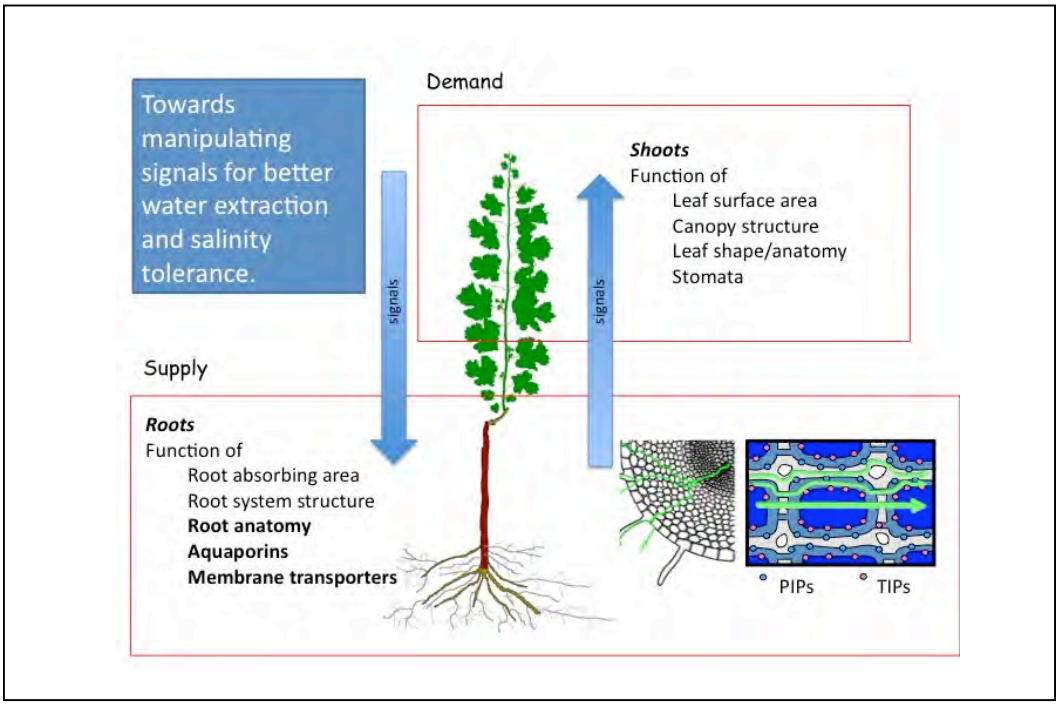
THE UNIVERSITY OF ADELAIDE
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Grape and Wine Research and Development Corporation

Root physiology and vine performance



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GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 04/03**

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1. Abstract

The physiology of the roots of grapevine varieties and rootstocks have been investigated in the context of water extraction from soil and salinity tolerance. Three major findings from the project are:

1) There are large diurnal changes in the capacity of roots to extract water from the soil, which is largely in tune with the shoot's capacity to use the water. This is variety dependent particularly after water stress and re-watering.

2) There are signals from the shoot going to the roots that communicates the shoot's demand for water so that sufficient supply is maintained. This is affected by shoot pruning. This is a general feature of plants first identified in grapevine in this project.

3) Salt tolerance in rootstocks can be attributed to major differences in chloride transport across specific membranes within the root. These differences occur in the main roots but not lateral roots.

2. Executive summary

There were two broad components to the project: 1) How roots control water flow to the shoot and how this control may be manipulated to improve water extraction from soil; 2) How roots control the delivery of chloride salts to the shoot and if this is linked to water flow and salinity tolerance of grapevines. This work was also partially subsidized by the Australian Research Council, which funded an Australian Professorial Fellowship to Professor Tyerman (2007-2011).

1) Control of root water transport

We report physiological and anatomical characteristics of water transport across roots grown in soil of two cultivars of *Vitis vinifera* L. differing in response to water stress (Grenache-isohydric; Chardonnay-anisohydric). Both cultivars have similar root hydraulic conductances (L_o, a measure of water transport capacity) that change diurnally. There is a positive correlation between L_o and transpiration indicating coordination between root and shoot. Under water stress both cultivars have reduced minimum daily L_o (predawn) attributed to development of apoplastic barriers. Water-stressed and well-watered Chardonnay had the same diurnal change in amplitude of L_o, while water-stressed Grenache showed a reduction in daily amplitude compared to well-watered plants. Hydraulic conductivity of root cortex cells (L_{pcell}) doubles in Chardonnay but remains unchanged in Grenache. Of the two most highly expressed PIP aquaporins (water protein channels) in roots (VvPIP1;1 and VvPIP2;2) only VvPIP2;2 functions as a water channel in *Xenopus* oocytes. VvPIP1;1 interacts with VvPIP2;2 to induce 3-fold higher water permeability. These two aquaporins are co-located in the root from *in situ* hybridisation, and immunolocalisation of VvPIP1 and VvPIP2 subfamily members. They occur in root tip, exodermis, root cortex (detected up to 30 mm), and stele. *VvPIP2;2* mRNA does not change diurnally or with water stress, in contrast to *VvPIP1;1*, where expression reflects the differences in L_o and L_{pcell} between cultivars in their responses to water stress and rewatering. VvPIP1;1 may regulate water transport across roots such that transpirational demand is matched by root water transport capacity. This occurs on a diurnal basis and in response to water stress that corresponds to the difference in drought tolerance between the cultivars. **This work has demonstrated that roots are far from passive structures in regulating water transport; the aquaporin water channels in roots are just as important as stomata in regulating water transport and there are large differences between varieties.**

Shoot topping is commonly used in vineyards to control vine vigour. It can also be utilised during experimentation to minimise variation in leaf area between grapevine (*Vitis vinifera* L.) plants. We have observed that shoot topping has a negative impact on root hydraulic conductance (L_o). Although first discovered by us in grapevines, this response was also observed for other plant species; soybean (*Glycine max* L.) and maize (*Zea mays* L.). L_o was not significantly reduced 1-2 h after shoot topping but was 24 h after shoot topping. The percentage reduction in L_o was not associated with the degree of leaf area reduction. A significant reduction occurred when young expanding leaves and the shoot apex were removed by cutting the stem, but no reduction occurred if just the shoot apex was removed. The reduction in L_o in response to shoot topping was variable and depended upon the initial L_o. We are yet to determine if the response is due to a negative signal from the shoot or the loss of a positive signal. It is also likely that a reduction in total plant transpiration contributes to the reduction. There was a significant reduction in the gene expression of *VvPIP1;1*, an aquaporin highly expressed in grapevine roots. This suggests that the signal acts upon aquaporin expression, which contributed to the reduction in L_o. **This work has demonstrated that there is a signal from the shoot going to roots that could be manipulated to improve water extraction by roots.**

Partial rootzone drying (PRD) is an irrigation technique that improves water use efficiency with minimal impact on yield and quality of grapes. The root systems of two grapevine (*Vitis vinifera*) cultivars, Chardonnay and Grenache, were divided between two pots. Plants subjected to the PRD treatment had water withheld from one pot, while the other pot remained well-watered. The control plants had both pots well-watered. PRD resulted in a

reduction in stomatal conductance, while the leaf water potential was maintained. The root hydraulic conductance (L_o) normalised to root dry weight declined in the half not watered. Grenache experienced a 4-fold decline, whereas Chardonnay only suffered a 2-fold decline. Compared to the control plants the roots of the well-watered half of PRD plants had a slight increase in L_o. This resulted in no change in the L_o of the complete root system of Chardonnay PRD plants. However, Grenache suffered a significant decline, most likely related to the large reduction in L_o on the dry side. Xylem sap ABA concentration increased in Grenache PRD plants, but the ABA content of Chardonnay PRD plants was not significantly different to the control plants. **This work has demonstrated that PRD has a profound effect on water extraction by roots and that roots compensate to different degrees, depending on variety, between the dry and wet side.**

2) Control of chloride transport to grapevine shoots.

Grapevine is moderately sensitive to salinity and accumulation of toxic levels of chloride (Cl^-) in leaves is the major reason for salt-induced symptoms. In this study apoplastic Cl^- uptake (movement in cell walls) and transport mechanism(s) were investigated in two grapevine (*Vitis* sp.) rootstock hybrids differing in salt tolerance; 1103 Paulsen (salt tolerant) and K 51-40 (salt sensitive). Increased external salinity caused high Cl^- accumulation in shoots of the salt sensitive K 51-40 in comparison to Paulsen. Measurement of $^{15}NO_3^-$ net fluxes under high salinity showed that by increasing external Cl^- concentrations K 51-40 roots showed reduced NO_3^- accumulation. This was associated with increased accumulation of Cl^-. In comparison to Paulsen, K 51-40 showed reduced NO_3^- / Cl^- root selectivity with increased salinity, but Paulsen had lower selectivity over the whole salinity range (0-45 mM). In order to examine if root hydraulic and permeability characterisations accounted for differences between varieties, the root pressure probe was used on excised roots. This showed that the osmotic L*pr* was significantly smaller than hydrostatic L*pr*, but no obvious difference was observed between the rootstocks. The reflection coefficient (σ) values (0.48-0.59) were the same for both rootstocks, and root anatomical studies showed no obvious difference in apoplastic barriers of the main and lateral roots. Comparing the uptake of Cl^- with an apoplastic tracer, PTS (3-hydroxy-5, 8, 10-pyrentrisulphonic acid), showed that there was no correlation between Cl^- and PTS transport. **These results indicated that by-pass flow of salts to the xylem is the same for both rootstocks (10.01±3.03 % and 12.1±1.21 %) and hence pointed to differences in membrane transport, and hence chloride transport proteins, to explain differences in Cl^- transport to the shoot.**

Root Cl^- transport was investigated in more detail using ^{36}Cl flux analysis. Initial $^{36}Cl^-$ influx to the root was greater in Paulsen than K 51-40. This flux, attributed to the Cl^- influx to the cytoplasm (Φ_{oc}) increased with increasing external concentrations of Cl^- for plants adapted to growth in 30 mM NaCl. The concentration kinetics in this high concentration range could be fit to a Michaeils-Menton equation, indicating saturable transport through a membrane transport protein. There was no significant difference between genotypes in Km (28.68 ± 15.76 and 24.27 ± 18.51 mM for Paulsen and K 51-40 respectively), but Paulsen had greater V_{max} (0.127 ± 0.042) compared to K51-40 (0.059 ± 0.026 μm g^{-1} F.W. min^{-1}). This indicates that Paulsen may have a higher density of transporters on the membrane. In Paulsen the main root had greater contribution to $^{36}Cl^-$ uptake than lateral roots; there being no significant difference in lateral root influx between the genotypes. $^{36}Cl^-$ transport to the shoot of K 51-40 was greater than for Paulsen. It was estimated that efflux rate from the xylem parenchyma cells to the xylem vessels (Φ_{cx}) in K 51-40 was twice that of Paulsen. Compartmental analysis from $^{36}Cl^-$ efflux kinetics confirmed the larger Φ_{oc} for Paulsen, and confirmed the higher ratio of main to lateral root Φ_{oc} for Paulsen. Efflux from the cytoplasm (Φ_{co}) was higher than 95% of Φ_{oc} indicating a high degree of cycling across the plasma membrane in roots at these high external Cl^- concentrations. Paulsen appears to keep the cytoplasmic Cl^- concentration in roots lower than K51-40 via greater efflux to the vacuole and to the outside medium. **The difference in salt tolerance between the genotypes can be attributed to different Cl^- transport properties at the plasma membrane and tonoplast and particularly in Cl^- efflux to the xylem.**

Overall the project has revealed considerable scope for manipulating roots to the benefit of better water extraction and better salt tolerance in grapevines.

3. Background

Grapevines like many other horticultural plants transpire large quantities of water as a necessary part of gaining carbon dioxide for photosynthesis and fruit production. In warm climates it would not be uncommon for 3 to 4 ML to be transpired per hectare of vineyard over a growing season. More water is applied than is transpired, and although some of this is required for leaching of salts, a variable proportion, depending on irrigation efficiency, is not productively used. Of the water that is transpired by grapevines, probably over 50% will traverse water channels (aquaporins) in root membranes. These water channels represent points of control in the soil-plant-atmosphere continuum, and like stomata they are regulated by the vine in response to environmental and internal conditions. Their activity in roots may have a profound effect on the extraction of water from the soil.

Although stomata play the major role in regulating transpiration and will determine the efficiency of water use (carbon gained/water loss), the roots must be able to carry the supply of water to match demand from the shoots. In this respect roots will determine the operating conditions of the shoot in terms of the shoot water potential and xylem solute concentrations. The hydraulic conductance of roots and the responses to soil conditions and shoot conditions, actually determine the operating *water use envelope* of the plant, that is, the amount of water that can be extracted from the soil over a range of plant water potentials. The root system hydraulic conductance is variable, the cause of the variability depending on the time scale. For longer time scales hydraulic conductance is determined by changes in the size of the root system, patterns of root growth and changes in root anatomy. Within the time scale of a day variability in root hydraulic conductance, as much as 2-10 fold, is mainly due to the activity of aquaporins. Aquaporins, either directly or indirectly respond to time of day (circadian rhythms), soil water potential, salinity, nutrient status, soil temperature, root metabolic status, and probably shoot water status via hormone signalling.

Now that we are beginning to understand the central role of aquaporins in determining root hydraulic conductance, we can consider the possibility of applying treatments of nutrients and timing of water applications to maximise the water use envelope of grapevines and improve water extraction from the soil under different conditions. This would be analogous to finding a treatment like PRD that “tricked” the roots into responding in a particular way that could be used to optimize water use and to improve yield and quality. Unlike PRD we are able to use more than two dimensions (ie time and space) in order to manipulate root hydraulic conductance. The matrix is likely to be time x space x nutrients x canopy leaf area x transpirational demand, resulting in a second generation of a “PRD type” manipulation.

Objectives

- To obtain the fundamental knowledge on grapevine root water and nutrient transport (and interactions) and the roles of aquaporins in these processes across major varieties and rootstocks.
- To determine the link between root reflection coefficients, specifically for NaCl, and root hydraulic conductivities across varieties and rootstocks.
- To determine the diurnal variability of root hydraulic conductivity and reflection coefficients and the degree to which these can be altered by potential field treatments.
- To investigate the influence of canopy manipulations on root water extraction.
- To develop an understanding of the role of plant hormones in shoot to root signaling in regulation of root water transport.
- To validate to field conditions the predictions from laboratory/glass-house experiments of the soil conditions for the major grapevine varieties and rootstocks that are beneficial or detrimental to efficient water extraction.
- To produce recommendations on practices to improve water extraction efficiency and nutrient use efficiency that are cognisant of climate, soil conditions and water quality.

4. Project Aims and Performance targets

Outputs and Performance Targets 2004-05

Outputs	Performance Targets
1. Information on the relationship between root aquaporins and root hydraulic conductance and conductivity for major varieties/rootstocks.	Analysis of data from first years-results available December 2005
2. Information on the diurnal variability of root hydraulic conductivity for selected varieties/rootstocks.	Analysis of data from first years-results available December 2005

Outputs and Performance Targets 2005-06

Outputs	Performance Targets
1. Information on the interactions between salinity and proportions of major nutrient types on root hydraulic conductivity and reflection coefficients.	Analysis of data from second years-results available December 06
2. Information on the interactions between major nutrient applications and proportions of major nutrient types on root hydraulic conductivity.	Analysis of data from second years-results available December 06

Outputs and Performance Targets 2006-07

Outputs	Performance Targets
1 Information on the diurnal variability of root hydraulic conductivity and reflection coefficients and how this can be varied by soil conditions.	Results available December 2007

Outputs and Performance Targets 2007-08

Outputs	Performance Targets
1 Information on the link between transpirational demand, canopy leaf area and root hydraulic conductance.	Results available Dec 2008
2. Information on the role of root ABA and auxin levels on root hydraulic conductance.	Results available Dec 2008

Outputs and Performance Targets 2008-09

Outputs	Performance Targets
1.Validation of predictions from pot experiments to field conditions for major treatment effects observed in pot experiments of nutrient/salinity interactions on root hydraulic conductance and water extraction ability.	Results from 2 years of experiments by Dec 09
2. Clear recommendations on best practices to induce high water extraction efficiencies and nutrient extraction efficiencies when these are linked to water uptake (eg N).	Production of information by Dec 2009

5. Method

5.1 Summary

Most of the basic root physiology was performed on glasshouse grown potted vines. Although the PD is aware that this has its limitations in terms of translation to field grown vines, all precautions were taken to reduce the effects of root restrictions in the pots by using large pots and maintaining vines at an appropriate shoot:root ratio. The advantage of using potted vines in controlled environment conditions was that more manipulations could be done on the roots, and work continued through winter. We were also aiming to find large effects that will more likely be also found in field grown vines.
Most measurements entailed using the Hydraulic Conductance Flow Meter (HCFM), which is a destructive sampling technique. The vines were de-topped and the hydraulic conductance of the root system was measured by applying positive pressure to the cut stem and measuring flow rate very accurately. The root length, surface area and weight was then determined to determine hydraulic conductivity. Shoot surface area was also determined to enable calculation of a shoot specific hydraulic conductivity. This technique can be applied in the field, but the shoot system of the plant is sacrificed using the current technique. We are currently undertaking these experiments in the Coombe Vineyard (at the time of writing this report). There are also other less direct methods whereby the root hydraulic conductivity can be derived. These require simultaneous measurements of sap flow and water potential gradients. Two field-based experiments were carried out using this technique.

Other methodologies used included the root pressure probe to measure hydraulic conductance and reflection coefficients of single roots and acoustic monitoring equipment to measure cavitation events in roots.

Measurements of nitrate and chloride transport used isotope methods and kinetic analysis of accumulation and loss of isotopes.

The molecular components of the project include basic gene cloning and sequencing and for expression analysis we used real time PCR.

5.2 Detailed materials and methods

5.2.1 Detailed material and methods for 6.1

Plant growth conditions
One year old *Vitis vinifera* (L.) rootlings, cv. Chardonnay (Clone I10V1) and Grenache (Clone BVRC38) were obtained from Yalumba Nursery (Nuriootpa, South Australia). Grapevines were grown in 20 cm diameter pots (4.7 L) and re-potted into 25 cm pots (9 L) 2-3 weeks prior to application of treatments, to prevent the grapevines from becoming root-bound. Grapevines were grown in University of California soil mix: 61.5 L sand, 38.5 L peat moss, 50 g calcium hydroxide, 90 g calcium carbonate and 100 g Nitrophoska® (12:5:1, N:P:K plus trace elements), per 100 L at pH 6.8. Pots were placed in a temperature controlled greenhouse, watered to field capacity every 2 days, and grown over spring and summer. Night/day temperatures were maintained at approximately 19/24ºC.

Additional grapevines were grown in a two-pot system to obtain roots for RNA extractions, *in situ* hybridisation, immunolocalisation, root anatomy and cell pressure probe measurements. The top pot containing UC mix had holes in its base and covered with plastic netting, and the bottom pot contained a 50:50 mix of vermiculite and perlite that enabled roots to be sampled easily when the top pot was raised. An additional 25 g Nitrophoska® was applied to the top pot approximately every 3 months. Roots were obtained for RNA extractions when the plants were in a growth chamber over winter. Growth chamber temperatures were identical to those in the glasshouse, with a 12 hour light period and average light intensity of 200 µmol m^{-2} s^{-1}. The root system hydraulic conductance of the cultivars was not significantly different to plants grown in the glasshouse.

Treatments
All treatments were applied in a completely randomised design. Grapevines were three months old, with only vegetative growth that was restricted to two main shoots. Diurnal variability of L_o of Chardonnay was measured every four hours in a 24 hour period, at 600 h, 1000 h, 1400 h, 1800 h and 2200 h. At 600 h and 2200 h the plants were in darkness. In addition, at 600 h and 1400 h, L_o was measured on water-stressed grapevines, from which water had been withheld for 8 days. In a separate experiment well-watered and water-stressed Grenache vines were measured at 600 h and 1400 h only.

Chardonnay and Grenache were used to examine the impact of water stress and rewatering on L_o. The two cultivars were examined in separate experiments to prevent diurnal variability impacting on the results. Control plants remained well-watered whereas water-stressed plants had water withheld for 8 days. Rewatered plants were stressed for 8 days before watering to field capacity 24 hours prior to measurements being taken. Additional well-watered plants were used as controls on the second day with the rewatered plants.

Water potential
A leaf, 8 nodes from the base, was placed in a plastic bag covered with aluminium foil for one hour prior to measurement in a Scholander pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) to determine the stem water potential (Begg and Turner, 1970). This was performed between 1100 h and 1300 h and is referred to as midday Ψ_{stem}.

Transpiration
An infrared gas analyser (Type LCA-4 ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) was used to measure the transpiration of leaves under ambient VPD at nodes 7, 8 and 9 between 1100 and 1200 h before plants were removed for hydraulic conductance measurements. A section of each leaf was placed in the broad leaf chamber whilst still attached to the plant. Measurements were taken once the sub-stomatal CO_2 concentration had reduced and stabilised.

Hydraulic conductance
Hydraulic conductance of whole root systems of potted plants was measured with a Dynamax (Houston, Texas, USA) Hydraulic Conductance Flow Meter (HCFM). This is a destructive technique whereby water is forced to flow into root systems from the cut stump at the base of the shoot, and has been shown to give hydraulic conductance values similar to the pressure chamber (Tyree et al., 1995) and the evaporative flux method (Tsuda and Tyree, 1997 & 2000). The grapevine stem was cut above the soil surface, covered with filtered (0.22 µM) deionised water and the stump was connected to the HCFM with a water tight seal. We used the transient ramp technique in HCFM in which pressure was ramped up to 0.5 MPa at a rate of approximately 7 kPa s^{-1} while simultaneously recording the flow through the roots. On account of the high pressures and the direction of water flow imposed, this technique will not detect changes in hydraulic conductance as the result of xylem embolisms or reduced root-soil contact. There is the possibility that osmotic gradients will be established as solutes are polarised on the inside of the endodermis in the root, because water flows out of the root in the reverse direction to normal flow (Knipfer et al., 2007). It would be predicted that successive application of ramps would polarise solutes further with each ramp because more volume is extruded from the root, which would manifest as a progressive reduction in hydraulic conductance (see Fig. 3 & 4, Knipfer et al., 2007). We closely examined both the linearity of each flow versus pressure ramp, and the changes that occur from one ramp to the next over three successive ramps done in quick succession for each measurement made under low transpiration (pre-dawn) and high transpiration (midday) conditions. There was no consistent pattern of changes in L_o; successive ramps differed on average by 10.03% (± 2.37; n = 16). Results were the same for both midday and predawn measurements and did not correlate with the magnitude of hydraulic conductance. The amount of water injected during the ramp was less than 1×10^{-6} m^3 which is small compared to the total estimated volume of the root system of 7×10^{-5} m^3. Generally an average of the second and third determination was taken for calculation of hydraulic conductance, L_o, as the slope of the plot of the water flow versus pressure. This was normalised by dividing the conductance by the total root dry weight. For both cultivars, there was a linear correlation between root dry weight and root surface area (which was more difficult to obtain in routine

measurements). Measurements were undertaken in the laboratory at a temperature of approximately 21-22ºC. The soil was washed from the roots before drying at 60ºC for > 48 h.

Cell Hydraulic Conductivity
A cell pressure probe was used to measure turgor pressure (*P*), cell elastic volumetric modulus (ε) and half times of pressure relaxations ($T_{½}$) to determine the hydraulic conductivity of cortical cells (L_{pcell}) (Tyerman *et al*., 1989; Steudle, 1993). Two to four cells from roots of at least four different plants were measured for each treatment, well-watered and water-stressed. Water stress was applied for 10 days to obtain midday Ψ_{stem} similar to those observed in hydraulic conductance experiments. A 40 mm piece of excised root that included the root tip was firmly held in a perspex holder. The roots used were 0.7-0.9 mm in diameter and obtained from the two-pot system. A peristaltic pump was used to pump a 1 mM $CaSO_4$ solution around the root at a constant flow rate. Roots were in position for approximately 10 min before measurements commenced and roots were discarded approximately 1.5 h later. There was no indication of time-dependent changes in turgor pressure after harvesting the roots and bathing them in 1 mM $CaSO_4$ for periods up to 1.5 h.

Microcapillaries were made from borosilicate glass with 1 mm OD x 0.58 mm ID (GC 100-15 Harvard Apparatus, SDR Clinical Technology, Middle Cove, NSW, Australia). Capillaries were filled with silicone oil and attached to the cell pressure probe with nitrile rubber seals. Roots were probed between 25-30 mm from the root tip, and when punctured cell sap formed a meniscus with the oil.

L_{pcell} was determined using hydrostatic pressure relaxations. Pressure was altered by less than 0.05 MPa via a metal rod (attached to an electric motor) that moved the meniscus to a new position, where it was held in place with small movements of the rod until the pressure equilibrated. Single exponential curves were fitted to pressure relaxations to obtain the $T_{½}$ for rate of water exchange across the cell membrane.

The ε ($\varepsilon = V\Delta P / \Delta V$) was measured by changing cell volumes (ΔV), which caused changes in cell turgor (ΔP). The meniscus was quickly moved and then returned to its original position. L_{pcell} was calculated by $L_{pcell} = V\ln(2)/ AT_{½}(\varepsilon + \pi_i)$, where *V* is the cell volume, *A* is the cell surface area, and π_i is the cell osmotic pressure estimated from steady state turgor pressure in a solution of known osmotic pressure. Standard error was determined from the standard errors of cell sizes, ε and $T_{½}$ using the differential equation of Gauss for the calculation of error propagation. Unpaired t-tests were performed to determine statistical differences between the well-watered and water-stressed cells of Grenache and Chardonnay, for the parameters measured.

Root Anatomy
Roots were sampled from the two-pot system from well-watered and water-stressed plants. Water stress was applied for 10 days to obtain midday Ψ_{stem} similar to those observed in hydraulic conductance experiments. Freehand cross sections were taken at 25 and 50 mm from the root tip using a total of six roots from at least 3 different plants. Suberin lamellae were detected by staining for 2 h with 0.1% (w/v) Sudan Red 7B (Sigma) then mounting in 75% (v/v) glycerol (Brundrett et al., 1991). Brightfield images were taken with a Zeiss Aixophot Pol Photomicroscope (Oberkochen, Germany).

RNA extractions
Grapevines were grown in a two pot system and the apical 50 mm of the roots from the bottom pot were carefully and quickly harvested, frozen in liquid nitrogen and stored at -70˚C. Replicate RNA samples were prepared from 350 mg of roots from three different plants per treatment. Roots were harvested every 4 h at 600, 1000, 1400, 1800 and 2000 h from well-watered Chardonnay vines. In two separate experiments roots were harvested from Grenache and Chardonnay vines that had been either well-watered, water-stressed for 10 days, or water-stressed for 10 days and then re-watered 24 h prior to harvest. Water stress was applied for 10 days to obtain midday Ψ_{stem} similar to those observed in hydraulic conductance experiments.

RNA was extracted with 5 M sodium perchlorate 0.2 M Tris pH 8.3, 8.5% (w/v) polyvinylpolypyrrolidone, 5% (w/v) SDS, 1% (v/v) β-mercaptoethanol for 30 minutes at room temperature. Samples were then processed with a modified protocol of the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) (Franks et al., 2006). Contaminating DNA was removed with Turbo DNase treatment for 20 minutes at 37ºC (Ambion, Austin, Texas, USA), and RNA was stored at -70ºC. Total RNA was quantified with a UV spectrophotometer. The presence of contamination from genomic DNA was tested for by RT-PCR.

Quantitative PCR

Primers for quantitative PCR were designed based on published sequences of aquaporins found in grapevines (Table M1), with the criteria of a melting temperature of 59 ± 1˚C, primer length of 20-24 base pairs (bp), a product size of 110-150 bp, and a GC content of 45-60%. To create stock solutions for each PCR product individual RT-PCR reactions were performed on total RNA extracted from well-watered Chardonnay roots. Amplified cDNA were separated on a 1.5% agarose gel, and correctly sized bands were excised and then eluted with the MinElute Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). This stock solution was used to create a dilution series covering five orders of magnitude ($x \times 10^{-3} - 10^{-7}$). Two replicates of each of the five standard concentrations were included with every Q-PCR experiment, together with no template controls. For *VvPIP1;1*, *VvPIP2;2* and *Vvactin* (reference gene) the concentration of each cDNA stock solution was determined using fluorescent PicoGreen Reagent (Invitrogen, Mount Waverly, VIC, Australia) with excitation at 480 nm and the emission at 520 nm, using a VersaFluor Fluorometer (Bio-Rad, Hercules, CA, USA) against a known DNA standard (Invitrogen, Mount Waverly, VIC, Australia).

For each RNA sample 1 µg was reverse transcribed using iScript^{TM}cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The thermocycler was programmed for 1 cycle of 5 min at 25 ˚C; 30 min at 42 ˚C; 5min at 85 ˚C. Q-PCR was performed with an iCycler (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 µL containing 10 µL SBYR Green Mix (Bio-Rad Hercules, CA, USA), 0.6 µM of primer and 1 µL cDNA. The PCR cycle profile was: 1 cycle of 2 min at 95˚C; 40 cycles of 30 s at 95˚C, 30 s at 57˚C, 15 s at 72˚C. Amplification data was collected during the extension step (72˚C). Melt curve analyses were made by elevating the temperature from 55˚C to 99˚C at a rate of 0.5˚C.s^{-1}. Only a single band with a characteristic melting point was observed for each sample indicating that the product was specific to the primers. Products were routinely checked by 1.5% (w/v) agarose gel electrophoresis.

To determine the relative gene expression of the eight aquaporins in the root tissue of well-watered Chardonnay, the method described by Muller et al. (2002) was used. In experiments examining diurnal variation and the impact of water stress, only changes in expression of *VvPIP1;1* and *VvPIP2;2* were determined. In these Q-PCR experiments standard curves using known amounts of cDNA were used to quantify the starting amounts of cDNA for each gene. The final value of relative gene expression is the ratio of the starting quantity of the gene of interest to the starting quantity of *Vvactin*, the reference gene, to account for differences in the original RNA concentration and the efficiency of cDNA transcription. *Vvactin* expression was not significantly different between treatments. For each treatment there were three biological replicates.

Table M1. Accession numbers of aquaporin genes and sequences of primer pairs used for Q-PCR.

Vitis Gene	Accession #	Forward/Reverse	Sequence
PIP1;1	EF364432	Forward	5'- AAGAGAAGAGAAGAGAGATGGAAGG -3'
		Reverse	5'- CACATTTCACAGCGTCACCT -3'
PIP1;2	EF364433	Forward	5'- CGCCATCGTCTACAACAAAG -3'
		Reverse	5'- CAGGCTCTGGTCTTGAATGG -3'
PIP1;4	EF364435	Forward	5'- TCTGTTTCTTCTTTTATTTGCTGCT -3'
		Reverse	5'- ATTCAAAAGCTGCCCATTGT -3'
PIP2;1	AY823263	Forward	5'- ACCTTCTCCTGAACCCCCTA-3'
		Reverse	5'- TCATGCCCTCATACATATCAATAAC -3'
PIP2;2	EF364436	Forward	5'- CCACGGTCATAGGCTACAAGAAG -3'
		Reverse	5'- CGAAGGTCACAGCAGGGTTG -3'
PIP2;3	EF364437	Forward	5'- GCCATTGCAGCATTCTATCA -3'
		Reverse	5'- TCCTACAGGGCCACAAATTC -3'
PIP2;4	EF364438	Forward	5'- TTCAGAAGCCTTTTGTACTGGA -3'
		Reverse	5'- GCAGATTGGAAGGCTTTGAC -3'
Actin	AM465189.1	Forward	5'- GCCTCCGATTCTCTCTGCTCTC -3'
		Reverse	5'- TCACCATTCCAGTTCCATTGTCAC -3'

Expression in Xenopus laevis oocytes

The cDNA of *VvPIP1;1* (accession EF364432) and *VvPIP2;2* (accession EF364436), obtained by RT-PCR, was cloned into the expression vector pGEMHE using the restriction enzymes *Bst* EII for *VvPIP1;1* and *Pvu* II for *VvPIP2;2*. PGEMHE carries the 5' and 3' untranslated sequences of the β-globin gene from *X. laevis* in order to promote translation efficiency of plant cRNA (Linman et al. 1992). The positive control, *HsAQP1* (accession P29972) was cloned into the vector pXBG using the restriction enzyme *Bgl* II. Capped cRNAs were synthesised from plasmids linearised with *Nhe* 1 for grapevine aquaporins and *Sma* I for AQP1 using a mCAP RNA capping kit (Stratagene, La Jolla, CA, USA). *X. laevis* oocytes were isolated and digested at room temperature for 70 minutes with 2 mg mL^{-1} collagenase in ND96 (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.5). Oocytes were defolliculated with a hypotonic buffer (100 mM KH_2PO_4-KOH, 0.1% BSA, pH 6.5), washed twice with ND96 and then with Ca-free Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.6). Prepared oocytes were stored in Ca Ringers (Ca-free Ringers + 0.6 mM $CaCl_2$) supplemented with horse serum (5%, Sigma Chemical Company) and antibiotics (100 units mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 0.05 mg mL^{-1} tetracycline) prior to injection (Nanoject II microinjector, Drummond Scientific Company, Broomall, PA, USA) with cRNA or sterile diethyl pyrocarbonate (DEPC)-treated water in a volume of 46 nL. The capillaries used were pulled in two stages with a capillary puller on heat settings 11.83 and 9 (Narishige Scientific Equipment Lab, Tokyo, Japan). There were 12 ng of either *VvPIP1;1* or *VvPIP2;2* injected or 12 ng of each injected together to create a 1:1 ratio. To create the 0.5:1, 2:1 and 3:1 ratios of *VvPIP1;1*:*VvPIP2;2* the amount of *VvPIP2;2* remained at 12 ng with the amount of *VvPIP1;1* adjusted accordingly. After injection oocytes were incubated in Ca-Ringers plus horse serum and antibiotics (as above) for 3 days at 18˚C. The osmotic water permeability was determined by transferring the oocytes to the same solution diluted fivefold (215 mOs to 43 mOs) and the changes in volume were captured with a Vicam colour camera (Pacific Communications, Melbourne, Australia) attached to a Nikon SMZ800 Microscope (Tokyo, Japan). Images were analysed using the computer programme Global Lab Image-2 (Data Translation, Marlboro, MA, USA), using the Blob Analysis Tool to determine the change in the total area of the oocytes captured in the AVI video file. The change in area was used to calculate change in volume assuming the oocytes were spheres. The osmotic water permeability (P_{os}) was determined from the initial rate of change of relative cell volume ($J_w = d(V/V_o)/dt$) using the equation $P_{os} = J_w / V_w \times A \times \Delta Osm$**,** where A = area of oocyte, ΔOsm = change in osmolarity and V_w = partial molar volume of water (18 mL mol^{-1}).

In situ hybridisation

DIG-labelled antisense and sense *VvPIP1;1* and *VvPIP2;2* probes were generated with a DIG RNA labelling kit as described by the manufacturer (Roche Diagnostics, Mannheim,

Germany), using template synthesised by in vitro transcription of PCR products with a T7 promoter sequence upstream (antisense) or downstream (sense), for each of the *VvPIP1;1* and *VvPIP2;2* fragments. Probes of 176 bp and 180 bp (*VvPIP1;1* and *VvPIP2;2*, respectively*)* were designed to target 3`UTR regions specific to each gene

Grenache and Chardonnay roots, sampled at the root tip, and 30 mm and 50 mm from the tip, were fixed for 2 h in FAA and processed as described in Sutton et al. (2007), with the following modifications. Probes were hybridised at final concentrations of 0.5 ng nL^{-1} and 1 ng nL^{-1} for *VvPIP2;2* and *VvPIP1;1*, respectively, in hybridization buffer (50% formamide, 2x SSC (standard saline citrate),10% dextran suphate, 1x Denhardt's solution, 1 µg $µl^{-1}$ tRNA). *In situ* hybridization was performed overnight at 50°C and 45°C for *VvPIP2;2* and *VvPIP1;1*, respectively, and washes were in 0.2 x SSC at the corresponding temperature. In addition to sense probe controls, controls were made with no probe. Images were taken with a Leica AS LMD microscope. *VvPIP2;2* longitudinal images were taken after a 3 h development, with the remaining images taken at 24 h.

Immunolocalisation
Custom designed KLH-peptide conjugated oligonucleotide sequences were synthesised and injected into New Zealand White rabbits to produce antibodies against all known plant PIP1s and all known PIP2s in grapevine (Sigma-Genosys, Castle Hill, NSW, Australia). For VvPIP1 we used a N-terminal sequence (GKEEDVRLGANKFPERQPIGSTAQ) and for VvPIP2 we used a C-terminal sequence (CRAGAIKALGSFRS).

Grenache and Chardonnay roots were sampled at the root tip, and 30 mm and 50 mm from the tip, fixed for 2 h in TEM fixative and processed, embedded and sectioned as described previously (Sutton *et al*., 2007). Sections were de-waxed for 2 x 10 mins in xylene and rehydrated through an ethanol series into phosphate-buffered saline pH 7.4 (PBS), then blocked for 30 min in 1% (w/v) bovine serum albumin in PBS. Primary antibody was applied for 1 h at room temperature, slides washed three times in blocking buffer, secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG (H+L), Molecular Probes, Eugene, OR, USA) applied for 1.5 h, slides washed three times in blocking buffer, then mounted in 90% glycerol:10% water (v/v). Controls were made with no primary and/or no secondary antibody. Images were taken with a Leica AS LMD microscope equipped with fluorescence filter N2.1 (excitation filter 515-560 nm BP, barrier filter 590 LP), with exposure standardized at 9 s.

Statistical analysis
Statistical analysis was performed using the statistics package, Genstat, version 6 (Numerical Algorithms Group, Oxford, UK). Differences were accepted as significant if $P < 0.05$.

5.2.2 Detailed material and methods for 6.2

Plant material and growth conditions
One year old grapevine (*Vitis vinifera* L.) rootlings, cv. Chardonnay were obtained from Yalumba Nursery (Nuriootpa, South Australia). Grapevines were grown in 20 cm diameter pots (4.7 L) and re-potted into 25 cm pots (9 L) 2-3 weeks prior to application of treatments to prevent the grapevines from becoming root-bound. Soybean (*Glycine max* L. cv. Stephen) and maize (*Zea mays* L. cv. Early Chief) were grown from seed in 20 cm diameter pots. Plants were grown in University of California soil mix: 61.5 L sand, 38.5 L peat moss, 50 g calcium hydroxide, 90 g calcium carbonate and 100 g Nitrophoska® (12:5:1, N:P:K plus trace elements), per 100 L at pH 6.8. Pots were placed in a temperature-controlled greenhouse with supplementary light. Night/day temperatures were maintained at approximately 19/24ºC. Pots were watered to field capacity every 2 days. Unless stated, grapevines were grown from one-year old rootlings and were approximately 4 months old. Soybeans were used 7-8 weeks after sowing and maize was used 8 weeks after sowing.

Additional grapevines were grown in a two pot system to obtain roots for RNA extractions. The top pot, with holes in its base and covered with plastic netting, contained UC mix, the bottom pot contained a 50:50 mix of vermiculite and perlite. Roots grew into the bottom pot,

enabling them to be sampled easily when the top pot was raised. An additional 25 g Nitrophoska® was applied to the top pot approximately every 3 months.

Stomatal conductance and transpiration
A Leaf Chamber Analyser, Type LCA-4 (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) was used to measure the transpiration and stomatal conductance of leaves at nodes 7, 8 and 9 prior to being sampled at midday. A section of each leaf was placed in the broad leaf chamber whilst still attached to the plant. Measurements of CO_2, H_2O and light intensity were made via an infrared gas analyser (IRGA) arrangement in the LCA-4 system. Ideally, measurements were taken in cloud-free situations. Measurements were taken once the sub-stomatal CO_2 concentration had reduced and stabilised.

Hydraulic conductance
Hydraulic conductance measurements were taken with a Hydraulic Conductance Flow Meter (HCFM) (Dynamax, Houston, Texas, USA) as detailed in Vandeleur *et al.* (2008). Hydraulic conductance (L_o) was normalised by dividing the conductance by the total root dry weight. L_o was measured between 1300 and 1500 h. The soil was washed from the roots before drying at 60ºC for > 48 h. The leaf area was measured with an Area Meter (ADC BioScientific Ltd. Hoddesdon, Hertfordshire, UK) before the shoot was also dried at 60ºC for > 48 h.

RNA extractions and Quantitative PCR
RNA extractions and quantitative PCR were performed as described in Vandeleur *et al.* (2008). Root samples used for RNA were obtained from Chardonnay plants grown in two-pots either shoot-topped or not 24 h prior to sampling. Shoot topping removed 30-40% of the leaf area. There were three biological replicates for each treatment.

Main Treatment
When plants were shoot-topped, the main stem and any laterals were cut to remove approximately 35-40% of the leaf area; this included all young expanding leaves and shoot tips. In all cases the basal section of laterals and the main stem remained. This was done at 1200 h the day preceding all measurements. In the first experiment there was an additional treatment of shoot topping performed 5 days before the measurement of L_o of grapevine roots. All treatments were applied in a completely randomised design.

Degree of shoot topping

There were 4 shoot topping treatments: a) removal of shoot tips only; b) removal of tips and young expanding leaves; c) removal of 35% of leaf area, including young leaves and tips; d) removal of 70% leaf area, including young leaves and tips (Fig. 1). In all cases the main stem and any laterals were cut. This experiment was first performed using grapevines and repeated using soybeans.

Ethylene
The leaves of the soybean plants were sprayed with 10 mM AVG (trans-2amino-4(2aminoethoxy)-3-betenoic acid hydrochloride) and 0.1% v/v Tween, prior to half the sprayed plants being shoot-topped to remove 35% of the leaf area. There was also a group of unsprayed plants, half of which were shoot-topped. This experiment was a two-way factorial design.

Abscisic acid
Two-pot grapevine plants were used to sample young roots (apical 5 cm) 24 h after plants were shoot-topped to remove 30-40% of leaf area. The roots of control and shoot-topped Chardonnay vines were harvested, weighed immediately and frozen in liquid nitrogen. Frozen tissue was ground into a powder in liquid nitrogen using a mortar and pestle. The powder was placed in a pre-weighed and pre-chilled glass centrifuge tube and weighed to determine the sample weights. ABA extraction was performed by adding 5 mL of boiling water to the tube and placing the tube in a boiling water bath for a further 10 min (Loveys & van Dijk 1988). The samples were cooled on ice and then 100 ng D_6-ABA internal standard was added. Samples were centrifuged (200 g × 3 min) and the supernatant collected. The supernatants were adjusted to pH 2.5 with 1 N HCL and loaded in 5 mL volumes onto Strata

–x 33 µm Polymeric Sorben 500mg/6mL columns (Phenomonex, Australia), which were conditioned according to the manufacturer's instructions. After loading, the columns were washed with 5 mL of 5% methanol which was discarded and then the ABA fraction was eluted with 5 mL of 100% methanol. The samples were injected onto the HPLC (HP 1100 series) using a Merck Hibar LiChrospher 100 RP-18 ODS 5 µm column (Adelab Scientific, Adelaide, Australia). The sample was eluted using a linear methanol/water (with 0.2% acetic acid) gradient running from 30-100% methanol over 11.8 min. The purified ABA fraction was collected with a fraction collector attached to the HPLC. The samples were then analysed by gas chromatography/ mass spectrometry (Provisor).

5.2.3 Detailed material and methods for 6.3

Plant growth conditions
One year old *Vitis vinifera* (L.) rootlings, cv. Chardonnay and Grenache were obtained from Yalumba Nursery (Nuriootpa, South Australia). Grapevines were grown in 20 cm diameter pots. Before planting all roots were removed except two strong primary roots opposite each other on the cane. Once established the grapevines were repotted into two 20 cm diameter pots, with the two separate root systems split between the pots. The top of the root was exposed and the base of the cane sat on the edge of the two pots. The cane was supported with a stake. Ideally there was a single shoot growing facing each pot. The grapevines were grown in University of California soil mix: 61.5L sand, 38.5L peat moss, 50g calcium hydroxide, 90g calcium carbonate and 100g Nitrophoska® (12:5:1, N:P:K plus trace elements), per 100L at pH6.8 . The pots were placed in a temperature controlled greenhouse with supplementary light. The night/day temperatures were maintained at approximately 19/24ºC. Pots were watered to field capacity every 2 days. Water stress was induced by withholding water for 8 days when the grapevines were approximately 3 months old. Soil water content was monitored using Time Domain Reflectometery (TDR). Measurements for the two cultivars were taken when pot water loss was similar. The soil water content at the conclusion of the experiments was determined volumetrically.

The leaves at nodes 7, 8 and 9 (from the apex) were sampled to measure ψ_{leaf} with a Scholander pressure bomb and xylem sap was collected from the petioles of the same leaves for determination of ABA. Leaves in the same position on each plant were used as a gradient in the xylem sap ABA concentration was found along the length of grapevines by Soar *et al*. (2004). The sap was stored at -70ºC. A Leaf Chamber Analyser, Type LCA-4 (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) measured the transpiration and stomatal conductance on the leaves prior to being sampled at midday.

Hydraulic conductivity
Hydraulic conductance measurements were taken with a Hydraulic Conductance Flow Meter (HCFM) (Dynamax, Houston, Texas, USA). The HCFM has been shown to give hydraulic conductance values similar to the pressure chamber (Tyree *et al*., 1995) and the evaporative flux method (Tsuda and Tyree, 1997 & 2000). The grapevine stem was cut above the soil surface and the stump was connected to the HCFM with a water tight seal. Transient measurements involved increasing the pressure from 0 – 0.5 MPa at a rate of approximately 7 kPa s^{-1}. The water flow was measured by the instrument every few seconds. The hydraulic conductance, L_o, was calculated as the slope of the plot of the water flow versus the pressure. The conductance of the whole plant was measured. Then the seal was attached to the root system in a single pot. All measurements were taken between 1pm and 3pm. The soil was washed from the roots before drying at 60ºC for > 48 hrs. The leaf area was measured with an Area Meter (ADC BioScientific Ltd. Hoddesdon, Hertfordshire, UK) before the shoot was also dried at 60ºC for > 48 hrs. The hydraulic conductance was normalised by dividing the conductance by relevant root dry weight.

ABA determination
The sap samples were thawed and weighed before adding 100 µL of methanol containing 9.727 ng of deuterated ABA as an internal standard. The samples were dried under vacuum with a Savant SC110A speed-vac plus (New York, NY, USA). The residue was redissolved

in 50 µL of acetone followed by the addition of 50 µL ethereal diazomethane. The samples were covered for 20 minutes and then air dried for 20 minutes. The residue was redissolved in 100 µL acetone and the samples centrifuged (30 seconds at 10 000 g). The supernatant was transferred to a 200 ìL gas chromatography vial, dried then redissolved in 20 µL acetone from which 1 ìL was analysed by gas chromatography/ mass spectrometry. The analysis was performed with an AGILENT GC-MS system with a ZB-5 W/Guardian column (Phenomonex, Lane Cove, NSW, Australia). The ion pairs 190/194 and 162/166 were monitored using the selective ion monitoring mode (SIM). The values were adjusted for the initial sample volumes.

Field experiments
Two field experiments were performed on Chardonnay vines in the Alverstoke Vineyard University of Adelaide in January 2008 and October 2008. Trenches were dug adjacent to the vines, filled with sand and drip irrigated to encourage root growth in to the sand (Fig 5.1). One month before the experiments a sharp spade was used to sever old roots and the trench was fertilised to encourage new roots to grow into the trench. This allowed easier sampling of young roots, analogous to the pot experiments for gene expression studies. The vines were set up so that 4 vines were used as control (no pruning) and 4 adjacent vines were pruned. Sap flow sensors (Green Span) were installed in each vine and midday and pre-dawn stem water potentials were measured before and after pruning (approx 30% leaf removal). Stomatal conductance and transpiration was measured with an ADC infra red gas analyser. Total vine hydraulic conductance was estimated as the sap flux divided by the difference between pre-dawn and midday leaf water potentials.



Figure 5.1 Trenches dug beside control and treated vines for rapid sampling of roots after shoot pruning. River sand was filled in to the trenches and drippers maintained irrigation to the trenches so that roots would be encouraged to grow into the trenches. The trenches were dug in the season prior to the experiments. This technique of root sampling was obtained from Brian Loveys and Jim Spiers.

Statistical analysis
Statistical analysis was performed using the statistics package, Genstat, version 6 (Numerical Algorhthyms Group, Oxford, UK).

5.2.4 Detailed materials and methods for 6.4

Plant material
Grapevine cuttings of two varieties, K 51-40 and 1103 Paulsen, were collected from the Coombe vineyard at Waite Campus, University of Adelaide, Australia. K 51-40 is salt sensitive (Nicholas, 1997) and originates from *Vitis champini x Vitis riparia*. 1103 Paulsen is a salt tolerant variety (Walker et al. 2004) and originates from *Vitis berlandieri x Vitis rupestris*. The basal parts of stem cuttings were immediately soaked in IBA (0.1 % w/v in 50% ethanol) for 5-10 s. They were fixed in a growing medium (a sheet of the engineered

foam root cubes) and put in a growth chamber (humid heat bed) to initiate roots. After 3-4 weeks, rooted cuttings were transferred to plastic pots with sand or hydroponic medium containing ¼ strength Hoagland solution depending on experimental design.

NO_3^-/Cl^- interaction

For the first 3-4 weeks the plants in pots were watered with ¼ strength Hoagland solution containing 0.25 mM $NH_4H_2PO_4$, 1.5 mM KNO_3, 1 mM Ca $(NO_3)_2$, 0.5 mM $MgSO_4$, and micronutrients 576.25 µM H_3BO_3, 114.25 µM $MnCl_2$, 9.5 µM $ZnSO_4$, 4 µM $CuSO_4$, 3 µM H_2MoO_4 and 88 µM iron tartrate (this composition was used in all experiments), to produce a single stem with 3-4 leaves. In order to increase N demands of the plants and also to measure net fluxes of ^{15}N later, plants were irrigated with N-depleted ¼ Hoagland solution (0.25 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.5 mM KCl and micronutrients as mentioned above) for another 3 weeks. Salinity (NaCl) was imposed as 15, 30 and 45 mM in ¼ strength Hoagland solution lacking N, for 7 days. The above mentioned range of salinity was selected according to the previous studies carried out on grapevine (i.e. Walker et al. 2004). After pre-treatment with NaCl, the plants were exposed to 4 mM $K^{15}NO_3$ in Hoagland solution lacking N compounds plus specified NaCl concentrations for 3 days to measure net fluxes of nitrate. During the ^{15}N treatment, all pots were irrigated with 300 mL of the solution two times per day to field capacity (pots drained).

Determination of NO_3, Cl and ^{15}N

At the end of the experiment (1 or 2 days after ^{15}N treatment period) plants were harvested and plant parts including leaves, stem, cane and roots were weighed separately and dried at 70^oC for 48 hours. Dried plants were well ground to increase sample homogeneity by a grinder (Labtech Essa LM1-P).

100 mg of ground plant materials were put in plastic vials containing 10 mL distilled water. The vials were boiled in a hot water bath for 20 min. After cooling the vials, their contents were transferred to 10 mL plastic tubes to centrifuge at 5000xg for 15 min. Then supernatants were poured in new 10 mL plastic tubes and made up to 10 mL with distilled water. The extracts were stored in a fridge until doing NO_3^- and Cl^- measurements.

Chloride concentration in water extracts was determined by silver ion titration using a chloride analyzer (Corning 926). The instrument was adjusted by standard solution (200 mg Cl/L). Then 0.5 ml of the above mentioned extracts was injected into the titration solution containing combined acid buffer. Cl^- concentration was read in mg L^{-1} and converted to mg Cl^- content per gram dry weight of the tissue.

Total nitrate concentration was measured using a spectrophotometer according to the modified method of Cataldo, (1975). Extracts (0.5 mL) were pipetted into 10 mL plastic tubes containing 0.3 mL 5% salicylic acid in concentrated H_2SO_4. After 10 minutes at room temperature, 7.2 mL NaOH (2M) was gently added to tubes. NaOH helps to make a solution with pH above 12 which is suitable for NO_3^- and acid interaction. Absorbance at 410 nm of each sample was measured by a spectrophotometer (BIO-RAD SmartSpec 3000). Standards containing 5, 10, 15, 20, 25 and 30 µg NO_3^--N in a 0.5 mL aliquot were analyzed with each set of samples. Blank solution was made of 0.5 mL H_2O, 0.3 ml 5% salicylic acid in concentrated H_2SO_4 and 7.2 mL NaOH (2N).

^{15}N contents of the tissues was determined using a mass spectrometer (Geo 20-20, Europa Scientific). 4-4.5 mg of well powdered plant parts was weighed and put in a small tin capsule (4x6 mm). The capsules were folded several times and put inside the sinks on a tray. References including 25 capsules of EDTA (0.9-1.5 mg) per 100 samples, 15 capsules of ammonium sulphate (20 uL of 0.05897 g in 2.5 mL distilled water) per 100 samples and 15 capsules of glycine (20 uL of 0.06699 g in 2.5 mL distilled water) per 100 samples were analyzed with each set of samples. ^{15}N contents of the plant tissues were measured as atom % :

$$\text{atom \%} = 1/(1+(^{15}N/^{14}N)) \times 100 \qquad \text{(Eq. 1)}$$

and then were calculated as μg g^{-1} (dry wt). All data were analysed statistically and plotted with GraphPad Prism-4 program.

Measurement of root hydraulic conductivity (Lpr) and reflection coefficient (σs)
Cuttings of K 51-40 and 1103 Paulsen were provided as described above. Rooted cuttings were transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution. After 1-2 weeks, the main roots of the plants (rooted cuttings with 3-4 leaves) before lateral roots were used in pressure probe experiments and root anatomical studies.

Water and solute transport between the external medium and the xylem were measured using a root pressure probe. Unbranched root segments (50-100 mm) from apical end were cut under the culture solution. The cut end of a segment was connected to the pressure probe using a silicone seal (about 10 mm long) provided from silicone material (Optosil-Xantopren, Heraeus Kulzer). In order to prevent solution leakage, the seal around the root was tightened gently by a screw around silicon seal until the root pressure started to increase. The capillary between probe and root was filled with distilled water and silicone oil and it was checked that no air bubbles were left in the system. A meniscus, formed between water and oil, was monitored using a microscope. Steady state root pressure (0.3-0.7 bars) was obtained after 10-20 min. After each experiment the root at the seal was cut and the fast decrease in pressure showed the proper functioning of the seal. 4-7 replicates were used in each experiment depends on the experiment conditions. The solutions used in all the experiments were circulated by a peristaltic pump along the root segments connected to the pressure probe.

In hydrostatic experiments, radial water flow was induced across the root by moving the meniscus either forward (exosmotic water flow caused by an increase in the xylem pressure) or backward (endosmotic water flow caused by a decrease in the pressure of the xylem) using a movable metal rod in the pressure probe. Doing hydrostatic pressure relaxation, water rate constant (K_{wr}) or relaxation time ($T_{1/2}$) was obtained and from which hydraulic conductivity (Lp_r) of a given root was calculated using the following equations (Steudle & Jeschke, 1983):

$$K_{wr} = \ln(2) / T_{1/2} \qquad \text{(Eq. 2)}$$

$$Lp_r = K_{wr} / A_r . \beta \qquad \text{(Eq. 3)}$$

Where, A_r is the geometric root surface area and β is the elastic modulus. A_r was calculated from the root length and diameter.

The root elastic modulus (β) was estimated by moving the metal rod by a certain amount, in the root pressure probe instantaneously determining the change in volume and recording the changes in the pressure.

In osmotic experiments with different permeating solutes (NaCl and $NaNO_3$), root pressure relaxations are usually biphasic (Steudle & Jeschke, 1983). The first rapid phase (water phase) is attributed to the change in osmotic pressure of the external medium. The second (slower) phase (solute phase) is attributed to the passive transport of the solutes into or out of the root. It in turn causes water uptake resulting from changes in the osmotic gradient across the root. Osmotic Lp_r was estimated from the first phase of relaxation curves using Eq. 3.
The reflection coefficient (σ_r) was calculated using the following relationships (Azaizeh & Steudle, 1991):

$$\sigma_s = \frac{\Delta P}{\Delta \pi} . \frac{\varepsilon + \pi_i}{\varepsilon} \exp(K_s . t_{\min}) \qquad \text{(Eq. 4)}$$

Where, σs is the reflection coefficient of the root, ΔP is the maximum change of root pressure P_{ro}-P_{min}, Δπ is the change of osmotic pressure in the external medium of

permeating solute (in this experiment NaCl or $NaNO_3$), ε is the elastic modulus of the xylem, π_i is the osmotic pressure in the xylem, K_s is the rate constant for the solute phase, τ_s is the time constant for the external solute flow, and t_{min} is the time of pressure reduction to a minimum level. It is assumed that the factor ($\varepsilon + \pi_i / \varepsilon$) is equal to unity because ε is much bigger than π_i of the xylem.

Anatomy
Root cross sections were made at 20-50 mm behind the root tip of K 51-40 and Paulsen. The plants were grown in hydroponics as mentioned in water relation section . Hand-cut root sections were stained using the berberine-aniline blue fluorescent staining procedure (Brundrett et al. 1988). The sections were put in mesh-bottomed holders and then transferred into staining plates. They were stained with 0.1% (w/v) berberine hemisulphate in distilled water for 1 h. The sections in the holders were rinsed with distilled water several times. The holders were transferred into 0.5% (w/v) aniline blue in distilled water for half an hour and then rinsed as above. The sections were placed in 0.1% $FeCl_3$ in 50% (v/v) glycerol for 2-3 min and then were put on slides and mounted in the same solution. A fluorescent microscope (Zeiss, Axiophot Pol, Photomicroscope) was used to observe the mounted sections.

Measurement of by-pass flow
Plants with 3-4 leaves (provided as described in plant material) were transferred to black plastic pots containing different solutions depending on experimental design.

Three different treatments with 4 replications were considered in this experiment:
1) control plants grown in ¼ strength Hoagland solution without NaCl and PTS; 2) plants that were grown in ¼ strength Hoagland solution with NaCl (30 mM) without PTS; 3) plants that were grown in ¼ strength Hoagland solution with NaCl (30 mM) + PTS (0.0125 % w/v). All pots were aerated and carefully sealed using plastic bags to prevent evaporation from the lids. Plants were grown in the above mentioned media for 4 days in glasshouse conditions. The pots were weighed before and after the four days incubation of the plants in the solutions to estimate total transpiration.

Chloride contents in different parts of the plants were extracted and measured as mentioned before (determination of NO_3, Cl and ^{15}N section). PTS concentrations in root, stem, cane and leaves were measured using a fluorescence spectrometer (excitation wavelength: 405 nm; emission wavelength: 510 nm). PTS extraction was carried out using the chloride extraction procedure mentioned in determination of NO_3, Cl and ^{15}N section.

Statistical analysis
In this study, all means are given with the Standard Error of the means (SEM). Error bars on graphs are SEM. When required significance tests were performed. These were either t-test (for equality of variance in each test), one-way analysis of variance with post tests for differences between means, or two-way analysis of variance with post tests. Different letters were used to indicate significant difference between the compared means within the columns. Significance level was $P< 0.05$ for all tests.

5.2.5 Detailed materials and methods for 6.5

Plant material
Grapevine cuttings of two varieties, K 51-40 and 1103 Paulsen, were collected from the Coombe vineyard at Waite Campus, University of Adelaide, Australia. K 51-40 is salt sensitive (Nicholas, 1997) and originates from *Vitis champini x Vitis riparia*. 1103 Paulsen is a salt tolerant hybrid (Walker et al. 2004) and originates from *Vitis berlandieri x Vitis rupestris*. The basal parts of stem cuttings were immediately soaked in indol 3-butyric acid (IBA) (0.1 % w/v in 50% ethanol) for 5-10 s. They were fixed in a growing medium (a sheet of the engineered foam root cubes) and put in a growth chamber (humid heat bed) to initiate roots. After 3-4 weeks, rooted cuttings were transferred to hydroponic medium containing ¼ strength Hoagland solution composed of 0.25 mM $NH_4H_2PO_4$, 1.5 mM KNO_3, 1 mM Ca

$(NO_3)_2$, 0.5 mM $MgSO_4$, and micronutrients 576.25 µM H_3BO_3, 114.25 µM $MnCl_2$, 9.5 µM $ZnSO_4$, 4 µM $CuSO_4$, 3 µM H_2MoO_4 and 88 µM iron tartrate.

In order to provide root material in a shorter time, leaf cuttings were also made from grapevines as described by Schachtman & Thomas (2003). The leaves with lignified petioles were selected, excised and immersed in 0.1% IBA for 5-10 s. They were then placed in a tray containing a moist medium of 50% vermiculite and 50% perlite. The lamina was located above the surface of the tray. The tray was put on a heat bed and covered with a clear dome to maintain high humidity around the shoot. The cuttings were watered with ¼ strength Hoagland solution. After 2 weeks callus and root initiations began to appear.

Rooted cuttings (stem and leaf) were then transferred to an aerated culture solution in plastic containers, containing ¼ strength Hoagland solution. Plants were pretreated with NaCl (20 and 30 mM depending on the experiment) for 5-7 days before used in flux experiments.

Measurements of $^{36}Cl^-$ influxes

Influx of $^{36}Cl^-$ in root segments (8-12 cm and including lateral roots and the tip) from plants pretreated for 5-7 days with 30 mM NaCl in ¼ Hoagland solution, were soaked in aerated loading solutions labelled with $^{36}Cl^-$ (9.25 MBq, 250 µCi, Amersham Biosciences) for different periods to determine the time over which unidirectional influx could be determined. Preliminary experiments developed a rinsing procedure that indicated removal of the apoplastic fraction of ^{36}Cl as follows: After the loading period, the root segments were transferred to aerated ice-cold non-labelled solutions in series for 1 and 5 min. After 1 min washing, the root segments were well blotted and then put in the second washing solution for 5 min. After rinsing root segments were blotted and weighed and put in 5 ml plastic scintillation vials containing 2 ml SDS (10% w/v) and boiled for 20-30 min in a water bath. After cooling, 3 ml scintillation liquid was added to the vials. Each vial was counted by a liquid scintillation counter (TRI-CARB 2100TR, PACKARD). Specific activity of $^{36}Cl^-$ in the external solution (S_o) which is defined as counts per minute (cpm) per µM of Cl^- in the external medium, and was the same for all experiments. Influx was calculated as tissue content (counts per gram fresh weight of tissue / counts per µmole of chloride in the external solution) divided by the loading time.

Concentration kinetics of unidirectional ^{36}Cl influx were performed on root segments that were pre-treated for 5-7 days with NaCl (30 mM) and then were placed for 10 min in the required concentrations of NaCl (1, 3, 5, 10, 15, 20, 30 mM) labelled with $^{36}Cl^-$. Rinsing, extraction and counting were performed as described above.

$^{36}Cl^-$ influx by main and lateral roots were separately estimated. Plants were pre-treated in NaCl (5 days in 30 mM and the last 24 h in 20 mM). Influx was nearly the same for 20 and 30 mM external concentration of NaCl therefore to prevent excessive build up of salt in the plants over a reasonable acclimation period, the 20 mM treatment was selected. The root segments were placed in the loading solution for 10 min and were then washed, blotted, weighed and counted the same as in previous experiments.

Uptake by rooted leaves was used as a surrogate for root to shoot $^{36}Cl^-$ transport. The plants were pre-treated with NaCl as described above and were loaded in a solution labelled with $^{36}Cl^-$ for various times (1, 3, 6 h). After the loading periods, root, lamina and petiole were separated, weighed, extracted and counted by liquid scintillation counting. Roots were rinsed, extracted and counted as above.

Efflux of $^{36}Cl^-$

To measure $^{36}Cl^-$ efflux from the whole root, intact plants (pretreated leaf or stem cuttings) were loaded with $^{36}Cl^-$ for 12 h under light and then transferred to aerated successive washing solutions for varying time periods (1-720 min) after 5 min wash in a non-labelled solution. Within 12 h, the cytosolic compartment, as well as the vacuole are labelled by $^{36}Cl^-$ and there will be enough time for the isotope to be transported to the shoot (Davenport et al. 2005; Cram & Laties 1971; Britto et al. 2004). The radioactivity in the rinse solutions and that remaining in the root and shoot (if applicable) tissue were counted. Data were plotted as $^{36}Cl^-$

remaining inside the root tissue and ion fluxes into / out of each root compartment were calculated using compartmental analysis (Walker & Pitman 1976).

$^{36}Cl^-$ efflux from lateral roots was measured after loading the pre-treated intact plants with $^{36}Cl^-$ for 12 h, lateral roots of each plant were separated from the main root and floated in aerated successive washing solutions for varying time periods (1-720 min) after 5 min wash in a non-labelled solution. Because of their small size and in order to handle them easily and quickly, the lateral roots of each plant were put inside a mesh-bottomed holder. The radioactivity counting and data calculation and plotting procedure was the same as for whole roots.

Analysis of efflux experiments

Data were analysed assuming a two compartment model for efflux to the external medium (cytoplasm and vacuole) and were analysed fitting to the sum of two exponentials (Walker & Pitman 1976). There will also be a component to the xylem (Pitman, 1972). The final slower part of the efflux curve is defined as vacuolar efflux (the slowest efflux component) and by extrapolation to zero time, the amount of $^{36}Cl^-$ in the vacuole at the beginning of the efflux can be determined. The efflux from the cytoplasm (the faster-exchanging component) is the first part of the curve and theoretically is defined as subtraction of the vacuolar part from the total tissue contents. It was assumed that the free space component of efflux was negligible because of the 5 min wash in a non-labelled solution before the main elution process (Macklon, 1975). Parameters obtained from two exponential fitting analysis of the curves were used to calculate $^{36}Cl^-$ fluxes. The amount of radioactivity in the vacuole (the intercept of the slowest efflux component or I_v) and that of cytoplasm (the intercept of the faster efflux component or I_c), the rate constant of the cytoplasmic efflux (K_c) and the rate constant of the vacuolar compartment (K_v) were used to calculate $^{36}Cl^-$ influx from outside to cytoplasm (Φ_{oc}), influx from cytoplasm to the vacuole (Φ_{cv}) , net influx (Φ_{net}), and efflux from cytoplasm to outside (Φ_{co}), and to the xylem vessel (Φ_{cx}) and from the vacuole to cytoplasm (Φ_{vc}) using the equations given by Macklon (1975), Macrobbie (1981), Walker & Pitman (1976). Assuming some parameters, it is possible to calculate the cytoplasmic Cl^- concentration in terms of mM/L. If cell water content (CW) is 0.8 ml g^{-1} FW and the fraction of cytoplasm as cell water (Cyt) is 0.1 ml g^{-1} FW (Dracup et al. 1989). To calculate tracer efflux from the cytoplasm to the xylem (Φ_{cx}), the slope of the linear regression of $^{36}Cl^-$ uptake to the shoot was used to indicate the rate of the tracer release to the xylem (Davenport et al. 2005).

6. Results & Discussion

6.1 The role of PIP aquaporins in water transport through roots: diurnal and drought stress responses reveal different strategies between isohydric and anisohydric cultivars of grapevine.

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6.6.1 Introduction

Root hydraulic conductance is usually lowest within the liquid component of the soil-plant-air continuum. The hydraulic conductance of roots can be highly variable in both time and space, which will impact on soil-water extraction and shoot water status (Steudle and Peterson, 1998; Steudle, 2000a,b). Steudle (2000a,b) explains variation in root hydraulic conductivity (L_p, hydraulic conductance normalised to root surface area) in terms of the composite transport model based on the composite anatomical structure of roots where water can move radially towards the xylem along three pathways; the apoplastic, symplastic and transcellular. The symplastic and transcellular pathways are difficult to separate experimentally and are collectively considered as the cell-to-cell pathway (Steudle, 2000b). The extent to which water flow predominates in either pathway will vary according to the relative hydraulic conductances of the pathways and the relative magnitude of hydrostatic versus osmotic gradients (Steudle, 2000a; Bramley et al., 2007a). Apoplastic flow can be altered irreversibly by anatomical changes, including Casparian bands and suberin lamellae (Steudle and Peterson, 1998). The conductance of the cell-to-cell pathway can be largely determined by the activity of aquaporins within the series array of membranes, which results in changes in conductance that can be relatively rapid and reversible. An example is the rapid reduction in root hydraulic conductance of Arabidopsis roots to anoxia, which is considered to be caused by inhibition of plasma membrane aquaporins due to reduced cytoplasmic pH (Tournaire-Roux et al., 2003; Alleva et al., 2006).

Aquaporins are members of the major (membrane) intrinsic protein (MIP) family. They are highly hydrophobic proteins with six membrane spanning domains and molecular weights of 26-34 kDa. In the genomic sequence of Arabidopsis 35 aquaporins have been identified (Johanson et al., 2001) while 28 are evident in the *Vitis* genome (Fouquet et al., 2008). The proteins are divided into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs). The PIPs are further divided into two subclasses. In general PIP1s have little or no water channel activity *in vitro*, whereas the PIP2s show high water permeability when expressed in *Xenopus laevis* oocytes (Chaumont et al., 2000). Water permeability of aquaporins can be regulated by cytosolic pH and pCa (Gerbeau et al., 2002; Alleva et al., 2006), phosphorylation (Maurel, 1995; Johansson et al. 1998), large pressure pulses (Wan et al., 2004), and osmotic solutes (Ye et al., 2004; Vandeleur et al., 2005). It has been demonstrated that PIP1 and PIP2 members may interact either within the membrane or by targeting to the plasma membrane (Fetter et al. 2004; Zelazny et al., 2007).

Root L_p has been shown to vary diurnally in *Lotus japonicus* and sunflower (*Helianthus annuus*) (Henzler et al., 1999; Tsuda and Tyree, 2000). Diurnal regulation of L_p has been associated with aquaporin gene expression (Henzler et al., 1999; McElrone et al., 2007). There can be a delay between changes in expression and subsequent changes in hydraulic conductivity (Lopez et al., 2003). Yamada et al. (1997) detected diurnal variation in *MIP* expression in the leaves of *Nicotiana excelsior*, while diurnal regulation was also observed in the permeability of motor cell protoplasts in relation to AQP2 expression of *Samanea saman* (Moshelion et al., 2002).

Root L_p is usually reduced when soil dries (North and Nobel, 1991; 1996). During drying conditions, L_p of roots of *Agave deserti* declined, partly because of collapse of cortical cells, increased suberisation, and embolism in xylem vessels (North and Nobel, 1991). Roots also shrink as a result of cortical cell collapse, which reduces contact between soil and roots. Use of mercuric chloride has demonstrated down-regulation of aquaporins in water-stressed desert plants and aspen seedlings (Martre et al., 2001; Siemens and Zwiazek, 2003; North et al., 2004). Gene expression studies in various plant species have shown variable response of aquaporin isoforms to water stress, with both up- and down-regulation of genes evident (Yamada et al., 1997; Mariaux et al., 1998; Sarda et al., 1999; Suga et al., 2002; Jang et al., 2004; Alexandersson et al., 2005). In leaves, roots and twigs of olive (*Olea europaea* L.) *OePIP1;1, OePIP2;1* and *OeTIP1;1* were significantly reduced 3 and 4 weeks after water was withheld (Secchi et al., 2007). Overexpression of *AtPIP1b* in transgenic tobacco plants caused plants to wilt faster when water was withheld (Aharon et al., 2003). In contrast Siefritz et al. (2002) observed reduced resistance to water stress in antisense tobacco plants with reduced expression of *NtAQP1*, the homologous aquaporin.

Grapevines have now become a model system for fruit trees (Troggio et al., 2008) based on the ease of clonal plant propagation and full genome sequence availability (Jaillon et al., 2007). There is also considerable phenotypic and genetic variation between cultivars of grapevine that are advantageous in comparative physiology and molecular studies (Debolt et al., 2006; Tilbrook and Tyerman, 2008). Grapevines contribute substantially to economies, and in order to achieve high fruit quality and efficient water use, deficit irrigation techniques are commonly used. These result in various degrees of water stress with roots exposed to cycles of drying and wetting over time scales ranging from diurnal to several days or weeks (Dry and Loveys, 1998).

In this paper we have undertaken a comparative study between the two cultivars Grenache and Chardonnay to determine to what extent the cell-to-cell pathway and aquaporins affect changes to root hydraulic conductance in response to time of day and water stress. We expected changes in aquaporin expression to match changes in whole root and cell hydraulic conductivity in a genotype x environment dependent manner, broadly reflecting the different strategies to drought stress in the two cultivars. Grenache has been shown to be near-isohydric, exerting a tight regulation of stomatal aperture that may contribute to drought tolerance (Schultz, 2003; Soar et al., 2006), and is considered to be more drought tolerant than Chardonnay (Alsina et al., 2007). Gibberd et al. (2001) presented data for transpiration efficiency and transpiration per unit leaf area for a number of cultivars including Grenache and Chardonnay, grown in the same well-watered glasshouse conditions. Grenache had a much lower transpiration rate per unit leaf area than Chardonnay, and shared similar characteristics of transpiration efficiency to that of some *Vitis* hybrids that are known to be drought tolerant.

We examined root hydraulic conductance (normalised to root dry weight, L_o) induced by water stress, taking into account variation that may be linked to transpiration rate of the shoots, to see if changes in L_o were consistent with observed changes in root apoplastic barriers, cortical cell hydraulic conductivity, and the mRNA expression of PIP aquaporins. We functionally characterised the two most highly expressed PIP aquaporins in the root (*VvPIP1;1* and *VvPIP2;2*) by expression in *Xenopus* oocytes to determine whether they interact, and we examined the sites of expression and protein location in the root for evidence of co-location.

6.1.2 Results

Variation in hydraulic conductance of whole root systems

There was a large degree of variation in the root L_o (normalised to root dry weight) of well-watered grapevines between experiments (different batches of plants grown at different times), and during a day within a batch of plants grown under the same conditions. During a 24-hour period, L_o of well-watered Chardonnay vines was measured five times; it varied diurnally, peaking in the middle of the day before declining during the evening (Fig. 6.1.1A). By combining L_o values of well-watered Chardonnay and Grenache plants from all experiments described in this paper, a positive relationship was observed between transpiration rate (E) measured before plants were harvested and root L_o (Fig. 6.1.1B). There

was no significant difference between cultivars in the regressions of L_o versus E and the regression line for the combined data is shown in Fig. 6.1.1B. Grenache also showed a similar diurnal variation under well-watered conditions (Fig. 6.1.2)

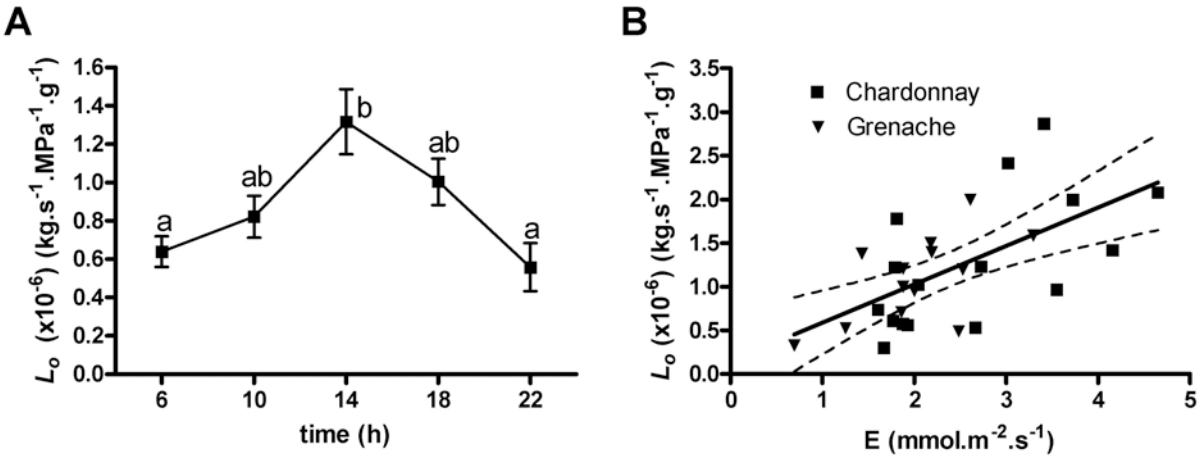


Figure 6.1.1. Variation in root hydraulic conductance normalised to root dry weight (L_o) (A) Diurnal change in L_o of well-watered Chardonnay plants within a 24 hour period. Values are mean ± SEM of four plants. Different letters indicates values are significantly different (Tukey's test $P < 0.05$). (B) L_o measured between 1300 h and 1500 h plotted against average transpiration rate (E) measured between 1100 h and 1200 h. The equation for the linear regression is $L_o = m \times E + b$, where $m = 0.47$ and $b = 0.03$ ($r^2 = 0.369$) for Chardonnay and $m = 0.45$ and $b = 0.18$ ($r^2 = 0.381$) for Grenache. Regressions for the two cultivars were not significantly different thus the combined regression, with 95% confidence levels, is shown.

Impact of water stress on diurnal variation

Compared to well-watered conditions, Chardonnay root systems in response to water stress, have an almost 2-fold reduction in L_o at 1400 h and a 4-fold reduction prior to sunrise (600 h) (Fig. 6.1.2). In contrast, water-stressed Grenache maintained a 4.5-fold lower L_o at both 600 h and 1400 h compared to the well-watered controls (Fig. 6.1.2). The similar magnitude of diurnal amplitude of L_o of water-stressed Chardonnay roots compared to the controls between 600 h and 1400 h contrasts with a large decrease in amplitude of diurnal variation in L_o for water-stressed Grenache root systems over the same period (Fig. 6.1.2). Although the stem water potential (Ψ_{stem}) at midday was slightly lower in Grenache than Chardonnay under water stress in this experiment, subsequent experiments confirmed that Grenache consistently gave a larger reduction in L_o for similar reductions in Ψ_{stem}, independently of growth conditions. With the two pot system there was a 5.5-fold reduction in L_o under water stress compared to a 5.5-fold reduction in a single pot experiment under the same growth conditions.

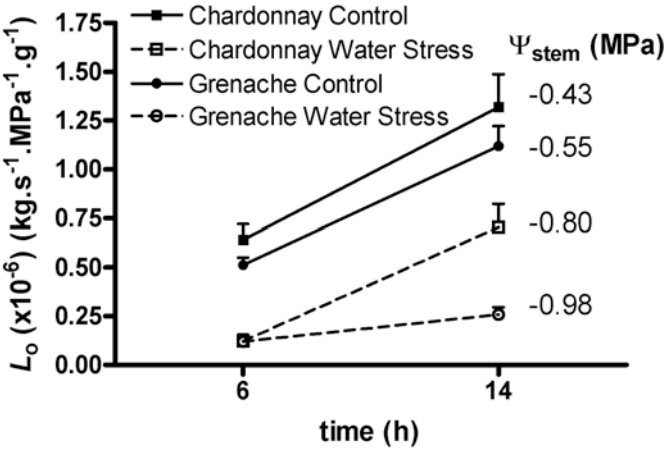


Figure 6.1.2. The effect of water stress on the change in amplitude of L_o between 600 h and 1400 h. Diurnal change in L_o for both cultivars, under well-watered and water-stressed conditions. Stem water potentials measured between 1100 and 1300 h on the day of harvest for L_o are indicated to the right of the associated points. The Chardonnay and Grenache plants were from different experiments. Values are mean ± SEM of four plants. Within each cultivar, the effect of time of measurement and water stress treatment were significant (2 Way ANOVA, $P <$ 0.05, interaction significant only for Grenache).

Response to subsequent rewatering after water stress

The impact of water stress and subsequent rewatering were investigated in another set of experiments. This was done in separate experiments for each cultivar to allow measurements of root L_o over a sufficiently narrow range of time in the middle of the day. Control values of root L_o for Grenache (1.43×10^{-6} kg s^{-1} MPa^{-1} g^{-1}) were similar to the experiment shown in Fig. 6.1.2, but Chardonnay in this case had almost twice the value of root L_o (2.90×10^{-6} kg s^{-1} MPa^{-1} g^{-1}) corresponding to higher transpiration rates in this particular experiment (data included in Fig. 6.1.1B). Chardonnay gave a 3.2-fold reduction in L_o when water-stressed, but Grenache showed a much larger 6.5-fold reduction consistent with the experiment reported in Fig. 6.1.2. There was no correlation between the extent of the reduction in L_o and the extent to which midday Ψ_{stem} was reduced ($r^2 = 0.592$, $P > 0.05$) at least for the range of midday Ψ_{stem} achieved under water stress.

One day after rewatering there was no significant increase in L_o above the value for water-stressed Chardonnay vines (fold difference between controls and drought & rewatered = 2.9). L_o of Grenache did show some recovery (fold difference between controls and drought & rewatered = 3.3) but the increase was not significant.

Cortical cell water relations

In order to examine how cortical cell hydraulic conductivity (L_{pcell}) responds to water stress and to examine differences between the cultivars, pressure probe experiments were performed. Plants were water-stressed in the same manner as above and to similar stem water potentials. The midday Ψ_{stem} of water-stressed Chardonnay used for cell pressure probe measurements and root anatomy (see below) decreased from -0.50 to -1.15 MPa, while Grenache Ψ_{stem} decreased from -0.55 to -1.20 MPa. Roots were harvested from a basal pot (see methods) and bathed in 1 mM $CaSO_4$ to perform pressure probe measurements on root cortical cells 25 mm to 30 mm from root apex. Steady turgor pressures recorded in cortex cells under these conditions ranged from very low values for roots from water-stressed plants (sometimes less than 0.08 MPa) up to 0.5 MPa for roots harvested from control plants (Table 6.1.1). Both varieties showed a significant positive relationship between $\Delta P/\Delta V$ and turgor pressure that was due to a turgor-dependent volumetric elastic modulus ($\varepsilon = mP + b$, where $m = 13.16 \pm 1.303$ and $b = 0.6787 \pm 0.2860$ MPa, $r^2 = 0.98$). This was not significantly different between the two cultivars.

The data in Table 6.1.1 summarise cell dimensions of cortical cells that were used to calculate L_{pcell}. For both cultivars cell dimensions were significantly altered in response to water stress. Both Grenache and Chardonnay had similar cell radii and showed a reduction in radius under water stress, but in contrast to Chardonnay, Grenache had a substantially increased cell length under water stress.

L_{pcell} increased in response to water stress in Chardonnay roots (Fig. 6.1.3) as indicated by a significant decrease in relaxation half-times (Table 6.1.1). The increase in L_{pcell} observed for Grenache roots (Fig. 6.1.3) was not significant, and there was a lack of impact of water stress on relaxation half-times (Table 6.1.1).

Table 6.1.1. Cell size and water relation parameters of root cortical cells of well-watered and water-stressed Chardonnay and Grenache. Measurements were made in the third or fourth layer of cortical cells, 25-30 mm from the root tip.

	Chardonnay		Grenache	
	Control	Water-stressed	Control	Water-stressed
Cell radius[1] (µm)	26.9 ± 1.2^{a} (36)	21.4 ± 0.9^{bc} (45)	24.3 ± 0.9^{ab} (79)	19.9 ± 0.8^{c} (90)
Cell length[2] (µm)	106.0 ± 4.1^{a} (65)	105.0 ± 3.0^{a} (69)	111.8 ± 4.9^{a} (35)	142.6 ± 3.9^{b} (55)
Water relations	n = 18	n = 19	n = 18	n =21
$T_{½}$ (s)	1.68 ± 0.16^{a}	0.96 ± 0.12^{b}	1.11 ± 0.13^{b}	1.12 ± 0.14^{b}
E (MPa)	4.45 ± 0.70^{a}	1.54 ± 0.10^{b}	4.65 ± 0.25^{a}	2.34 ± 0.18^{c}
π_i (MPa)	0.30 ± 0.02^{a}	0.08 ± 0.01^{b}	0.29 ± 0.02^{a}	0.11 ± 0.03^{b}

Values with different letters within a row are significantly different ($P < 0.05$).
[1]Cells of 6 roots from 3 plants were measured; total number of cells measured in brackets.
[2]Cells of 4 roots from 2 plants were measured; total number of cells measured in brackets.

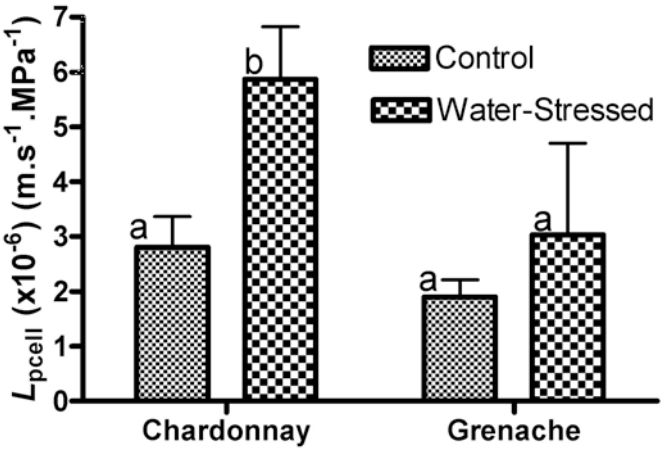


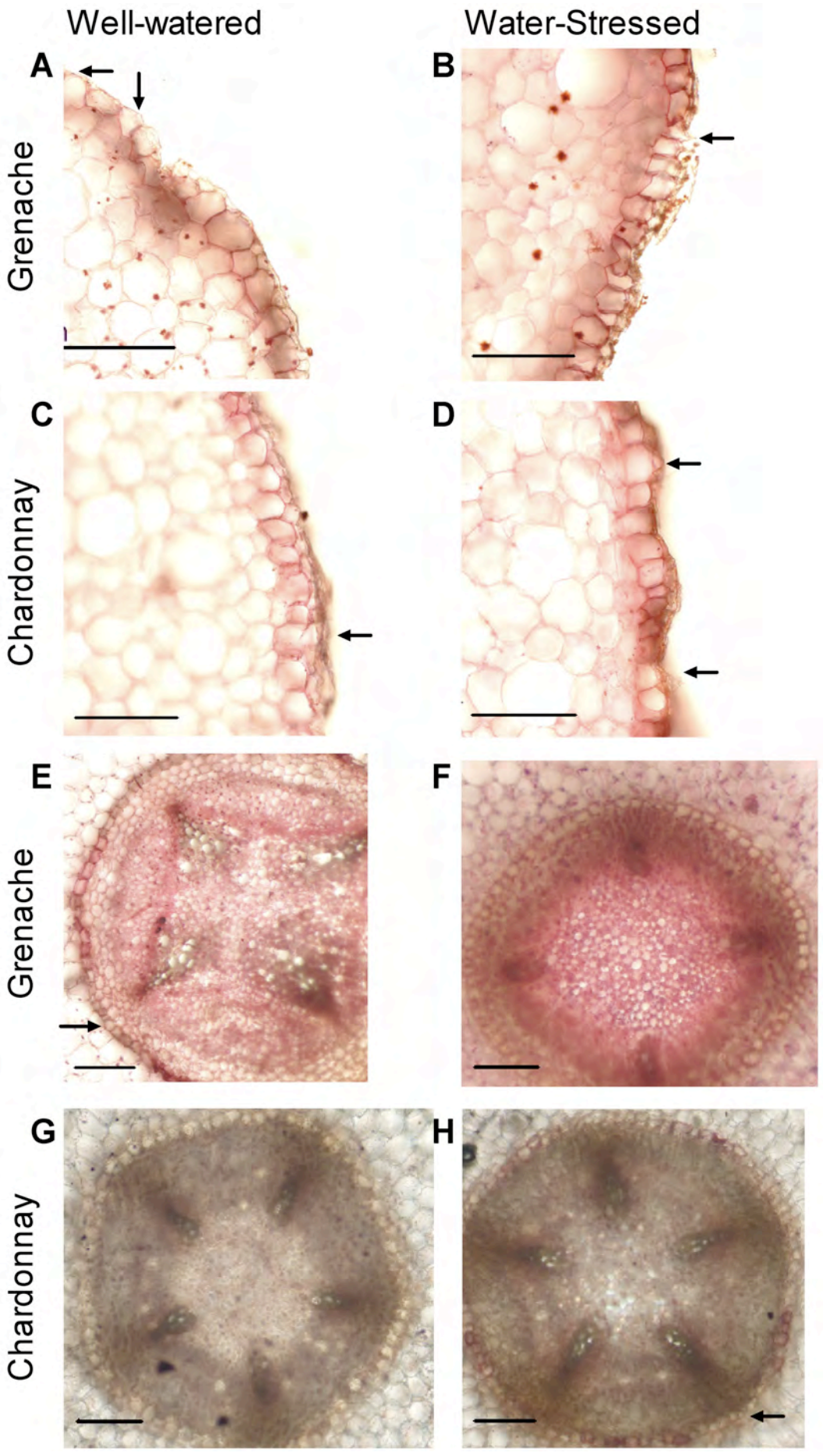
Figure 6.1.3. Cell hydraulic conductivity (L_{pcell}) of third- and fourth-layer cortical cells of Chardonnay and Grenache roots. L_{pcell} for both cultivars is shown for control and water-stress treatments. Measurements were taken 25-30 mm from the root tip. Values are means ± SEM (calculated for error propagation of component measured variables) of 18-23 cells. Different letters indicates values are significantly different within cultivar (T test $P < 0.05$).

Changes in suberin deposition in response to water stress

Qualitative changes in suberin lamellae deposition in the roots in response to water stress were examined at 50 mm from the root tip. The Ψ_{stem} at midday of the plants used were the same as those used for cortical cell water relations. In the cell layer beneath the epidermis that contains Casparian bands (exodermis, Fig. 6.1.4 A-D), there was a greater intensity of suberin deposited in cell walls of water-stressed roots than in well-watered roots, for both cultivars. (Chardonnay, Fig. 6.1.4C,D; Grenache, Fig. 6.1.4A,B). Exodermal cells of water-stressed roots of both cultivars had suberin lamellae deposited on the outer tangential walls in addition to the radial and inner tangential walls observed in both treatments. Passage cells were still evident in the water-stressed roots of both cultivars.

In well-watered roots at 50 mm from the root tip only a limited number of endodermal cells had suberin lamellae (Fig. 6.1.4E,G). In some roots there was deposition of suberin lamellae, but passage cells still remained, aligned with the xylem poles. Water-stress roots appeared to have more endodermal cells with suberin lamellae (Fig. 6.1.4F,H). In the case of Chardonnay, passage cells were generally still evident (Fig. 6.1.4H), but in Grenache all cells of the endodermis appeared to have become suberised (Fig. 6.1.4F).

Figure 6.1.4 (over page). Root suberin lamellae. Cross sections of Grenache (A,B,E,F) and Chardonnay (C,D,G,H) roots taken 50 mm from root tip and stained with Sudan Red 7B for 2 h to show suberin lamellae. Roots from well-watered plants are on the left, and roots from water-stressed plants on the right. The exodermis (A-D) and endodermis (E-H) are shown. Examples of passage cells are indicated by the arrows. Bars = 100 µm


Well-watered
Water-Stressed
A
B
Grenache
C
D
Chardonnay
E
F
Grenache
G
H
Chardonnay

Gene expression of PIP aquaporins

Five *VvPIP1* members and four *VvPIP2* members of the PIP aquaporin sub-family were identified as full length sequences from the sequenced genome of *Vitis vinifera* (Jaillon *et al*., 2007). Recently eight members of the PIP sub-family were identified from the sequenced genome (Fouquet et al, 2008). Screening of a cDNA library constructed from Cabernet Sauvignon vegetative tissue resulted in the identification of six PIP1 aquaporins and five PIP2 aquaporins (Shelden, 2007). Of these, *VvPIP1;1* and *VvPIP2;2* were the most highly expressed relative to *Vvactin* in roots sampled at midday of well-watered Chardonnay plants (Fig. 6.1.5). A similar result was found for Grenache roots (results not shown).

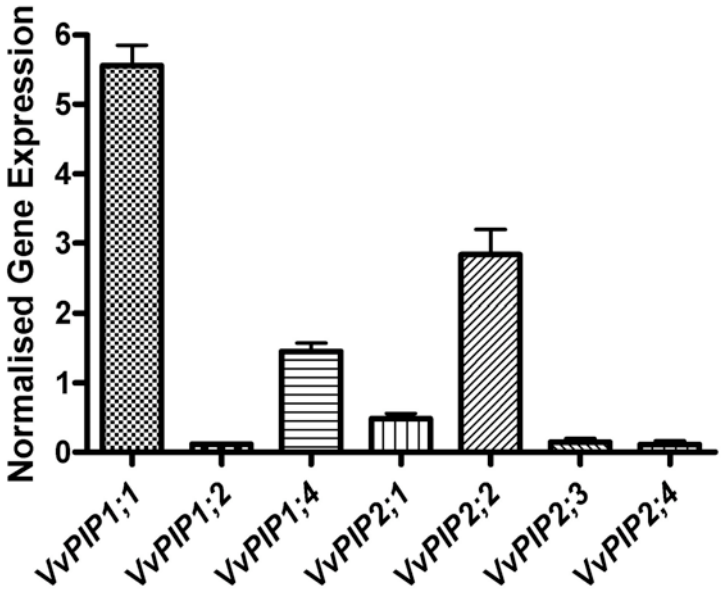


Figure 6.1.5. The relative gene expression of seven PIPs in well-watered Chardonnay roots. Values are mean ± SEM of two biological replicates sampled at midday. Relative gene expression was normalised to expression of *Vvactin* according to the method of Muller et al. (2002).

The responses of these two aquaporins diurnally and to water stress were examined in more detail. *VvPIP2;2* appeared to be constitutively expressed irrespectively of time or treatment with only slight changes in the level of expression relative to *Vvactin* (Fig. 6.1.6A, B), but relative expression of *VvPIP1;1* changed significantly (Fig. 6.1.6A,C). Diurnally, *VvPIP1;1* expression levels peaked at 1000 h in Chardonnay roots and a similar expression level was maintained while the lights were on, then significantly reduced during the dark period (Fig. 6.1.6A). Grenache and Chardonnay differed in *VvPIP1;1* expression response to water stress and rewatering with similar changes in Ψ_{stem}. In this experiment midday Ψ_{stem} of water-stressed Chardonnay decreased from -0.30 to -0.90 MPa and increased to -0.30 MPa, 24 hours after rewatering. Grenache Ψ_{stem} decreased from -0.35 to -0.90 MPa and increased to -0.35 MPa. *VvPIP1;1* showed an approximately 3-fold increase in level of expression in response to water stress in the roots of Chardonnay (Fig. 6.1.6C). This declined to the level of the control plants upon rewatering. In contrast to Chardonnay, Grenache did not show a significant increase in *VvPIP1;1* expression due to water stress (Fig. 6.1.6C); and transcript level was significantly higher in rewatered roots compared to control roots (Fig. 6.1.6C). The L_o of Grenache roots was measured in the double-pot configuration for this experiment. There was an approximately 5-fold reduction in L_o in response to water stress and a slight, but non-significant recovery 24 h after rewatering. This result was similar to that observed in the single pot experiments described above.

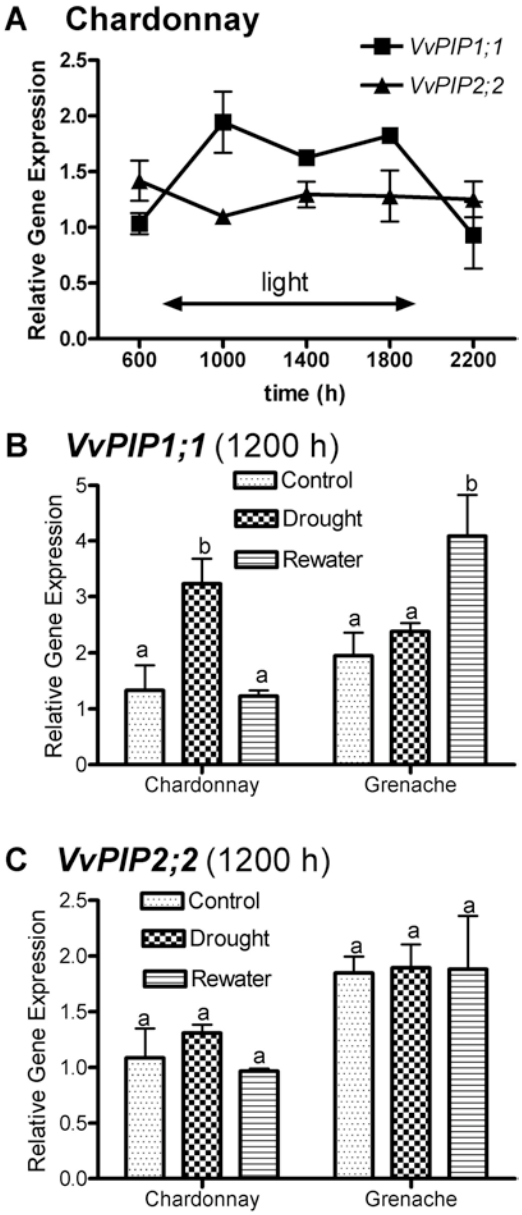


Figure 6.1.6. *VvPIP1;1* and *VvPIP2;2* gene expression. (A) Relative gene expression of *VvPIP1;1* and *VvPIP2;2* in response to time of day for well-watered Chardonnay roots. Lights were on between 700 and 1900 h. Values are mean ± SEM of three biological replicates. (B) Relative expression of *VvPIP1;1* in response to water stress and rewatering for Chardonnay and Grenache at 1200 h. (C) Relative expression of *VvPIP2;2* in response to water stress and rewatering for Chardonnay and Grenache at 1200 h. Relative gene expression is the ratio of the starting quantity of the gene of interest and the starting quantity of *Vvactin*. Values are mean ± SEM of three biological replicates. For each cultivar, columns with a different letter are significantly different ($P < 0.05$).

Water channel activity of VvPIP1;1 and VvPIP2;2

Water transport activity of VvPIP1;1 and VvPIP2;2 was examined in *Xenopus laevis* oocytes. Data shown in Fig. 6.1.7 are the combined results of two separate experiments, each with five oocytes. Water permeability (P_{os}) was calculated from the rate of increase in volume of oocytes when exposed to a hypotonic solution. The P_{os} of oocytes expressing *VvPIP1;1* was not significantly greater than those injected with water (Fig. 6.1.7). Oocytes injected with *VvPIP2;2* had a P_{os} two-fold larger than those expressing *VvPIP1;1*. When *VvPIP1;1* and *VvPIP2;2* cRNA were injected together there was a three-fold increase in P_{os} above the level of *VvPIP2;2* alone. Varying the amount of *VvPIP1;1* (6 to 36 ng) injected with *VvPIP2;2* (12 ng) did not significantly alter this increase in P_{os}.

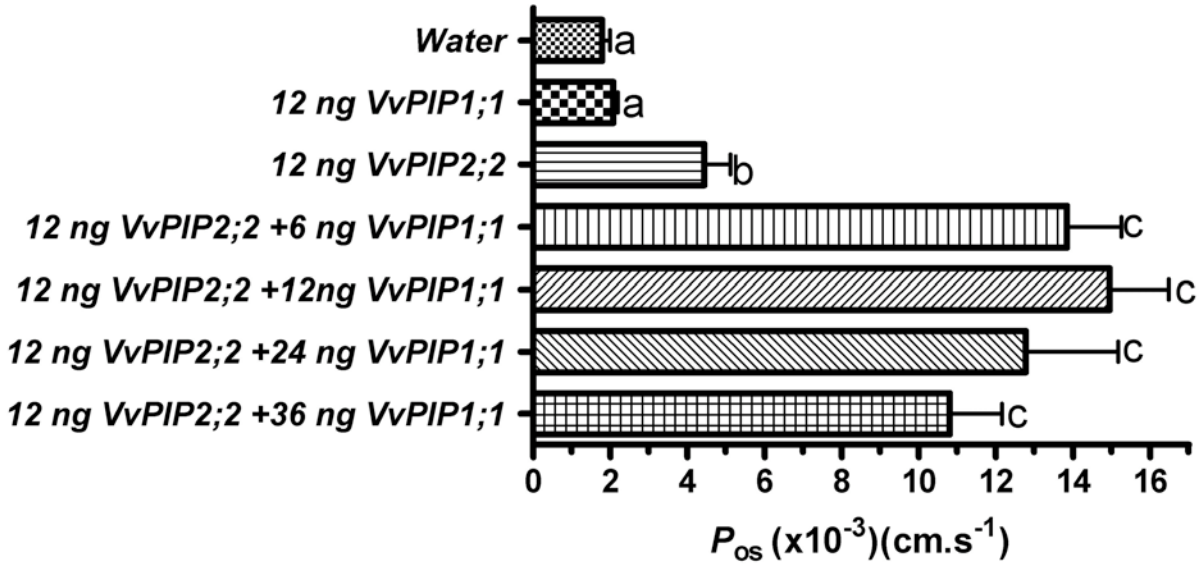


Figure 6.1.7. Functional expression of *VvPIP1;1* and *VvPIP2;2* in *Xenopus laevis* oocytes. P_{os} of oocytes was measured from swelling kinetics. Oocytes were injected with 46 nL cRNA solution or water. The quantity of *VvPIP1;1* and *VvPIP2;2* cRNAs used is shown on the left. Values are means of measurements of 10 oocytes ± SEM. Columns with a different letter are significantly different ($P < 0.05$).

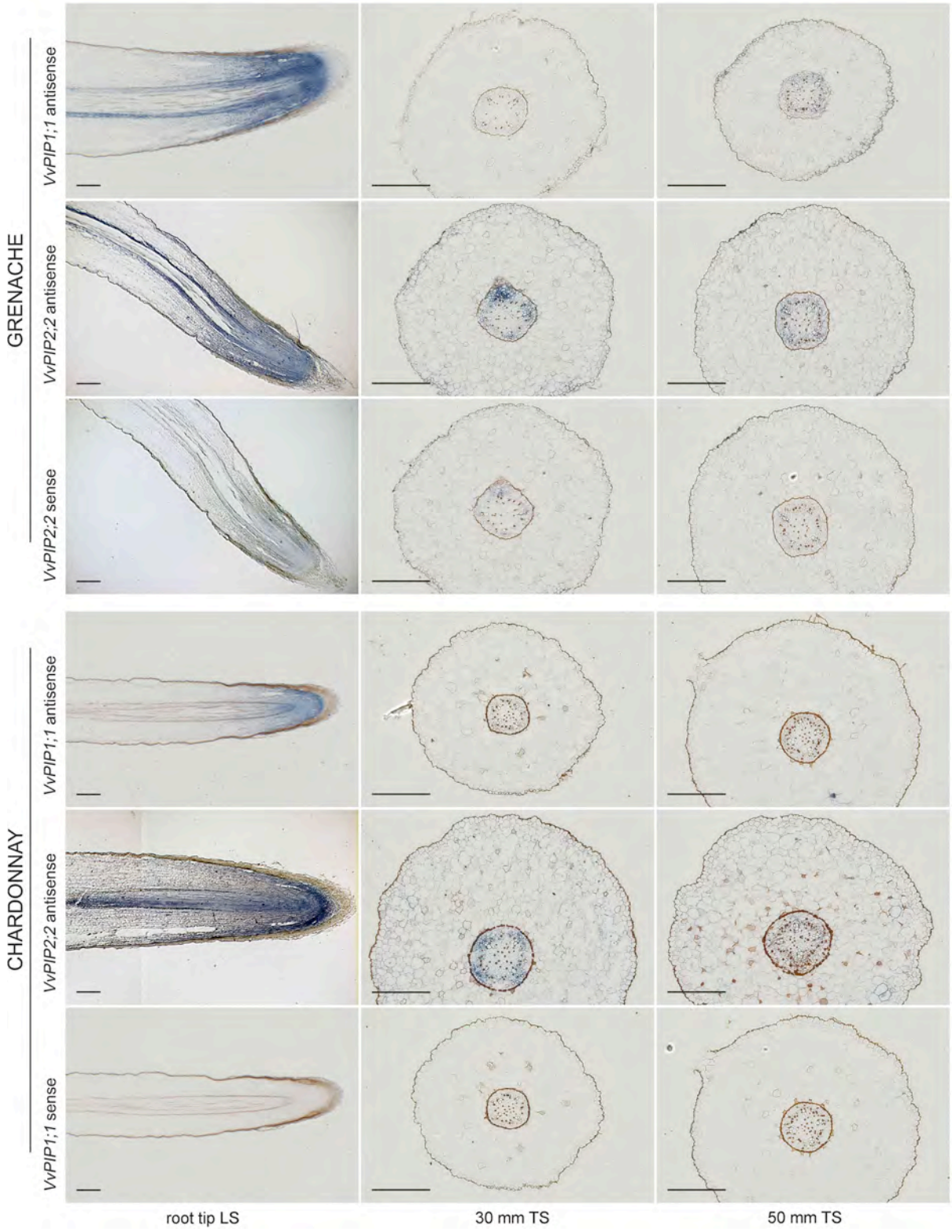
Location of VvPIP1;1 and VvPIP2;2 in roots.

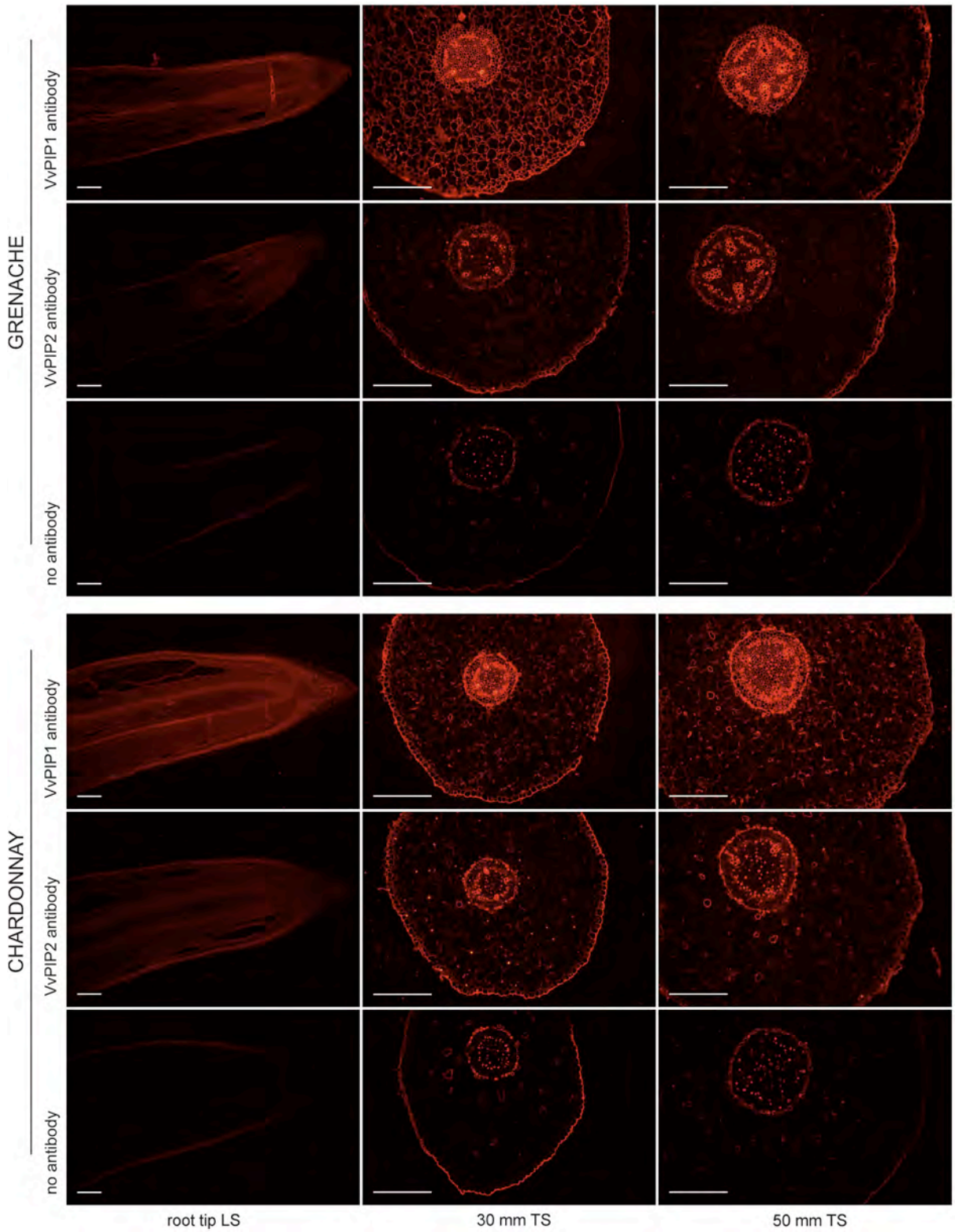
Figure 8 shows *in situ* hybridisation of *VvPIP2;2* and *VvPIP1;1* antisense and sense (controls) probes for both cultivars. All sense probe controls showed minimal background hybridisation so for the sake of brevity images were only included for one gene per cultivar. Controls made with no probe showed no signal in any tissues, and are therefore not presented. Grenache and Chardonnay roots displayed similar localised patterns of *VvPIP1;1* and *VvPIP2;2* mRNA expression. For both genes, strong signal was detected in elongating cortical tissue and vascular tissue of root tip longitudinal sections with strongest signal in the root apex (Fig. 6.1.8). At 30 mm and 50 mm from the root apex, transverse sections revealed that *VvPIP2;2* expression occurred in vascular tissue adjacent to and between the xylem poles, and also in the cortex. Expression of *VvPIP1;1* at 30 and 50 mm from the root apex was not consistently detected. The brown material evident in the root, in particular in the epidermis and endodermis, is likely to be phenolic compounds.

To examine the general patterns of protein expression of VvPIP2 and VvPIP1 subgroups, antibodies were raised to peptides designed to detect grapevine PIP1 or PIP2 members based on the conserved N- and C-termini that differ between the two subgroups (Schäffner, 1998). Longitudinal sections showed signal throughout the elongation zone and in the cortical tissue and vascular tissue for both antibodies (Fig. 6.1.9). At 30 and 50 mm from the root tip strong VvPIP1 signal was detected in the vascular tissue and exodermis, with reduced signal in the cortex (Fig. 6.1.9). VvPIP2 signal pattern was similar to VvPIP1 but weaker, and more consistently seen in the vascular tissue and exodermis than the cortex (Fig. 6.1.9). There was a strong signal for both groups of proteins in cells closely associated with xylem vessels, and phloem cells. Controls with no primary and secondary antibodies revealed the background autofluorescence of grapevine root tissues (Fig. 6.1.9). Controls with a single antibody (no primary, or no secondary) did not differ from the latter controls.

Figure 6.1.8 (over page). *In situ* localisation of *VvPIP1;1* and *VvPIP2;2* mRNA in Grenache and Chardonnay root. Blue signal indicates the location of antisense *VvPIP1;1* and *VvPIP2;2* probes on each grape cultivar. Sense probe controls are only presented for one gene per grape cultivar, to indicate background staining. Transverse sections (TS) were taken 30 mm and 50 mm from the root tip. Longitudinal section = LS, and scale bars = 250μm.

Figure 6.1.9 (over page). Immunolocalisation of VvPIP1 and VvPIP2 proteins in Grenache and Chardonnay root. VvPIP1 antibody is specific to all PIP1s, and VvPIP2 antibody is specific to all PIP2s. Controls with no primary or secondary antibody indicate cells with background auto-fluorescence. Transverse sections (TS) were taken 30 mm and 50 mm from the root tip. Longitudinal section = LS, and scale bars = 250μm.


GRENACHE
CHARDONNAY
VvPIP1;1 antisense
VvPIP2;2 antisense
VvPIP2;2 sense
VvPIP1;1 antisense
VvPIP2;2 antisense
VvPIP1;1 sense
root tip LS
30 mm TS
50 mm TS


GRENACHE
CHARDONNAY
VvPIP1 antibody
VvPIP2 antibody
no antibody
root tip LS
30 mm TS
50 mm TS

6.1.3 Discussion

Changes in root hydraulic conductance
Root L_o of both Chardonnay and Grenache grapevines under well-watered conditions showed considerable variation in the various experiments described in this study. This variation may be linked to differences in transpiration from one experiment to another, and to diurnal variation associated with changes in transpiration rate during the day, which is partly dependent on light intensity.

At low water flow (low transpiration) the composite transport model predicts some circulation flow of water across the root, because osmotic gradients may become more significant and there should be a large difference in the reflection coefficients for the cell-to-cell and apoplastic pathways. It has been proposed that with increasing transpiration and xylem tension, there will be an increase in root L_o because a greater proportion of radial flow occurs via the apoplast pathway in response to increasing hydrostatic gradients (Steudle and Heydt, 1997). A positive relationship between apparent L_o and stomatal conductance has been shown previously (Meinzer and Grantz, 1990: Sperry et al., 1993; Saliendra et al., 1995). Modelling by Franks et al. (2007) showed that a dependence of hydraulic conductance on transpiration rate would explain the responses they observed in leaf water potential of *Eucalyptus gomphocephala* in response to changes in soil water. However, there is a crucial difference between most of these studies and our measurements. We have measured L_o under identical pressure gradients and identical conditions for all experiments. Thus the explanation for an increase in L_o based on the composite transport model where gradients are different probably does not account for our observations. Solute polarisation was not evident because there was not a change in conductance obtained from successive pressure ramps. The volume of fluid injected into the root was also small relative to the total root volume. Therefore we conclude from our measurements that the variation in L_o is a function of intrinsic changes to permeability barriers in the roots, which are affected by the conditions that the plants were under at the time of harvest. These changes appear to persist for at least the time that it takes to decapitate the shoot and take the root systems into the laboratory for measurement with the HCFM.

Grapevines along with a number of other plant species demonstrate a reduction in root hydraulic conductivity in response to water stress. This has been intensively investigated in desert plants (North and Nobel, 1995, 1996, 2000; Martre et al., 2001; North et al., 2004;). In some of these cases reduction was associated with a closure of aquaporins, as evidenced by the inability of mercuric chloride to further reduce hydraulic conductivity under water-stressed conditions (Martre et al., 2001; North et al., 2004). This was suggested as a mechanism to prevent water loss to the soil, which has a lower water potential than the plant. Similar results were seen for severely stressed aspen seedlings (Siemens and Zwiazek, 2003).

The present study discovered a remarkable difference in diurnal change in L_o between the two cultivars in response to water stress (Fig. 6.1.2). A reasonable assumption is that relatively rapid (daily) and reversible changes in L_o can only occur via changes in cell membrane water permeability, probably via aquaporins as we discuss below. In Grenache the apparent scale of the change in L_o would suggest that the same relative changes in cell membrane permeability occur over a diurnal period, but that much less root surface area (or dry weight) is able to conduct water under water stress. Under water stress, Chardonnay showed a similar reduction in pre-dawn L_o as Grenache, but a smaller reduction in midday L_o indicating that cell membrane water permeability increased to a much larger extent between pre-dawn and midday under water stress.

One day after rewatering neither cultivar showed a significant increase in L_o. An increase in L_o following rewatering may be delayed while significant changes in root anatomy are overcome by new lateral roots and the resumption of apical root growth. *Olea oleaster* appeared to recover only after 48-72 hours of rewatering, when new lateral roots had emerged and root tips resumed growth (Lo Gullo et al., 1998). In contrast to our results with grapevine, there were significant increases in hydraulic conductivity when desert plants were rewatered (North

et al., 2004). This is likely to be an adaptation to the environmental conditions with limited rainfall events in which desert plants grow.

Suberisation increased in the roots of both cultivars as a consequence of water stress. Passage cells remained in the endodermis of Chardonnay, whereas the endodermis was completely suberised 50 mm from the root tip of Grenache. The association between increased suberisation in the roots and reduced hydraulic conductivity has been observed previously in *Agave deserti* (North and Nobel, 1991) and sorghum (*Sorghum bicolour* L.) (Cruz et al., 1992). The increased suberisation under water-stress for both cultivars of grapevine probably accounts for the reduced "baseline" L_o measured at pre-dawn.

Changes in cortical cell water relations
From a comparison of diurnal changes in L_o under water replete and water-stress conditions, it was expected that Chardonnay would have increased cell-to-cell conductance to water under water-stress at midday, compared to Grenache. We observed a significant increase in L_{pcell} of water-stressed Chardonnay cortical cells and no significant change in L_{pcell} of water-stressed Grenache roots. These results indicate clear cultivar differences that are in line with the proportional changes in diurnal amplitude of root L_o under water stress. These changes also correlate with the different pattern of expression of *VvPIP1;1* between cultivars in response to water stress discussed below. These measurements were obtained for roots growing into a different media than the majority of roots in the two pot system. Therefore we cannot exclude the possibility that the roots in the bottom pot may behave differently to the majority of the root system in the top pot. However, given that there was no difference in response of root L_o between single pot and two pot cultivation, and given the correlations between whole root system L_o and *VvPIP1;1* expression and cell L_p, we think that an entirely different qualitative behaviour between sampled roots and the entire root system is unlikely.

The reduced cortical turgor pressure seen in water–stressed roots was not expected. Assuming cells had accumulated solutes for osmotic adjustment, it would be expected that water would rapidly move into cells once the root was placed in a solution of low osmotic pressure (necessary in our case to perform the measurements). This would cause turgor pressure to increase compared to control roots. Another possibility is that the osmotic concentration of the apoplast around the cortical cells is increased under water stress and that there is reduced exchange between the apoplast and the external media because of the dermal apoplastic barriers. This may lead to a decrease in measured turgor even if the osmotic concentration in the cells was maintained or increased compared to controls. Using osmotic pressure of control roots for the calculation of L_{pcell} in water-stressed roots did not strongly affect the magnitude of the L_{pcell}.

There is minimal evidence of osmoregulation in grapevine roots in the literature. Düring and Dry (1995) observed osmoregulation in the apical 3 mm of grapevine roots (cv. Kober 5BB). Osmoregulation was observed in other parts of the root, but only after a number of cycles of severe and rapid water stress. The work of Sharp and others with turgor regulation in root cells has concentrated on the apical 10 mm of maize roots (Sharp et al., 1990, 2004; Voetberg and Sharp, 1991). At 25 mm from the root tip the radius of cortical cells in water-stressed grapevine roots was reduced, which may suggest a loss of turgor, although cell extensibility will have an important role and is known to change under water stress (Wu et al., 1996; Fan et al., 2006). Therefore, it is possible that osmoregulation in grapevine roots occurs mostly in the root apices to enable root growth to recommence when the plants are rewatered or to maintain root elongation in drying soil.

Changes in expression of VvPIP1;1 and VvPIP2;2
Diurnal variation in L_o for Chardonnay root systems was associated with changes in the level of *VvPIP1;1*. However, *VvPIP2;2* appeared to be constitutively expressed. In *Lotus japonicus* the diurnal change in hydraulic conductivity of excised roots was associated with changes in the abundance of a putative *PIP1* aquaporin (Henzler et al., 1999). The highly variable response to water stress of aquaporins at the transcript level depends on species, type of water stress, degree of water stress and the plant organ (Tyerman et al., 2002; Bramley et al., 2007b). Individual isoforms also vary in their response all of which makes interpretation of the role of aquaporins during water stress rather difficult. However, in our case the expression

data does assist interpretation of the different responses of Grenache and Chardonnay to water-stress.

The observation of similar diurnal change in amplitude of Chardonnay L_o under water-stress and well-watered conditions suggests that cell-to-cell conductance increased to a larger extent during the day under water stress. At one extreme where most of the radial flow may occur through the cell-to-cell pathway, the fold changes possible for cell-to-cell conductance from pre-dawn to mid-day are calculated to be from 2.5-fold under water replete conditions, to 6.6-fold under water stress. In Grenache there was no indication that the diurnal change in cell-to-cell conductance would need to be different. This is supported by the increase in transcript level of *VvPIP1;1* in the roots of water-stressed Chardonnay vines at midday compared to no change in Grenache.

A number of researchers have previously observed the up-regulation of aquaporins in response to water stress in other plant species (Jang et al., 2004; Alexandersson et al., 2005; Aroca et al., 2006). The use of transgenic plants with over- or under-expressing PIP1 also supports the importance of PIP1 for tolerance to water stress (Siefritz et al., 2002; Yu et al., 2005). Arabidopsis plants expressing *VfPIP1* had longer roots and a greater number of lateral roots, which may have contributed to improved drought resistance (Cui et al., 2008). Our results for Chardonnay support the view that increased aquaporin levels can be associated with adaptation to water-stress. This contrasts to the strategy suggested for desert plants and aspen seedlings where aquaporins are down regulated to prevent water loss to the soil (Martre et al., 2001, Siemens and Zwiazek, 2003; North et al., 2004).

The more drought tolerant Grenache cultivar showed a different response to Chardonnay, with a reduction in diurnal change in root L_o due to water stress, indicating either a maintained or reduced diurnal amplitude of cell-to-cell conductance. This was associated with a lack of change in transcript level of *VvPIP1;1* and *VvPIP2;2*. A similar result for homologues of the two genes was observed by Galmés et al. (2007) in the roots of a drought tolerant grapevine rootstock, Richter 110 (*Vitis berlandieri* ×*V. rupestris*), with the exception that after 7 days maintained at a soil water deficit there was an increase in *PIP2;2*. It is possible that other aquaporin isoforms not examined in our study were down-regulated, or there were post-translational changes causing a reduction in aquaporin activity. Grenache seems to have a more conservative approach in its response to drought stress, similar to the desert plants. A combination of anatomical changes and reduced aquaporin gene expression or activity is likely to be the cause of the much larger reduction in hydraulic conductivity at midday observed for Grenache.

When Grenache plants were rewatered, the slight recovery in L_o, though not significant, was associated with an up-regulation of *VvPIP1;1*. In the distal regions of the desert plants *Agave desertii* and *Opuntia acanthocarpa*, a significant recovery in hydraulic conductivity was associated with an increase in aquaporin activity, determined by the impact of mercuric chloride (Martre et al., 2001; North et al., 2004). Arabidopsis plants with reduced (antisense) expression of *PIP1* and *PIP2* aquaporins were slower to recover hydraulic conductance and transpiration rates four days after rewatering (Martre et al., 2002).

As shown in a number of other plant species, VvPIP2;2 had much higher water permeability in oocytes than VvPIP1;1. Moshelion et al. (2002) observed that SsAQP1 (from the PIP1 group) had a permeability that was twice that of the control, but SsAQP2 (from the PIP2 group) was 10 fold higher again. ZmPIP1a and ZmPIP1b had no water channel activity in oocytes (Chaumont *et al*., 2000). Positive interaction between a PIP1 and PIP2 has been demonstrated in other plant species, but is not always observed (Zhou et al., 2007). Fetter et al. (2004) and Temmei et al. (2005) both demonstrated interaction between aquaporins from the PIP1 sub-class and the PIP2 sub-class. In living maize cells it appears that interaction is required to traffic PIP1 from the endoplasmic reticulum to the plasma membrane (Zelazny et al., 2007). In lilly pollen protoplasts expression of AtPIP1;1 and AtPIP1;2 did not increase water permeability in contrast to the increase observed with AtPIP2;1 or AtPIP2;2 (Sommer et al., 2008). Co-expression of AtPIP1;1 or AtPIP1;2 with AtPIP2;1 or AtPIP2;2 did not elevate protoplast water permeability above that when AtPIP2;1 or AtPIP2;2 were expressed alone. However, the authors did point out that a native PIP1 may have interacted with AtPIP2 to

increase water permeability. Varied levels of VvPIP1;1 protein may influence the water permeability of the transcellular pathway across grapevine roots when interacting with VvPIP2;2. Our results with co-expression in *Xenopus* showed a positive interaction, but there was no indication over the range of RNA amounts that we tested that this was a graded response. It could be that a graded response between transcript abundance and water permeability occurs at lower ratios of PIP1;1 to PIP2;2 than we tested. Alternatively co-expression may be required *in planta* to achieve a certain threshold of water permeability and thereafter post translational control may give finer regulation.

We performed *in situ* hybridisation and immunolocalisation to determine if *VvPIP1;1* and *VvPIP2;2* genes were expressed in the same cell type, thereby indicating the possibility of interactions between the two proteins. Both genes had a similar mRNA expression pattern in Grenache and Chardonnay, particularly in the longitudinal sections at the root tip. This pattern of gene expression for PIPs was similar to that observed in maize longitudinal sections by Hachez et al. (2006). Unfortunately we were unable to consistently detect *VvPIP1;1* mRNA in the cortical cells at 30 mm from the root tip, but in this tissue it is possible that the presence of large vacuoles effectively reduces signal intensity. Conversely VvPIP1 protein in particular could be detected in the cortical cells at 30 mm from the root tip. Otto and Kaldenhoff (2000) also detected little *NtAQP1* mRNA in the older parts of the root in contrast to strong protein expression.

Due to the large number of cortical cell layers in grapevine roots this cell type would likely contribute the greatest quantity of RNA, and most likely also account for a large portion of the radial hydraulic resistance. This is supported by the correlation between changes in *VvPIP1;1* expression levels between cultivars and the hydraulic conductivity of cortical cells, which in turn matches with the different diurnal amplitudes that we observe in root L_o between cultivars under water stress. However, we cannot exclude the possibility that endodermal and exodermal cells may have a different response to that which we observed in the cortex. The significant increase in *VvPIP1;1* in response to water stress was associated with a significant increase in L_{pcell} of Chardonnay, whereas there was no significant change for Grenache. Increased expression of *ZmPIP1;2*, an aquaporin that does not transport water, and *ZmPIP2;4* at 5-6 mm compared to 1.5-2.5 mm from the root tip was associated with sensitivity of L_{pcell} to a mercury treatment (Hukin et al., 2002). Javot et al. (2003) observed a decrease in L_{pcell} of cortical cells in one line of transgenic Arabidopsis plants with AtPIP2;2 knocked out. This indicated that a single aquaporin isoform had a significant contribution to the hydraulic conductivity of cortical cells, which was associated with a reduction in osmotic hydraulic conductivity of the roots.

In conclusion, the two grapevine cultivars showed contrasting responses to water stress and rewatering. Aquaporins appear to be important contributors to the overall L_o of the root system as evidenced by the large diurnal change in L_o. These responses were associated with changes in the expression of *VvPIP1;1*. *VvPIP2;2* appeared to be constitutively expressed in the roots in the situations examined. Even though *VvPIP1;1* resulted in low water channel activity when expressed in *Xenopus* oocytes, water permeability increased when *VvPIP1;1* was co-injected with *VvPIP2;2*. Reduction in root conductance under water stress seems to be constrained in Chardonnay by an increase in the expression of *VvPIP1;1* resulting in an increased contribution of the cell-to-cell pathway to the radial transport of water during the day. Chardonnay appears to be an “optimistic” cultivar, only reducing L_o in the middle of the day by 2-3 fold. This is also consistent with the more anisohydric behaviour of this cultivar. The smaller reduction in root hydraulic conductance may also be important in maintaining a small water potential gradient between the xylem and the soil, which could be associated with the lower vulnerability of Chardonnay to embolisms relative to Grenache (Alsina et al., 2007). In contrast, L_o of Grenache was reduced by about 6-fold by water stress in the middle of the day and there was no up-regulation of *VvPIP1;1*. Grenache had a relatively “pessimistic” response, possibly to restrict water loss to the soil. However, this response would require greater stomatal control in order to prevent excessively negative xylem water potentials. This behaviour is consistent with the more isohydric stomatal control in this cultivar (Schultz, 2003). However, Grenache may be able to better respond to rainfall and irrigation events by up-regulation of *VvPIP1;1*.

We have established clear differences in the way roots respond to water stress that correlate with different water use strategies between closely related cultivars of the same species. This indicates that root water transport is closely coupled to shoot transpiration, evident both in the correlation between L_o and transpiration, and in the way shoot and root conductances are controlled under different strategies of response to water stress. The challenge now will be to determine the signalling pathways and master switches that coordinate these molecular and anatomical changes within the plant.

6.2 Shoot topping impacts on root hydraulic conductivity

This section forms the basis of a manuscript in preparation by the following authors:
R.K. Vandeleur, B.N. Kaiser, P.R. Dry, W. Sullivan and S.D. Tyerman

6.2.1 Introduction

Shoot topping or shoot trimming is a canopy management technique applied during the growing season to reduce vine vigour and improve canopy ventilation (Koblet 1987, cited in Poni & Giachino 2000). Increased ventilation and exposure to sunlight can reduce disease incidence (Petrie *et al*. 2003) and can also improve fruit quality (Smart,1987).

Under well watered conditions the root system is the main limitation to water uptake (Nobel & Cui 1992). Hydraulic conductivity (L_p) of roots is highly variable. There are three pathways along which water can move radially towards the xylem; the apoplastic, symplastic and transcellular (cell to cell) Steudle (2000). Switching between the cell-to-cell and apoplastic pathways causes the variability and is dependent on the forces, hydrostatic or osmotic, acting (Steudle 2000). Apoplastic flow can be altered irreversibly by anatomical changes, including Casparian bands and suberin lamellae. The cell-to-cell pathway is regulated by aquaporins (proteinaceous water pores). Aquaporins allow for reversible, rapid and fine control of water flow. Aquaporins are members of the major intrinsic protein (MIP) family. Aquaporins have six membrane spanning loops and molecular weights of 26-34 kDa. The function of aquaporins was first determined by Preston *et al*. (1992) when AQP1 was expressed in *Xenopus laevis* oocytes. The proteins are divided into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs). The PIPs are further divided into two subclasses. In general PIP1s have little or no water channel activity, whereas the PIP2s show high water permeability when expressed in *Xenopus* oocytes (Chaumont *et al*., 2000). It has also been shown that aquaporins may transport other small solutes such as glycerol, carbon dioxide (Tyerman *et al*. 2002; Uehlein *et al*. 2003) and ammonia (Niemietz & Tyerman 2000).

In previous experiments to obtain consistent leaf areas across treatments grapevines were initially shoot-topped. A preliminary experiment was conducted to test the effect of shoot topping on L_o of roots. The substantial reduction in L_o led to further experiments to understand the physiological basis of the response. The signal from shoots to roots that may mediate the response was also investigated. Other species were also examined to determine if the response was consistent amongst monocots and dicots.

6.2.2 Results

Impact of shoot topping on grapevine, and other plants
Shoot topping (Fig. 6.2.1) caused the L_o to be reduced by approximately 50% for grapevine (Fig. 6.2.2). This was also observed in other plants, soybean and maize (Fig. 6.2.3). The reduced L_o was still measurable 5 days after shoot topping of grapevine (Fig. 6.2.2).

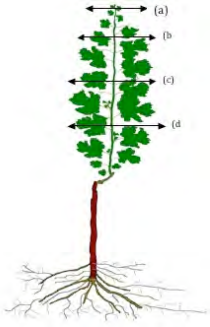


Figure 6.2.1 Drawing indicates the locations of the cuts for the degree of leaf removal experiment: a) removal of shoot tips only; b) removal of tips and young expanding leaves; c) removal of 35% of leaf area, including young leaves and tips; d) removal of 70% leaf area, including young leaves and tips. The plants were shoot-topped 24 h before measuring L_o.

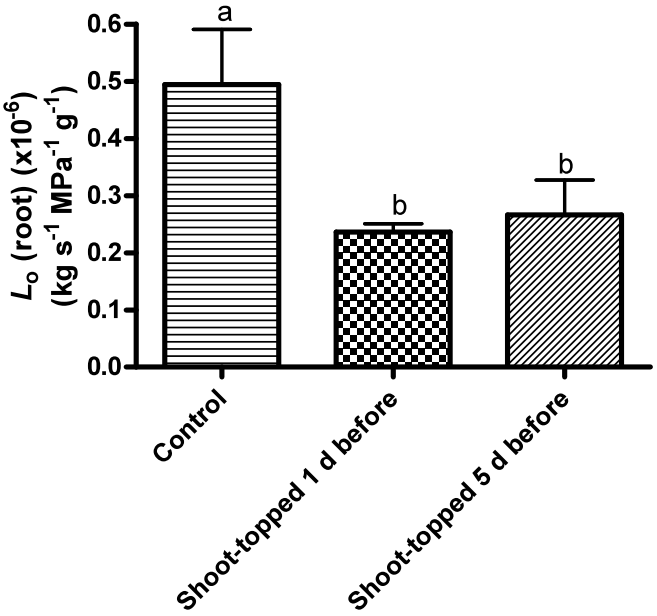


Figure 6.2.2 The impact of shoot topping on L_o of grapevine. The L_o was measured between 1300 and 1500 h, 1 and 5 days after shoot topping. The values are means ± SEM of 5 replicate plants. Columns with different letters are significantly different ($P < 0.05$).

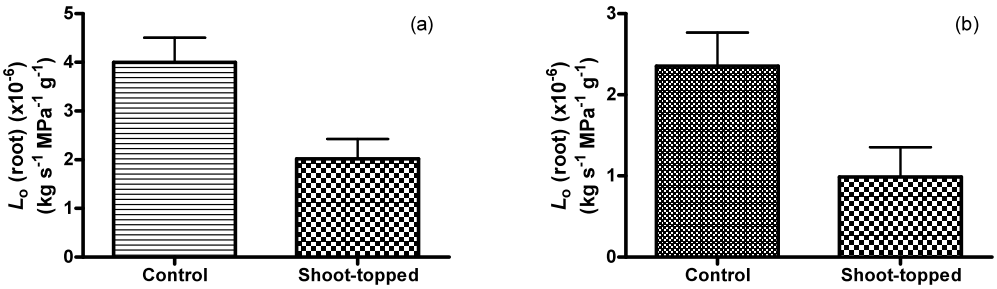


Figure 6.2.3 The impact of shoot topping on L_o of soybean (a) and maize (b) demonstrating that this phenomena is more general amongst plants. The L_o was measured between 1300 and 1500 h, 1 day after shoot topping. Values are means ± SEM and 4 replicate maize plants.

Degree of leaf removal

To determine if the response due to shoot topping was related to the remaining leaf area, both soybean and grapevine were shoot-topped at various positions on the shoot. The relationship between leaf area remaining after shoot topping and L_o was weak for grapevine (Fig. 6.2.4a) and soybean (Fig. 6.2.5a). However, there was a significant difference between the treatments; with a similar response observed for grapevine and soybean (Figs 6.2.4b & 5b). There was no significant difference between the L_o of plants not shoot-topped and those which had just the shoot tips removed. However, those plants which had their young leaves removed when the shoots were topped had a L_o 48 & 58% lower than the control plants for grapevine and soybeans, respectively (Figs 6.2.4b & 5b). When 35 or 70% of the leaf area was removed by shoot topping the L_o was no lower than when just the young leaves were removed. For grapevine the L_o when 70% of the leaf area was removed was not significantly different to that of the control plants (Fig. 6.2.4b).

There was no difference between control and shoot-topped (removal of 35% of leaf area) soybeans for either transpiration rate per unit leaf area (E) or stomatal conductance (g_s) (Table 6.2.1). When calculated for the entire plant, transpiration was 34% lower in shoot-topped soybean, which corresponds to the magnitude of reduction in leaf area.

Table 6.2.1 The impact of shoot topping (removal of 35% of leaf area) on transpiration rate (E) and stomatal conductance (g_s) in the degree of leaf area removal experiment. Values are means ± SEM of 4 replicate plants.

	Control	Shoot-topped
E (mmol s^{-1} m^{-2})	1.12 ± 0.38[a]	1.00 ± 0.22[a]
g_s (mmol s^{-1} m^{-2})	53.3 ± 23.3[a]	45.0 ± 10.2[a]

Values with different letters within a row are significantly different ($P < 0.05$).

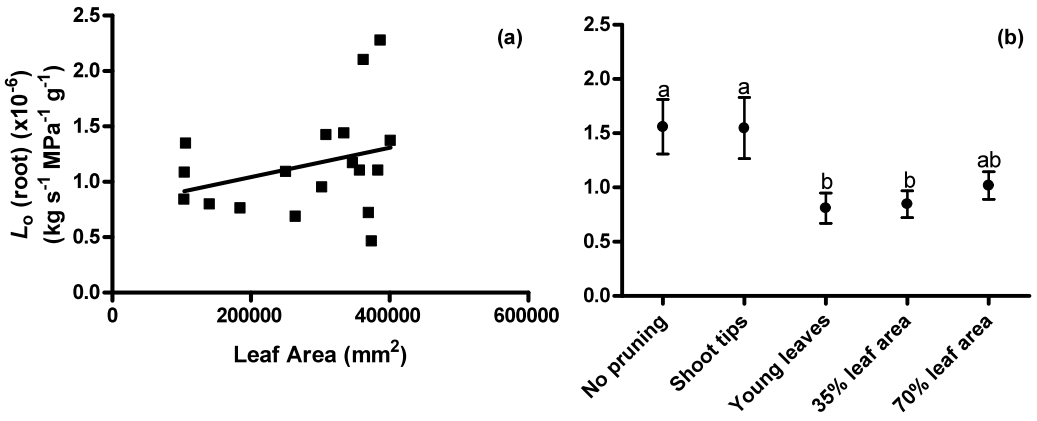


Figure 6.2.4 Relationship between final leaf area after shoot topping and L_o of grapevine. The linear regression line is fitted, $r^2 = 0.093$ and $P = 0.217$ (a). The effect of shoot topping at various positions along the grapevine shoot on L_o (b). The x-axis indicates the part of the shoot that was removed but cutting the stem. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different ($P < 0.05$).

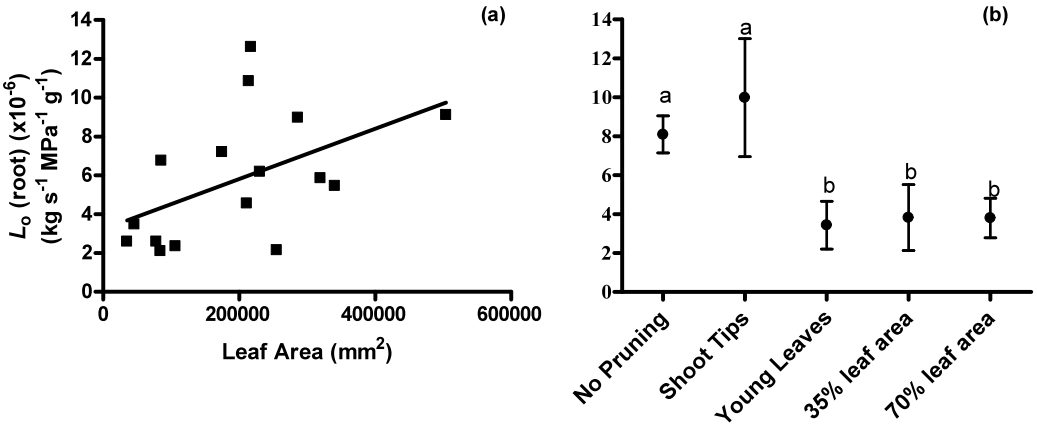


Figure 6.2.5 Relationship between final leaf area after shoot topping and L_o of soybean. The linear regression line is fitted, $r^2 = 0.247$ and $P = 0.051$ (a). The impact of shoot topping at various positions along the soybean shoot on L_o (b). The x-axis indicates the part of the shoot that was removed by cutting the stem. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different ($P < 0.05$).

Ethylene

Shoot topping may cause a wounding response in the plant, causing the synthesis of ethylene. An inhibitor of the synthesis of the ethylene precursor ACC (1-aminocylcopropane-1-carboxylic acid) was utilised. The use of an ACC synthase inhibitor (AVG) did not prevent the reduction in L_o of soybean plants when shoot topped (Fig. 6.2.6). However, when sprayed with AVG the L_o of shoot-topped plants was not significantly different from that of control plants. The L_o of plants sprayed with AVG and not shoot-topped was 77% higher than the control plants. The ethylene transport inhibitor altered L_o independently of the effect due to shoot topping; there was no interaction between the two treatments.

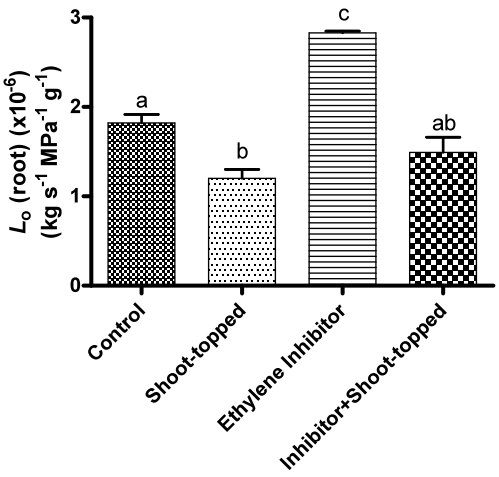


Figure 6.2.6 The impact of the ACC synthase inhibitor (AVG) on L_o of soybeans. Plants sprayed with the inhibitor were either not shoot-topped or shoot-topped to remove approximately 35% of leaf area. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different ($P < 0.05$).

Abscisic acid

ABA is another possible hormone whose supply to the roots may be reduced by shoot topping. The concentration of ABA in the roots was not significantly altered by shoot topping 24 h prior to sampling of the roots.

Auxin

Auxin is another hormone that is transported from the shoots to the roots. The application of the auxin transport inhibitor, NPA, to soybean stems caused a significant reduction in L_o but there was also non-significant reduction in the plants which had only ethanol applied. There was no significant difference in L_o between the ethanol only and NPA treated plants.

Variability in response

The possible influence of L_o prior to shoot topping on the reduction in L_o due to shoot topping led to a comparison of the different experiments using soybeans. The mean L_o of the shoot topped soybeans was plotted against the mean L_o of the control plants for each experiment (Fig. 6.2.7a). The control L_o ranged from 2 to 20 kg s^{-1} MPa^{-1} g^{-1}, whereas the L_o of the shoot topped plants ranged from 1 to 6 kg s^{-1} MPa^{-1} g^{-1}. There was a hyperbolic relationship between the percentage reduction in L_o due to shoot topping and the mean L_o of the control plants (Fig. 6.2.7b). In those experiments where the L_o of the control plants was much higher there was a greater percentage reduction due to shoot topping. It appears that the response may saturate at higher control L_o.

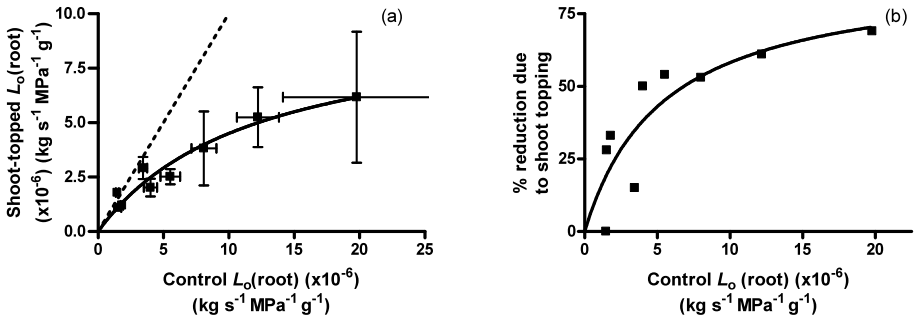


Figure 6.2.7 The association between the mean L_o of the control plants and the mean L_o of the shoot-topped plants of each experiment using soybean plants (a). The fitted line is a hyperbola, $r^2 = 0.43$. The dashed line indicates the potential relationship if there was no reduction in L_o due to shoot topping. The association between the mean L_o of the control plants and the percentage reduction due to shoot topping in each experiment using soybean plants (b). The fitted line is a hyperbola, $r^2 = 0.72$

Impact of shoot topping on expression of VvPIP1;1 and VvPIP2;2
Shoot topping of Chardonnay, to remove 40% of the leaf area including the young leaves and shoot tips, caused a significant 3-fold reduction in the relative gene expression of *VvPIP1;1* (Fig. 6.2.8). However, there was no change in the expression levels of *VvPIP2;2* 24 h after shoot topping.

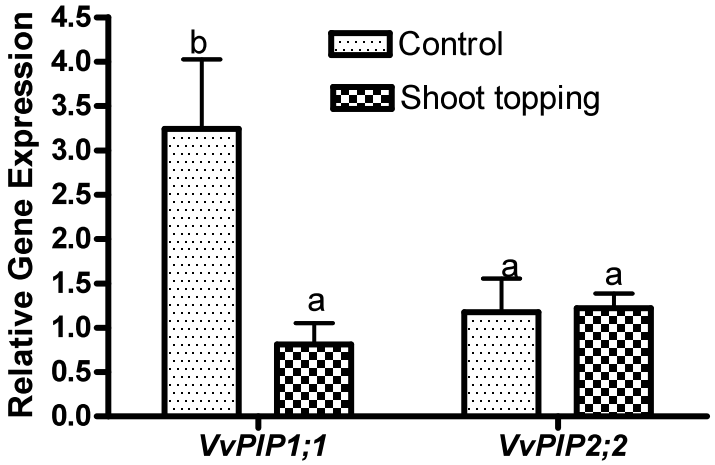


Figure 6.2.8 *VvPIP1;1* and *VvPIP2;2* gene expression in Chardonnay roots. Relative gene expression is the ratio of the starting quantity *VvPIP1;1* or *VvPIP2;2* and the starting quantity of *VvActin*. Plants were shoot-topped to remove approximately 30-40% of leaf area 24 h prior to root sampling. Roots were sampled at 1200 h. Values are mean ± SEM of three biological replicates. Columns with a different letter are significantly different ($P < 0.05$).

Two experiments were carried out on field grown vines in the Alverstoke Vineyard (Waite Campus University of Adelaide) during 2008 to test the impact of shoot pruning (30 % of shoot removed) on vine hydraulic conductance. These experiments were set up with Chardonnay vines on own roots that had sap flow sensors installed prior to shoot pruning. Predawn and midday stem water potentials were measured on control (non pruned) and pruned vines before and after pruning. Conductance was determined as the sap flow rate divided by the difference between pre-dawn and midday stem water potential. Root conductance is normally smaller than shoot conductance, therefore if there is a decrease in root conductance this should be reflected in a reduced overall vine conductance (shoot and root together). However, the results indicated if anything an increase in vine conductance due to pruning, which was largely due to a decrease in the water potential gradient for the same sap flux (Fig. 6.2.9). A second experiment was carried out on vines earlier in vegetative development and in this case aquaporin expression was measured in roots sampled from adjacent trenches filled with sand. No change in vine stem water potential was observed after pruning (Fig. 6.2.10), though there was a decrease in the expression of bother *VvPIP1;1* and *VvPIP2;2*, both of which are implicated in control of root hydraulic conductance. This decrease however was not statistically significant.

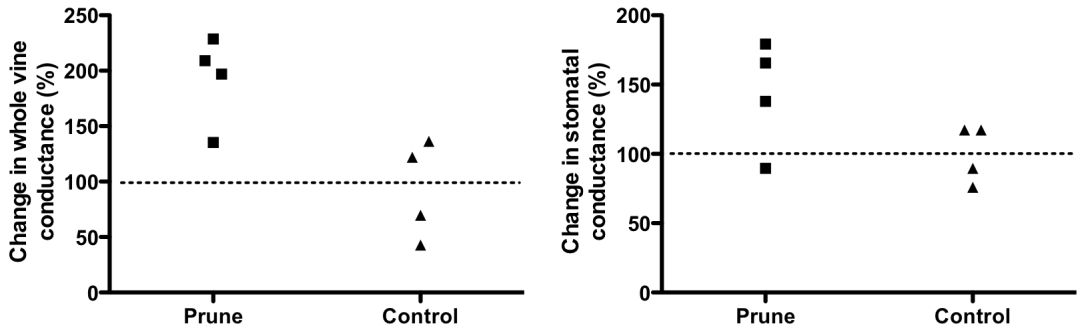


Figure 6.2.9 Change in whole vine conductance and stomatal conductance of pruned field vines and control (unpruned vines) carried out on the 29 and 30 Jan 2008. Whole vine conductance was determined from water

potential gradient (pre-dawn – midday) and sap flow rate using Greenspan sap flow sensors. The increase in conductance observed for pruned vines was largely due to a reduced water potential gradient, but with no change in transpiration.

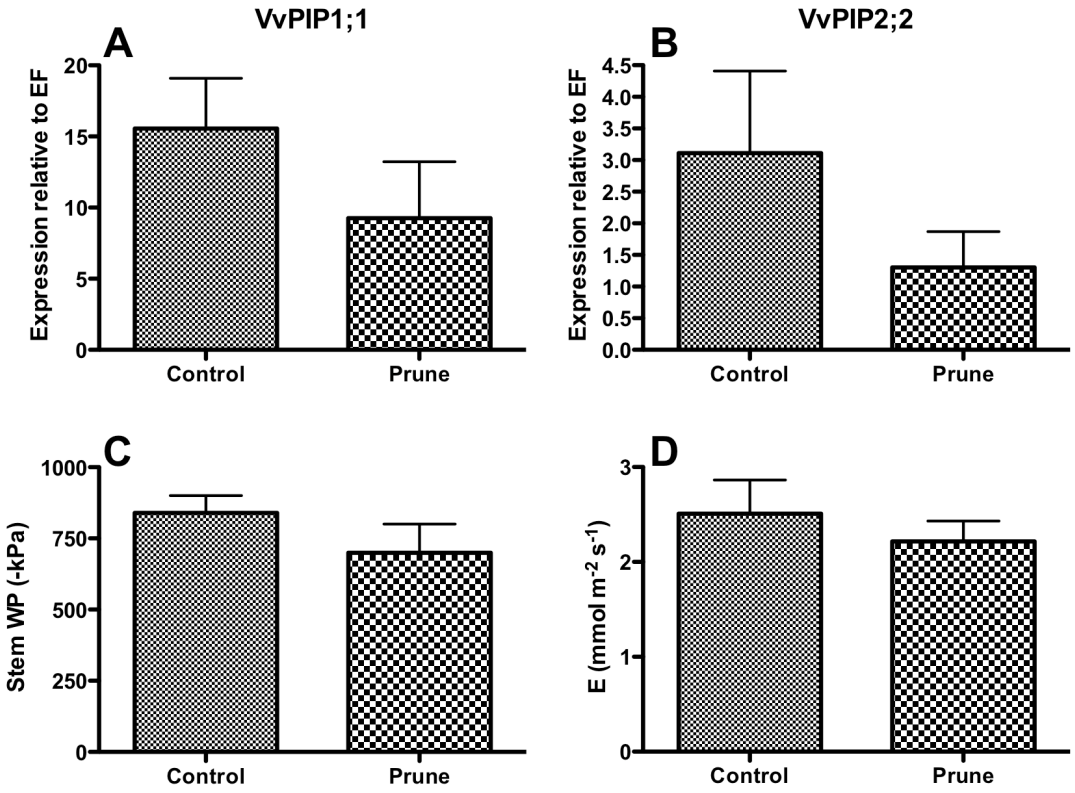


Figure 6.2.10 A second experiment on field grown vines (29th and 30th Oct, 2008) to examine the effect of pruning on vine conductance and aquaporin expression in roots. In this case a trench beside the vines was established (filled with sand, see methods) that enabled easier access to roots. Roots were sampled from the trench to compare the changes in expression of two key aquaporin genes (A: *VvPIP1;1* and B: *VvPIP2;2*) in response to pruning. Although both decreased in expression relative to elongation factor (which did not change expression) this was not significant at the 0.05 level. In this experiment there was no change in water potential gradient or transpiration (on leaf basis) to pruning.

6.2.3 Discussion

Shoot topping does affect the L_o of roots of potted vines. Therefore, there must be a signal from shoots to roots, either a negative signal or the loss of a positive signal. The identity of this signal or possibly multiple signals remains elusive. It appears to be a general plant phenomenon with three species examined - grapevine, soybean and maize - all demonstrating a lower L_o in shoot-topped plants. This lower L_o did not correlate with leaf area remaining after shoot topping in soybean and grapevine. Therefore it did not directly correlate with transpirational demand of the plant, assuming that transpiration per unit leaf area remained constant. However, it is likely that lower transpirational demand does contribute to the lower value of L_o (see 6.1). Reduced transpiration may be important in sustaining the lower value of L_o over time, as seen for grapevines (Refer 6.1 above). The influence of transpiration on L_o was evident when soybeans were bagged and kept in the dark to reduce transpiration, resulting in a substantially lower L_o.

A reduced response to shoot topping when L_o of control plants was relatively low suggests the involvement of aquaporins confirmed by the reduced expression of *VvPIP1;1*. When transpiration is low, the activity or number of aquaporins may be low. If shoot topping also caused a reduction in the number or activity of aquaporins this reduction would be limited if the L_o was low prior to application of treatments. Carvajal *et al*. (1996) observed a similar effect with wheat: when plants were covered to reduce transpiration there was a 50%

reduction in L_o (normalised to root fresh weight). Plants that were deprived of nitrogen or phosphorus did not have their L_o affected by covering the plants. This was also suggested to be due to aquaporins already having been switched off such that no further reduction in L_o could occur (Carvajal *et al*. 1996).

There was a large degree of variability of the L_o of control grapevines and soybeans. There was a large difference in the transpiration rate in the 'darkness' and 'degree of leaf removal' experiments. The large transpiration rate in the 'darkness' experiment was associated with high L_o. Franks *et al*. (2006) also found that whole plant leaf specific hydraulic conductance was significantly correlated with transpiration rate. Hubbard *et al*. (2001) systematically reduced the conductance of the soil to leaf pathway with an air injection technique to cause cavitation in the stem. This reduction in conductance was associated with reduced stomatal conductance and assimilation of ponderosa pine (*Pinus ponderosa*). It is possible that there is a two-way regulation or feedback between transpiration and root hydraulic conductance, such that changes in either one can alter the other. A positive relationship between apparent root hydraulic conductance (calculated from E and hydrostatic pressure gradients) and stomatal conductance has also been found by Saliendra and Meinzer (1989) and Meinzer and Grantz (1990).

The effect of shoot topping was evident when just the young, expanding leaves were removed by cutting the stem. These young leaves are a major sink for sugar and carbohydrates in the plant and also a source of hormones. Auxin is synthesised in the young dividing leaves (Palme & Gäweiler 1999). In addition, in young Arabidopsis plants, synthesis was shown to occur in the expanding leaves, cotyledons and the roots (Ljung *et al*. 2001). ABA has been shown to be synthesised in the leaves of grapevines, with higher concentrations of ABA in the leaves closer to the shoot apex (Soar *et al*. 2004).

There was no difference in ABA concentration in roots of control and shoot-topped grapevine. It is possible that ABA concentrations may be lowered by shoot topping as it has been shown that the leaves of grapevines do synthesise ABA (Soar *et al*. 2004). ABA recirculation has been detected in castor bean (*Ricinus communis* L.), *Xanthium strummarium* L. (Zeevaart & Boyer 1984) and maize (Jeschke *et al*. 1997). The ABA synthesised in the leaves is loaded into the phloem to be transported to the roots where it is either stored or recirculated to the xylem vessels and transported to the young leaves in the transpiration stream. ABA is sequestered in the symplast and is only mobilised into the apoplast if there are changes in pH (Wilkinson & Davies 1997). Therefore, movement of ABA to the roots may only occur under certain conditions, such as drought stress, when pH is elevated (Stoll *et al*. 2000). Therefore, in the well-watered grapevines utilised in these experiments, ABA translocation to the roots may be quite low resulting in minimal impact of shoot topping on ABA movements to the roots.

It is possible that cutting of the shoot caused a wounding response, resulting in ethylene synthesis. There has also been a link between decreased light intensity and ethylene synthesis in Arabidopsis (Vandenbussche *et al*. 2003). Therefore, ethylene is a possible candidate to reduce L_o in response to shoot topping and darkness. Although this cannot be ruled out completely, it does not appear that ethylene is the signal causing a lower L_o. The ACC synthase inhibitor acted independently of the response to shoot topping. Soybeans sprayed with the inhibitor 24 h previously had a higher L_o. The AVG may have opened stomata and increased transpiration resulting in a higher L_o than the unsprayed controls. Kamaluddin and Zwiazek (2002) have reported that the addition of ethylene to the hydroponic solution of aspen (*Populus tremuloides*) seedlings increased the L_o of hypoxic plants. This response is in contrast to that expected based on the use of the ethylene inhibitor in this experiment.

Another possible form of a negative signal is hydraulic. Air may enter the xylem when the shoot is cut, causing an embolism that may be transmitted to the root. Shoot topping did not alter either transpiration rate or stomatal conductance on a per unit leaf area basis of soybean. Within 24 h there was no compensatory increase in transpiration rate per unit leaf area to overcome an overall reduction in whole plant transpiration due to reduced leaf area. Increased photosynthesic rate in response to leaf removal has been previously recorded for

grapevine (Candolfi-Vasconcelos 1991; Petrie *et al*. 2000). Petrie *et al*. (2003) did not observe an increase in photosynthesic rate per unit leaf area 2 days after a shoot topping treatment that reduced leaf area by 8%. The reduction in L_o may limit any compensatory increase in transpiration rate and photosynthesis of the remaining leaves.

The results of the shoot topping experiments imply that shoot-to-root signals may be important. At this stage it is still unclear what the exact signal is that causes the reduction in L_o when the plants were shoot-topped. The response may be due to the loss of a positive signal or the production of a negative signal that reduces L_o. It appears that the signal is most likely not ABA, ethylene or due to changes in transpirational leaf area. Auxin is another possibility yet to be tested. Auxin is a good candidate as it is synthesised in the young leaves, whose removal did cause a reduction in L_o, and auxin can be transported by auxin carrier-mediated transport in t vascular tissue, which would not be affected by girdling, and via the phloem (Guo *et al*. 2005; Palme & Gälweiler 1999). Additionally, auxin has been demonstrated to increase the water permeability of leaf epidermal cells of *Allium cepa* bulbs and *Rhoeo discolour* (Loros & Taiz 1982). Other possibilities include an electrical signal (Fromm & Lautner 2007). It is possible that longer-term reduction in L_o may be due to reduced leaf area, thereby reducing the demand for water. The reduction in L_o 24 h after shoot topping suggests that, in a field situation, it may not be ideal to perform summer pruning operations during periods of high evaporative demand. The reduced L_o may cause a large reduction in the water potential of the plant and cavitation may occur in the xylem.
The lack of a significant response to pruning in field grown vines could be due to several factors, but we believe that the major problem with these measurements is the indirect way in which hydraulic conductance is measured. Whole vine conductance is determined from sap flow and the difference between pre-dawn and midday water potentials. There was a response in aquaporin expression, which is tantalizing and suggesting that further experiments should be carried out.

6.3 Impact of partial root drying on root hydraulic conductivity of grapevines

This section forms the basis of a manuscript in preparation by the following authors:
R.K. Vandeleur, B.N. Kaiser, P.R. Dry, W. Sullivan and S.D. Tyerman

6.3.1 Introduction

Grapevines often require supplementary water to maximise their potential. Due to environmental and economic concerns it has become imperative to reduce the use of water for irrigation. Deficit irrigation methods have been developed to achieve this goal. Reduced deficit irrigation (RDI) reduces the water applied during certain growth stages to create a mild stress. It is mostly utilised from flowering to veraison for grapevines (Mitchell and Goodwin, 1996). This method requires constant monitoring of soil and plant water status. Sudden increases in temperature or low humidity can cause the plants to quickly become severely water stressed, possibly causing yield penalties. This problem is overcome using partial root-zone drying (PRD). The technique of applying water to only half the root system has been developed commercially for vineyards (Loveys et al. 1997, Dry 1997). PRD is also a technique to reduce vine vigour to optimise grape quality and yield. This improves water use efficiency. PRD takes advantage of the fact that the chemical signal, abscicic acid (ABA), from the roots controls shoot growth and transpiration. Dry and Loveys (1999) observed that shoot function recovers without rewatering of the dry half, due to the transient nature of ABA accumulation. Therefore the dry side needs to be alternated between the 2 halves every 10-14 days under field conditions.

The impact of PRD on hydraulic conductivity of the roots is unknown. Whole plant conductance of Pinot Noir grafted to *V. riparia* × *V berlandei* was not altered by only watering half the root system in a split-root experiment (Lovisolo et al. 2002). Stoll et al. (2000) demonstrated using deuterium enriched water that water moved from the roots on the wet side to roots in the dry half. It has been shown that there is increased root growth in the dry container of grapevines (Poni et al 1992 (in Dry and Loveys 98). This may be in response to ABA which is known to maintain root growth in drying soil (Sharp et al. 1994).

The aim of this work was to examine the impact of PRD on the hydraulic conductivity of the root system and the individual root systems in the separate halves. Two different cultivars, which differ in their response to water stress were used.

6.3.2 Results

Leaf water potential of PRD plants was not significantly different to well watered plants of both cultivars (Table 6.3.1 & 6.3.2). The stomatal conductance was reduced significantly ($F_{pr}<0.01$) for both cultivars in response to withholding water from half the root system for 7 days (Table 6.3.1 & 6.3.2). The reduction in g_s was approximately 36-38 %. There was a 20-22% reduction in leaf transpiration rate. This reduction was only significant for Grenache ($F_{pr}<0.05$) (Table 6.3.2). The xylem sap of Grenache had a higher concentration of ABA in PRD plants compared to well-watered plants (Table 6.3.2). There was no significant difference in the ABA content of control and PRD Chardonnay plants (Table 6.3.1).

Table 6.3.1. Effect of PRD on leaf water potential (ψ_{leaf}), stomatal conductance (g_s), transpiration (E) and ABA content of xylem sap of Chardonnay plants. Values are means ± SE of six replicate plants.

Treatment	ψ_{leaf} (kPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	ABA ($nmol.ml^{-1}$
Control	910±30[a]	111±9[a]	2.26±0.0.21[a]	2.16±0.0.38[a]
PRD	872±30[a]	72±10[b]	1.77±0.22[a]	1.78±0.0.29[a]

Values with different letters within a column are significantly different ($P<0.05$).

Table 6.3.2. Effect of PRD on leaf water potential (ψ_{leaf}), stomatal conductance (g_s), transpiration (E) and ABA content of xylem sap of Grenache plants. Values are means ± SE of six replicate plants.

Treatment	ψ_{leaf} (kPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	ABA ($nmol.ml^{-1}$
Control	861±75[a]	120±10[a]	2.97±0.0.19[a]	1.82±0.0.17[a]
PRD	910±92[a]	75±6[b]	2.4±0.07[b]	2.68±0.0.23[b]

Values with different letters within a column are significantly different (P<0.05).
The total root L_o of Chardonnay (Fig. 6.3.1) was not affected by watering treatment, whereas there was a significant (Fpr<0.05) reduction in L_o of Grenache plants (Fig. 6.3.2) with only half the root system watered.

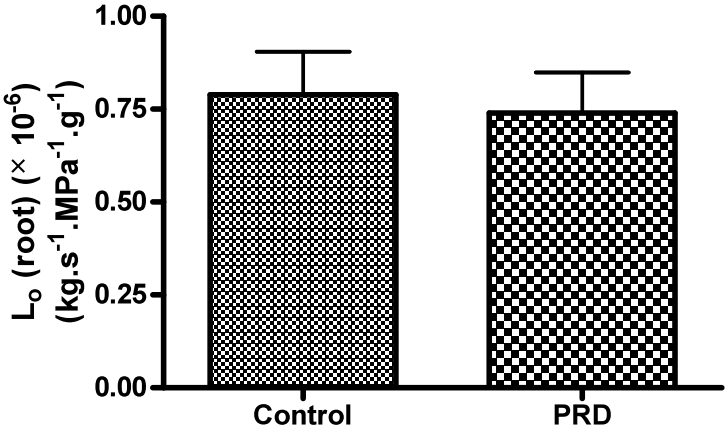


Figure 6.3.1. Impact of partial rootzone drying (PRD) on hydraulic conductance (normalised to root dry weight) of the total root system of Chardonnay. Measurements were taken between 1 and 3 pm. Values are mean ± SE of six plants.

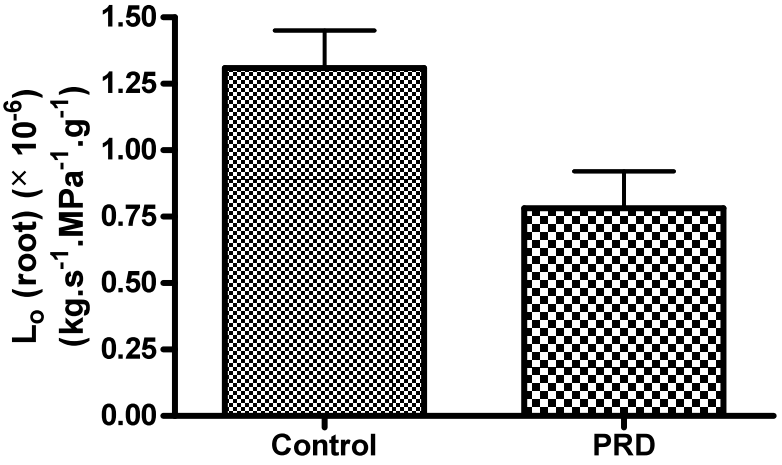


Figure 6.3.2 Impact of partial rootzone drying (PRD) on hydraulic conductance (normalised to root dry weight) of the total root system of Grenache. Measurements were taken between 1 and 3 pm. Values are mean ± SE of six plants.

When the two halves of PRD plants were examined, both Chardonnay and Grenache roots on the wet side had an increase in L_o of 30-40 %, compared to plants with both halves watered (Fig 6.3.3, 6.6.4, & 6.3.5A). This increase was significant at the 5 % level in the second experiment on Chardonnay (Fig. 6.3.5), and at the 10 % level in the first experiment (Fig. 6.3.3). The L_o on the dry side of PRD plants declined significantly (Fpr<0.05) for both cultivars. Compared to well watered control plants, Grenache roots had a 4-fold decline in L_o, while Chardonnay roots had a 2-fold decline. In the second experiment on Chardonnay this decline was less (Fig. 6.3.5A). To test for the activity of aquaporins, acid inhibition was used. This entailed watering the pot with a weak acid (acetate) at pH 5.5 and then measuring the

change in Lo. This technique has been used to measure involvement of aquaporins because they are strongly inhibited by slight acidification of the cytoplasm. There was a significantly larger inhibition of the dry roots of Chardonnay, despite the Lo being smaller (Fig. 6.3.5B). This experiment was also used to check on possible changes in overall root structure since some changes in Lo may be a consequence of changed proportions of small roots or total root mass (though Lo is normalised to root dry weight). There was no significant difference in the proportion of fine roots or in root dry weight between the wet and dry sides of the PRD system (Fig. 6.3.5C,D).

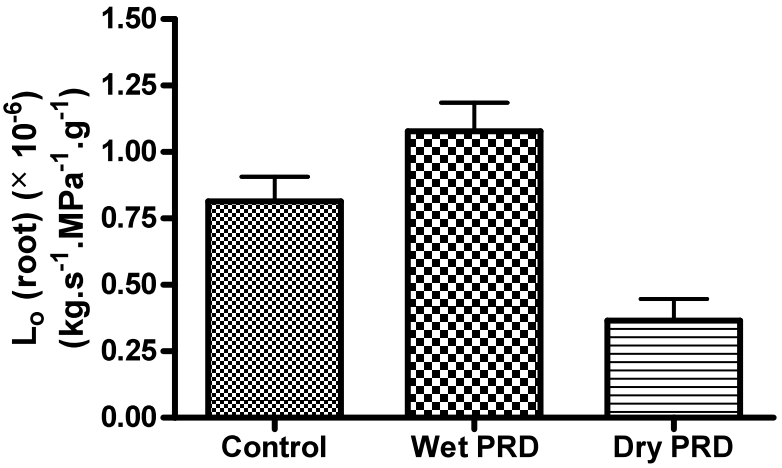


Figure 6.3.3 The hydraulic conductance (normalised to root dry weight) of Chardonnay roots in individual pots. Control is the pots that were well-watered on both halves. Values are mean ± SE of twelve pots. Wet PRD is the wet half of PRD plants, while Dry PRD is the dry half of the PRD plants. Values are mean ± SE of six pots.

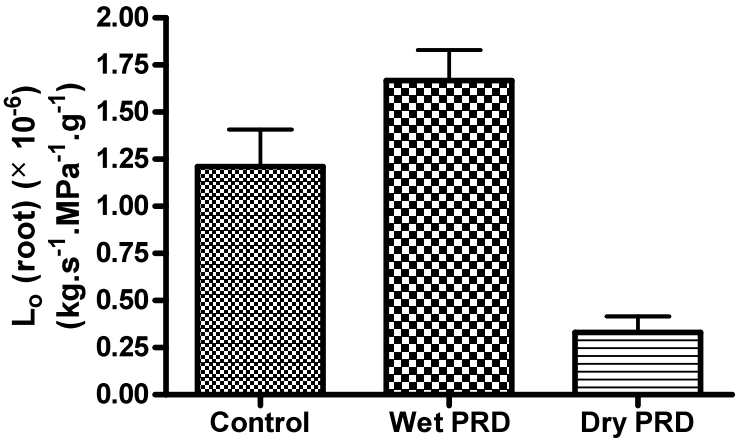


Figure 6.3.4 The hydraulic conductance (normalised to root dry weight) of Grenache roots in individual pots. Control is the pots that were well-watered on both halves. Values are mean ± SE of twelve pots. Wet PRD is the wet half of PRD plants, while Dry PRD is the dry half of the PRD plants. Values are mean ± SE of six pots.

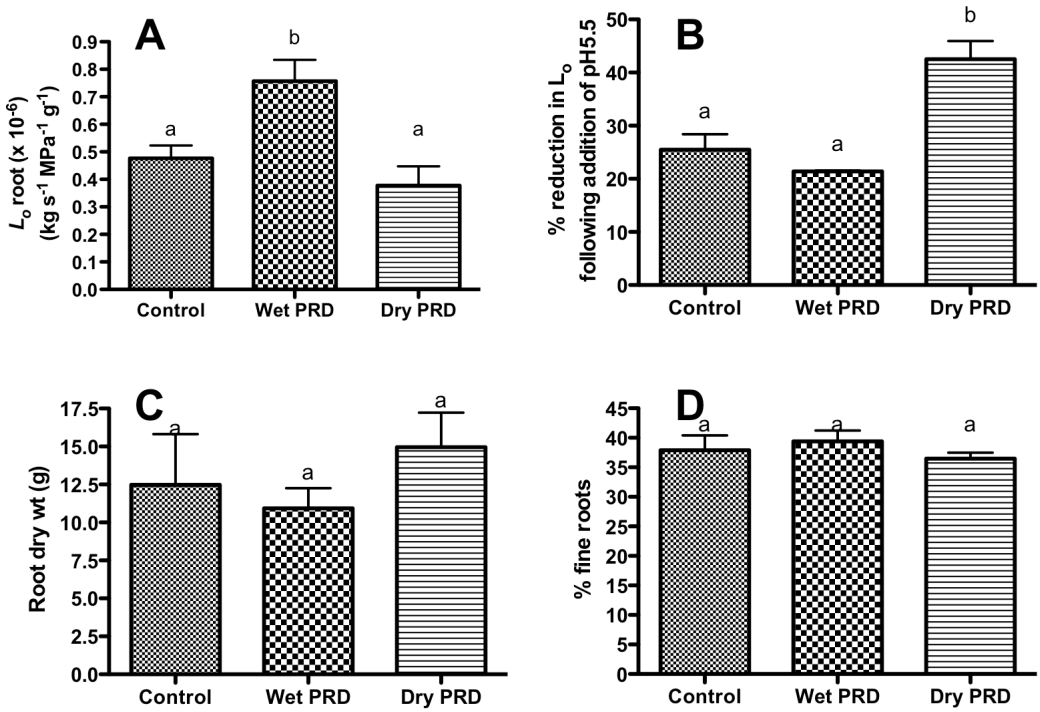


Figure 6.3.5 A, The hydraulic conductance (normalised to root dry weight) of Chardonnay roots in individual pots. Control is the pots that were well-watered on both halves. Wet PRD is the wet half of PRD plants, while Dry PRD is the dry half of the PRD plants (8 days). B. In this case the inhibition caused by weak acid (acetate) treatment at pH 5.5 was used as a test of the involvement of aquaporins. Changes in root structure were also assessed with total dry weight (C) and proportion of fine roots (less than 1 mm) (D). Values are mean ± SE of 4 to 6 plants.

Using a double pot system so that roots in both wet and dry pots could be sampled, we determined the change in expression of 3 aquaporin genes (Fig. 6.3.6). These genes were chosen from there expression profiles (the most highest expressed genes in roots) and there association with changes in root hydraulic conductivity in response to diurnal time, drought and shoot topping (see 6.1, and 6.2). It was expected that *VvPIP1;1* would increase in expression in the wet side of PRD treatments, since this side had higher Lo. However, the expression of *VvPIP1;1* was not different between controls, wet PRD and Dry PRD. There was also no change in *VvPIP1;1*. However, *VvPIP2;2* decreased significantly in both the dry and wet side of PRD plants (Fig. 6.3.6, B,C).

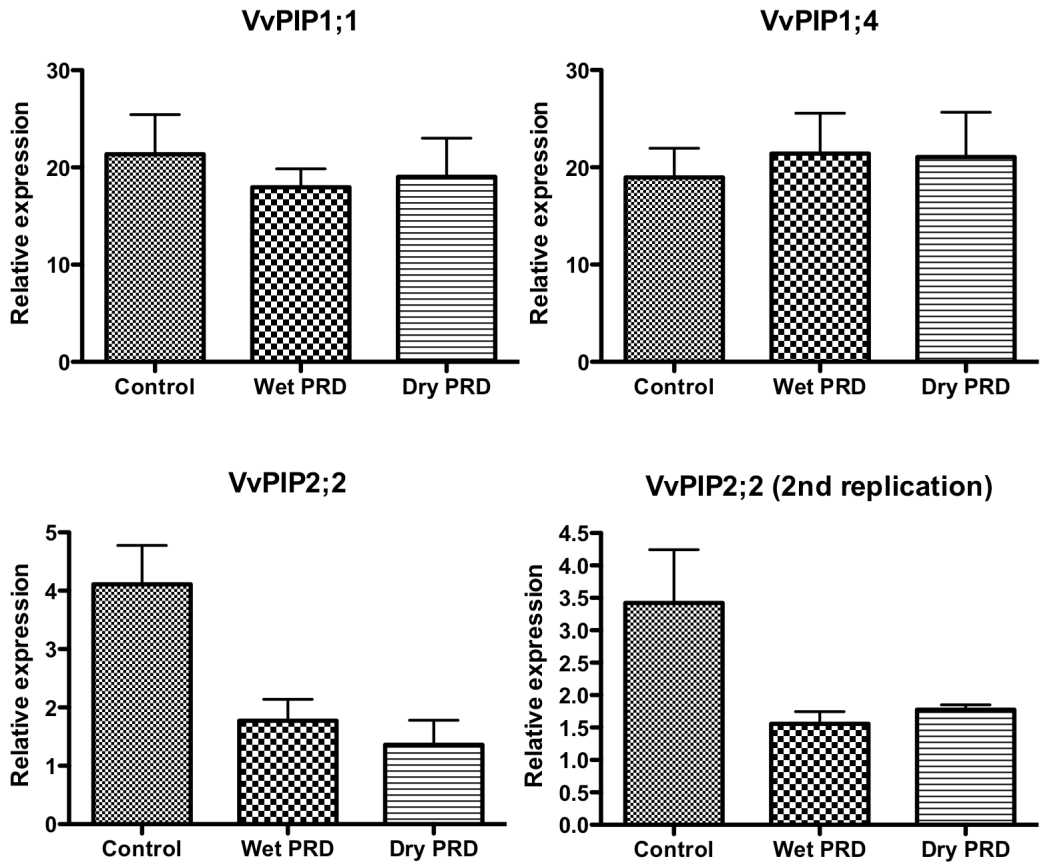


Figure 6.3.6 Relative expression of key aquaporin genes (relative Vvactin) in Chardonnay roots sampled from the bottom pot in double pot experiments where PRD was imposed in split pot experiments. Mean +/- SEM of 4 biological replicates.

6.3.3 Discussion

The effect of PRD on shoot functions were similar to those observed by Dry and Loveys (1998), Dry et al (2000) and Stoll et al (2000). There was partial closure of stomata of approximately 35 %, which is almost the same as that found using the cultivars Chardonnay and Shiraz (Dry and Loveys 1999). Generally, when grapevines are water stressed there is almost complete closure of stomata. There was an approximately 20 % decline in transpiration, compared to a halving of the amount of water applied, indicating an improvement in the transpiration efficiency. There was no effect of PRD on ψ_{leaf} as reported previously (Dry and Loveys 1999, Dry et al. 2000). This has supported the fact that the signal was chemical rather than hydraulic. The ABA content of the xylem sap did vary between the two cultivars. Grenache showed the expected increase in ABA in response to PRD. This increase was much smaller than observed for water stressed vines under similar conditions (Vandeleur et al. to be published). Stoll et al. (2000) suggest that under water stressed conditions ABA is imported from the roots and is also synthesised in the leaf due to a reduction in leaf water potential. The data of Stoll et al. (2000) also indicates the transient nature of ABA in the leaf and the small increase in the wet/dry treatment compared to the well watered control. The non significant difference in ABA content for Chardonnay could be due to the ABA levels already declining from their peak once the soil water content had stopped declining further.

There was no influence of PRD on the root and shoot size or the ratio of shoot to root dry matter. This may be due to the short time period of the experiment. Over an extended time period you may expect a decline in the shoot size of PRD plants (Dry and Loveys 1999).

The reduction in the overall L_o of Grenache was due to the large reduction in L_o on the dry side which was not overcome by the increase on the wet side. This larger reduction due to water stress of Grenache compared to Chardonnay has been observed previously (Vandeleur et al. 2009, see 6.1 above). Due to the small decline on the dry side of Chardonnay and slight increase in L_o on the wet side there was no overall decline in L_o due to the implementation of PRD. The response of Chardonnay supports the results of Lovisolo et al. (2002) using Pinot Noir grafted to *V. riparia* × *V berlandei.* Lovisolo et al. (2002) measured the conductance of the whole plant. Due to the lack of reduction in plant L_o Lovisolo et al (2002) proposed it was not regulated by ABA. Whereas whole plant conductance was reduced in split rooted, shoot inverted grapevines with no change in ABA accumulation. We have now shown that imposing water stress to one half of split rooted grapevines does modify the hydraulic conductance of the roots of the individual halves and in the case of Grenache the overall root L_o. We did not measure the shoots to determine the overall plant hydraulic conductance. Hose et al (2000) has shown in maize roots that ABA increases the water permeability of individual cells. It is possible that the ABA produced in response to water stress on the dry side is transported to the wet side where it acts to up-regulate the activity of aquaporins resulting in an increase in L_o.

Green and Clothier (1995) changed the water distribution to kiwifruit vines and monitored water uptake using heat pulses. The water uptake moved away from the roots in the dry soil with the water being taken up preferentially from the wet soil. When the soil was rewetted the previously inactive roots in the dry soil recovered activity and new root growth was observed. Plants appear to be able to adapt their water uptake to the locations of water availability. This was also the case when split-rooted wheat plants were deprived of nitrogen and phosphorus. Even though transpiration wasn't effected L_o was significantly reduced on the nutrient deprived side. In this case the L_o quickly recovered when nutrients were resupplied.

There maybe an effect of cultivar on the grapevine's ability to compensate and also the degree of reduction in L_o of any roots exposed to dry soil appears to be important. Removing roots of Shiraz on 101-14 Mgt rootstock had a significant effect on leaf and stem water potential in addition to g_s and A and a decline in leaf specific L_o (Smart et al 2006). In this case the remaining roots could not compensate. It is possible that there may have been some increase in L_o if it had been normalised to the size of the remaining root system. The reduction in water potential though suggests otherwise. However, the remaining root of wheat plants could compensate for the loss of four roots (Vysotskoya et al. 2004). Five minutes after root excision there was an increase in L_o (normalised to root fresh weight) in the presence of a hydrostatic pressure gradient, but not due to an osmotic pressure suggesting an increase in apoplastic flow only. After 1.5 hours there was an increase in L_o under both conditions, evidence that aquaporins contributed to root water permeability. The increase in L_o resulted in no impact of root excision on transpiration and stomatal conductance (Vysotoskoya et al. 2004). The differences observed between Grenache and Chardonnay may be important in selecting cultivars appropriate for PRD implementation.

The increase in L_o on the wet side of PRD plants was not associated with a change in expression of *VvPIP1;1* in Chardonnay. This goes against the general pattern observed in other experiments where changes in *VvPIP1;1* correlates with changes in root L_o. However, *VvPIP2;2* decreased in PRD plants in both the wet and dry sides. This would cause the ratio of *VvPIP1;1* to *VvPIP2;2* to increase, and this ratio may be more important than the absolute amounts of each individual aquaporin because of the interaction that appears to occur (See 6.1). This ratio does seem to correlate with root L_o in other experiments, but it does not explain the apparent increased importance of aquaporins in the dry PRD roots as indicated by the acid inhibition. Here it is possible that increased lignification and suberisation has reduced the apoplastic pathway of radial water transport across roots, thereby increasing the proportion that traverses the cell-cell pathway via aquaporins. The presence of new lateral roots or root hairs would likely not cause a significant difference in the root size between the wet and dry side during such a short period of time and this was indicated by no significant change in the proportion of fine roots. It is also unclear how a number of cycles of drying and rewatering may influence the L_o of the wet and dry sides. We have observed previously that the recovery in L_o after 24 hours or rewatering is minimal. When the watering is switched to the dry half there may be a further reduction in transpiration, g_s and possibly in leaf water

potential until the water permeability of the roots is improved either through new root growth or up regulation of aquaporins.

6.4 Root apoplastic transport and water relations cannot account for differences in Cl^- transport and Cl^-/NO_3^- interactions of two grapevine rootstocks differing in salt tolerance.

This section forms the basis of a manuscript in preparation by the following authors:
Nasser Abbaspour, Brent Kaiser and Steve Tyerman

6.4.1 Introduction

Excessive soil and water salinity reduces plant growth by salt accumulation on the outside of roots (osmotic effect) and within the plant (ion toxicity) as well as ion imbalance (Munns and Tester, 2008). In a large percentage of studies, Na^+ and Cl^- have been taken as the major salinisation factors in soils and plants (Gratten & Grieve, 1999). $Chloride^-$ can be the more toxic component in some plants (Munns & Tester, 2008; Teakle and Tyerman, 2009) and compared to Na^+ transport, the mechanisms of Cl^- transport to the root and its uptake to the shoot and / or efflux under salt stress are less understood (White & Broadley, 2001; Teakle and Tyerman, 2010). Chloride transport to and accumulation in the shoot is a main indication of Cl^- sensitivity in many plants including *Vitis vinnifera* (grapevine) and *Vitis* root stock species (Greenway, 1965; Downton & Millhouse 1983).

Grapevines are frequently grown and irrigated in semi-arid zones where drought and salinity are common problems (Cramer et al. 2007). Salinity causes growth reduction in grapevine (Walker, 1994) and excessive accumulation of Cl^-, particularly in leaves, is the main reason for salt damage (Downton & Millhouse, 1983; Hardie & Cirami, 2000). Studies show that photosynthesis and stomatal conductance (Downton, 1977), ion composition in different parts of grapevine (Garcia & Charbaji 1993; Fisarakis et al. 2004) and grapevine yield (Downton, 1985; Prior et al. 1992b; Stevens et al. 1999) are affected by salinity. Walker et al. (2002a) found a strong correlation between salt tolerance in Sultana grapevine and rootstocks vigor. Many studies have shown that certain grapevine rootstocks for example Salt Creek (Ramsey) (Bernstein et al. (1969) or 1103 Paulsen (Walker et al. 2004) are able to decrease Cl^- accumulation within scions.

Nitrate (NO_3^-) is an important source of plant nitrogen nutrition and there are many reports that show that under salt (NaCl) stress conditions, uptake of nitrogenous inorganic ions is severely reduced (Pessarakli & Tucker 1985; Frota & Tucker, 1987; Palfi, 1965; Mahajan & Sonar, 1980). Cl^- and NO_3^- have nearly similar size and identical charge and may interact (e.g. competition for transport sites) (Kafkafi et al. 1982). It is likely that Cl^- in high concentrations may interact with transport of NO_3^- across membranes since both anion channels and some anion transporters may transport both ions (reviewed in Teackle and Tyerman, 2010).

In considering different ion selectivities between genotypes that differ in salt tolerance, it is important to consider the different pathways through which ions and water can traverse the root. There are three pathways: apoplastic, symplastic and transmembrane pathways (Flowers & Yeo, 1992). Among the different possible ways for salt (NaCl) entry from epidermis to the xylem during high salinity conditions, direct apoplastic continuity is a pathway without biological selectivity for ion transport in the regions of the root where endodermis has not been formed or has been interrupted (bypass flow) (Yeo et al. 1987). The importance of this pathway becomes more dominant in high external concentrations of salt (Pitman, 1982) and at high transpirational rates (Sanderson, 1983) and has not be examined in grapevines or other woody rooted plants.

The bypass flow pathway can be assessed in different ways. One way is to examine the osmotic properties of the root based upon the composite transport model using the root pressure probe. The root consists of a complex composite osmotic barrier built up of several layers (Steudle & Peterson 1998). In contrast to a perfect osmometer which has a reflection coefficient close to unity, the reflection coefficient measured in roots of different species is between 0.2-0.8 (Steudle & Frensch 1989). This indicates that ions may pass through the root cylinder (Steudle & Peterson 1998). According to the composite transport model, which is

based on observations with the root pressure probe reconciled with anatomical studies, the low reflection coefficients of roots can be attributed to by-pass flow of water and solutes via the apoplastic pathway. The apoplast has high hydraulic conductance and very low reflection coefficient.

Another technique to measure the impact of bypass flow, is to measure the flux of a compound that moves quantitatively with water flow in the apoplast. It should also not pass cell membranes and not stick to cell walls (Yeo et al. 1987). A fluorescent dye, 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS), which includes all of the above mentioned characteristics, has been used successfully to study the apoplastic pathway of Na^+ transport under high salinity in rice roots (Yeo et al. 1987 & 1999), rice and wheat (Garcia et al. 1997), red pine root (Hanson et al. 1985), mangrove root (Moon et al. 1986), and it has been used to trace water flow (Zimmerman & Steudle 1998).

Using PTS, Yeo et al. (1987) found that rice plants with high Na^+ transport to the shoot had high shoot concentrations of PTS. They suggested that Na^+ was transported to the xylem probably via the same pathway for PTS uptake. They showed that there was a correlation between PTS uptake and Na^+ uptake amongst different varieties. They also concluded that bypass flow can increase under high salinity and it is a major contribution to Na^+ transport in rice roots under salt stress conditions.

In the present study we have used various techniques to estimate the extent of apoplastic transport of Cl^- under high salinity in two grapevine rootstocks differing in salt tolerance; K 51-40 a salt sensitive variety and 1103 Paulsen a moderately salt tolerant variety (Hardie and Cirami 2000). Using $^{15}NO_3$, the selectivity between Cl^- and NO_3^- uptake into plants in the presence of different NaCl concentrations was investigated. The root pressure probe technique was used to measure the hydraulic conductivity of detached roots (L_{pr}) and the reflection coefficient (σ_s) for NaCl and $NaNO_3$ in K 51-40 and 1103 Paulsen for the purpose of testing if there were differences between the two rootstocks from the view point of bypass flow of salts to the xylem. Root anatomical studies also were carried out to find differences in apoplastic barriers or root branching for the rootstocks. We also assessed potential differences in bypass flow between rootstocks using PTS in the presence of high external concentrations of NaCl.

6.4.2 Results

NO_3^-/Cl^- interaction

Concentrations of Cl^- in both varieties increased in shoots and roots after 7 days treatment with different concentrations of NaCl (Fig 6.4.1). In comparison to the salt tolerant Paulsen, the salt sensitive K 51-40 accumulated more Cl^- in the shoot (Fig. 6.4.1 A) and this was already evident in the basal growth solution. Cl^- accumulation in roots of both varieties was similar although it significantly increased with increased NaCl concentrations in Paulsen (Fig. 6.4.1B).

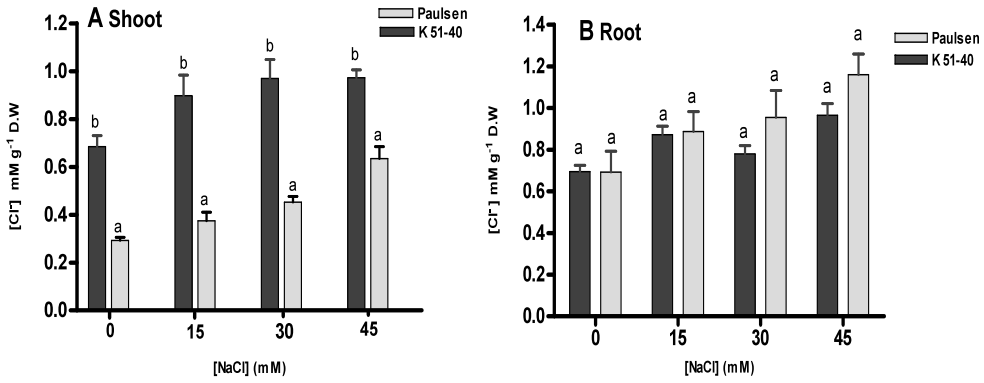


Figure 6.4.1. Chloride concentration in shoot (A) and root (B) of K 51-40 and Paulsen treated with different concentrations of NaCl for 7 days. Bars are SE of the means (n=4). Different letter indicates significant difference between the varieties in each treatment..

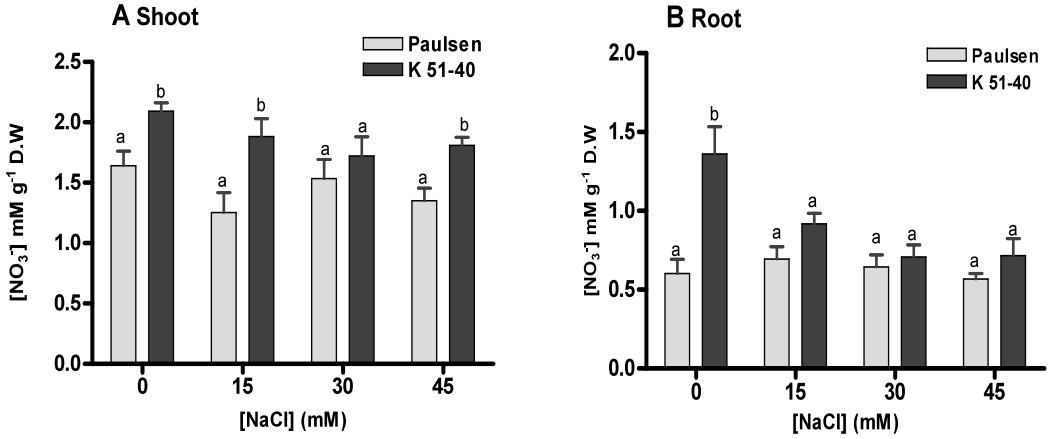


Figure 6.4.2. Total NO_3^- concentration in shoot (A) and root (B) of K 51-40 and Paulsen treated with different concentrations of NaCl for 7 days. Bars are SE of the means (n=4) (two way ANOVA, $P < 0.05$). Different letter indicates significant difference between the varieties in each treatment (t-test).

Total NO_3^- concentration of the shoot in the salt sensitive K 51-40 was higher than salt tolerant Paulsen, but with increasing NaCl concentrations no significant difference between different salt treatments was observed (Fig. 6.4.2A). However, NO_3^- concentrations in roots were reduced in K 51-40 while it had no significant effect on Paulsen (Fig. 6.4.2 B).

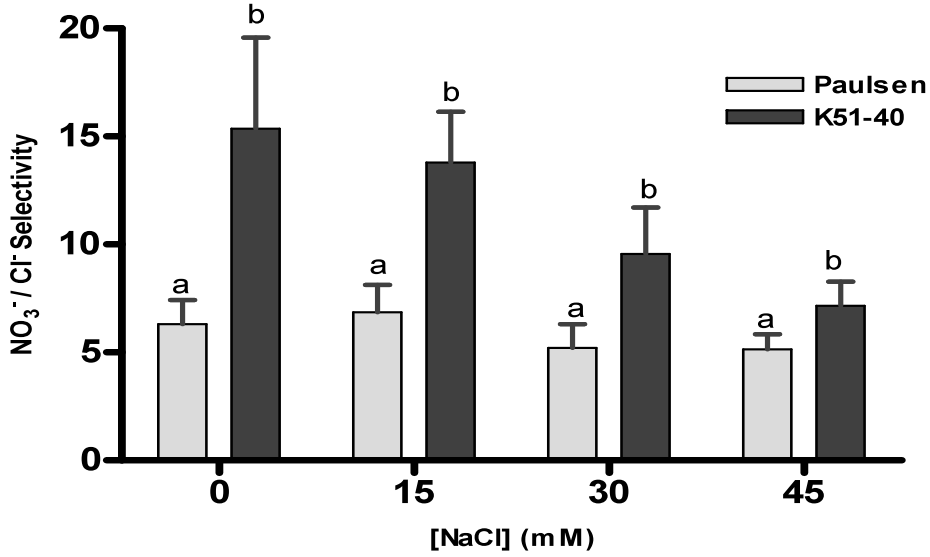


Figure 6.4.3. NO_3^-/Cl^- selectivity in root of K 51-40 and Paulsen. Bars are SE of the means (n=4). Different letter indicates significant difference between the varieties in each treatment.

A NO_3^- / Cl^- selectivity (SNO_3^-/Cl^-) ratio was calculated as the ratio of tissue (root) NO_3/Cl to external NO_3/Cl. This showed significant differences between the two varieties with K 51-40 showing reduced root SNO_3^-/Cl^- with increased salinity, though it initially had a higher SNO3/Cl (Fig. 6.4.3).

Root hydraulic conductivity and reflection coefficients

Hydraulic root pressure relaxations were measured in K 51-40 and Paulsen roots yielding monophasic exponential curves. From the half-time ($T_{1/2}$) of water exchange obtained, hydraulic conductivity (Lp_r) of the roots was calculated using Eq. 3. Hydrostatic Lp_r for K 51-40 and Paulsen was 5.7 x 10^{-7} (m $s^{-1}MPa^{-1}$) and 6.1 x10^{-7} (m $s^{-1}MPa^{-1}$) respectively. There was no significant difference between the Lp_r of the rootstocks (t-test) (Table 6.4.1).

Table 6.4.1. Water and salt transport properties of grapevine roots comparing K 51-40 and Paulsen rootstocks. Hydraulic conductivity (Lp_r) and the reflection coefficient (σ_s) was calculated using root surface area (Ar), the elasticity coefficient (β), the half-time of water ($T^w_{1/2}$) and solute ($T^s_{1/2}$) exchange. The rate constants of water (K_w) and solute (K_s) transport are also given. (n=15) (t-test was used to compare the mean values of Lp_r and σ_s , $P< 0.05$). Different letters indicate significant difference between rootstocks.

	Ar (mm^{-2})	β (MPa/µl)	$T^w_{1/2}$ (s)	K_w (s^{-1})	$T^s_{1/2}$ (s)	K_s (s^{-1})	Lpr Hydrostatic ($ms^{-1}MPa^{-1}$)	Lpr Osmotic ($ms^{-1}MPa^{-1}$)	σ_s NaCl	σ_s $NaNO_3$
K51-40	183.9 ±7.0	0.27 ±0.019	33.0 ±4.8	0.026 ±0.003	123 ±15	0.008 ±0.001	$^a5.40x10^{-7}$ $\pm0.05 x10^{-7}$	$^b2.16x10^{-7}$ $\pm0.03 x10^{-7}$	^{a}0.51 ±0.05	^{a}0.48 ±0.05
Paulsen	171.4 ±11.1	0.31 ±0.03	41.5 ±6.8	0.032 ±0.01	237 ±70	0.007 ±0.001	$^a6.1x10^{-7}$ $\pm0.8 x10^{-7}$	$^b1.95x10^{-7}$ $\pm0.23 x10^{-7}$	^{a}0.59 ±0.05	^{a}0.51 ±0.05

In osmotic experiments conducted on the roots of both rootstocks, NaCl and $NaNO_3$ were applied as permeating salts. The half time ($T_{1/2}$) of the water phase in relaxation curves was used to calculate the osmotic hydraulic conductivity (Lp_r) of the roots. The osmotic Lp_r for K 51-40 and Paulsen was 2.6 $x10^{-7}$ (ms^{-1} MPa^{-1}) and 1.95 $x10^{-7}$ (ms^{-1} MPa^{-1}) respectively. It showed that the Lp_r values were almost similar for the both rootstocks (Table 6.4.1).

The reflection coefficient (σ_s) of the applied solutes (NaCl and $NaNO_3$) on the roots was calculated from the ΔP and half-time of solute phase obtained from the biphasic root pressure relaxation curves using Eq. 5 and 6.

As shown in Table 6.4.1 the reflection coefficient (σ_s) of NaCl (30 mM) for K 51-40 and Paulsen roots was 0.51 and 0.59 respectively and of $NaNO_3$ (30 mM) was 0.48 and 0.51 for K 51-40 and Paulsen respectively which were not significantly different (t-test, $p< 0.05$).

Root anatomy

Transverse sections of main roots at the distance of 20-50 mm behind the root tip, showed that there were no obvious differences in endodermis and exodermis between the two rootstocks (Fig. 6.4.4). At the distance of 20-50 mm, endodermis and exodermis with Casparian bands in radial walls were developed around vascular tissue and under the epidermis (hypodermis) respectively.

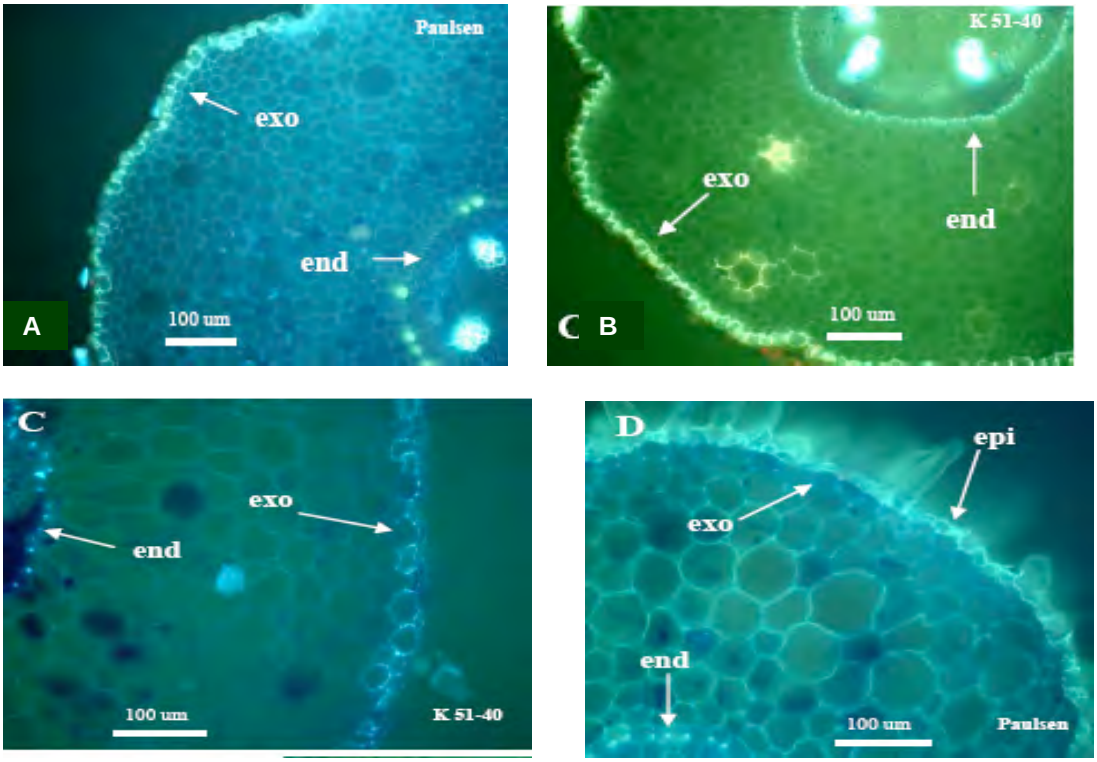


Figure 6.4.4. Photomicrographs of K 51-40 and Paulsen main root (A and B) and lateral root (C and D). The cross sections of the roots were taken at a distance of 20-50 mm from the root tip. Casparian bands in radial wall of endodermis (end) and immature exodermis (exo) were stained by berberine hemisulfate (0.1% w/v) and counterstained with aniline blue (0.5% w/v). Epidermis (epi) is observed in photograph D.

PTS uptake and association with Cl^-

Similar to the previous results obtained for Cl^- (Fig. 6.4.1A), shoot Cl^- content of K 51- 40 was higher than that of the root in all treatments (Fig. 6.4.5B). Unlike K 51-40, shoot Cl^- contents of Paulsen showed no significant difference with root in NaCl treatments (Fig. 6.4.5A). As expected, Cl^- concentrations in shoot and root of Paulsen and K 51-40 in the treatments containing NaCl were higher than that of the control plants (Fig. 6.4.5A and B).

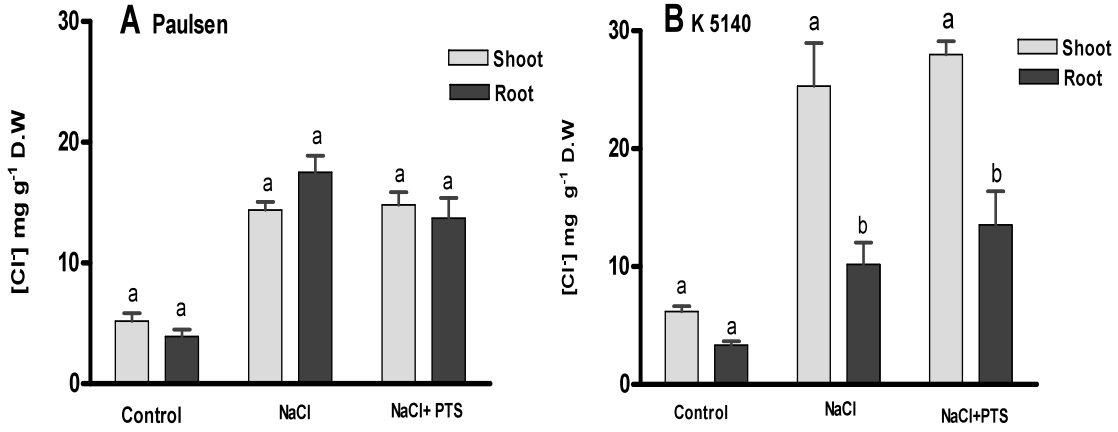


Figure 6.4.5. Shoot and root chloride contents of Paulsen (A) and K 51-40 (B). Plants were grown under different treatments: Control, NaCl (30 mM) and NaCl (30 mM) + PTS (0.0125 % w / v). Bars are SEM of the means (n=4). Different letters indicate significant difference between shoot and root in each treatment.

Determination of the fluorescent dye (PTS) contents showed that there was no significant difference between root and shoot PTS contents of K 51-40 and Paulsen. Both rootstocks also had no difference in accumulation of PTS in their root and shoot (Fig. 6.4.6A). Total transpiration rate measured under the glasshouse conditions demonstrated that both rootstocks had similar rates of transpiration particularly in the third treatment (NaCl+PTS) (Fig. 6.4.6B).

Shoot and root PTS and Cl^- concentrations were compared in both rootstocks. The results showed that there was no positive correlation between Cl^- and PTS transport to the shoot. Cl^- accumulation in the shoot of K 51-40 was higher than that of Paulsen (Fig. 6.4.5A and B) whereas PTS content in the shoot of K 51-40 tended to be lower than that of Paulsen (Fig. 6.4.6A).

Similar to the shoot, no positive correlation was observed between Cl^- and PTS accumulation in the root. The results demonstrated that Cl^- content in the root of K 51-40 was lower than that of Paulsen (Fig. 6.4.5 A and B) while for PTS concentrations there was no significant difference between the two rootstocks in roots (Fig. 6.4.6A).

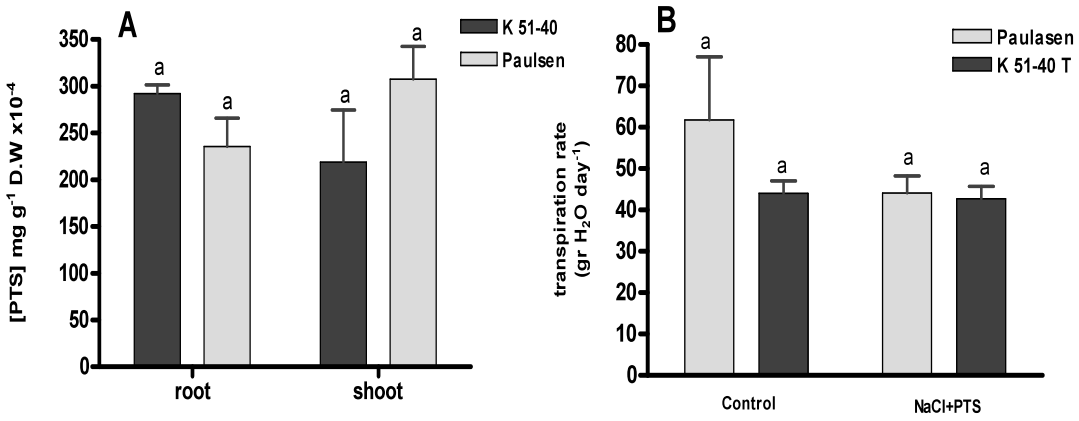


Figure 6.4.6. The apoplastic tracer (PTS) concentrations in shoot and root (A) and transpiration rate (B) of K 51-40 and Paulsen. Plants were grown in hydroponic culture contain NaCl (30 mM) and PTS (0.0125 % w / v). Bars are SE of the means (n=4) (two way ANOVA, *P*<0.05). Different letters indicate significant difference between the varieties in each treatment (t-test).

In order to estimate the fraction of Cl^- and PTS transport to the shoot via an apoplastic pathway, the transpirational bypass flow as a percentage of the total water flow over the experimental period and Cl^- concentration in the xylem were calculated using the procedure described by Yeo et al. (1987) and Garcia et al. (1997). Briefly, the percentage contribution of bypass flow to water transport was estimated by dividing the fluorescent (PTS) contents in the plant shoot by the volume of water transpired. It gave the apparent concentration of PTS in the transpiration stream or $PTS_{(xyl)}$. Dividing $PTS_{(xyl)}$ by the external concentration of $PTS_{(ext)}$ gives the leakage of PTS to the xylem as a percentage. Then, an empirical correction factor was applied (7.75; obtained for rice by Yeo et al. 1987) to give bypass flow of water expressed as a percentage (Table 6.4.2). PTS is a larger molecule (diameter of 1.1 nm) in comparison to water molecule (diameter of 0.39 nm). PTS penetrates through the cell wall microfibrillar spaces slower than water molecules. So, the correction factor is applied to minimize underestimation of bypass flow of water.

Table 6.4.2. Measure of apoplastic bypass flow using PTS transport in K 51-40 and Paulsen. The shoot PTS contents [$PTS_{(Sh)}$] for the rootstocks were divided by the volume of water transpired (J_t) to give the apparent concentration of PTS in the xylem [$PTS_{(xyl)}$]. The leakage of PTS [$PTS_{(xyl)}$ / $PTS_{(ext)}$] was given by dividing $PTS_{(xyl)}$ by the external PTS concentration [$PTS_{(ext)}$]. An empirical correction factor (7.57; see discussion for details) was applied to the PTS leakage values to estimate the water bypass flow (J_{BP}) as a percentage of total flow (n=4) (t-test, $P < 0.05$). No significant difference was observed between the varieties.

Rootstock	J_t (ml)	$PTS_{(Sh)}$ (mg/g.DW)	$PTS_{(xyl)}$ (mg/ml)	$PTS_{(ext)}$ (mg/ml)	$PTS_{(xyl)}/PTS_{(ext)}$ (%)	J_{BF} (%)
K51-40	[a]170.75 ±12.14	[a]0.28[a] ±0.06	0.0017 ±0.0005	0.125	[a]1.34 ±0.400	[a]10.1 ±3.03
Paulsen	[a]176.50 ±16.45	[a]0.340 ±0.004	[a]0.0020 ±0.0002	0.125	[a]1.60 ±0.160	[a]12.1 ±1.21

Furthermore, the apparent concentration of Cl^- in the xylem [$Cl_{(xyl)}$] was calculated by dividing Cl^- concentration of the shoot by the volume transpired. The result then divided by the external concentration of Cl^- [$Cl_{(ext)}$] to give the apparent net concentration of Cl^- in the xylem which is expressed as a percentage (Table 6.4.3).

The results shown in Table 6.4.2 and Table 6.4.3 revealed that the values of the PTS leakage and the percentage of water bypass flow were not significantly different between the rootstocks (Table 6.4.2). In contrast, the apparent net concentration of Cl^- in the xylem was two times bigger in K 51-40 than that of Paulsen (Table 6.4.3).

Table 6.4.3. Cl^- transport by K 51-40 and Paulsen. The shoot Cl^- concentrations [$Cl_{(Sh)}$] for the rootstocks were divided by the volume of water transpired (J_t) by the plants to give the apparent concentration of Cl^- in the xylem [$Cl_{(xyl)}$]. The apparent net concentration of Cl^- in the xylem [$Cl_{(xyl)}$ / $Cl_{(ext)}$] was given dividing [$Cl_{(xyl)}$] by the external Cl^- concentration [$Cl_{(ext)}$]. It is expressed as a percentage of total Cl^- transport (n=4) (t-test, $P < 0.05$).

Rootstock	J_t (ml)	$Cl_{(Sh)}$ (mM)	$Cl_{(xyl)}$ (mM/ml)	$Cl_{(ext)}$ (mM)	$Cl_{(xyl)}/Cl_{(ext)}$ (%)
K51-40	[a]170.75 ±12.14	[a]7.88 ±0.32	[a]0.047 ±0.002	30	[a]0.16 ±0.01
Paulsen	[a]176.50 ±16.45	[b]4.18 ±0.24	[b]0.025 ±0.003	30	[b]0.08 ±0.01

6.4.3 Discussion

Paulsen (salt tolerant), in comparison to K 51-40 (salt sensitive) is able to maintain lower Cl- accumulation in the shoot (Fig. 6.4.1 A). This is in line with a finding reported by Walker et al. (2004). They showed that Paulsen is the best Cl^- excluder rootstock because it had the lowest Cl^- concentrations in petiole, lamina and grape juice of field grown vines. The higher root/shoot Cl^- concentrations ratio indicates the Cl^- retaining ability of the Paulsen roots, which can be a determinant of Cl^- tolerance (Miklos et al. 2000). High Cl^- accumulation in the shoot of K 51-40 is the most likely reason for salt sensitivity in this variety.

In grapevine (various cultivars), it has been shown that by increasing NaCl concentrations in the external medium the NO_3-N concentrations in all parts of the tested grapevine cultivars were decreased after 60 days (Fisarakis et al. 2004). Miklos et al. (2000) treated grapevine cultivars with high NO_3^- and high Cl^- concentrations and showed interaction between NO_3^- and Cl^- and also the inhibitory effects of high NO_3^- and Cl^- on the transport of other ions.

NO_3^- and Cl^- interference could occur via various transport mechanisms. Generally anion channels are more selective to NO_3^- than to Cl^- but it is not clear what competitive effects occur between these two anions (Teakle and Tyerman 2010). The interaction between

anions can be attributed to the competition of anions for transport across the tonoplast and/or at the location of anion release to the xylem (Jaenicke et al. 1996).

Using the root pressure probe technique, hydrostatic and osmotic L_{p_r} and σ_s of two grapevine rootstocks, K 51-40 and Paulsen, were measured. The results showed that L_{p_r} of the two rootstocks was similar when the driving force of water flow was hydrostatic or osmotic. The nature of the driving force affected the L_{p_r} values in both rootstocks similarly. The hydrostatic L_{p_r} measured using the pressure relaxation technique was significantly greater than the osmotic L_{p_r} for the rootstocks (Fig. 6.4.5 A). It has been found that there are up to three orders of magnitude differences between osmotic and hydrostatic water flow (Steudle, 2000). This variable L_{p_r} of roots has been already reported in many herbaceous and woody plants as summarized by Steudle & Peterson (1998). It has been suggested that a dilution of the xylem contents due to water uptake and consequently a decrease of osmotic driving force (Fiscus, 1975) and an opening of valve like plasmodesmata in the presence of hydrostatic pressure gradients (Passioura, 1988) can be the reasons for the above mentioned differences in L_{p_r}. The composite transport model has also been proposed to explain the difference between hydrostatic and osmotic L_{p_r} based on the difference in selectivity of the membrane pathway and apoplastic pathways (Steudle et al. 1993).

Recently, a different explanation for the higher Lpr obtained by pressure relations was proposed by Bramley et al. (2007). They carried out a series of pressure clamps by increasing 5 KPa in root pressure at each clamp for 60-120 s. L_{p_r} was determined as the slope of linear regression between water flow rate and applied pressure, which showed no difference for inward and outward flows. They suggested that the difference in the values of hydrostatic and osmotic L_{p_r} can be minimized by using the pressure clamp method. Because the pressure clamp method measures L_{p_r} under steady-state conditions compared with transition conditions observed in the pressure relaxation method, it was argued that there will be a uniform pressure change along the root xylem. On the other hand, Knipfer et al. (2007) has argued against the application of pressure clamp technique to measure hydraulic L_{p_r}. According to this explanation, the pressure clamp forces larger amounts of water across the root in a direction opposite to the transpiration stream and hence causes a concentration polarization or unstirred layer (USL) effect due to considerable accumulation of solutes in front of the endodermis. This in turn, will reduce water flow and show an underestimation of L_{p_r}. They concluded that measuring the initial phase of water flow through the first step of pressure relaxation in root pressure probe experiments could be used to determine the real L_{p_r}. Currently there is no clear consensus on how to measure hydrostatic L_{p_r}, and in addition osmotic L_{p_r} may be also be underestimated due to the presence of unstirred layers. However, in the context of our comparison between the two grapevine rootstocks we can conclude that there is likely to be no substantive difference in Lpr between the salt tolerant and salt sensitive genotypes.

The reflection coefficient (σ_s) values reported in this study for K 51-40 (0.51 and 0.48 using NaCl and $NaNO_3$ respectively) and Paulsen (0.59 and 0.51 using NaCl and $NaNO_3$ respectively) were not remarkably different and were less than unity, thus it could be consistent with the composite model. Furthermore, low reflection coefficient indicates apoplastic by-passes in the absence of Casparian bands, for example in endodermis disrupted by lateral root initiation and before differentiation of the endodermis in young roots or water movement through Casparian bands (Steudle & Peterson, 1998). The nearly similar reflection coefficients (σ_s) for Cl^- and NO_3^- salts used in this measurement showed that by-pass flow of salts to the xylem is the same for both rootstocks and anions.

Root anatomy

The endodermis of all vascular plants and the exodermis of many angiosperms possess a Casparian band that is developed in the radial and tangential wall of their cells (Karahara et al. 2004). The Casparian band is made of suberin and /or lignin that is deposited in the cell wall (Zeier & Schreiber, 1998).

Water permeability in a root is mostly related to changes in suberisation of roots that increases with age and stressful environments (Steudle, 2000). Since suberisation in woody roots is greater than herbaceous plants, the L_{p_r} of trees is often lower than that of herbs

(Steudle & Frensch, 1996). Miyamoto et al. (2001) found identical values of Lp_r for two rice varieties (IR64 and Azucena) and suggested that it can be due to the same root anatomy.

The root anatomy did not obviously differ for K 51-40 and Paulsen (Fig. 6.4.7). No obvious differences were observed in apoplastic barriers (endo- and exodermis) of the main and lateral roots between the two rootstocks. Thus the similar Lp_r and the reflection coefficient (σ_s) of the rootstocks could be related to the similar root anatomy.

By-pass flow

There have been some studies in which the contribution of transpirational bypass flow to water and solute uptake has been assessed (Moon et al. 1986; Yeo et al. 1987; Kamaludin & Zwiazek 2001; Garcia et al. 1997; Hanson et al. 1985). There are variable results reported about the relative extent of apoplastic bypass flow of water and ions. Hanson et al. (1985) and Moon et al. (1986) demonstrated that the apoplastic pathway in *Pinus resinosa* roots contributes less than 1% of total uptake. In contrast, using La^{3+} uptake, Lawton et al. (1981) showed that the apoplastic route in *Avicennia marina* was significant. However, they did not present any estimation in terms of symplastic or total ion uptake. Gracia et al. (1997) estimated the importance of the transpirational bypass flow in rice and wheat. They concluded that the transpirational bypass flow in rice is 10 times greater than that of wheat and found a high correlation between Na^+ uptake and the bypass flow magnitude in rice but not in wheat.

The results reported in this investigation showed that this difference does not exist between the grapevine genotypes, K 51-40 and Paulsen. PTS contents of the shoot and the root in both rootstocks were nearly similar and the results obtained for the leakage of PTS [$PTS_{(xyl)}$ / $PTS_{(ext)}$] and the bypass flow of water (J_{BF}), were also not significantly different in the two rootstocks. The leakage of PTS and J_{Bf} for K 51-40 were 1.34 % and 10.1 % and for Paulsen were 1.6 % and 12.1 % respectively. It can be compared to the values obtained for rice by Garcia et al. (1997), which were 0.722 % and 5.74 % for the leakage of PTS and J_{BF} respectively. The large difference between the leakage of PTS and the bypass flow of water (J_{BF}) in grapevine (at least in K 51-40 and Paulsen) can possibly be due to smaller spaces distributed between cell wall microfibrills whose diameters are less than the diameter of PTS molecule (1.1 nm; Moon et al. 1986). Thus water molecules with diameter of 0.39, Na^+ with 0.6 and Cl^- with 0.5 nm (Lauchli, 1979) will freely move through the spaces. This was also the reason that Yeo et al. (1987) used the empirical correction factor (7.57; see results) in calculations to correct underestimation of bypass flow of water.

PTS is a highly fluorescent dye that can move through unmodified cell walls, but it is blocked by walls with Casparian bands (Vesk et al. 2000). Similar values of PTS transport obtained for K 51-40 and Paulsen is also consistent with the anatomical findings

Although there is evidence that shows a strong correlation between PTS transport and ion transport, for example for Na^+ transport in rice (J_{BF} =5.47 % and the leakage of Na^+ = 5.65 %) (Garcia et al. 1997; Yeo et al. 1987; Gong et al. 2006), no correlation was observed in the grapevine rootstocks (K 51-40 and Paulsen) between PTS and Cl^- transport to the shoot (J_{BF} = 10.1 % and 12.1 % and the apparent net concentration of
Cl^- = 0.16 % and 0.08 % for K 51-40 and Paulsen respectively). The net concentration of Cl^- in the xylem of K 51-40 is two times greater than that of Paulsen. In fact the higher apparent net concentration of Cl^- in the xylem [$Cl^-_{(xyl)}$] for K 51-40 can be attributed to higher Cl^- loading into the xylem in K 51-40 when compared to Paulsen.

In conclusion, Paulsen is a Cl^- excluder rootstock that accumulates low Cl^- in the shoot. As salinity increased in the external medium the NO_3^-/Cl^- selectivity was maintained in Paulsen by increase in NO_3^- uptake by roots and a greater proportion being transferred to the shoots. Since root concentrations of Cl^- and NO_3^- were nearly similar in both varieties, it is likely that the xylem-loading for Cl^- and NO_3^- differs between the two varieties in terms of regulation and/or selectivity. Both genotypes have similar root Lp and similar σ_s for Cl^- and NO_3^- salts indicating that bypass flow of salts to the xylem is similar for both rootstocks. According to the anatomical study, there was no noticeable difference in apoplastic barriers between the two rootstocks.

PTS transport showed that there was an apoplastic bypass flow and it was similar for both rootstocks. There was no correlation between PTS and Cl^- transport. Although, there is a bypass flow for Cl^- transport, it does not account for differences observed between the rootstocks. These results taken together with the results from NO_3^- and Cl^- interaction experiments, point to differences in membrane transport properties between the rootstocks as the underlying cause for the difference in Cl transport to the shoot and salt tolerance.

6.5 Chloride transport and compartmentation within main and lateral roots of two grapevine rootstocks differing in salt tolerance.

This section forms the basis of a manuscript in preparation by the following authors:
Nasser Abbaspour, Brent Kaiser and Steve Tyerman

6.5.1 Introduction

Chloride$^-$ can be the more toxic component in some plants under salinity stress (Munns & Tester, 2008; Teakle and Tyerman, 2009), but the mechanisms of Cl^- transport to the root and its uptake to the shoot and / or efflux under salt stress are poorly understood (White & Broadley, 2001; Teakle and Tyerman, 2010). *Vitis vinnifera* (grapevine) and *Vitis* root stock hybrids are Cl^- sensitive (Greenway, 1965; Downton & Millhouse 1983), where excessive accumulation of Cl^-, particularly in leaves, is the main reason for salt damage (Downton & Millhouse, 1983; Hardie & Cirami, 2000). In a previous report we have shown that the differences between root stock hybrids of *Vitis sp* that are salt tolerant (Paulsen) and salt sensitive (K51-40) could not be explained by differences in fluxes of Cl^- salts across the apoplastic pathway in roots, nor in coupling to water flow to the shoot. Rather the interactions with NO_3^- accumulation suggested differences in membrane transport in the root that resulted in higher retention of Cl^- in the roots and lower Cl^- flux to the shoot of the salt tolerant (Paulsen) hybrid.

Measurement of ion fluxes in roots is an important first step in finding the underlying differences in transporters/channels and their location when comparing closely related genotypes that differ in salt tolerance (Essah et al., 2003; Britto et al, 2004; Davenport et al 2005; Wang et al, 2006), yet there are few comparative studies of Cl^- fluxes in roots between salt sensitive and salt tolerant genotypes. Roots of salt tolerant *Populus euphratica* grown in 100 mM NaCl exhibited a net efflux of Cl^- from a specific region of the root, whereas no efflux of Cl^- was observed in the salt-sensitive *Populus popularis* (Sun, Chen & Dai 2009). More generally, important information about mechanisms of Cl^- transport has been obtained by using Cl^- isotopes, Cl^- sensitive microelectrodes, or a Cl^- indicator located in the cytoplasm. Lorenzen et al. (2004) indirectly demonstrated that passive Cl^- influx could occur into Arabidopsis roots under salt stress using the recombinant fluorescent probe CLOMELEON, which was consistent with an anion channel in wheat roots that could allow influx under certain circumstances (Skerrett & Tyerman, 1994; Teakle and Tyerman, 2010). In contrast, Cl^- influx would need to be active in low external concentrations, or with hyperpolarized membrane potentials, or when the cytoplasm has loaded with Cl^- under high salinity, and is accomplished by a ΔpH-driven Cl^- / nH^+ symport (Felle, 1994; Yamashita et al. 1997; Babourina et al. 1998a).

Using $^{36}Cl^-$, Cram & Laties (1971) studied Cl^- influx and efflux in barley root segments and showed saturation kinetics for Cl^- uptake to the vacuole at external Cl^- concentrations higher than 10 mM, while the plasmalemma influx increased linearly. Pitman, (1971) and (1972) suggested a two-pump model for Cl^- transport from the external medium to the xylem. Britto et al. (2004) undertook compartmental analysis of Cl^- transport in intact barley root systems. They showed that by increasing the external concentrations of Cl^- to salinity levels, cytoplasmic content of Cl^- increased to rather high levels; from about 6 mM in 0.1 mM external Cl^- to 360 mM with 100 mM external Cl^-. Cl^- flux to the xylem showed saturation kinetics with low affinity . An interesting feature of the plasma membrane flux was the high apparent futile cycling where unidirectional efflux was up to 90% of the unidirectional influx in the highest external Cl^- concentration (*ibid*.). This cycling has been discussed in relation to energy requirements for Na^+ by Britto and Kronzucker (2009), and for Cl^- in Teakle and Tyerman (2010).

Here we report analysis of Cl^- fluxes in both intact root systems and detached root segments in order to determine where and to what extent Cl^- transport differed between the salt tolerant Paulsen and salt sensitive K51-40. We found that unidirectional influx to the cytoplasm was about twice as high in main roots of the salt tolerant Paulsen, but cytosolic Cl^- concentration determined from compartmental analysis was lower in Paulsen than K51-40. This was likely due to a higher flux to the vacuole and greater vacuolar accumulation, as well as high efflux

from the cytoplasm to the outside. Paulsen also had about half the flux to the xylem compared to K51-40. Lateral roots showed no differences between the hybrids.

6.5.2 Results

$^{36}Cl^-$ accumulation in the root of Paulsen was higher than that of K 51-40 over a long period of loading (2 h) and influx rate in the two rootstocks decreased remarkably after the first 10 min (Fig 1). The initial $^{36}Cl^-$ influx was estimated as the rate of ^{36}Cl accumulation over the first 10 min loading period. Paulsen initial $^{36}Cl^-$ influx was twice that of K 51-40 (Table 1).

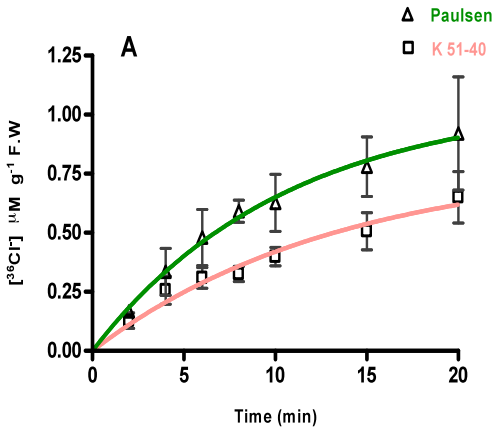


Figure 6.5.1. (A) Root $^{36}Cl^-$ influx as $^{36}Cl^-$ accumulation in K 51-40 (□) and Paulsen (Δ) root segments. Data were fitted using one phase exponential association. (B) Root $^{36}Cl^-$ influx rate. Whole plants were pretreated with NaCl (30 mM) and root segments (including lateral roots) were cut 8-12 cm from root tip. They were loaded by the labelled solution for 20 min and washed for 1 and 5 min. Bars are SE of the means (n = 4).

Table 6.5.1. $^{36}Cl^-$ transport to the cytoplasm estimated as $^{36}Cl^-$ influx rate (slope of linear regressions) to the root segments of K 51-40 and Paulsen over the first 10 min of radioisotope loading in two experiments.

	Cytoplasmic net influx (µmol $g^{-1}_{F.W.}$ min^{-1})	
Experiment	K51-40	Paulsen
1	0.050±0.005	0.120±0.013
2	0.030±0.007	0.059±0.013

Influx increased with increasing external Cl^- concentration in both Paulsen and K5-40, however the increase in Paulsen was higher than K 51-40 (Fig. 2). The data could be fit to the Michaelis-Menten equation where V_{max} and K_m values for K 51-40 were 0.059 ± 0.026 (µm g^{-1} F.W. min^{-1}) and 24.27 ± 18.51 (mM) and for Paulsen were 0.127 ± 0.042 (µm g^{-1} F.W. min^{-1}) and 28.68 ± 15.76 (mM) respectively. The large error in K_m is probably because the flux still had not saturated at 30 mM external Cl^-.

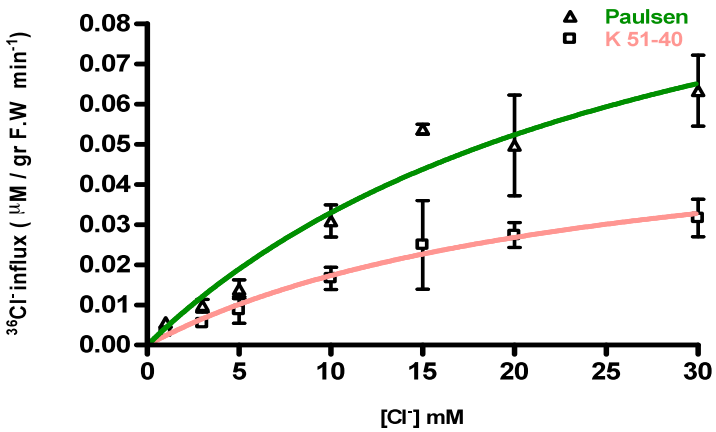


Figure 6.5.2. Unidirectional cytoplasmic $^{36}Cl^-$ influx in K 51-40 (□) and Paulsen (Δ) estimated as $^{36}Cl^-$ influx rate over 8 min loading time in the presence of different external Cl^- concentrations. Whole plants were pretreated with NaCl (30 mM) and root segments (including lateral roots) were cut 8-12 cm from the root tip. They were loaded by the labelled solution for 10 min and washed for 1 and 5 min. Data were analysed fitting to Michaelis-Menten. Bars are SE of the means (n = 4). Comparison of fits indicated significant difference between the curves. V_{max} was significantly different between the varieties at 30 mM [Cl^-] but no difference observed for K_m.

The previous experiments showed differences between the two rootstocks in $^{36}Cl^-$ accumulation and influx rate. One of the possibilities for describing this difference could be different physiological capability of lateral roots in their contribution to Cl^- uptake. To test this hypothesis, the cytoplasmic $^{36}Cl^-$ accumulation was measured in the main and lateral roots separately. The results showed that the lateral roots $^{36}Cl^-$ influx is the same in the two rootstocks; however the main root of Paulsen accumulated $^{36}Cl^-$ about three times faster than for K 51-40. $^{36}Cl^-$ accumulation in lateral root was higher than main root in K 51-40, whereas main root of Paulsen significantly accumulated more $^{36}Cl^-$ than lateral roots (Fig. 3) during a 10 min load.

The rootstocks showed a large difference in $^{36}Cl^-$ uptake to the shoot. As shown in (Fig. 4A), accumulation of $^{36}Cl^-$ to the shoot of K 51-40 and Paulsen increased over time, however $^{36}Cl^-$ content and influx rate (slope of linear regression) in K 51-40 (0.83 ± 0.33 µmol g^{-1}F.W h^{-1}) was higher in comparison to Paulsen (0.52 ± 0.14 µmol g^{-1}F.W h^{-1}).

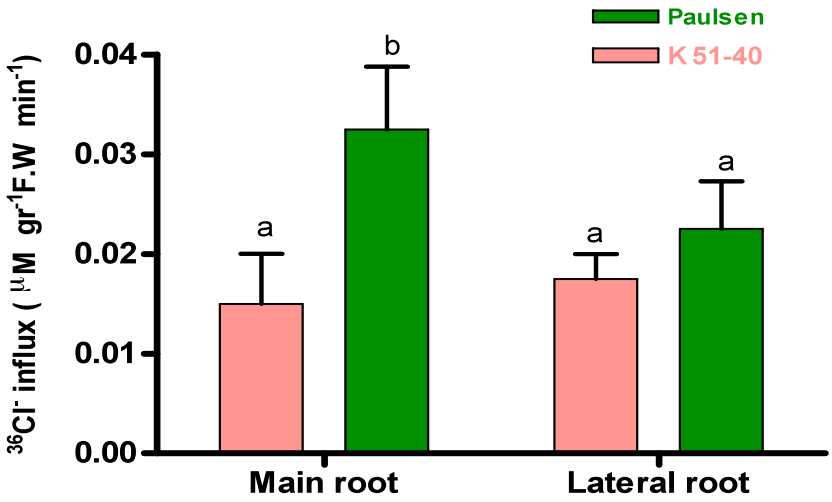


Figure 6.5.3. Main and lateral root $^{36}Cl^-$ influx in K 51-40 and Paulsen. Plants were pretreated by NaCl (30 mm for 5 days and 20 mM for 24 h) and root segments (main and lateral roots separately) were loaded by $^{36}Cl^-$ for 10 min. Bars are SE of the means (n = 4) (t-test, $P < 0.05$). Different letters indicate significant difference between the varieties.

The same trend was observed in the values of $^{36}Cl^-$ uptake per root g F.W estimated in the rootstocks (Fig. 4B). Similar results were obtained for petiole and lamina separately (data not shown). In K 51-40, $^{36}Cl^-$ transport to petiole and lamina was higher than that of Paulsen.

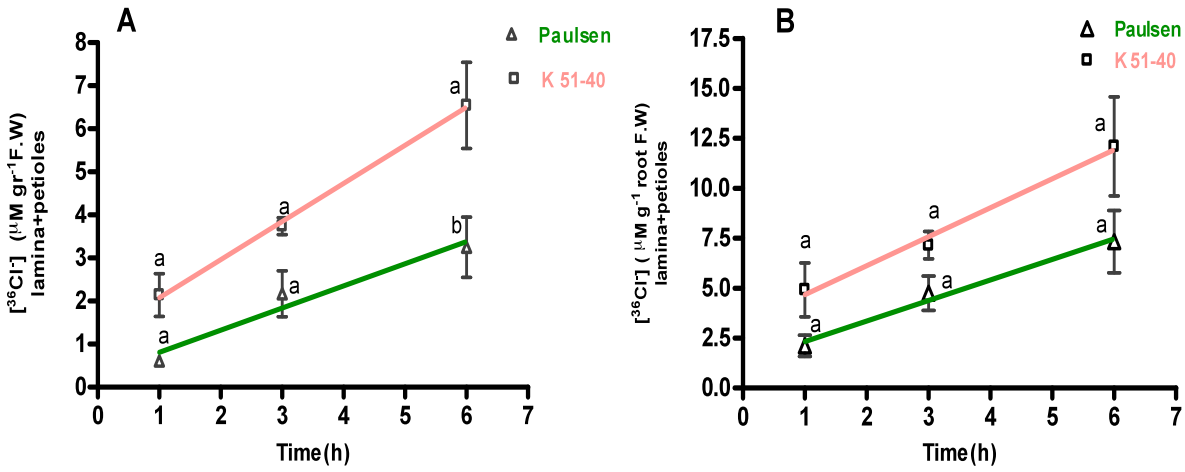


Figure 6.5.4. Shoot (petiole+lamina) $^{36}Cl^-$ uptake estimated as shoot $^{36}Cl^-$ content (A) and shoot $^{36}Cl^-$ uptake per root FW (B) in K 51-40 (□) and Paulsen (Δ). Plants (rooted leaves) were pretreated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with radiolabelled solution for various times (1, 3, and 6 h). Data (A) were analysed using linear regression. Bars are SE of the means (n = 4) (two way ANOVA, $P < 0.05$). Different letters indicate significant difference between the varieties within the columns.

Results from efflux experiments carried out on whole and lateral roots of K 51-40 and Paulsen are summarized in Table 2 and as a diagram (Fig. 7). The efflux curves for whole roots of K 51-40 and Paulsen (Fig. 5) showed that the initial rate of loss of the tracer (R) in Paulsen (0.99 µmol g^{-1} FW min^{-1}) was about three times higher than that of K 51-40 (0.34 µmol g^{-1} FW min^{-1}). In Paulsen, $^{36}Cl^-$ influx from the external medium to the cytoplasm (Φ_{oc}) (1.04 µmol g^{-1} FW min^{-1}) was much higher than K 51-40 (0.368 µmol g^{-1} FW min^{-1}). Similar results were observed for $^{36}Cl^-$ efflux from cytoplasm to the external medium (Φ_{co}).

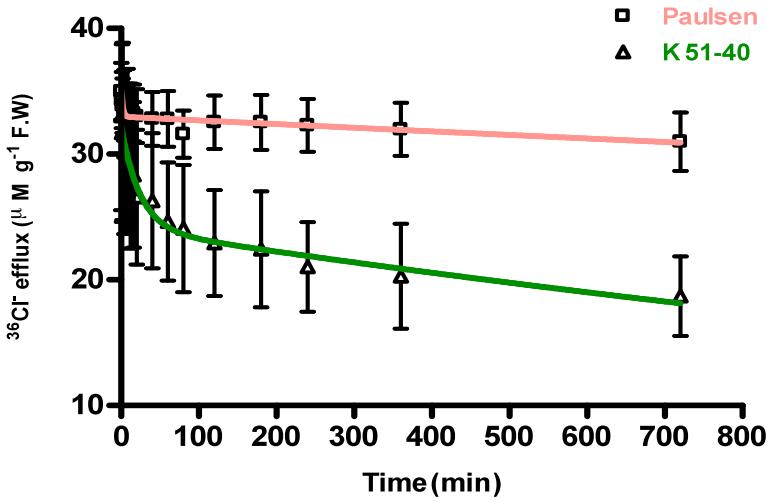


Figure 6.5.5. Efflux of $^{36}Cl^-$ from intact whole roots of K 51-40 (Δ) and Paulsen (□) plotted as decrease in tissue $^{36}Cl^-$ content expressed as µM g^{-1} F.W. after transfer to non-labelled solution. Plants were pre-treated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with $^{36}Cl^-$ for 12 h. Plants then were transferred to aerated successive washing solutions for 1-720 min. Data were fitted with a double exponential equation to calculate fluxes. Rate constant for Bars are SE of the mean (n = 6). Comparison of fits indicated significant difference between the curves.

The results also showed that the tracer influx from the cytoplasm to the vacuole (Φ_{cv}) in Paulsen was higher than K 51-40 but unlike influx rate, the efflux rate from the vacuole to the

cytoplasm (Φ_{vc}) in K 51-40 was higher than Paulsen. The $^{36}Cl^-$ efflux from cytoplasm to the xylem in K 51-40 (0.014 µmol g^{-1} FW min^{-1}) was about two times greater than that of Paulsen (0.0087 µmol g^{-1} FW min^{-1}).

In comparison to whole roots, the efflux kinetics for lateral roots were very similar between the two hybrids (Fig. 6) and the fluxes estimated for the lateral roots did not show significant differences between the two rootstocks, although the values for the lateral roots of K 51-40 were slightly higher than those of Paulsen (Table 2)

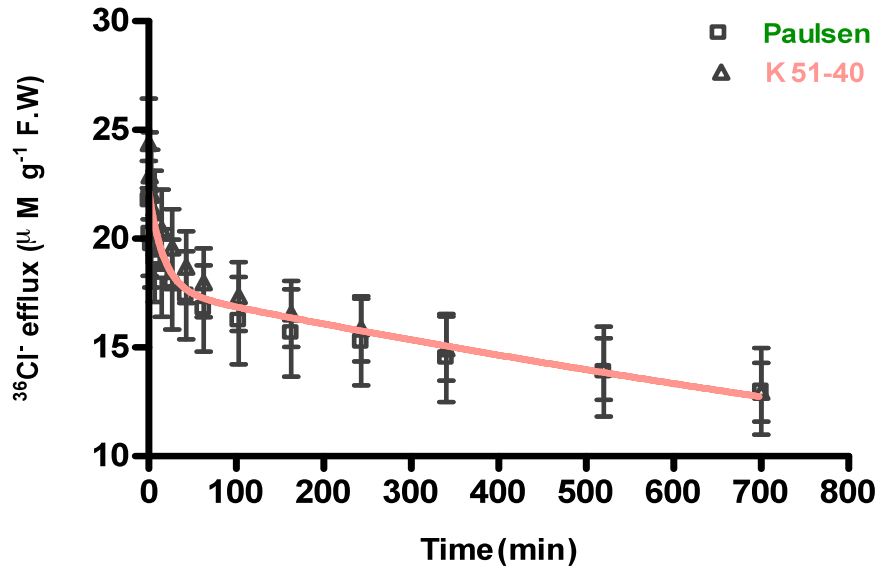


Figure 6.5.6. Efflux of $^{36}Cl^-$ from lateral root segments of K 51-40 (Δ) and Paulsen (◊) plotted as decrease in tissue $^{36}Cl^-$ content expressed as µM after transfer to non labelled solution. Plants were pretreated by NaCl (30 mM for 5 days and 20 mM for 24 h) and loaded with $^{36}Cl^-$ for 12 h. The root segments were then transferred to aerated successive washing solutions for 1-720 min. Data were fitted with a double exponential equation to calculate fluxes. Bars are SE of the mean (n = 6). No significant difference observed between the varieties.

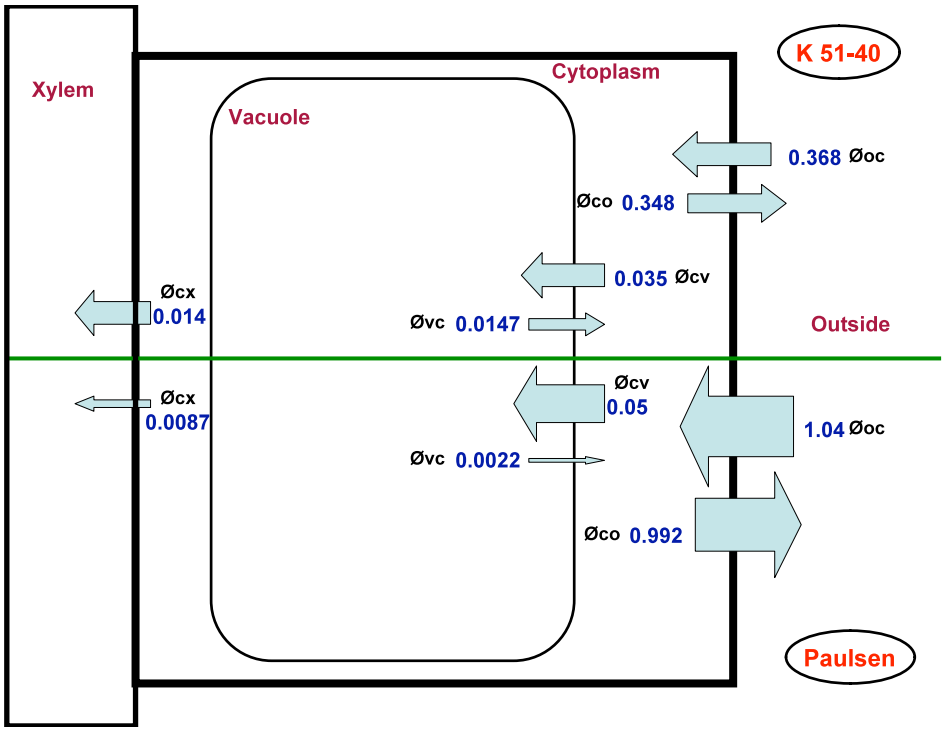


Figure 6.5.7. Schematic demonstration of the whole root fluxes values estimated as µM g^{-1} FW min^{-1} from the influx and efflux experiments (compartmental analysis) summarized in Table 2. The growth condition as described for experiment 6.

Table 6.5.2. Compartmental analysis of $^{36}Cl^-$ fluxes yielding influx from outside to cytoplasm (Φ_{oc}), cytoplasm to vacuole (Φ_{cv}) and from cytoplasm to outside (Φ_{co}), vacuole to cytoplasm (Φ_{vc}), net influx (Φ_{in}), net flux to cytoplasm (Φ_{net}), efflux from cytoplasm to the xylem (Φ_{cx}) and the rate of loss of tracer (R) in whole and lateral roots estimated as μM g^{-1} FW min^{-1} using parameters which were obtained from fitting efflux data (Supplemental Table). The plants were pretreated with 30 and 20 mM NaCl for 5 days and 24 h respectively. The plant roots (K 51-40 and Paulsen) were loaded in a $^{36}Cl^-$- labelled solution for 12 h and then washed in successive non-labelled solutions for various times (1-720 min) (n = 6). The radioactivity in the tissue and those of the washing solutions were counted. Errors are SEM.

	Φ_{oc} (μM $g^{-1}_{F.W.}$ min^{-1})	Φ_{co} (μM $g^{-1}_{F.W.}$ min^{-1})	Φ_{vc} (μM $g^{-1}_{F.W.}$ min^{-1})	Φ_{cv} (μM $g^{-1}_{F.W.}$ min^{-1})	R (μM $g^{-1}_{F.W.}$ min^{-1})	Φ_{in} (μM $g^{-1}_{F.W.}$ min^{-1})	Φ_{net} (μM $g^{-1}_{F.W.}$ min^{-1})	$^{\dagger}\Phi_{cx}$ (μM $g^{-1}_{F.W.}$ min^{-1})
Whole root								
K51-40	0.368±0.005	0.348±0.007	0.015±0.002	0.035±0.001	0.34±0.02	0.034±0.005	0.039±0.004	0.014±0.002
Paulsen	1.04±0.09	0.992±0.083	0.0022±0.0002	0.050±0.002	0.99±0.02	0.05±0.01	0.06±0.01	0.009±0.001
Lateral root								
K51-40	0.228±0.003	0.215±0.004	0.016±0.002	0.029±0.002	0.21±0.04	0.027±0.005		
Paulsen	0.198±0.005	0.182±0.004	0.010±0.002	0.026±0.004	0.18±0.02	0.025±0.004		

† is the slope of the linear regression of $^{36}Cl^-$ uptake to the shoot (experiment 5)

6.5.3 Discussion

The main aim of this investigation was to compare component Cl^- fluxes in roots of two grapevine rootstock hybrids, K 51-40 and Paulsen that differ in salt tolerance and Cl^- accumulation in the shoot. Since differences in the passage of Cl^- salts via apoplast pathways in the root and coupling to water flow were not evident between the hybrids (Abbaspour et al. submitted) we expected that membrane transport of Cl^- would show differences between the hybrids. This investigation has revealed large differences in component fluxes that can account for greater accumulation of Cl^- in the primary root and lower flux to the shoot via the xylem in Paulsen compared to K51-40.

The differences between the hybrids can be mainly attributed to the primary roots since the fluxes in lateral roots did not differ as seen from influx measurements and efflux analysis on detached roots. However, we have not explored the flux to the xylem in lateral roots, which may differ between the hybrids. Such a difference between primary and secondary roots does not appear to have been reported previously in the literature related to salinity stress and the fluxes of NaCl, and has implications in terms of when secondary roots develop and the proportion of primary roots to lateral roots that occur under salinity stress. The difference in Cl^- fluxes between primary and lateral roots should be taken into account when searching for the transporter/channel genes that account for the differences between the hybrids. More investigations are required to clearly indicate the effect of lateral roots in Cl^- uptake by grapevine under different conditions and at different developmental stages.

There are precedents for lateral roots displaying different transport properties to primary roots for other ions. Lateral roots of grapevine have previously been reported to have high capacity for NO_3^- uptake and that NO_3^- uptake and respiration rate decreased rapidly as lateral roots aged (Volder et al. 2005). This high transport capacity for NO_3^- influx in lateral roots was not reflected in the Cl^- fluxes for either hybrid as shown in this investigation, though the effects of external NO_3^- on Cl^- influx would need to be examined. In other plants lateral roots can show very different behaviour to primary roots. Ma et al. (2001) investigated the role of root hairs and lateral roots in the Si uptake by two mutants of rice. They showed that silicon uptake by RH2 mutant (defective in the formation of root hairs) was similar to wild type but RM109 mutant (defective in the formation of lateral roots) had much less Si uptake than wild type. The most spectacular example is the small tightly packed lateral roots that occur in some plants (cluster roots or proteoid roots) that efflux organic anions to mobilise inorganic phosphate in the soil (Zhang et al., 2004).

In this investigation $^{36}Cl^-$ fluxes across different membranes (plasma membrane and tonoplast) were estimated from influx and efflux experiments on roots of whole plants and root segments of K 51-40 and Paulsen. While there was some considerable variation between experiments in the magnitude of influx (compare Φ_{oc} in Table 2 with influxes in Table 1 and Fig 3), which may be explained by different treatments (ie detached or intact roots for plants in the light). Paulsen always displayed about twice the Cl^- influx than K 51-40, which is surprising given the lower transport of Cl^- to the shoot by Paulsen. However, it is consistent with our previous finding of higher Cl^- content in roots of Paulsen compared to K 51-40.

The higher unidirectional influx across the plasma membrane in Paulsen was matched by an almost equally high efflux, but in both hybrids the efflux was about 0.95 that of the influx in 20 mM external NaCl. This is similar, but somewhat higher than the ratio of 0.90 observed for Cl^- influx:efflux in barley roots in a much higher external concentration of 100 mM NaCl, a concentration which is lethal to even the most salt tolerant Vitis rootstocks. Interestingly the magnitude of unidirectional influx in barley roots in 10 mM NaCl is somewhat larger (about 6 μmol g^{-1} h^{-1} depending on external NO_3^- concentration) to what we measure for grapevines at the same concentration (1.2 to 1.8 μmol g^{-1} h^{-1}). Since the current paradigm is that Cl^- influx is active via proton symport under steady state conditions (when the cytoplasm has loaded with Cl^-), and efflux is passive via anion channels, it would seem that this cycling across the plasma membrane is a futile waste of energy (Britto and Kronzucker 2009). The estimated energy consumption could be a considerable proportion of that of the respiratory output of the root (Britto and Kronzucker, 2009; Teackle and Tyerman, 2010), and thus it has been suggested that there may be less energy depleting exchange processes occurring across the plasma membrane, or electroneutral transport of Cl^- coupled to the movement of other cations, such as Na^+ and K^+, as occurs with the cation-Cl^- cotransporter (Britto and Kronzucker, 2009; Colmenero-Flores et al. 2007).

Cram, (1973) and Cram & Laties (1971) showed for isolated maize root cortex that influx across the plasmalemma increased with increasing external concentrations of Cl^-, and they suggested that in low external Cl^- concentrations the plasmalemma influx can be the rate-limiting step for influx to the vacuole. Furthermore, at high external concentrations of Cl^-, influx to the cytoplasm continued to increase but the Cl^- influx to the vacuole remained constant. In the two rootstocks examined here the concentration kinetics for influx showed saturation where only differences in Vmax between the hybrids could be determined from fits to the Michaelis-Menton equation. This might imply that the transport system responsible may only differ in density within the membrane between the hybrids.

Davenport et al. (2005) showed that $^{22}Na^+$ accumulation in roots of two durum wheat genotypes (salt tolerant line149 and salt sensitive Tamaroi) was fast over the first 5 min as we observe for $^{36}Cl^-$ in the grapevine hybrids. However in contrast to the grapevine hybrids the wheat genotypes did not show any difference in unidirectional influx to the cytoplasm; the main difference being the efflux to the xylem which was lower in the salt tolerant wheat genotype, and efflux from the root to the outside which was higher in the salt tolerant genotype. There are some similarities to the wheat study in comparisons between the grapevine hybrids in our study particularly in the flux to the xylem and efflux from the root. The salt sensitive K 51-40 had high $^{36}Cl^-$ flux to the shoot (petiole +lamina) than the salt tolerant Paulsen. Compartmental analysis of $^{36}Cl^-$ efflux experiments showed efflux from the cytoplasm to the xylem in K 51-40 that was nearly twice that in Paulsen. This difference may be due to a difference in the number of transporters or channels that load Cl^- into the xylem of main roots. Also, higher cytoplasmic Cl^- concentration in K 51-40 could be another reason for higher Cl^- efflux to the xylem. The lower estimated cytoplasmic Cl^- concentration in Paulsen may result from a higher vacuolar storage combined with the high efflux to the external medium.

In conclusion, $^{36}Cl^-$ flux experiments showed that the cytoplasmic $^{36}Cl^-$ influx for primary roots was higher in Paulsen than K 51-40 and similar results were observed for the cytoplasmic $^{36}Cl^-$ efflux. The $^{36}Cl^-$ influx from outside to cytoplasm increased with increases in external Cl^- concentrations with a saturating effect at high external Cl^-. Lateral roots were not different between the varieties at least in terms of the internal fluxes within the root. In comparison to Paulsen, $^{36}Cl^-$ transport to shoot and uptake rate in K 51-40 was significantly higher. Compartmental analysis of $^{36}Cl^-$ fluxes showed that the cytoplasmic $^{36}Cl^-$ efflux rate to the medium in Paulsen was greater than K 51-40. It also showed higher efflux from the vacuole to the cytoplasm in K 51-40. Furthermore, high efflux rate from the cytoplasm to the xylem (Φ_{cx}) in K 51-40 appears to be the reason for high $^{36}Cl^-$ transport to the shoot. These differences between the rootstocks can be due to a difference in the number of transporters or channels in the cell membranes and their response to the external and cytoplasmic concentrations of Cl^-. Electrophysiological experiments along with positional cloning (Davenport et al. 2005) could indicate Cl^- transport mechanism(s) across different cell membranes in the two rootstocks.

7. Outcome/Conclusion

7.1 Comparison of project performance against planned outputs.

The project largely met all outputs as initially proposed. These are detailed in the Table below.

Table 7.1 Comparison of planned outputs against project performance for each year.

Outputs and Performance Targets 2004-05

Outputs	Project Performance
1. Information on the relationship between root aquaporins and root hydraulic conductance and conductivity for major varieties/rootstocks.	We have shown that root aquaporins can account for changes in root hydraulic conductance/conductivity. This depends on variety (Grenache vs Chardonnay) and conditions (drought vs well-watered vs PRD).
2. Information on the diurnal variability of root hydraulic conductivity for selected varieties/rootstocks.	Root capacity to extract water varies diurnally and can more than double from pre-dawn to midday. This is accounted for by changed activity of root aquapoprins. This has major implications for irrigation timing.

Outputs and Performance Targets 2005-06

Outputs	Project Performance
1. Information on the interactions between salinity and proportions of major nutrient types on root hydraulic conductivity and reflection coefficients.	Salts reduce root hydraulic conductivity, but differences between rootstocks in their salt tolerance cannot be accounted for by differences in physical barriers and cell wall anatomy as indicated by similar reflection coefficients and similar permeability to apoplastic tracers.
2. Information on the interactions between major nutrient applications and proportions of major nutrient types on root hydraulic conductivity.	There were interactions between nitrate and chloride. A more salt tolerant rootstock (Paulsen) had a lower selectivity for nitrate over chloride but this did not decrease with increased salinity. This contrasted to the salt sensitive rootstock (K51-40) which showed reduced selectivity with increased salinity. Membrane transport of chloride to the root vacuole and to the xylem accounted for differences between root stocks, but this only occurs in primary roots, not secondary roots.

Outputs and Performance Targets 2006-07

Outputs	Project Performance
1 Information on the diurnal variability of root hydraulic conductivity and reflection coefficients and how this can be varied by soil conditions.	The diurnal changes in hydraulic conductivity were increased under drought conditions, but this only occurred in a more anisohydric (less conservative) variety (Chardonnay). The diurnal variation was decreased in the more isohydric (conservative) variety (Grenache). Root reflection cooefficents could not be explicitly determined under these conditions but we did observed a much lower root turgor (implying lower reflection cooefficient) for both varieties.

Outputs and Performance Targets 2007-08

Outputs	Project Performance
1 Information on the link between transpirational demand, canopy leaf area and root hydraulic conductance.	There is an increase in root hydraulic capacity with increased demand for transpiration and this occurs across many varieties and root-stocks. Reduction in shoot area indicated a novel signal that comes from shoot topping. Shoot topping can reduce root capacity to transport water by 50%.
2. Information on the role of root ABA and auxin levels on root hydraulic conductance.	Xylem ABA concentration is correlated with the root response to drought, but was not altered in roots due to shoot topping. Auxin transport from shoot to root does not seem to be the signal for shoot to root coordination, since phloem girdling did not prevent the response and the effect of auxin transport inhibitor was inconclusive. There is some evidence for the involvement of ethylene.

Outputs and Performance Targets 2008-09

Outputs	Performance Targets
1.Validation of predictions from pot experiments to field conditions for major treatment effects observed in pot experiments of nutrient/salinity interactions on root hydraulic conductance and water extraction ability.	Field trials have shown that shoot topping does have an effect on root capacity to transport water as demonstrated by altered expression of the major aquaporin genes. Shoot responses were unexpected and indicate more experimentation is required.
2. Clear recommendations on best practices to induce high water extraction efficiencies and nutrient extraction efficiencies when these are linked to water uptake (eg N).	These are listed in this report

7.2 Practical implications of the research results for the Australian grape and wine industry.

Practical Outcome 1 Diurnal variation in capacity of roots to extract water.

We have shown that the roots of grapevine (Chardonnay and Grenache) have greater capacity to extract water from the soil during the day, with the lowest capacity being pre-dawn. Based on these findings and from anecdotal reports from grapegrowers that early evening irrigation appears to give better results than other times, it would be worthwhile carrying out field trials to examine irrigation timing in terms of efficiency of water extraction by roots.

Practical Outcome 2 Signals from shoot to root that can change water extraction by roots.

This is a completely new finding not only for grapevines but for plants in general. It opens the possibility that shoot derived signals could be manipulated in order to increase/decrease the capacity of the roots to extract water, depending on the desired outcome. The common use of summer pruning to control vigor of vines needs to be re-examined in terms of how the roots are responding and whether de-vigorating vines is actually a response to reduced capacity of the roots to extract water. This also has implications for the quantum of irrigation water delivered to vines that have been shoot pruned.

Practical Outcome 3 Membrane transport identified as reason for differences in salt tolerance in grapevine rootstocks.

We have shown that the underlying difference between rootstocks in variable salinity tolerance is a result of different activities (or types) of membrane transport within the root. It would be worthwhile identifying the trasnporter genes responsible. This would allow more rapid assessment of salinity tolerance for new rootstocks as well as opening the possibility of genetic engineering of roots stocks for increased salinity tolerance.

8. Recommendations:

8.1 Identification of future research directions.

There are three clear research directions that should be considered linked to the practical outcomes listed above:

1) Assessing timing of irrigation (diurnal) on the efficiency of water extraction by vines. There would be a variety effect in this since we have shown that different varieties have very large differences in amplitudes of diurnal ability to extract water depending on the degree of water stress. Since there can be a two fold difference during 24 hours in the ability to extract water this could translate to a large difference in the efficiency of use of irrigation water by vines. The longer water is sitting in the spoil ie when vines are not efficiently extracting the water, the more likely water will be lost by other means. This should be linked to the propensity of soil evaporation, which would be higher during the day. An hypothesis to test would be: Water extraction and outcomes from irrigation in early evening is more efficient than other times of the day/night.

2) Manipulation of the shoot-derived signal that controls water extraction by roots: This would involve:
    a. Discover the nature of the signal (chemical or hydraulic) that communicates water stress information from the shoots to the roots.
    b. Discover the way in which this signal is transduced by the roots to up-regulate water channel activity.
    c. Carry out field trials to further characterise when the shoot pruning response is maximal in altering root water transport.
    d. Using the information in 1 & 2, devise methods to alter the signalling so that water extraction by roots is maximised or minimised depending on the desired strategy.
    e. Examine the response in grafted vines, in order to examine the effect of root stock and to extend the prospects of manipulation of root water transport to various scion rootstock combinations.

3) Identify the genes for the membrane transporters that confer salninty tolerance to rootstocks. This ouwl be a relatively standard molecular project that could be undertaken by a PhD student. The outcomes would be that these genes could be used as markers for salt tolerance when identifying new rootstocks. It would also be possible to increase the expression of these genes in a desirable root stock to increase salt tolerance.

8.2 Research outcomes related to broader industry practices and priorities for further R&D, extension and policy.

The research outcomes are closely aligned with:

Program 4, Sustainability of Industry, Environments and Communities
Sub Program a) Sustainable Production

AND

Program 5 - Smart Science, Practical Solutions
Sub Program a) Knowledge Development

With the following benefits:

Economic

1. Reduction in water use by shoot manipulations and/or in combination with hormonal applications is exiting. The cost of water is likely to increase, thus reduction in water use is a direct economic benefit.

2. Australia would be on the forefront of innovation through adaptation, which indirectly will benefit all parties linked to wine production in Australia.
3. Benefits would flow to users of water for irrigating any crop produced on an arboreal structure. Reduced water usage is a cost saving but may also be an “enabler” for an entire industry. The outcomes may also produce significant quality gains with water being able to be applied more precisely.
4. Higher skill levels, knowledge or expertise developed in this project may lead to further opening of pathways in our scientific/technical communities.
5. Opportunities exist to sell patented technology to other parts of the world.

Environmental

1. Less use of water, more or less saline, traditionally applied, may improve soil structures (or at least reduce the rate of decline) and hence fertility over time.
2. Less use of water industry-wide may enable crops to continue to be grown in an increasingly hostile (hotter) environment.

Social Benefits

1. There are wide areas of Australia, as elsewhere, that are struggling with reduced water availability. Given SOME water is available to these regions, then these communities may be maintained, not just the growers but also the people that provide the support.
2. Healthier environment;
3. More water available for other uses.

Appendix 1: Communication

PhD Theses

Three students supported the project have obtained their PhDs and graduated on the 5th of August 2008. Each student was partially supported by funding from this grant.

Rebecca Vandeleur, Grapevine root hydraulics: the role of aquaporins. PhD Thesis, University of Adelaide

Nasser Abbaspour, A comparative study of Cl transport across the roots of two grapevine rootstocks, K51-40 and Paulsen, differing in salt tolerance. PhD Thesis, University of Adelaide

Megan Shelden, A comparison of water stress-induced xylem embolism in two grapevine cultivars, Chardonnay and Grenache, and the role of aquaporins. PhD Thesis, University of Adelaide

Journal Publications

Vandeleur, R., Niemietz, C.M. Tilbrook, J., and Tyerman, S.D. (2005) Roles of aquaporins in root responses to irrigation. Plant and Soil (2005) 274:141–161

Vandeleur RK, Mayo G, Shelden MC, Gilliham M, Kaiser BN, Tyerman SD (2009) The role of plasma membrane intrinsic protein aquaporins in water transport through roots: diurnal and drought stress responses reveal different strategies between isohydric and anisohydric cultivars of grapevine. Plant Physiology 149: 445-460.

Shelden MC, Howitt SM, Kaiser BN, Tyerman SD (2009) Identification and functional characterisation of aquaporins in the grapevine, *Vitis vinifera.* Functional Plant Biology.

Plus four more publications currently in draft to be submitted (as detailed in the body of the report):

Vandeleur R.K., Kaiser B.N., Dry P.R., Sullivan W. and Tyerman S.D. (2010) Shoot topping impacts on root hydraulic conductivity. (in prep)

Vandeleur R.K., Kaiser B.N., Dry P.R., Sullivan W. and Tyerman S.D. (2010) Impact of partial root drying on root hydraulic conductivity of grapevines. (in prep)

Abbaspour, N., Kaiser, B. and Tyerman S.D. (2010) Root apoplastic transport and water relations cannot account for differences in Cl^- transport and Cl^-/NO_3^- interactions of two grapevine rootstocks differing in salt tolerance. (in prep)

Abbaspour, N., Kaiser, B. and Tyerman S.D. (2010) Chloride transport and compartmentation within main and lateral roots of two grapevine rootstocks differing in salt tolerance. (in prep)

Book Chapter

Tyerman S. D., Vandeleur R. K., Shelden M. C., Tilbrook J., Mayo G., Gilliham M., and Kaiser B. N. (2008) Water transport and aquaporins in grapevines. Chapter 5 *In*: Grapevine Molecular Physiology and Biotechnology. Ed: Kalliopi A. Roubelakis-Angelakis, Kluwer Dordrecht pp 73-104.

Published conference proceedings

Tyerman S.D., Kaiser, B.N., Coleman, R., Shelden, M.C. and Tilbrook J. (2005) Investigating the hydraulics of grape vines: from the genes for the pores to the physiology of the pipes. Proceedings of the Twelfth Australian Wine Industry Technical Conference (eds R. Blair, P. Williams, S. Pretorius) pp 179-181.

Other

Anderson, K., Findlay, C., Funetes, S., Tyerman S.D. (2008) Viticulture, wine and climate change. *In:* Ganaut Climate Change Review.
http://www.garnautreview.org.au/CA25734E0016A131/WebObj/01-HViticulture/$File/01-H%20Viticulture.pdf

Seminars including to the grape and wine industry*

Gordon Conference on Cellular Basis of Adaptation to Salt and Water Stress in Plants. Oxford 2006 (Invited Speaker on Plant Aquaporins)

International Workshop on Soil-Plant Interactions and Sustainable Agriculture in Arid Environments July 11-18, 2008 Keynote Speaker: The role of aquaporins in controlling root hydraulic conductance in grapevine.

Boden Conference, Plant Energy and Water Productivity, Canberra 18th-20 Sept 2008 (Invited Speaker, on plant aquaporins)

Sensing, Response and Adaptation to Altered Water Status Conference, Academia Sinica, Taipei, Taiwan March 12-13 2009 . Invited Keynote: The role of aquaporins in controlling root hydraulic conductance.

Water transport and control of water channels in grapevine. 16-18th November 2009 Genomics of Salinity (GRDC & ACPFG).

*Oral Presentation at the 8th International Symposium on Grapevine Physiology and Biotechnology: Dr Rebecca Vandeleur – University of Adelaide (Australia) The role of aquaporins in controlling root hydraulic conductance in grapevine

*5th Australian Wine Industry Environment Conference 23 Sept. 2009. Invited Keynote: Viticulture and Climate Change. Known unknowns and unknown unknowns that might catch us by surprise.

*Plant Physiology session (3 hours, 29th Oct, 2009) Mildura - Murray Valley Winegrowers Inc. (Organised by Liz Singh) within a GWRDC funded project: Advanced Viticulture Information Sessions.

*Oral presentations at all Soil and Water Initiative yearly meetings.

*Tyerman as been invited to present this work at the AWITC 2010 conference.

A poster entitled **Grapevine root hydraulics: The role of aquaporins**, was presented at COMBIO 2005, Adelaide.

A poster entitled **Selectivity between chloride and nitrate in grapevine roots and the relationship to salt tolerance** Nasser Abbaspour, Brent N. Kaiser and Stephen D. Tyerman

Conference abstracts

Vandeleur, R.K., Kaiser, B.N., Dry, P.R., Tyerman, S.D. (2007) The impact of PRD on root hydraulic conductance of Chardonnay and Grenache. *In* Proceedings of 13th Australian Wine Industry Conference. pp 302.

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Appendix 2: Intellectual Property

All IP resulting from this project has been, or will be published, and is in the public domain. A list of publications arising from this project is attached.

Appendix 3: References

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Appendix 4: Staff

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