOS           VFEVKEGEMLFGYQPFATKDPKIFE-RSEEFVPDRFVGE-GEKLLKHVLWSNGPETENPT 420
HvAOS1          VFAVQKGEMLFGYQPCATKDPRVFGSTAREFVGDRFVG-EGSKLLQYVYWSNGRETESPS 422
HvAOS2          VFAVKKGEMLFGYQPCATKDPRVFGPTAREFVGDRFVGKEGSKLLKYVYWSNGRETESPS 428
OsAOS2          SFAIKKGEMLFGYQPCATRDPRVFGATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPS 423
OsAOS3          SFAIKKGEMLFGYQPCATRDPRVFGATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPS 423
OsAOS1          SFAIKKGEMLFGYQPCATRDPRVFGATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPS 423
CsHPL1          CFKIKKGETIFGYQPFATKDPKIFK-DSEKFVGDRFVGEEGEKLLKYVYWSNERETVEPT 423
CmHPL           SFKIKKGETIFGYQPFATKDPKIFK-DSEKFVGDRFVGEEGEKLLKYVYWSNERETVEPT 426
VvHPL2          AFVIKKGEMIFGYQPFATKDPKVFD-NPEEFVAHRFMGD-GEKLLEYVYWSNGRESDDAT 368
VvHPLE          AFEIKKGEMIFGYQPFATKDPKVFD-NPEEFVAHRFMGD-GEKLLEYVYWSNGRESDDPT 368
VvHPLD          AFEIKKGEMIFGYQPFATKDPKVFD-NPEEFVAHRFMGD-GEKMLEYVYWSNGRESDDPT 368
VvHPLF          AFEIKKGEMIFGYQPFATKDPKVFD-NPEEFMGNRFMGE-GERLLKYVYWSNGRESGNPT 433
VvHPLB          AFEIKKGEMIFGYQPFATKDPKVFE-NPEDFVAHRFMGE-GEKLLKYVYWSNGRETDNPT 429
VvHPLC          AFEIKKGEMIFGYQPFATKDPKVFE-NPEEFVAHRFMGE-GEKLLKYVYWSNGRETDNPT 429
MtHPL1          SFEIKKGEMIFGYQPFATKDAKIFD-KPEDFIAERFIGD-GEKLLKHVFWSNGRETDEAT 427
MtHPL2          AFEIKKGEMIFGYQPFATKDPRVFD-DPEVFVAKRFVGE-GEKLLKYVLWSNGKETEEPS 431
StDES           SYKINKGQFVVGYQPMASRDPKIFA-NPDEFVPDRFMND-GEKMLKHVLWSNGRETENPA 425
LeDES           SYKINKGQFVVGYQPMASRDPKIFA-NPDEFVPDRFMND-GEKMLKHVLWSNGRETESPA 425
CaDES           SYKINKGQFLVGYNPMASRDPKIFA-NPDEFVPDRFMGD-GEKMLKHVLWSNGRETENPA 425
NtDES           SYMIKKGQFLVGYQPMASRDPKIFD-KPDDFIPDRFMGE-GVKMLKHVLWSNGRETENPA 426
StAOS3          SFLIKKDELIFGYQPLATKDSKVFK-NAEEFNPDRFVGY-GEKLLKYVYWSNGKETDNPT 438
LeAOS3          SFLIKKDELIFGYQPLATKDSKVFK-NAEEFNPDRFVGG-GEKLLKYVYWSNGKEIDNPS 438
MsHPL1          AFNVKKGELLCGFQKLVMRDPVVFD-EPEQFKPERFTKEKGAELLNYLYWSNGPQTGSPT 436
MsHPL3          AFNVKKGELLCGFQKLVMRDPVVFD-EPEQFKPERFTKEKGAELLNYLYWSNGPQTGSPT 436
MsHPL2          AFNVKKGELLCGFQKLIMRDPVVFD-EPEQFKPERFTKEKGAELLNYLYWSNGPQTGSPT 436
AtHPL           VFEVKKGELLCGYQPLVMRDANVFD-EPEEFKPDRYVGETGSELLNYLYWSNGPQTGTPS 414
LeHPL           VYEIKKGELLCGYQPLVMKDPKVFD-EPEKFVLERFTKEKGKELLNYLFWSNGPQTGRPT 432
StHPL           VYEIKKGELLCGYRPLVMKDPKVLD-EPEKFVLERFTKEKGKELLNYLFWSNGPQTGRPT 436
CaHPL           VYEIKKGELLCGYQPLVMKDPKVFD-EPEKFMLERFTKEKGKELLNYLFWSNGPQTGSPT 436
NaHPL           VYEIKKGELLCGYQPLVMRDPKVFD-DPEKFVLERFTKEKGKELLNYLFWSNGPQTGRPT 412
VvHPL1          VFEIKKGDLLCGFQKVAMTDPKIFD-DPETFVPDRFTKEKGRELLNYLFWSNGPQTGSPS 412
VvHPLA          VFEIKKGDLLCGFQKVAMTDPKIFD-DPETFVPDRFTKEKGRELLNYLFWSNGPQTGSPS 379
PgHPL           VFDVKKGELLCGYQKVVMTDPKVFD-EPESFNSDRFVQNS--ELLDYLYWSNGPQTGTPT 406
HvHPL           GFSVAGGEMLCGYQPLAMRDPEVFE-RPEEFVADRFVGAGGEALLRYVYWSNGPETGEPA 426
ZmHPL           AYQVSAGEVLCGYQPLAMRDPEVFE-RPEEFVPERFLGDEGARLLQHLFWSNGPETAQPG 388
MaHPL           AFKVEKGELLCGYQPLVMRDPAVFD-DPETFAPERFMGS-GKELLKYVFWSNGPETGTPT 339
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PaAOS1          VENKQCAGKDFVVLITRLFVIELFRRYDSFEIELGESPLG------AAVTLTFLKRASI- 473
AaAOS           AGNKQCAGKDFVVLITRLFVIELFRRYDSFDIEVGASPLG------AKITLTSLKRARV- 491
AtAOS           VGNKQCAGKDFVVLVARLFVIEIFRRYDSFDIEVGTSPLG------SSVNFSSLRKASF- 486
LeAOS1          INNKQCAGKDFVVLVSRLLLVELFLRYDSFEIEVGASPLG------AAITLTSLRRASF- 465
StAOS1          INNKQCAGKDFVVLVSRLLLVELFLRYDSFEIEVGASPLG------AAITLTSLRRASF- 492
InAOS           VNNKQCAGKDFVVLVSRLMVVELFLRYDSFDIEVGTSPLG------ASVTVTSLKRASF- 486
StAOS2          VGNKQCAGKDFVVMVSRLFVTEFFLRYDTFNVDVGKSALG------ASITITSLKKA--- 478
StAOS           VGNKQCAGRDFVVMVSRLFVTEFFLRYDTFNVDVDKSALG------ASITITSLKKA--- 479
LeAOS2          VGNKQCAGKDFVVMVSRLFVTEFFLRYGTLNVDVGTSALG------SSITITSLKKA--- 510
NaAOS           VENKQCAGKDFVVLVSRLLVTEFFLRYDTLDIDVGTSPLG------AKITITSLKRA--- 475
GmAOS           LGNKQCAGKDFVTLVSRLFVVEFFLRYDSFEIQVGTSPLG------SSVTITSLKRASF- 488
GmAOS1          IGNKQCAGKDFVTLVSRLLVVEFFLRYDSFEIQVGTSPLG------SSVTITSLKRASF- 488
MtAOS           VGNKQCAGKDFTTLISRLLVVELFLRYDSFEIQVGNSPLG------PSITLTSLKRSSF- 487
CmAOS           VQNKQCAGKDFIVFISRLLVVELFLRYDSFDIEASNTPLGAAVTVSAAVTVTSLKKASF- 493
LuAOS           VANKQCAGKDFVVMAARLFVVELFKRYDSFDIEVGTSSLG------ASITLTSLKRSTF- 493
VvAOS           LGNKQCAGKDFVVLAARLFVVELFLRYDSFDIEVGTSLLG------SAINLTSLKRASF- 473
HvAOS1          VDNKQCPGKNLVVLVGRLLVVELFLRYDTFTADVGVDLLG------PKVEFTGVTKATSG 476
HvAOS2          VHNKQCPGKNLVVLVGRLLVVELFLRYDTFTAKVGLDLLG------TKVEFTGVTKATSG 482
OsAOS2          VDNKQCPGKNLVVLVGRLLLVELFLRYDTFTAEA-----G------KKVVITGVTKASTS 472
OsAOS3          VDNKQCPGKNLVVLVGRLLLVELFLRYDTFTAEA-----G------KKVVITGVTKASTS 472
OsAOS1          VDNKQCPGKNLVVLVGRLLLVELFLRYDTFTAEA-----G------KKVVITGVTKASTS 472
CsHPL1          AENKQCPGKNLVVMMGRIIVVEFFLRYDTFTVDVADLALG------PAVKFKSLTRATAS 477
CmHPL           PENKQCPGKNLVVLIGRIMVVEFFLRYDTFTVEVADLPLG------PAVKFKSLTRATDM 480
VvHPL2          VENKQCPGKDLVVLLSRVMLVEFFLHYDTFDIEYGTLLLG------SSVTFKSLTKQPTF 422
VvHPLE          VENKQCPGKDLVVLLSRVMLVEFFLHYDTFDIECGTLLLG------SSVTFKSLTKQPTF 422
VvHPLD          VENKQCPGKDLVVLLSRVMMVEFFLRYDTFNIECGTLLLG------SSVTFKSLTKQPTF 422
VvHPLF          VENKQCAGKDLVLLLSRVMLVEFFLRYDTFDIESGTLLLG------SSVTFKSITKATDS 487
VvHPLB          AENKQCSGKDLVVLISKLMLVEIFLRYDTFEVESGTMVLG------SAVLFKSLTKSSYT 483
VvHPLC          AENKQCSGKDLVVLISRLMLVEIFLRYDTFEVESGTMLLG------SSLLFKSLTKTSYT 483
MtHPL1          PDNKICPAKNLVVLLCRLYLVEFFLNYDTFTFDFKPSVLG------PTITIKSLVKASST 481
MtHPL2          VGNKQCPGKNLVVLLCRLLLVEFFLRYDTFENETKNNAFG------AAVSITSLTKASSV 485
StDES           PDNKQCPGKDLVHLLGRLILVEFFMRYDTFTVEITPLFRA------PNVAFKTLTKASK- 478
LeDES           PDNKQCPGKDLVHLLGRLILVEFFIRYDTFTLEITPLFRA------PNVAFNTLTKASK- 478
CaDES           PENKQCAGKDLVQLLGRLILVEFFMRYDTFTVEITPLFRA------PNVAIKTLTKATS- 478
NtDES           PDNKQCAGKDLVHLLGRLMLVEFFLRYDTFTVEITPLFRA------PNVAIKTLTKAT-- 478
StAOS3          VNDKQCPGKDLIVLLGRLLVVEFFMRYDTFEIEFGKLLLG------SKVTFKSLTKATS- 491
LeAOS3          VNDKQCPGKDLIVLMGRLLVVEFFMRYDTFEVEFGKLLLG------SKVTFKSLTKATS- 491
MsHPL1          VSNKQCAGKDIVTFTAALIVAHLLRRYDLIKGDG--------------SSITALQKAK-- 480
MsHPL3          VSNKQCAGKDIVTFTAALIVAHLLRRYDLIKGDG--------------SSITALRKAK-- 480
MsHPL2          VSNKQCAGKDIVTFTAALIVAHLLRRYDLIKGDG--------------SSITALRKAK-- 480
AtHPL           ASNKQCAAKDIVTLTASLLVADLFLRYDTITGDS--------------GSIKAVVKAK-- 458
LeHPL           ESNKQCAAKDMVTLTASLIVAYIFQKYDSVSFSS--------------GSLTSVKKAS-- 476
StHPL           ESNKQCSAKEIVTLTASLIVAYIFQKYDSVSFSS--------------GSLTSVKKAS-- 480
CaHPL           ESNKQCAAKDAVTLTASLIVAYIFQKYDSVSFSS--------------GSLTSVKKAC-- 480
NaHPL           ESNKQCAAKDVVTLTASLIVAYIFQRYDSVSFSS--------------GSLTSVKKAS-- 456
VvHPL1          DRNKQCAAKDYVTMTAVLFVTHMFQRYDSVTASG--------------SSITAVEKAN-- 456
VvHPLA          DRNKQCAAKDYVTMTAVLFVTHMFQRYDSVTASG--------------SSITAVEKAN-- 423
PgHPL           ESNKQCAAKDYVTLTACLFVAYMFRRYNSVTGSS--------------SSITAVEKAN-- 450
HvHPL           LGNKQCAAKDVVIATACMLVAELFRRYDDFECTG--------------TAFTSLKKRPQP 472
ZmHPL           PGNKQCAAKEVVVDTACMLLAELFRRYDDFEVEG--------------TSFTKLVKR-QA 433
MaHPL           PANKQCAAKDYVVETACLLMAEIFYRYDEFVCAD--------------DAIS-VTKLDRA 384


PaAOS1          ---------------
AaAOS           ---------------
AtAOS           ---------------
LeAOS1          ---------------
StAOS1          ---------------
InAOS           ---------------
StAOS2          ---------------
StAOS           ---------------
LeAOS2          ---------------
NaAOS           ---------------
GmAOS           ---------------
GmAOS1          ---------------
MtAOS           ---------------
CmAOS           ---------------
LuAOS           ---------------
VvAOS           ---------------
```

```
HvAOS1          PGAV---------- 480
HvAOS2          VADAV--------- 487
OsAOS2          AVNRTA-------- 478
OsAOS3          AVNRTA-------- 478
OsAOS1          AVNRTA-------- 478
CsHPL1          V------------- 478
CmHPL           V------------- 481
VvHPL2          DHKSIKHVS------ 431
VvHPLE          DHKSIKHVS------ 431
VvHPLD          DHKSITHVS------ 431
VvHPLF          ---------------
VvHPLB          ---------------
VvHPLC          ---------------
MtHPL1          V------------- 482
MtHPL2          ---------------
StDES           ---------------
LeDES           ---------------
CaDES           ---------------
NtDES           ---------------
StAOS3          ---------------
LeAOS3          ---------------
MsHPL1          ---------------
MsHPL3          ---------------
MsHPL2          ---------------
AtHPL           ---------------
LeHPL           ---------------
StHPL           ---------------
CaHPL           ---------------
NaHPL           ---------------
VvHPL1          ---------------
VvHPLA          ---------------
PgHPL           ---------------
HvHPL           QPSS---------- 476
ZmHPL           SPSVAQAAAAAGAQQ 448
MaHPL           REWE---------- 388
```

Figure C.3 Multiple alignment of CYP74 enzymes polypeptide sequences

Multiple alignment sequence of CYP74 enzymes family member polypeptide sequences to generate cladogram diagram relationship (figure 3.5). Deduce amino acid sequences of CYP74 enzymes members is a collection of previously identified in other species (Howe and Schilmiller, 2002; Mei et al., 2006; Stumpe and Feussner, 2006; Kongrit et al., 2007; Pajerowska-Mukhtar et al., 2008; Stumpe et al., 2008; Wu et al., 2008; Podolyan, 2010; Zhu et al., 2012). Sequence details a shown on the Table 3.3

C.4 Multiple alignment of CYP74A enzymes polypeptide sequences

```
HvAOS1          ----------MNQSAI-----------------------------------------------
HvAOS2          ----------MNQSGMA----------------------------------------------
OsAOS2          ----------ME--------------------------------------------------
OsAOS3          ----------ME--------------------------------------------------
OsAOS1          ----------ME--------------------------------------------------
PaAOS1          ------------------------------------------------------------
AaAOS           ----------MSTSSLT-FP-SLHHHRKNNLPTSKTTIHRR-----------RPTTI---
LeAOS1          ----------MASTSLS-LP-SL----KLQFPSHTSSSSRK--NSSSYRVSIRPIQA---
StAOS1          ----------MASTSLS-LP-SL----KLQFPSHKSSSSRK--NSSSHRVSIRPIQA---
InAOS           ----------MASSSL-----AV----HFQIPSQKSSLTLK---PSSRRFKICPVSA---
LeAOS2          MA-------LTLSFSLP-LP-SL----HQKIPSKYS--------------TFRPIIV---
StAOS2          MA-------LTLSFSLP-LP-SL----HQQFPSKYS--------------TFRPIIV---
StAOS           ----------MASFSLP-LP-SL----HQQFPSKYS--------------TFRPIIV---
NaAOS           MAVATATATLSSSSSLP-FH-SL----HQQFPSKY--------------FTVRPITV---
VvAOS           ----------MASPSLT-FP-SL----QLQFPTHTKSS-----KPSKHKLIVRPIFA---
MtAOS           ----------MASSTLS-TP-SPNLLKHQNRPSSTTSSRRS-------STFLPPIRS---
GmAOS           MAS-------SASTTLS-SP-FL----RLEFPSSTKQRSS------------ISIRA---
GmAOS1          MAS-------SASTTLS-SP-FL----RLELPSRTKKRSSS-------IIPVPSIRA---
CmAOS           ----------MSSIVIP----SLQ--PHLRFPSSQETPQRS--RSRVGFVSIRPIYATDG
LuAOS           ----------MASSALNNLV-AVN--PNTLSPSPKSTPLPNTFSNLRRVSAFRPIKA---
AtAOS           ----------MASISTP-FPISL----HPK-------TVRS--KPLKFRVLTRPIKA---
StAOS3          ----------MA--------------------------------------------------
LeAOS3          ----------MA--------------------------------------------------

HvAOS1          -------------------------------GSLVPRQAPGSYGLPFVSAIRDRLDFYYFQ
HvAOS2          --------------------------RSDEGSLVPREVPGSYGLPFVSAIRDRLDFYYFQ
OsAOS2          ----------------------------LGVPLPRRPVPGSYGVPFVSAVRDRLDFYYLQ
OsAOS3          ----------------------------LGVPLPRRPVPGSYGVPFVSAVRDRLDFYYLQ
OsAOS1          ----------------------------LGVPLPRRPVPGSYGVPFVSAVRDRLDFYYLQ
PaAOS1          --------------------------MDPSSKPLREIPGSYGIPFFQPIKDRLEYFYGT
AaAOS           -RFSATSPD-TTTTTTTT------GSNTDNKNLPIRPIPGSYGIPFYQPLKDRFEYFYGP
LeAOS1          -SVSEIPPY---ISSPSQSPSSSSSPPVKQAKLPAQKVPGDYALPLVGPWKDRLDYFYNQ
StAOS1          -SVSERPPY---ISSPSPSP----SPPVKQAKLPTRKVPGDYGLPLVGPWKDRLDYFYNQ
InAOS           -TVSDTPP----SVSLSP----------VPEKLPKRKIPGDYGLPLIGPWKDRLDYFYNQ
LeAOS2          -SLSDKSTI---EIT-------------QPIKLSTRTIPGDYGLPGIGPWKDRLDYFYNQ
StAOS2          -SLSEKPTI---VVT-------------QPTKLPTRTIPGDYGLPGIGPWKDRLDYFYNQ
StAOS           -SLSEKPTI---VVT-------------QPTKLPTRTIPGDYGLPGIGPWKDRLDYFYNQ
NaAOS           -SLSEKIP----AVTQSS----------EFTKLPIRTIPGDYGLPLIGPWKDRQDYFYNQ
VvAOS           -SVSEKPSV---PVSQSQ----------VTPPGPIRKIPGDYGLPFIGPIKDRLDYFYNQ
MtAOS           -SVSEKPPF---QVSISQ-P--------QTTKLPIRKIPGDYGIPFIQPYKDRLDYFYNQ
GmAOS           -SVSEKPPL--PAVSVTS-P--------EPSKLPIRKIPGDCGFPVIGPFKDRQDYFYKQ
GmAOS1          -SVSEKPPL--PAVSVTS-P--------EPSKLPIRKIPGDCGFPVIGPLKDRQDYFYKQ
CmAOS           VSSSSSSSLQVPQRIVSP-P--------EPTKLPLRKVPGDYGPPMFGALKDRHDYFYNQ
LuAOS           -SLFGDSPIKIPGITSQP-P-----PSSDETTLPIRQIPGDYGLPGIGPIQDRLDYFYNQ
AtAOS           -SGSETPDL---TVATRT----------GSKDLPIRNIPGNYGLPIVGPIKDRWDYFYDQ
StAOS3          -NTKDSYHI--ITMDTKE-S--------SIPNLPMKEIPGDYGVPFLGAIKDRYDFHYNQ
LeAOS3          -NTKDSYHI--ITMDTKE-S--------SIPSLPMKEIPGDYGVPFFGAIKDRYDFHYNQ

HvAOS1          -GEAKYFESRVEKHGSTVLRINVPPGPFMARDPRVVAVLDAKSFPVLFDVDKVEKKNLFT
HvAOS2          -GQDKYFESRVEKYGSTVVRINVPPGPFMARDPRVVAVLDAKSFPVLFDVTKVEKKNLFT
OsAOS2          -GQDKYFESRAERYGSTVVRINVPPGPFMARDPRVVALLDAKSFPVLFDVAKVEKRDVFT
OsAOS3          -GQDKYFESRAERYGSTVVRINVPPGPFMAREPRVVALLDAKSFPVLFDVAKVEKRDVFT
OsAOS1          -GQDKYFESRAERYGSTVVRINVPPGPFMARDPRVVALLDAKSFPVLFDVAKVEKRDVFT
PaAOS1          GGRDEYFRSRMQKYQSTVFRANMPPGPFVSSNPKVIVLLDAKSFPILFDVSKVEKKDLFT
AaAOS           GGRDEFFKTRVQKHQSTVFRTNMPPGPFISKNPNVVVLLDAKSFPTLFDVTKVEKKDLFT
LeAOS1          -GKNEFFKSRIQKHQSTVFRTNMPPGPFISFNPNVVVLLDGKSFPVLFDVSKVEKKDLFT
StAOS1          -GKNEFFKSRIQKHQSTVFRTNMPPGPFISFNPNVVVLLDGKSFPILFDVSKVEKKDLFT
InAOS           -GREEFFRSRVQKYGSTVFRTNMPPGPFISFSPNVVVLLDGKSFPTLFDPGKVEKRDLFT
LeAOS2          -GKNDFFESRIAKYKSTIFRTNMPPGPFITSNPKVIVLLDGKSFPVLFDASKVEKKDLFT
StAOS2          -GKDEFFESRVVKYKSTIFRTNMPPGPFISSNPKVIVLLDGKSFPVLFDVSKVEKKDLFT
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StAOS           -GKDEFFESREVKYKSTIFRTNMPPGPFISSNPKVIVLLDGKSFPVLFDVSKVEKKDLFT
NaAOS           -GKEEFFRSRIQKYKSTVFKTNMPPGNFISSNPNVVVLLDGKSFPTLFDVSKVEKKDLFT
VvAOS           -GREEFFRSRAQKHQSTVFRSNMPPGPFISSNSKVIVLLDGKSFPVLFDVSKVEKKDVFT
MtAOS           -GRDEYFKSRIQKYQSTIFRTNVPPGPFIAQNPNVVVLLDGKSFPVLFDASKIDKTDVFT
GmAOS           -GRDEFFKSRIQKYQSTVFRTNMPPGPFLAPDPNVVVLLDAKSFPVLFDNSKVEKKDVFT
GmAOS1          -GRDEFFKSRIQKYQSTVFRTNMPPGPFLAPNPNVVVLLDAKTFPILFDNSKVDKRDVFT
CmAOS           -GREEYLKSRMLRYESTVYRTNMPPGPFITSDSRVVVLLDGKSFPVLFDHSKVEKKDLFT
LuAOS           -GREEFFKSRLQKYKSTVYRANMPPGPFIASNPRVIVLLDAKSFPVLFDMSKVEKKDLFT
AtAOS           -GAEEFFKSRIRKYNSTVYRVNMPPGAFIAENPQVVALLDGKSFPVLFDVDKVEKKDLFT
StAOS3          -GADEFFRSRMEKHDSTIFRTNVPPGPFNARNSKVVVLVDAVSYPILFDNSQVDKENYFE
LeAOS3          -GADEFFRSRMKKYDSTVFRTNVPPGPFNARNSKVVVLVDAVSYPILFDNSQVDKENYFE

HvAOS1          GTYMPSTSLTGGFRVCAYLDPSEPTHTKVKQLLFSLLASRKDAVIPAFRSHFSSLLATVE
HvAOS2          GTYMPSTSLTGGFPVCSYLDPSEPTHTKVKQLLFSLLASRKDAFIPAFRSHFSSLLATVE
OsAOS2          GTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFVPVFRSNFGALLDTVE
OsAOS3          GTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFVPVFRSNFGALLDTVE
OsAOS1          GTFMPSTSLTGGYRVCAYLDPSEPNHAKIKQLLLSLLVSRKDAFVPVFRSNFGALLDTVQ
PaAOS1          GTYMPSTKLTGGYRVLSYLDPSEPRHAQLKNLLFFMLKNSSNRVIPQFETTYTELFEGLE
AaAOS           GTYMPSTELTGGHRVLSYLDPSEPKYAPLKNMVFFMLKNSIKKIIPEFQKTYNELFDELE
LeAOS1          GTFMPSTDLTGGYRVLSYLDPSEPNHAKLKKLMFYLLSSRRNEVIPEFHNSYSELFETLE
StAOS1          GTFMPSTDLTGGYRVLSYLDPSEPNHAKLKKLMFYLLSSRRNEVIPEFHNSYSELFETLE
InAOS           GTFMPSTELTGGYRILSYLDPSEPKHAQLKQLMFFLLSSRRGHVIPEFHRSFTEMFEGLE
LeAOS2          GTFVPSTELTGGYRILSYLDPSEPNHEKLKKLMFFLLSSRRDHVIPEFHETYTELFETLD
StAOS2          GTYMPSTELTGGYRVLSYLDPSEPNHEKLKKLMFFLLSSRRDHVIPKFHETYTEFFETLD
StAOS           GTYMPSTELTGGYRVLSYLDPSEPNHEKLKKLMFFLLSSRRDHVIPKFHETYTEFFETLD
NaAOS           GTFMPSTELTGGYRVLSYLDPSEPTHEKLKKLLFFLLSSRRDYIIPQFHESYTELFKTLE
VvAOS           GTFMPSTEFTGGFRVLSYLDPSEPDHTKLKRLLFFLLQSSRDRIIPEFHSCFSELSETLE
MtAOS           GTYTPSTELTGGYRVLSYLDPSEPKHEQLKKLMFFLLKSRSRHVIPEFQSCYREFFNALE
GmAOS           GTFMPSTELTGGYRVLSYLDPSEPKHALLKQLMFFLLKSRRAHVISEFHASYKELFHALE
GmAOS1          GTFMPSTQLTGGYRVLSYLDPSEPKHSLLKQLMFFLLKSRRAHVISEFHASYKDLFHELE
CmAOS           GTYMPVTELTGGYRVLSYIDPSEPDHAKLKQLIFFLLKHRRDKIMPEFHSTFSELFETLE
LuAOS           GTYMPSTELTGGYRILSYLDPSEPNHTKLKQLLFNLIKNRRDYVIPEFSSSFTDLCEVVE
AtAOS           GTYMPSTELTGGYRILSYLDPSEPKHEKLKNLLFFLLKSSRNRIFPEFQATYSELFDSLE
StAOS3          GTFMSSPSFNGGYKVCGFLGTTDPKHTTLKGLFLSTLTRLHDKFIPIFTTSITQMFTSLE
LeAOS3          GTFMSSPSFNGGYKVCGFLGTSDPKHTTLKGLFLSTLTRLHDKFIPIFTTSITSMFTSLE

HvAOS1          SQLVL-SGKSNFNTLNDFTSFEFIADTYF-GVLPS-ASDLGTTGPAKAAKWLIFQLHPLV
HvAOS2          SQLLL-SGKSNFNTLNDATSFEFIGDGYF-GVLPS-ASDLGTTGPAKAAKWLIFQLHPLV
OsAOS2          SQLASGGGKSDFTALNDATSFEFIGEAYF-GVRPSASSSLGTGGPTKAALWLLWQLAPLT
OsAOS3          SQLASGGGKSDFTALNDATSFEFIGEAYF-GVRPSASSSLGTGGPTKAALWLLWQLAPLT
OsAOS1          SQLASGGGKSDFTALNDATSFEFIGKAYF-GVRPSASSSLGTGGLDQGRLWLLWQLAPLT
PaAOS1          AELAK-NGKAAFNDVGEQAAFRFLGRAYF-NSNPE-ETKLGTSAPTLISSWVLFNLAPTL
AaAOS           AELSN-KGKAFFNDVGEQTAFRFLGRAYL-NTNPE-ETKIGKDGPKLIGTWVLFNLGPLL
LeAOS1          NELST-KGKAGLNAANDQAAVNFLARSLY-GINPQ-DTELGTDGPKLIGKWVLFQLHPLL
StAOS1          NELST-KGKARLNAANDQAAFNFLARSLY-GINPQ-DTKLGTDGPKLIGKWVLFQLHPLL
InAOS           KEVAS-KGKVGLNAANDQAAFNFLARSWF-GVDPA-GTKIGNDGPNLVGKWVVFNLHPLL
LeAOS2          KEMEE-KGTVGFNSGSDQAAFNFLARSLF-GVNPV-ETKLGTDGPALIGKWILLQLHPVI
StAOS2          KEMAE-KGTAGLNSGNDQAAFNFLARSLF-GVNPV-ETKLGTDGPTLIGKWVLLQLHPVL
StAOS           KEMAE-KGTAGLNSGNDQAAFNFLARSLF-GVNPV-ETKLGTDGPTLIGKWVLLQLHPVL
NaAOS           KEMEK-NGKADLNSANDQAAFNFLARSLY-GANPV-ETKLGTDGPTLIGKWVLFQLHPLL
VvAOS           SELAA-KGKASFADPNDQASFNFLARALY-GTKPA-DTKLGTDGPGLITTWVVFQLSPIL
MtAOS           NQLAE-NGHASFADNNDQAAFNFLNRALF-GVNPV-DTELGLDGPKMVQKWVLFQLGPVL
GmAOS           ANLAE-AGKASFGDANDQAAFNFLSRSLF-NSNPA-DTKLGLDGPKIVQKWVLFQIGPIL
GmAOS1          ANLAE-AGKASFGDANDQAAFNFLARSLF-NSNPA-DTKLGRDGPKIVQKWVLFQLGPIL
CmAOS           KDLAA-AGRAEYNASGEQAAFNFLARSLF-GADPV-DSKLGRDAPKLIAKWVLFQLGPVL
LuAOS           YDLAT-KGKAAFNDPAEQAAFNFLSRAFF-GVKPI-DTPLGKDAPSLISKWVLFNLAPIL
AtAOS           KEAFP-LRESGFRRFQRRNRLLFLGSSFL-RDESR-RYKLKADAPGLITKWVLFNLHPLL
StAOS3          KELSE-KGTSYFNPMSDNLSFEFLFRLFCEGKNPV-DTSVGTNGPKIVDKWVFLQLAPLI
LeAOS3          KELSE-KGTSYFNPIGDNLSFEFLFRLFCEGKNPI-DTSVGPNGPKIVDKWVFLQLAPLI

HvAOS1          TFGL---PMILEEPLLHTVLLPPIFVSGDYKALYKYFYAAATKALDMAESLGLNRDEACH
HvAOS2          TLGL---PMILEEPLLHTVHLPPFLVSGDYKALYKYFFAAATKALDTAEGLGLKRDEACH
OsAOS2          TLGL---PMIIEDPLLHTLPLPPFLISSDYKALYAYFAAAASQALDAAEGLGLSREEACH
OsAOS3          TLGL---PMIIEDPLLHTLPLPPFLISSDYKALYAYFAAAASQALDAAEGLGLSREEACH
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OsAOS1          TLGL---PMIIEDPLLHTLPLPPFLISSDYKALYAYFAAAASQALDAAEGLGLSREEACH
PaAOS1          DLGL---PWFLQEPLLHTFRLPAFLIKSTYNKLYDYFQSVATPVMEQAEKLGVPKDEAVH
AaAOS           RLGL---PWFVEEPLLHTFRLPAALVKKNYNKLYDFFESCSGEIIEHAKSLGLEKDEAVH
LeAOS1          ILGL---PKVLEDLVMHTFRLPPALVKKDYQRLYNFFYENSTSVLDEAEKIGISREEACH
StAOS1          ILGL---PKVLEDLVMHTFRLPPALVKKDYQRLYNFFYENSTSVLDEAEKIGISREEACH
InAOS           VLGL---PKGLEEALLHTFRLPAALVKKDYQRLYEFFYANSTEILDEAENLGLSREEACH
LeAOS2          TLGL---PKFLDDVLLHTFRLPPILVKKDYQRLYDFFYTNSANLFIEAEKLGISKDEACH
StAOS2          TLGL---PKFLDDLILHTFRLPPFLVKKDYQRLYDFFYTNSASLFAEAEKLGISKEEACH
StAOS           TLGL---PKFLDDLILHTFRLPPFLVKKDYQRLYDFFYTNSASLFAEAEKLGISKEEACH
NaAOS           TLGL---PKVLDDFLLHNFRLPPALVKKDYQRLYDFFYESSTAVLNEAGNFGISRDEACH
VvAOS           TLGL---PKFIEEPLIHTFPLPAFLAKSSYQKLYDFFYDASTHVLDEGEKMGISREEACH
MtAOS           KLGL---PKFVEDSMIHNFRLPFRLIKKDYQRLYDFFYASSGFALEEAERLDVSKEEACH
GmAOS           RLGL---PQFLEESTIRTFRLPFSLIKKDYQRLYDFFYESSGLVLDEAERLGITRDEACH
GmAOS1          RLGL---PQFLEESTIRSFRLPFSLIQKDYQRLYDFFYQSSGSVLDEAERLGITRDEACH
CmAOS           SLGL---PKVVEELLLRTVRLPPALIKADYRRLYDFFYKSSEAVFEEADRLGISREEACH
LuAOS           SVGL---PKEVEEATLHSVRLPPLLVQNDYHRLYEFFTSAAGSVLDEAEQSGISRDEACH
AtAOS           SIGL---PRVIEEPLIHTFSLPPALVKSDYQRLYEFL-RIRGEILVEADKLGISREEATH
StAOS3          SLGLKFVPNFLEDLVLHTFPLPYFLVKGDHQKLYNAFYNSMKDILDEAEKLGVKREEACH
LeAOS3          SLGLKFVPNFLEDLVLHTFPLPYILVKRDHQKLYNAFYNSMKDILDEAEKLGVKRDEACH

HvAOS1          NLLFATVFNSYGGLKVMLPGFLGRIAEAGEKFHQRLAAEVRTAVADAG-GKVTIEALEKM
HvAOS2          NLLFATVFNSYGGLKVLLPGILARIADSGEKFHKKLVTEIRAAVAEAG-GKVTIEALEKM
OsAOS2          NLLFATVFNSYGGFKLLLPQILSRVAQAGEKLHERLAAEIRSAVADAG-GNVTLAALEKM
OsAOS3          NLLFATVFNSYGGFKLLLPQILSRVAQAGEKLHERLAAEIRSAVADAG-GNVTLAALEKM
OsAOS1          NLLFATVFNSYGGFKLLLPQILSRVAQAGEKLHERLAAEIRSAVADAG-GNVTLAALEKM
PaAOS1          NILFAVCFNTFGGVKILFPNTLKWIGLAGENLHTQLAEEIRGAIKSYGDGNVTLEAIEQM
AaAOS           NILFTLCFNTFGGIKILFPNTLKWLGRAGTNLHTQLAEEIRNAIKVHGGGKVTMAAMEQM
LeAOS1          NLLFATCFNSFGGIKIFFPNMLKWIGRAGAKLHSQLAQEIRSVISSNS-GKVTMAAMEKM
StAOS1          NLLFATCFNSFGGIKIFFPNMLKWIGRAGAKLHSQLAQEIRSVISSNS-GKVTMAAMEKM
InAOS           NLLFATCFNSFGGMKIFFPNMIKWIGRGGAKLHAQLAREIRSVVKSNG-GKVTMAGMEQM
LeAOS2          NLLFATCFNSFGGMKIFFPNMLKSIAKAGVEIHTRLANEIRSEVKSAG-GKITMSAMEKM
StAOS2          NLLFATCFNSFGGMKIFFPNMLKSIAKAGVEVHTRLANEIRSEVKSAG-GKITMSAMEKM
StAOS           NLLFATCFNSFGGMKIFFPNMLKSIAKAGVEVHTRLANEIRSEVKSAG-GKITMSAMEKM
NaAOS           NLLFATCFNSFGGMKIFFPNMLKWIARAGVELHIRLANEIRSAVKSAG-GKITMSAMEKM
VvAOS           NLLFATCFNSFGGMKIIFPTILKWVGRGGVKLHTQLAQEIRSVVKSNG-GKVTMASMEQM
MtAOS           NLLFATCFNSFGGMKLFFPNLMKWIGRGGVRLHTKLATEIREAVRSAG-GEITMAAMENM
GmAOS           NLLFATCFNSFGGMKLFFPNVLKWIGRAGVKLHARLAEEIRSAVRSGG-GEISMAAMEKM
GmAOS1          NLLFATCFNSFGGMKLFFPNVLKWIGRAGVKLHARLAEEIRSAVRGAG-GEITMAAMENM
CmAOS           NLLFTTCFNSFGGMKIFFPNMIKWIGRAGVNLHTRLAREIRTAVKANG-GKITMGAMEQM
LuAOS           NILFAVCFNSWGGFKILFPSLMKWIGRAGLELHTKLAQEIRSAIQSTGGGKVTMAAMEQM
AtAOS           NLLFATSFNTWGGMKILFPNMVKRIGPGGHQVHNRLAEEIRSVIKSNG-GELTMGAIEKM
StAOS3          NFIFLAGFNSYGGMKVFFPSLIKWIGTSGPTLHTRLVKEIRTAVKEAG-G-VTLSAIDKM
LeAOS3          NFVFLAGFNSYGGLKVFFPSLIKWIGTSGPSLHARLVKEIRTAVKEAG-G-VTLSAIDKM

HvAOS1          ELTKSAVWEALRLEPPVKFQYGRAKVDMNIESHDAVFAVQKGEMLFGYQPCATKDPRVFG
HvAOS2          ELTKSAVWEALRLDPAVKFQYGRAKADMNIESHDAVFAVKKGEMLFGYQPCATKDPRVFG
OsAOS2          ELTRSVVWEALRLDPPVRFQYGRAKADLEIESHDASFAIKKGEMLFGYQPCATRDPRVFG
OsAOS3          ELTTSVVWEALRLDPPVRFQYGRAKADLEIESHDASFAIKKGEMLFGYQPCATRDPRVFG
OsAOS1          ELTRSVVWEALRLDPPVRFQYGRAKADLEIESHDASFAIKKGEMLFGYQPCATRDPRVFG
PaAOS1          PLTKSVVYESLRIEPPVPPQYGKAKSNFTIESHDATFEVKKGEMLFGYQPFATKDPKVF-
AaAOS           PLMKSVVYESLRIEPPVALQYGKAKKDMTIESHDAVFKVKEGEMLFGYQPFATKDPKIF-
LeAOS1          PLMKSVVYESLRIEPPVASQYGRAKHDMVIESHDASFEIKEGELLYGYQPFATKDPKIF-
StAOS1          PLMKSVVYESLRIEPPVASQYGRAKHDMVIESHDASFEIKEGELLYGFQPFATKDPKIF-
InAOS           PLMKSVVYEALRIEPPVPAQYGRAKRDFVVESHDAVFEVKEGEMLFGFQPFATKDPKIF-
LeAOS2          PLMKSVVYEALRVDPPVASQYGRAKQDLKIESHDAVFEVKKGEILFGYQPFATKDPKIF-
StAOS2          PLMKSVVYEALRVDPPVASQYGRAKQDLKIESHDAVFEVKKGEMLFGYQPFATKDPKIF-
StAOS           PLMKSVVYEALRVDPPVASQYGRAKQDLKIESHDAVFEVKKGEMLFGYQPFATKDPKIF-
NaAOS           PVMKSVVYEALRIDPPVASQYGRAKRDLMIESHDGVFEVKKGEMLFGYQPFATRDPKIF-
VvAOS           PLMKSTVYEAFRIEPPVALQYGKAKQDLVIESHDSVFEVKEGEMLFGYQPFATKDPKIF-
MtAOS           PLMKSVVYEAFRIDPPVPLQFGRAKRDMVIENHENGFLVKKGELLLGYQPFATKDPKIF-
GmAOS           PLMKSVVYEAFRIDPPVALQFGRAKRDLIIESHDHAFQVKEGEMLFGYQPFATKDPRIF-
GmAOS1          PLMKSVVYEAFRIDPPVPLQFGRAKRDLIIESHDHAFQVKEGEMLFGYQPFATKDPRIF-
CmAOS           PLMKSVVYEALRIEPPVPVQYGRAKKDLVVESHDAAFEIKEGEVICGYQPFATRDPKIF-
LuAOS           PLMKSVVYETLRIEPPVALQYGKAKKDFILESHEAAYQVKEGEMLFGYQPFATKDPKIF-
AtAOS           ELTKSVVYECLRFEPPVTAQYGRAKKDLVIESHDAAFKVKAGEMLYGYQPLATRDPKIF-
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StAOS3          PLVKSVVYETLRMDPPVPFQTVKARKNIIVSNHEASFLIKKDELIFGYQPLATKDSKVF-
LeAOS3          PLVKSVVYETLRMDPPVPFQTVKARKNIIITNHESSFLIKKDELIFGYQPLATKDSKVF-


HvAOS1          STAREFVGDRFVG-EGSKLLQYVYWSNGRETESPSVDNKQCPGKNLVVLVGRLLVVELFL
HvAOS2          PTAREFVGDRFVGKEGSKLLKYVYWSNGRETESPSVHNKQCPGKNLVVLVGRLLVVELFL
OsAOS2          ATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPSVDNKQCPGKNLVVLVGRLLLVELFL
OsAOS3          ATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPSVDNKQCPGKNLVVLVGRLLLVELFL
OsAOS1          ATAREFVGDRFVGEEGRKLLQYVYWSNGRETENPSVDNKQCPGKNLVVLVGRLLLVELFL
PaAOS1          DRPEEYVPDRFVG-DGEALLKYVWWSNGPETESPTVENKQCAGKDFVVLITRLFVIELFR
AaAOS           DRPEESVPDRFVG-EGEKLLKYVTWSNGPETETPTAGNKQCAGKDFVVLITRLFVIELFR
LeAOS1          DRSEEFVADRFKGEEGEKLLKHVLWSNGSETENASINNKQCAGKDFVVLVSRLLLVELFL
StAOS1          DRSEEFVADRFIGEEGEKLLKHVLWSNGSETENPSINNKQCAGKDFVVLVSRLLLVELFL
InAOS           DRAEEFVPDRFTGENANELLSHVLWSNGPETESPTVNNKQCAGKDFVVLVSRLMVVELFL
LeAOS2          DRPGEFVADRFVGEEGEKLLKHVLWSNGPETESPTVGNKQCAGKDFVVMVSRLFVTEFFL
StAOS2          DRPEEFVADRFVG-EGEKLLKYVLWSNGPETESPTVGNKQCAGKDFVVMVSRLFVTEFFL
StAOS           DRPEEFVADRFVGEEGEKLLKYVLWSNGPETESPTVGNKQCAGRDFVVMVSRLFVTEFFL
NaAOS           DRPDEFVPDRFVGEEGEKLLKHVLWSNGPETESPTVENKQCAGKDFVVLVSRLLVTEFFL
VvAOS           ERSEEFVPDRFVG-EGEKLLKHVLWSNGPETENPTLGNKQCAGKDFVVLAARLFVVELFL
MtAOS           ERAEEFVADRFVGDEGEKLLKHVLWSNGPESQSPTVGNKQCAGKDFTTLISRLLVVELFL
GmAOS           ERAEEFVGDRFVGEEGEKLLKHVLWSNGPETESPTLGNKQCAGKDFVTLVSRLFVVEFFL
GmAOS1          ERAEEFVGDRFVGEEGEKLLKHVLWSNGPETESPTIGNKQCAGKDFVTLVSRLLVVEFFL
CmAOS           DRADELVPDRFTG-EGEELLKHVIWSNGPETQSPSVQNKQCAGKDFIVFISRLLVVELFL
LuAOS           DRPEEFVADRFVG-EGVKLMEYVMWSNGPETETPSVANKQCAGKDFVVMAARLFVVELFK
AtAOS           DRADEFVPERFVGEEGEKLLRHVLWSNGPETETPTVGNKQCAGKDFVVLVARLFVIEIFR
StAOS3          KNAEEFNPDRFVG-YGEKLLKYVYWSNGKETDNPTVNDKQCPGKDLIVLLGRLLVVEFFM
LeAOS3          KNAEEFNPDRFVG-GGEKLLKYVYWSNGKEIDNPSVNDKQCPGKDLIVLMGRLLVVEFFM


HvAOS1          RYDTFTADVGVDLLG------PKVEFTGVTKATSGPG--AV
HvAOS2          RYDTFTAKVGLDLLG------TKVEFTGVTKATSGVAD-AV
OsAOS2          RYDTFTAEAG-----------KKVVITGVTKASTSAVNRTA
OsAOS3          RYDTFTAEAG-----------KKVVITGVTKASTSAVNRTA
OsAOS1          RYDTFTAEAG-----------KKVVITGVTKASTSAVNRTA
PaAOS1          RYDSFEIELGESPLG------AAVTLTFLKRASI-------
AaAOS           RYDSFDIEVGASPLG------AKITLTSLKRARV-------
LeAOS1          RYDSFEIEVGASPLG------AAITLTSLRRASF-------
StAOS1          RYDSFEIEVGASPLG------AAITLTSLRRASF-------
InAOS           RYDSFDIEVGTSPLG------ASVTVTSLKRASF-------
LeAOS2          RYGTLNVDVGTSALG------SSITITSLKKA---------
StAOS2          RYDTFNVDVGKSALG------ASITITSLKKA---------
StAOS           RYDTFNVDVDKSALG------ASITITSLKKA---------
NaAOS           RYDTLDIDVGTSPLG------AKITITSLKRA---------
VvAOS           RYDSFDIEVGTSLLG------SAINLTSLKRASF-------
MtAOS           RYDSFEIQVGNSPLG------PSITLTSLKRSSF-------
GmAOS           RYDSFEIQVGTSPLG------SSVTITSLKRASF-------
GmAOS1          RYDSFEIQVGTSPLG------SSVTITSLKRASF-------
CmAOS           RYDSFDIEASNTPLGAAVTVSAAVTVTSLKKASF-------
LuAOS           RYDSFDIEVGTSSLG------ASITLTSLKRSTF-------
AtAOS           RYDSFDIEVGTSPLG------SSVNFSSLRKASF-------
StAOS3          RYDTFEIEFGKLLLG------SKVTFKSLTKATS-------
LeAOS3          RYDTFEVEFGKLLLG------SKVTFKSLTKATS-------
```

Figure C.4 Multiple alignment of CYP74A enzymes polypeptide sequences

Multiple alignment sequence of CYP74A enzymes Sub-family member polypeptide sequences to generate cladogram diagram relationship (Figure 2.6). Deduce amino acid sequences of CYP74A enzymes members is a collection of previously identified in other species (Howe and Schilmiller, 2002; Mei et al., 2006; Stumpe and Feussner, 2006; Kongrit et al., 2007; Pajerowska-Mukhtar et al., 2008; Stumpe et al., 2008; Wu et al., 2008; Podolyan, 2010; Zhu et al., 2012). Sequence details a shown on the Table 3.3

C.5 Predicted chloroplast transit peptide region (cTP) of CYP74 enzymes polypeptide sequences

CYP74 (ID)	Length	Score	cTP	CS-score	cTP-length
MsHPL1	480	0.487	-	3.271	59
MsHPL2	480	0.484	-	2.624	59
MsHPL3	480	0.483	-	2.624	59
AtHPL	458	0.496	-	9.162	33
LeHPL	476	0.490	-	0.069	17
CaHPL	480	0.492	-	3.781	82
StHPL	480	0.498	-	3.781	82
NaHPL	456	0.497	-	3.781	58
HvHPL	476	0.475	-	1.489	42
MaHPL	388	0.446	-	2.207	64
ZmHPL	448	0.473	-	4.885	32
CsHPL1	478	0.440	-	1.333	64
PgHPL	450	0.479	-	1.768	40
StDES	478	0.429	-	0.729	25
LeDES	478	0.429	-	0.729	25
NtDES	478	0.428	-	1.954	7
HvAOS1	480	0.452	-	3.395	12
HvAOS2	487	0.437	-	1.695	4
OsAOS2	478	0.439	-	2.199	13
PaAOS1	473	0.450	-	6.737	53
LeAOS1	465	0.490	-	0.698	45
LeAOS2	510	0.483	-	1.584	31
StAOS1	492	0.482	-	0.698	72
StAOS2	478	0.436	-	1.617	92
AtAOS	486	0.437	-	2.137	11
CmAOS	493	0.475	-	-0.191	28
LuAOS	493	0.439	-	-2.799	20
MtAOS	487	0.440	-	0.390	2
CmHPL	481	0.450	-	6.737	57
MtHPL1	482	0.436	-	2.662	3
MtHPL2	485	0.458	-	0.698	61
StAOS3	491	0.431	-	0.516	12
LeAOS3	491	0.435	-	4.498	57
NaAOS	475	0.433	-	0.256	47
StAOS	479	0.433	-	-1.601	8
CaDES	478	0.430	-	0.729	25
GmAOS	488	0.457	-	0.698	68
GmAOS1	488	0.457	-	2.124	10
AaAOS	491	0.481	-	-1.701	2
InAOS	486	0.461	-	2.183	56
OsAOS1	478	0.439	-	2.199	13
OsAOS3	478	0.441	-	2.199	13
VvHPL1	456	0.490	-	-0.033	33
VvHPL2	431	0.463	-	1.542	56
VvHPLA	423	0.487	-	-3.007	10
VvAOS	473	0.444	-	3.922	44
VvHPLB	483	0.457	-	1.928	8
VvHPLC	483	0.475	-	1.804	2
VvHPLD	431	0.475	-	1.542	56
VvHPLE	431	0.468	-	1.542	56
VvHPLF	487	0.465	-	6.737	65

Figure C.5 Multiple alignment of CYP74A enzymes polypeptide sequences

Chloroplast transit peptide region of CYP74 enzymes family member based on ChloroP1.1 software (Emanuelsson et al., 1999) prediction. Details sequences a shown on the Table 3.4

C.6 Multiple alignment of nucleotide sequences to assessed grapevine AOS and Arabidopsis AOS gene insert in pARTBGW binary vector

C.6.1 pARTBGW:35S promoter:VvAOS

```
Reference seq   GACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGG
5S-3'end        GACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGG
VvAOS-5'end     ------------------------------------------------------------
pARTBGw-VvAOS   ----------------------------------TGACATCTCCACTGACGTAAGG

Reference seq   GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT
35S-3'end       GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT
VvAOS-5'end     ------------------------------------------------------------
pARTBGw-VvAOS   GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT

Reference seq   CATTTGGAGAGGACACGCTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTGG
35S-3'end       CATTTGGAGAGGACACG-------------------------------------------
VvAOS-5'end     ------------------------------------------------------------
pARTBGw-VvAOS   CATTTGGAGAGGACACGCTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTGG

Reference seq   ATCCTCTAGATC-ACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCAT
35S-3'end       ------------------------------------------------------------
VvAOS-5'end     ------------------------------------------------------------AT
pARTBGw-VvAOS   ATCCTCTAGATCAACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCAT

Reference seq   GGCGTCCCCTTCTCTAACTTTCCCTTCCCTGCAACTACAATTCCCAACACACACAAAATC
35S-3'end       ------------------------------------------------------------
VvAOS-5'end     GGCGTCCCCTTCTCTAACTTTCCCTTCCCTGCAAcTaCaATTcCCAACACACACAAAATC
pARTBGw-VvAOS   GGCGTCCCCTTCTCTAACTTTCCCTTCCCTGCAACTACAATTCCCAACACACACAAAATC

Reference seq   ATCTAAGCCATCCAAGCATAAGCTCATTGTTCGCCCGATATTTGCCTCTGTTTCTGAGAA
35S-3'end       ------------------------------------------------------------
VvAOS-5'end     ATCTAAGCCATCCAAGCATAAGCTCATTGTTCGCCCGATATTTGCCTCTGTTTCTGAGAA
pARTBGw-VvAOS   ATCTAAGCCATCCAAGCATAAGCTCATTGTTCGCCCGATATTTGCCTCTGTTTCTGAGAA

Reference seq   ACCATCGGTACCGGTTTCTCAGT
35S-3'end       ----------------------
VvAOS-5'end     ACCATCGGTACCGGTTTCTCAGT
pARTBGw-VvAOS   ACCATCGGTACCGGTTTCTCAGT
```

Figure C.6 pARTBGW:35S promoter:VvAOS
Binary vector pARTBGW incorporated with VvAOS was sequenced using pART 35S primer (Forward primer at 3'end region of 35S promoter) in order to confirm gene insertion and it direction within the plasmid vector. Underline "CACC" region indicate a starting point for the VvAOS gene insert. Multiple alignment sequencing was carried out between Reference sequence (pARTBGW:35S-promoter:VvAOS constructed bioinformatically via Lasergene software by DNASTAR Inc, Madison, USA), AtAOS gene, CaMV 35S promoter gene sequences and pARTBGW:35S-promoter:VvAOS sequencing result to confirm insertion

C.6.2 pARTBGW:35S promoter:AtAOS

```
Reference seq      GACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGG
35S-3'end          GACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGG
AtAOS-5'end        ------------------------------------------------------------
pARTBGW-AtAOS      ----------------------------------ATGTGAAATCTCCACTGACGTAAGG

Reference seq      GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT
35S-3'end          GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT
AtAOS-5'end        ------------------------------------------------------------
pARTBGW-AtAOS      GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTT

Reference seq      CATTTGGAGAGGACACGCTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTGG
35S-3'end          CATTTGGAGAGGACACG------------------------------------------
AtAOS-5'end        ------------------------------------------------------------
pARTBGW-AtAOS      CATTTGGAGAGGACACGCTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTGG

Reference seq      ATCCTCTAGATC-ACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCAT
35S-3'end          ------------------------------------------------------------
AtAOS-5'end        ------------------------------------------------------------AT
pARTBGW-AtAOS      ATCCTCTAGATCAACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCAT

Reference seq      GGCTTCTATTTCAACCCCTTTTCCGATTTCTCTCCACCCAAAAACCGTACGATCAAAGCC
35S-3'end          ------------------------------------------------------------
AtAOS-5'end        GGCTTCTATTTCAACCCCTTTTCCGATTTCTCTCCACCCAAAAACCGTACGATCAAAGCC
pARTBGW-AtAOS      GGCTTCTATTTCAACCCCTTTTCCGATTTCTCTCCACCCAAAAACCGTACGATCAAAGCC

Reference seq      GTTGAAATTCCGAGTTTTGACCCGTCCGATCAAAGCTTCCGGGTCAGAAACTCCTGATCT
35S-3'end          ------------------------------------------------------------
AtAOS-5'end        GTTGAAATTCCGAGTTTTGACCCGTCCGATCAAAGCTTCCGGGTCAGAAACTCCTGATCT
pARTBGW-AtAOS      GTTGAAATTCCGAGTTTTGACCCGTCCGATCAAAGCTTCCGGGTCAGAAACTCCTGATCT

Reference seq      AACCGTAGCGACACGAACCGGAT
35S-3'end          -----------------------
AtAOS-5'end        AACCGTAGCGACACGAACCGGAT
pARTBGW-AtAOS      AACCGTAGCGACACGAACCGGAT
```

Figure C.7 pARTBGW:35S promoter:AtAOS

Binary vector pARTBGW incorporated with AtAOS was sequenced using pART 35S primer (Forward primer at 3'end region of 35S promoter) in order to confirm gene insertion and it direction within the plasmid vector. Underline "CACC" region indicate a starting point for the AtAOS gene insert. Multiple alignment sequencing was carried out between Reference sequence (pARTBGW:35S-promoter:AtAOS constructed bioinformatically via Lasergene software by DNASTAR Inc, Madison, USA), AtAOS gene, CaMV 35S promoter gene sequences and pARTBGW:35S-promoter:AtAOS sequencing result to confirm insertion

Appendix D

Supplementary protocols

D.1 Heat shock transformation of plasmid vector into chemically competent *Escherichia coli* (E.coli)

Transformation plasmid into E.coli was carried out based on the heat shock standard protocol (Sambrook and Russell, 2001). Briefly, 100 µL of chemically competent E.coli (DH5α) were thawed on ice. Approximately 1-2 µL of ligation reaction mixture (or plasmid vector) was added and incubated on ice for 30 min. Mixture was heat shock at 42^{o}C for 45 sec and continue incubated on ice for another 5 min. After 5 min, 900 µL of SOC medium was added at room temperature followed by shacking incubation at 37^{o}C, 200 rpm for 1 hour. After incubation, 50 µL of cell culture were spread on LB agar plates with appropriate antibiotic selection. Cell culture were incubated for 16 hours at 37°C to allow colony growth.

D.2 Electroporation transformation of plasmid vector into *Agrobacterium tumefaciens*

Transformation of plasmid vector into *Agrobacterium tumefaciens* was carried out based on the methodology described by Wise et al. (2006). Briefly, 50 µL of electro-competent *Agrobacterium tumefaciens* (GV3101) cells were thawed on ice. Approximately 1-2 µL of purified plasmid vector was added into electro-competent *Agrobacterium tumefaciens* and mixed gently. Mixture then were transferred to an ice cold electroporation cuvette. Electroporation of cells was performed with a MicroPulser (Bio-Rad) using the *Agrobacterium* selection, and electroporation cuvette immediately returned on ice. Mixture was incubated for two minutes, before 450 µL of SOC medium was added to the cells room temperature. Cells were incubated at 28°C, 200 rpm for 1 hour before 100 µL of cell culture were spread on separate LB agar plates with appropriate antibiotics selection. Plates were incubated in the dark condition for 48 hours at 28°C to allow colony growth.

D.3 Colony PCR

In order to screen bacterial colonies following transformation, colony PCR was used to identify successfully transformed with desired plasmid vector of gene insert in the correct orientation. Generally, primer pair used are one specific primer targeted plasmid vector and another specific primer targeted insert to amplify only insertion in the correct orientation. Single colony was picked by touching them with sterile tooth pick and then swirled into already prepared PCR mix into 10 µL of sterile dH_20 in 0.2 mL of PCR tube and discarded. Cells in 10 µL then was incubated at 95°C for 10 min in order to let all bacterial cell completely lyse. Lyse bacterial cell was used as a template for PCR

reaction. Colony PCR reaction was carried out based on the standard reaction suggested by the polymerase manufacturer and PCR parameter respected to the insert gene.

Appendix E

Supplementary material

E.1 Botrytis infection on table grape berry

Figure E.1 Botrytis infection on table grape berry
Isolate of *Botrytis cinerea* used for Arabidopsis detach leaf assay was maintained on table grape before inoculated on PDA agar plates.

E.2 Mechanical wounding applied on Arabidopsis leaves

Figure E.2 Mechanical wounding on Arabidopsis leaves
Arabidopsis leaves were wounded using small plier.

E.3 PCR method to assessed of homozygous and complemented (AOS function) Arabidopsis *aos* mutant

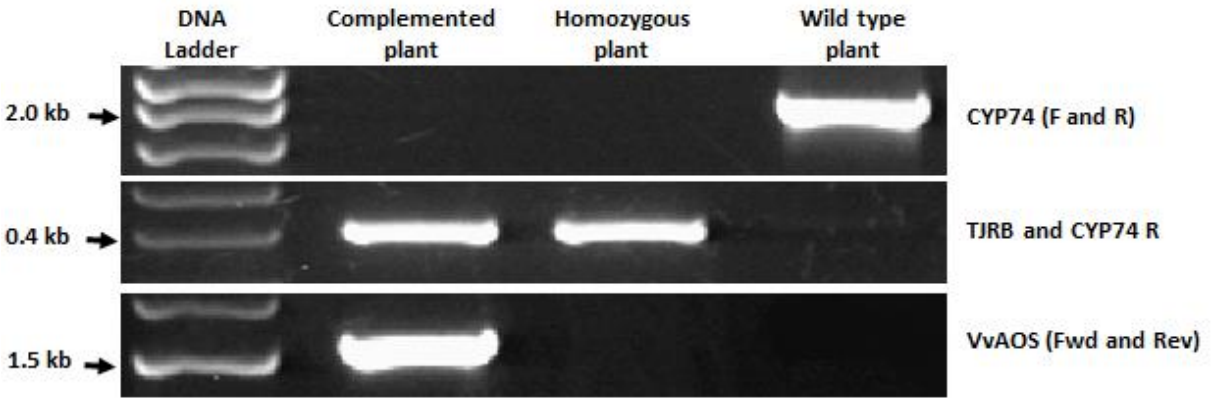

Figure E.3 PCR method to assess of homozygous and complemented (AOS function) Arabidopsis *aos* mutant
PCR methods to assessed complemented (AOS function) to separate from homozygous Arabidopsis *aos* mutant progenies. Arabidopsis was used as a control comparison.

E.4 Quantification of derivatized Jasmonic acid (Methyl jasmonate) via GC/MS

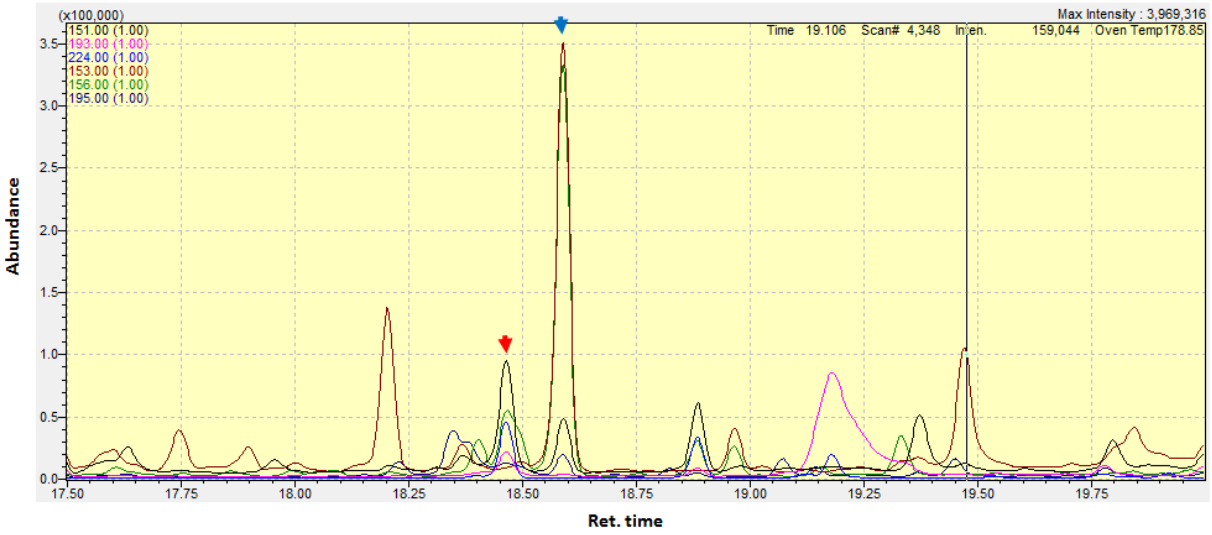

Figure E.4 Quantification of derivatized Jasmonic acid
Jasmonic acid was derivatized and quantified via GC-MS method. Dihydrojasmonic acid (blue arrow) was used as an internal standard to quantify jasmonic acid (red arrow) in the sample.