A comparison of water stress-induced xylem embolism in two grapevine cultivars, Chardonnay and Grenache, and the role of aquaporins

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ABSTRACT

Aquaporins (AQP) are membrane bound proteins that facilitate the movement of water and other small neutral solutes across cellular membranes. Plant aquaporins belong to a large family of highly conserved proteins called the Membrane Intrinsic Protein (MIP) superfamily. In many plant species the expression of aquaporin genes and their regulation has been linked to water stress. Grapevines respond to water stress with a variety of physiological mechanisms, including the susceptibility to xylem embolism. The formation of embolised vessels can lead to a reduction in hydraulic conductivity of the xylem. Recently, it has been hypothesised that aquaporins may contribute to the water movement required for embolism recovery of xylem vessels thus restoring the hydraulic pathway. Molecular and physiological techniques have been combined to study the putative role of plasma membrane and tonoplast membrane aquaporins in response to water stress induced xylem embolism in two cultivars of grapevine (*Vitis vinifera* cv. Chardonnay and Grenache).

Water-stress induced cavitation was measured in the stems and petioles of pot grown grapevines of a drought tolerant (Grenache) and a drought sensitive variety (Chardonnay) by the detection of ultrasonic acoustic emissions (UAEs) over both a drying and diurnal cycle. Vulnerability curves were generated by correlating the UAEs with the leaf water potential (ψ_L). Varietal differences in cavitation vulnerability and hydraulic properties were observed. Grenache was more susceptible to water-stress induced xylem embolism than Chardonnay, and displayed a higher hydraulic capacity (measured by maximum hydraulic conductivity). This is most likely due to anatomical differences of the xylem vessels. Chardonnay displayed vulnerability segmentation, with cavitation occurring first in the petiole and later in the stem, before developing into “runaway” cavitation under severe water stress.

Vulnerability segmentation was not observed in Grenache, with both petioles and stems equally vulnerable to the formation of xylem embolism. Under severe water stress, Grenache did not develop runaway cavitation indicating that they must have some mechanism to prevent the onset of runaway cavitation.

To determine the role of aquaporins, candidate genes were identified, by screening a *Vitis vinifera* cv. Cabernet Sauvignon cDNA library, for aquaporin cDNAs encoding members of the Plasma membrane Intrinsic Protein (PIP) and Tonoplast Intrinsic Protein (TIP) subfamilies. The screen resulted in the identification of 11 full-length and two partial aquaporin cDNAs. Sequence analyses of these cDNAs reveal five are homologous to PIP2 aquaporins, six to PIP1 and two to the TIP aquaporins. Functional expression of the full-length AQP cDNAs in *Xenopus* oocytes showed PIP2 members have significantly higher water permeability compared to PIP1 aquaporins. VvPIP2;1 showed very high water permeability which was reduced by acidic cytosolic pH, as has been reported for other members of the PIP2 family. Transcript analysis of some of these aquaporin genes provides preliminary evidence that aquaporins may contribute to differences in the hydraulic response of these two grapevine varieties to conditions of water stress.

DECLARATION

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

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Megan Cherie Shelden

2nd of October, 2007

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I would like to dedicate this thesis to my late grandmother, Valmai Shelden and Grandfather, Ronald Henderson.

ABBREVIATIONS

ψ	Water Potential
Amp	Ampicillin
AQP	Aquaporin
BLAST	Basic Local Alignment Sequence Tool
BLASTn	Basic Local Alignment Sequence Tool nucleotide
bp	base pairs
BSA	Bovine Serum Albumin
cDNA	complimentary deoxyribonucleic acid
CRCV	Cooperative Research Centre for Viticulture
cRNA	capped ribonucleic acid
CS	cleavage site
cUAE	cumulative Ultrasonic Acoustic Emission
DEPC	diethylpyrocarbonate
EST	Expressed Sequence Tag
FUE	far upstream element
Kan	Kanamycin
LB	Luria Broth
MCS	multiple cloning site
MIP	major intrinsic protein
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
NPA motif	asparagine, proline, alanine motif
NUE	near upstream element

PCR	Polymerase Chain Reaction
Pf	Water permeability
PIP	plasma membrane intrinsic protein
QPCR	Quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
TAE	Tris Acetic Acid EDTA
TBE	Tris Boric Acid EDTA
TIP	Tonoplast intrinsic protein
TMD	transmembrane domain
UAE	ultrasonic acoustic emission
UTR	untranslated region
VAC	vessel associated cell

Chapter 1: Introduction

1.1 Introduction

The monitoring of grapevine water stress is crucial for the ongoing management of vineyard irrigation. There are both economic and environmental reasons for the improvement of irrigation, including, the sustainable use of water resources, prevention of rising water tables and salinity, reducing the costs of irrigation and sustainable grape production. Vineyard irrigation must be optimised to maintain a balance between vegetative and reproductive growth, ultimately to enhance berry production and quality. Although grapevines are able to survive over a range of soil moisture conditions, their growth and yield is determined by their total water use (McCarthy *et al.*, 2001). Nutrient availability is subsequently affected by soil moisture as nutrient ions are dissolved in the soil solution (Keller, 2005). One of the few ways of influencing grapevine vigour is the management of irrigation (Dry and Loveys, 1998). An increased knowledge of the physiological mechanisms that influence shoot growth and transpiration rates in plants has allowed the development of novel irrigation techniques such as Partial Rootzone Drying (PRD) to control vine vigour. In this process, irrigation is scheduled such that the root system is partially dried, stimulating the release of a chemical signal (ABA) by the plant roots that ultimately results in a decrease in shoot growth and water use (Dry and Loveys, 1998). Deficit irrigation requires a careful management strategy, to allow a balance between vine mineral nutrition and water availability, and vine growth and vigour.

The effect of water stress on growth and yield is dependent on a number of factors including the vine's stage of development. For example, water stress during budburst, flowering and veraison can lead to irregular budburst, fewer flowers, poor setting and small berries (McCarthy *et al.*, 2001). Grapevines respond to water stress and water deficit with a variety

of physiological and molecular mechanisms including modifications to the transpiration pathway (root, shoot and stomata). Under conditions of water stress, grapevines are susceptible to xylem embolism (air in the water conducting vessels) (Schultz and Matthews, 1988b; Tyree and Sperry, 1989b). Xylem embolism is likely to occur if the water deficit becomes such that the vine can no longer maintain the xylem water potential above a threshold value (Tyree *et al.*, 1994). The formation of embolisms in the water conduction pathway reduces the overall hydraulic conductivity of the vine, therefore restricting water flow to the leaves.

The discovery of membrane intrinsic channels permeable to water (aquaporins) in plants has led to an exciting development in plant water transport. In many species, aquaporin expression has been linked to water stress (reviewed by Tyerman *et al.*, 2002). A number of aquaporins have been identified in grapevine (Baiges *et al.*, 2001; Picaud *et al.*, 2003) but their role in response to vine water deficit and recovery is unknown. In this project I plan to measure cavitation in grapevine and determine if this is a useful method to monitor vine water stress. I also hope to elucidate the functional role of aquaporins in response to water stress and correlate this with water stress induced xylem embolism and embolism recovery.

1.1.1 Water Transport in Plants and the Cohesion-Tension (CT) Theory

Water transport in higher plants is an intricate process requiring the movement of water over both long distances ie; roots to the transpiring surfaces of leaves, and short distances ie; cell to cell water movement. In the long distance pathway, water is taken up by the roots from the soil, and moves radially through the roots to the xylem vessels, either by the apoplastic, symplastic or transcellular pathway (reviewed by Steudle and Peterson, 1998). Steudle and

Peterson (1998) have proposed that when a plant is actively transpiring during the day water predominately moves radially across the roots by way of the apoplastic pathway. At night, when plants are not actively transpiring, the symplastic pathway is utilized (Steudle and Peterson, 1998). The Casparian band, located between the cortex and the vascular tissue in grapevine, consists of suberised tissue that restricts the flow of water across the apoplast (Steudle and Peterson, 1998). The movement of water across the Casparian strip, by the transcellular and symplastic pathway (collectively known as the cell to cell pathway), requires that water be transported across cellular membranes. The presence of water channels (aquaporins) in cellular membranes, facilitate this movement of water into and out of cells, and across intracellular membranes. It has been suggested that aquaporins may be involved in the regulation of water movement across roots (Javot and Maurel, 2002; Tyerman *et al.*, 2002; Lopez *et al.*, 2003).

Water moves through the plant via the water conducting xylem vessels forming a continuous system from the roots to the cell walls of the leaf mesophyll (known as the soil-plant-air-continuum; SPAC). The ascent of water flow in plants can be explained by the Cohesion Tension (CT) theory. The CT Theory is usually ascribed to Dixon (1914) but the idea of the xylem being under negative pressure was first proposed by Bohm (1893) over 100 years ago. The ascent of sap in higher plants is driven by transpiration in the leaves, providing a pressure gradient for the water to flow. The water conducting vessels are maintained under constant negative pressure such that the water column remains in a metastable state. According to the CT theory, this is deemed possible by the physical properties of water and the hydraulic architecture of plants (Tyree, 1997; Tomos and Leigh, 1999; Steudle, 2001). The validity of the Cohesion-Tension theory has been questioned resulting in a lively ongoing debate (Wei *et al.*, 1999; Wei *et al.*, 2000; Zimmermann *et al.*, 2000).

1.2 Xylem Embolism

1.2.1 Vulnerability of Xylem Vessels to Cavitation

The water conducting cells are principally responsible for the transport and distribution of large quantities of water and solutes throughout the plant. The xylem vessels and xylem parenchyma cells have evolved an intricate structure – function relationship to satisfy the demands of water flow within higher plants; by controlling and facilitating the movement of water and ions into (loading) and out (unloading) of the xylem vessels. According to the CT theory, the xylem vessels are maintained under negative pressure, typically around –1 to –2 MPa but pressure can fall as low as –10 MPa (Tyree and Sperry, 1989b). For the plant to sustain a constant water flow to the leaves, interruption to the water column must be avoided. An increase in tension in the xylem vessels, usually as a result of stress, can cause the water column to suddenly break or "cavitate". A vacuum is formed and this results in air being drawn into the vessels resulting in the formation of an embolism, thus the vessel can no longer be used for water transport. The loss of xylem conduits for water transport results in a reduction in the overall hydraulic conductivity of the stem. Different plant species have a varying vulnerability to cavitation and this is dependent on the hydraulic architecture of the plant (Tyree *et al.*, 1994). In the woody species, *Juniperus ashei* and *Bumelia lanuginose*, vulnerability to water stress-induced xylem embolism was found to be greater in the roots than in both the stems and shallow roots (McElrone *et al*., 2004). This difference in cavitation vulnerability was accounted for by variations in both xylem anatomy and hydraulic properties (McElrone *et al.*, 2004).

In 1983, Zimmermann proposed the segmentation hypothesis to explain how plants were able to adapt to different environmental conditions (Zimmermann, 1983). The segmented

structure of plants allows plants to "sacrifice" organs or tissues under stressful conditions. In dicotyledonous plants, water must be transported long distances from the roots to the leaves via the main stem, therefore maintaining stem function is of critical importance for the survival of the plant. Under conditions of water stress, there is a decrease in pressure in the xylem vessels. The more negative xylem pressure increases the tension in the xylem vessel and this can result in cavitation. According to the segmentation hypothesis plants will more likely sacrifice renewable regions of the plant such as the leaves or lateral branches of trees (Zimmermann, 1983). Recently, Tyree *et al.* (1991) elaborated upon this hypothesis and proposed that plants have both the capacity to segment based on 1) hydraulic segmentation and 2) vulnerability segmentation. The concept of "vulnerability segmentation" describes how distal portions of a plant are more vulnerable to cavitation of the xylem vessels than basal portions. This has been shown in a number of species including *Juglans regia L.* (Tyree *et al.*, 1993) and *Acer saccharinum* (Tsuda and Tyree, 1997).

1.2.2 Water Stress-Induced Embolism

Cavitation in plants occurs as a result of water stress, winter freezing or mechanical stress (pathogens, herbivory) (Tyree and Sperry, 1989b). There have been numerous mechanisms proposed to describe how water stress causes embolism in plants. Zimmermann's (1983) "air seeding" hypothesis has gained the most experimental support (Cochard *et al.*, 1992; Jarbeau *et al.*, 1995; Pockman *et al.*, 1995). The mechanism of air-seeding relies on the structural properties of the xylem vessels and the associated pit membrane. Air seeding will occur when the tension in the water column (as a result of water stress) increases such that air is forced through the pits into the water filled vessel, resulting in cavitation of the water column.

Grapevines have been classified as an isohydric species based on their ability to maintain leaf water potential at a minimum through stomatal control of transpiration (During, 1987; Winkel and Rambal, 1993). A large diversity in drought tolerance is observed in different *Vitis vinifera* L. (Smart and Coombe, 1983) by their varied ability to maintain leaf water potential (ψ_{leaf}) under conditions of water stress. This suggests that a classification of isohydric and anisohydric may be appropriate within the grapevine species (Chaves *et al.*, 1987; Winkel and Rambal, 1993; Schultz, 1996). Schultz (2003) compared the hydraulic architecture of two different grapevine cultivars, Grenache and Syrah (Shiraz). Grenache demonstrated near-isohydric behaviour and Syrah anisohydric behaviour when subject to water stress.

The hydraulic conductivity of grapevines is usually high due to the presence of large xylem vessels (Scholander *et al.*, 1955; Essau, 1965). Early work in grapevines has shown that cavitation occurs in the xylem vessels when the xylem is under negative tensions (Scholander *et al.*, 1955). Cavitation and embolism has been shown to occur in grapevine (Lovisolo and Schubert, 1998; Holbrook *et al.*, 2001; Schultz, 2003). It has previously been shown that under moderate water stress, the hydraulic conductance of grapevine shoots is decreased, most likely due to the formation of embolisms (Schultz and Matthews, 1988b). Water stress in grapevines results in a decrease in the stem water potential. Stem water potential is maintained just above the point at which cavitation occurs (Cochard *et al.*, 1997). Schultz (2003) detected cavitation by measuring the acoustic emissions of two grapevine cultivars, Syrah and Grenache, under water stress.

Lovisolo and Schubert (1998) studied the effect of water stress on vessel size and xylem hydraulic conductivity in grapevine. Shoot hydraulic conductivity and vessel size were both

reduced when plants were subjected to water stress. Moderately water stressed vines (ψ_{leaf} –0.6 MPa) showed a reduction in vessel size but had no detectable embolisms. Under more severe water stress (ψ_{leaf} –0.8 MPa), the hydraulic conductivity was further reduced due to embolism formation in the xylem vessels. This observation was similar to that previously reported by Schultz and Matthews (1988a).

1.2.3 Detection of Cavitation

Milburn and Johnson (1966) found that cavitation could be detected by measuring the acoustic emissions from plants using audio (low frequency) detection. When cavitation occurs in the xylem vessels under tension, this causes a rapid relaxation producing an acoustic emission (AE) of energy. Subsequently, Tyree adapted this method by measuring the ultrasonic frequency in stems of *Thuja occidentalis, Tsuga and Acer* (Tyree and Dixon, 1983; Tyree *et al.*, 1983; Tyree *et al.*, 1984a; Tyree *et al.*, 1984b; Tyree and Dixon, 1986). A strong correlation between cavitation events in the xylem with acoustic emissions has been shown for both methods in a number of plant species (Tyree and Dixon, 1983; Nardini *et al.*, 2001; Kikuta *et al.*, 2003; Nardini and Salleo, 2003).

1.3 Mechanism of Embolism Repair

For plants to maintain their hydraulic capacity they must have a mechanism to repair or replace embolised vessels. Numerous studies have shown that the xylem is able to recover from embolism although the mechanism for recovery is still under debate (Salleo *et al.*, 1996; Canny, 1997; McCully *et al.*, 1998; Zwieniecki and Holbrook, 1998; McCully, 1999; Tyree *et*

al., 1999; Zwieniecki and Holbrook, 2000; Clearwater and Goldstein, 2005). In studies on plant species able to recover from winter embolism, it has been found that these plants have the ability to either replace embolised vessels with new functional vessels every year, and/or, refill embolised vessels by generating positive xylem pressures (Holbrook and Zwieniecki, 1999; Tyree *et al.*, 1999; Ameglio *et al.*, 2002). The generation of positive xylem pressures has only been reported in a few species such as *Acer pseudoplatanus* (Omalley and Milburn, 1983); (Tyree and Dixon, 1983; Sperry *et al.*, 1987; Sperry *et al.*, 1994), birch (Sperry *et al.*, 1994; Zhu *et al.*, 2000), walnut (Ameglio *et al.*, 2001; Ewers *et al.*, 2001) and *Vitis vinifera* (Sperry *et al.*, 1987). Evidence has shown that the refilling of embolised vessels in grapevine not only occurs under positive root pressures but can also occur when the vine is actively transpiring (Holbrook *et al.*, 2001). Holbrook and Zwieniecki (1999) have proposed a mechanism for embolism repair involving the movement of water from the surrounding living xylem cells based on xylem structure and their known physical properties. Recently, Konrad and Roth-Nebelsick (2003) presented a theoretical analysis of the interfacial processes of bubbles in the xylem conduit and found their analysis supported the mechanism suggested by Holbrook and Zwieniecki (1999). The identification of water channels located in plant cellular membranes may help to elucidate the possible pathway of water movement into the xylem vessels and may implicate aquaporins in repair of embolism.

1.4 Aquaporins

Aquaporins or water channels are membrane intrinsic proteins that facilitate the movement of water and other small neutral solutes across cellular membranes. The first aquaporin identified, AQP1 (CHIP28), was isolated from the erythrocyte membrane (Denker *et al.*, 1988) and was later functionally characterised in *Xenopus* oocytes where it was shown to

function as a water selective channel (Preston *et al.*, 1992). Aquaporins consist of six putative transmembrane alpha helices with a predicted molecular mass between 26 - 34 kDa. AQP1 has been crystallised confirming the homotetrameric assembly and structurally resolved to 3.8Å using electron crystallography (Murata *et al.*, 2000). The discovery of AQP1 has led to the identification of aquaporins in animals, fungi, bacteria and plants (Borgnia *et al.*, 1999; Tyerman *et al.*, 2002). In the last decade, the atomic structure of a number of mammalian aquaporins selective for water have been determined, including AQP1 (Murata *et al.*, 2000; Sui *et al.*, 2001a), AQP0 (Gonen *et al.*, 2004; Harries *et al.*, 2004) and AQPZ from *E.coli* (Savage *et al.*, 2003). The crystal structure of the glycerol channel, GlpF, has also been determined (Fu *et al.*, 2000). The first aquaporin from plants to be cloned and functionally expressed was a tonoplast integral membrane protein (γ-TIP) from *Arabidopsis thaliana* (Maurel *et al.*, 1993). Plant aquaporins constitute a large gene family with 35 members identified in Arabidopsis (Johanson *et al.*, 2001), 31 in maize (Chaumont *et al.*, 2001) and 33 in rice (Sakurai *et al.*, 2005). Recently, a spinach plasma membrane intrinsic protein, SoPIP2;1, has been crystallised in both the open and closed conformation yielding new insights into the structural characteristics of plant aquaporins (Tornroth-Horsefield *et al.*, 2006). The identification of aquaporins in plants has resulted in many studies to ascertain the importance of aquaporins in the transport of water through plants. There has been significant progress on the structural characterisation of AQPs and their functional role at the cellular level. Despite large quantities of data in the literature about aquaporin expression patterns in plant tissues (see below), and their expression in response to environmental stimuli, there is still much information to be gained about the functional role of aquaporins in plant water transport.

1.4.1 Structural Characteristics

Much of the early structural information about aquaporin proteins has come from studies on the human red cell aquaporin, AQP1 and the ancestral aquaglyceroporin, GlpF. The AQP1 monomer consists of 269 amino acid residues (Preston and Agre, 1991) that form tandem repeats of three membrane spanning α-helical domains (Figure 1-1). Comparison with other AQP members show that they also have six membrane spanning domains with the amino (N) and carboxyl (C) termini located on the cytoplasmic face (Preston *et al.*, 1994). Each tandem repeat has the highly conserved asparagine – proline – alanine (NPA) sequence motif (Jung *et al.*, 1994). In the "hourglass" model, proposed by Jung *et al.*, (1994), connecting loops B (cytoplasmic) and E (extracellular) form short hydrophobic helices that correspond to two hemipores. These dip into the membrane and overlap forming a single transmembrane aqueous pathway. Although aquaporins are known to form homotetramers in the membrane (Engel *et al.*, 2000), each monomer functions as a single water pore. A second region of the AQP1 protein, the aromatic/arginine (ar/R) region, is located 8Å above the NPA motif and is thought to constitute the selectivity filter (Fu *et al.*, 2000). The movement of glycerol and water through the pore has been elucidated and is thought to involve both the NPA motif and the ar/R region of the protein (Fu *et al.*, 2000).

AQPs may function as water selective (aquaporins) or non-selective channels for water and other small non-electrolytes (aquaglyceroporins). Based on the predicted structure of AQP1 (Mitsuoka *et al.*, 1999; Murata *et al.*, 2000), specific amino acid residues conserved in maize have been identified, and their involvement in helix interactions and stabilisation of the aqueous pore predicted (Chaumont *et al.*, 2001). Electron cryo-crystallography of 2D crystals of the bean α-TIP (PvTIP3;1) (Daniels *et al.*, 1999) and SoPIP2;1 from spinach

(Fotiadis *et al.*, 2001; Kukulski *et al.*, 2005) provided evidence that homotetramerisation of aquaporins is conserved in the plant MIP family. More recently, X-ray crystallography on 3D crystals of SoPIP2;1 (Tornroth-Horsefield *et al.*, 2006) showed that the protein crystallised as a homotetramer. Based on functional data in *Xenopus* oocytes, Fetter *et al.* (2004) proposed that the PIP1 and PIP2 isoforms may interact to form heterotetramers. Furthermore, an interaction of the two subgroups has been confirmed using FRET imaging in living maize cells over expressing both these isoforms (Zelazny *et al.*, 2007).

NOTE: This figure is included on page 1-13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-1: The Hourglass Model of AQP1 (Agre *et al.*, 1998). *Top* Model of the AQP1 monomer showing a tandem repeat of 3 membrane spanning alpha helical domains (TMH1-3) and (TMH4-6) with the amino and carboxy terminus facing inside the cell. Each tandem repeat has the highly conserved NPA motif. *Bottom* In the hourglass model it is proposed that loops B and E dip into the membrane and overlap to form a single aqueous pathway.

1.4.2 Plant Aquaporins

Plant aquaporins belong to a large superfamily of membrane proteins called the Major Intrinsic Proteins (MIPs) based on their homology to the major intrinsic protein of bovine lens cells, AQP0, the first sequenced member of the family (Gorin *et al.*, 1984). The MIP superfamily consists of four subgroups (Santoni *et al.*, 2000; Johanson *et al.*, 2001; Zardoya and Villalba, 2001), the Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Membrane Intrinsic Proteins (TIPs), Small Intrinsic Proteins (SIPs), and NOD26-like intrinsic proteins (NIPs). Historically, the PIP and TIP subfamily were named based on the putative location of these proteins within the cell. As more members were identified and their subcellular location determined it became apparent that not all members of these subfamilies are located in the plasma membrane and tonoplast, respectively (Barkla *et al.*, 1999). Plant MIP genes have been divided into subfamilies based on homology of their amino acid sequence (Johanson *et al.*, 2001). Wallace and Daniels (2004) have used computational modelling based on the structural characteristics of the pore regions of the mammalian AQP1 and the bacterial glycerol permease, GlpE to construct homology models of the pore regions of the Arabidopsis MIPs. Based on homology of the aromatic/Arg [ar/R] filter the plant MIPs can be classified into eight subgroups. The transport selectivity of the plant aquaporins have been functionally characterised by expression in both *Xenopus* oocytes and yeast, and the reconstitution into proteoliposomes. MIP members have a broad range of transport selectivity's including the permeability to water (Biela *et al.*, 1999; Santoni *et al.*, 2000; Tyerman *et al.*, 2002), glycerol (Biela *et al.*, 1999; Weig and Jakob, 2000; Moshelion *et al.*, 2002), urea (Gerbeau *et al.*, 1999; Liu *et al.*, 2003), CO_2 (Uehlein *et al.*, 2003), NH_3 (Jahn *et al.*, 2004; Loque *et al.*, 2005), H_20_2 (Bienert *et al.*, 2007) and silicon (Ma *et al.*, 2006).

Phylogenetic analysis reveals the PIP subfamily differs from the TIP subfamily by the presence of an extended N-terminal tail comprising of 20-38 amino acid residues. The PIPs can be subdivided into two groups; PIP1 and PIP2. Expression of PIP2 and PIP1 proteins in *Xenopus* oocytes show high and low water channel activity, respectively (Chaumont *et al.*, 2000). The reason for this difference remains unclear but high water channel activity has been observed in some PIP1 members including NtAQP1 (Biela *et al.*, 1999) and BoPIP1b1 and BoPIP1b2 (Marin-Olivier *et al.*, 2000).

The NOD26-like intrinsic proteins were named based on homology to NOD26, the first member identified in the peribacteroid membrane of the nitrogen-fixating symbiosomes of root nodules in soybean (Fortin *et al.*, 1987). The NIPs can be divided into two subgroups (I and II) based on structure function studies of the selectivity determining ar/R filter (Wallace *et al.*, 2006). More recently a fourth group, the small basic intrinsic proteins has been proposed (Chaumont *et al.*, 2001; Johanson *et al.*, 2001). SIPs are quite divergent from the other MIP members. In particular the NPA motif in loop B is not conserved and instead consists of the motif asparagine–proline–threonine (NPT) or asparagine-proline-leucine (NPL) (Chaumont *et al.*, 2001). In Arabidopsis the SIPs are the smallest subgroup of the MIP superfamily with only three members identified. These have been localised on the ER membrane in Arabidopsis cells and both AtSIP1;1 and AtSIP1;2 had water channel activity when expressed in yeast cells (Ishikawa *et al.*, 2005)

The Arabidopsis Genome Project has resulted in the identification of 35 full length MIPs that have been categorised into the four subfamilies (Johanson *et al.*, 2001). MIP genes have been identified in over 30 plant species of both monocots and dicots. In maize 31 full length cDNA's have been identified, four of which have been functionally characterised (Chaumont

et al., 2001). A comparitive homology modelling analysis of the ar/R selectivity filter in MIP members from rice, maize and Arabidopsis shows the diversity in the ar/R region is most likely linked to the functional diversity of plant MIPs (Bansal and Sankararamakrishnan, 2007). Evidence is also provided that both rice and maize MIPs may have higher diversity for solute transport than the Arabidopsis MIPs (Bansal and Sankararamakrishnan, 2007). In the published literature, 10 putative aquaporins with homology to Arabidopsis MIPs have been identified in grapevine (Table 1-1). A number of others have recently (2007) been included on the Genbank database. Eight cDNA's encoding putative aquaporins were identified in *Vitis* rootstock Richter-110 by screening a leaf cDNA library with homologous probes (Baiges *et al.*, 2001). Using reverse Northerns, tissue specific differential expression patterns were analysed for each of the putative aquaporins. Two plasma membrane aquaporins (PIPs) have been cloned from ripening grape berry of *Vitis vinifera* cv Pinot Noir (Picaud *et al.*, 2003). The large diversity of aquaporins found in plant species can in part be explained by differences in subcellular and cellular location (Santoni *et al.*, 2000).

Table 1-1: MIP members identified in grapevine. Shown are PIP and TIP genes identified from *Vitis* Richter-110 (Baiges *et al*., 2001) and *Vitis vinifera* cv Pinot Noir (Picaud *et al*., 2003), their Genbank accession numbers and closest homolog in Arabidopsis.

cDNA NAME	ACCESSION NUMBER	ARABIDOPSIS HOMOLOG
Vitis-PIP1-1	AF141643	PIP1
Vitis-PIP1-2	AF141898	PIP1
Vitis-PIP1-3	AF141899	PIP1
Vitis-PIP2-1	AF141642	PIP2
Vitis-PIP2-2	AF141900	PIP2
Vitis-TIP1	AF271661	δTIP
Vitis-TIP2 (Partial)	AF271662	γ-TIP
Vitis-TIP3	AF271660	γ-TIP
VvPIP1a	AF188843	PIP1
VvPIP1b	AF188844	PIP1

1.5 The Role of Aquaporins in Response to Water Stress

Water transport in plants is a highly developed and intricate process. The large diversity of aquaporins in plants can be explained by their subcellular localisation, tissue specific expression, developmental expression, response to environmental stimuli and different functional selectivity. The discovery of aquaporins in plants has provided an important insight into our understanding of plant water relations. The expression patterns of aquaporin mRNA and protein, and their localisation within cellular tissues can be used to gain a greater understanding about the role of MIPs in the water transport pathway of plants. Over the past decade a number of review articles (Schaffner, 1998; Maurel and Chrispeels, 2001; Maurel *et al*., 2002; Tyerman *et al*., 2002; Kaldenhoff and Fischer, 2006; Maurel, 2007) have

examined the literature on AQP expression and postulated potential functional roles for the diversity of MIPs *in planta*. Broadly, the expression patterns of both PIP and TIP proteins have provided evidence of their involvement in both the long and short distance water transport pathway in plants.

1.5.1 Regulation of Aquaporins in Response to Water Stress

Aquaporins are highly regulated in response to many environmental signals including both biotic and abiotic factors (reviewed by Maurel *et al.*, 2002). Abiotic factors include drought, salt and cold stress, and biotic factors include symbiotic and pathogen interactions and hormonal factors. Evidence for aquaporin regulation in response to water stress has been documented to include transcriptional and post-transcriptional regulation, and post-translational modification of aquaporin proteins.

1.5.1.1 Transcriptional and Post-Transcriptional Regulation

Over the last decade numerous studies have examined the effect of water stress on the mRNA expression patterns of aquaporin genes (reviewed by Tyerman *et al.*, 2002). Several aquaporin genes have been found to be upregulated under conditions of water stress including *MipA* from *Brassica oleracea* (Barrieu *et al.*, 1998), *rd28* gene from *Arabidopsis thaliana* (Yamaguchi-Shinozaki *et al.*, 1992), *OsPIP1a* and *OsPIP2a* from rice (Malz and Sauter, 1999), tomato-ripening-associated membrane protein (TRAMP) (Fray *et al.*, 1994), and *CpPIPa6* and *CpPIPa2* from the dessication tolerant resurrection plant *Craterostigma plantagineum* (Mariaux *et al.*, 1998). Aquaporins have also been shown to be downregulated

under water stress in a number of species including a family of PIPs from the iceplant *Mesembryanthemum crystallium* (Yamada *et al.*, 1995), *SunTIP18* from sunflower (Sarda *et al.*, 1999) and *CpTIP* from *Craterostigma plantagineum* (Mariaux *et al.*, 1998). Recently, Suga *et al.* (2002) studied the response of aquaporin isoforms in radish seedlings to salt and water stress. The mRNA levels of *RsPIP2* members were upregulated under water stress. Protein levels were also measured but these did not correlate directly with the mRNA levels indicating that these genes may be regulated post-transcriptionally. The stress hormone ABA, which is known to be critical in plants as a key indicator of water stress, has been shown to increase expression of aquaporin genes in a number of species. In rice, *OsPIP1a* (Malz and Sauter, 1999) and *rTIP1* (Liu *et al.*, 1994) showed higher expression in plants treated with ABA.

Many studies have attempted to correlate AQP expression in response to biotic and abiotic factors, by localising these genes to help elucidate their functional role in the plant. A review by Maurel *et al.* (2002) has attempted to link the current literature on aquaporin expression and localisation to assess functional roles in response to environmental stimuli. The expression of PIP homologs in Arabidopsis showed significantly higher expression in the vascular tissues, consistent with a role in the transport of water from the roots into the stele (Schaffner, 1998). Consistent with these findings, numerous other studies have shown high expression of both MIP and TIP genes in the vascular tissues of both the roots and shoots. For example, *ZmTIP* is highly expressed in the parenchyma cells that surround the early and late metaxylem in roots and shoots of maize (Barrieu *et al.*, 1998). Recently, Siefritz *et al.* (2004) found high expression of *NtAQP1* in the xylem and epidermal cells of petioles associated with the leaf unfolding mechanism in tobacco. *Pip1b* from Arabidopsis leaves has

also been found to be highly expressed in the parenchyma cells and vascular bundles of the protoxylem and protophloem (Kaldenhoff *et al.*, 1995).

1.5.1.2 Post-Translational Modification

Until recently the gating of plant aquaporins was not well understood. The X-ray crystal structure of the plant aquaporin, SoPIP2;1, in both the open and closed conformation, has provided novel insights into the molecular mechanism of aquaporin gating (Tornroth-Horsefield *et al.*, 2006). Previous studies have shown that one mechanism of post-translational regulation of aquaporins is reversible phosphorylation. In spinach leaves, the plasma membrane aquaporin, SoPIP2;1 (previously called PM28A) is dephosphorylated under drought conditions and therefore inactivated (Johansson *et al.*, 1996). Dephosphorylation occurs at two serine residues, Ser115 in cytosolic loop B and, Ser274 in the C terminus, by a Ca^{2+}-dependent protein kinase (Johansson *et al.*, 1996; Johansson *et al.*, 1998). Serine phosphorylation of the bean α-TIP has also been demonstrated (Maurel *et al.*, 1995). Recently, Tournaire-Roux *et al.* (2003) found that during anoxic stress PIPs may be gated by the cytosolic pH in Arabidopsis. The first report of methylation of membrane proteins in plants has been shown for the Arabidopsis PIP2s. Using mass spectrophotometry, two N terminal residues, Lys3 and Glu6 were shown to be dimethylated and monomethylated, respectively (Santoni *et al.*, 2006).

1.5.1.3 The Role of Aquaporins *in Planta*

The role of aquaporins in root water uptake from the soil has been looked at in some detail and reviewed by (Javot and Maurel, 2002). The effect of mercury (mercuric chloride) was first assessed on plant aquaporins functionally expressed in *Xenopus* oocytes (Daniels *et al.*, 1994; Biela *et al.*, 1999). Mercury compounds inhibit aquaporin activity by targeting the cysteine residues in the aqueous pore. This finding has allowed the use of mercurials to assess the contribution of aquaporins in water uptake in whole plant roots (reviewed by Javot and Maurel, 2002). Not all aquaporins are sensitive to mercurial compounds, such as NtAQP1 (Biela *et al.*, 1999). Mercurial compounds are not specific and may also target other membrane proteins making the results of these studies somewhat ambiguous (Javot and Maurel, 2002).

Recently, reverse genetics has been used to attempt to assess the functional role of individual aquaporin isoforms *in planta* (reviewed in Hachez *et al.*, 2006). In a recent study the role of plasma membrane aquaporins was assessed in *Arabidopsis* by the construction of a double antisense line (*pip1* and *pip2*) (Martre *et al.*, 2002). The results of this study showed that during soil drying the double antisense plants (dAS) suffered a greater water stress than control wildtype plants. After rewatering the dAS plants took longer to recover their hydraulic conductance and transpiration rates suggesting that PIPs may play a role in the recovery from water deficit. In tobacco, antisense *NtAQP1* plants had a significantly reduced root hydraulic conductivity corresponding to decreased aquaporin expression (Siefritz *et al.*, 2002). By over expressing Arabidopsis *Pip1b* in transgenic tobacco plants, Aharon *et al.* (2003) was able to show that transgenic plants had an improved growth response under favourable conditions. However, under drought stress, constitutive

expression of *Pip1b* was detrimental to the plant causing a faster wilting response. Inhibition of *AtTIP1;1* using RNAi resulted in plant death (Ma *et al.*, 2004).

1.5.2 Involvement of Aquaporins in Embolism Recovery

In recent years it has been hypothesised that aquaporins may contribute to the water movement required for embolism recovery of xylem vessels thus restoring the hydraulic pathway (Holbrook and Zwieniecki, 1999; Tyree *et al.*, 1999). In walnut, aquaporins have been postulated to be involved in the recovery from winter embolism (Sakr *et al.*, 2003). By using semi-quantitative RT-PCR Sakr *et al.*, (2003) showed high levels of *JrPIP2* mRNA expression in the winter months, corresponding to, a simultaneous increase in sucrose levels. The accumulation of soluble sugars in walnut has previously been shown to correlate with recovery from winter embolism (Ameglio *et al.*, 2001). Immunolocalisation studies showed expression of the corresponding JrPIP2 proteins localised mainly in the vessel associated cells (VAC's) indicating a potential role in controlling water movement between the parenchyma cells and the embolised vessels.

1.6 Conclusion

The detection of cavitation in grapevines using acoustic monitoring could potentially allow the development of a novel method for measuring water stress in grapevines and across a vineyard. This will be beneficial in managing vineyard irrigation and ultimately will result in enhanced fruit development and wine quality. There is little information known about the molecular mechanisms that co-exist with the physiological mechanisms for the regulation of

water transport in grapevines. This project will not only allow the establishment of a method for directly sensing water stress in the vine, but also provide novel information about the water relations of grapevines particularly in regards to the role aquaporins play in response to cavitation and their recovery from drought.

1.7 Significance of the Project

The aim of irrigation management is to optimise soil water content so that vine water deficit is controlled and vine water logging is avoided. The occurrence of either of these two physical constraints is likely to result in a reduced efficiency or altered fruit quality. Currently, there are three methods used to manage irrigation scheduling. These are plant and soil-based methods and weather based modelling. Soil-based methods are by far the most common method of measuring the water status of a grapevine/vineyard. A variety of factors can influence a vines ability to utilise soil water including varietal differences, soil properties, root distribution, root-soil interactions, spatial heterogeneity, nutrient availability and vineyard management. Thus, measuring soil water status does not always give an accurate measure of vine water status. Plant-based methods include monitoring vine appearance and the measurement of vine water status using the pressure bomb or sap flow measurements. Although these methods are useful a more accurate and sensitive method of measuring vine water status is needed.

Cavitation occurs in the xylem vessels of plants when there is an increase in xylem tension, causing the water column to suddenly break or “cavitate”. Eventually the cavitated vessel fills with air resulting in the formation of an embolism. Cavitation can be induced by freezing, water stress and pathogens (reviewed by Tyree and Sperry, 1989b). The occurrence of

cavitation in the xylem vessels reduces the overall hydraulic conductivity of the stem. Using a specialized microphone attached to the stem, the expansion of air in the xylem can be registered as an acoustic emission (AE) or cavitation click (Milburn and Johnson, 1966; Tyree and Dixon, 1983). This method has been used for other woody plants (Jackson *et al.*, 1995) and recently Schultz (2003) used this method to compare cavitation in two grapevine cultivars, Shiraz and Grenache. To use this technology as a measure of water stress in grapevine it is important to first determine the relationship between water stress and cavitation and link this to the acoustic emissions. It will also be important to determine where in the grapevine cavitation is likely to occur first, which is dependent upon the hydraulic architecture of the vine.

1.8 Project Aims

The objective of this PhD project was to compare water-stress induced xylem embolism in two grapevine cultivars, Chardonnay and Grenache. These two varieties are known to have differences in drought tolerance. To achieve this, ultrasonic acoustic emissions (UAE's) were measured in these two varieties, allowing *in planta* monitoring of water-stress induced cavitation of the xylem vessels. Molecular biology techniques were used to correlate the expression of water channels (aquaporins) in the petioles with cavitation events under water stress. The specific aims of the project were to:

- Characterise the correlation between cavitation events in xylem vessels and acoustic emissions.
- Determine the correlation between UAEs and water potential over a diurnal cycle.

- Compare grapevine varieties to determine differences in cavitation thresholds and correlate this to drought tolerance.
- Identify and clone aquaporin genes from grapevine.
- Functionally characterise grapevine aquaporins using *Xenopus* oocytes as a heterologous expression system.
- Localise expression of aquaporins in the petioles.
- Determine the expression of AQPs in the petioles under water stress.
- Correlate expression of AQPs with cavitation and refilling of embolised xylem vessels.

Chapter 2:
Water Stress-Induced Xylem Embolism in Two Grapevine Cultivars, Chardonnay and Grenache

2.1 Introduction

Xylem embolism is a well-known phenomenon that occurs in plants as a result of freezing, water stress and mechanical stress. Xylem embolism induced by drought stress occurs when the tension in the water column becomes so negative, that it causes the water column to cavitate. Xylem cavitation as a result of water stress is a common occurrence and is widely reported in woody plants including grapevines (Milburn and Johnson, 1966). According to the air seeding hypothesis (Zimmermann, 1983) air enters through the conduit pit membranes, resulting in the vessel rapidly filling with water vapour and becoming embolised. Evidence for this mechanism has been supported by a number of studies in woody plants including grapevines (Sperry *et al.*, 1987; Sperry and Tyree, 1988). Previous studies on grapevine have shown that even in conditions of moderate drought stress, cavitation can occur resulting in an increase in the hydraulic resistance within the plant (Scholander *et al.*, 1955; Schultz and Matthews, 1988a). Schultz and Matthews (1988a) reported that cavitation can occur in well-watered grapevines over a diurnal cycle when transpiration rates are high. The hydraulic capacity of a plant under water stress is determined not only by the formation of xylem embolisms, but also by the size and structure of the xylem vessels (Lovisolo and Schubert, 1998). Grapevines have large xylem vessels (Scholander *et al.*, 1955; Essau, 1965; Zimmermann and Jeje, 1981) making them more susceptible to the formation of embolised vessels under conditions of water stress, thus resulting in a reduction in their ability to transport water. It is also well established that plants can exhibit vulnerability segmentation (Tyree and Ewers, 1991). This is when renewable parts of the plant are more vulnerable to cavitation such as was shown for petioles in both walnut and *Acer saccharinum* L. (Tyree *et al.*, 1993; Tsuda and Tyree, 1997).

For plants to maintain their hydraulic capacity a mechanism must exist to either replace or repair embolised vessels. For many years cavitation was viewed as being catastrophic to a plant's hydraulic pathway because it was believed that embolised vessels were unable to refill, or could only refill over a long period of time (such as over a season) or by the generation of positive root pressure by active solute transport (Tyree and Dixon, 1986; Sperry *et al.*, 1994). Sperry *et al.* (1987) reported that during spring, grapevines use positive root pressure to refill gas filled vessels that form during winter. Many recent studies have provided evidence for the rapid refilling of embolised vessels over much shorter time frames, even while vessels are under tension (McCully *et al.*, 1998; McCully, 1999; reviewed in Meinzer *et al.*, 2001; Clearwater *et al.*, 2004; reviewed in Clearwater and Goldstein, 2005). Recently, Holbrook and Zwieniecki (1999) proposed a refilling mechanism involving the surrounding living xylem parenchyma cells combined with the anatomical features of the xylem vessels to repair embolised xylem vessels. Using magnetic resonance imaging (MRI), Holbrook *et al.* (2001) was able to show both cavitation and embolism repair in the xylem vessels of grapevine stems. Cavitation occurred whilst the plant was actively transpiring, while embolism repair only occurred when the lights were turned off and xylem tension was close to zero. They proposed that the movement of water back into the embolised vessels may require the presence of membrane bound water channels (aquaporins) in the surrounding xylem parenchyma cells.

Grapevines have previously been classified as a drought avoiding species (Smart and Coombe, 1983) or isohydric species, which means they control their water loss and midday leaf water potential by closing their stomata. A recent study compared differences in the hydraulic architecture of two grapevine varieties, Grenache and Syrah (Shiraz) in response to soil water deficit (Schultz, 2003). The authors classified Grenache as a near-isohydric

variety and Syrah as anisohydric, and that this behaviour may be based on differences in the water conducting capacity of the stems and petioles. Soar *et al.* (2006) further investigated the stomatal response of these two varieties by exposing them to varying vapour pressure deficit (VPD). The same isohydric / anisohydric behaviour was apparent in response to high VPD, as was seen for soil moisture deficit. It was further suggested that ABA physiology may be a key process in determining this stomatal response. Stomatal closure is thought to control drought induced cavitation in grapevine (Lovisolo and Schubert, 1998).

The aim of this project was to compare the cavitation vulnerability of two grapevine varieties, Chardonnay and Grenache, in response to water stress. Grenache is a drought tolerant variety and Chardonnay is comparably more sensitive to water stress. In order to determine the correlation between cavitation and leaf water potential, experiments were conducted on potted Chardonnay and Grenache vines. Measurements of leaf water potential and ultrasonic acoustic emissions (UAEs) were recorded in the short-term over a diurnal cycle and in the longer-term over a drying cycle. Acoustic emissions have previously been used to measure cavitation in other woody plants including grapevine (Milburn and Johnson, 1966; Tyree and Dixon, 1983; Tyree *et al.*, 1984b; Tyree and Sperry, 1989a). In this study, the cavitation vulnerability was determined for the stem and petioles in Chardonnay and Grenache. The hydraulic properties of the petiole of each variety were also determined and this was related to xylem anatomy.

2.2 Methods

2.2.1 Plant Material and Growth Conditions

Vitis vinifera cv. Grenache SA38 and Chardonay I10V1 one year old rootlings were purchased from Glen Avon Nursery (Langhorne Creek, SA) or Yalumba Nursery (Barossa Valley, SA), and stored at 4 °C until required. One year old cuttings of the same varieties were also taken from dormant vines in the Coombe Vineyard (Waite Campus, Urrbrae, SA) during July, treated with 0.5% (v/v) Chinosol solution overnight and stored at 4 °C until required. Canes were calloused for 2-3 weeks in a heated propagation bed at 25 °C. Rootlings were planted in 4.7 L pots containing a modified University of California (UC) soil mix (61.5 % (v/v) sand, 38.5 % (v/v) peat moss, 0.50 g/L calcium hydroxide, 0.90 g/L calcium carbonate, 100 g/L Nitrophoska® (12:5:1 N:P:K plus trace elements) pH 6.8) and fertilised with 0.06 g/L per month of Osmocote®. Vines were spur pruned to have one shoot (unless indicated otherwise) (Tassie and Freeman, 2001). Plants were grown in controlled temperature glasshouses at 25 °C day / 20 °C night with night break lighting provided by halide lamps for 1 hour during the night period (Plant Research Centre, Waite Campus, Adelaide). Pot grown vines were subjected to a drying cycle to impose a water stress. Plants were watered to field capacity in the evening prior to starting drought experiments. Water was withheld for the remainder of the experiment. All physiological experiments were conducted between October and March, over seasons 2004/05 and 2005/06. Where indicated, a number of physiological experiments were conducted in temperature controlled growth rooms during winter. Plants grown in chambers were exposed to 12.5 h of lighting, day / night temperatures were set at 25 °C and 15 °C respectively.

2.2.2 Detection of Cavitation by Acoustic Emissions

The vulnerability to cavitation in petioles and stems was measured by detection of UAEs in response to water stress. Using a specialized microphone attached to the stem, the expansion of air in the xylem can be registered as an UAE or cavitation click (Milburn and Johnson, 1966; Tyree and Dixon, 1983). This method has been used for other woody plants (Jackson *et al.*, 1995) including grapevines (Schultz, 2003). Acoustic emissions were measured using an acoustic monitoring system (Model 4615 DSM, Physical Acoustic Corporation, NJ, USA) with I151 sensors. Signals were amplified in the 150 - 400 kHz range. Data was recorded automatically on a computer. Only two sensors were available so only one plant could be monitored at a time. At least five individual plants were monitored over the growing season for each variety. Sensors were positioned on the basal portion of the plant, between nodes 2-6, on fully developed leaves. One specialised microphone was clamped to the middle of an internode and one to a petiole (Figure 2-1). A thin layer of silicon grease was put onto the transducer to allow better acoustic contact with the plant. The plants were watered to field capacity at the start of the experiment and UAEs were recorded continuously over the drought period.

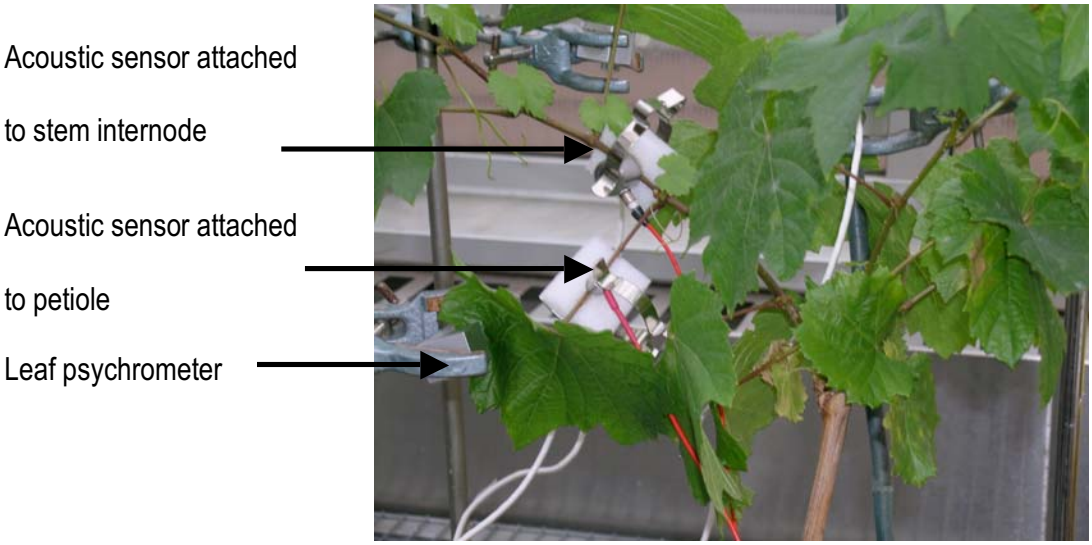


Figure 2-1: Photo showing the acoustic sensors attached to the petiole and internode of a mature Chardonnay vine. Leaf psychrometer sensors are attached to the abaxial side of the leaves.

2.2.3 Data Analysis

Each cavitation click detected by the acoustic sensors was recorded at the time at which it occurred. The cumulative UAEs (cUAE) were determined and plotted against time of drought stress (Figure 2-5). In addition, cUAEs were normalised to the maximum number of cavitated vessels in each organ (petiole or stem) and plotted against the mean leaf water potential. A sigmoidal dose-response curve (variable slope) was fitted to the data using GraphPad Prism® Version 4.0. Leaf water potential was determined as described in section 2.2.4. The cavitation threshold ψ_{CAV} value is the leaf water potential value determined as the point at which cavitation is triggered and is taken as 10 % of the maximum cUAEs (Salleo *et al.*, 1996; Nardini *et al.*, 2001). The mean threshold ψ_{CAV} value was determined for the stem and petiole of at least three plants of each variety. An unpaired t-test was used to determine if values were significantly different.

2.2.4 Measurement of Leaf Water Potential

Leaf water potential (ψ_L) measurements were made using the PSYPRO™ (WESCOR, INC, Utah, USA) data logger with leaf psychrometer sensors. The PSYPRO™ water potential system determines water potential by measuring the relative humidity in equilibrium with the object. The following equation is used to calculate the water potential:

$$\psi = (RT/ V_w) e/e_o \qquad \text{Equation 2-1}$$

where:

ψ is the water potential,

R is the universal gas constant (8.3143 J mol^{-1} K^{-1}),

e/e_0 is the relative humidity,

T is the absolute temperature (K),

V_w is the molar volume of water (1.8 x 10^5 m^3mol^{-1}).

Psychrometers were calibrated with NaCl solutions at the following concentrations; 207 mOs kg^{-1}, 414 mOs kg^{-1}, 621 mOS kg^{-1}, as described by Campbell and McInnes (1999). The corresponding water potential was calculated using the Van't Hoff Equation:

$$\psi = - MRT \qquad \text{Equation 2-2}$$

where:

R is the universal gas constant,

T is the temperature (K) and

M is the osmolarity.

Correction factors were determined for each psychrometer by plotting the actual water potential versus expected water potential. The slope of this line gives the correction factor.

Four L-51(A)-SF leaf psychrometer sensors were positioned on four basal leaves surrounding the acoustic sensors. Prior to attachment the cuticle was removed from the abaxial side of the leaf with 1200 grit sandpaper, as described by Campbell and McInnes (1999). The scan sequence programme was set as follows: cooling current time 15 s, measurement period seconds 20 s, delay seconds after cooling 5.2 s and read average seconds 6 s. For each psychrometer a measurement was taken every 15 min and recorded with the PSYPRO™.

Where indicated, leaf water potentials were also measured with a pressure chamber (PMS Instruments, Oregon, USA). For experiments conducted in growth chambers, predawn water potential measurements were taken 30 min prior to lighting being switched on.

2.2.5 Measurement of Petiole Hydraulic Conductance

The hydraulic conductance (K) of grapevine petioles was measured using the XYL'EM apparatus (Instrutec Company, H. Cochard and T. Ameglio, INRA-PIAF Laboratory, Clermont-Ferrand, France). The XYL'EM apparatus was equipped with a pressure transducer and two flow meters (Liquiflow, Instrutec), 5 and 50 g h^{-1}, that measure the thermal mass flow rate. Degassed glass distilled water (LABGLASS CASCADE, Graintech, Australia) was used to fill the captive air tank (Figure 2-2). Petioles were cut parallel to the node under MilliQ water and immediately attached to Luer tubes that were connected to the XYL'EM apparatus. The leaf blade was removed from the petiole after connection to the Luer tube. The water flow entering the petiole was measured when exposed to a positive pressure of -0.15 MPa. This pressure was sufficient to remove embolisms (as determined by preliminary experiments). The flow rate was measured every second until a stable reading was obtained, usually between 2-5 min (Figure 2-3). XYL_WIN was loaded into a PC and was used to log the data. The XYL'EM software automatically corrects for air temperature and the viscosity of water. Air temperature varied from 20 - 22 °C. Hydraulic conductance, was calculated automatically by the XYL'EM software, with the following formula:

$$K = F / \Delta P \qquad \text{Equation 2-3}$$

where:

K is the conductance,

F is the volumetric flow rate ($m^3\ s^{-1}$),

$\Delta P = P_{in} - P_{out}$ is the pressure difference across the sample (MPa).

An example of the raw data for thermal mass flow rate and K plotted against time for a Chardonnay and Grenache petiole is shown in Figure 2-3. The petiole specific hydraulic conductivity (K_h) was computed as:

$$K_h = F * L / \Delta P \quad \text{Equation 2-4}$$

where L is the length of the petiole segment (m).

At the completion of the experiment the leaf area (LA, m^2) for the corresponding leaf blade, was measured with a Leaf Area Meter (AM200, ADC Bioscientific Ltd, Herts, England). Leaf area specific conductivity (LSC) was determined by:

$$LSC = K_h / LA \quad \text{Equation 2-5}$$

Attempts were made to measure the percent loss conductance (PLC) in grapevines using the XYL'EM apparatus. However, during preliminary measurements it was found that the time required to conduct the low and high pressure perfusions of the petioles resulted in consistently decreasing flow rates, generating negative PLC values. The reason for this is unknown but it may be a result of tyloses forming, blockage of the petiole end, or rapid changes in the hydraulic pathway of the petiole as a result of the experimental technique. Due to this unexpected result, PLC values were not reported. Similar problems using the XYL'EM have been reported previously (Cochard *et al.*, 2007).

Hydraulic conductance experiments were performed on glasshouse grown Chardonnay and Grenache vines. Plants were moved to a temperature controlled growth room one week

prior to experiments. Controlled temperature growth rooms were maintained at 25 °C day and 15 °C night, with lighting from 7:30 am until 8 pm. Day length was 12.5 hours. Lighting was provided by OSRAM Powerarc® 400 W lamps (M59/E). The PAR at pot level was approximately 130 μmol m^{-2} s^{-1}. Plants used for the measurements in the dark (7 am and 10 pm), were covered with black plastic bags for transport to the laboratory and remained covered until the measurements were taken. Petioles from three plants were used for each time point and measurements were made on two petioles from each plant. Petioles were harvested from the same nodal positions on all plants, at the basal portion of the stem (nodes 3-5).

A.

B.

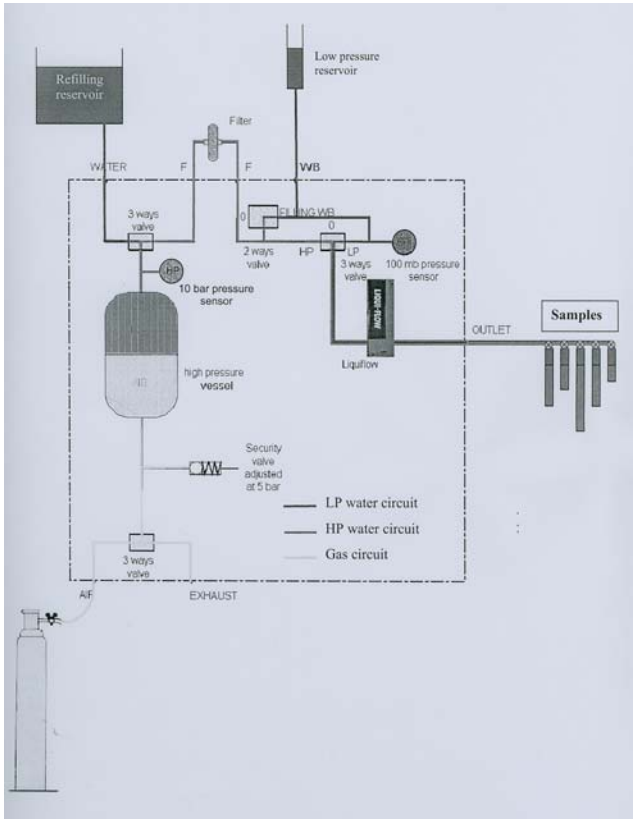


Figure 2-2: XYL'EM apparatus used to measure hydraulic conductance. A. XYL'EM apparatus. B. Schematic diagram of the XYL'EM apparatus (XYL'EM instruction manual, INSTRUTEC HI-TEC, Clermont-Ferrand, France). The high pressure vessel is filled with degassed water and pressurised with nitrogen gas. A 3-way valve allows control of water flow into the attached petiole sample.

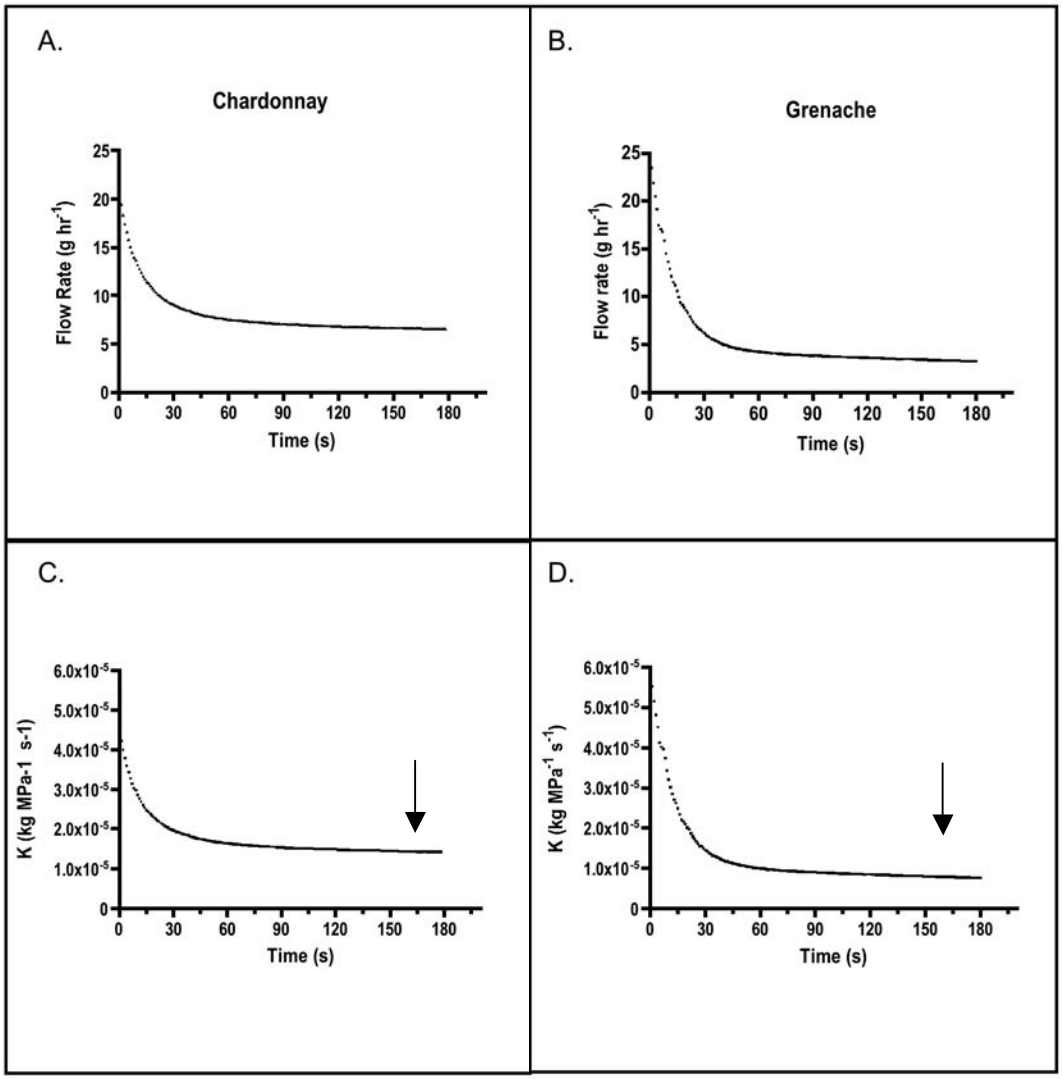


Figure 2-3: Conductance and flow rate of water over time though grapevine petioles. Flow rate of water plotted against time through A. Chardonnay and B. Grenache petioles at 7 am. Hydraulic conductance (K) plotted against time for C. Chardonnay and D. Grenache petioles. Measurements of conductance were taken once flow rate had stabilised as indicated by the arrow. Representative raw data is shown for one petiole from each variety.

2.2.6 Xylem Anatomy

Xylem vessel diameters were measured on petioles from Grenache and Chardonnay vines. Transverse sections were made from petioles collected from glasshouse grown vines used for acoustic monitoring and hydraulic conductance experiments. Hand sections were made using a single blade razor blade in the middle of the petiole. Sections were stained with toluidine blue O (0.05 %) for 1 min, rinsed with distilled water and mounted onto slides in distilled water. Sections were viewed under a light microscope (Zeiss Aixophot Pol Photomicroscope, Oberkochen, Germany) at 5 x and 10 x magnification. Images were captured using Nikon DXM1200F digital video (Coherent Life Sciences, Adelaide, Australia) and Nikon ACT-1 software (version 2.62, Nikon Corporation, Coherent Life Sciences, Adelaide, Australia). The lignified secondary walls of the xylem vessels were identified by their blue-green staining (O'Brien *et al.*, 1964). The mean xylem vessel diameter was determined for each variety by sampling petioles from four vines and measuring the diameter of 100 xylem vessels for each vine (n=400).

2.2.7 Data Analysis

Data was analysed using Microsoft® Office X Excel and GraphPad Prism® Version 4.0. Statistics were performed using GraphPad Prism® Version 4.0. One-Way and Two-Way ANOVAs were performed where indicated. Two-tailed unpaired t-tests were used to compare mean values.

2.3 Results

2.3.1 Correlation of Pressure Chamber with Leaf Psychrometers

Leaf water potential was measured on Chardonnay vines using leaf psychrometers and the pressure bomb to determine if the two techniques gave similar values. The use of leaf psychrometers was preferential for monitoring over drought periods, as these would allow continuous *in situ* monitoring of leaf water potential. This method was also non-destructive so the leaf area of the plant was not altered over the course of the experiment, and removal of leaves may have resulted in induced cavitation. Figure 2-4 shows the leaf water potential of a Chardonnay vine over six days of water stress. The leaf psychrometer sensors took measurements every 15 min over the drought period while readings with the pressure bomb were taken at 1 pm each day. A linear regression of the two methods for readings taken at 1 pm shows a good correlation (r^2 = 0.9086) with no significant difference observed (p = 0.0032). More variability was observed with the leaf psychrometers than the pressure bomb. Under well-watered conditions the psychrometers measured a less negative mean leaf water potential. The high variability and less negative ψ_L measured by the psychrometers, results in a slope of less than one. Attachment of the psychrometers to the leaf results in a localised area of darkness possibly reducing stomatal conductance and transpiration rates. Measurement with the psychrometers may therefore more closely reflect the water potential in the vessels and this is usually less negative than the whole leaf. The variability measured with the psychrometers may be a result of stomatal patchiness which has previously been reported to occur in grapevine (During and Loveys, 1996). Given the high correlation

between the two methods, the pyschrometer sensors were used for measurement of leaf water potential in drought experiments unless indicated otherwise.

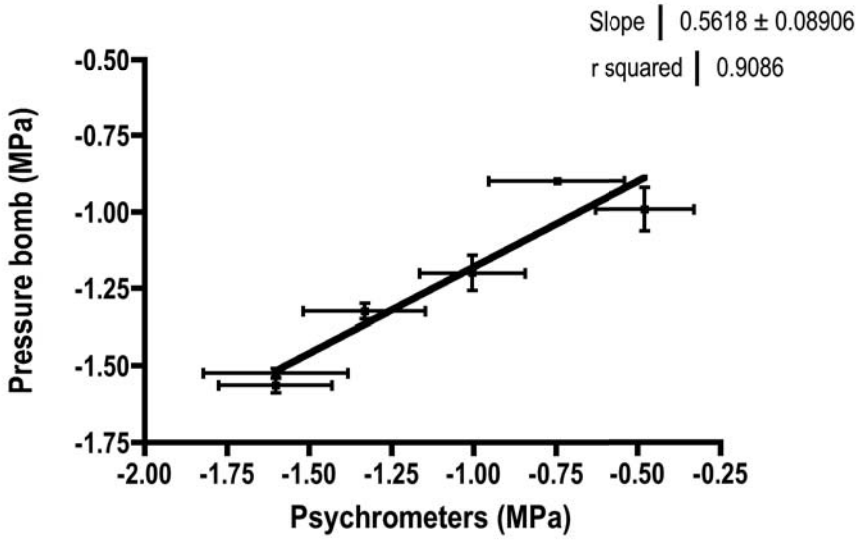


Figure 2-4: Correlation between the pressure bomb and leaf psychrometers. Leaf water potential was measured continuously (readings taken every 15 min with each psychrometer) over a drought period on Chardonnay vines with leaf psychrometers (n=3-5) attached to basal leaves on three different vines. One leaf from each of the vines was used to measure leaf water potential with the pressure bomb at 1 pm. Data shows the mean ± SEM for the psychrometers and pressure bomb (n=3) for values obtained at 1 pm.

2.3.2 Leaf Water Potential

Leaf water potential measurements were made continuously over the course of drought experiments on Chardonnay and Grenache vines (Figure 2-5). Diurnal changes in leaf water potential were observed for both varieties. The predawn leaf water potential of well-watered Chardonnay vines was -0.26 MPa (Table 2-1). Leaf water potential decreased steadily during the day, reaching midday values of around -1.0 MPa, and then increasing to similar values as seen at predawn. As the water stress increased, the predawn water potentials decreased and midday water potentials reached values close to -1.5 MPa. In Grenache, the daily leaf water potential reached a minimum (maximum water stress) during the morning. Midday leaf water potentials were around -0.7 MPa under moderate water stress. As the day progressed the leaf water potential was maintained at a fairly constant rate. As water potential was recorded every 15 min, oscillations were observed throughout the day, indicating the continuously changing water status of the leaf. Increases in water potential (more positive) were sometimes observed throughout the day. This is most likely due to localised changes in water availability in the leaf tissue, possibly as a result of cavitation events in the petiole and leaf traces.

Table 2-1: Predawn and midday leaf water potential (MPa) for Chardonnay and Grenache vines. WW – well watered vines, WS – moderate water stress. The mean values of three individual experiments ± SEM (n=12) are shown.

Variety	WW		WS	
	Predawn ψ_L (MPa)	Midday ψ_L (MPa)	Predawn ψ_L (MPa)	Midday ψ_L (MPa)
Chardonnay	-0.259 ± 0.066	-1.17 ± 0.104	-0.313 ± 0.013	-1.437 ± 0.011
Grenache	-0.288 ± 0.103	-0.7 ± 0.092	-0.340 ± 0.114	-1.047 ± 0.058

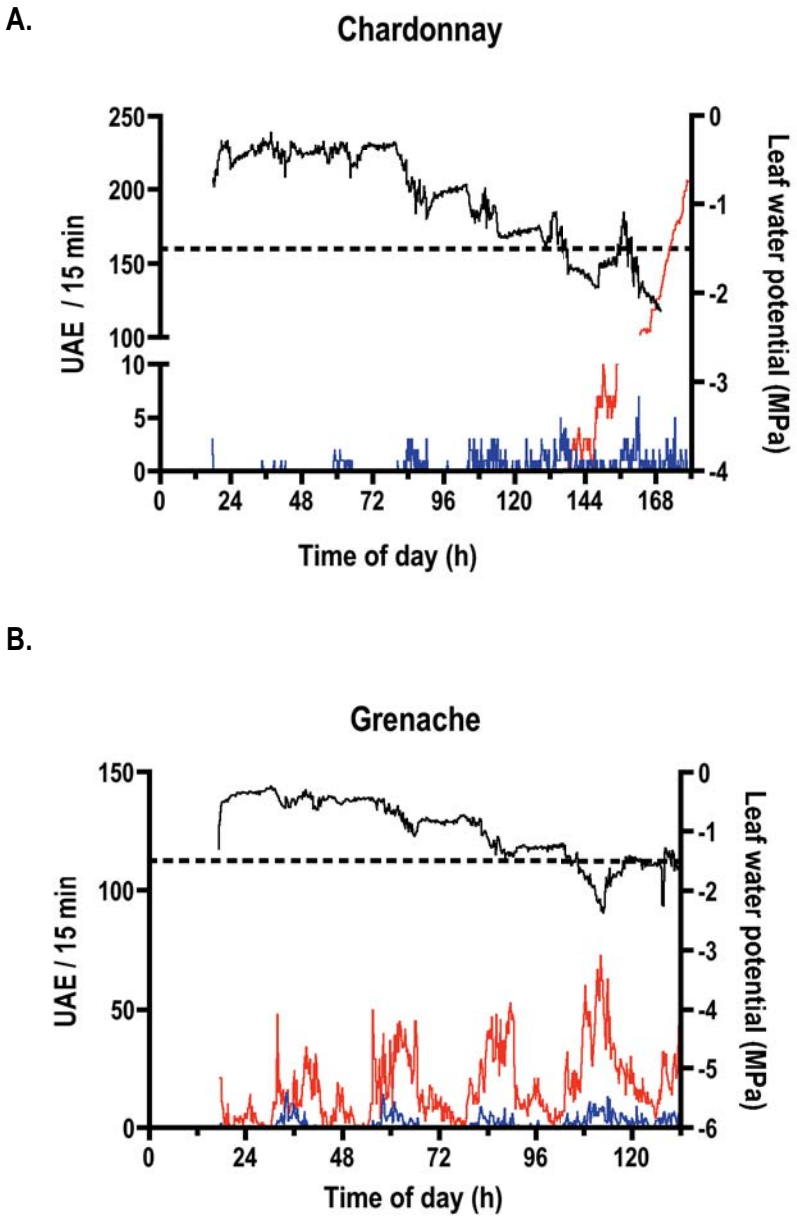


Figure 2-5: Leaf water potential and cavitation over a drought period in A. Chardonnay and B. Grenache vines. Leaf water potential (black line) was measured continuously (readings taken every 15 min with each psychrometer) over the drought period with leaf psychrometers. Shown is the mean leaf water potential recorded with four psychrometers on one plant (n=4). Midday is represented as a tick with no number. Cavitation was detected simultaneously in the petiole (blue) and stem (red) by measuring the UAEs. The dotted line indicates leaf water potential at -1.5 MPa. Note the scaling is different for each graph.

2.3.3 Cavitation

Cavitation was measured in the internodes and the petioles of both Chardonnay and Grenache vines, by the detection of UAEs using specialised microphones. In well-watered Chardonnay vines, cavitation was only detected in the petiole and not in the stem internode. As the water stress increased (to values of approximately -1.5 MPa), UAEs were detected in both the stem and petiole. In Grenache, acoustic emissions were detected simultaneously in both the petioles and stems of well-watered vines. The number of emissions per day increased over time as the water stress became more severe.

Vulnerability curves were generated by plotting the cumulative UAEs (cUAEs) against the leaf water potential, for three independent drying experiments for both Chardonnay and Grenache. From the vulnerability curves, it is possible to determine the ψ_{CAV} threshold value, taken as 10% of the maximum cUAEs, for the point at which cavitation is triggered (Salleo *et al.*, 1996; Nardini *et al.*, 2001). In Grenache vines, the petiole and stem had very similar ψ_{CAV} values of - 0.132 ± 0.095 and - 0.191 ± 0.114 MPa (n=3), respectively. As can be seen in Figure 2-5, this value of leaf water potential would be reached during a normal diurnal period when transpiration rates were high. In Chardonnay vines, the leaf water potential at which cavitation was triggered in the petioles and stems was significantly different ($p < 0.05$), with threshold ψ_{CAV} = - 0.53 ± .035 MPa and – 1.813 ± 0.23 MPa for the petioles and stems, respectively.

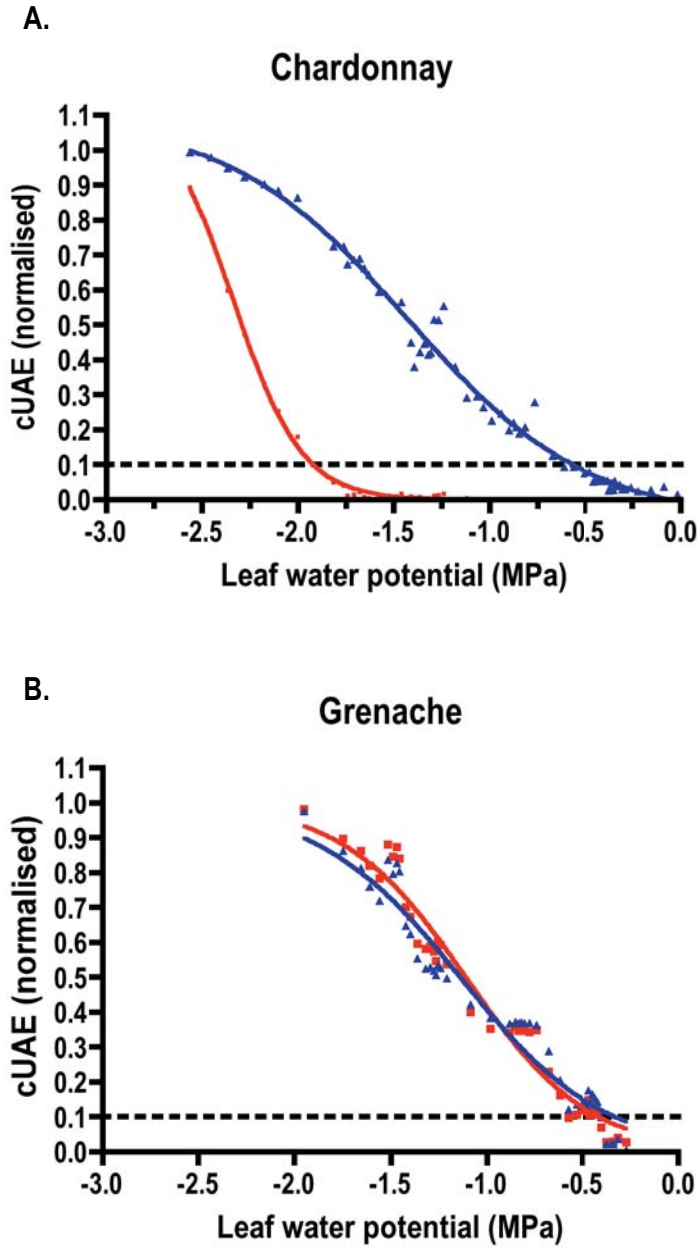


Figure 2-6: Vulnerability curves for A. Chardonnay and B. Grenache vines. Shown is the normalised cUAE plotted against mean leaf water potential for both the petiole (blue) and stem (red) (n=1). Curves were generated by fitting a sigmoidal dose-response curve to the data. The hillslope and r^2 (shown in brackets) for Chardonnay petiole and stem is -0.9 (0.99) and -2.4 (0.99), respectively and for Grenache petiole and stem, -1.2 (0.94)and -1.4 (0.97) respectively. The dotted black line represents 10 % of the maximum cUAEs.

Table 2-2: Threshold ψ_{CAV} for petioles and stems of Chardonnay and Grenache vines. Data presented is the mean ± SEM of at least three independent experiments. Superscript letters indicate significant differences ($p<0.05$).

Variety	$\psi_{CAV\ Petiole}$ (MPa)	$\psi_{CAV\ Stem}$ (MPa)
Chardonnay	- 0.53 ± 0.035[a]	- 1.813 ± 0.23[b]
Grenache	- 0.132 ± 0.095	- 0.191 ± 0.114

2.3.4 Petiole Hydraulic Conductance

Petiole conductance was measured in both Grenache and Chardonnay vines over a diurnal period. Petiole hydraulic conductivity varied diurnally in both varieties (Figure 2-7). A similar diurnal pattern for leaf water potential, K_h and LSC was observed for both varieties. K_h was higher in Grenache than in Chardonnay vines over the diurnal period, When data was normalised to leaf area (LSC) the differences between the two varieties was much less, although at 10 pm, the LSC was significantly higher in Grenache vines ($p < 0.05$). In Chardonnay K_h was significantly lower at 10 am than at 1 pm and 10 pm ($p < 0.01$). In Grenache both the K_h and LSC was significantly lower at 5 pm than at 1 pm and 10 pm.

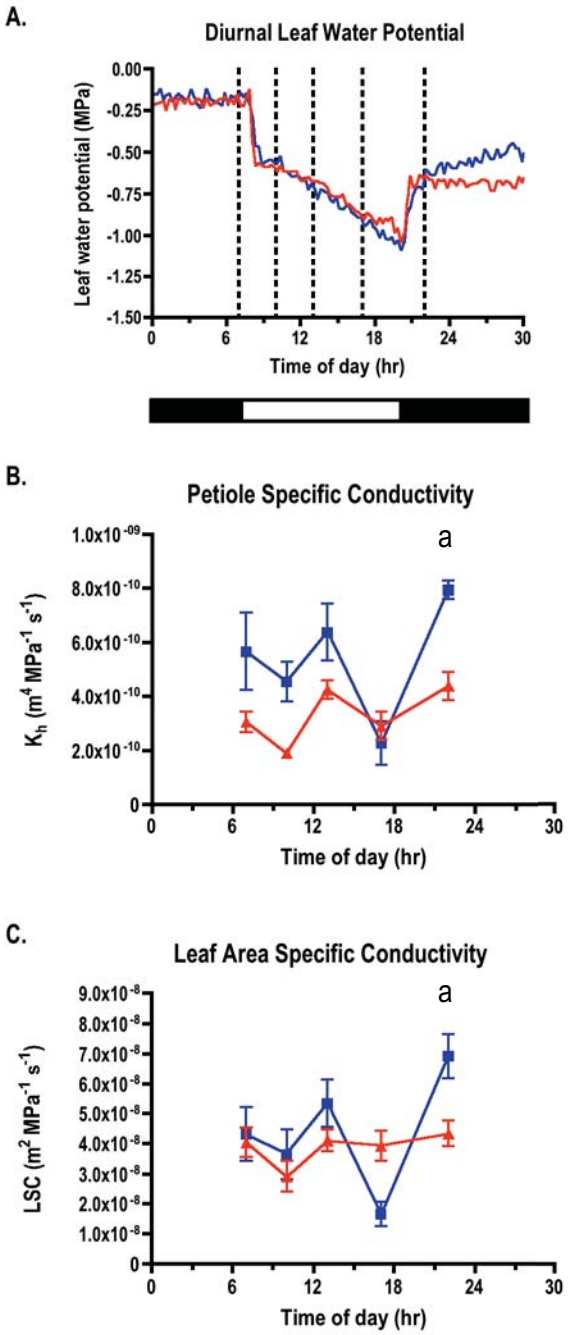


Figure 2-7: Hydraulic Conductance**.** A. Mean diurnal leaf water potential of Chardonnay (red) and Grenache (blue) (n=3). Dotted lines indicate time points at which conductance measurements were made B. Mean petiole specific hydraulic conductivity (K_h). C. Mean leaf area specific conductivity (LSC). Data shows the mean ± SEM (n=6). Hydraulic conductance measurements were performed on plants grown in a growth chamber. Letter "a" denotes a significant difference between varieties ($p<0.05$). In Chardonnay, K_h was significantly lower at 10 am than at 1 pm and 10 pm ($p < 0.01$). In Grenache, both the K_h and LSC was significantly lower at 5 pm than at 1 pm and 10 pm ($p < 0.01$).

2.3.5 Xylem Anatomy

Transverse sections of petiole tissues were stained with toluidine blue O and examined to determine the size (vessel diameter) of mature xylem vessels in Chardonnay and Grenache vines. Figure 2-8 shows the mean diameter of xylem vessels in mature petioles for Grenache and Chardonnay. The average vessel diameter was significantly larger in mature Grenache petioles than Chardonnay petioles ($P < 0.0001$).

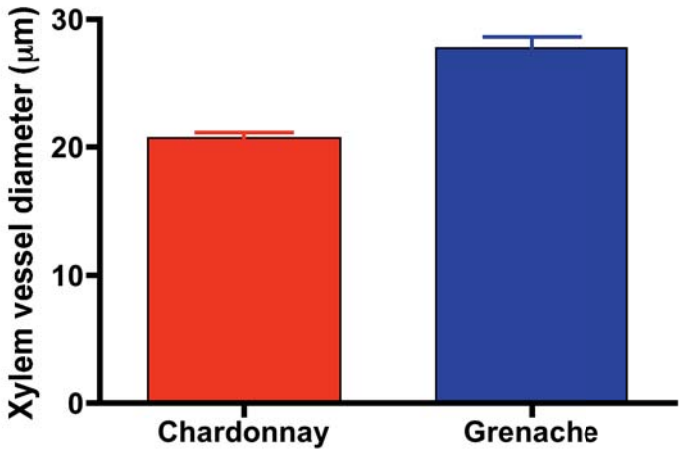
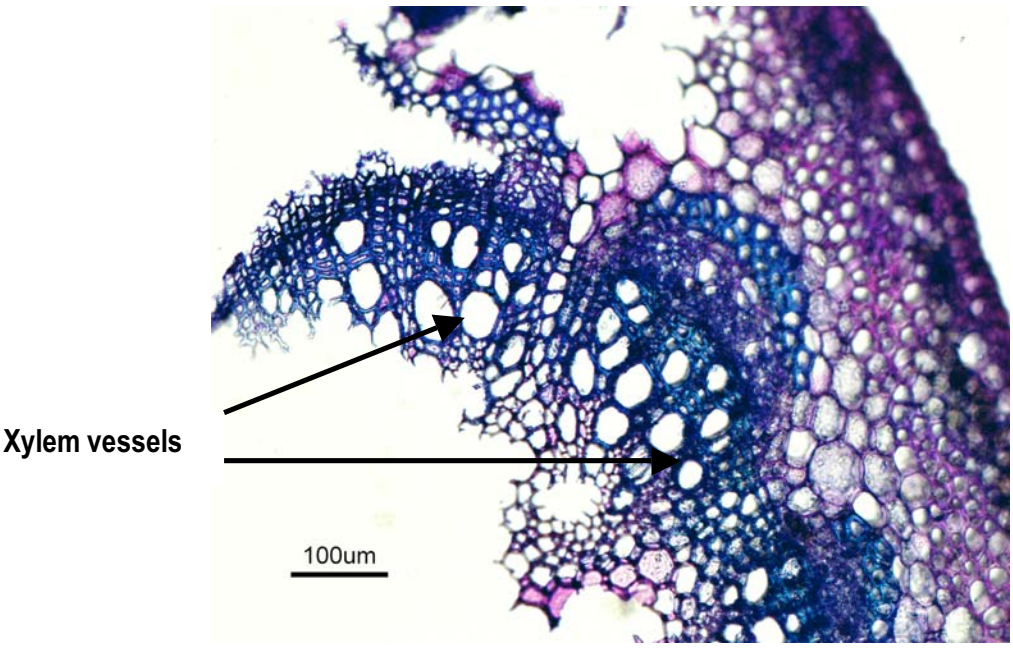


Figure 2-8: Average xylem vessel diameter in mature petioles from Grenache and Chardonnay vines. Bars represent the mean ± SEM (n=400) calculated from transverse sections of four petioles from each variety.

A.



B.

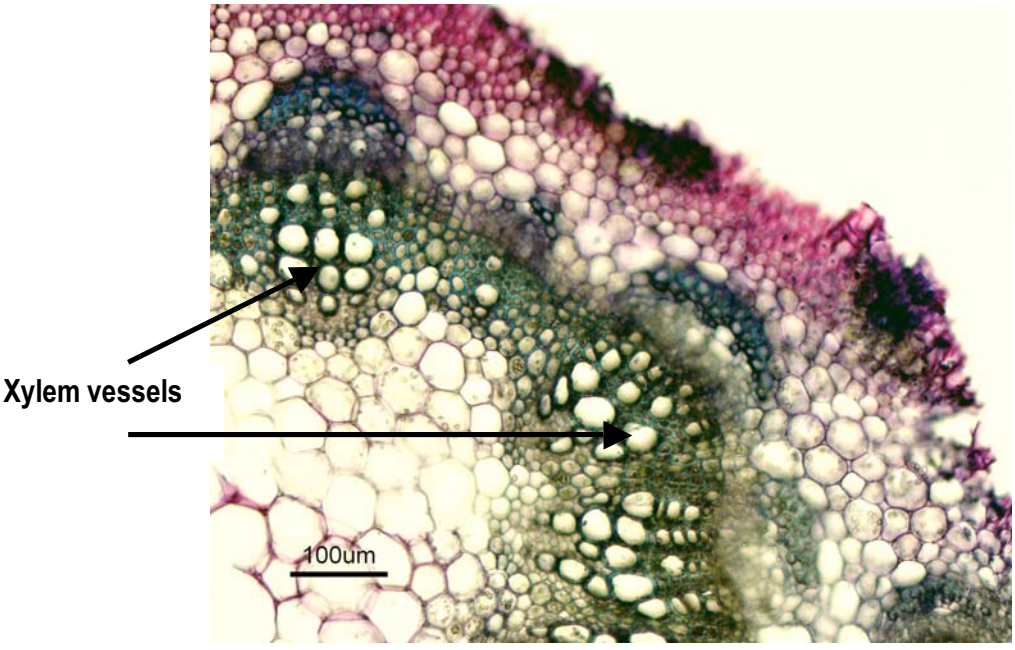


Figure 2-9: Images showing transverse sections of petiole tissue from A. Grenache and B. Chardonnay vines. Sections were stained with toluidine blue O. The arrows indicate the position of xylem vessels. Scale bars = 100 μm.

2.4 Discussion

2.4.1 Water Stress-Induced Xylem Embolism

This study compared the vulnerability of two grapevine varieties to water stress-induced cavitation. Grapevines were found to be susceptible to water stress-induced xylem embolism as has been reported previously (Scholander *et al.*, 1955; Tyree and Sperry, 1989a; Holbrook *et al.*, 2001; Schultz, 2003; Alsina *et al.*, 2007). This study has shown that Grenache is more susceptible to water-stress induced xylem embolism than Chardonnay, as indicated by the higher leaf water potential at which cavitation began to occur in both the petioles and stems (Figure 2-6). Varietal and species differences have previously been observed in *Vitis* in their physiological responses to drought stress (Schultz, 2003; Soar *et al.*, 2006; Alsina *et al.*, 2007). A preliminary study by Schultz (2003) reported that Grenache was more susceptible to the formation of embolised vessels than Syrah in both the petioles and internodes. More recently, Alsini *et al.* (2007) compared vulnerability curves of eight grapevine varieties and found that the stems of Black Grenache vines were more susceptible to the formation of embolised vessels than Chardonnay stems.

In Chardonnay vines, the petioles were more susceptible to water stress-induced cavitation (ψ_{CAV} = -0.53 ± 0.035 MPa) than the stems (ψ_{CAV} = -1.813 ± 0.23 MPa). Cavitation was observed in the petioles of well-watered Chardonnay vines, when the plant was actively transpiring. As shown by the leaf water potential data (Figure 2-5), a threshold ψ_{CAV} of - 0.53 MPa would be reached at midday during a normal diurnal period even under well-watered conditions. Chardonnay vines therefore maintain their midday leaf water potential at values

close to the cavitation threshold ψ_{CAV} for petioles, as has been reported for other species (Tyree and Sperry, 1989b; Nardini *et al.*, 2001). This is most likely a mechanism to balance transpiration, through stomatal regulation with the hydraulic conductivity and plant water status (Salleo *et al.*, 2000). Both petioles and leaves have been reported to be more vulnerable to xylem embolism in other woody species such as *Juglans regia,* some *Quercus* species (Cochard *et al.*, 1992; Tyree *et al.*, 1993) and in *Laurus nobilis* L. (Salleo *et al.*, 2000). This is not surprising as according to Zimmermann's segmentation hypothesis, the plant will sacrifice renewable parts of the plant to protect more crucial organs (Zimmermann, 1983). Cavitation occurred in the stems of Chardonnay vines at much higher tensions (more negative ψ) and resulted in the rapid formation of "runaway cavitation" (steeper slope vulnerability curve compared to the petiole). A high degree of embolism formation in the stems of grapevines would be catastrophic, as this would reduce the hydraulic conductivity of the shoot and result in less water flow to the transpiring leaves. Thus from the data attained here, it is apparent that Chardonnay exhibit vulnerability segmentation, with cavitation occurring first in the petiole, whilst protecting the stem from embolism formation and therefore maintaining the hydraulic capacity of the shoot.

In Grenache, the ψ_{CAV} was very similar for both the petioles (-0.132 ± 0.095 MPa) and stems (-0.191 ± 0.114 MPa) indicating no difference between these organs in their vulnerability of the xylem vessels to water-stress. The onset of cavitation occurred at leaf water potential values of ~-0.2 MPa. Unlike Chardonnay and other woody species, Grenache did not exhibit "runaway cavitation" or vulnerability segmentation. This suggests that these two grapevine varieties have evolved different mechanisms to cope with the onset of water stress. Grenache is a drought tolerant variety that has recently been classified as an isohydric species (Schultz, 2003). From this study it appears that Grenache has some mechanism to

prevent the rapid formation of embolised vessels under drought stress. Recently it has been proposed that early cavitation events, such as what occurs in Grenache, may act as a hydraulic signal for stomatal closure (Salleo *et al.*, 2000). It is unknown exactly what the hydraulic signal for stomatal closure is but it may involve chemical signals such as ABA. Under conditions of high VPD, Grenache exhibit a tighter control over stomatal conductance than Shiraz (Soar *et al.*, 2006). Stomatal closure during the day prevents water loss and allows leaf water potential to be maintained at a more constant level. Soar *et al.* (2006) suggested a possible link between the hydraulic properties of grapevines with the abundance of ABA in the xylem sap and the level of stomatal control. Another possibility is that stomata may respond to transient changes in leaf water potential. Cavitation events in the petioles or leaves can result in a transient release of water that can increase the water availability in the leaf. Transient changes in leaf water potential were observed for both Chardonnay and Grenache during the transpiration period. Stomata may respond to local changes in leaf water status (Meinzer *et al.*, 2001) and this may influence the occurrence of stomatal patchiness (During and Loveys, 1996).

2.4.2 Hydraulic Properties

The petiole hydraulic conductivity was found to be 2-4 times higher in Grenache than Chardonnay over a diurnal period (with the exception of 5 pm). K_h values varied between 2 - 8 x 10-10 m^4 MPa^{-1} s^{-1} for Grenache vines and between 2 - 4 x 10-10 m^4 MPa^{-1} s^{-1} for Chardonnay vines. The LSC showed a similar trend with values between 2 – 7 m^2 MPa^{-1} s^{-1} for Grenache and between 3 - 4 m^2 MPa^{-1} s^{-1} for Chardonnay vines. Values obtained for Grenache are similar to those reported by Schultz (2003) where Grenache petioles had a 2-3 times higher K_h than Syrah and LSC was 4-6 times higher. Significant anatomical

differences were observed between the two varieties with mature Grenache petioles having a larger mean xylem vessel diameter compared to Chardonnay vessels. Differences in the frequency of xylem vessels of different diameters, has been reported for different internodes of grapevine shoots (Lovisolo and Schubert, 1998). Only small differences in the mean xylem diameter were observed between internode positions, although differences between irrigated and water stressed vines was apparent (Lovisolo and Schubert, 1998). In the current study, petioles were taken from a similar internode position from well-watered vines, thus it is unlikely that either of these factors are influencing the observed anatomical differences between Chardonnay and Grenache. The anatomical differences observed in the petioles are consistent with the higher hydraulic conductivity of Grenache and the increased sensitivity to the formation of embolised vessels under increasing xylem tensions.

The formation of embolised xylem vessels decreases the number of functional vessels for water flow, resulting in a reduction in the hydraulic conductivity of the plant. The long distance pathway moves water from the roots to the shoots of the plant primarily through xylem conduits. Water that is taken up by the roots moves radially across the roots to the xylem vessels. Root hydraulic conductivity is a measure of both water movement through the xylem vessels and radial water movement which can be apoplastic or by way of the cell to cell pathway (symplastic or transcellular) (Steudle and Peterson, 1998). Rapid changes in root hydraulic conductivity is thought to be due to the regulation of water flow across cell membranes mediated by aquaporins (reviewed in Javot and Maurel, 2002; Tyerman *et al.*, 2002; Lopez *et al.*, 2003; Lovisolo and Schubert, 2006) . If water movement from the roots to the cell wall of the leaf mesophyll (SPAC) is primarily through xylem conduits, then the hydraulic capacity of the shoot should remain unaltered throughout a diurnal period. The maximum specific petiole hydraulic conductivity (at pressures of 0.1 - 0.2 MPa) was

measured in both Grenache and Chardonnay petioles. Diurnal changes in the hydraulic conductivity were observed for both varieties. In other woody plants K_h has been found to remain constant over a diurnal period (Bucci *et al.*, 2003). A significant decrease in K_h was observed at 10 am in Chardonnay and 5 pm in Grenache. The reason for this decrease is unknown. One possibility is that embolisms are present, although this seems unlikely as the perfusion pressure used should have been sufficient to remove all air emboli. In well-watered Chardonnay plants cavitation was shown to occur later in the day (midday), therefore if embolised vessels were present we would expect to see a lower K_h value in the afternoon when cavitation is occurring. The other possibility is that water flow through the petiole is not solely through xylem conduits and may involve movement across transcellular membranes. Many authors have suggested that xylem parenchyma cells may be involved in the active repair process of embolised vessels by the movement of water and other solutes (Holbrook *et al.*, 2001; Sakr *et al.*, 2003; reviewed in Clearwater and Goldstein, 2005). Xylem vessels and xylem parenchyma cells have an intricate structure-function relationship to satisfy the demands of the water flow within the plant, by controlling and facilitating the movement of ions into and out of xylem vessels. Diurnal transcriptional expression and/or post-translational regulation of aquaporins in the plasma membrane and tonoplast membrane of xylem parenchyma cells in the petiole could contribute to the diurnal changes of K_h and LSC measured in both Chardonnay and Grenache vines. Cochard *et al,* (2007) reported that changes in the hydraulic conductance of walnut leaves, in response to light, correlated with changes in the transcript abundance of PIP2 aquaporins. The low light environment used in this study to measure the petiole hydraulic conductivity could therefore be influencing the expression of aquaporins, and thus measurements of K_h. Plants were acclimated to the low light environment for a week prior to experiments, and both varieties

were grown under the same light regime. It is therefore unlikely that this is a factor in the observed diurnal fluctuations in K_h.

Both *in situ* analysis and immunolocalisation has detected high expression of aquaporins in the plasma membrane and tonoplast membrane of xylem parenchyma cells (Barrieu *et al.*, 1998; Sakr *et al.*, 2003). In walnut the expression of aquaporins has been correlated with freeze induced xylem embolism refilling (Sakr *et al.*, 2003). The use of sulfhydryl compounds such as $HgCl_2$ to inhibit aquaporin function has been widely used in both heterologous expression systems and in whole plants (Preston *et al.*, 1993; Daniels *et al.*, 1994; Jung *et al.*, 1994; Javot and Maurel, 2002). It is generally accepted that the use of mercurial compounds can affect other cellular metabolic processes that can negatively impact on water transport (Zhang and Tyerman, 1999). Inhibition with $HgCl_2$ has provided evidence that the living cells surrounding the xylem vessels may influence K_h. Diurnal changes in petiole specific conductivity (ks) of red maple and tulip tree petioles were attributed to the formation of xylem embolisms (Zwieniecki *et al.*, 2000). The addition of $HgCl_2$ and girdling resulted in a reduction in ks, indicating that living cells were contributing to the petiole conductivity. Lovisolo and Schubert (2006) showed that $HgCl_2$ impeded the recovery of both the root and shoot hydraulic conductivity after rewetting following drought stress in *V. vinifera*. The affects were reversed with the addition of β-mercaptoethanol that acts as a mercury scavenger. The diurnal changes in K_h observed in this study may be related to aquaporin expression in the xylem parenchyma cells.

In conclusion, the data presented here show cultivars of *V. vinifera* differ in their response to water stress. Grenache exhibited greater susceptibility to the formation of embolised xylem vessels in both the stems and petioles. The greater susceptibility to embolism formation and

the higher hydraulic conductivity compared to Chardonnay could be attributed to the larger vessel size observed in Grenache petioles. Diurnal variation in the hydraulic properties of grapevine petioles may be linked to aquaporin expression.

Chapter 3:

Identification of Grapevine Aquaporins

3.1 Introduction

Aquaporins are membrane bound proteins that facilitate the movement of water and other small neutral solutes across cellular membranes. Plant aquaporins belong to a large family of highly conserved proteins called the Membrane Intrinsic Protein (MIP) superfamily. Phylogenetic analyses reveals the MIP superfamily can be divided into the four subfamilies, the Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin-like Intrinsic Proteins (NIPs) and the Small Basic Intrinsic Proteins (SIPs) (Johanson *et al.*, 2001; Zardoya and Villalba, 2001; Quigley *et al.*, 2002). The divergence of plant aquaporins is believed to have occurred early, with recent findings that aquaporins in the moss, *Physcomitrella patens*, fall into the same subfamilies as the angiosperms (Borstlap, 2002). In angiosperms a large number of aquaporins have been identified. The Arabidopsis genome sequencing project (Arabidopsis Genome Initiative, 2000; http://www.arabidopsis.org/) has resulted in the identification of 38 aquaporin sequences with homology to the MIP superfamily (Quigley *et al.*, 2002). Of these, 35 are believed to encode complete aquaporin proteins (Johanson *et al.*, 2001). The amino acid identity of the 13 members of the Arabidopsis PIP aquaporin subfamily ranges from 72 – 97 % (Quigley *et al.*, 2002). The TIP subfamily is more diverse than the PIPs, and is broken into five subclasses (Chaumont *et al.*, 2001; Johanson *et al.*, 2001), having identities between 44 and 93% in Arabidopsis (Quigley *et al.*, 2002). The large diversity of aquaporins has also been shown by screening of *Zea mays* (maize) ESTs leading to the identification of 31 aquaporin genes, encoding 30 amino acid sequences (Chaumont *et al.*, 2001). When this project began, a database search (http://www.ncbi.nlm.nih.gov/) revealed a total of ten AQP cDNAs identified in grapevine. Eight of these cDNAs were identified from a *Vitis* rootstock Richter-110 leaf cDNA library (Baiges *et al.*, 2001). Five of these were PIP cDNAs and three TIP cDNAs.

Two PIP1 cDNAs were also identified from berry of *Vitis vinifera* cv. Pinot noir (Picaud *et al.*, 2003). Aquaporins have recently been identified in other woody plants such as *Juglans regia* (walnut) (Sakr *et al.*, 2003) and *Olea europaea* (olive) (Secchi *et al.*, 2007b).

Aquaporins were originally assigned different subgroups based on their subcellular localization with PIPs abundantly expressed on the plasma membrane and TIPs on the tonoplast membrane (reviewed in Maurel *et al.*, 2002). The classification of plant aquaporins based solely on their subcellular localization has recently been challenged (Barkla *et al.*, 1999) and classification of MIPs should primarily be based on sequence analysis. Sequence analysis of the MIP superfamily shows they have a number of distinct signature motifs that allow classification of these genes into the four subfamilies. For example the N-terminus of PIPs is extended compared to TIPs, and the PIPs are also divided into two subgroups, PIP1 and PIP2, identified by distinct residues located in the N and C termini (Schaffner, 1998). The coding region of MIPs is typically between 250 and 300 amino acid residues in length and they have a molecular mass between 26 - 34 kDa. Aquaporins have a highly conserved structure found throughout the AQP family, consisting of six transmembrane alpha helices that span the membrane. Loops B and E contain the highly conserved NPA motif that forms the aqueous pore (Fu *et al.*, 2000).

In order to study the role that aquaporins play in response to water stress induced xylem embolism (chapter 2), it was necessary to first identify the aquaporin genes present in the grapevine, *Vitis vinifera*. To achieve this, a *Vitis vinifera* cv. Cabernet Sauvignon cDNA library was constructed from shoot and root tissues. The library was screened for members of the PIP and TIP subfamilies. When I began this project, The International Grapevine Genome Project (UC Davis) had begun to sequence the genome of *V. vinifera* cv. Cabernet

Sauvignon. To stay in line with the grapevine genome project I choose to use the same variety, Cabernet Sauvignon, as the plant material to make the cDNA library. During the process of writing my thesis (late 2006), a number of new grapevine aquaporins were deposited onto the database. These sequences were not available when this screen was done in 2004 but have been included in the analysis.

3.2 Materials and Methods

3.2.1 Plant Material

V. vinifera L. cv. Cabernet Sauvignon clone LC14 one year old rootlings were purchased from Yalumba Nursery (Barossa Valley, SA). Rootlings were planted in 12 inch pots containing a modified UC soil mix (61.5 % (v/v) sand, 38.5 % (v/v) peat moss, 0.50 g/L calcium hydroxide, 0.90 g/L calcium carbonate, 100 g/L Nitrophoska® (12:5:1 N:P:K plus trace elements) pH 6.8) and fertilised with 0.08 g/L per month of Osmocote®. Plants were grown in controlled temperature glasshouses at 25 °C day / 20 °C night with night break lighting provided by halide lamps for 1 hour during the night period. Plant material was collected between 10 am and 12 pm, frozen immediately in liquid nitrogen and stored at –80 °C.

3.2.2 Solutions and Media

All components of solutions and media were analytical or molecular biology grade and purchased from BDH and SIGMA unless indicated otherwise. The following companies were used to purchase chemicals, enzymes and general molecular biology consumables:

Applied Biosystems	Foster City, CA, USA
BDH	Kilsyth, Victoria, Australia
Biorad	Hercules, CA, USA

Fermentas	Distributed by Quantum Scientific
Fisher Biotech	Adelaide, Australia
Invitrogen	Mount Waverly, Victoria, Australia
New England Biolabs	Ipswich, MA, USA
Progen	Toowong, QLD, Australia
Promega	Annandale, NSW, Australia
Qiagen	Doncaster, Victoria, Australia
Quantum Scientific	Murarrie, QLD, Australia
Roche Diagnostics	Castle Hill, NSW, Australia
Sigma-Aldrich	Castle Hill, NSW, Australia

All solutions and media were made with MilliQ water and when required, autoclaved at 121 °C for 20 min. The components of general solutions and media are listed in Table 3.1.

Table 3-1: General solutions and growth media.

Media / Solution	Components
Luria Broth (LB)	1% (w/v) Yeast Extract (Difco), 1% (w/v) Sodium Chloride, 0.5% (w/v) Tryptone pH 7, + 2% (w/v) agar for solid media
LB / Kan	Luria Broth + 50 μg ml^{-1} of kanamycin
LB / Amp	Luria Broth + 100 μg ml^{-1} of ampicillin
SOC	2% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM Mg SO_4, 20 mM glucose.
SOB	2% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$
10 x TAE	0.4 M Tris, 0.01 M EDTA Disodium Salt, 0.2 M Acetic Acid
10 x TBE	0.89 M Tris, 0.02 M EDTA Disodium Salt, 0.89 M Boric Acid
10 x MOPS	0.2 M MOPS (pH 7.0), 20 mM NaAC, 10 mM EDTA (pH 8)
DNA loading dye (10x)	78% w/v glycerol, 0.25% w/v bromophenol blue, 0.25% xylene cyanel, 10 mM EDTA
Agarose gels	0.8 – 2.0% (w/v) agarose dissolved in 1 x TAE buffer

3.2.3 RNA Solutions

General RNA protocols were conducted as described in Sambrook and Russel (2001). All glassware was baked at 180 °C for 4 hours. MilliQ water was treated with 0.1% DEPC, stirred continuously overnight, then autoclaved at 121° C for 20 min. RNA solutions were made with DEPC treated MilliQ water or treated directly with 0.1 % DEPC, depending on the solution. Rnase Zap (Ambion) was used to clean gel electrophoresis equipment.

3.2.4 Bacterial Transformation

Chemically competent *Escherichia coli* XL1-B cells (Stratagene) or DH5α cells were used for bacterial transformations unless indicated differently. Chemically competent cells were made using a modified version of the Ultra-competent E.coli Cells Protocol (Inoue *et al.*, 1990) available at (http://www.hybtech.org/Liu/uccell.html). Plasmid DNA was transformed into 200 μl of chemically competent cells. Cells were heat shocked at 37 °C for 45 s and placed on ice for 2 min. 500 μl of LB was added to cells and these were grown at 37 ° C for 1 hour to allow recovery. 100 μl of transformation mix was spread onto LB agar plates with the appropriate antibiotic selection and grown at 37 °C overnight.

3.2.5 Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving 1 - 2% (w/v) agarose in 1 x TAE buffer with the addition of 0.5 μg ml^{-1} of ethidium bromide. DNA samples were loaded in sample buffer and

electrophoresed at ~ 80 V. Agarose gels were visualised under UV light using the Molecular Imager ChemiDoc XRS System (Biorad).

3.2.6 Total and Poly (A)$^+$ RNA Isolation

Total RNA was extracted from stems, leaves, tendrils, petioles and roots using a combination of a sodium perchlorate extraction method and modified protocol of the RNeasy Extraction Kit (Qiagen) (Franks *et al.*, 2006). Between 200-500 mg of fresh plant tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. 1 ml of sodium perchlorate solution (5 M $NaCl0_4$, 0.2 M Tris-Cl pH 8.3, 2% (w/v) SDS, 8.5% (w/v) PVPP, 1% (v/v) B-mercaptoethanol was added per 250 mg of ground tissue and this mixture was further ground until a smooth paste was obtained. From here on the protocol was followed as described in Franks *et al.* (2006). Total RNA was eluted from the Qiagen spin column in 60 μl of RNase free water. Poly (A)$^+$ RNA was purified from 0.5 – 1.0 mg of total RNA using Oligo (dT)-Cellulose spun column chromatography following the manufacturer's instructions (mRNA Purification Kit, Amersham Biosciences). The quality and purity of mRNA was checked on a denaturing agarose gel (1.2 % (w/v) agarose, 1 x MOPS, 8% (v/v) formaldehyde) prior to construction of the cDNA library and by measuring the absorbance at 260nm.

3.2.7 cDNA Library Construction

The cDNA library was constructed using the Cloneminer™ cDNA Library Construction Kit according to the manufacturer's instructions (Invitrogen, Life Technologies, CA). Briefly, 3

μg of poly (A) $^{+}$ RNA was used in the first strand synthesis catalysed by Superscript™ II Reverse Transcriptase, following hybridisation of the Biotin-*att*B2-Oligo(dT) primer to the mRNA poly(A) tail. Second strand synthesis of cDNA was catalysed by *Escherichia coli* DNA Polymerase I. The *att*B1 adapter was then ligated to the 5' end of the cDNA. The cDNA pool was size fractionated using column chromatography and then recombined into the Gateway® vector, pDONR222 using the Gateway® BP recombination reaction, to create an entry library. The cDNA library was transformed into Electromax DB10B T1 Phage Resistant Cells using the Micropulser Electroporator (Bio-Rad), according to the manufacturers instructions. Electroporated cells were grown at 37 °C for 1 hour in SOC media to allow recovery of transformed cells. An equal volume of sterile freezing media was added (60% v/v SOC, 40 % v/v glycerol) and the cDNA library was then stored at – 80 °C. The cDNA library titre was determined using the plating assay as outlined in the Gateway® manual. 48 positive colonies from the plating assay were selected and cultured overnight in LB containing 50 μg / ml of kanamycin. Plasmid DNA was isolated using the SIGMA Genelute kit and digested with *Bsr* G1 following manufacturer's instructions. Samples were electrophoresed on a 1 % agarose gel in 1 x TAE buffer, allowing the percentage of recombinants and average insert size to be determined.

3.2.8 Macroarray

20 ml LB containing 50 μg ml^{-1} of kanamycin was inoculated with 20 μl of the *Escherichia coli* cDNA library transformants and grown shaking at 250 rpm at 37 ºC until the absorbance at 600 nm was approximately 0.5. Aliquots of 10 fold serial dilutions were made and plated onto LB + Kan plates to obtain a cell density of 4000 - 5000 colonies per plate. 50,000 *E. coli* transformants containing cDNA inserts were subsequently spotted into 384 well microtitre

plates containing 64 μl of sterile LB + 7.5% glycerol + Kan using the VersArray Colony Picker and Arrayer System (Biorad) located at the Australian Centre for Functional Plant Genomics (Waite Campus, University of Adelaide). Plates were sealed in plastic bags and cells grown at 37 ºC for 20 - 24 hour. Prior to storing at -80 ºC, plates were covered with an aluminium seal (AlumaSeal II, Excel Scientific, CA). When required for spotting onto nylon membranes, plates were thawed, shaken for 30 seconds on a plate shaker then spun briefly at 900 rpm and the foil cover carefully removed. Hybond N^+ nylon membranes (Pharmacia Biosciences) were placed onto large plastic plates and using the VersArray Colony Picker robot, colonies were spotted from 384 well plates onto the membranes. Membranes were then carefully placed, colony side up, onto large plates containing solid LB media + Kan and grown overnight at 37 ºC.

3.2.9 Fixation of DNA to Membrane

Membranes were removed from plates and the cells lysed, DNA denatured and fixed to the membrane following the protocol of Sambrook and Russel (2001). Briefly, 3MM paper was wetted with the following solutions and the membrane placed on top, colony side up: 10 % (w/v) SDS for 5 minutes, denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 15 minutes, neutralising solution (1.5M NaCl, O.5 M Tris.HCl [pH 7.4]) for 15 mins and 2 x SSC (0.2 M NaCl, 0.03 M sodium citrate [pH 7.0]) for 10 min. Membranes were placed on a dry sheet of 3MM paper and air dried for 30 min. DNA was fixed using a UV transilluminator (Amersham), using settings specified by the manufacturer. Membranes were stored in a cool dark place until required.

3.2.10 Screening of cDNA Library

PIP and TIP PCR amplified probes were used to screen the library under high stringency conditions. Forward and reverse PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to the 5' and 3' end of *Vitis* Rootstock Richter 110 aquaporin cDNAs and *Vitis vinifera* Pinor Noir AQP cDNAs (Table 3-2). Desalted primers were ordered from Proligo (SIGMA).

Table 3-2: PCR primer combinations (forward and reverse) are shown for the synthesis of aquaporin probes. Previously identified grapevine aquaporins from *Vitis* rootstock Richter 110 (Baiges *et al.*, 2001) and *V. vinifera* cv. Pinot Noir (Picaud *et al.*, 2003) are listed with their accession number. Primer3 was used to design forward and reverse primers for each of these genes.

PRIMER NAME	FORWARD AND REVERSE PRIMERS (5' – 3')	Tm °C
*Vitis*PIP1;1 (AF141643)	Forward 5'-AAGAGAAGAGAAGAGAGATGGAAGG-3'	53
	Reverse 5'-CACATTTCACAGCGTCACCT-3'	60
*Vitis*PIP1;2 (AF141898)	Forward 5'-AAGCTCTGAACTCTCAGTGTTTTC-3'	51
	Reverse 5'-CATTCAAAAGCTGCCCATTG-3'	58
Vitis PIP1;3 (AF141899)	Forward 5'-TTTGAGTGGTGCTGAGTTGC-3'	60
	Reverse 5'-GGGCAGGGAAGGATAAAAGA-3'	60
Vitis PIP2:1 (AF141642)	Forward 5'-ACCTTCTCCTGAACCCCCTA-3'	62
	Reverse 5'-CAACAAGACAAAGCCCAACA-3'	58
Vitis PIP2;2 (AF141900)	Forward 5'-GGGATAAGTGAGAAGAGAGAACAGA-3'	52
	Reverse 5'-GCCCAAAGCTAACAAAGAAGG-3'	53
Vitis TIP1 (AF271661)	Forward 5'-TTCATCTTCAATAGTTGCTTCCA-3'	51
	Reverse 5'-CACAGCTTGAACCAAAGCAA-3'	58
Vitis TIP2 (AF271662)	Forward 5'-TTCAGAAGCCTTTTGTACTGGA-3'	51
	Reverse 5'-CACCATCGAAGGCACCAC-3'	58
Vitis TIP3 (AF271660)	Forward 5'-GGCCTAGAGCTTGAGGAGGA-3'	64
	Reverse 5'-TGATTGCAAACAAACCAGACA-3'	51
VvPIP1b (AF188844)	Forward 5'-TTTGAGTGGTGCTGAGTTGC-3'	60
	Reverse 5'-TCAAAGGGGGTCCAACATAA-3'	58
VvPIP1a (AF188843)	Forward 5'-TGTTTTCAGATCTGTAGAGGGAGA-3'	52
	Reverse 5'-ATTCAAAAGCTGCCCATTGT-3'	56

Using the appropriate PCR primers, cDNA fragments were amplified from purified plasmid cDNA library template with the Digoxygenin (DIG) - labelling PCR kit (Roche) and TaqTi polymerase (Fischer Biotech). The PCR cycling conditions were as follows: 95 ºC for 15 min for 1 cycle; 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 1.5 min for 35 cycles; extension at 72 ºC for 7 min. The DIG-labelled PCR products were separated by gel electrophoresis on a 1.2 % (w/v) agarose gel in 1 x TAE buffer, stained with ethidium bromide and visualised under UV light using the Molecular Imager ChemiDoc XRS System (BioRad). Correct size products were gel purified (Qiagen QIAXII Gel Purification Kit, Qiagen) and cloned into pGEMT-Easy (Promega). Inserts were sequenced to confirm the amplified product and then later used to screen the printed cDNA library for homologous cDNAs. Probe specificity was determined against dot blots of known amounts of serially diluted unlabelled PCR products spotted onto a Hybond N^+ membrane (Amersham Biosciences). Spotted DNA was fixed with the UV transilluminator (as described previously) and the membrane probed under high stringency conditions (according to Roche Molecular Biochemicals Dig Application Manual) with the DIG-labelled DNA. Appropriate amounts of DIG-labelled DNA probes were then used to screen the cDNA library under high stringency conditions (Roche Molecular Biochemicals Dig Application Manual). Briefly, membranes were prehybridised in DIG-Easy Hyb solution in roller bottles and placed for 1 hr at 42 °C in a hybridisation oven. Probes were denatured at 100 °C for 5 min and then immediately placed on ice. The probe mixture was added to the membranes and these were hybridised at 42 °C overnight in roller tubes. Membranes were washed in high stringency wash buffer (0.1 x SSC, 0.1 % SDS) at 68 °C, twice for 15 min each, followed by washing in 2 x SSC. DIG-labelled DNA was detected by chemilumiscence using an anti DIG – alkaline phosphotase antibody and CDP star (Roche Diagnostics). Positive colonies were detected using the Molecular Imager ChemiDoc XRS System (Biorad) and ImaGene 6.0 software was used to locate these on the macroarray.

3.2.11 Plasmid Purification and Sequencing

Positive colonies identified using macroarrays were grown aerated overnight in 5 ml cultures of LB + Kan at 37 ºC to amplify plasmid DNA. Plasmid DNA was purified using the Sigma Genelute Kit then digested with *Bsr* G1 to confirm insert size. The cDNA in the isolated plasmids were sequenced using Dye Terminator 3 (Applied Biosystems) and M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse primers (5'-CAGGAAACAGCTATGAC-3') and analysed by the Institute of Medical and Veterinary Services (IMVS, Adelaide). Where necessary internal sequencing primers were designed to obtain the full-length sequence of the cDNA.

3.2.12 Cloning of *VvPIP2;1* and *VvTIP1;1* by RT-PCR

Reverse transcriptase-PCR (One Step RT-PCR Kit, Qiagen) was used to amplify *VvPIP2;1* and *VvTIP1;1* cDNAs from 25 ng of total RNA isolated from Cabernet Sauvignon petiole tissue (section 3.2.6). RNA was DNase treated with DNase 1 according to manufacturers instructions (DNase free, Ambion) prior to RT-PCR. PCR primers (Table 3-2) for *VitisPIP2;1* and *VitisTIP3*, were used to amplify products using the following cycling conditions: the reverse transcriptase reaction was performed at 50 °C for 30 min, followed by activation of the HotStar Polymerase at 95 °C for 15 min (and inactivation of the reverse transcriptase), denaturation at 94 °C for 30 s, annealing 55 °C for 30 s, and extension at 72 °C for 1 min and cycled 35 times, followed by an extension time of 72 °C for 10 min. PCR products were gel purified (Qiagen) and subsequently cloned into pGEMT-easy (Promega). Positive colonies were selected on LB + Amp plates using blue white colour selection according to the

manufacturer's instructions. Plasmids were purified and the inserts sequenced as described above.

3.2.13 5' RACE PCR

Rapid amplification of cDNA ends (RACE-PCR) was performed to amplify the 5' end of two putative aquaporin cDNAs. Primers were designed to amplify the 5' end of the partial length aquaporin cDNAs, 1-6-G10 and 7-39-L11, using 5' RACE-PCR (Table 3-3). The forward primer was designed in the MCS of pDONR222 and specific reverse primers were designed to the 5' end of each partial length cDNA (Figure 3-1). 5' RACE-PCR was performed on the cDNA library template using High Fidelity Platinum Taq Polymerase (Invitrogen). PCR cycle for isolate 1-6-G10 and 7-39-L11 was as follows: 1 cycle of 95 ºC for 2 min; 35 cycles of 95 ºC for 30 s, 60.3 ºC for 30 s, 68 ºC for 1 min; 1 cycle of 68 ºC for 7 for min. PCR products were electrophoresed on a 2 % (w/v) agarose / 1 x TAE gel, and the correct size band gel purified (Qiagen QIAXII Gel Purification Kit, Qiagen). Inserts were ligated into pGEMT-easy (Promega), transformed into chemically competent DH5α *E. coli* cells and positives selected using blue white colour selection. Positives colonies were selected and grown overnight in liquid LB + Amp media to amplify plasmid DNA, miniprepped (Gelelute Miniprep Kit, SIGMA) and digested with Eco R1 to confirm correct size. Once correct size was confirmed the insert was sequenced with T7 primer (5'-TAATACGACTCACTATAGGG-3').

3.2.14 Cloning Full-length cDNAs

To obtain the full-length cDNA of isolate 1-6-G10 and 7-39-L11, gene specific *att*B primers were designed to the 5' and 3' end of the full-length sequences, according to the Gateway® Technology manual (Invitrogen).

Table 3-3: Primers designed for 5' RACE-PCR of isolates 1-6-G10 and 7-39-L11. Primers were designed to the MCS of pDONR222 and to the 5' end of each partial cDNA to allow amplification of the 5' end of each cDNA. attB primers were subsequently designed to the 5' and 3' ends of the full-length sequence. Each attB primer contains four guanine residues at the 5' end followed by the 25 bp attB1 site (red) followed by 18-25 bp of gene-specific sequence. F - forward primer, R-reverse primer. The melt temperature (Tm) for each primer is also shown.

Isolate	Primer (5'-3')	Tm (°C)
pDONR222	F CTGGCAGTTCCCTACTCTCG	64
1-6-G10	R CCAACCCCGTTGCATTCATTTC	60
7-39-L11	R GCAAAGATCATACCCCCAAA	58
1-6-G10	F GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTCAAGCTCACTGAGAAG	79
	R GGGGACCACTTTGTACAAGAAAGCTGGGTGGAGTTTATTAGAGCAGAGTTGTTG	78
7-39-L11	F GGGGACAAGTTTGTACAAAAAAGCAGGCTGGAGAAAAATGGAGGGGAAG	79
	R GGGGACCACTTTGTACAAGAAAGCTGGGTGAGCTCAACCCAGATTCAG'	80

Using petiole cDNA as a template (0.5, 0.2 and 0.1 μg per reaction) the following PCR reaction was performed with Pwo DNA Polymerase (Roche): 1 cycle of 94 ºC for 2 min; 10 cycles of 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 1 min; 25 cycles of 94 ºC for 1 min, 55 ºC for 30 s, 72 ºC for 1 min + 5 s per cycle, followed by an extension at 72 ºC for 7 min. Quality

of the PCR was checked by running a small aliquot on a 1.2 % agarose gel / 1 x TAE buffer. The attB PCR product was PEG purified (Gateway manual) and recombined using BP Clonase. 1 μl of the BP clonase reaction was transformed into chemically competent DH5α cells. Transformants were selected by their ability to grow at 37 ºC on LB + Kan and screened by purifying the plasmid DNA and digesting with *Bsr* G1 (NEB) at 37 ºC for 1 hour.

3.2.15 Bioinformatics

Sequences identified in the library screen were analysed using BLASTn and tBLASTn searches at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997). The grapevine EST database was used to compare cDNAs identified in the library screen with ESTs identified by the grapevine genome project. MacVector 9.0 (Oxford Molecular Ltd, Oxford) was used to translate sequences and ClustalW was used to generate multiple sequence alignments using blosum30 matrix and slow mode. The open gap penalty and extended gap penalty was set at 10.0 and 0.05, respectively. Phylogenetic analysis was performed using a neighbour joining analysis of the deduced amino acid sequences (bootstrap 1000 repetitions) using MacVector 9.0. Hydropathy plots were calculated using the Goldman, Engelman and Steitz (GES) algorithm, with a window size of 19. All novel aquaporin sequences identified were submitted to GenBank (accession numbers shown in Table 3.5). Genbank flatfiles are shown in Appendix A.

3.3 Results

3.3.1 Grapevine cDNA library

The titre of the cDNA library in *E.coli* was determined to be 2.1 x 10^7 colony forming units / ml with insert size ranging from 500 bp to 4000 bp as determined by digesting 48 plasmids with *Bsr* G1.

3.3.2 Screening of the cDNA Library for Grapevine Aquaporin cDNAs

Approximately 30,000 *E. coli* transformants containing cDNAs were screened under high stringency conditions. A total of 30 positive colonies were identified from the macroarray screen. These were selected and grown in liquid culture, the plasmid purified and subsequently digested. Plasmids containing cDNAs greater than 300 bp were sequenced. Sixteen cDNAs were positively identified as being members of the MIP super family by BLASTn searches using Genbank (Table 3-4). Of these eight were found to be full-length cDNAs and eight were identified as partial length cDNAs. The cDNAs ranged in size from 278 bp – 1216 bp. The sequences were compared to previously identified MIP genes from both grapevine and *Arabidopsis thaliana* and were annotated according to Johanson *et al.* (2001). A number of cDNAs were found to be identical at the nucleotide level indicating degeneracy in the library and potentially high expression of this gene within the plant (discussed below). Aquaporins identified as novel grapevine sequences were submitted to Genbank and designated an accession number (Table 3-5).

Table 3-4: Aquaporin cDNAs identified from screening a *Vitis vinifera* cv. Cabernet Sauvignon cDNA library. Nucleotide sequences were analysed using the BLASTn algorithm (Altschul *et al.*, 1997). The table shows the first two BLASTn hits for each submitted sequence. Details shown for each BLAST hit include gene name, accession number, organism, % identity and e-score. Isolates in bold show full-length cDNAs.

Isolate	cDNA	ORF (amino acids)	Relatedness of Query Sequence to 1st Two BLAST Hits (Blastn)				
			Gene Name	Organism	Genbank Accession	% Identity	E Score
1-6-G10	Partial	216	VvPIP2;3	*Vitis vinifera*	DQ834700.1	99	0.0
	648 bp		JrPIP2	*Juglans regia (walnut)*	AY18997.1	87	5e-86
1-6-H18	Full-length	285	VvPIP2;1	*Vitis vinifera*	DQ834698	99	0.0
	1215 bp		VvPIP2;1	*Vitis vinifera*	AY823263	98	0.0
1-6-H24	Partial	20	VvPIP1;2	*Vitis vinifera*	DQ834695	100	6e-36
	278 bp		PIP1;2	*Vitis berlandieri x Vitis rupestris*	AF141898	98	4e-36
1-4-E21	Full -length	250	VvTIP2;1	*Vitis vinifera*	DQ934703	100	0.0
	1093 bp		VvTIP	*Vitis berlandieri x Vitis rupestris*	AJ271661	100	0.0
4-23-A20	Partial	142	VvPIP1;3	*Vitis vinifera*	DQ834696	99	0.0
	588 bp		VvPIP1b	*Vitis vinifera*	AF188844	99	0.0
4-24-G12	Full length	286	PIP1;2	*Vitis berlandieri x Vitis rupestris*	AF141898	99	0.0
	1083 bp		VvPIP1a	*Vitis vinifera*	AF188843	98	0.0
5-25-018	Partial	251	VvPIP1;3	*Vitis vinifera*	DQ834696	99	0.0
	822 bp		PIP1;3	*Vitis berlandieri x Vitis rupestris*	AF141899	99	0.0

4-19-A19	Full-length	280	VvPIP2-2	*Vitis vinifera*	DQ834699	100	0.0
	1169 bp		PIP2-2	*Vitis berlandieri x Vitis rupestris*	AF141900	100	0.0
7-39-L11	Partial	231	VvPIP1a	*Vitis vinifera*	AF188843	99	0.0
	693 bp		VvPIP1	*Vitis vinifera*	DQ834695	100	0.0
8-45-E16	Full length	284	VvPIP2	*Vitis vinifera*	DQ834698	99	0.0
	1139 bp		VvPIP2;1	*Vitis vinifera*	AY823263	99	0.0
7-42-K11	Full length	250	VvTIP2;1	*Vitis vinifera*	DQ934703	100	0.0
	1017 bp		VvTIP	*Vitis berlandieri x Vitis rupestris*	AJ271661	100	0.0
6-32-I1	Full length	250	VvTIP2;1	*Vitis vinifera*	DQ934703	100	0.0
	1039 bp		VvTIP	*Vitis berlandieri x Vitis rupestris*	AJ271661	100	0.0
6-32-N3	Partial	64	PIP1;2	*Vitis berlandieri x Vitis rupestris*	AF141898	98	1e-112
	419 bp		VvPIP1;2	*Vitis vinifera*	DQ834695	97	6e-108
7-42-M5	Partial	32	VvPIP2;1	*Vitis vinifera*	DQ834698	98	4e-167
	360 bp		VvPIP2;1	*Vitis vinifera*	AY823263	98	3e-162
7-40-M5	Partial	137	VvTIP2;1	*Vitis vinifera*	DQ934703	100	0.0
	673 bp		VvTIP	*Vitis berlandieri x Vitis rupestris*	AJ271661	100	0.0
7-38-A2	Full length	250	VvTIP2;1	*Vitis vinifera*	DQ934703	100	0.0
	1041 bp		VvTIP	*Vitis berlandieri x Vitis rupestris*	AJ271661	100	0.0
VvPIP2;1	Full length	285	VvPIP2;1	*Vitis vinifera*	DQ834698	98	0.0
	1216 bp		VPIP2;1	*Vitis berlandieri x Vitis rupestris*	AF141642	98	0.0
VvTIP1;1	Full-length	251	VvTIP1;1	*Vitis vinifera*	DQ834701	98	0.0
	993 bp		VvTIP3	*Vitis berlandieri x Vitis rupestris*	AF271660	98	0.0

Table 3-5: Genbank accession numbers for identified *Vitis vinifera* aquaporin cDNAs. Shown is the proposed gene name for each identified aquaporin, the subgroup, isolate name, source of the cDNA, length of cDNA (complete or partial) and the GenBank accession number. A number of novel cDNAs were identified using PCR based methods as detailed in the table. Phylogenetic analyses was used to determine aquaporin subgroups and these were then classified accordingly, based on their homology to other MIP members, and named using the nomenclature proposed by Johanson et al. (2001). Identical isolates are listed with the same gene name.

Gene name	Subgroup	Isolate	Source	cDNA	Genbank Accession Number
VvPIP1;1	PIP1	VvPIP1;1probe	PCR-library	Complete	EF364432
VvPIP1;2	PIP1	4-24-G12 1-6-H24 6-32-N3	cDNA library cDNA library cDNA library	Complete Partial Partial	EF364433
VvPIP1;3	PIP1	VvPIP1;probe	PCR-library	Complete	EF364434
VvPIP1;4	PIP1	7-39-11	cDNA Library	Complete	EF364435
VvPIP1;5	PIP1	5-25-018	cDNA library	Partial	EF364440
VvPIP1;6	PIP1	4-23-A20	cDNA library	Partial	Not submitted to Genbank
VvPIP2;1	PIP2	VvPIP2;1	RT-PCR	Complete	AY823263
VvPIP2;2	PIP2	4-19-A19	cDNA library	Complete	EF364436
VvPIP2;3	PIP2	1-6-G10	cDNA library	Complete	EF364437
VvPIP2;4	PIP2	8-45-E16 7-42-M5	cDNA library cDNA library	Complete Partial	EF364438
VvPIP2;5	PIP2	1-6-H18	cDNA library	Complete	Not submitted to Genbank
VvTIP1;1	TIP1	VvTIP1;1	RT-PCR	Complete	AY839872
VvTIP2;1	TIP1	1-4-E21 7-42-K11 6-32-I1 7-38-A2 7-40-M5	cDNA library cDNA library cDNA library cDNA library cDNA library	Complete complete complete complete Partial	EF364439

Table 3-6: Pairwise identity matrix of *V. vinifera* AQP cDNA nucleotide sequences. Pairwise alignments were performed by comparing the open reading frames for each cDNA, using ClustalW in slow mode, with an open gap penalty of 10 and an extended gap penalty of 0.05.

	VvPIP1;1	**VvPIP1;2**	**VvPIP1;3**	**VvPIP1;4**	**VvPIP1;5**	**VvPIP1;6**	**VvPIP2;1**	**VvPIP2;2**	**VvPIP2;3**	**VvPIP2;4**	**VvPIP2;5**	**VvTIP1;1**	**VvTIP2;1**
VvPIP1;1	100	79	78	79	68	39	63	63	61.5	63	63	37	38
VvPIP1;2		100	85	99	74	42.5	64	62	61.5	64	64	37	39
VvPIP1;3			100	85	87	49	62.5	61	60	62	62	38	39
VvPIP1;4				100	74	43	64	62	62	64	64	37	39
VvPIP1;5					100	57	61	61	59	61	61	42	43
VvPIP1;6						100	35	35	34	35	35	24	23
VvPIP2;1							100	72	77	99	99	41	42
VvPIP2;2								100	69	72	72	38	42
VvPIP2;3									100	76	76	41	43
VvPIP2;4										100	99.6	42	42-
VvPIP2;5											100	42	42
VvTIP1;1												100	61
VvTIP2;1													100

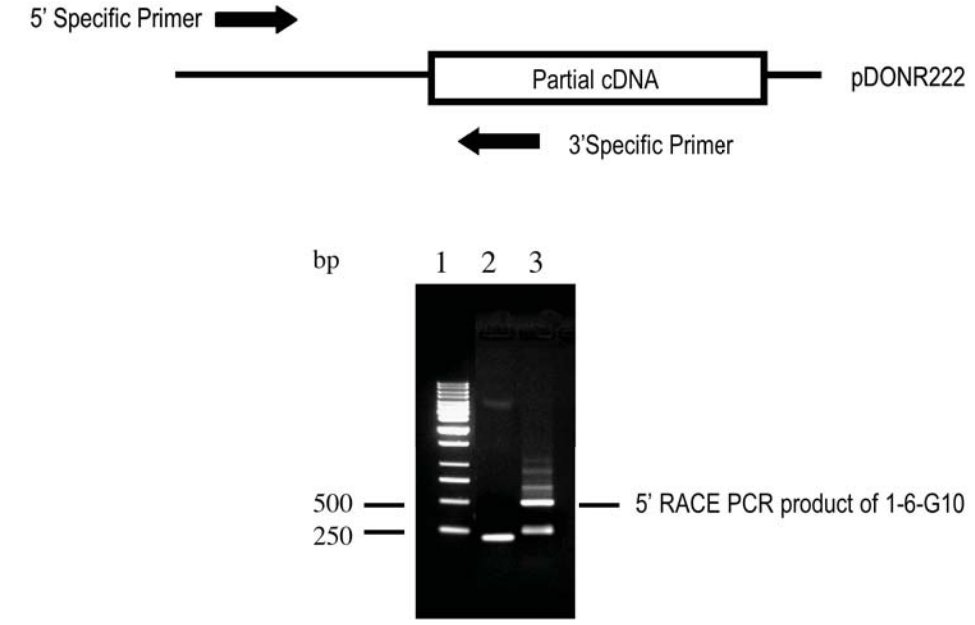
Table 3-7: Pairiwise identity (black) and similarity (red) matrix of *V. vinifera* aquaporins. Pairwise alignments were performed by comparing the deduced amino acid sequence for VvPIP1;1, VvPIP1;2, VvPIP1;3, VvPIP1;4, VvPIP1;5, VvPIP1;6, VvPIP2;1, VvPIP2;2, VvPIP2;3, VvPIP2;4, VvPIP2;5; VvTIP1;1, VvTIP2;1, using ClustalW in slow mode, with an open gap penalty of 10 and an extended gap penalty of 0.05.

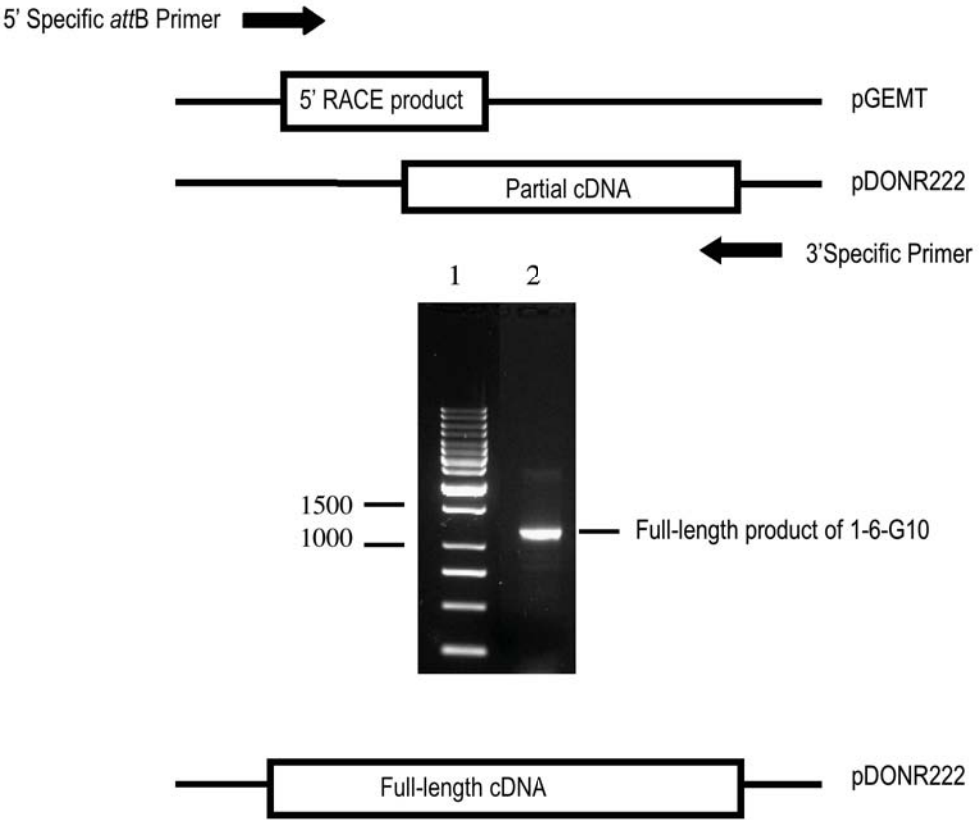
	VvPIP1;1	**VvPIP1;2**	**VvPIP1;3**	**VvPIP1;4**	**VvPIP1;5**	**VvPIP1;6**	**VvPIP2;1**	**VvPIP2;2**	**VvPIP2;3**	**VvPIP2;4**	**VvPIP2;5**	**VvTIP1;1**	**VvTIP2;1**
VvPIP1;1	100	89	85	89	75	44	64	64	63.5	64	64	27	27
VvPIP1;2	95.5	100	89.5	99	79	45	65	63	63	64	64	28	29
VvPIP1;3	94	97	100	90	87	49	63.5	62	61	63	63	28	28
VvPIP1;4	96	100	97	100	80	46	65	63	63	65	64	28	29
VvPIP1;5	82	85	87	85	100	57	64	63	62	64	64	32	31
VvPIP1;6	47	48	49	48	57	100	36	36	34	36	36	18	17
VvPIP2;1	74	73	73	73	73	42	100	80	83	99	99	30	29
VvPIP2;2	73	72	71	72	72	41	86	100	74	79	79	30	31
VvPIP2;3	72	71	71	71	71	41	90	82	100	83	82	32	29
VvPIP2;4	74	73	73	73	73	42	100	86	90	100	99	31	30
VvPIP2;5	73	73	72	73	72	42	99	85	89	99	100	30	29
VvTIP1;1	43	43	43	43	47.5	26	47	49	49	47	47	100	61
VvTIP2;1	44	45	46	45	51	27	47	50	47	47	47	75	100

3.3.3 5' RACE PCR of Partial Aquaporin cDNAs

5' RACE-PCR was performed on a selection of partial cDNAs to obtain a full-length cDNA. Primers designed to the multiple cloning site (MCS) of pDONR222 and the 5' end of 1-6-G10 and 7-39-L11 partial clones successfully amplified major products from the cDNA library at the expected size of 500 bp and 600 bp respectively. These fragments were cloned into pGEMT- easy and sequenced to confirm the correct product. A number of clones were sequenced and aligned to the corresponding partial cDNA, allowing primers to be designed to the 5' and 3' end of the full-length fragment. The full-length fragment was then amplified from the library using Gateway *att*B primers and recombined into pDONR222 to create entry clones. The full-length construct was subsequently sequenced to confirm the correct cDNA. The full-length cDNA of 1-6-G10 is 1225 bp, encoding a polypeptide of 280 amino acids. The cDNA of 7-39-L11 is 1061 bp, encoding a PIP1 aquaporin, 287 amino acids in length.

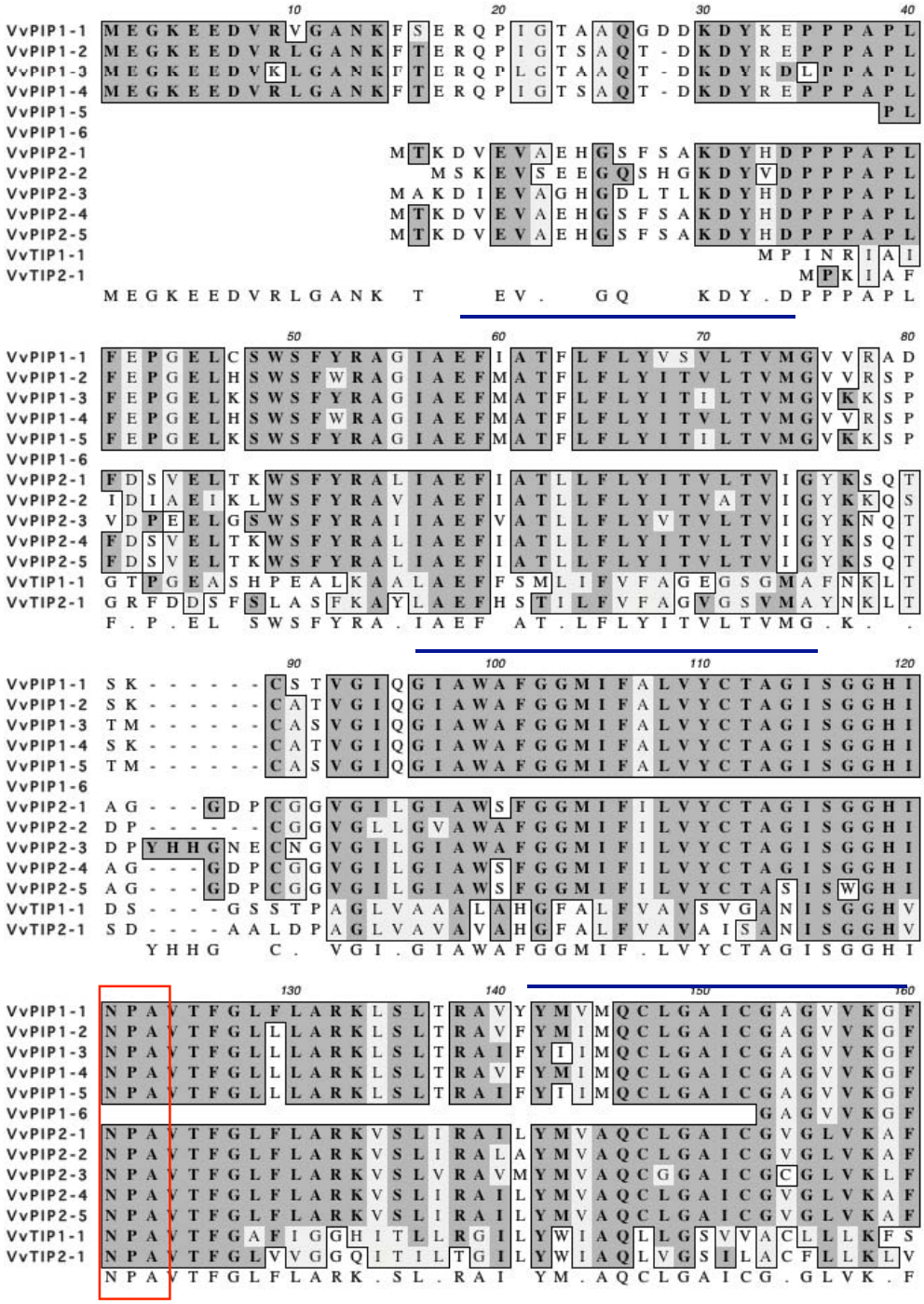
Figure 3-1: Schematic diagram showing the method used for 5' RACE-PCR and the subsequent cloning of the full-length cDNA of isolate 1-6-G10. A. The 5' primer was designed to the MCS of pDONR222 (Invitrogen) and a gene specific 3' primer was designed to the 5' end of the partial AQP cDNA. B. Gel picture of 5' RACE-PCR product of isolate 1-6-G10. Lane 1 – 1 kb DNA ladder (Fermentas), Lane 2 – PCR product amplified from partial cDNA of 1-6-G10 in pDONR222 (positive control), Lane 3 – 5' RACE-PCR product of 1-6-G10. C. Gel picture of full length PCR product of 1-6-G10 amplified from cDNA library with attB gene specific primers designed to the 5' end of the 5' RACE PCR product and the 3' end of the partial cDNA. Lane 1 – 1 kb DNA ladder (Fermentas), Lane 2 - Full-length PCR product of 1-6-G10 ~1200 bp in length.


5' Specific Primer
Partial cDNA
pDONR222
3'Specific Primer
bp
1 2 3
500
250
5' RACE PCR product of 1-6-G10


5' Specific attB Primer
5' RACE product
pGEMT
Partial cDNA
pDONR222
3'Specific Primer
1 2
1500
1000
Full-length product of 1-6-G10
Full-length cDNA
pDONR222

2.3.6 Cloning of *VvPIP2;1* and *VvTIP1;1* by RT-PCR

Reverse Transcriptase-PCR (RT-PCR) was used to clone the full length-cDNA of *VvPIP2;1* and *VvTIP1;1*. RT-PCR was performed on total RNA extracted from petiole tissue with primers designed to *Vitis* PIP2;1 and *Vitis* TIP3 (Table 3-2), producing DNA fragments of 1200 and 1000 bp, respectively. These fragments were ligated into pGEMT-easy and subsequently sequenced. Database searches using the BLAST algorithm (blastn and tblastx) confirmed both cDNAs encoded aquaporins based on their homology with other members of the MIP superfamily (Altschul *et al.*, 1997). The sequences of these two cDNAs have been included in the analysis with the aquaporin cDNAs identified in the library screen (Table 3-6, Table 3-7).


VvPIP1-1
VvPIP1-2
VvPIP1-3
VvPIP1-4
VvPIP1-5
VvPIP1-6
VvPIP2-1
VvPIP2-2
VvPIP2-3
VvPIP2-4
VvPIP2-5
VvTIP1-1
VvTIP2-1

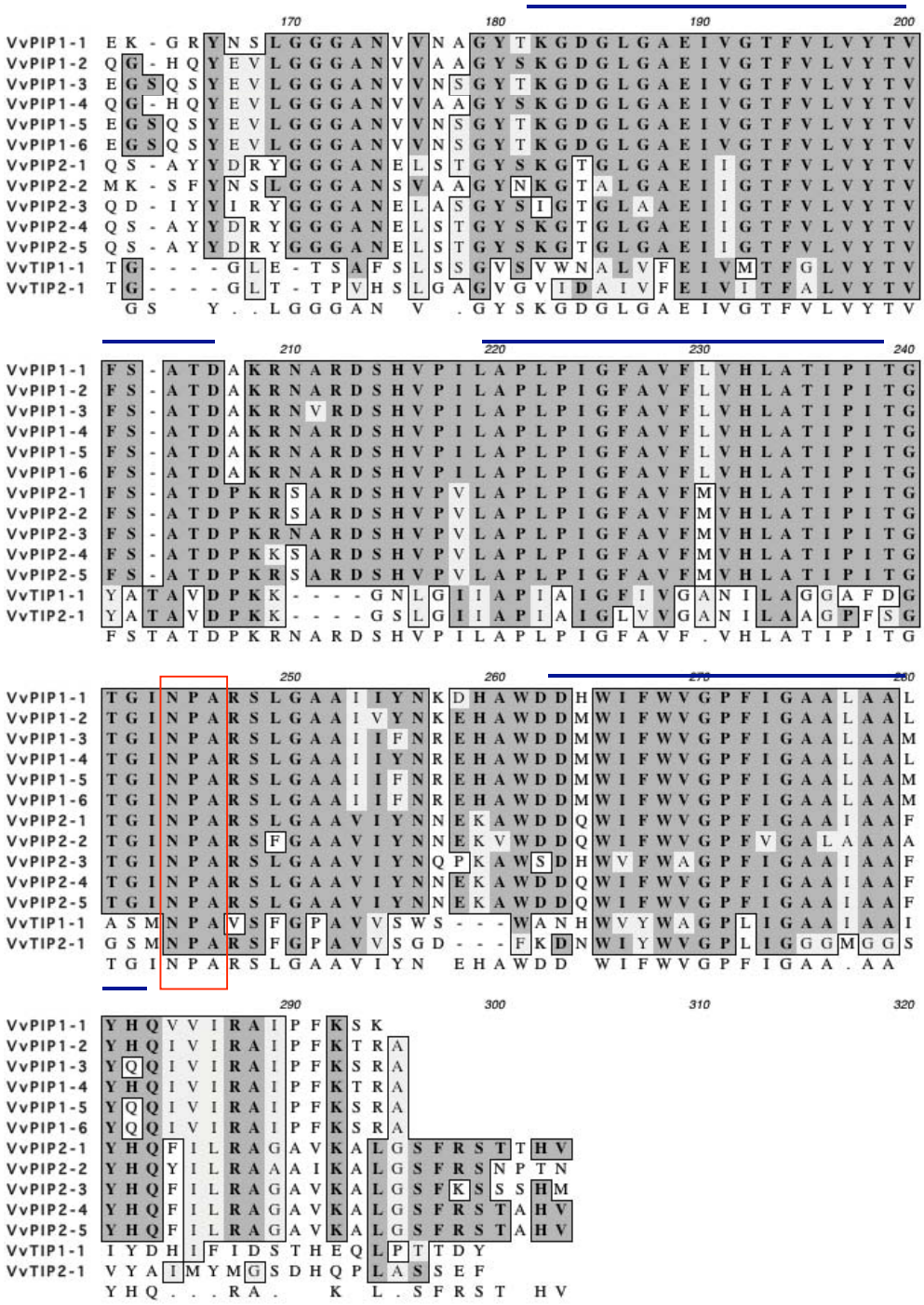
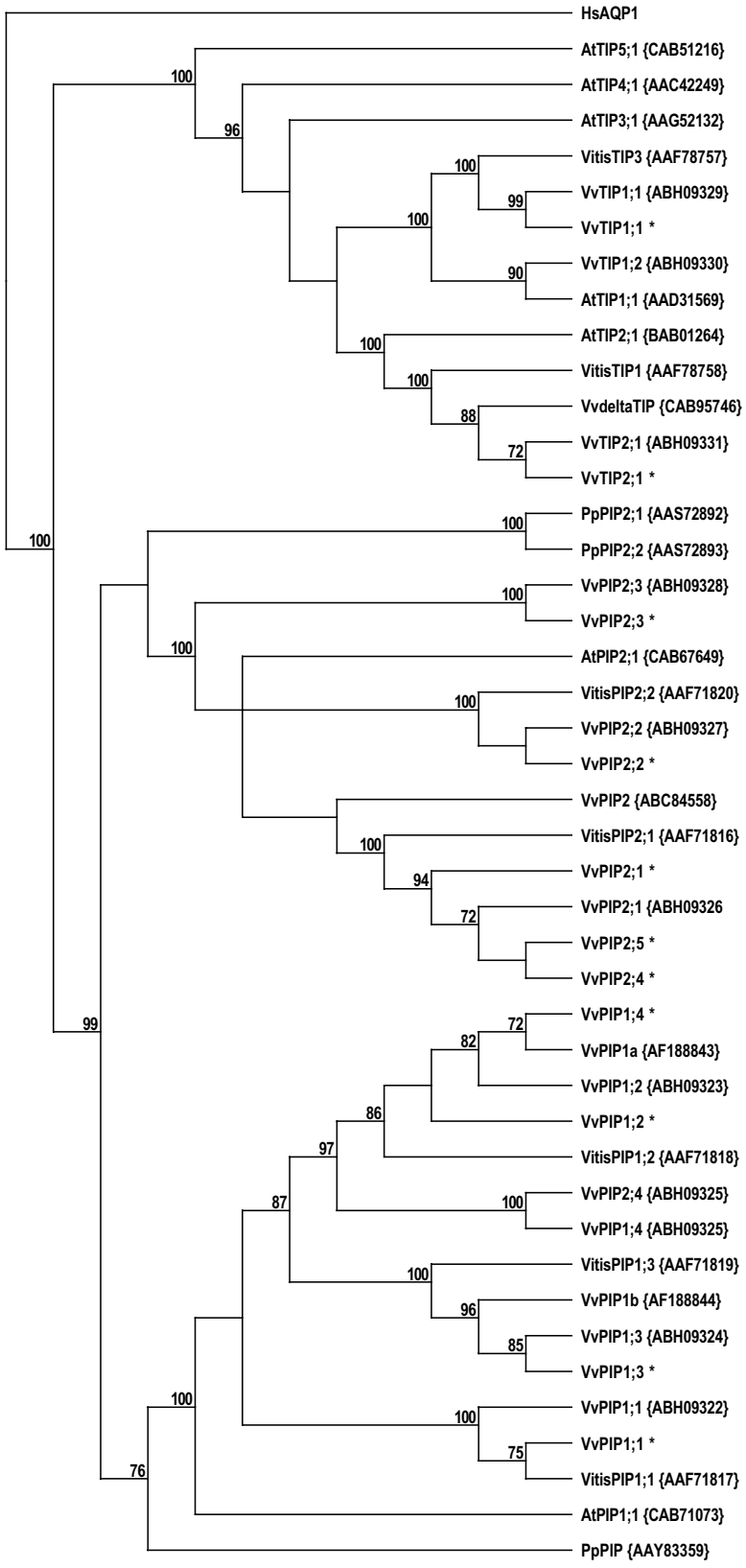


Figure 3-2: Alignment of the deduced amino acid sequences of the 13 putative *Vitis vinifera* aquaporin cDNAs. ClustalW (MacVector 9.0) was used to generate multiple sequence alignments using blosum30 matrix with an open gap penalty of 10 and extended gap penalty of 0.05. The black bars indicate the position of the six transmembrane spanning domains. The dark shaded boxes show identical residues and the light shaded boxes show similar residues in over 50 % of sequences. The consensus sequence is shown below the cDNAs. The red box highlights the NPA motif conserved in all sequences.

Figure 3-3: Phylogenetic analysis of the grapevine and other plant MIPs. ClustalW (MacVector 9.0) was used to perform multiple sequence alignments of the deduced amino acid sequences and the phylogenetic tree was generated using the neighbour joining method. HsAQP1 was used as the outgroup. The number next to the nodes represents the bootstrap value of 1000 repetitions (a bootstrap value of 100% indicates that branches were supported in all replicates of resampling). The MIPs identified in this study are highlighted with black stars. Only amino acid sequences of full- length cDNAs were included in the phylogenetic analysis. Accession numbers are shown in brackets.

Method: Neighbor Joining; Bootstrap (1000 reps); tie breaking = Random
Distance: Uncorrected ("p")
Gaps distributed proportionally



3.3.4 Phylogenetic Analysis of Grapevine PIPs and TIPs

The following sequence analysis includes all MIP members identified in this study, including those identified by PCR based methods (*VvPIP1;1, VvPIP1;3, VvPIP2;1* and *VvTIP1;1*).

All full-length PIP2 cDNAs identified are approximately 1200 bp in length and when compared at the nucleotide level show between 59 - 99 % homology (Table 3-6). *VvPIP2;4* and *VvPIP2;5* are 99% homologous at the nucleotide level. At the amino acid level *VvPIP2;1, VvPIP2;4* and *VvPIP2;5* are 99 % homologous (Table 3-7). VvPIP2;1 and VvPIP2;4 are 100 % similar with only one conservative amino acid substitution at position 193 from arginine to lysine. VvPIP2;1 and VvPIP2;5 are also 99% identical in the coding region. These differences are a glycine residue to serine (position 97) and glycine to tryptophane (position 100), both of which are located in the highly conserved loop B. VvPIP2;3 and VvPIP2;2 are 77 and 80 % homologous to VvPIP2;1, respectively. The PIP1 cDNAs are approximately 1100 bp in length and between 68-99% homologous (Table 3-6). *VvPIP1;2* and *VvPIP1;4* are 99% homologous at both the nucleotide (Table 3-6) and at the amino acid level (Table 3-7) with only 2 amino acid differences in the coding region, isoleucine to valine (position 247) and arginine to lysine (position 250).

3.3.5 Expression of Grapevine cDNAs

In some cases, individual cDNA populations within a cDNA library can give a guide to the abundance of the transcript in that plant tissue under those particular growing conditions. As the library was constructed from many different tissues (shoot and root) it was not possible to

obtain more specific information about putative gene expression patterns based on macroarray hybridisation. A number of cDNA isolates (1-4-E21, 6-32-IP, 7-38-A2, 7-40-M5 and 7-42-K11) were found to have identical nucleotide sequences in the coding region. The deduced amino acid sequence of these cDNAs encode the protein, VvTIP2;1. Interestingly, the only difference observed between the isolates was in the 3' UTR. Isolates 6-32-I1 and 7-42-K11 were both found to have a 15 bp deletion in the 3' UTR, immediately upstream of the poly(A) tail, compared to the other 3 isolates. *VvTIP2;1* is 72% identical to *VvTIP1;1* at the nucleotide level (Table 3-6) and 72% at the amino acid level. Multiple cDNA isolates of *VvPIP1;2* and *VvPIP2;4* were also identified in the library screen.

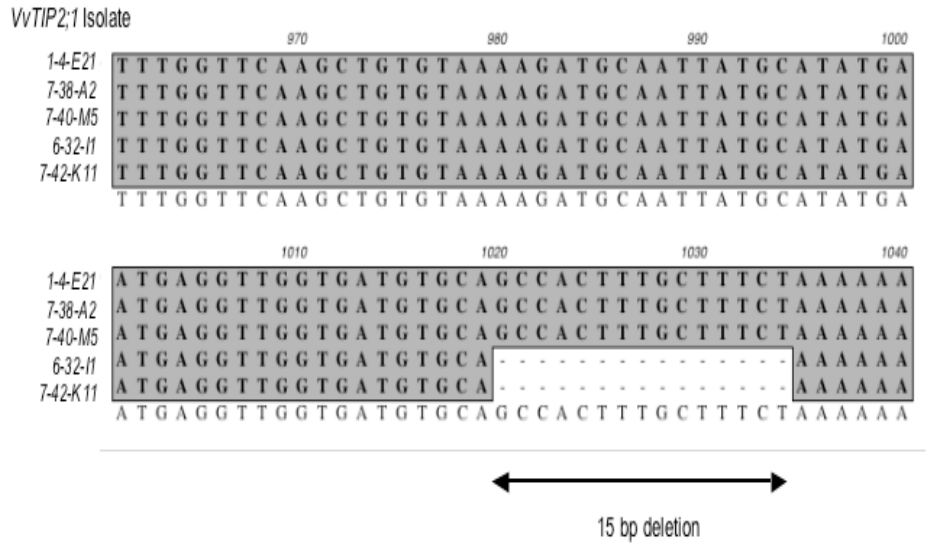


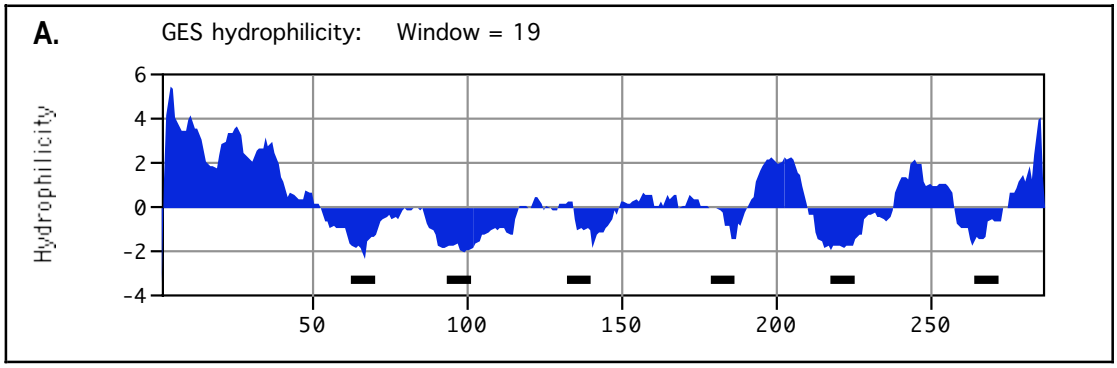
Figure 3-4: Sequence alignment of the 3' untranslated region of the 5 isolates encoding the protein, *VvTIP2;1*. The 15 bp deletion in isolates 7-40-M5, 6-32-I1 and 7-42-K11 is indicated with the black arrow.

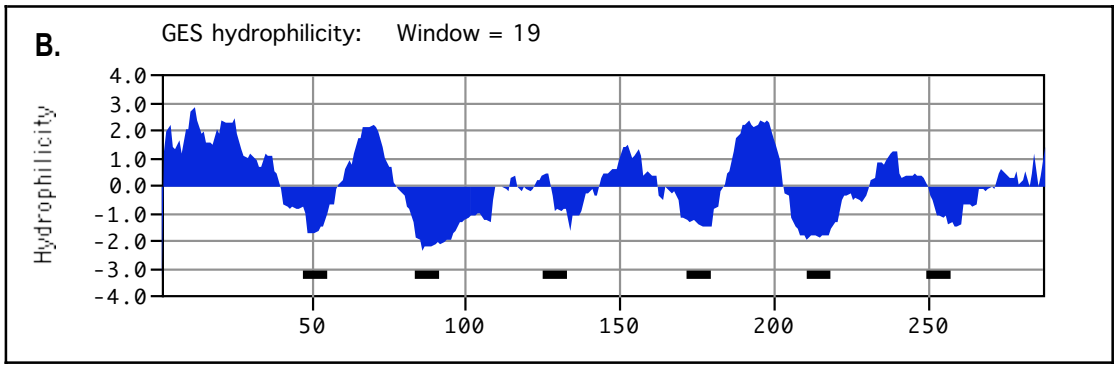
3.3.6 Structural Characteristics and Conserved Motifs

All MIP members identified in *V. vinifera* show characteristic sequence motifs found throughout the MIP superfamily. Multiple sequence alignments of the deduced amino acid sequences, performed using ClustalW, show the high homology between the PIP and TIP members identified in this study (Figure 3-2). The PIP1 members show an extended N-terminal tail of 14 amino acids compared to the PIP2 members. Conversely, the PIP2 members have an extended C-terminal tail comprising eight amino acids. TIP members have a much shorter N-terminal tail than all the PIPs. A number of residues (58) are found to be completely conserved in all grapevine MIPs identified, with particularly high conservation found in the functional loops B and E. A number of these residues are also conserved in loops B and E of mammalian aquaporins (Murata *et al.*, 2000). Shown are hydropathy plots, determined using the GES algorithm with a window size of 19, for proteins VvPIP1;1, VvPIP2;1 and VvTIP1;1 (Figure 3-5). The grapevine aquaporins are predicted to have 6 transmembrane alpha spanning domains that are separated by hydrophilic loops. The C and N termini are predicted to be cytoplasmic. The position of the 6 predicted transmembrane spanning domains are shown on the alignment and on the hydropathy plots. The loop between TMD1 and TMD2, designated loop B, and loop E between TMD5 and TMD6 contain the highly conserved hydrophobic NPA motif.

The phylogenetic relationship of the grapevine aquaporins with other plant aquaporins is shown in Figure 3-3. The grapevine aquaporins cluster into the distinct groupings of the PIP and TIP subfamilies with members from both Arabidopsis and the moss, *Physcomitrella patens*. The PIP members also show divergence into the two subgroups, PIP1 and PIP2. The length of the branches indicates the relatedness of the proteins. A number of grapevine

aquaporins cluster on one branch (VvPIP2;1, VvPIP2;4 and VvPIP2;5) separate to those identified in Arabidopsis and moss which form separate branches. This multiplicity of highly related aquaporins has been seen for both monocots (Chaumont *et al.*, 2001; Sakurai *et al.*, 2005) and dicots (Johanson *et al.*, 2001). The grapevine TIPs cluster into two groups, TIP1 and TIP2. No grapevine aquaporins have yet been identified in the TIP3, 4 and 5 subfamilies.





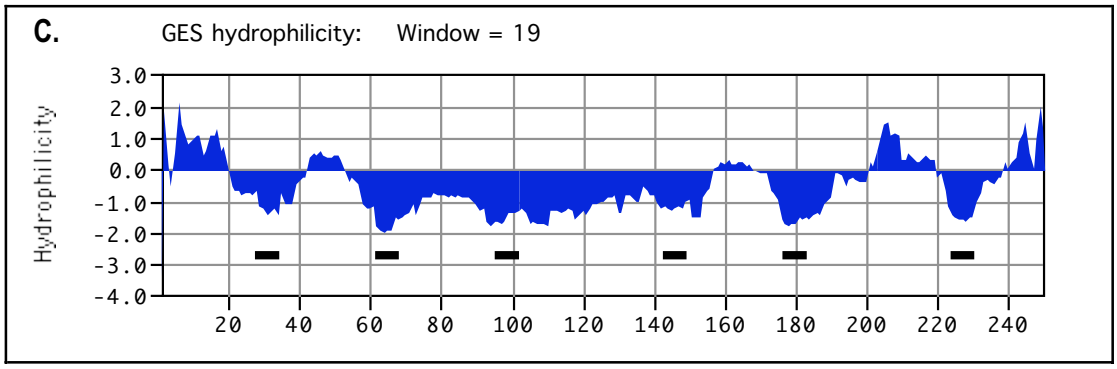


Figure 3-5: Hydropathy plots of (A) *VvPIP1;1*, (B) *VvPIP2;2* and (C) *VvTIP1;1* respectively. Hydrophilicity plots were determined using GES algorithm and a window size of 19, using MacVector 9.0. Negative regions are hydrophobic. Putative transmembrane domains are indicated with a black horizontal bar.

3.4 Discussion

3.4.1 Identification and Phylogenetic Analysis of Grapevine Aquaporins

The screening of a *V. vinifera* cv. Cabernet Sauvignon cDNA library has resulted in the identification of 13 cDNAs, 11 of which are full-length and two are partial. These were identified as members of the MIP superfamily based on homology to other plant aquaporins. Of these cDNAs, five are PIP2 aquaporins, six are PIP1 and two are TIP aquaporins. The completion of the sequencing of the Arabidopsis and maize genomes provides us with important information about the members of the MIP superfamily in both monocots and dicots. In both Arabidopsis and maize a total of 13 PIPs have been identified (Chaumont *et al.*, 2001; Johanson *et al.*, 2001). Based on this, it is likely that there are a similar number in grapevine, and we have most likely isolated the majority of the PIP genes found in this cultivar. The PIPs identified in this study are highly homologous at both the nucleotide and amino acid level. This is characteristic of PIP aquaporins with very high homologies found in other species such as Arabidopsis (Johanson *et al.*, 2001; Quigley *et al.*, 2002), Brassica (Marin-Olivier *et al.*, 2000), walnut (Sakr *et al.*, 2003), maize (Chaumont *et al.*, 2001) and rice (Sakurai *et al.*, 2005). The large number of aquaporin genes found in plants can be partially explained by their presence throughout the plant, in different tissues and different organelles. However functional roles of each remain poorly understood.

A phylogenetic analysis of the PIPs from the monocot maize and the dicot Arabidopsis, indicate that the divergence of these aquaporin genes occurred after the monocot - dicot divergence (Chaumont *et al.*, 2001). Comparison of the *V. vinifera* aquaporins with

Arabidopsis and *Physocomitrella patens* aquaporins clearly shows that the divergence of plant aquaporins into the four subfamilies occurred early in evolution. The grapevine aquaporins segregate onto distinct branches within each subfamily indicating that highly homologous genes most likely arose from recent gene duplications. An example of this is the high homology that exists between the cDNAs for *VvPIP2;1*, *VvPIP2;4* and *VvPIP2;5* (Table 3-6).

This high nucleotide homology has been seen for aquaporins in other species such as maize, where *ZmPIP1-3* and *ZmPIP1-4* have 98% nucleotide homology and encode identical proteins. In maize, these genes were also shown to have a similar transcript distribution pattern, and the authors hypothesised that the role of having two genes encoding identical proteins may in fact be to increase the expression levels of the duplicated genes (Chaumont *et al.*, 2001). *VvPIP1;2* and *VvPIP1;4* are 99 % identical at the nucleotide level and 100% similar at the amino acid level. The transcript abundance and tissue location in grapevine of each of these isoforms is unknown.

The *V. vinifera* TIP aquaporins have lower homologies than observed for the PIP aquaporins. This has been seen previously in both Arabidopsis (Johanson *et al.*, 2001) and maize TIPs (Chaumont *et al.*, 2001). The full-length cDNA of VvTIP2;1 was independently identified in the library screen five times, indicating that this gene is most likely highly expressed throughout the plant. The Arabidopsis homolog δ-TIP (renamed AtTIP2;1), has been shown to be highly expressed in the leaves (Alexandersson *et al.*, 2005). Interestingly, two of the cDNAs identified had a 15 bp deletion at the 3' end of the cDNA, immediately prior to the poly(A) tail. Polyadenylation of mRNA is an important process that most likely provides stability, enhances the translation of the mRNA, and maybe required for correct trafficking

from the nucleus to the cytoplasm (Zhao *et al.*, 1999). In plants, poly(A) signals are comprised of the far upstream elements (FUE), near upstream elements (NUE) and cleavage sites (CS) (Rothnie, 1996; Li and Hunt, 1997; Rothnie *et al.*, 2001). Plant polyadenylation cleavage sites have a consensus signal which comprises a PyA (pyrimidine A) dinucleotide (Hunt, 1994) within a U-rich region. The presence of multiple CS in the 3' UTR can result in the addition of the poly(A) tail at different positions. The choice of which cleavage site is used can be dependent on many factors including cell type and growth conditions. The position at which polyadenylation occurs will effect the sequence of the 3' UTR as has occurred with *VvTIP2;1*. This may affect the stability of the transcript, translation of the protein and trafficking of the transcript to the cytoplasm, and ultimately may lead to changes in protein expression.

3.4.2 Structural Characteristics of Grapevine Aquaporins

The protein structure of AQP1 has been confirmed by both X-ray (Sui *et al.*, 2001b) and electron crystallography (Murata *et al.*, 2000) to a resolution of 2.2 Å. The predicted AQP topology consists of six transmembrane alpha helices with connecting hydrophilic loops. The protein consists of two tandem repeats, with hydrophobic loops B and E forming half helices that dip back into the membrane to essentially form a seventh helix. Recently, the spinach plasma membrane aquaporin, SoPIP2;1, was crystallised in the open confirmation at 3.9 Å resolution and in the closed confirmation at 2.1 Å resolution (Tornroth-Horsefield *et al.*, 2006). SoPIP2;1 crystallised as a tetramer with the four monomers predicted to be held together by hydrophobic interactions, as observed with all other aquaporins crystallised to date (Murata *et al.*, 2000; Sui *et al.*, 2001b). As has been seen previously, the two half helices of loops B and E with the conserved NPA motif, fold into the membrane to create a

seventh transmembrane helix. This "hour glass" model (Jung *et al.*, 1994) is completely preserved throughout the evolutionary lines. In all *V. vinifera* MIPs identified the NPA motif is completely conserved in loops B and E (Figure 3-2). Hydropathy plots predict these aquaporins to also have six transmembrane domains connected by hydrophilic loops.

Loop D in the PIP aquaporin members differ to other plant aquaporins, in that there are usually four to seven additional amino acid residues. A number of these residues in loop D of SoPIP2;1 have been identified as being involved in gating of the channel (Tornroth-Horsefield *et al.*, 2006). The amino acid alignment of the *Vitis vinifera* aquaporins shows that all the PIPs identified have four additional amino acid residues in loop D, not found in the TIP aquaporins. In SoPIP2;1, Leu 197 was identified as a key residue, thought to effectively form a lid to cap the channel, and in combination with other conserved residues His99, Val 104 and Leu 108, contribute to the hydrophobic barrier that blocks the pore region. Leu 200 in VvPIP2;1, corresponding to Leu 197 in SoPIP2;1, is also fully conserved in all the PIP members, as are His 102, Val 108 and Leu 111. In the closed conformation loop D occludes the pore from the cytosol.

Phosphorylation of two serine residues, Ser 115 and Ser 274, in SoPIP2;1 (previously called PM28A) has been shown to open the channel. Dephosphorylation results in channel closure (Johansson *et al.*, 1996; Johansson *et al.*, 1998) and dephosphorylation has been shown to occur in response to drought stress. Ser 118 and Ser 277 (conserved only in PIP2 homologues) in VvPIP2;1 are most likely phosphorylated resulting in channel opening as observed in SoPIP2;1. Recently, a tonoplast integral protein, PvTIP3;1 (α-TIP), has been shown to be phosphorylated at Ser 7 in the N-termini by protein kinase A (Daniels and

Yeager, 2005). This serine is not present in the *V. vinifera* TIPs identified, but it is possible that other Ser residues may be phosphorylated.

A highly conserved His residue in loop D has been implicated in channel gating under anoxic conditions in an Arabidopsis PIP2 aquaporin (Tournaire-Roux *et al.*, 2003). The corresponding His residue in loop D is conserved in all *V. vinifera* PIP aquaporins. The crystal structure of SoPIP2;1 has confirmed the involvement of the His residue in pH regulated gating (Tornroth-Horsefield *et al.*, 2006).

VvPIP2;5, identified in the cDNA library screen, has two amino acid substitutions, Gly 97 is substituted for Ser, and Gly 100 is substituted for Trp, in the highly conserved region of loop B. Both glycine residues are completely conserved in all plant PIP members. Gly 100 is highly conserved in the MIP superfamily and it is also conserved in mammalian aquaporins. The structural model of AQP1 shows this glycine faces the inside of the aqueous pore (Murata *et al.*, 2000). This combined with the conservation of this residue throughout the aquaporin family indicates it most likely plays a crucial role in water transport through the channel. Ser 99 is conserved in all identified PIP and TIP aquaporins from *V. vinifera* and the corresponding serine residue in AQP1 forms a hydrogen bond with Tyr 137 which helps to stabilise loop B (Mitsuoka *et al.*, 1999). The substitution of the glycine residue by tryptophane residue at position 100 may also affect the stability of this interaction. Therefore, it is expected that the addition of the Trp 100 will significantly affect the expression and functionality of the protein (Chapter 4). This gene is most likely a pseudogene, although for this to hold true it must be non-functional. If these mutations are deleterious to the functionality of the protein, it will remain in the genome unless there is some selective

pressure to remove it. Aquaporin pseudogenes have previously been identified in both Arabidopsis and maize (Chaumont *et al.*, 2001; Johanson *et al.*, 2001; Quigley *et al.*, 2002).

In conclusion, 11 full-length aquaporin cDNAs have been identified from *V. vinifera* cv. Cabernet Sauvignon. These encode aquaporin proteins from the PIP and TIP subfamilies that appear to be structurally similar to previously identified aquaporins.

Chapter 4:
Functional Characterisation of Grapevine Aquaporins

4.1 Introduction

The first aquaporin identified, AQP1 (CHIP28) was isolated from the erythrocyte membrane (reviewed in Denker *et al.*, 1988), and subsequently shown to function as a water selective channel by expression in *Xenopus laevis* oocytes (Preston *et al.*, 1992). In plants, the first aquaporin to be cloned and functionally expressed in *Xenopus* oocytes was a tonoplast integral membrane protein, AtTIP1:1 (previously γ -TIP) from *Arabidopsis thaliana* (Maurel *et al.*, 1993). When AtTIP1;1 was expressed in *Xenopus* oocytes the water permeability was 6-8 fold higher than water-injected oocytes. Since this time, the use of *Xenopus* oocytes as a heterologous expression system has become a convenient method to assess the functionality of aquaporins from different organisms. Recently, a novel AQP, designated AQPxlo, (AY120934), was isolated and cloned from *Xenopus laevis* oocytes (Virkki *et al.*, 2002). AQPxlo has highest homology to the aquaglyceroporins, and when overexpressed in oocytes, is permeable to water, glycerol and urea (Virkki *et al.*, 2002). In native oocytes, AQPxlo is highly transcribed, but the water permeability coefficient (Pf) remains low (as does glycerol and urea uptake), allowing the use of oocytes as a heterologous expression system for other aquaporins.

Members of the MIP superfamily have been shown to have different functional properties when expressed in *Xenopus* oocytes, with various members permeable to water (Biela *et al.*, 1999; Santoni *et al.*, 2000; Tyerman *et al.*, 2002), glycerol (Biela *et al.*, 1999; Weig and Jakob, 2000; Moshelion *et al.*, 2002), urea (Gerbeau *et al.*, 1999; Liu *et al.*, 2003), CO_2 (Uehlein *et al.*, 2003), NH_3 (Jahn *et al.*, 2004; Loque *et al.*, 2005) and silicon (Ma *et al.*, 2006). In plants, the PIP2 subgroup, have been shown to have high water permeability while most PIP1 AQPs have low water permeability or are completely inactive. In maize, all

ZmPIP2 proteins analysed show high water permeability and ZmPIP1a and ZmPIP1b low water permeability (Chaumont *et al.*, 2000). The reason for this difference remains unclear, but these inactive proteins were expressed in the plasma membrane of oocytes, indicating correct targeting to the plasma membrane (Chaumont *et al.*, 2000). Recently, Fetter *et al.* (2004) showed that co-expression of a non-functional maize aquaporin, ZmPIP1;2 with ZmPIP2 isoforms in *Xenopus* oocytes resulted in an increase in Pf, greater than observed for ZmPIP2 when expressed alone (Fetter *et al.*, 2004). The authors proposed that when these two isoforms are co-expressed in *Xenopus* oocytes, they can interact to form heterotetratamers. High water channel activity has also been observed in some PIP1 members including NtAQP1 (Biela *et al.*, 1999), BoPIP1b and BoPIP1;2 (Marin-Olivier *et al.*, 2000) and the Arabidopsis aquaporins, AtPIP1a, AtPIP1b and AtPIP1c (Kammerloher *et al.*, 1994). Only two AQPs identified from grapevine have been functionally characterised. Both of these AQPs, VvPIP1a and VvPIP1b from grape berry, have low Pf values of 14.4 and 8.2 x 10^{-3} cms^{-1}, respectively, but have been shown to transport glycerol (Picaud *et al.*, 2003).

In order to assess the functionality of the grapevine aquaporins identified from *Vitis vinifera* (Chapter 3), we expressed these in *Xenopus laevis* oocytes. An oocyte expression vector pGEMHE, was converted to a Gateway-enabled oocyte expression vector, pGEMHE-DEST. Full-length AQP cDNAs were expressed in *Xenopus* oocytes and the water permeability determined. The effect of cytosolic pH on the regulation of two aquaporins, VvPIP2;1 and VvTIP1;1, was also examined.

4.2 Materials and Methods

4.2.1 Conversion of pGEMHE to Gateway Vector

The oocyte expression vector, pGEMHE (Liman *et al.*, 1992) was converted to a Gateway vector using the Gateway Vector Conversion System according to the manufacturers instructions (Invitrogen). pGEMHE was linearised with *Bam* HI restriction enzyme (Promega), that cuts within the polylinker site of pGEMHE. The polylinker is flanked by the 5' and 3' β-globin untranslated regions (Krieg and Melton, 1984). The linearised vector was treated with Klenow (Roche) to blunt end the double-stranded DNA, and the 5' phosphates removed with antartic phosphotase (NEB). The reading frame cassette A (RfA) was blunt end ligated to the linearised pGEMHE vector, destroying the original *Bam* H1 restriction site, and subsequently transformed into DB3.1 chemically competent cells (Invitrogen). The reading frame cassette A contains the chloramphenical resistance gene (CmR) and the *ccdB* gene flanked by the *att*R1 and *att*R2 sites (Figure 4-1). Plasmid preparations of putative pGEMHE / Gateway transformants resistant to chloramphenical and ampicillin selection were digested with *Bsr* G1 (New England Biolabs) and sequenced with the Gateway primers 5'CACATTATACGAGCCGGAAGCAT-3' and 5'-CAGTGTGCCGGTCTCCGTTATCG-3' to confirm conversion to a Gateway vector. The resulting plasmid was designated pGEMHE-DEST (Figure 4-1).

4.2.2 Amplification of *att*B PCR Products

*Att*B PCR primers were designed according to the Gateway® Technology manual (Invitrogen) to amplify *att*B PCR products of *VvPIP2;1* and *VvTIP1;1* (Table 4-1). Pwo DNA Polymerase (Roche) was used to amplify *att*B/*VvTIP1;1* by PCR from a *Vitis vinifera* cDNA library (Chapter 3). *Att*B/*VvPIP2;1* was amplified from *VvPIP2;1*/pGEMT. The PCR cycling conditions were as follows: 94 °C for 2 min; 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1 min cycled 10 times; 94 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1 min + 5 sec each cycle for 15 cycles; and extension at 72 °C for 7 min. The *att*B PCR products were PEG purified (according to Gateway® manual, Invitrogen) and recombined into pDONR222 using BP Clonase (Invitrogen). The Gateway BP reaction facilitated recombination of the *att*B PCR products into pDONR222 to create *att*L entry clones of *VvPIP2;1* and *VvTIP1;1*. LR Clonase was used to recombine *VvPIP2;1* and *VvTIP1;1* into pGEMHE-DEST. Entry clones were digested and sequenced to confirm correct PCR product.

Primer name	**Primer Sequence 5'-3'**	**Tm (°C)**
attB1/VvPIP2;1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTACCTTCTCCTGAACCCCCTA	78
attB1/VvPIP2;1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACAAGACAAAGCCCAACA	81
attB1/VvTIP1;1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCCTAGAGCTTGAGGAGGA	80
attB1/VvTIP1;1R	GGGGACCACTTTGTACAAGAAAGCTGGGTTGATTGCAAACAAACCAGACA	80

Table 4-1: *attB* primers for amplification of *attB* PCR products. Each primer contains four guanine residues at the 5' end followed by the 25 bp attB1 site (red) followed by 18-25 bp of gene-specific sequence. The melt temperature (Tm) for each primer is also shown.

4.2.3 Cloning of AQP cDNAs into Oocyte Expression Vector

Full-length *Vitis vinifera* aquaporin cDNAs identified in the library screen (Chapter 3) were recombined into the pGEMHE-DEST vector using the LR recombination reaction (Invitrogen). The LR reaction allows recombination of the *att*L entry clones with the *att*R destination vector (pGEMHE-DEST). The LR reaction was set up as per the manufacturers instructions (100-300 ng of *att*L entry clone and 300 ng of destination clone). The LR recombination reaction was stopped by the addition of proteinase K solution. 1 μl of the LR reaction was transformed into chemically competent DH5α cells. The pGEMHE-DEST vector contains the ampicillin resistance gene allowing selection of transformants on LB plates containing 100 μg/ml of ampicillin. Single colonies were selected and grown overnight in 5 ml LB + Amp at 37°C shaking at 200 rpm. Plasmid was purified using the Sigma Genelute Plasmid Purification Kit and digested with *Hind* III and *Sac* I restriction enzymes (Promega) to confirm the correct cDNA. The cDNA of *AQP1* from human red blood cells was obtained from Daniel M. Roberts (University of Tennessee, Knoxville, Tennessee). The *AQP1* cDNA was cloned into the vector XβG-ev1 between the 5' and 3' untranslated region (UTR) of the *Xenopus* β-globin cDNA (Preston *et al.*, 1992).

4.2.4 cRNA Transcription

1 μg of each pGEMHE-DEST vector was digested with *Nhe* 1 (Promega) at 37°C overnight to linearise the plasmid. 1 μg of *AQP1* XβG-ev1 plasmid was digested with *Bam* H1. Plasmids were checked for complete digestion by running 100 ng on a 1 % (w/v) agarose gel / 1 x TAE buffer. After linearisation the reaction was terminated with the addition of 1/10th volume of 3 M NaOAc pH 5.2, 1/20th 0.5 M EDTA and 2 volumes of 100% ETOH,

precipitated at – 20°C for 15 min, and spun at 14,000 rpm for 20 min to recover digested DNA. Linearised DNA was resuspended in DEPC water at a concentration of 0.5 – 1.0 μg μl-1. Complimentary RNA was transcribed using 1 μg of linearised DNA with the mMESSAGE mMachine Kit (Ambion) utilizing the T7 promoter of pGEMHE, according to the manufacturers instructions. The mMESSAGE MACHINE kit allows the *in vitro* synthesis of capped RNA. The 7-methyl guanosine cap structure is incorporated at the 5' end of the transcript and when injected into *Xenopus* oocytes, mimics eukaryotic mRNAs thus allowing *in vivo* translation to occur. Phenol: chloroform: isoamyl alcohol (25:24:1 v/v) (SIGMA) was used for extraction and isopropanol for precipitation of the synthesised cRNA. cRNA was quantitated and checked for purity using a spectrophotometer (Smartspec, Biorad) and by running on a denaturing RNA gel.

4.2.5 Harvesting Oocytes

Oocytes were harvested from *Xenopus laevis* frogs by Christa Niemietz using the protocol described by Hill et al. (Hill *et al.*, 2005). Following harvest, oocytes were washed twice in cold Calcium-free Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl2; 5 mM HEPES, pH 7.6), then defolliculated in 1.7% (wt/vol) collagenase in Calcium-free Ringers solution for 90 min with rotation. Oocytes were washed five times in Calcium-Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl2, 6 mM CaCl2, 5 mM HEPES, pH 7.6) to remove all traces of collagenase. Oocytes were transferred to Ca-Ringers solution + antibiotics (100 μg / ml tetracycline-HCl, 100 units / ml penicillin and 100 μg / ml streptomycin) and kept at 18°C until required.

4.2.6 Expression of Grapevine Aquaporins

3.5" capillary glass pipettes (Drummond Scientific) were pulled with the Pipette Puller (Narishige, Japan) and bevelled with the EG-400 Microgrinder (Narishige, Japan) to an angle of 30 °. Defolliculated oocytes were microinjected with the Nanoject II Auto-Nanoliter Injector (Drummond Scientific) into the cytoplasm with 50 ng of capped cRNA or with DEPC treated water (control) and incubated for 3 days at 18°C in Ca-Ringers solution + antibiotics (100 μg / ml tetracycline-HCl, 100 units / ml penicillin and 100 μg / ml streptomycin).

4.2.7 Oocyte Swelling Assay

Oocytes were preincubated at room temperature in an iso-osmotic solution (Ca-Ringers solution) for 5 min, 64 - 72 hours after cRNA injection, and then transferred to a hypoosmotic solution (Ca-Ringers solution diluted 5 fold with sterile water) at which time swelling was measured for 2 min. Oocytes were viewed with a Nikon SMZ800 light microscope (Japan) with Vikam colour camera at 2 x magnification and imaged with Global Lab® Image/2 software (Data Translation, Marlboro, MA). Images were acquired every 4 s for 2 min. Osmotic permeability (Pf) was calculated for water injected and cRNA injected oocytes from the increase in volume with time (n = 5-8 ± SEM). At least two independent experiments were conducted for each gene. Osmolarity of each solution was determined using a Vapour Pressure Osmometer 5500 (Wescor). The Pf was calculated using equation 4-1.

$$Pf = V_o(d(V/V_o)/dt) / S*V_w (Osm_{in}-Osm_{out}) \quad \text{Equation 4-1}$$

where:

V_o is the initial oocyte volume,

S the initial surface area

V_w is the molar volume of water, given as 18 cm^3 mol^{-1}.

4.2.8 Acidification of the Cytosol

Cytosolic pH was lowered using an external solution of 50 mM Na-acetate at pH 5 + Ca-Ringers solution (Tournaire-Roux *et al.*, 2003). Controls contained 50 mM Na acetate at pH 7. Swelling assays were conducted as above (Section 4.2.7) with oocytes incubated in 50 mM Na-acetate pH 5 solution for 5 min to allow acidification of the oocyte cytosol. Oocytes were then transferred to a hypotonic solution (5 times diluted with MilliQ water) and swelling imaged for 2 min.

4.2.9 Modelling of AQP

Homology modelling was used to generate a predicted structure for the VvPIP2;5 protein. The model was generated using SWISS-MODEL (Schwede *et al.*, 2003) with the structure of the open conformation of SoPIP2;1 (PDB number 285F) selected as a template. Pictures were generated with Deep View - Swill PDB viewer by Dr. Susan Howitt (Australian National University, Canberra). Using the spinach plasma membrane aquaporin, SoPIP2;1 model (PDB number 2B5F), the mutate function was used to change the two glycine residues to serine and tryptophane.

4.3 Results

4.3.1 Cloning of Full-length Constructs into pGEMHE

The ooctye expression vector pGEMHE was successfully converted to a Gateway vector by insertion of the reading frame cassette A. Correct orientation of the reading frame cassette A into pGEMHE was confirmed by sequencing across the insertions sites. The resulting plasmid was designated pGEMHE-DEST and is shown in Figure 4-1. Based on the sequences obtained in chapter 3, clones identified as full-length aquaporin cDNAs were recombined into the oocyte expression vector pGEMHE-DEST. Correct inserts were confirmed using restriction enzyme analysis. To demonstrate functionality of the aquaporin genes, each gene was expressed in *Xenopus* oocytes and the water permeability determined.

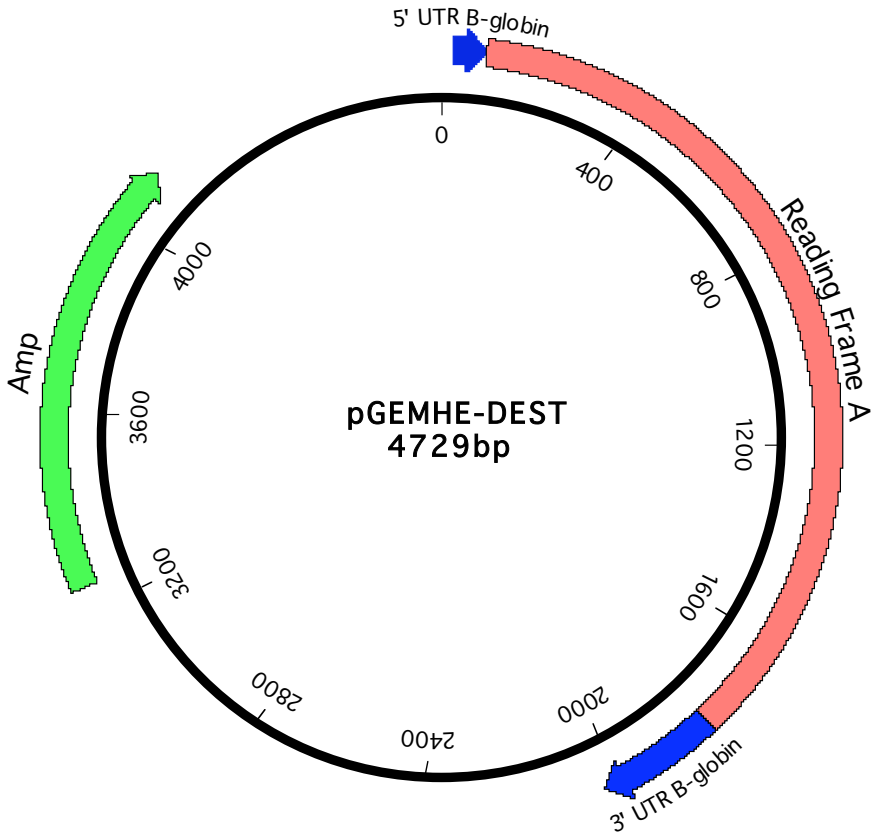


Figure 4-1: Expression vector pGEMHE-DEST, constructed as detailed in section 4.2.1. 5' and 3' UTRs from *Xenopus* β-globin gene flank a polylinker (Krieg and Melton, 1984) enabling the recombination of the reading frame cassette A containing the *ccdB* gene and chlorophenical resistance gene.

4.3.2 Oocyte Permeability in Response to Hypotonic Shock

The functionality of full-length *Vitis vinifera* AQP cDNAs was assessed by expression in *Xenopus* oocytes. Swelling was measured in response to bathing in a hypo-osmotic solution, approximately three days after injection with cRNA. Figure 4-2 shows the increase in oocyte volume plotted against time. The water permeability (Pf) was calculated and compared to the water-injected oocytes (control) (Figure 4-3). AQP1, from human red cells, was used as a positive control. The mean Pf value for AQP1 was $22 \pm 4 \times 10^{-3}$ cm s^{-1}. The mean Pf of VvPIP2 proteins ranged from $8.0 – 27.0 \times 10^{-3}$ cm s^{-1}. The Pf value for VvPIP2;5 was 0.6×10^{-3} cm s^{-1}, higher than the water injected control 0.1×10^{-3} cm s^{-1} (although not significant $p > 0.05$). PIP1 proteins (VvPIP1;2 and VvPIP1;4) also showed low permeability with no significant difference compared to the water injected controls, 0.5×10^{-3} cm s^{-1}. The Pf value for VvTIP2;1 was $9.0 \pm 5.0 \times 10^{-3}$ cm s^{-1}, whilst VvTIP1;1 was significantly lower at $5.0 \pm 2.0 \times 10^{-3}$ cm s^{-1}.

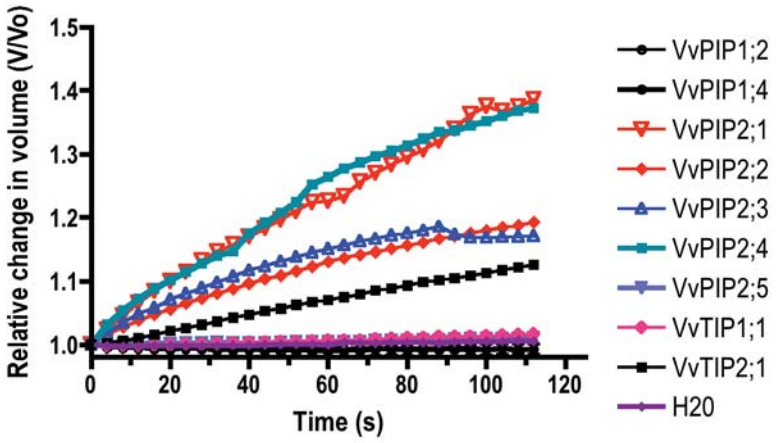


Figure 4-2: Swelling rates of *Xenopus* oocytes. Oocytes were separately injected with 50 ng of cRNA (mESSAGE mACHINE, Ambion) from cloned cDNAs (*VvPIP1;2, VvPIP1;4, VvPIP2;1, VvPIP2;2, VvPIP2;3, VvPIP2;4, VvPIP2;5, VvTIP1;1, VvTIP2;1*) or with water (control) and incubated for three days at 18°C. Oocytes were preincubated in an iso-osmotic solution and then transferred to a hypo-osmotic solution at which time swelling was measured. The mean value of the relative volume (V/V_o) is shown plotted against time (n=5-10).

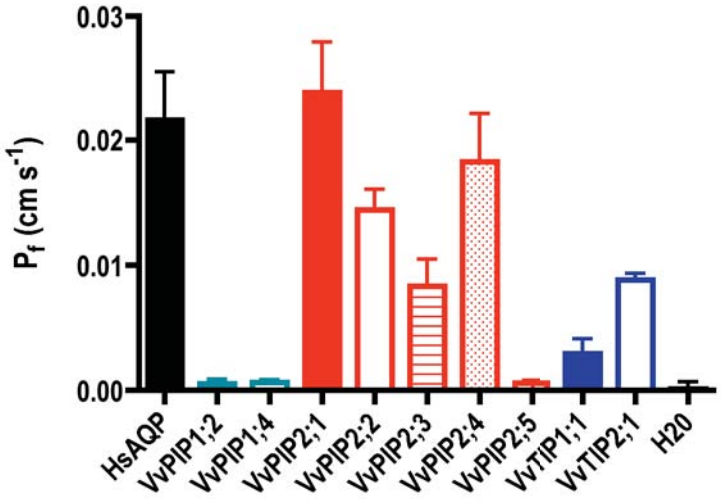


Figure 4-3: Swelling rates of *Xenopus* oocytes. Oocytes were separately injected with 50 ng of cRNA (mESSAGE mACHINE, Ambion) from cloned cDNAs (*VvPIP1;2, VvPIP1;4, VvPIP2;1, VvPIP2;2, VvPIP2;3, VvPIP2;4, VvPIP2;5, VvTIP1;1, VvTIP2;1*) or with water (control) and incubated for three days at 18°C. Oocytes were preincubated in an iso-osmotic solution and then transferred to a hypo-osmotic solution at which time swelling was measured. The mean value of the relative volume (V/V_o) is shown plotted against time (n=5-10).

4.3.3 Effect of Cytosolic pH on Aquaporin Water Permeability

In order to check the effect of cytosolic pH on regulation of aquaporins, oocytes expressing AQP1, VvPIP2;1, VvTIP1;1 and H_20 injected controls, were pre-incubated in an isotonic solution containing 50 mM Na-acetate for 5 min prior to the assay. This allowed time for the Na-acetate to freely diffuse the membrane and acidify the cytosol. This was diluted five-fold to approximately 80 mosmols. The results are shown in Figure 4-4. An eleven-fold decrease in Pf was observed for VvPIP2;1 when exposed to Na-acetate pH 5, compared to Na-acetate pH 7, although this was not significant ($p > 0.05$). The Pf value of VvTIP1;1 was unaltered.

The mammalian AQP1 showed a two-fold increase in Pf when exposed to Na-acetate pH 5 solution, compared to the pH 7 control solution.

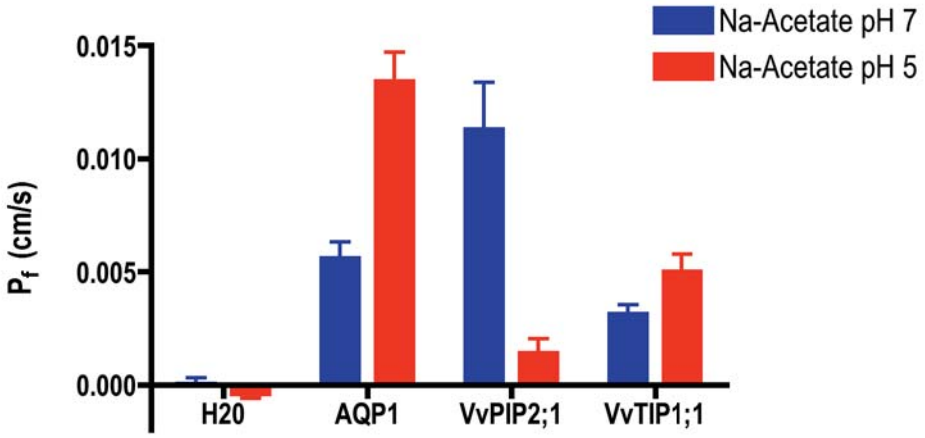


Figure 4-4: Pf values of oocytes expressing grapevine aquaporins and the effect of cytosolic pH. Pf was calculated for water injected, *AQP1, VvPIP2;1, VvTIP1;1* cRNA injected oocytes in response to bathing in a hypotonic solution. Cytosolic pH was lowered using an external solution of Ca-Ringers with the addition of Na-acetate, pH 5. Control solutions contained Ca- Ringers + 50 mM Na-acetate at pH 7. Data represents the mean ± SE (n=5-8).

4.4 Discussion

Eleven full-length AQPs from the grapevine *Vitis vinifera* were identified and nine have been assessed for functionality by expression in *Xenopus laevis* oocytes. All VvPIP2 AQPs tested (with the exception of VvPIP2;5) function as water channels with high water permeability, as has been shown previously for other plant PIP2 aquaporin members (reviewed by Tyerman *et al.*, 2002). Water permeability varied slightly between the different *V. vinifera* PIP2 isoforms with VvPIP2;1 and VvPIP2;4 having the highest Pf values. These two genes are very closely related (99% homologous) with only one very conservative amino acid substitution at position 209 from arginine to lysine. VvPIP2;3 and VvPIP2;2 are between 74% and 82% homologous to the other Vitis vinifera PIP2 aquaporins and have slightly lower Pf values. Both the VvPIP1 AQPs examined showed low or no water transport activity. VvPIP1 AQPs, VvPIP1;2 and VvPIP1;4 are 89% identical to the previously characterised VvPIP1b, and 98% and 99% identical to PIP1a, respectively (Picaud *et al.*, 2003). Both of these PIP1 AQPs had no permeability for water, but PIP1a facilitated glycerol uptake (Picaud *et al.*, 2003). Given their high homology to VvPIP1a and VvPIP1b, it is likely that VvPIP1;4 and VvPIP1;2 may also be permeable to glycerol. The inactivity of VvPIP1 aquaporins when expressed in *Xenopus* oocytes may also be a result of incorrect trafficking and/or insertion into the oocyte membrane. Although, inactive maize PIP1 aquaporins were shown to be trafficked correctly to the plasma membrane (Chaumont *et al.*, 2000), this would need to be confirmed for VvPIP1 members using localisation studies. It has also been postulated that the inactivity of PIP1 aquaporins in oocytes, may be that they require a positive regulator for functionality that is not present in oocytes (Fetter *et al.*, 2004). Using affinity chromatography, Fetter et al. (2004) showed that co-expression of PIP1 and PIP2 aquaporins resulted in both being targeted to the oocyte membrane, forming

heterotetramers. A role *in planta* for the formation of heterotetramers has yet to be demonstrated. Mammalian AQP1 was used as a positive control for the *Vitis* aquaporins. Similar Pf values for AQP1 were observed to those shown previously (Pf ~20 x 10^{-3} cm s^{-1}) (Preston *et al.*, 1992).

The water permeability of VvPIP2;5, was not significantly higher than the control oocytes. VvPIP2;5, has 2 amino acid differences, Ser97 and Trp100, in the highly conserved loop B region, compared with all other PIP and TIP members which have glycine residues at both these positions. An alignment of loop B with VvPIP2;1 and SoPIP2;1 is shown with the two residues highlighted (Figure 4-5).

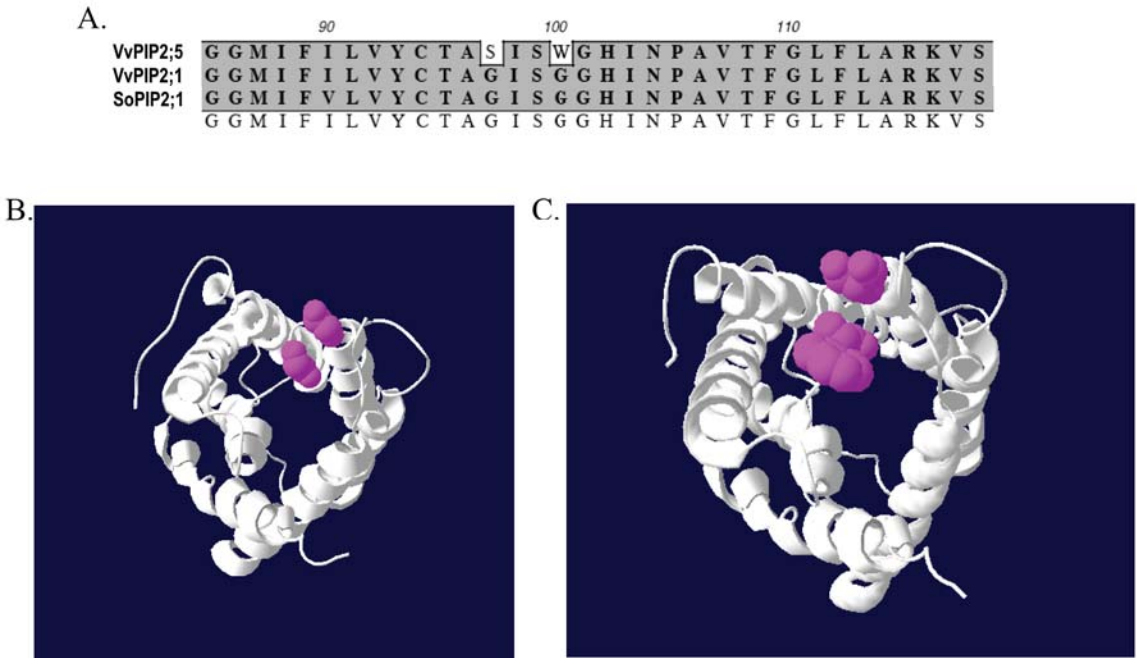


Figure 4-5: A. Sequence alignment of the conserved loop B of VvPIP2;5, VvPIP2;1 and SoPIP2;1. The two different residues in VvPIP2;5, G97S and G100W are highlighted. B. The open conformation of SoPIP2;1 (PDB number 285F) looking down the pore from the extracellular side. Gly97 and Gly100 are shown in pink. C. The same view of the modelled structure of VvPIP2;5 with Ser97 and Trp100 shown in pink.

A homology model of the VvPIP2;5 structure was generated using the open structure of SoPIP2;1 as a template. As shown in Figure 4-5, the two structures are very similar, as expected from the high overall homology between the two proteins (75 % identical). As both serine and tryptophan are larger than the glycine residues they replace, the pore mouth becomes more crowded in VvPIP2;5 and both residues make contact with other parts of the protein that are not present in the SoPIP2 structure. In particular, because Trp100 is large and hydrophobic, it may block the pore. This would explain the very low water permeability observed for VvPIP2;5 when expressed in *Xenopus* oocytes. It is also possible that these substitutions could interfere in correct protein folding and / or trafficking to the plasma membrane. Given that VvPIP2;5 appears to have two deleterious amino acid substitutions it is interesting that the gene is still transcribed in grapevines (as evidenced by obtaining the cDNA from library). This gene is most likely a pseudogene, and thus has been designated as such, *VvPIP2;1pseudo*. Three putative aquaporin pseudogenes (*TIP2;x-pseudo, NIP2;1pseudo* and *NIP3;1pseudo*) have previously been identified in Arabidopsis (Quigley *et al.*, 2002). *TIP2:x-pseudo* encodes a truncated TIP-like protein, whilst both the NIP pseudo genes appear to arise from a gene duplication, as does *VvPIP2;1pseudo* (see Chapter 3, designated as *VvPIP2;5*).

Until recently the molecular basis of aquaporin gating in both plants and mammals remained unknown. However, it is known that plant aquaporins are gated by pH (Tournaire-Roux *et al.*, 2003) and phosphorylation (Johansson *et al.*, 1996; Johansson *et al.*, 1998). Mammalian aquaporins, have been shown to be regulated by external pH, when expressed in *Xenopus* oocytes (Nemeth-Cahalan and Hall, 2000; Nemeth-Cahalan *et al.*, 2004; Hedfalk *et al.*, 2006). The effect of cytosolic pH on gating of human AQP1, VvPIP2;1 and VvTIP1;1 was examined by expression in *Xenopus* oocytes (Figure 4-4). Our results show a two-fold

increase in Pf of AQP1. Human AQP1 is insensitive to external pH (Nemeth-Cahalan *et al.*, 2004) but it is unknown how internal pH affects the water permeability of this AQP. Tournaire-Roux *et al.* (2003) recently showed plant AQPs to be regulated by cytoplasmic pH. Mutation of a conserved His residue (His197) in the intracellular loop D of the Arabidopsis plasma membrane aquaporin, AtPIP2, showed reduced effects in water permeability to a reduction in cytosolic pH. This histidine residue is conserved throughout the PIP family and is found in all *Vitis vinifera* PIP members identified in this study. Reduction of cytosolic pH with Na-acetate pH 5, resulted in an eleven-fold decrease in water permeability of VvPIP2;1 not observed for VvTIP1;1. Recently, a spinach plasma membrane aquaporin, SoPIP2;1 was crystallised to 2.1 Å in its closed conformation and 3.9 Å in its open conformation (Tornroth-Horsefield *et al.*, 2006). The X-ray structure of SoPIP2;1 in the closed confirmation supports the proposed mechanism of pH regulated PIP (Tornroth-Horsefield *et al.*, 2006). Conservation of this histidine residue (His196) in VvPIP2;1, combined with the decrease in Pf observed by acidifying the cytosol, is evidence that His196 is most likely protonated as is the case for SoPIP2;1 and AtPIP2. Due to the inactivity in oocytes of the VvPIP1 members identified, these were not examined for gating by cytosolic pH. Only one Arabiopdsis PIP1 isoform investigated had significant water transport activity in oocytes and this was also blocked by Na-acetate (Tournaire-Roux *et al.*, 2003). Although mammalian AQP1 does not contain a His residue in loop D, there is a stretch of highly charged residues in this region which may respond to the presence of cytosolic protons and result in the increase in Pf measured under these conditions.

In conclusion, I have shown that when expressed in *Xenopus* oocytes, VvPIP2 and VvTIP aquaporins are highly permeable to water. VvPIP1 aquaporins showed no water permeability when expressed in *Xenopus* oocytes. This may be due to incorrect trafficking or

incorrect insertion into the oocyte membrane, or a positive regulator may be required for activity. Alternatively, these may facilitate movement of another small neutral solute such as glycerol as has been shown for other grapevine aquaporins (Picaud *et al.*, 2003).

Chapter 5:
Expression of Aquaporin Transcripts in Response to Water Stress in Chardonnay and Grenache Petioles

5.1 Introduction

Aquaporins are highly regulated at the transcriptional level in response to both biotic and abiotic factors. Abiotic factors include water stress, salt stress, cold stress and light (reviewed by Maurel *et al.*, 2002). Aquaporin, have been documented to be regulated in response to water stress transcriptionally, post-transcriptionally and by post-translational modifications. By studying the expression of aquaporin genes in response to environmental stimuli, more information can be obtained about the role of aquaporins in the water transport pathway in plants. In the hope of elucidating the functional role of aquaporins *in planta*, a number of studies have attempted to correlate AQP expression and localisation *in planta* with physiological data obtained in response to environmental stimuli (Sakr *et al.*, 2003; Cochard *et al.*, 2007; Secchi *et al.*, 2007b). Many studies have examined the response of aquaporin expression to water stress with responses differing markedly between plant species (Tyerman *et al.*, 2002).

Physiological evidence presented here (Chapter 2), and in other studies, suggest that not only does xylem embolism occur over extended periods of water stress in grapevines, but it can also occur over a normal diurnal period when plants are well-watered and transpiration rates are high (Holbrook *et al.*, 2001; Schultz, 2003; Alsina *et al.*, 2007). Water stress-induced xylem embolism, can result in a reduction in the plants' hydraulic capacity, therefore plants must have some mechanism to be able to recover from water stress-induced xylem embolism. Recently, it has been hypothesised that aquaporins located in the surrounding xylem parenchyma cells may contribute to the water movement required for embolism recovery thus restoring the hydraulic pathway (Holbrook and Zwieniecki, 1999; Tyree *et al.*, 1999). In walnut, increased expression of two aquaporin genes, *JrPIP2;1* and *JrPIP2;2* was

found to correlate with winter embolism (Sakr *et al.*, 2003). Localisation of these genes in the surrounding xylem parenchyma cells provided evidence that these aquaporins may modulate water movement into the xylem vessels. The movement of water back into embolised vessels from the surrounding parenchyma cells whilst the cells are under such negative pressures has become quite a contentious debate (reviewed in Clearwater and Goldstein, 2005). The key to gaining an understanding of the role of aquaporins in xylem embolism is to examine the molecular mechanisms in combination with whole plant physiology.

In order to assess if aquaporins have a role in water-stress induced xylem embolism in grapevines, transcript analysis was used to examine their expression pattern in the petiole tissue. Initially, the abundance of mRNA transcript in the shoot tissues of Cabernet Sauvignon was examined using semi-quantitative PCR. This was used as a guide, combined with the functional expression data in *Xenopus* oocytes (Chapter 4), to determine candidate aquaporin genes for further transcript analysis. Functional expression in *Xenopus* oocytes showed high water permeability of the VvPIP2 aquaporins and moderate activity of the VvTIP aquaporins. Of particular interest was VvPIP2;3 that has high sequence homology with the walnut AQPs, JrPIP2;1 and JrPIP2;2, both recently postulated to be involved in embolism refilling (Sakr *et al.*, 2003). Based on these reasons, transcript abundance was determined for the following aquaporin genes, *VvPIP2;1, VvPIP2;3, VvTIP1;1* and *VvTIP2;1* in the petioles of both Chardonnay and Grenache vines, over a diurnal cycle and under conditions of water stress.

5.2 Materials and Methods

5.2.1 Plant Material

V. vinifera cv. Chardonnay I10V1 and Grenache 38 one year old rootlings were obtained from Yalumba Nursery (Barossa Valley, Australia) and grown in 4.7 L pots in UC soil. The growth conditions for *V. vinifera* cv. Cabernet Sauvignon, Chardonnay and Grenache vines are outlined in section 2.2.1. Plants were watered to field capacity every two days. All diurnal experiments were conducted in a temperature controlled growth chamber in the Plant Research Centre (Waite Campus, University of Adelaide). The temperature of the growth room was set to 25 ° C day and 15 ° C night. Day length was 12.5 hours. Lighting was provided by OSRAM Powerarc® 400 W lamps (M59/E). The PAR at pot level was approximately 130 μmol m^{-2} s^{-1}. For semi-quantitative PCR, tissue was harvested from *V. vinifera* cv. Cabernet Sauvignon glasshouse grown plants (as described in Chapter 2) between 10 am and 12 noon and frozen immediately in liquid N. Tissue was sampled from the shoot tips down to the 8th node.

5.2.2 Drought Experiments

Drought experiments on Chardonnay and Grenache were conducted in growth rooms as outlined above. Plants were spur pruned to have two shoots. Plants were watered to field capacity at the beginning of the experiment, at which time psychrometers were attached to the leaves of four plants to allow continuous monitoring of leaf water potential over the whole

drought period. Petioles were sampled from older leaves at the basal portion of the stem (as described in Chapter 2) over a diurnal period at the following times 7 am (predawn), 10 am, 1 pm, 5 pm and 10 pm. Water was withheld from the plants for the next 2 - 3 days, until midday water potentials were predicted to be approximately -1.2 MPa, at which time plants were sampled again over the diurnal period. The same plants were sampled for each time point under well watered (WW) and water stressed conditions (WS). One leaf was taken to measure leaf water potential from each vine at each time point. Leaf water potential was measured using the Scholander pressure bomb. Sampling of the petioles involved cutting the petiole from the stem with a sharp razor blade, parallel to the shoot axis. The leaf was removed and petioles were immediately frozen in liquid nitrogen.

5.2.3 RNA Extractions

RNA was extracted from petiole tissue using a modified sodium perchlorate extraction method as described in Chapter 3. All total RNA samples were treated with DNase 1 (Ambion) following the manufacturers instructions prior to cDNA synthesis. The quantity and purity of total RNA was determined with a Nanodrop Spectrophotometer ND-1000 (Biolab Ltd, Australia). Very low quantities of total RNA were extracted from the petioles with amounts varying from 20 – 50 µg / g fresh weight. Total RNA was of a high quality with 260 / 280 ratios in the range of 1.8 – 2.0. A major problem linked to studying gene expression in xylem tissues is the low transcript abundance of mRNA (Sakr *et al.*, 2003). The petioles that were used in this study had very low quantities of total RNA, making it very difficult to obtain high quantities of RNA for QPCR.

5.2.4 cDNA Synthesis

First-strand cDNA synthesis from normalised total RNA was synthesised using the iScript(TM) cDNA Synthesis Kit according to the manufacturers' instructions (Biorad, CA, USA). The kit uses a combination of oligo (dT) and random primers with RNase H^+ iScript reverse transcriptase (Biorad). The cDNA synthesis reaction contained 1 x iScript Buffer, iscript reverse transcriptase and 1 μg of total RNA. Cycling conditions were as follows: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, cool to 4 °C.

5.2.5 Design and Optimisation of Primers for Quantitative Real-Time PCR

Due to the high homology at the nucleotide level between the grapevine aquaporins identified in the library screen, primers were designed by careful sequence analysis and the use of Primer3 (Rozen and Skaletsky, 2000) to regions of the sequence where nucleotide differences were observed. Primers were designed to amplify amplicons between 100 - 230 bp in length, with melting temperatures between 58 and 65 °C and GC content not higher than 55 % (Table 5.1). Primers designed to highly homologous genes were designed with different amplicon size to allow easier identification of the correct amplicon. Primer combinations were checked for gene specificity by performing PCR on 100 pg of purified plasmid DNA for each cDNA, with primer combinations of closely related genes. HiFi Taq DNA polymerase (Invitrogen, Mt Waverly, Vic) was used and annealing temperatures were increased until only one product was obtained for each primer pair.

5.2.6 Semi-Quantitative Polymerase Chain Reaction

Semi-quantitative PCR was performed on cDNA synthesised from total RNA, isolated from *V. vinifera* cv. Cabernet Sauvignon leaf, petiole, tendril and stem tissues (Section 3.2.6 and 5.2.4). Primer pairs designed for QRT-PCR were used for semi-quantitative PCR (Table 5.1). Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Mt Waverly, Vic) was used in 20 μl reactions containing the following: 1x High Fidelity PCR Buffer, 0.2 mM each dNTP (dATP, dCTP, dGTP dTTP), 0.2 mM of each primer, 1.5 mM $MgSO_4$, template cDNA equivalent to 5 ng of total RNA and 1 unit of Platinum Taq DNA Polymerase High Fidelity. Cycling conditions were as follows: denaturing at 94 °C for 2 min, 94 °C for 30 s, 61 °C for 30 s, 68 °C for 1 min for 35 cycles, extension at 68 °C for 7 min. PCR products were run on a 2.5% (w/v) agarose gel / 1 X TAE. To ensure saturation had not occurred, samples were taken at 15, 25 and 35 cycles and compared by DNA gel electrophoresis. PCR products were sequenced to confirm correct products.

5.2.7 Quantitative Real-Time Reverse Transciptase Polymerase Chain Reaction

Primer pairs were used to amplify PCR products for each gene using Platinum Taq DNA Polymerase High Fidelity as outlined above (Invitrogen, Mt Waverley, Australia). Petiole cDNA synthesised from the equivalent of 5 ng of total RNA from Cabernet Sauvignon was used as the template. PCR products were run on a 2.5% w/v agarose gel / 1 xTAE, gel purified and sequenced to confirm correct product and that only one amplicon was being synthesised. Gel eluted products were used as standards for real time PCR reactions. 10-

fold serial dilutions were made for each standard with the addition of acetylated BSA (Promega, Annandale, Australia) at a final concentration of 1 μg ml^{-1}.

A 1x mix of IQ™ SYBR® Green Supermix (Biorad, Hercules, CA, USA) was used for all real-time PCR reactions. The reaction mix contained 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), iTaq DNA polymerase (50 units ml^{-1}), 3 mM $MgCl_2$, SYBR® green I, 20 nM fluoresein and stabilizers, 200 nM of each primer and an amount of cDNA template equivalent to 15 ng of total RNA. 20 μl reactions were used and each reaction was performed in triplicate. Thermocycling was conducted in an iCycler IQ (Biorad, Hercules, CA) by denaturation at 95 °C for 2 min, 45 cycles consisting of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s and extension 72 °C for 15 s. Prior to melt curve analysis a final denaturation step was performed at 95 °C for 30 s. The specificity of amplification was determined with melt curve analysis. The melt curve analysis was performed between 57 °C and 97 °C at 0.5 °C increments for 30 s. Amplification efficiencies varied between 95 - 100% (Table 5-3).

Table 5-1: Forward and reverse primers for quantitative real-time PCR of aquaporin genes. Primers were designed to amplify fragments between 100 - 230 bp in length using Primer3 (Rozen and Skaletsky, 2000) with Tm between 58 -65 °C.

Primer name	**Primers 5'-3'**	**Tm (°C)**	**Amplicon Size (bp)**
VvPIP1;2	Forward 5'-CGCCATCGTCTACAACAAAG-3' Reverse 5'-CAGGCTCTGGTCTTGAATGG-3'	65 64	228
VvPIP1;4	Forward 5'-GCCGCCATCATCTACAACAG-3' Reverse 5'-GCTCAACCCAGATTCAGGAG-3'	63 64.7	135
VvPIP2;1	Forward 5'-CAGGAGCACCACTCATGTATG-3' Reverse 5'-TCATGCCCTCATACATATCAATAAC-3'	63 62	152
VvPIP2;2	Forward 5'- AAAGTTTGGGACGACCAGTG Reverse 5'-TTTTTAGTTGGTGGGGTTGC	64 63.5	141
VvPIP2;3	Forward 5'- GCCATTGCAGCATTCTATCA-3' Reverse 5'-TCCTACAGGGCCACAAATTC-3'	58 60	199
VvPIP2;5 (VvPIP2;1pseudo)	Forward 5'- GCACTGCCAGCATTTCTTG Reverse 5'- GCAGATTGGAAGGCTTTGAC	65 64	157
VvTIP1;1	Forward 5'-CATTGCCGCCATCATCTAC-3' Reverse 5'-AGAAATCTCAACCCCACCAG-3'	64 63	156
VvTIP2;1	Forward 5'-GGAGGAAGAGCAAGTTGTGC-3' Reverse 5'-GCACATCACCAACCTCATTC-3'	60 59	157
VveEF1γ (AF176496)	Forward 5'-CGGGCAAGAGATACCTCAAT-3' Reverse 5'-AGAGCCTCTCCCTCAAAAGG-3'	60 62	147
VvUbq (TC38636)	Forward 5'-GTGCTGTCAACTGCAGGAAA-3' Reverse 5'-GTAGCCATGGCACATCCAAT-3'	60 60	103

5.2.8 Calculation of Changes in Transcript Abundance

Changes in transcript abundance were calculated using a relative quantitation method normalised against a reference (housekeeping) gene. The method chosen was the $2^{-\Delta\Delta CT}$ (Livak) method (Livak and Schmittgen, 2001). This method assumes the amplication efficiencies are between 95-105% for all genes Table 5-3. The $2^{-\Delta\Delta CT}$ gives the normalised expression ratio and this is computed by the following equations:

$$\Delta CT_{WS} = C_{T(AQP, WS)} - C_{T (ELF, WS)} \qquad \text{Equation 5-1}$$

$$\Delta CT_{WW} = C_{T(AQP, WW)} - C_{T (ELF, WW)} \qquad \text{Equation 5-2}$$

$$\Delta\Delta CT = \Delta CT_{WS} - \Delta CT_{WW} \qquad \text{Equation 5-3}$$

$$2^{-\Delta\Delta CT} = \text{normalised expression ratio} \qquad \text{Equation 5-4}$$

This method gives the ratio of aquaporin gene expression under conditions of water stress (WS) relative to well-watered (WW) conditions, normalised to the reference gene (*VveELF*). The fold change in transcript abundance is expressed relative to the basal condition, whereby a fold change of one represents no change in gene expression. To determine the fold change in diurnal expression, data are expressed relative to the 7 am time point. For the water stress data, expression is calculated relative to well-watered for each time point. $2^{-\Delta\Delta CT}$ was determined for each biological representative. Data are presented as the mean fold change in transcript abundance of the three biological replicates ± SEM. In some cases, transcript levels were undetectable by QRT-PCR indicating very low – nil transcript abundance under these condtions. In this case, no CT value could be determined and $2^{-\Delta\Delta CT}$ could not be computed. Where this occurred, only 2 biological reps are shown. To

determine if the effect of the treatment (diurnal or water stress) on transcript abundance was statistically significant, a one sample t-test to compare the mean against a hypothetical value of 1 was computed. A two-way ANOVA was performed to determine statistical differences between the two varieties (variety x time). Statistical analysis was performed with GraphPad Prism® (GraphPad Software Inc, San Diego,CA).

5.3 Results

5.3.1 Optimisation of Quantitative Real-Time RT-PCR Primers

Due to the high nucleotide homology between the aquaporin isoforms identified in chapter 3, it was necessary to firstly test primers for their specificity to amplify only one amplicon. This was achieved by conducting PCR on all constructs (pDNA) using gradient PCR. Primers designed for *VvPIP2;1, VvPIP2;2, VvPIP2;3, VvTIP1;1* and *VvTIP2;1* were successfully able to amplify the correct PCR product at an annealing temperature of 61 °C. Primers designed for *VvPIP1;2* and *VvPIP1;4* were able to amplify both amplicons even at high annealing temperatures. Primers for *VvPIP2;5* were able to amplify both *VvPIP2;4* and *VvPIP2;1.* Based on this result, quantitative real-time PCR was only conducted on the following genes, *VvPIP2;1, VvPIP2;3, VvTIP1;1* and *VvTIP2;1,* Primers for these genes were only able to amplify the gene of interest.

5.3.2 Optimisation of Housekeeping Genes

A reference gene or housekeeping gene is required to allow determination of the relative expression level of the target gene (normalised gene expression). Two housekeeping genes were selected based on previous studies, *VveELF* and *VvUBQ.* It is important to validate the housekeeping gene to ensure that expression is not influenced by the experimental condition. The transcript abundance of both *VveELF* and *VvUBQ,* were determined in the shoot tissues of *V. vinifera* Cabernet Sauvignon using semi-quantitative PCR. Figure 5-2 shows the transcript abundance of these genes in the shoot tissues. Both *VveELF* and *VvUbq* show constitutive expression in the tissues examined. It was also necessary to confirm that treatment did not affect the expression levels of the housekeeping gene. Figure 5-1 shows the expression of *VveELF* in response to time and in response to treatment. No significant differences were observed in CT values for *VveELF* over a diurnal cycle or under conditions of water stress.

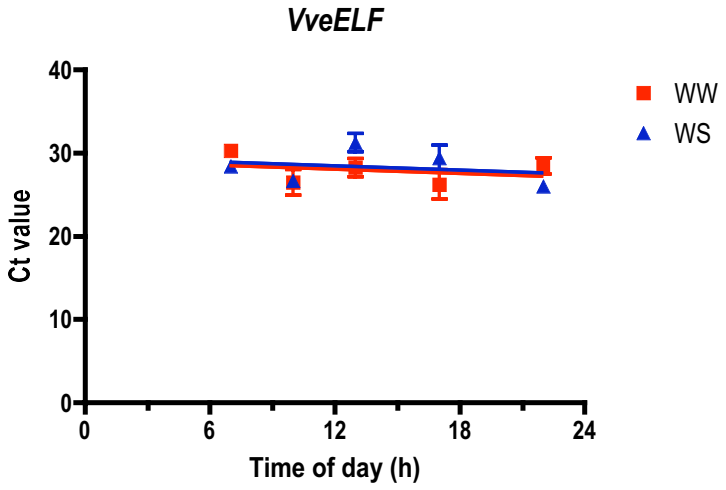


Figure 5-1: Validation of housekeeping genes under experimental conditions. Shown is the mean CT values ± SEM (n=9) for *VveELF* over time and under WW and WS conditions.

5.3.3 Tissue specific Expression in Cabernet Sauvignon by Semi-Quantitative PCR

To identify possible candidate aquaporin genes that may play a role in cellular water balance in the petioles of grapevines, semi-quantitative PCR was used to examine tissue specific expression. Using semi-quantitative PCR, large differences in the transcript abundance of the different aquaporin genes were observed in the shoot tissues of Cabernet Sauvignon (Figure 5-2). Transcript abundance of aquaporins was found to be very low, with transcript virtually undetectable after 15 and 25 PCR cycles (results not shown), hence 35 PCR cycles was used. Under these conditions very little difference is seen between tissues for each aquaporin gene, although slightly higher transcript abundance is apparent for *VvPIP2;3* in the petiole tissue compared to the other shoot tissues. Expression of *VvPIP1;4* is higher than all other genes examined. Due to the very high nucleotide homology between *VvPIP1;2* and *VvPIP1;4* (99 % homologous) and the results obtained in the optimisation of the primers, it is likely that if both mRNAs were present in the tissue, both of these cDNAs would be amplified. Both *VvTIP1;1* and *VvTIP1;2* also have relatively high expression compared to the PIP2 genes. Based on these results, further transcript analysis was conducted on the PIP2 and the TIP isoforms. As the PIP1 aquaporins were impermeable to water when expressed in *Xenopus* oocytes (Chapter 4) and it was possible that both transcripts were being amplified, no further transcript analysis was conducted on these genes.

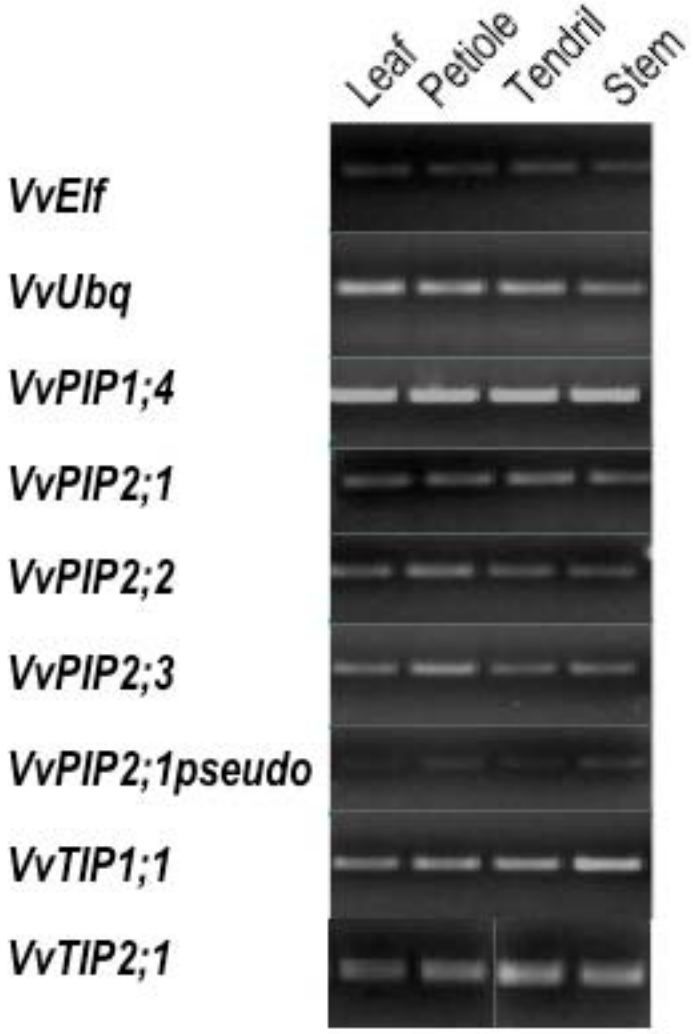


Figure 5-2: Expression of aquaporin genes in the shoot tissues of *V. vinifera* cv. Cabernet Sauvignon. Semi-quantitative PCR was used to amplify the following aquaporin genes *VvPIP1;4, VvPIP2;1, VvPIP2;2, VvPIP2;3, VvPIP2;1pseudo (VvPIP2;5), VvTIP1;1, VvTIP2;1, VveEF1* and *VvUBQ* from stem, petiole, tendril and leaf cDNA. Bands show detection of transcripts after 35 PCR cycles.

5.3.4 Measurement of Leaf Water Potential

The leaf water potential was measured at all time points to give an indication of the level of water stress at the time of sampling (Figure 5-3). The midday leaf water potential for the well-watered plants was -0.75 and -0.78 MPa, and for moderate water stress after 2-3 days of drying, -1.12 MPa and -1.18 MPa for Chardonnay and Grenache, respectively. In Chardonnay, the leaf water potential under water stress conditions was significantly different compared with well-watered conditions at 1, 5 and 10 pm ($p<0.05$). The ψ_L was not significantly different under moderate stress at 7 am and 10 am compared with well-watered vines. A difference in the diurnal patterns is evident between Chardonnay and Grenache. Although similar midday water potential values were attained, the predawn and 10 am ψ_L value in the water stress vines was significantly different between the two varieties ($p<0.05$). This suggests that the Grenache vines were more water stressed than the Chardonnay vines. Leaf water potential was at a minimum at 10 am in Grenache, some recovery was seen in the middle of the day and then decreases were observed in the afternoon. The recovery in the middle of the day is most likely due to stomatal closure as has been reported previously for this variety (Schultz, 2003; Soar *et al.*, 2006)). Grenache does not recover as well as Chardonnay in the dark. All time points except for 7 am were significantly more water stressed compared with well-watered vines for Grenache. The minimum ψ_L in Grenache vines occurred at 10 am (-1.6 MPa) and in Chardonnay at 1 pm (-1.12 MPa). Large variations in ψ_L were observed in Grenache vines at 5 pm and 10 pm, possibly due to differences in stomatal closure between biological representatives.

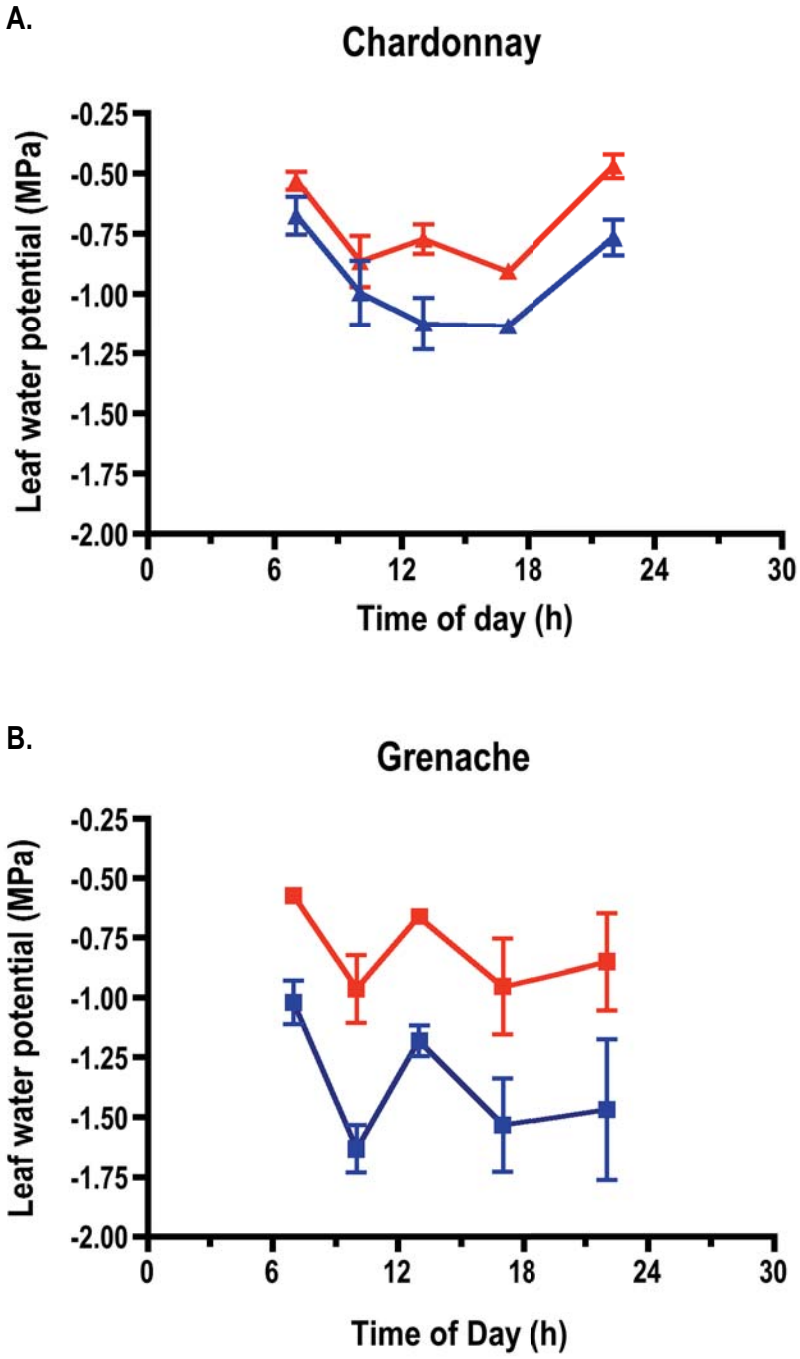


Figure 5-3: Mean Diurnal Leaf Water Potential of A. Chardonnay and B. Grenache vines. Shown is the mean leaf water potential ± SEM (n=3) measured at the time of collecting petioles samples for RNA extractions, under well watered (WW) and water stressed (WS) conditions.

Table 5-2: Predawn and midday leaf water potential values for Chardonnay and Grenache vines under well-watered and water stressed conditions. Values shown are the mean ± SEM (n=3). Superscript letters indicate significant differences between values ($p<0.05$).

Variety	WW ψ_L (MPa)		WS ψ_L (MPa)	
	Predawn ψ_L	Midday ψ_L	Predawn ψ_L	Midday ψ_L
Chardonnay	-0.527 ± 0.037 [a]	-0.77 ± 0.061 [a]	-0.673 ± 0.079 [a]	-1.123 ± 0.107 [b]
Grenache	-0.573 ± 0.007 [a]	-0.66 ± 0.012 [a]	-1.02 ± 0.092 [b]	-1.18 ± 0.064 [b]

5.3.5 Quantitative Real-Time RT-PCR

Quantitative Real Time Reverse-Transcriptase PCR was used to determine the transcript abundance *of VvPIP2;1, VvPIP2;3, VvTIP1;1* and *VvTIP2;1* in the petioles of Chardonnay and Grenache vines. Standard curves were generated for each gene using 10-fold serial dilutions and gene specific primers (Table 5-1). An example of a typical standard curve is shown in Figure 5-4A. The correlation coefficient and the PCR (amplification) efficiency was determined for each set of primers. The threshold cycle (CT) values were obtained from the amplification curve (Figure 5-4B). A melt curve was generated for each reaction and checked to ensure only one product was amplified for each primer set. An example of the melt curve analysis for *VveELF* is shown in Figure 5-4C.

Table 5-3: A typical example of PCR amplification efficiencies and correlation coefficients obtained for each primer set.

Gene	Amplification Efficiency (%)	Correlation Coefficient
VveELF	98.2	0.994
VvPIP2;1	97.9	0.995
VvPIP2;3	94.4	0.995
VvTIP1;1	95.5	0.996
VvTIP2;1	96.9	0.996

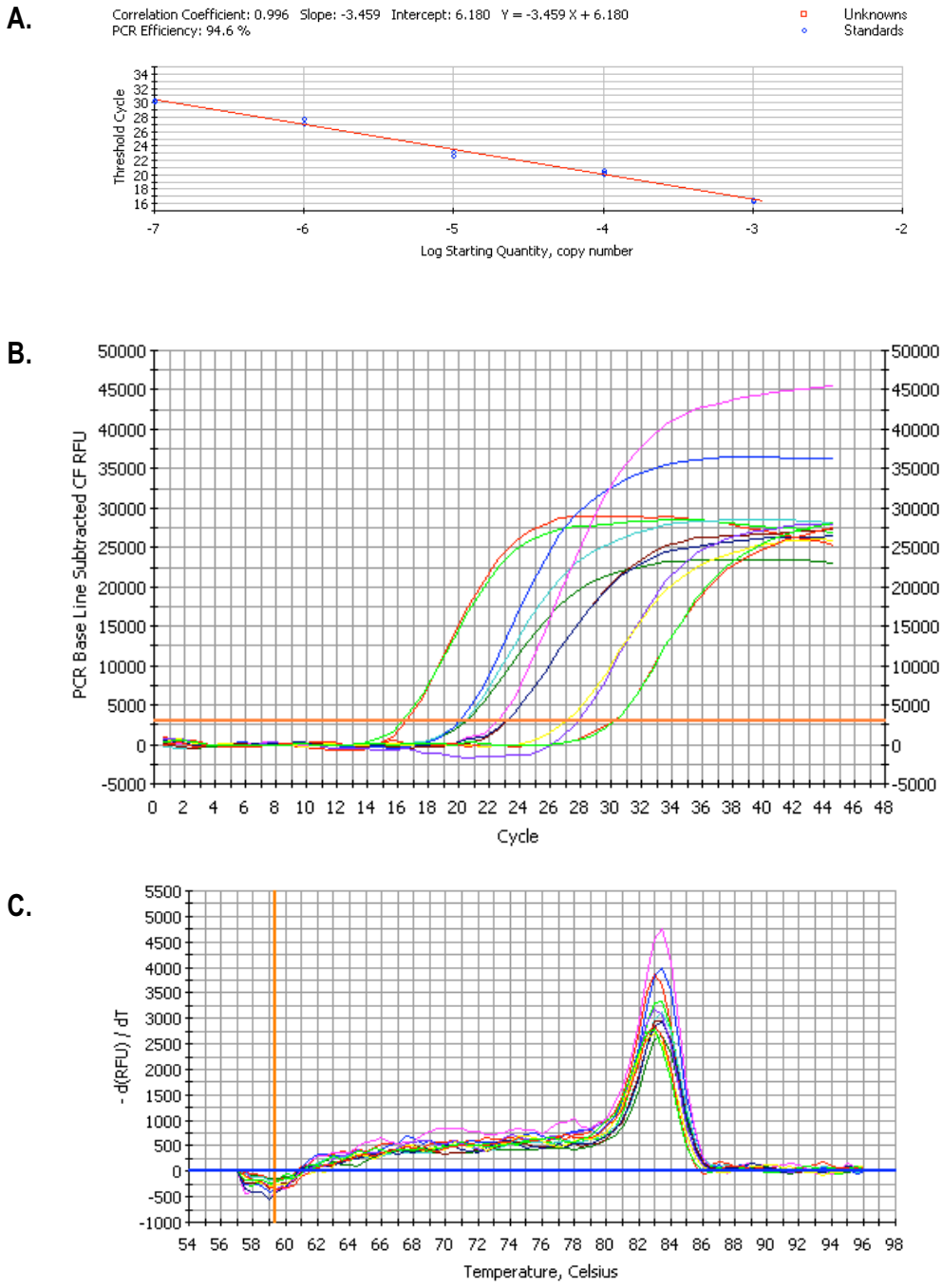


Figure 5-4: Typical data generated for real-time PCR using SYBR-490 and the iCycler. (A). Standard curve generated from the *VveELF* internal standard using 10-fold serial dilutions with gene specific primers (r = 0.996, PCR efficiency = 94.6%). (B). Plot of fluorescence versus cycle number using *VveELF* primers with the *VveELF* standards. (C). Melt curve data for the *VveELF* standards.

5.3.6 Diurnal Regulation of Aquaporin Transcript Abundance

In order to assess whether aquaporin expression was responding to a diurnal rhythm, petioles were harvested from well-watered Chardonnay and Grenache vines at 7 am, 10 am, 1 pm, 5 pm and 10 pm. As can be seen in Figure 5-5, different aquaporin genes have different diurnal responses. In Chardonnay petioles all the genes examined have increased expression during the day with both *VvPIP2;1* and *VvPIP2;3* having maximum expression at 1 pm. Expression of *VvPIP2;3* at 1 pm was significantly higher (approximately 100 fold) in Chardonnay vines. *VvTIP1;1* has increased expression at both 1 pm and in the late afternoon at 5 pm. *VvTIP2;1* transcript abundance did not significantly change over the diurnal period.

In Grenache petioles, a very different diurnal regulation of aquaporin expression was observed. Both the PIP2 isoforms were down regulated during the day. *VvPIP2;3* transcript decreased during the day and then subsequently increased in the evening to levels similar to those observed predawn. *VvPIP2;3* expression is significantly higher (~ 100 fold) in Chardonnay petioles compared to Grenache petioles. *VvPIP2;1* continued to decrease during the afternoon and evening. *VvTIP2;1* expression varied less than 10 fold throughout the day with peaks at 10 am and 5 pm when leaf water potential was lowest. Expression of *VvTIP2;1* was significantly higher at 10 am than in Chardonnay petioles. *VvTIP1;1* expression remained fairly constant throughout the day and then decreased rapidly during the evening.

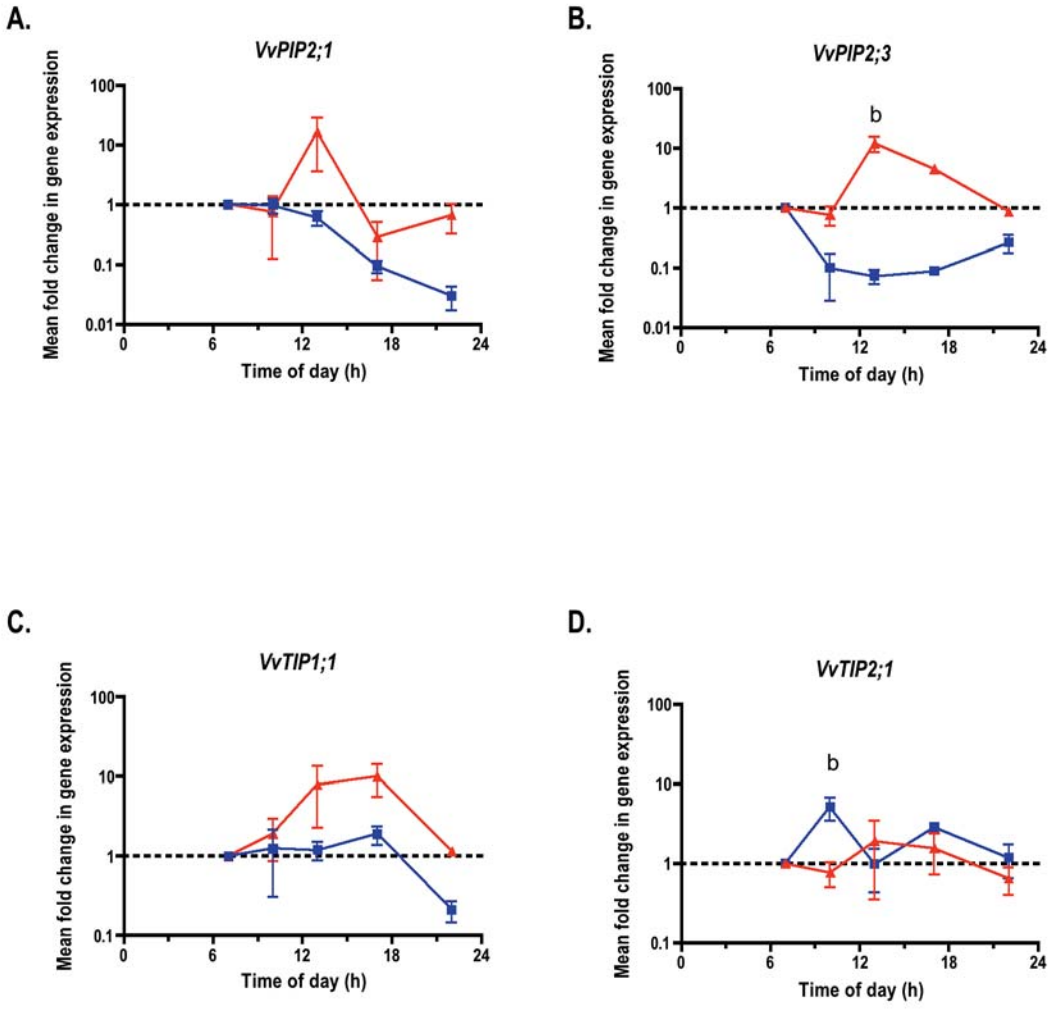


Figure 5-5: Diurnal regulation of aquaporin transcript abundance in the petioles of well-watered Chardonnay (red triangles) and Grenache (blue squares) vines. Petioles were harvested over a diurnal period from well-watered vines. Quantitative real time PCR was used to determine the mean fold change in transcript abundance of aquaporins over the diurnal period relative to the 7 am time point. Shown is the mean relative abundance ± SEM (n=3) of the following genes A. *VvPIP2;1*. B. *VvPIP2;3* C. *VvTIP1;1* and D. *VvTIP2;1*. Note the y-axis is shown as a log10 scale. The letter "b" indicates a significant difference between varieties ($p < 0.05$).

5.3.7 Transcriptional Regulation of Aquaporins in Response to Water Stress

In order to determine the transcriptional response of aquaporins to water stress in the petioles of grapevine, petioles were harvested under moderate water stress conditions as determined in Chapter 2 (ψ_{midday} ~ -1.25 MPa), total RNA extracted and quantitative real-time PCR performed. Two PIP2 isoforms and two TIP isoforms were examined (Figure 5-6). In Grenache, *VvPIP2;1* was significantly down regulated in response to water stress at 1 pm and 5 pm compared to well-watered vines (p=0.0089). *VvPIP2;3* was significantly decreased at 7 am but no significant change was observed over the remaining diurnal period. The TIPs also showed a general down regulation under water stress. In this variety, *VvTIP2;1* was significantly down regulated in response to water stress at 7 am (p=0.0125) and 5 pm (p=0.0142). No change however was observed at 10 am and 10 pm. *VvTIP1;1* appeared to be down regulated in the morning although such changes were not significantly different to well-watered plants and no changes were observed in the afternoon or evening.

In Chardonnay the responses to water stress were more variable over the diurnal period. The transcript abundance of both *VvPIP2;3* and *VvTIP2;1* were not influenced by the moderate water stress imposed with no significant changes found over the diurnal period. A down-regulation is seen at 10 pm for *VvTIP2;1*, but due to very low transcript abundance of this gene, data was obtained for only one biological representative. It is therefore difficult to evaluate the significance of this time point. *VvPIP2;1* was significantly down regulated in response to water stress at 1 pm (p=0.0102) and then subsequently upregulated later in the afternoon at 5 pm. At 10 pm no fold change in expession was observed. A similar pattern is observed for *VvTIP1;1* with significant down regulation at 10 am (p=0.0001) followed by upregulation at 5 pm before decreasing at 10 pm to levels of well-watered vines.

Significantly higher expression of *VvPIP2;1* and *VvTIP1;1* is observed under water stress in Chardonnay compared to Grenache vines.

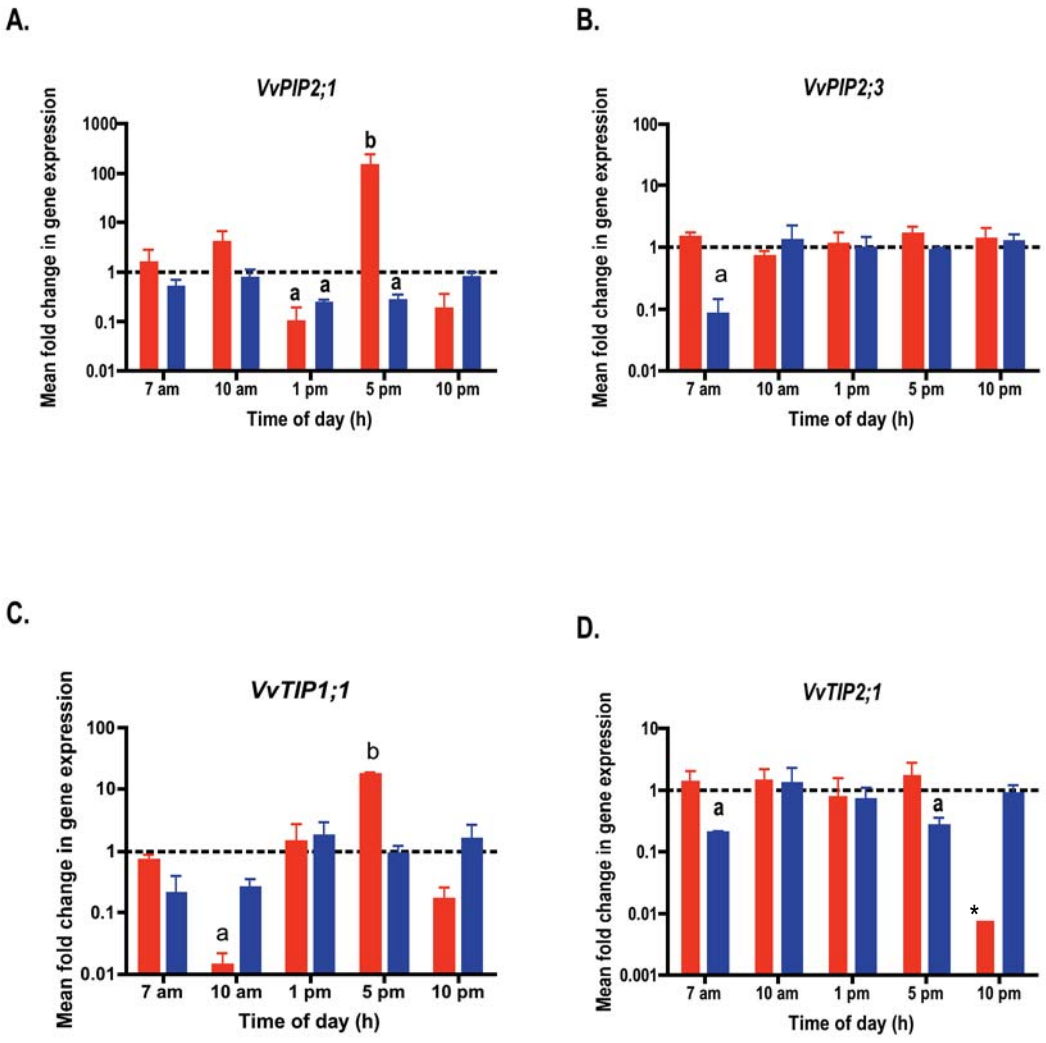


Figure 5-6: Transcriptional response of Chardonnay (red bars) and Grenache (blue bars) petioles to a moderate water stress. Petioles were harvested over a diurnal period form well watered vines and then again after a drying period of 2-3 days. Real-time PCR was used to determine the mean fold change in transcript abundance of aquaporins in the petioles of water stress vines relative to well-watered vines (set at 1) for each time point. Shown is the mean relative abundance ± SEM (n=2-3) for the following genes A. *VvPIP2;1*. B. *VvPIP2;3* C. *VvTIP1;1* and D. *VvTIP2;1*. Note the y-axis is a log10 scale. The letter "a" denotes a significant change in gene expression ($p<0.05$) as determined by a one sample t-test relative to no change in gene expression (as indicated by the dotted black line). The letter "b" indicates a significant difference between the varieties ($p<0.05$). The * indicates only one biological representative.

5.4 Discussion

The high diversity of aquaporins found *in planta* can partly be attributed to their presence in different tissues and subcellular compartments (Maurel *et al.*, 2002). In this study, 13 MIPs have been identified from *V. vinifera* indicating that grapevines also have a high diversity of aquaporins as has been shown for both maize and Arabidopsis (Chaumont *et al.*, 2001; Johanson *et al.*, 2001). To obtain information about the possible physiological role of aquaporins in the cell-to-cell pathway of water movement in the petioles of grapevine, a combination of both semi-quantitative PCR and quantitative real-time PCR were employed to analyse transcript abundance. The expression profile of aquaporins was found to differ between the two grapevine varieties, Chardonnay and Grenache, in response to both water stress and over a diurnal cycle. The data reveal that there were two apparent responses in the transcriptional regulation of aquaporins in the petioles of grapevine. In the first instance, a diurnal response was observed under favourable conditions when the vines were well-watered. A separate mode of transcriptional regulation was apparent in response to water stress. Both these responses differed in the genes targeted and between varieties, indicating that the transcriptional regulation of aquaporins in grapevines is quite complex and is unique between varieties. A recent study by Galmés *et al.* (Galmes *et al.*, 2007) reported differing transcriptional response of aquaporins dependent on both water stress and organ in the drought tolerant rootstock *Vitis* Richter 110.

5.4.1 Tissue Specific Expression of Grapevine Aquaporins

The large number of aquaporin isoforms present in plants has often been associated with different expression profiles throughout the plant tissues (Maurel *et al.*, 2002). Many aquaporin isoforms have been shown to have tissue specific expression in different plants including grapevine. Baiges *et al.* (2001) reported higher expression of both PIPs and the TIPs in grapevine roots of Richter-110, compared with the shoot tissues. More recently, Galmés *et al.* (2007) reported *PIP1;1* to have higher expression in the roots of grapevine, whilst five other PIP isoforms had higher expression in the leaves. The aquaporin genes examined in this thesis by semi-quantitative PCR in Cabernet Sauvignon shoot tissues did not exhibit organ specificity, with all genes expressed in all the shoot tissues examined (tendrils, stem, leaf and petiole). Isoform *VvPIP2;3* was found to have slightly higher expression in the petioles than the other shoot tissues. *VvPIP2;3*, shares high homology with the walnut aquaporins *JrPIP2;1* and *JrPIP2;2,* both postulated to be involved in embolism refilling and expressed in the xylem parenchyma cells of walnut twigs (Sakr *et al.* 2003). Expression levels in the shoot tissues were highest for the PIP1 isoform, *VvPIP1;4,* that had constitutive expression in all aerial tissues. Due to the high homology of *VvPIP1;4* with *VvPIP1;2* (99% homologous at nucleotide level), it is likely that if both of these isoforms are expressed in the shoot tissues, both these genes would be amplified using these primers. *VvPIP1;4* has very high homology with *VvPIP1a (*99%), previously identified in the grape berries of *V. vinifera* cv. Pinot Noir (Picaud *et al.*, 2003). Expression of *VvPIP1a* was found to vary over the development of the grape berry, with expression highest following veraison (Picaud *et al.*, 2003). High expression of the VvPIP1 isoforms could imply increased water permeability through cellular membranes *in planta*. It is interesting to note that although PIP1 genes were highly expressed in the shoot tissues, when these were

expressed in *Xenopus* oocytes no water permeability was observed (Chapter 4). This was also the case for VvPIP1a, which showed only a small increase in water permeability but had a large increase in glycerol permeability (Picaud *et al.*, 2003). Therefore, it is possible that the VvPIP1 proteins may play a role in the movement of a small neutral solute other than water, or that post-translational modification may be required *in planta* for the formation of a functional protein. PIP1s have been shown to be abundantly expressed in other plants, with expression in the roots often higher than the leaves (Weig *et al.*, 1997; Lopez *et al.*, 2003; Galmes *et al.*, 2007). Expression of *VvTIP1;1* was highest in the stem, whilst *VvTIP2;1* was constitutively expressed in all shoot tissues. The internal controls, *VveELf* and *VvUbq* were both constitutively expressed in all the shoot tissues examined.

5.4.2 Diurnal Regulation of Aquaporin Gene Expression

In Chardonnay vines, a diurnal pattern was apparent, with all the aquaporins examined having increased expression levels in the petioles during the day, peaking around midday and then decreasing in the late afternoon / evening. Transcriptional regulation of aquaporins in response to both a diurnal rhythm and circadian rhythm have been previously reported in maize roots (Lopez *et al.*, 2003) and in the leaves of *Nicotiana excelsior* (Yamada *et al.*, 1997). A circadian regulation of aquaporins was also apparent in the leaves of *Samanea saman* (Moshelion *et al.*, 2002). In all these studies, aquaporin expression levels were higher during the day than at night, similar to what was observed in Chardonnay petioles. The increased expression levels in the middle of the day in Chardonnay petioles, indicates aquaporins may play a role in modulating water flow through the petiole via a trans-cellular pathway. In tobacco petioles, high expression of *NtAQP1* in the cells surrounding the xylem vessels, was attributed to a trans-cellular pathway for water movement through the petiole

(Otto and Kaldenhoff, 2000). Increased expression may also be attributed to refilling of cavitated vessels as has been postulated previously for grapevine (Holbrook *et al.*, 2001). Interestingly, in Grenache, a completely different diurnal pattern was observed. Both VvPIP2 aquaporins were down-regulated during the day time and *VvTIP1;1* expression had little change throughout the day but decreased in the evening. *VvTIP2;1* fluctuated throughout the day with expression highest in the morning and late in the afternoon. If increased aquaporin expression in the petioles of grapevine is required for the refilling of embolised vessels, the transcriptional data is consistent with Chardonnay potentially having the capacity to refill during the day under well-watered conditions. The down-regulation of aquaporins in Grenache during the day, combined with the physiological parameters of stomatal closure is consistent with this variety not being able to refill and instead employing a molecular and physiological strategy to conserve cellular water. In *V. lumbrusca* cv. Concord, embolised vessels formed in the grapevine stem while the plant was actively transpiring and under considerable water stress (ψ_L = -1.5 to -2.1 MPa) (Holbrook *et al.*, 2001). Refilling of embolised vessels in the stem occurred but only when combined with an increase in the leaf water potential and a cessation of sap flow. Given the physiological and molecular differences observed between Chardonnay and Grenache it is probably unwise to assume the same refilling mechanisms exist in all grapevine varieties.

5.4.3 Transcriptional Regulation of Aquaporins in Response to Water Stress

The differences observed in AQP transcript abundance between Chardonnay and Grenache is most likely a reflection of the differences in the physiological properties of these two varieties under water stress. Changes in aquaporin gene expression in plants have been reported previously in response to abiotic stresses including drought (Yamaguchi-Shinozaki

et al., 1992; Yamada *et al.*, 1995; Mariaux *et al.*, 1998; Barrieu *et al.*, 1999; Sarda *et al.*, 1999; Secchi *et al.*, 2007b), salt stress (Kirch *et al.*, 2000; Suga *et al.*, 2002), light (Cochard *et al.*, 2007), cold stress (Li *et al.*, 2000), and diurnal fluctuations (Lopez *et al.*, 2003). The response of aquaporins to water stress appears to be species dependent and isoform dependent. Upregulation of MIPs in response to water stress has been shown in the leaves of *Nicotiana excelsior* (Yamada *et al.*, 1997) and down regulation of PIPs and TIPs has been shown in sunflower, *Nicotiana glauca,* and olive (Sarda *et al.*, 1999; Smart *et al.*, 2001; Secchi *et al.*, 2007b). A number of studies have shown different isoforms in the same species are up or down-regulated in response to drought stress (Alexandersson *et al.*, 2005; Aroca *et al.*, 2006). Downregulation of transcript would presumably result in a decrease in the amount of protein, which in turn would decrease the cell membrane permeability to water. In this study, aquaporins are shown to be upregulated, downregulated, or unchanged in response to water stress in the petioles of grapevine. In general, most aquaporin genes were unchanged or downregulated in response to water stress. *VvPIP2;3* remained fairly constant in response to water stress except for a downregulation in Grenache at predawn. This limited response to water stress, is interesting considering this gene showed diurnal regulation in both varieties. *VvPIP2;1* and *VvTIP2;1* was significantly down-regulated during the afternoon in Grenache. The down-regulation of aquaporins in Grenache petioles may therefore act as a mechanism to conserve water in the cells. Down-regulation in the afternoon could possibly contribute to the reduction in hydraulic conductivity measured in the late afternoon in Grenache petioles. In Chardonnay vines, the down-regulation of aquaporins was observed in all genes but at different times of the day, with the exception of *VvPIP2;3* where expression remained unchanged. In Chardonnay, two genes, *VvPIP2;1* and *VvTIP1;1,* were strongly upregulated late in the afternoon. This upregulation of aquaporin expression in response to water stress could be triggered by cavitation events or changes in

the leaf water potential. An increase in expression of aquaporin genes may facilitate cellular water movement and this may indicate a potential role for aquaporins in refilling of embolised vessels in the petioles. This would help to restore the hydraulic pathway under conditions of moderate water stress. Several studies have provided hydraulic evidence for the refilling of xylem vessels in the petioles, while under tension (Zwieniecki *et al.*, 2000; Bucci *et al.*, 2003). Upregulation of AQPs in response to water stress was not observed in Grenache petioles. Although expression data is shown for the whole petiole, much of the petiole is comprised of xylem vessels and xylem parenchyma cells. The xylem vessels and xylem parenchyma cells have an intricate structure-function relationship to modulate the movement of water and solutes between them. High expression levels of both PIP and TIP aquaporins in the vascular tissues have been reported in a number of plant species (Kaldenhoff *et al.*, 1995; Yamada *et al.*, 1995; Barrieu *et al.*, 1998; Otto and Kaldenhoff, 2000). *ZmTIP1* expression was localised to the xylem parenchyma cells in the roots and stem of maize (Barrieu *et al.*, 1998) and *MIP-B* was highly expressed in xylem parenchyma cells of *Mesembryanthemum crystallinum* (Kirch *et al.*, 2000). Clearwater and Goldstein (2005) provide a comprehensive review on the possible mechanisms for refilling of xylem vessels under tension. In all four proposed mechanisms, the adjacent living xylem parenchyma cells are thought to be involved in both water and solute movement. The data presented in this thesis forms a good basis for continuing experiments to examine the expression patterns of aquaporins in the petioles of grapevines using *in situ* analysis and immunolocalisation (see Chapter 6 for a more detailed discussion).

The complicated transcriptional data is difficult to interpret given the complexities of the diurnal and water stress response. Transcriptional regulation of aquaporins is most likely a long-term response to modulate drought stress and may only play a minor role in the

regulation of aquaporins over a diurnal cycle. It is important to remember that aquaporins are also post-transcriptionally and post-translationally regulated in response to water stress. Under conditions of drought stress, the plasma membrane aquaporin PM28A, is dephosphorylated and therefore inactivated (Johansson *et al.*, 1996). Post- translational regulation would most likely play a significant role in the short term regulation of aquaporins.

The results of the transcriptional analysis of aquaporins in grapevine petioles, indicates a complex transcriptional regulation of aquaporin genes in response to both a diurnal rhythm and water stress. From the data obtained, it is apparent that Chardonnay and Grenache have different molecular mechanisms to cope with water stress.

Chapter 6:
General Discussion and Future Directions

The role of plant aquaporins in cellular water movement in response to water stress-induced xylem embolism, and refilling of embolised vessels, is largely unknown. Grapevines respond to water stress with a variety of physiological mechanisms, including the susceptibility to xylem embolism (Scholander *et al.*, 1955; Holbrook *et al.*, 2001; Schultz, 2003). Recently, it has been hypothesised that aquaporins may contribute to the water movement required for embolism recovery of xylem vessels thus restoring the hydraulic pathway (Holbrook *et al.*, 2001; Clearwater and Goldstein, 2005). In this thesis, molecular and physiological techniques were combined to study the putative role of plasma membrane and tonoplast membrane intrinsic proteins in response to water-stress induced xylem embolism in two cultivars of grapevine (*Vitis vinifera* cv. Chardonnay and Grenache). The two varieties were chosen to allow a varietal comparison between a drought tolerant variety, Grenache and a drought sensitive variety, Chardonnay. Grapevines have generally been classified as an isohydric species, meaning they maintain tight regulatory control of stomatal opening and closing. Recently, Schultz (2003) has proposed that different varieties of grapevines may be classified as either near-isohydric or anisohydric. Water-stress induced xylem embolism was measured in the petioles and stems of grapevines by measuring UAEs over a drying cycle (Chapter 2). To determine the role of aquaporins in these physiological processes, a grapevine cDNA library was constructed and screened for members of the MIP superfamily (Chapter 3). The water permeability of the identified grapevine aquaporins was determined using the heterologous expression system, *Xenopus* oocyte (Chapter 4). Transcript abundance in response to water stress was determined using quantitative real-time PCR and correlated to physiological measurements (Chapter 5).

It is apparent from the results obtained in this thesis that Grenache and Chardonnay have different physiological and molecular mechanisms to cope with the gradual onset of water

stress. At the onset of this project, there was very little information in the literature about aquaporins in grapevines with only two studies conducted (Baiges *et al.*, 2001; Picaud *et al.*, 2003). During the course of this project a number of new studies have investigated aquaporins in grapevines with a particular emphasis on water stress (Cramer *et al.*, 2007; Galmes *et al.*, 2007).

6.1 Water Stress-Induced Xylem Embolism in Grapevines

In order to study water-stress induced xylem embolism, potted grapevines were subjected to a drying cycle to impose a water stress. Cavitation was detected in the petioles and stems by measuring the UAEs (cavitation clicks) in Chardonnay and Grenache vines over a diurnal and drying cycle. Vulnerability to xylem embolism was determined by correlating leaf water potential and cavitation in the petioles and stems of both cultivars. Grenache exhibited a greater sensitivity to the formation of xylem embolism compared to Chardonnay under conditions of moderate water stress. Varietal differences in cavitation vulnerability has been reported previously in grapevine (Schultz, 2003; Alsina *et al.*, 2007). Chardonnay displayed vulnerability segmentation, with cavitation occurring first in the petioles, and then later in the stem (at a more negative water potential). Vulnerability segmentation has been reported previously in walnut (Tyree *et al.*, 1993) and in *Acer saccharinum* (Tsuda and Tyree, 1997).

6.2 The Identification and Functional Characterisation of Grapevine Aquaporins

To identify candidate aquaporin genes, a *V. vinifera* cv. Cabernet Sauvignon cDNA library was constructed from root and shoot tissues. The library was screened for members of the PIP and TIP subfamilies. The library screen resulted in the identification of 13 aquaporin cDNAs. Eleven of these were full-length cDNAs and two were partial-length cDNAs. Phylogenetic analysis of the grapevine aquaporins with other members of the MIP superfamily, show they cluster into two distinct subfamilies, the PIPs and TIPs. The PIP genes are further classified into the PIP1 and PIP2 subgroups. Of the cDNAs identified, five are PIP2 aquaporins, six PIP1 aquaporins and two TIP aquaporins. It is unknown how many aquaporins are in the grapevine genome. Sequencing of the maize, arabidopsis and rice genomes has revealed these species have 31, 35 and 33 MIPs, respectively (Chaumont *et al.*, 2001; Johanson *et al.*, 2001; Sakurai *et al.*, 2005). A similar number of MIPs would be expected in the grapevine genome. During the completion of this thesis, the grapevine genome sequencing project of *Vitis vinifera* was completed and published in Nature (Jaillon *et al.*, 2007). *In silico* anaysis of the genome database in the future will allow the determination of the number of MIP genes in this species. To date no members of the NIP or SIP subfamilies have been identified in grapevine.

The first crystal structure of a plant aquaporin was recently determined for the spinach plasma membrane protein, SoPIP2;1, in both the open and closed conformation (Tornroth-Horsefield *et al.*, 2006). This has provided novel insights into the structure of plant aquaporins. All aquaporins have a conserved structure of six transmembrane spanning

domains, with a highly conserved NPA motif in loops B and E that forms the aqueous pore (Fu *et al.*, 2000). In all grapevine aquaporins identified the NPA motif is completely conserved. Predictions of membrane topology using hydropathy plots, suggest these aquaporins also have six transmembrane spanning domains connected by hydrophilic loops, with the N and C termini in the cytoplasm. A number of other key residues identified in the crystal structure of SoPIP2;1 are conserved in the grapevine aquaporins (as detailed in Chapter 3).

The full-length aquaporin cDNAs identified in this study were functionally characterised in a heterologous expression system, *Xenopus* oocytes. The osmotic water permeability (Pf) was determined for each aquaporin. The water permeability of *Xenopus* oocytes expressing VvPIP2 aquaporins was high, with values comparable to those obtained for human AQP1 in both this study and in the published literature (Preston *et al.*, 1992). The VvTIP aquaporins showed moderate water permeability and the VvPIP1 aquaporins had water permeability comparable to that of the water-injected control. Many other studies have shown PIP1 aquaporins to be impermeable to water when expressed in oocytes, including aquaporins identified from maize (Chaumont *et al.*, 2000) and grapevine (Picaud *et al.*, 2003). A number of other aquaporins identified in the grapevine, *Vitis* rootstock Richter 110, have not been functionally characterised. Further functional characterisation of grapevine aquaporins in the *Xenopus* oocyte heterologous expression system is required, to provide more comprehensive information about the transport specificities of each of these proteins. Given the impermeability of the VvPIP1 proteins to water when expressed in *Xenopus* oocytes, it is likely that these may mediate transport of another solute such as glycerol, as has been shown for VvPIP1a (Picaud *et al.*, 2003). It is has been well documented that aquaporins have varied transport specificities for numerous solutes. For example NtAQP1, a PIP1

aquaporin, has transport specificity for CO_2 (Uehlein *et al.*, 2003), water, urea and glycerol (Otto and Kaldenhoff, 2000). The functional diversity of aquaporins has recently been reviewed by Kaldenhoff and Fischer (2006). The availability of an antibody would allow determination of whether VvPIP1 proteins are trafficked to the oocyte membrane. If they are trafficked to the oocyte membrane but are folded incorrectly, this would still result in non-functional proteins. Maize PIP1 aquaporins have been shown to be trafficked to the oocyte membrane, but are impermeable to water when expressed alone (Chaumont *et al.*, 2000). Recently it has been postulated that PIP1 and PIP2 aquaporins may form functional heterotetramers when co-expressed in *Xenopus* oocytes, thus modulating water permeability (Fetter *et al.*, 2004). Data from FRET imaging in maize protoplast cells led the authors to suggest that the co-expression of PIP1 and PIP2 proteins and their interaction may be required for PIP1 aquaporins to be trafficked to the plasma membrane (Zelazny *et al.*, 2007). The functionality of PIP1 aquaporins may require this interaction between isoforms in all plant species.

6.3 The Physiological Role of Aquaporins in Grapevine Petioles

The long distance pathway of water movement through plants has been well documented. Many studies have examined the contribution of aquaporins in the cell-to-cell pathway of water movement in the roots of plants (Steudle and Peterson, 1998; Javot and Maurel, 2002). There is substantial evidence in the roots that increases in root hydraulic conductance are correlated with aquaporin expression (Javot and Maurel, 2002; Tyerman *et al.*, 2002). Very little information is available on the cell-to-cell pathway of water movement in the shoots of plants, although an aquaporin contribution to the hydraulic conductivity in response to light has been reported in the leaves of walnut (Cochard *et al.*, 2007). In olive

twigs increased hydraulic resistance was correlated with increased xylem embolism and decreased expression of the aquaporin gene, *OePIP2;1* (Secchi *et al.*, 2007a). A role of aquaporins in xylem refilling has also been proposed in the twigs of walnut (Sakr *et al.*, 2003) and in the stems of grapevine (Holbrook *et al.*, 2001).

Grapevines exhibit high hydraulic conductivity and this is most likely due to the presence of large xylem vessels (Scholander *et al.*, 1955; Essau, 1965). In this study, significant anatomical differences were observed between the two cultivars, Chardonnay and Grenache. Mature Grenache petioles had larger xylem vessel diameter compared to Chardonnay petioles. According to the Hagen-Poiseuille law, the theoretical conductivity of a vessel is directly proportional to the fourth power of the vessel radius. Accordingly, the larger vessel diameter in Grenache petioles is consistent with the higher petiole specific hydraulic conductivity, and the increased sensitivity to the formation of embolised vessels under increasing xylem tensions. This relationship is also dependent on the total number of xylem vessels in the petiole segment, and this would need to be determined in the future to accurately quantitate the relationship between vessel diameter and hydraulic conductivity. The size and structure of xylem vessels can also influence the cavitation vulnerability (Lovisolo and Schubert, 1998), although a study by Holbrook *et al.* (2001) in grapevines showed no correlation with vessel size and the formation of xylem embolisms under tension.

Under favourable conditions (well-watered) it would be expected that the expression of aquaporins would facilitate trans-cellular water movement across plant membranes and contribute to the maintenance of cellular water balance. In Chardonnay petioles, *VvPIP2*;1, *VvPIP2;3* and *VvTIP1;1* aquaporin genes were transcriptionally unregulated during the day. In the evening, the transcript abundance was similar to what was observed predawn. Diurnal

and circadian changes in aquaporin expression have been observed in maize roots (Lopez *et al.*, 2003) and in the leaves of *Nicotiana excelsior* (Yamada *et al.*, 1997) and *Samanea saman* (Moshelion *et al.*, 2002). The increased AQP expression in Chardonnay petioles during the day indicates that aquaporins may play a role in modulating water flow across cellular membranes. Increased expression of the tonoplast intrinsic protein, *VvTIP1;1*, during the day indicates a potential role in modulating water movement between the vacuole and the cytoplasm. The diurnal changes in aquaporin expression in Chardonnay may indicate that trans-cellular water movement through aquaporin proteins is contributing to the petiole hydraulic conductivity. Diurnal changes in the maximum petiole specific hydraulic conductivity were observed in both Chardonnay and Grenache vines. Although a direct correlation is not seen between the petiole specific hydraulic conductivity and aquaporin gene expression in the petiole, the fact that both are responding to a diurnal cycle provides some evidence that a membrane step may be involved in the water movement through the petiole. It is not surprising that there is not a direct correlation between the transcriptional expression of aquaporins and the hydraulic conductivity, as transcript abundance does not always reflect the protein abundance. A number of other factors may be influencing petiole hydraulic conductivity. As mentioned above the size and structure of the xylem vessels can affect the rate of water flow through the petioles. In grapevines, it has been reported that under conditions of prolonged moderate water stress, there was a reduction in vessel size and no detectable embolisms (Lovisolo and Schubert, 1998). The leaf water potential in Chardonnay reached a minimum of - 0.75 MPa during the day, more negative than the cavitation threshold for petioles, indicating that some vessels would most likely be embolised. The increased expression during the day of *VvPIP2* aquaporins may facilitate water movement during periods of active transpiration, maintaining the flow of water movement through the petioles to the leaves, and hence maintaining transpiration and photosynthetic

rates. Increased expression in the afternoon would maximise cellular water movement, and increase photosynthesis to perhaps maximise photosynthetic capacity. Overexpression of PIP1b in tobacco, under favourable conditions resulted in increases in photosynthesis and higher transpiration rates (Aharon *et al.*, 2003).

The xylem vessels in Grenache petioles and stems were found to be more sensitive to the formation of xylem embolism than Chardonnay with embolisms forming even under favourable conditions (well-watered). A down regulation in gene expression of *VvPIP2* aquaporins was observed in Grenache petioles over a normal diurnal period. Given, VvPIP2 proteins are highly permeable to water when expressed in *Xenopus* oocytes, the reduction in transcript abundance of these genes may result in a decrease in cellular water movement, thus conserving water. A significant reduction in petiole hydraulic conductivity was observed in Grenache during the afternoon. This reduction in hydraulic conductivity may be linked to the decrease in transcript abundance of the plasma membrane aquaporins. This would indicate movement of water across cellular membranes in the petioles is contributing to the overall hydraulic conductivity in grapevines. The rapid increase in K_h in the evening in Grenache petioles does not seem to correlate with increased aquaporin expression, although expression of *VvPIP2;3* does increase at night time compared with the daytime measurements. It is important to remember that rapid changes in water flow across cellular membranes may also be a result of post-translational regulation. To more thoroughly examine the contribution of aquaporins in water movement in the petiole, further research is necessary.

The use of an aquaporin inhibitor, such as a mercurial compound (Daniels *et al.*, 1994), to perfuse the petioles may provide insight into the cell-to-cell pathway in the petioles. If a

decrease in K_h was observed with the addition of $HgCl_2$ this could be attributed to the potential inhibition of aquaporins; although care needs to be taken when evaluating this data as mercurials will bind to cysteine residues and could therefore be inhibiting other transporters (such as ion channels). The addition of mercury chloride, after rewetting following drought stress in grapevine, impeded recovery of the root and shoot hydraulic conductivity, indicating that living cells were contributing to the hydraulic conductivity (Lovisolo and Schubert, 2006). Measurement of hydraulic conductivity under water stress conditions in grapevines may provide more insight into the role that aquaporins play in maintaining the hydraulic conductivity of the petiole. Problems measuring hydraulic conductivity under low pressure were encountered using the XYL'EM apparatus, thus PLC could not be determined. Similar problems were reported in measuring hydraulic conductance in the leaves of walnut (Cochard *et al.*, 2007). This needs to be further investigated in the future.

The transcriptional regulation of aquaporins may help to provide a balance in maintaining cellular water balance, to optimise photosynthesis and prevent water loss under conditions of water stress. A proteomic analysis comparing differences between *V. vinifera* cv Chardonnay and Cabernet Sauvignon in response to water deficit and salinity found varietal differences were the main source of variation in protein expression (Vincent *et al.*, 2007). Aharon *et al.* (2003) speculated that in tobacco, the downregulation of aquaporins may be a long term response to modulate water conservation and reduce drought stress. The overexpression of PIP1b in tobacco increased photosynthetic rates but also resulted in faster wilting. Grenache, although more susceptible to water-stress induced xylem embolism than Chardonnay, maintain drought tolerance by closing their stomata (Schultz, 2003; Soar *et al.*, 2006) thus preventing runaway cavitation but resulting in a lower rate of photosynthesis.

Under conditions of water stress, grapevines can display isohydric behaviour as a result of closing their stomata during the day (Schultz, 2003; Soar *et al.*, 2006). In the drought tolerant rootstock, *Vitis* Richter 110, when stomatal conductance was low the midday leaf water potential was reported to be higher (less negative) than in plants where the stomatal conductance was high (Galmes *et al.*, 2007). Thus stomatal closure during the day helps to maintain higher leaf water potential. This was observed in Grenache vines, where midday ψ_L was higher than in the morning, thus indicating midday stomatal closure. A tight regulatory control of stomatal closure has been reported previously in Grenache (Soar *et al.*, 2006).

The expression pattern of some of these aquaporins suggests that these genes may be involved in maintaining the hydraulic conductivity in the petiole. To further examine this possibility, *in situ* hybridisation could be utilised to show cell specific expression patterns of these genes. Of particular interest would be to examine the expression pattern of these genes in the xylem parenchyma cells especially those associated directly with the xylem vessels (vessel-associated cells). Development of an antibody specific for the isoforms present in the xylem parenchyma cells would allow immunolocalisation studies which would provide a more quantitative approach to protein expression. A combination of these approaches to show expression of the PIP and TIP aquaporins in the xylem parenchyma cells would strongly support a role of these proteins in maintaining the hydraulic conductivity of the petiole. The Tyerman lab has recently had peptides synthesised to PIP1 and PIP2 proteins and these could be used for future analysis of expression in the petioles. A common problem arising from the high homology between aquaporin isoforms, is that of cross-reactivity. Using these antibodies, would only enable AQPs to be distinguished by subgroups as has been shown previously (Sakr *et al.*, 2003).

6.4 Are Aquaporins Involved in Refilling of Embolised Xylem Vessels?

From the data obtained in this study, it is difficult to assess if aquaporins play a role in embolism recovery. Holbrook *et al.* (2001) has previously shown, using MRI, embolism refilling in the stems of grapevine. In this thesis, increases in AQP gene expression in Chardonnay petioles was correlated with increased petiole specific hydraulic conductivity under well-watered conditions. Under well-watered conditions, xylem tension was high enough to result in some cavitation events in the petioles. The increased diurnal expression of both PIP and TIP isoforms during the light period suggests that the refilling of embolised vessels in Chardonnay petioles may be plausible over a diurnal cycle when water availability is high. Other authors have proposed that refilling of embolised vessels can occur when transpiration rates are high (McCully *et al.*, 1998; McCully, 1999; Clearwater *et al.*, 2004). Increased expression may also help to maintain the hydraulic pathway in the petioles, by maintaining the flow of water around embolised xylem vessels. The increased expression of some AQP isoforms under a moderate water stress also indicates a possible mechanism for refilling in Chardonnay petioles. In Chardonnay, *VvPIP2;3* has increased expression under well-watered conditions. This gene is highly homologous to the walnut aquaporins, *JrPIP2;1* and *JrPIP2;2* that have been postulated to be involved in recovery from winter embolism (Sakr *et al.*, 2003), and therefore may be a good candidate gene for refilling in grapevines. Grenache vines appear to conserve their cellular water balance during periods of high transpiration, even under well-watered conditions. The results suggest that Grenache may not have a mechanism to refill their embolised vessels even under favourable conditions, thus a more conservative strategy for conserving water is required.

6.5 Conclusion

The results obtained in this study provide the first detailed comparative study of aquaporins in two cultivars of grapevine, and their role in water-stress induced xylem embolism. The main finding in this thesis is that the two grapevine varieties, Chardonnay and Grenache exhibited different physiological and molecular responses, under conditions of water stress. The drought tolerant variety, Grenache exhibits a more conservative water use strategy compared to Chardonnay which has implications for vineyard management. Grenache can tolerate drought conditions better than Chardonnay, in the long term closing stomata and down regulating aquaporins to preserve cellular water. Long-term water stress may result in a decrease in photosynthesis and thus may have a negative affect on fruit development and yield.

Advances of microarrays and large scale proteomic studies will allow direct comparisons on the regulation of aquaporins and other genes under the same conditions. In the long term, the use of transgenic grapevines, either over or under expressing specific aquaporin genes, would allow a more precise evaluation into the role that aquaporins play in response to environmental stimuli such as water stress.

Appendix A

LOCUS EF364432 1032 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP1;1 (PIP1;1) mRNA, complete cds.
ACCESSION EF364432
VERSION EF364432.1 GI:124702459
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1032)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1032)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
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/protein_id="ABN14347.1"
/db_xref="GI:124702460"

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VYCTAGISGGHINPAVTFGLFLARKLSLTRAVYYMVMQCLGAICGAGVVKGFEKGRYN
SLGGGANVVNAGYTKGDGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPILAPLPIGF
AVFLVHLATIPITGTGINPARSLGAAIIYNKDHAWDDHWIFWVGPFIGAALAALYHQV
VIRAIPFKSK"
ORIGIN
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      121 cgccaccggc gccgctgttc gagcctggag agctgtgttc gtggtcgttt tacagggctg
      181 ggatagcgga gttcatagcc acttttctgt tcctgtacgt ctccgttttg acggtgatgg
      241 gggtggttag ggctgactcc aagtgttcca cggttgggat tcaagggatt gcttgggctt
      301 ttggtggtat gatcttcgct ctcgtctact gcaccgccgg aatctcaggt gggcatataa
      361 atccagcggt gacctttggg ctgttcttgg caaggaagct gtcgctgacg agggcagtgt
      421 actacatggt gatgcagtgc ctgggtgcga tatgtggtgc gggtgtagtg aagggtttcg
      481 agaagggcag gtacaactcg ctgggtggtg gtgccaatgt ggtgaacgct ggctacacca
      541 aaggagatgg gcttggtgct gagattgttg gcaccttcgt ccttgtctac actgtcttct
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      841 aggtggtcat cagagccatt cccttcaagt ccaagtgatg atgagtggtg ctgggcgttg
      901 atcaaggggc atcaaaaacc tactctttgt cttttgtccc acttttatcc gtttgcttat
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LOCUS EF364433 1115 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP1;2 (PIP1;2) mRNA, complete cds.
ACCESSION EF364433
VERSION EF364433.1 GI:124702485
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1115)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1115)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..1115
/organism="*Vitis vinifera*"
/mol_type="mRNA"
/cultivar="Cabernet Sauvignon"
/db_xref="taxon:29760"
/clone="LC14"
/tissue_type="leaves; petioles; shoots; tendrils; roots"
gene 1..1115
/gene="PIP1;2"
CDS 31..891
/gene="PIP1;2"
/note="putative plasma membrane intrinsic protein; VvPIP1;2"
/codon_start=1
/product="aquaporin PIP1;2"
/protein_id="ABN14348.1"
/db_xref="GI:124702486"

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VFLVHLATIPITGTGINPARSLGAAIVYNKEHAWDDMWIFWVGPFIGAALAALYHQIV
IRAIPFKTRA"
ORIGIN
        1 atctgtagag ggagaatttg agggagaaaa atggagggga aggaagagga tgtgaggttg
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      241 actgttatgg gtgttgtcag gtcccccagc aagtgtgcca ctgttggtat tcagggtatt
      301 gcttgggctt ttgggggtat gatctttgcc cttgtctact gcactgctgg tatctcagga
      361 ggacacataa acccagcggt gacatttggc ctgctcttgg ccagaaagct gtctctaacc
      421 cgagccgtgt tctacatgat catgcaatgc ctgggcgcca tctgtggcgc aggtgtggtg
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      661 gctcctctcc cgattgggtt tgcagtgttc ttggttcatc tggcaaccat ccccatcact
      721 ggaaccggca tcaaccctgc taggagtctg ggggccgcca tcgtctacaa caaagagcat
      781 gcctgggatg acatgtggat tttctgggtt ggacccttca ttggagctgc tcttgctgcc
      841 ttgtaccacc agatagtaat cagagccatc ccattcaaga ccagagcctg agaaattcct
      901 ccattttctg tttcttcttt tatttgctgc ttctatcttc ttcttcttgt tcatcttcct
      961 cctctgggat ctgggttgag atcagaatag aggtgggggt ggttattatg tgtataagtt
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     1081 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaa
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LOCUS EF364434 1083 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP1;3 (PIP1;3) mRNA, complete cds.
ACCESSION EF364434
VERSION EF364434.1 GI:124702508
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1083)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1083)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..1083
/organism="*Vitis vinifera*"
/mol_type="mRNA"
/cultivar="Cabernet Sauvignon"
/db_xref="taxon:29760"
/clone="LC14"
/tissue_type="leaves; petioles; shoots; tendrils; roots"
gene 1..1083
/gene="PIP1;3"
CDS 52..915
/gene="PIP1;3"
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VLGGGANVVNSGYTKGDGLGAEIVGTFVLVYTVFSATDAKRNVRDSHVPILAPLPIGF
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VIRAIPFKSRA"
ORIGIN
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      901 aagagcaggg cttgagactt ccatcgcctt tctctgttgt gcattttgtt tctctcatgt
      961 ggatttggtg ttttaatgta ctcctactac ctaagtgtgt aaattattga gtattcttgt
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     1081 ccc
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LOCUS EF364435 1061 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP1;4 (PIP1;4) mRNA, complete cds.
ACCESSION EF364435
VERSION EF364435.1 GI:124702522
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1061)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1061)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..1061
/organism="*Vitis vinifera*"
/mol_type="mRNA"
/cultivar="Cabernet Sauvignon"
/db_xref="taxon:29760"
/clone="LC14"
/tissue_type="leaves; petioles; shoots; tendrils; roots"
gene 1..1061
/gene="PIP1;4"
CDS 42..902
/gene="PIP1;4"
/note="putative plasma membrane intrinsic protein; VvPIP1;4"
/codon_start=1
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/protein_id="ABN14350.1"
/db_xref="GI:124702523"

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IRAIPFKTRA"
ORIGIN
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     1021 gtgtgtaagt tatctgggct gcttctatta caatgggcag c
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LOCUS EF364440 951 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP1;5 (PIP1;5) mRNA, partial cds.
ACCESSION EF364440
VERSION EF364440.1 GI:124702536
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 951)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 951)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..951
/organism="*Vitis vinifera*"
/mol_type="mRNA"
/cultivar="Cabernet Sauvignon"
/db_xref="taxon:29760"
/clone="LC14"
/tissue_type="leaves; petioles; shoots; tendrils; roots"
gene <1..951
/gene="PIP1;5"
CDS <1..753
/gene="PIP1;5"
/note="putative plasma membrane intrinsic protein; VvPIP1;5"
/codon_start=1
/product="aquaporin PIP1;5"
/protein_id="ABN14355.1"
/db_xref="GI:124702537"

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DAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGAAIIFNREHAWDD
MWIFWVGPFIGAALAAMYQQIVIRAIPFKSRA"
ORIGIN
        1 cccttgtttg agcccgggga gttgaagtca tggtcttttt acagagccgg gattgctgag
       61 ttcatggcca ctttcctgtt tctctatatc accattttga ctgttatggg tgtgaaaaag
      121 tcacccacca tgtgtgccag tgttggtatt caggggattg cttgggcttt tggtggtatg
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      901 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa a
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LOCUS AY823263 1216 bp mRNA linear PLN 28-NOV-2004
DEFINITION *Vitis vinifera* aquaporin (PIP2-1) mRNA, complete cds.
ACCESSION AY823263
VERSION AY823263.1 GI:55982652
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1216)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1216)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (10-NOV-2004) Wine and Horticulture, Waite Campus, University of Adelaide, Plant Research Centre, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..1216
/organism="*Vitis vinifera*"
/mol_type="mRNA"
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/db_xref="taxon:29760"
/clone="LC14"
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gene 1..1216
/gene="PIP2-1"
CDS 76..930
/gene="PIP2-1"
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recorded"
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/protein_id="AAV69744.1"

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                     /db_xref="GI:55982653"

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GYSKGTGLGAEIIGTFVLVYTVFSATDPKRSARDSHVPVLAPLPIGFAVFMVHLATIP
ITGTGINPARSLGAAVIYNNEKAWDDQWIFWVGPFIGAAIAAFYHQFILRAGAVKALG
SFRSTTHV"
ORIGIN
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LOCUS EF364436 1169 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP2;2 (PIP2;2) mRNA, complete cds.
ACCESSION EF364436
VERSION EF364436.1 GI:124702518
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1169)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1169)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
source 1..1169
/organism="*Vitis vinifera*"
/mol_type="mRNA"
/cultivar="Cabernet Sauvignon"
/db_xref="taxon:29760"
/clone="LC14"
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gene 1..1169
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LOCUS EF364437 1225 bp mRNA linear PLN 12-FEB-2007
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ORGANISM *Vitis vinifera*
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Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1225)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1225)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
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LOCUS EF364438 1139 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin PIP2;4 (PIP2;4) mRNA, complete cds.
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SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1139)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1139)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
FEATURES Location/Qualifiers
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VERSION     AY839872.2  GI:123965224
KEYWORDS    .
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  ORGANISM  Vitis vinifera
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Tracheophyta;
            Spermatophyta;     Magnoliophyta;     eudicotyledons;     core
eudicotyledons;
            Vitales; Vitaceae; Vitis.
REFERENCE   1  (bases 1 to 993)
  AUTHORS   Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
  TITLE     Identification of Vitis vinifera aquaporins
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 993)
  AUTHORS   Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2004) Wine and Horticulture, Waite Campus,
            University of Adelaide, Plant Research Centre, Hartley Grove,
            Urrbrae, SA 5064, Australia
REFERENCE   3  (bases 1 to 993)
  AUTHORS   Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JAN-2007) Wine and Horticulture, Waite Campus,
            University of Adelaide, Plant Research Centre, Hartley Grove,
            Urrbrae, SA 5064, Australia
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COMMENT     On Jan 22, 2007 this sequence version replaced gi:56608596.
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LOCUS EF364439 1093 bp mRNA linear PLN 12-FEB-2007
DEFINITION *Vitis vinifera* aquaporin TIP2;1 (TIP2;1) mRNA, complete cds.
ACCESSION EF364439
VERSION EF364439.1 GI:124702532
KEYWORDS .
SOURCE *Vitis vinifera*
ORGANISM *Vitis vinifera*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
Vitales; Vitaceae; *Vitis*.
REFERENCE 1 (bases 1 to 1093)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Identification of *Vitis vinifera* aquaporins
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1093)
AUTHORS Shelden,M.C., Kaiser,B.N. and Tyerman,S.D.
TITLE Direct Submission
JOURNAL Submitted (17-JAN-2007) Wine and Horticulture, University of Adelaide, Hartley Grove, Urrbrae, SA 5064, Australia
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