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Genetic and mechanistic characterisation of rootstock traits conferring abiotic stress tolerance to grapevines

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Genetic and mechanistic characterisation of rootstock traits conferring abiotic stress tolerance to grapevines

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Front cover: Grapevine plants loaded onto the Plant Accelerator, December 2013

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1 Abstract

Modelling of climate change predicts that the grape-growing areas of southern Australia are likely to experience higher temperatures, and reduced quality and quantity of irrigation water and rainfall resulting in increased salinity problems in the future. We utilised the Plant Accelerator as a tool for screening young rootstock rooted cuttings for their ability to withstand these stresses. As a result, we mapped and characterised genes involved in sodium exclusion. We compared the water use of rootstocks grown in the Plant Accelerator and the field. Other experiments identified candidate genes for chloride exclusion traits and more essential information about mechanisms of ion exclusion.

2 Executive summary

Predictions of the effects of climate change on southern Australia suggest that there is likely to be an increase in temperatures and extreme temperature events, a reduction in rainfall and increased evaporation leading to reduced water availability for irrigation. The quality of the available water is also likely to be reduced. As a result, salinity will be an increasing problem, impacting on many of the irrigated grape growing areas. Hence, vineyards will probably be faced with a combination of abiotic stress factors: higher temperatures, reduced water and increased salinity. To assist in the protection of the Australian winegrape industry, CSIRO, in partnership with Wine Australia, is developing new rootstocks that can tolerate these stresses to a higher degree than currently available commercial rootstocks.

This project is built on a solid foundation of salinity tolerance research in grapevines carried out at CSIRO over many years and took advantage of a unique new facility, the Australian Plant Phenomics Centre's Plant Accelerator, to facilitate the screening of large numbers of young rooted cuttings for their ability to withstand imposed stresses of salt, high temperature and water deficit, alone or in combination.

Salinity tolerance has generally been considered the result of ion exclusion, a trait conferred by rootstocks, as previously identified in many studies. By utilising the progeny of a cross between K51-40 (that takes up chloride) and 140 Ruggeri (a chloride excluder) and amazing serendipity, we were able to map a gene responsible for sodium exclusion, which proved to be highly variable in this population. In collaboration with our colleagues at the University of Adelaide, Dr Sam Henderson and Prof. Matt Gilliham, functional characterisation of the major gene encoding a protein essential for sodium exclusion, HKT1;1 has led to a publication in New Phytologist. However, further studies in glasshouse-grown plants have also implicated other mechanisms of sodium exclusion between different tissues, so this trait does require elucidation. The identification of this gene is a major step forward in developing a suite of markers for abiotic stress tolerance to aid in the breeding of new resilient rootstocks.

The Plant Accelerator experiments also confirmed that chloride exclusion is a complex trait, a conclusion previously reached by other studies. A second approach delivered a breakthrough in identifying a genetic locus responsible for chloride exclusion. Description of this work has been redacted from this report, as it is commercial-in-confidence.

Tolerance of soil water deficit is another trait well known to be complex and multi-genic. The Plant Accelerator experiments that examined this found significant differences between genotypes in their water use and their water use efficiency (in terms of growth per unit water transpired), but they did not find any difference between genotypes in their response to water deficit. In other words, a high vigour genotype when well-watered was a high-vigour genotype when water stressed, even though growth was reduced by water deficit. This may have been a result of the cross used for this work (*V. cinerea* x *V. vinifera*) or may be a feature of *Vitis* spp more generally.

A unique feature of this project was our ability to examine multiple combinations of abiotic stresses that are likely to be more common in a future Australian climate. Results from a glasshouse experiment brought a greater understanding of the interactions between root temperature and ion exclusion traits; however, it raised questions about how to discover the genes that identify good exclusion at high root temperature for use in a marker assisted approach to rootstock breeding.

While we have made significant strides towards providing markers for salt tolerance to the rootstock breeding program, we have also examined the extent to which traits observed in the Plant Accelerator are conferred to grafted mature vines in the field. For example, the relative growth rate of salt treated rooted cuttings was significantly and strongly correlated with pruning weights of 10 year old grafted vines in a salt treated vineyard. Our ability to test tolerance of water deficit in the same way was hampered by difficulties in generating soil moisture differences in field experiments. These experiments did demonstrate that ungrafted rootstock vigour in the Plant Accelerator was a poor indicator of conferred vigour in grafted plants in the field, demonstrating that using the Plant Accelerator for assessment of rootstock genotypes is best employed for traits that are clearly based in the root system with a minimal impact of the shoot.

Despite this, it is clear that the unique imaging facilities and scale of the Plant Accelerator provide a valuable new tool for predicting abiotic stress tolerance of new material in the CSIRO rootstock breeding program.

3 Background

Australian grape growers are facing increased environmental pressures commensurate with the challenges of climate change (Webb et al., 2013; Webb et al., 2008). From our understanding using climate modelling techniques, weather will become more unstable with more extreme events becoming more common. There is a very high probability that this will include increased average temperatures, increased frequency and severity of high temperature events, and reduction in the quantity and quality of irrigation water in southern Australia due to lower rainfall and higher evaporation rates (IPCC report 2013 http://www.ipcc.ch/report/ar5/wg1/). To future-proof the Australian wine grape industry, it is essential to develop rootstocks with the capability to protect the scion and fruit from these harsh conditions of high temperature, reduced water availability and reduced water quality.

In the previous decade there has been a severe drought, the 'Millennium drought', resulting in poor fruit quality and reduced yields. The Millennium drought that impacted southern Australia during the late 1990s and early 2000s resulted in harsh water restrictions to large areas of wine grape production in Australia. In many areas of the Murray Darling Basin, such restrictions led to soil water deficit and insufficient leaching fractions with consequent build-up of salts within soil. In other regions, e.g. Langhorne Creek, this was exacerbated by irrigation water becoming highly saline and lower than usual winter rainfall led to reduced winter flushing of the soil profile. Such conditions intensified the negative impacts of heat waves on plants.

Climate change predictions suggest that grapevines will be exposed to greater abiotic stress in the form of increased temperatures, greater water deficit and increased exposure to salt through a general change in weather patterns together with more extreme events such as prolonged drought and extreme heatwaves. Resilient rootstocks, capable of dealing with these climate extremes and protecting the scion and fruit from the associated abiotic stresses, are essential to the Australian wine grape industry as identified in the Wine Australia Strategic Plan 2015-2020.

CSIRO has had a longstanding rootstock breeding and evaluation program. This project is a continuation of that work and aims to provide new tools to assist in the selection of rootstocks, fit for Australia's harsh conditions. The project seeks to achieve this in two ways: 1) to develop perfect genetic markers for traits of abiotic stress tolerance to aid in marker-assisted selection, and 2) to develop new screening tools to rapidly assess rootstocks for key growth characteristics that correlate to field responses, to speed up the process of selecting elite rootstocks.

In this project we have built on outputs from project CSP 0901, where we demonstrated the ability of rootstocks to improve crop water use index (Edwards et al., 2014) and project CSP 1002, which has resulted in an enhanced characterisation of the chloride exclusion mechanism. The multidisciplinary team utilised extensive CSIRO and University of Adelaide

expertise in genetics and physiology of grapevine response to abiotic stress to formulate novel strategies for screening potential rootstocks at an early stage; we aimed to develop and validate genetic and phenotypic markers for drought, heat and salt tolerance. Tools created in this project will revolutionise and expedite rootstock breeding.

4 Project aims and performance targets

1. Develop assays for fast and early screening of rootstock cuttings for water use efficiency, drought tolerance and salinity tolerance as measured by biomass, leaf size, leaf temperature, water use and ion composition of leaves using the controlled conditions in the Plant Accelerator, Smart House, Australian Plant Phenomics Centre, Waite Campus.

2. Validate the efficacy of early screening by correlating the range of glasshouse responses to those in the field. This will include fruit-bearing grafted vines, utilising the trial at the CSIRO Irymple Farm, Victoria, established as part of CSP 0503 and will complement the assessment of those vines under CSP 1304.

3. Utilise information gained in 1&2 to identify rootstocks with different abilities to cope with stress and use these plant lines to identify new genes via a transcriptomics approach.

4. Identify genetic markers for use in future rootstock breeding and assessment of currently available rootstocks, using assays for tolerance to heat, drought and salinity stress and linkage map developed in CSP 1304.

5. Utilise markers already identified for chloride exclusion and markers developed in (3) to screen breeding populations of *V. cinerea* x *V. vinifera* and rootstock selections already in the CSIRO rootstock breeding pipeline.

6. Identify potential mechanisms for observed drought tolerance trait variability and determine their relevance to optimising current irrigation management practices for specific rootstock/soil combinations.

7. Identify interactions between root temperature and salt exclusion to inform management of soil cover in salt-affected areas.

8. Further characterise the genes identified as potentially responsible for chloride and sodium exclusion to provide robust, perfect markers for smart breeding of salt-excluding rootstocks.

Output	Target Date	Activities
1A Novel assay developed	31 October 2013	Test different soil and substrate mixtures. Define watering and salt conditions for screen. Select 4-5 genotypes including K51-40, 140 Ruggeri, and Shiraz for preliminary experiment. Perform small scale salt and water deficit experiment to test Plant Smart House function and determine best strategies for data and material collection.
1B Collaboration with Natalie Ollat, IRNA established	31 December 2013	Visit of two scientists to INRA for detailed discussion with Natalie Ollat and colleagues, who are established scientists studying abiotic stress responses in different rootstocks.
1C Smart House experiment 1 (Salt and Heat) completed	30 June 2014	Collect cuttings of genotypes for salt experiment, parents 140 Ruggeri, K51-40 and up to 80 progeny lines. Root cuttings and then plant in defined, weighed soil substrate. Apply stresses heat and salt to planted cuttings and collect leaf temperature and growth data over about 7 days. Harvest samples for ion analysis and gene expression experiments. Ion analysis (Na^+ and Cl^-) completed. Lines for glasshouse experiments and gene expression analysis identified.
1D Preliminary understanding of interaction of salt and heat	30 June 2014	Selection of five progeny lines used in smart house experiment representing full spectrum of response made. Selections grafted with Shiraz and utilised in a glasshouse experiment to examine physiological responses of grafted vines to combined heat and salt stress. Results from smart house experiment and glasshouse experiment on selected lines contrasted and compared. Preliminary conclusion on transferability of observed traits in the smart house to grafted vines made.
1E Four new genes for chloride exclusion characterised	30 June 2014	Functional analysis of at least four genes identified in previous project CSP 1002 as candidates for chloride exclusion. Analysis to include subcellular localisation and transient functional assays utilising Xenopus oocytes.
2A Smart House Experiment 2 completed	31 March 2015	Cuttings from *V. cinerea* x *V. vinifera* population (up to 120 lines) tested for ability to withstand temperature and reduced water using techniques as in 1A. Samples collected for further gene expression analysis.

Output	Target Date	Activities
(Heat and water deficit effect on V. cinerea x V. vinifera population)		Data sent to Cornell for correlation with SNIP analysis to identify new markers.
2B Transcriptomic analysis planned and performed to identify new genes	30 June 2015	Samples identified and high quality RNA extracted. Microarray experiments performed in Verona in collaboration with Mario Pezzotti.
2C Five putative genes for sodium exclusion identified	30 June 2015	Using database and literature searches, find genes in other species involved in sodium exclusion/uptake. Isolate similar grapevine genes and determine expression pattern matches suggested role.
2D Interaction between salt uptake and root temperature investigated	30 June 2015	Small vines of Shiraz grafted to K51-40, 140 Ruggeri or own roots subjected to hydroponic experiments to assess the combination of heat applied to roots and salt stress on plant growth, leaf physiology and salt uptake.
2E Transfer of rootstock traits observed in smart house screens to grafted plants demonstrated	30 June 2015	Glasshouse experiment examining response of a selection of five lines from the *V. cinerea* x *V. vinifera* population grafted with Shiraz to combined heat and drought stress completed. Nursery experiment with at least ten lines utilised in the smart house experiments, grafted with Shiraz, established at CSIRO's Irymple Farm. Preliminary results obtained of response to drought stress.
2E Stop/Go decision	30 June 2015	In conjunction with GWRDC conduct an internal review of progress to date and planned outputs and activities.
3A Smart House experiment 3 completed (Understanding the combined effect of temperature, water deficit and salt on selected rootstock lines)	30 June 2016	Plant lines (20-50) identified from previous 2 Smart House experiments tested for ability to withstand salt, heat and water stress combined. Ion analysis performed to determine effect of heat and drought on salt exclusion.
3B New candidate genes identified	30 June 2016	Transcriptomic data analysis completed, characterisation of genes initiated.
3C Effect of root temperature on salt exclusion confirmed	30 June 2016	Hydroponic experiments with Shiraz grafted to two elite rootstocks completed to assess the effect of root zone temperature on salt uptake, leaf physiology and plant growth.
3D Project findings shared with French collaborators	30 June 2016	Meeting with INRA held. Results from both Australian and French projects discussed in detail and plans for last year confirmed.

Output	Target Date	Activities
3E Five genes responsible for sodium exclusion/uptake identified	30 June 2016	Functional characterisation of putative sodium exclusion genes completed including subcellular localisation and transient functional assays.
3F Preliminary validation of results from Smart House and glasshouse to represent responses in field conditions.	30 June 2016	Response of established nursery vines at CSIRO's Irymple Farm to combined heat and drought stress, utilising simple custom built chambers designed to accommodate multiple small, densely planted vines, to generate heat stress. Initial examination of the response of a selection of mature *V. cinerea* x *V. vinifera* population, already established at Irymple Farm, to deficit irrigation determined. Collection of stem and other material from vines in nursery to assess for structural differences that might correlate with stress responses.
4A DNA markers developed for abiotic stress resistance	30 June 2017	Genes identified in transcriptomic analysis characterised and DNA markers developed and tested for robustness. Chromosomal regions identified in SNP by phenotype analysis used to generate DNA markers.
4B New salt exclusion genes characterised	30 June 2017	Genes identified from marker work and transcriptomic analysis in previous years characterised functionally.
4C Viability of using rapid glasshouse screens of ungrafted rootstock selections to determine desired traits of mature grafted vines established	30 June 2017	Physiological measurements on mature *V. cinerea* x *V. vinifera* selection at Irymple Farm completed. Full analysis of physiological and related molecular data across the experiments from smart house to field demonstrating the extent to which traits determined in new rootstock selections are likely to be transferred to mature, field grown vines.
4D Mechanistic properties of stress resistant vines determined	30 June 2017	Anatomical structure of plant material collected from Nursery experiments examined and correlated with data from all experiments on clones of same lines.
4E Extension of project outcomes through multiple extension mechanisms.	30 June 2017	Research outcomes to be compiled and prepared for at least two industry journal articles and at least two scientific papers published in refereed journals. At least one extension event held to communicate project outcomes to the broader industry (e.g. through a GWRDC Innovators Network event). Final report submitted to GWRDC.

Output	Target Date	Activities
1E Four new genes for chloride exclusion characterised	30 June 2015	Functional analysis of at least four genes identified in previous project CSP 1002 as candidates for chloride exclusion. Analysis to include subcellular localisation and transient functional assays utilising Xenopus oocytes.
2C Five putative genes for sodium exclusion identified	30 June 2014	Using database and literature searches, find genes in other species involved in sodium exclusion/uptake. Isolate similar grapevine genes and determine if expression pattern matches suggested role.
3D Project findings shared with French and American collaborators	31 December 2017	Meeting with collaborators held. Results from both Australian, French and American projects discussed in detail and plans for last year confirmed.
4D Mechanistic properties of stress resistant vines determined	31 March 2018	Anatomical structure of plant material collected from Nursery experiments examined and correlated with data from all experiments on clones of same lines.
4E Extension of project outcomes through multiple extension mechanisms.	31 March 2018	Research outcomes to be compiled and prepared for at least two industry journal articles and at least two scientific papers published in refereed journals. At least one extension event held to communicate project outcomes to the broader industry (e.g. through a GWRDC Innovators Network event). Final report submitted to GWRDC.

5 General methods

5.1 Plant material and genotyping

The family used for most of the investigation into salt exclusion was the result of a cross between K51-40 and 140 Ruggeri and had been used for many experiments previously (e.g. Gong et al., 2011), together with the parental lines. Dormant canes were collected from the CSIRO Irymple Farm, Victoria where possible (subjected to hot water treatment before import to SA) or PIRSA Research Centre, Nuriootpa, South Australia in July of each year. Following the completion of experiments 1 and 3, genotyping of these 68 hybrid lines by the Australian Genome Research Facility, revealed that 28 did not have 140 Ruggeri as the male parent (see results in 6.1.4).

Cuttings for the experiment with a range of different species and cultivars (diverse phenotypes) were collected as dormant canes from CSIRO Irymple Farm, Victoria (subjected to hot water treatment before import to SA) in July 2015.

For experiments 5 and 6, a family of F1 progeny from a cross between *V. cinerea* and *V. vinifera* Riesling was used, as this family has also been utilised for study in Wine Australia project CSP 1304. Dormant canes were collected from the CSIRO Irymple Farm, Victoria and subjected to hot water treatment before import to SA or from Nuriootpa, SA in July of each year.

Some experiments included grafted material with Shiraz clone ENTAV-INRA® No. 470 or clone 1654 as the scion and supplied by Yalumba Nursery. These canes were omega-grafted at Yalumba Nursery, Nuriootpa, Barossa Valley, SA.

All canes were callused by Yalumba Nursery resulting in successful and prompt rooting of most cuttings. Callused canes were planted in a perlite/sand mixture and maintained in a glasshouse with frequent misting with rainwater until roots were visible at the bottom of the pot.

5.2 Major glasshouse, Accelerator and field experiments

5.2.1 Experiment 1. The Plant Accelerator 2013. K51-40 x 140 Ruggeri progeny, salt x heat

The lines used in this experiment were 68 lines of K51-40 x 140 Ruggeri progeny or hybrids, the parents of the cross, K51-40 and 140 Ruggeri, and two CSIRO selections, previously identified for their ability to exclude chloride from the scion (Walker et al., 2014).

Twenty five dormant canes of each line were callused at Yalumba Nursery, planted in a 50:50 sand:perlite mix, placed in a glasshouse with the shades drawn and misted every hour with rainwater for a few days then gradually reduced over the next week (Figure 1A). During this period the sprouting cuttings were watered with Seasol Seaweed Concentrate (0.55 mL/L, Seasol, Bayswater, Victoria) and Aquasol fertiliser (110 mg/L, Yates Australia) mixture once a

week. After four weeks lateral shoots were removed and 15 selected rooted canes of each line were transferred to 1.7 kg of Biogro soil mix (Van Schaik, Mount Gambier, South Australia) and placed on trolleys in the end of the southern Smart Houses of the Plant Accelerator facility with shades closed and humidifiers operating for three days and plants watered every second day with rainwater (Figure 1B). Shades were removed after approximately one week, once the plants had become established at 25^{o}C. All vines were trimmed to a single shoot and axillary growth was removed as it appeared. After seventeen days, the 12 most uniform plants were selected, blue support frames added and labelled for loading on the conveyor belt (Figure 1C). Each line consisted of three replicates for each of the four treatments: cool, cool + salt, hot, hot + salt. Each Smart House was split into three randomised blocks. The two Smart Houses were programmed for different temperatures with a maximum/minimum of 27/18^{o}C (cool) in south east (SE) and gradually rising to 43/30^{o}C for three days then dropped back to 37/27^{o}C for three days (hot) in south west (SW).

Salt treatments consisted of a final pot concentration 100 mM chloride with cations of Na^{+}, Ca^{2+}, Mg^{2+} in a ratio of 6:1:1 was added to the salt treated plants over a seven day interval with three applications on days 2, 4 and 8 of 33, 33 and 35 mM mixed chloride respectively. As each pot was in its own tub, salts were not flushed through but remained in the enclosed system. Control treatment plants received a similar volume of rainwater on each of the three salt treatment days. Pots were imaged and watered to weight each day until day 15 when they were manually harvested.

Plants were imaged on all days except the three days of salt application and watered to specified, recorded weight with rainwater.

At harvest, all plant meristems with about two visible leaves were snap-frozen in liquid nitrogen, and mature fully expanded lamina were removed and dried for ion analysis.

A subsample of 12 genotypes (x12 replicates = 144 vines), were destructively harvested to determine total leaf dry weights, stem + petiole dry weights and root dry weights. The leaf area of these vines was also measured by passing harvested leaves through a digital leaf area meter (LiCor Biosciences, Nebraska, USA). Young leaves and stems + petioles were bagged separately for drying. Root samples were dried to obtain a root dry weight and provide material for ion analysis. All plants in replicate 2 from both the cool and hot sides were gently up-ended and a small representative root sample was taken and snap-frozen in liquid nitrogen, and the root system photographed for further analysis if required.

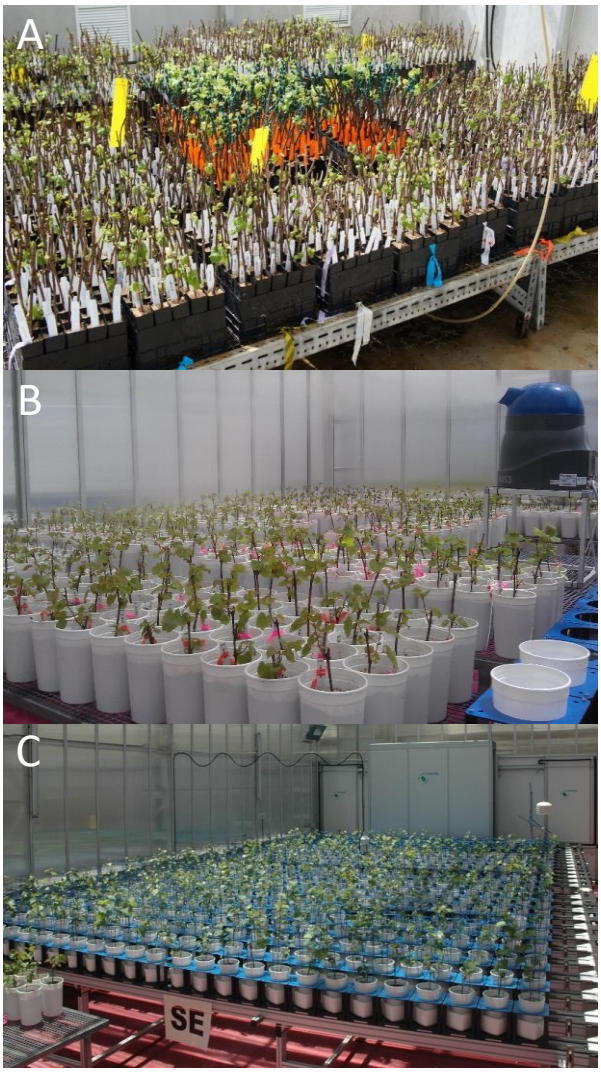


Figure 1. Rootstock cuttings growing for experiment 1. A) Callused canes being established in glasshouse (flagging tape and plastic labels used to identify lines), B) Canes with shoots planted in 2 litre pots with humidifier, and C) Plants growing on conveyor belt of the Smart House.

5.2.2 Experiment 2. The Plant Accelerator 2014. Salt exclusion by rootstocks grafted with Shiraz

The aim of this experiment was to determine the extent to which salinity tolerance traits of the rootstock are passed on to a grafted scion, Shiraz. Six hybrid genotypes displaying a wide range of chloride and sodium exclusion abilities from experiment 1 were selected, also accounting for the probability of grafting being successful, based on the thickness of the cuttings available. Together with K51-40 and 140 Ruggeri, at least 60 cuttings of each genotype were callused and grafted with a Shiraz scion at Yalumba Nursery. Grafted cuttings were then established as in experiment 1 and Figure 1. The most similar 16 vines from each genotype were selected and loaded onto the conveyor belt system. Each genotype consisted of four replicates of each of the four treatments: cool, cool + salt, hot, hot + salt. The experiment was split into three randomised blocks, mirrored in the two Smart Houses, with control and salt treatments of each genotype placed next to each other. Mixed chloride (final

concentration of 100 mM Cl^-, 6:1:1 ratio of Na^+:Ca^{2+}:Mg^{2+}) was added to the salt treatment plants with 33 mM Cl^- applications occurring on days 1, 3 and 5. Control treatments received an equal volume of rainwater. Plants were imaged on all days except the three days of salt application and watered to weight with rainwater. On day 19, vines were destructively harvested as in experiment 1.

The two Smart Houses were programmed for different temperatures with a set maximum/minimum temperature of 25/20°C (cool) in south west (SW) Smart House and in the south east (SE) Smart House the set maximum temperature was ramped to 35/23°C. However, the actual temperatures recorded in the two Smart Houses showed large deviation from the set temperatures due to several problems including air-conditioner issues with the average temperatures across the experiment being 25.6°C in the cool and 28.8°C in the hot Smart House.

5.2.3 Experiment 3. Glasshouse 2015 salt uptake by K51-40 x 140 Ruggeri progeny with heated roots, grafted with Shiraz

The aim of this experiment was to examine the influence of root zone temperature on chloride and sodium exclusion in the same six hybrid genotypes from Experiment 3 as well as the two parents K51-40 and 140 Ruggeri, grafted with Shiraz. In a glasshouse (average temperature 21°C) genotypes were grown in clay bead-filled vine tubes that were suspended in the air space of large fully-insulated 200 L tubs half-filled with special 1/4 strength Hoagland's nutrient solution (including 90 μM Fe), which was continually recirculated over the clay beads (Figure 2A). The four treatments consisted of cool (18°C), hot (30°C) and cool + salt and hot + salt. Salt treatment was 25 mM mixed chloride for one day then 50 mM mixed chloride for the remaining 26 days. Solutions were changed weekly to reduce bacterial and algal growth. Each genotype consisted of four replicates per treatment. Harvest of this experiment included measuring leaf area and new stem length and collecting each tissue for drying, weighing and ion analysis. Roots were sampled from the pots and also from those that had grown out into the hydroponic solution (Figure 2B).

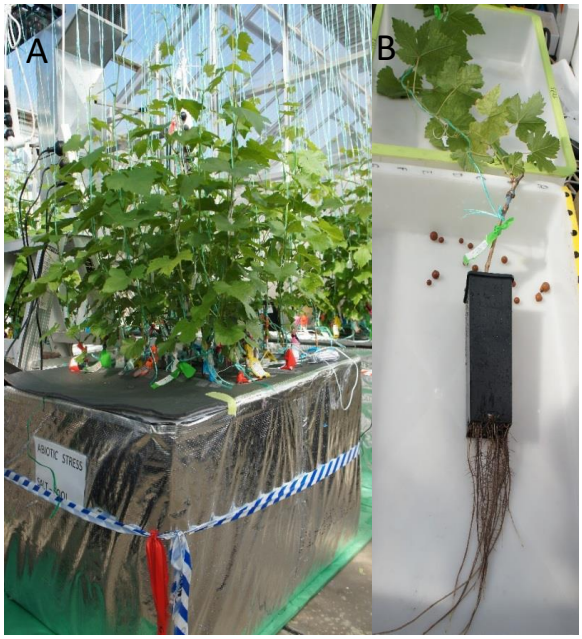


Figure 2. Heated root experiment. A) Plants were acclimatised in the tubs for one week before adding mixed chloride. B) Plant at harvest showing hydroponically grown roots.

5.2.4 Experiment 4. Glasshouse 2014. Comparison of ion accumulation in different plant organs

For this experiment six hybrid genotypes were selected based on a range of abilities to exclude chloride, including MC 215-13, MC 215-28, MC 215-30, MC 215-76 and MC215-78. One line, MC215-59 was subsequently shown to have a different pollen parent from the others. Rooted cuttings of these lines, together with the parents K51-40 and 140 Ruggeri, were grown for 12 months in 2.8 L pots filled with Mount Compass sand topped with perlite (Figure 3). The pots were lined with frost cloth to stop the sand falling through the drainage holes. Glasshouse temperature was maintained at 22-28^{o}C.

Special 1/4 strength Hoagland's nutrient solution (Gong et al., 2011) was applied daily. Mixed chloride (25 mM) in the nutrient solution was applied by hand-watering on day one of the experiment, followed by 50 mM mixed chloride on subsequent days. Volumes were sufficient to allow a substantial flow of watering solution through the pot to prevent build-up of salts. All tissues of the plants were harvested after three weeks, dried, weighed and ground for ion analysis.



Figure 3. Rooted cuttings grown in 200 mm diameter pots filled with Mount Compass sand and topped with perlite in glasshouse for experiment 4.

5.2.5 Experiment 5. The Plant Accelerator 2014 water deficit and heat

This experiment was set up in a very similar way to experiment 1, again utilising the Plant Accelerator Smart Houses, but to investigate the combination of heat and water deficit stress rather than salt. F1 progeny derived from a *V. cinerea* x *V. vinifera* cv. Riesling were assessed in this experiment. Twenty five cuttings of 90 F1 progeny and Riesling lines were callused at Yalumba Nursery and established as in Experiment 1. This was the first study at the Plant Accelerator to completely fill two Smart Houses to capacity and was therefore a major undertaking, requiring the establishment of over 1,500 cuttings. The *V. cinerea* parent failed to callus and upon potting cuttings into Biogro mix, six progeny genotypes had failed to strike roots. Thus 84 hybrid genotypes together with the Riesling parent, Shiraz, K51-40, 140 Ruggeri, *V. candicans* and *V. longii* were used in the experiment in the Smart Houses. Each genotype consisted of three replicates of each of the four treatments: cool, cool + water deficit, hot, hot + water deficit. The control water treatment consisted of watering to a target pot weight of 2,100 g (approximately 1050 g soil dry weight and 1050 g water), while the deficit treatments were watered to 1,700 g (approximately 1050 g soil dry weight and 650 g water). The two Smart Houses were programmed for different temperatures with a set maximum of 25^{o}C (cool) in south west (SW) and in the south east (SE) the set maximum was ramped from 33 to 40^{o}C across the three weeks of the experiment.

5.2.6 Experiment 6. Nursery experiment 1 planted 2014

The aim of this experiment was to investigate whether grafted cuttings grown in a field nursery setup would show similar growth patterns to that seen in the Plant Accelerator water deficit experiment 5. Four *V. cinerea* x *V. vinifera* cv. Riesling progeny, together with Riesling, K51-40 and 140 Ruggeri were included in the experiment, as they were part of the Plant Accelerator water deficit experiment 5. All cuttings were callused and grafted with Shiraz at Yalumba Nursery, and established in sand/perlite for six weeks before planting in the nursery

setup at the CSIRO Irymple Farm, Victoria. The randomised nursery trial consisted of 16 replicates of each genotype planted across two rows at a spacing of 0.5 metres. Control irrigation treatment was applied to eight replicates and deficit irrigation to another eight replicates. Two Shiraz vines were planted as buffers between each treatment block.

5.2.7 Experiment 7. Nursery experiment 2 planted 2015

A second nursery experiment was established to investigate the response of genotypes in the field under a combination of both water deficit and high heat treatment. For this planting, seven of the *V. cinerea* x *V. vinifera* cv. Riesling hybrids were used, together with Riesling and Shiraz. As previously, all lines were used as rootstocks and grafted with Shiraz. Vines were planted across two rows with vine and row spacing and vine training as described for Nursery experiment 1.

5.3 Plant Accelerator data collection and processing

5.3.1 Calculation of projected shoot area

Vine growth was captured using a LemnaTec 3D Scanalyzer (LemnaTech, GmbH, Wuerselen, Germany). On the days of imaging three Red/Green/Blue (RGB) images were taken of each plant, one from a top view, and two from the side with a 90^{o} rotation. At the end of the experiment the collection of images was processed by staff at the Plant Accelerator to count the total number of green pixels per plant. This effectively captures the growth of both the new stem and leaves but eliminated the existing cane which is brown in colour. The total vine pixels from each of the three daily images were summed to give a projected shoot area (PSA) of each vine.

5.3.2 Calculation of relative growth rates

In the 2013 'ungrafted' experiment, growth rate analysis was performed over the period from days 5 to 12. Images from day 13 were excluded from the analysis because a malfunction in the automated watering system resulted in some plants not receiving watering on that day. In the 2014 Grafted experiment, growth rate analysis was performed over a longer period from day 6 to day 18. Relative growth rates were calculated as the slope of the natural log of projected shoot area over the respective periods. Relative growth rate thus reflects the average increase in pixel per pixel per day across these periods.

5.3.3 Calculation of relative water use

On each of the imaging days, vines would also be automatically weighed and then watered to a target weight. Six pots without plants (blank) were also included in each Smart House to get an estimate of the average amount of water lost to surface evaporation each day (average evaporation). The daily water use of each vine was calculated as the difference in pot weight between successive days minus the average evaporation in that Smart House. Daily water use was divided by the PSA of that vine on each day to give a relative water use across the

experiment. Mean relative water use was calculated over the days of relative growth rate analysis (day 5-12, for the ungrafted experiment and day 6-18 for the grafted experiment) and is expressed as grams water use per 100,000 pixels per day.

5.4 Ion analysis

Upon harvest, samples for ion analysis were dried at 60^{o}C for at least 72 h and then finely powdered in a hammer mill. Small samples were ground by hand in a mortar and pestle.

5.4.1 Chloride concentration

Dried samples were examined in duplicate for chloride concentration by silver ion titration with a chloridometer (model 442-5150, Digital Chloridometer, Lenexa, KS, USA). Samples (50 mg for laminae, 25 mg for petioles and roots) were weighed into 4 mL glass vials and 3 mL of acid reagent (100 mL glacial acetic acid and 6.4 mL of concentrated nitric acid per litre) was added and allowed to extract for 30 min. Four drops of gelatin reagent (Labonco Buchler Instruments, Labonco Corporation, Kansas City, MO, USA) were added just prior to analysis.

5.4.2 Cation concentration

Dried samples were examined in duplicate for the concentration of the cations, K^{+}, Na^{+}, Ca^{2+} and Mg^{2+}. Extracts of approximately 100 mg powdered samples were weighed into 15 mL polypropylene digestion vessels (SC415) (Environmental Express, Mt Pleasant, SC, USA) and 2 mL of 15 mol/L nitric acid was added. Samples were incubated in a fumehood overnight at room temperature. A disposable polypropylene (SC417) watch glass was added to each tube to minimise evaporation. The tubes were sequentially heated on a 96 well HotBlock Pro Digestion System (SC189-240) with a Pro Controller-Touch Screen (SC180-240) (Environmental Express, Mt Pleasant, SC, USA) increasing the temperature at 3^{o}C/min initially to 60^{o}C, with a holding period at 60^{o}C for 20 min, then at 85^{o}C for 270 min. Ninety-six vessels were heated per batch. The tubes were allowed to cool, then made up to 15 mL with MilliporeR reverse osmosis water and then inverted to mix thoroughly. The solutions were then filtered through 125 mm of Whatman No 42 filter paper. Aliquots of approximately 10 mL were transferred to polypropylene tubes for cation analyses and sent to Adelaide Analytical Services Unit, CSIRO Land & Water, Waite Campus.

Cation concentration was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Thermo Fisher Model: iCAP6500 Radial, Cambridge, England) using appropriate standards for calibration and recalibration to correct for any drift that may occur during analytical runs.

For each ion and for each organ, concentration was expressed on a dry mass basis. The concentration of a specific ion on a whole tissue basis was calculated for each biological replicate (rep) as the sum of the content of that ion (µmol) in the dry mass of each organ type (lamina, petiole, roots), expressed relative to the sum of all dry mass for all the organs and presented as µmol/g dry mass.

5.5 ABA xylem sap collection and ABA (abscisic acid) analysis

Xylem sap ABA measurements in the 2014 Grafted experiment were carried out as described in (Degaris et al., 2017). Immediately following the harvest of lower mature leaves for Cl^- analysis, the next most mature leaf was placed in a Scholander pressure chamber (Soil Moisture Equipment, Santa Barbara, California, USA) for the extraction of xylem sap. Xylem sap exuded from the cut petiole was immediately pipetted into a 1.5 mL Eppendorf tube and frozen in liquid nitrogen. Upon thawing, sample volumes were measured by pipette and 30 µl of deuterated ABA (Plant Biotechnology Institute, Saskatoon, Canada) was added as an internal standard at a concentration of 100 ng/mL. ABA was separated on a 75 mm x 4.5 mm x 5 µm Phenomenex C18 column (Phenomenex, Torrance, California, USA) on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, California, USA) and detected on an Agilent 6410 QQQLC-MS/MS. A linear 15 min gradient of acetonitrile starting at 10% + 0.05% acetic acid and finishing at 90% + 0.05% acetic acid was used to elute from the column. ABA peaks were identified by retention times and mass/charge ratio and quantified relative to the deuterated standard.

5.6 Molecular analysis

5.6.1 Nucleic acid extractions

Extractions of genomic DNA were carried out using three different methods. For large quantities of DNA for Southern analysis or for a large number of experiments, 1 g of young leaves were frozen and ground under liquid nitrogen and extracted using a nuclear isolation method (Thomas and Scott, 1993). DNA was extracted from small quantities of leaf material using an Isolate II Plant DNA Kit (Bioline). When multiple small samples were required, AGRF (Australian Genome Research Facility, Adelaide) was nominated to perform this service.

For RNA extraction, tissues, snap frozen at the time of sampling, were ground under liquid nitrogen to a fine powder. 60-80 mg of ground tissue was used with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) with some minor modifications to the manufacturer's instructions. PVP-10 (Sigma-Aldrich) (8%w/v) was added to the lysis buffer to assist with the prevention of phenolic compounds and polysaccharides adhering to the nucleic acids, particularly important when recovering RNA from roots. Binding buffer (300 µL) was added to the flowthrough prior to adding it to the binding column. On column DNase digest was performed following the manufacturer's optional instructions (DNase I, Sigma-Aldrich). RNA was eluted with 40 µL elution buffer and quantified by spectrophotometry.

5.6.2 Measurement of gene expression

First strand cDNA was synthesised from 500 ng total RNA using the Transcriptor First Strand cDNA synthesis kit (Roche), utilising Oligo-d(T) primer. The resulting cDNA solutions were then diluted 1:20 in water and stored at -20°C.

For the standard curve, PCR products of each gene using primers listed in the relevant section, were purified using the Qiagen PCR purification kit, quantified by spectrophotometry and diluted to a concentration of 1 ng/µL. This stock solution was used to create a dilution series (10^{-1} to 10^{-8}). Four different standard dilutions were included in each qPCR experiment (usually 10^{-4} to 10^{-7} or 10^{-5} to 10^{-8} depending on the expression levels of the target gene) to establish a standard curve.

Quantitative PCR (qPCR) was performed using three technical reps with a Rotorgene 3000 (Corbett) in a reaction volume of 10 µL containing 5 µL SYBR Green (Roche), 0.5 µM of each primer and 2.5 µL cDNA from each of the biological reps. The PCR cycle profile was as follows: 1 cycle of 5 min at 95°C, 45 cycles of 20 sec at 95°C, 20 sec at 58°C, 20 sec at 72°C, and a final cycle of 5 min at 72°C. Melting curves were performed by elevating the temperature to 95°C for 15 sec, then cooling to 50°C for 45 sec and elevating again to 95°C at a rate of 0.11°C/sec.

5.6.3 Sequence analysis

Sequencing reactions were carried out at AGRF and analysis was preformed using Geneious (Biomatters Ltd) and software and databases available in the public domain including NCBI.

5.6.4 Other molecular techniques

Other techniques such as gene isolation and cloning were carried out using standard methods. Primers specific to a particular gene sequence are referred to in the relevant section.

5.6.5 Southern analysis

Genomic DNA (2µg) was restricted with 10 units EcoRV (Roche) overnight at 37°C. Gel electrophoresis, blotting and hybridisation methods were carried out as described in Walker et al., 2006. Blots were made in duplicate and probed with either p27RB right border fragment, RB (F1 cgctcttttctgttaggtttacc; R1 tggatgcataggttgagtattct) or the Arabidopsis Ubiquitin promoter probe, Ubi term (F gactaaagcctccacattcttcaga; R caagtgggcttataaaatcagtgaa), which allows detection of the left border. The size of EcoRV fragment that hybridises to each probe is unique to the independently transformed line as one site is located in the genomic DNA and will be a different distance from the site of vector depending on the position of integration in the genome. The number of bands on the blot was used as an indication of copy number of the inserted DNA. Plants with one insertion of independent origin (unique pattern) were chosen where possible for further study, together with a small number of lines with multiple inserts.

5.6.6 Short sequence repeat (SSR) markers

SSR marker analysis was carried out at AGRF to determine the true parentage of the K51-40 x 140 Ruggeri progeny (hybrids) and compared to marker scores from known grapevine accessions.

5.7 Plant transformation

Grapevine transformation was performed as described in detail in project report CSP 1306. Essentially, a binary construct containing the gene of interest together with selectable markers, is generated and used to transform Agrobacterium. The Agrobacterium is then mixed with pre-embryogenic Shiraz callus (Figure 4A) and callus is then sub-cultured using kanamycin as a selection agent. Green fluorescent protein (GFP) was used to as a visual marker for initial transformation of callus (Figure 4B) and later to transformed embryos (Figure 4C).

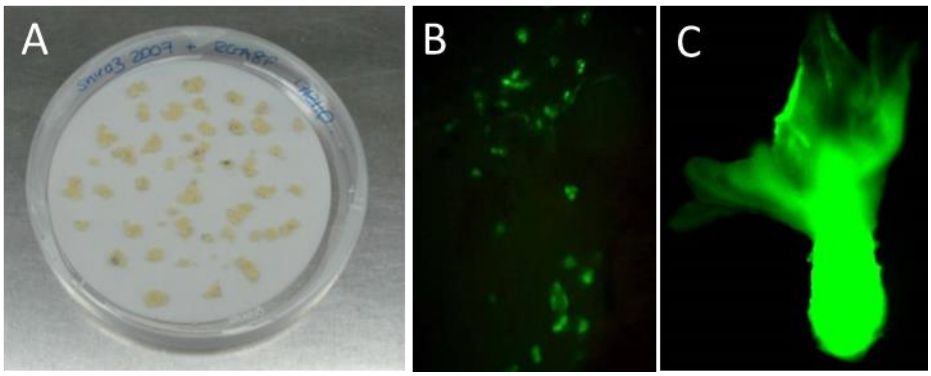


Figure 4. Transformation of Shiraz callus. A) calli mixed with Agrobacterium incubated together for two days. B) GFP spots five days after transformation. C) transformed embryo with GFP fluorescence.

5.8 Other methods

Methods that are specific to a particular section are included in that section.

6 Salinity tolerance in grapevine rootstocks

6.1 The Plant Accelerator as a novel tool to screen rootstock genotypes for salt tolerance traits

6.1.1 Objective

There were two major aims of this experiment: firstly, to test the image-based phenotyping at the Plant Phenomics Centre (the Plant Accelerator) to elucidate salinity tolerance traits in grapevine rootstocks, secondly, to map genetic loci linked with traits to provide genetic markers for selection of the best genotypes for breeding purposes.

6.1.2 Background

Climate change models predict that south-eastern Australia is likely to become hotter and drier with decreased rainfall and increased evaporation. This will affect the major winegrape growing areas such as the Riverland, Riverina and Murrumbidgee Irrigation Area (Phogat et al., 2018). Grapevines can generally withstand high heat if well-watered; however reduced quality and quantity of irrigation water is predicted (Connor et al., 2012) and this could be a major problem for irrigated regions.

Salinity is already a significant problem in a number of viticultural regions of Australia. The build-up of salts in the soil, mostly sodium and chloride, results from the ongoing application of irrigation water containing these ions, which then become concentrated in the soil due to the plant transpiration (vines and cover crops) and water evaporation from the soil surface. A primary management tool to combat the build-up of salts requires an annual heavy irrigation during the rainy winter periods to effectively leach salts from the root zone. With the predicted reduction of availability of good quality water, an increase in the occurrence of soil salinity is expected.

When exposed to saline conditions, grapevines suffer from decreased growth and yield as well as reduced berry quality due to the accumulation of chloride and sodium ions (Downton, 1977; Prior et al., 1992; Li et al., 2013). When present at high concentrations in wine, these ions are associated with an undesirable salty or brackish taste. The use of appropriate rootstocks provides growers with an avenue to combatting the effect of soil salinity. Certain rootstocks display a strong salt tolerance and/or the ability to limit the accumulation of chloride and sodium ions from the above ground parts of the vine. Referred to as chloride exclusion and sodium exclusion, these separate traits are important for minimising the extent of leaf burn symptoms associated with ion accumulation in leaves as well as avoiding berries with high chloride and sodium concentrations.

The CSIRO rootstock breeding program is currently working towards producing new salt tolerant rootstocks to ensure the Australian wine industry is capable of sustaining yields and quality in the face of increasing salinity in the future. Traditionally, rootstock breeding has

been a slow process with a low likelihood of creating a new genotype with superior traits to existing varieties. Many hundreds of new genotypes must be assessed, which requires large and expensive vineyard trials. In the case of screening for salt tolerance, this generally requires the application of both control and salt treatments which is a challenge to achieve in the field. Furthermore, the inconsistencies in soil profiles of vineyards makes assessment of salt tolerance traits difficult, as the salt concentrations in the soil can be highly variably causing misleading results.

To overcome these hurdles that have previously hampered the screening of salt tolerance traits across a large number of rootstock genotypes, we have utilised the Australian Plant Phenomics Facility's Adelaide node, the Plant Accelerator.

6.1.3 Materials and Methods

6.1.3.1 2013 Ungrafted experiment

The lines used in this experiment were K51-40 and 140 Ruggeri, 40 of their hybrid progeny, 18 hybrids derived from K51-40 x Schwarzmann, 10 lines derived from K51-40 x open pollinated and two CSIRO rootstock hybrids, selected for good capacity to exclude salt (Walker et al., 2014). The salt treatment was applied at a final concentration of 100 mM mixed chlorides.

Details of methods of establishment, the Plant Accelerator imaging and harvest of material are given in section 5.2.1 and Figure 5A.

6.1.3.2 2014 Grafted experiment

The aim of this experiment was to determine the extent to which salinity tolerance traits of the rootstock are passed on to a grafted scion, Shiraz. This study included eight different rootstock genotypes (K51-40, 140 Ruggeri, MC215-09, MC215-19, MC215-30, MC215-37, MI07-33 and MI07-36), each grafted with Shiraz as the scion and received a 100 mM mixed Cl^- treatment.

Details of methods of establishment, the Plant Accelerator imaging and harvest of material are given in section 5.2.2 and Figure 5B.

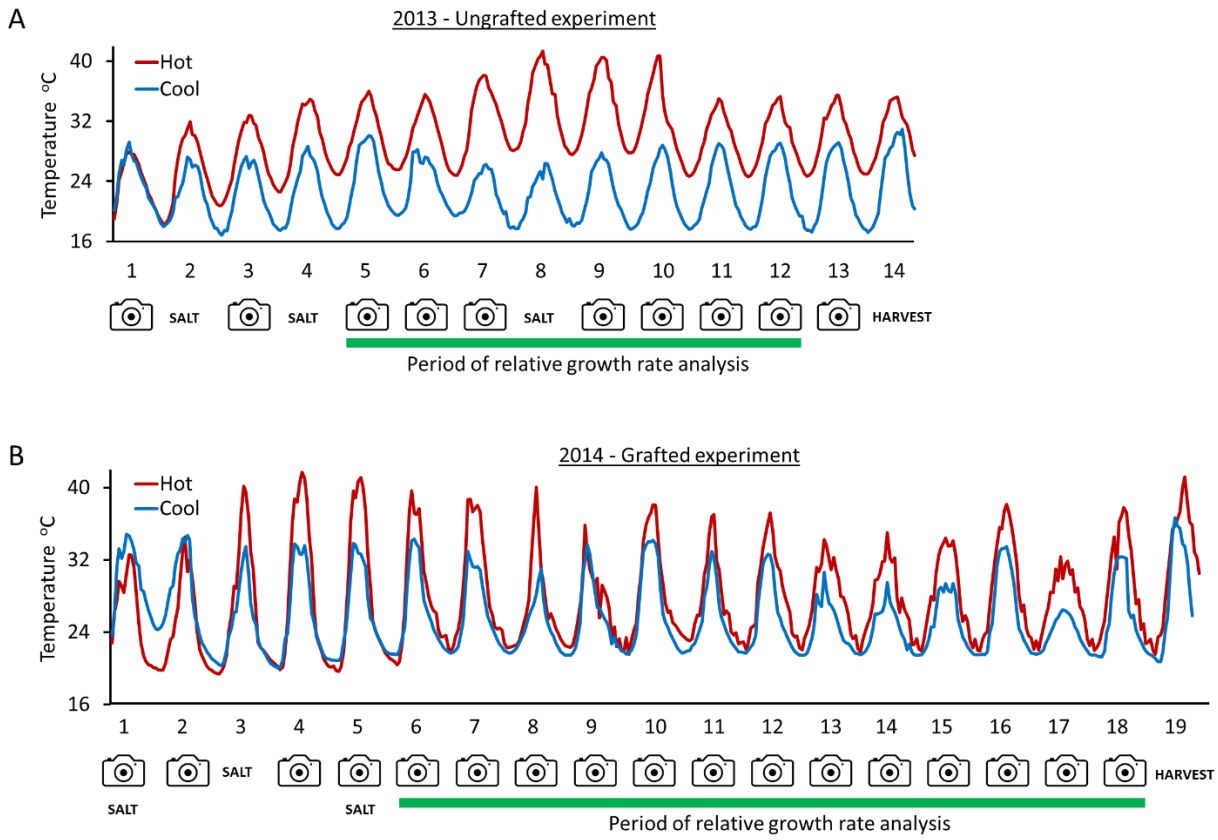


Figure 5. The Plant Accelerator experimental timelines and growing temperatures. A) Experiment 1 2013, K51-40 progeny ungrafted, and B) Experiment 2 2014, grafted K51-40 progeny.

6.1.4 Results

6.1.4.1 Determination of genetic constitution of K51-40 progeny

In this study, a family of interspecific hybrids derived from a cross between the rootstocks K51-40 and 140 Ruggeri was screened for salinity tolerance traits. Following experiment 1, each line of the progeny was genotyped using short sequence repeat (SSR) markers (AGRF). This analysis unexpectedly revealed that a number of the previously reported progeny (Gong et al., 2011; Gong et al., 2014) are in fact derived from either the rootstock Schwarzmann, or other unknown pollen parents. The resulting numbers of hybrids derived from K51-40 x 140 Ruggeri is thus reduced to 40 individuals, with a further 18 derived from K51-40 x Schwarzmann and the remaining 10 hybrids designated as K51-40 x Unknown male parent.

6.1.4.2 Generation of a genetic map for salt exclusion traits

The random segregation of plant traits in breeding populations means that only a small fraction of new genotypes will possess all desirable traits. Use of marker assisted selection can significantly improve the chances of selecting new genotypes that possess all key traits; however genetic loci linked with each trait must first be determined. The aim of this study was to utilise the K51-40 x 140 Ruggeri hybrid progeny to identify genetic markers that are

associated with Cl^- exclusion, Na^+ exclusion and relative growth rate and water use efficiency under salt stress to provide the basis of predictive genetic markers.

Single nucleotide polymorphisms present between the 40 hybrids and two parents were identified by Genotype by Sequencing performed by Diversity Arrays Technology (Canberra, Australia) as part of the 2016 Science and Innovation Award for Young People in Agriculture, Fisheries and Forestry. Detailed methodology of this analysis and the construction of a linkage map for identifying quantitative trait loci associated with traits is described in Henderson, Dunlevy et al., (2018).

6.1.4.3 Cl^- exclusion in rootstock rooted cuttings

A major problem with comparing the Cl^- exclusion of genotypes in the vineyard is that, due to natural variations in soil profiles, it is not always possible to achieve a uniform concentration of salt treatments applied to each vine. To overcome this variation a high number of replicate cuttings per genotype are required which limits the number of genotypes it is feasible to screen in the field. One benefit of the automated watering system of the Plant Accelerator is that it enables a large number of genotypes to receive equivalent salt treatments, and this can be maintained throughout the experiment.

The K51-40 x 140 Ruggeri population (now known to be a mixed population) was initially created with the purpose of studying the genetics of Cl^- exclusion. This population had been previously characterised and found to display a continual distribution in the Cl^- exclusion trait, which is indicative of control by multiple loci (Gong et al., 2011; Final Report project CSP 1002). A major aim of screening this population in the Plant Accelerator, and its highly accurate watering system, was to achieve an as uniform salt treatment as possible to produce accurate data for mapping genomic loci associated with this trait.

The mean Cl^- accumulation for each genotype under salt treatment in the cool and hot Smart Houses, ranked from lowest Cl^- to highest in the cool Smart House was determined. The continual variation present in progeny of both 140 Ruggeri and Schwarzmann is consistent with that reported by Gong et al. (2011) and is indicative of a trait controlled by multiple genes. We attempted to identify quantitative trait loci (QTLs) linked with this trait in the K51-40 x 140 Ruggeri population but were unable to detect any significant associations. This could be due to the small size of the population (n=40) and the complex control of the trait.

Relative to the cool treatment, the hot treatment increased Cl^- in the lamina of nearly all lines including 140 Ruggeri. Four hybrids, MC 215-04, MC 215-23, MC 215-40 and MC 215-71, accumulated lower Cl^- in the hot Smart House. The ability of these lines to maintain the same levels of chloride at the two temperatures could be an important trait for further investigation. The magnitude of the increase in Cl^- accumulation under hot conditions is highly variable amongst these genotypes. The significant ($p<0.001$) interaction of genotype x temperature on this trait may explain why past studies have repeatedly found that the relative Cl^- exclusion ability of different genotypes readily shifts between experiments (Sykes,

1987; Fort et al., 2013; Fort et al., 2015). The reason for the predominantly higher Cl^- accumulation in the heat is unknown. We searched for links between increased Cl^- in response to heat and that of both total water use and relative water use across all genotypes, but could find no meaningful correlations (data not shown). One possibility is that temperature alters the expression of transporter genes involved in the Cl^- exclusion mechanisms. Regardless of the cause, the varying response of genotypes to different temperature treatments highlight the value of using the Plant Accelerator for breeding purposes as it would be too expensive and time consuming to screen the equivalent number of genotypes in vineyard trials under different temperature conditions. The facilities at the Plant Accelerator provide an ideal tool for rapidly assessing the Cl^- exclusion abilities of new rootstock breeding material under multiple temperature conditions and could be included as a screening step in the breeding pipeline to reduce the number of selections required in a field trial. This type of experiment can also inform about the conditions that different rootstocks can withstand and thrive in.

6.1.4.4 Cl^- exclusion trait conferred to a grafted Shiraz scion

We compared the Cl^- accumulation of eight genotypes when screened ungrafted in 2013 and when grafted with a Shiraz scion in 2014. Strong positive correlations were seen between the two experiments for both the cool and hot Smart Houses. This is consistent with the theory that Cl^- exclusion is controlled by the rootstock and confirms that it is valid to screen ungrafted rootstock material for its Cl^- exclusion traits.

6.1.4.5 Association between Cl^- exclusion and ABA

A recent study found that an exogenous ABA treatment (50-100 µmol/L) applied to the roots of potted Shiraz cuttings significantly decreased the accumulation Cl^- in all above ground tissues (leaves, petiole, stem and trunk), but had no significant effect on root Cl^- concentration (Degaris et al., 2017). The physiological mechanism by which the exogenous ABA treatment led to a reduction in Cl^- exclusion is unclear; however the authors found that it could not be accounted for by reduced transpiration.

To investigate this apparent link between ABA and Cl^- exclusion, we measured the ABA concentration of xylem sap collected from all salt treated vines in the 2014 Grafting study. This study included eight different rootstock genotypes (K51-40, 140 Ruggeri, MC 215-09, MC 215-19, MC 215-30, MC 215-37, MI 07-33 and MI 07-36), each grafted with Shiraz as the scion and which received a 100mM mixed cation Cl^- treatment. For each genotype, four replicate cuttings were grown in the Smart House set to cool temperature and four replicates grown in the Smart House set to hot temperature. Immediately following the harvest of lower mature laminae for Cl^- analysis the next most mature leaf was placed in a pressure bomb for the extraction of xylem sap. Exuded xylem sap was immediately pipetted into a 1.5mL Eppendorf tube and frozen in liquid nitrogen. ABA extraction was performed as described in methods in section 5.5.

A significant strong negative correlation was seen between the Cl^- concentration in the laminae and the ABA concentration in the xylem sap in this population when expressed as the means of all eight replicates for each genotype. This negative correlation was still strong when expressed as four replicates under either cool or hot temperatures; however, both correlations were not statistically significant.

The negative correlation observed between laminae Cl^- and xylem ABA is consistent with the findings of Degaris et al., 2017 in which artificially increased soil ABA led to a decrease in Cl^- concentrations in all above ground tissues, including laminae. Our results add weight to their observations, and also suggest that natural variations in the ABA levels of different rootstock genotypes might have an influence on Cl^- exclusion ability. This is a potentially important finding because in most populations, Cl^- exclusion appears to be a complex trait, controlled by more than a single locus. Therefore, previously characterised genes involved in the biosynthesis of ABA could have an indirect effect on Cl^- exclusion ability. It is also possible that ABA positively regulates the expression of the unknown Cl^- transporter genes responsible for the primary mechanism of Cl^- exclusion and this information could be useful in supporting the hypothesised role of identified candidate genes. However, further evidence is needed to establish this link between ABA levels and Cl^- exclusion before these scenarios are thoroughly investigated.

6.1.4.6 Na^+ exclusion in rootstock rooted cuttings

Unlike the continual variation in Cl^- accumulation seen in this population, Na^+ accumulation showed a skewed pattern of variation, particularly in the K51-40 x 140 Ruggeri family. This indicates the trait in this family is highly influenced by a single gene. Indeed, we were able to successfully map a single major QTL, *NaE* (for Na^+ Exclusion) linked with Na^+ exclusion, which explains up to 70% of the genetic variation in the K51-40 x 140 Ruggeri family under cool conditions (detailed in Henderson, Dunlevy et al., 2018, Appendix 5).

As with the case with Cl^- accumulation in this population, there is an obvious interaction between genotype and temperature on Na^+ accumulation. Approximately half of the lines show no difference or a slight decrease in Na^+ accumulation under hot conditions, whereas the remaining half show an increase in Na^+ compared to cool conditions. This interaction had no impact on the influence of the *NaE* locus which explained a similar proportion of the variance in Na^+ accumulation in both the cool and hot Smart Houses.

No correlations were found between Na^+ accumulation and Cl^- accumulation in this population in either the cool or hot Smart Houses. This highlights that Na^+ exclusion and Cl^- exclusion are two individual traits, and therefore under different genetic control.

To investigate whether both Cl^- and Na^+ accumulation were responding to the same heat response signal, we calculated each genotype's heat response for the accumulation of these two ions (i.e. accumulation in the hot divided by accumulation in the cool Smart Houses). No correlation was seen between the Cl^- accumulation heat response vs the Na^+ accumulation

heat response across all genotypes, suggesting that increased heat influences each trait via separate mechanisms.

The fact that there is an interaction of genotype and temperature on both Cl^- and Na^+ exclusion, and that these are not related, further highlights the value of the Plant Accelerator as a tool to rapidly screen for each of these traits in a large number of genotypes.

6.1.4.7 Na^+ exclusion conferred to a grafted scion

The lamina Na^+ accumulation of ungrafted vines in 2013 was compared with the Na^+ accumulation when grafted with Shiraz in 2014. A strong correlation is seen between the accumulation of Na^+ in the cool treatment of each experiment. A weaker correlation is seen between the same comparison in the hot treatment, yet this seems to be largely influenced by a single genotype MI 07-33, which accumulated higher levels of Na^+ when grafted. The poorer relationship between ungrafted and grafted Na^+ accumulation under hot conditions could also simply be the result of the mild heat treatment applied during the 2014 experiment.

6.1.4.8 Growth rates of genotypes captured by digital images

The initial aim was to validate the accuracy of the projected shoot area values calculated from the captured grapevine images obtained from the Plant Accelerator experiments. To achieve this, a subset of 144 vines from 12 lines across all four treatments was destructively harvested and the leaf area of these vines was measured by passing harvested leaves through a leaf area meter. This validation check was performed in both the 2013 ungrafted and 2014 grafted experiments. Strong correlations were seen between the measured leaf area and the projected shoot area in both experiments, indicating that the projected shoot area is a valid measure of vine growth. Strong concentrations were also seen between the projected shoot area and dry weights of leaves, shoots, roots and whole plant dry weights across both years. These correlations were consistently stronger in the experiment in which vines were grafted with Shiraz than in the ungrafted experiment. This is to be expected as the leaf morphology in the ungrafted rootstocks was highly variable which probably included differences in leaf thickness, leaf curvature and the ratio between leaf and stem growth, which would all affect the relationship between projected shoot area and leaf area respectively. When all rootstocks were grafted with Shiraz, this genetic variability of leaf morphology is eliminated thus resulting in a stronger correlation with projected shoot area. There was no apparent effect of heat or salt treatment on the correlations between projected shoot area and biomass measures.

6.1.4.9 Analysis of relative growth rates

In these large experiments there was an unavoidable level of variation in the size of the seven week old rooted cuttings at the beginning of treatments and imaging, which could be related to the size of the rooted cutting and the levels of carbohydrate levels in the dormant cutting as well as genetic influences on early growth. The total growth of vines at the end of the experiment was largely driven by the initial size of each vine, as a strong correlation was evident between the initial and final projected shoot areas. Thus, final projected shoot area is not a suitable measure for comparing growth rates across genotypes. Instead, we calculated the relative growth rate of each vine to give a more valid measure for comparing the growth of different genotypes.

Relative growth rate of each vine was calculated by taking the slope of the natural log of the projected shoot area values between days 5 to 12. The relative growth rate essentially describes the average increase in plant size on a daily basis expressed as a percentage.

A single vine, MI 07-33, in the heat salt treatment died during the experiment and was therefore excluded from the analysis. A general analysis of variance was performed using the relative growth rate of individual vines to determine the effect of each treatment and treatment interactions. The interaction effect of each treatment on the relative growth rates and mean relative growth rates were calculated for each genotype x treatment combination.

Genotype had a highly significant effect on relative growth rate, as expected. Under cool and control conditions an approximately three-fold difference was seen between the minimum and maximum mean relative growth rates. The overall effect of temperature was not significant with overall mean growth rates in the hot. Salt treatment had a highly significant effect on relative growth rate with overall means of under control treatment and when exposed to a salt treatment.

There was a significant interaction between genotype and temperature. This result highlights that the ranking of genotypes based on their relative growth rate is highly variable depending on temperature. It also demonstrates the value of being able to screen large numbers of rootstocks under different temperatures as different grapevine lines grow differently in hot and cool conditions.

Although there was not a significant interaction of genotype and salt treatments across the population, a number of lines showed increased relative growth rate under salt treatment in one of the Smart Houses. A single genotype, MC 215-64, showed a higher relative growth rate when exposed to a salt treatment than control treatment in both the cool and hot Smart Houses. Thus MC 215-64 appears to be able to maintain its relative growth rate under saline conditions, effectively displaying high salt tolerance. A number of other genotypes, such as MC 215-01 in the cool Smart House and MC 215-39 in the hot Smart House, have higher relative growth rates under salt treatment than MC 215-64, and therefore may actually be

better suited to growth under saline conditions in the field, despite the effect of salt treatment being greater in these genotypes.

No significant correlations were seen between the relative growth rates of genotypes and their lamina accumulation of either Cl^-, Na^+ or the sum of both Cl^- and Na^+ when exposed to salt treatment. The ratio of relative growth rate between salt treated and control vines was also compared against Cl^- and Na^+ accumulation yet no meaningful correlations were seen. This suggests that across this population there is genetic variation for other unknown salt tolerance mechanisms, such as osmotic or tissue tolerance, which ultimately contribute to differences in relative growth rates under salt stress.

6.1.4.10 Comparison of growth rate traits of ungrafted and Shiraz-grafted rooted cuttings

The correlation in growth rates between the eight rootstock genotypes when ungrafted or grafted with a Shiraz scion varied depending on treatments. In both the cool control and hot salt treatments there were positive correlations. However, for cool salt and hot control treated vines only weak correlations were evident. The degree to which relative growth rates of ungrafted vines are conferred to a grafted vine is much less than that of either Cl^- or Na^+ exclusion, which are predominantly root based traits. This suggests that in the ungrafted vines screened in 2013, the genetics and physiology of the leaves and shoots contributes to the differences observed in relative growth rates and once grafted with a common scion some of these differences are lost. This implies that rootstock genotypes should be grafted before comparing their relative growth rates.

6.1.4.11 Relative water use in experiment 1

To compare water use across genotypes that differ in their size, relative water use was calculated as the grams of water used per 100,000 pixels of PSA per day. Genotype had a highly significant effect on relative water use, however the magnitude of differences was small with less than a two-fold difference between the lowest and highest values in both the cool and hot Smart Houses. Salt treatment also had a highly significant effect in which the relative water use was reduced by salt treatment which is consistent with reports in the literature. There was no evidence of an interaction between genotype and salt treatment. Temperature also had a highly significant effect on relative water use, with vines in the hot Smart House using more water per leaf area than the cool Smart House as would be expected. There was a highly significant interaction of genotype and temperature.

6.1.4.12 Comparison of water use in Shiraz-grafted and ungrafted plants

The relative water use of these eight genotypes was compared between the ungrafted and grafted experiments. Under both cool control and hot salt treatments there were weak correlations, however neither of these were significant. No correlations were seen in either the cool salt or hot control treatments. Furthermore, the difference in relative water use between genotypes was less when grafted than when ungrafted which suggests that the scion

could play a large role the relative water use trait. This result would need to be verified in an experiment in which both grafted and ungrafted vines were grown together.

6.1.5 Discussion

Studies in other plants have shown that when exposed to a salt stress, plants suffer from reduced growth which occurs in two distinguishable phases (Munns and Tester, 2008). The first is a rapid reduction in growth rate which has been attributed to the osmotic stress imposed by salts (Na^+ and Cl^-) on the outside of the plant which effectively forces the plant to expend more energy to extract water from the soil (Munns et al., 2006). The second phase of growth reduction results from the ionic toxicity of elevated Na^+ and Cl^- concentrations that gradually accumulate within the plants tissues. The onset of the second phase in growth reductions is slower and can therefore be distinguished from the initial osmotic stress (Munns et al., 2006). The timing and severity of the ionic phase is dependent on many factors including the plant's ability to exclude Na^+ and Cl^- from accumulating in photosynthetic tissues, as well as the ability to tolerate these ions in those tissues. The complex trait of salt tolerance can thus be broken down into three sub-traits, osmotic (or non-ionic) tolerance, ion exclusion and tissue tolerance (Gilliham et al., 2017). The degree to which each of these tolerance mechanisms contributes to overall salinity tolerance is not easy to assess. However recently the Plant Accelerator has been shown to be a valuable tool for dissecting salt tolerance sub-traits in numerous plant species including wheat, rice, chickpea and *Arabidopsis* (Rajendran et al., 2009; Hairmansis et al., 2014; Awlia et al., 2016), by accurately quantifying the initial non-ionic phase of salt stress via digital imaging, combined with the measurement of ion accumulation upon harvest.

In grapevines, the accumulation of high concentrations of Cl^-, which can manifest as visual leaf burn symptoms, is regarded as the main contributor to decreased growth and yield associated with salt stress (Woodham, 1956). This, combined with its simplicity of measure, has led to Cl^- exclusion being the most relied upon trait for scoring and selecting rootstocks for salt tolerance. Yet a recent study found that in a Cl^- excluding rootstock, yield reductions due to salt treatment were associated with accumulation of Na^+ in the leaves as opposed to Cl^- (Stevens et al., 2011). It thus follows that in genotypes capable of excluding both Cl^- and Na^+, the differences in the non-ionic tolerance sub-trait would have an increased influence on salt tolerance. Indeed, in numerous short term pot experiments where ion accumulation has not yet reached a toxic level, mechanisms other than ion exclusion appear to be responsible for the differences in salt tolerance between genotypes. One study comparing the response of two rootstock genotypes, M4 and 101-14, to a 21 day salt treatment found that M4 accumulated approximately twice the amount of Na^+ and Cl^- in the leaves compared with 101-14, yet it exhibited a greater overall tolerance to salt stress by maintaining higher photosynthetic activity (Meggio et al., 2014). In another experiment Sultana vines exposed to various levels of salinity for 60 days exhibited higher rates of photosynthesis and increased dry matter when on own-roots than when grafted onto either of five rootstocks, despite own

rooted vines having the highest content of both Cl^- and Na^+ in the leaves (Fisarakis et al., 2001). Furthermore, when the salts were flushed from the root zone over a 10 day period, photosynthetic rates rapidly returned to control levels despite the Cl^- and Na^+ levels in the leaves increasing in concentration throughout this period (Fisarakis et al., 2001), suggesting that factors other than Cl^- and Na^+ in the leaves were affecting photosynthesis.

In our experiment we found no correlation between reductions in relative growth rates and the accumulation of either Cl^- or Na^+ in the laminae after a short 13 day salt treatment. The threshold at which Cl^- ions become toxic and impact photosynthetic activity has been estimated to be 1.5% dry weight in Sultana (Walker et al., 1981). In our experiment only five genotypes accumulated Cl^- above this threshold, therefore it fits that tolerance mechanisms other than ion exclusion have an impact on relative growth rates under salt treatment. It is also possible that these ungrafted genotypes differ in their relative tissue tolerance abilities which could effectively mask the influence of Cl^- accumulation on growth rates. However, when grafted with a Shiraz scion, there was still a significant effect of genotype on the relative growth rates under salt treatment and these were not correlated with Cl^- accumulation, suggesting mechanisms other than ion exclusion or tissue tolerance are responsible for these growth rate differences.

Before employing the Plant Accelerator as a tool for screening for non-ionic tolerance mechanisms, it first must be confirmed that relative growth rates measured in the Plant Accelerator are indicative of vine performance in the field. However, no such field trial exists for the K51-40 derived hybrids screened in this experiment. In the following Section 6.2 we performed a similar experiment as described here, utilising nine genotypes that were previously included in a long term salt treated vineyard experiment, to gauge how well growth rate data in the Plant Accelerator is correlated with the ability to withstand salt stress in the vineyard.

6.1.6 Summary

The screening of rootstock genotypes in the Plant Accelerator has proved very successful particularly in the assistance of mapping a Na^+ exclusion locus, which led to a major publication and an excellent marker for ensuring Na^+ exclusion traits are maintained in our rootstock breeding program. Strong interactions of temperature on both Na^+ and Cl^- accumulation were seen in these experiments, which highlights the value of being able to simultaneously screen material for these traits under different temperatures. Furthermore, the ability to rapidly assess genotypes for their potential non-ionic tolerance should assist in the selection of future rootstocks with enhanced overall salt tolerance.

6.2 Heat x Salt screen of diverse genotypes in the Plant Accelerator

6.2.1 Objectives

A third major Plant Accelerator experiment was performed in 2015, which involved a heat x salt factorial treatment of 49 *Vitis* genotypes and species of diverse genetic backgrounds. There were multiple objectives to this study: to determine the variability of growth rate, water use and Cl^- and Na^+ exclusion traits in a diverse population together with well-known *V. vinifera* wine grapes, and to compare this to the variation in the closely related K51-40 x 140 Ruggeri F1 sibling population studied in the first Plant Accelerator experiment. Another objective was to identify genotypes that display extreme phenotypes for any of these four traits, which could then provide the basis for further analysis of underlying genetic and/or physiological mechanisms. It was also possible that this data set could be used to identify loci linked with these traits via an association mapping approach. A number of genotypes were also chosen as they are represented in a long term salt-treated field study, providing another data set to ascertain if growth rate traits in the Plant Accelerator reflect growth measures in the field.

6.2.2 Background

The first heat x salt experiment conducted using progeny of crosses from K51-40 X 140 Ruggeri and K51-40 X Schwarzmann, showed promise for using the Plant Accelerator as a rapid tool for screening for growth and water use traits when exposed to a salt stress under different heat treatments. However, these genotypes were not planted in replicated field trials and hence were not available to validate the reliability of the data obtained in the Plant Accelerator with that obtained in the field. In this current experiment, nine genotypes were chosen that had also been part of a long term salinity trial at the former CSIRO site at Merbein, Victoria, where rootstocks were grafted with both Shiraz and Chardonnay scions.

In the first heat x salt experiment we identified a major locus responsible for Na^+ exclusion in the K51-40 x 140 Ruggeri family. Genetic markers at this locus could be used in marker assisted selection of new material to select for predicted Na^+ excluders, but the utility of these markers in a broad range of genomic backgrounds needs to be tested. Screening a diverse collection of *Vitis* genotypes will provide suitable phenotypic data on Na^+ exclusion to ascertain how well these genetic markers predict Na^+ exclusion ability in genotypes derived from sources other than K51-40 and 140 Ruggeri.

6.2.3 Materials and Methods

Collection, callusing and establishment of dormant cutting material was as described in section 5.2.1. A factorial combination of cool and hot temperature, and control and salt treatments was applied. Temperature traces for the cool and hot Smart Houses are shown in Figure 6. For salt treated vines, a concentrated solution of mixed Cl^- (Na^+:Ca^{2+}:Mg^{2+} in a ratio of 6:1:1) was applied on days 4, 6 and 11 equating to a final pot concentration of 100 mM

mixed Cl^- from day 11 onwards. On each other day, vine growth was captured by three digital images, which were used to calculate a projected shoot area, as described in section 5.2.1. On day 27, the fully expanded mature laminae of each vine were harvested for Cl^- and Na^+ analysis as described in sections 5.4.1 and 5.4.2 respectively.

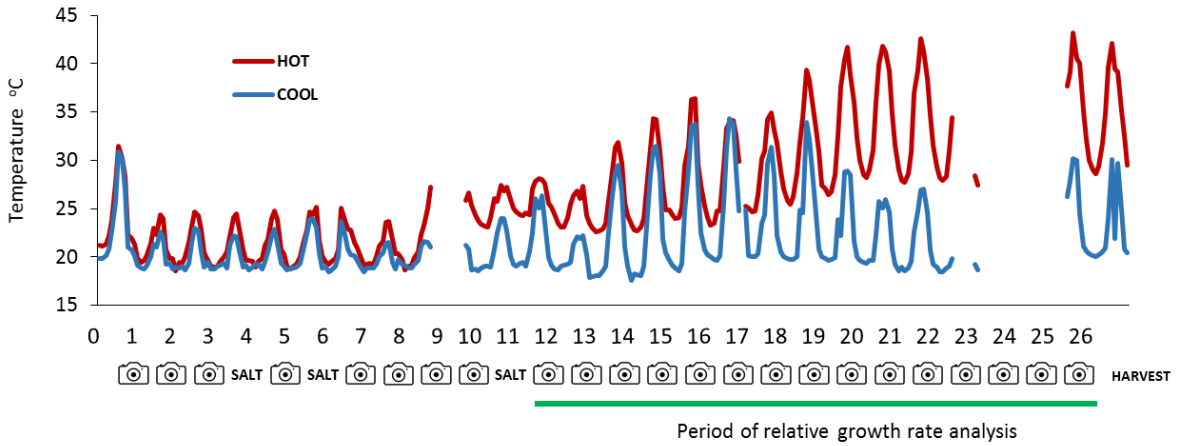


Figure 6. Recorded temperatures in the cool and hot Smart Houses throughout the experiment. Some temperature data are missing due a failure of digital thermometers. SALT signifies the days on which salt treated plants received one of three 33 mM mixed Cl^- applications. On all other days, vines were imaged as indicated by the camera icon. The green bar indicates the period over which relative growth rates and water use efficiency were calculated.

6.2.4 Results

During the establishment phase and throughout this experiment the two closely related *V. vinifera* cultivars, Merlot and Malbec, did not grow properly and they were not included in the analysis.

6.2.4.1 Relative growth rates

Relative growth rates were calculated for each individual vine over the period of day 12 to 26. Analysis of variance revealed that the three treatments of genotype, temperature and salt all had highly significant effects on relative growth rates. This is slightly different from 2013 where the effect of temperature was not significant. In this diverse collection of genotypes there were no significant interactions of any treatments.

Mean genotypic relative growth rates were calculated from the three replicates of each genotype by treatment combination. In the cool control treatments Ramsey, which is classed as a high vigour rootstock, had the second highest relative growth rate. A CSIRO rootstock selection had the highest relative growth rates in the control salt treatment as well as in the hot control and hot salt treatments. Of the *V. vinifera* cultivars, Pinot Noir and the table grape Cardinal consistently had the highest relative growth rates under all treatments.

6.2.4.2 Correlation between growth of cuttings and that of field grown vines.

A long term salinity field trial at Merbein, Victoria was used to search for correlations between field pruning weights and relative growth rates obtained in the Plant Accelerator. Genotypes that overlapped in the two studies include K51-40, 140 Ruggeri, Ramsey, Schwarzmann, SO4, *V. rupestris* St George, Shiraz and Chardonnay. Details of the field trial are described in the final report of project CSH8GW, and in Walker et al., (2010a) and Walker et al., (2014). In short, six replicates of each genotype were grafted with a Shiraz scion and six replicates grafted with a Chardonnay scion. In 1992 vines were planted in a randomised block design across four rows, two rows per scion. From 1996 to 2007 a salt treatment was applied to all vines through the addition of a concentrated salt solution into irrigation lines via a fertiliser injector pump. The mean electrical conductivity of irrigation water throughout this period was 2.1 dS/m equating to approximately 22 mM NaCl. Pruning weight data were collected in the winters of 1996, 1997, 2000 and 2007.

Correlations were sought between relative growth rates in the Plant Accelerator and the average pruning weights of these genotypes when grafted with Shiraz and Chardonnay in the field. Significant correlations were seen between average field pruning weights and relative growth rates of hot salt treated vines of the Plant Accelerator, but such correlations were not evident from vines in the hot control treatment or either of the cool treatments of the Plant Accelerator. Since Merbein has a relatively hot climate, and vines in the field trial were maintained under sustained salt treatment, it is logical that pruning weights from this trial would correlate best with the hot and salt treated vines in the Plant Accelerator. It is also interesting to note than in the ungrafted vs grafted comparison of K51-40, 140 Ruggeri and six hybrids in the Plant Accelerator, the strongest correlations were observed under Hot and Salt treatments.

When comparing correlations between relative growth rates and the averaged Shiraz and Chardonnay scion pruning weights in the field, the strength of the correlation improves throughout the seasons of the field trial. From samples taken in 1996 and 1997 the correlation is not significant, but in 2000 the correlation is significant. The strongest correlation is seen in 2007, 15 years after planting. The reason for the improvement of this correlation with time appears to be due to a single genotype K51-40 which had high vigour in 1996 and 1997. Yet in 2000 and 2007 the K51-40 vines had low pruning weights, which is in line with the relative growth rates of the Plant Accelerator, thus improving the correlation. Thus, it seems likely that K51-40 could sustain growth in the initial years following the onset of salt treatment in the field, but given its poor Cl^- exclusion ability succumbed to salt stress and reduced vigour after a number of years.

When K51-40 is removed from the analysis the correlation on the remaining eight genotypes becomes highly significant over all four years.

When the pruning weights of Shiraz and Chardonnay grafted vines are analysed separately, and in the absence of K51-40, significant correlations are present in only the first two seasons

of 1996 and 1997. While in the 2000 and 2007 seasons these correlations were relatively strong, they were below the level of significance.

Fruit yield was also measured in each of the seasons of the Merbein field trial. Significant correlations were seen between the relative growth rates of ungrafted genotypes and the fruit yield of both Shiraz and Chardonnay grafted to them in the 2007 season. Weaker correlations that were not statistically significant were evident in the 1996, 1997 and 2000 seasons.

6.2.4.3 Relative water use

The relative water use of each vine was calculated as the average daily water use in grams per 100,000 pixels of PSA as described in 6.1.4.11. A general analysis of variance on individual vines revealed a highly significant effect of all treatments, which is consistent with the 2013 experiment. Significant interactions between Genotype x Temperature were also present in both experiments, while a significant interaction by Genotype x Salt was seen in this experiment which was not present in experiment 1. This could be due to the genetic backgrounds of genotypes in this experiment being more diverse than that of the K51-40 derived hybrids in experiment 1.

Although genotype had a significant effect on relative water use, less than a two-fold difference was seen between the minimum and maximum values in the cool Smart House and three-fold difference in the hot Smart House. As expected, high temperature resulted in greater relative water use. Salt treatment resulted in reduced relative water use in both Smart Houses.

6.2.4.4 Cl^- exclusion

The concentration of Cl^- accumulated in the mature laminae of each vine at harvest was used as a measure of their ability to exclude Cl^-. As in the 2013 experiment, all treatments and interactions were highly significant with the exception of the Genotype x Temperature x Salt treatment which was not significant.

The mean of laminae Cl^- accumulation for each genotype and treatment combination was measured. *V. rupestris* St George and 140 Ruggeri were very strong excluders under cool conditions but accumulated significantly more under hot conditions. Surprisingly, Cabernet Sauvignon accumulated less Cl^- under hot conditions than did 140 Ruggeri, one of the best known excluders. At the other end of the spectrum the genotype 3309 Couderc accumulated double the amount of Cl^- than the accumulator, K51-40, under cool conditions. In the hot conditions, K51-40 accumulated Cl^- at levels equivalent to 3309 Couderc which were not significantly greater than what it accumulated in the cool conditions.

With the exception of *V. longii*, every other genotype accumulated higher levels of Cl^- in the hot conditions than in the cold conditions. For some of these genotypes, in particular *V. rupestris* St George, four CSIRO rootstock hybrids and K51-40, this represented a greater than

two-fold increase in Cl^- accumulation between cool and hot conditions. Interestingly, none of the *V. vinifera* cultivars displayed the same magnitude of increase in Cl^- accumulation between the two temperatures. Thus *V. vinifera* cultivars may lack the heat response mechanism leading to greatly increased Cl^- accumulation shown by the other species and this difference could be utilised to create a mapping population to identify loci linked with this heat response trait.

As in the 2013 experiment, no evidence could be found that suggests that the magnitude of increased Cl^- uptake under hot conditions is related to increased water use of individual genotypes.

6.2.4.5 Na^+ exclusion

The concentration of Na^+ in the mature laminae of each genotype at harvest was used as a measure of their relative Na^+ exclusion abilities. For this experiment Na^+ ion analysis was only conducted on vines growing in the cool Smart House. A general analysis of variance shows that both genotype and salt treatment have a highly significant effect on Na^+ concentration as does the interaction between the two, consistent with the 2013 experiment.

6.2.4.6 Association mapping of Cl^- and Na^+ exclusion traits

The ion exclusion data generated as part of this experiment were used in an attempt to identify associated genomic loci through an association mapping approach. This was conducted as part of the 2016 Science Award for Young People in Agriculture, Forestry and Fisheries project conducted by Jake Dunlevy, Appendix 4.

6.2.5 Summary

The dataset produced by this screen of the 49 diverse genotypes allowed the identification of genotypes with strong Cl^- exclusion, such as *V. rupestris* St George and *V. longii*, and one with extremely poor Cl^- exclusion, 3309 Couderc, which may assist in the understanding of this complex trait. The interaction of genotype x heat on Cl^- exclusion seen in the K51-40 x 140 Ruggeri family was also evident in this diverse collection of *Vitis* genotypes. This further highlights the value of the Plant Accelerator as a tool to compare large numbers of genotypes for their ability to exclude Cl^- under multiple temperature treatments. Furthermore, the relative growth rate of young salt treated cuttings was significantly correlated with the pruning weight of mature field grown vines that had been exposed to a prolonged salt stress. This initial observation lends some validation to the use of the Plant Accelerator as a tool to initially screen new rootstocks for their ability to tolerate salinity in a more efficient and timely manner than traditional long term field trials.

6.3 Rootstock salt exclusion at elevated root zone temperature

6.3.1 Objectives

The aim was to compare the effect of two root zone temperature treatments (high and cool) under a common glasshouse air temperature on growth, stomatal conductance and Cl^- accumulation by control and salt-treated Shiraz on 140 Ruggeri (good Cl^- excluder), K51-40 (poor Cl^- excluder) and selected 140 Ruggeri x K51-40 hybrid rootstocks.

6.3.2 Background

Rootstocks with good capacity for salt exclusion are important for salt tolerance of grapevines. In addition to reducing the impacts of salinity on growth and development, they minimise any potential effects of salt accumulation in grape berries and wine.

140 Ruggeri is an effective excluder of Cl^- relative to other rootstocks in both short term (Walker et al., 2010a) and longer term (Walker et al. 2014) field trials. It is also an effective excluder of Cl^- relative to other rootstocks when grown as propagated cuttings or rooted leaves in either potting mix or hydroponics (Tregeagle et al., 2010; Gong et al., 2011).

K51-40, on the other hand is a poor excluder of Cl^-. When moderate levels of salt are present in irrigation water under field conditions, scions grafted to K51-40 may accumulate high concentrations of Cl^- in laminae, potentially leading to 'leaf burn' and exhibit reduced growth measured by pruning wood weight and reduced yield (Tregeagle et al., 2006; Walker et al., 2010a; Walker et al., 2010b).

The impact of high root zone temperatures on salt exclusion by grapevine rootstocks has not previously been addressed. To address this question, Cl^- accumulation by Shiraz grafted to 140 Ruggeri, K51-40, and a subset of hybrid siblings from a cross between the two rootstocks, selected for their range of capacity for Cl^- exclusion, were compared in the glasshouse during early autumn 2015 at two different root zone temperatures and a common air temperature.

To confirm the results, a second experiment was conducted in early autumn 2016, under conditions as close as possible to those of the first experiment, again comparing Cl^- accumulation of Shiraz grafted to 140 Ruggeri, K51-40 and a smaller number of hybrid sibling rootstocks at the different root zone temperatures.

6.3.3 Materials and Methods

Plant establishment: Grafted rooted cuttings of Shiraz on 140 Ruggeri, K51-40 and hybrid sibling rootstocks were initially established in a 50% sand and perlite mix in a mist house and irrigated with rainwater. After four weeks, the plants were transferred to plastic tubes (vine tubes) containing expanded clay balls. The vines were left in the mist house for another week to harden off before being transferred to a specifically designed recyclable hydroponic system

in 160 litre tubs. The plants were irrigated for six days with nutrient solution only (1/4 strength special Hoaglands).

Salt treatment applications: On completion of the six day treatment with nutrient solution only, a salt solution was added to two tubs to achieve a concentration of 25 mM mixed Cl^- (Na^+:Ca^{2+}:Mg^{2+} = 6:1:1). After three days, extra salt was added to increase the salt concentration to 50 mM mixed Cl^-. Two control tubs contained 1/4 strength special Hoaglands in rainwater. The solutions were changed weekly. The plants were maintained as single leader shoots.

Root temperature: The hydroponics were set up with two root zone temperature treatments, a cooler treatment where the 24 h average temperature was 18.7°C, and a warmer treatment, where the 24 h average temperature was 30.3°C. Hence there were four tubs designated; control cool, salt cool, control hot, and salt hot. The aerial environment of all plants was at the glasshouse 24 h average temperature of 21.0°C. The set-up is shown in Figure 7.



Figure 7. Photograph of the experimental set-up. Chillers are on left side of the photo. Each tub contained a submersible pump to deliver solution to the top of the vine tubes which ensured mixing of the solution. Ambient heat from the pumps provided the higher temperature in two hot tubs, while the cool tubs were chilled by the chillers, adjusted to achieve a desired solution outflow temperature, to achieve the target root zone temperature.

Plant harvest and sampling: After 28 days, the plants were harvested and separated into shoot (laminae, petioles, stem), cane and roots (separately for roots in aeroponics and those in hydroponics). The separated organs from each plant were washed, blotted dry with paper towel, weighed, then dried at 60 °C for at least 72 h. Dry weights were obtained and the dried material was ground to a powder in a hammer mill. Chloride ion analyses of the dried processed material was carried out as in section 5.4.

Stomatal conductance: Stomatal conductance measurements were taken on days 12 and 23 of the experiment using an AP4 porometer (Delta-T Devices, Cambridge, UK). For each vine, two separate fully expanded, sun-exposed leaves were used for measurement (a single measure on each leaf on each day) and the average used for analysis.

6.3.4 Results

6.3.4.1 Laminae and total plant dry mass

There were rootstock differences in the laminae and total dry mass response of control plants to the temperature treatments but not for the response of the salt-treated plants. Three rootstock genotypes, 140 Ruggeri, MC 215-19, MI 07-36 and MC 215-30 resulted in higher laminae dry mass in the control at the hot treatment relative to the cool treatment. Two rootstock genotypes, 140 Ruggeri and MI 07-36, resulted in higher total dry mass in the control at the hot treatment relative to the cool treatment.

6.3.4.2 Organ Cl^- concentrations

First experiment (2015): The hot root zone treatment resulted in lower concentrations of Cl^- in Shiraz laminae and petioles of salt-treated 140 Ruggeri relative to that of the cool root zone treatment. The opposite was observed for salt-treated Shiraz on K51-40, where the hot root zone treatment resulted in higher concentrations of Cl^- in laminae and petioles relative to that of the cool root zone treatment. Shiraz on salt-treated hybrids MC 215-37, MC 215-09, MC 215-19, MI 07-36 and MC 215-30 behaved similarly to Shiraz on 140 Ruggeri, showing lower concentrations of Cl^- in laminae and petioles at the hot root zone treatment, while Shiraz on hybrid genotype MI 07-33 (a poorer Cl^- excluder at the cool root zone treatment) behaved similarly to salt-treated Shiraz on K51-40 with higher concentrations of Cl^- in laminae and petioles at the hot root zone treatment.

Root zone temperature had no effect on Cl^- concentrations in roots or in the cane (cutting) from which the plants were propagated.

These data show that when subjected to salt treatment, 140 Ruggeri resulted in significantly enhanced Cl^- exclusion capacity at the higher root zone temperature, while K51-40 resulted in significantly reduced Cl^- exclusion capacity. In order to confirm this response, a second experiment was conducted.

Second experiment (2016): As in the first experiment, the hot root zone treatment resulted in lower concentrations of Cl^- in laminae and petioles of Shiraz on salt-treated 140 Ruggeri relative to that of the cool root zone treatment. Similarly, the opposite was observed for Shiraz on salt-treated K51-40, where the hot root zone treatment resulted in higher concentrations of Cl^- in laminae and petioles relative to that of the cool root zone treatment.

As in the first experiment, Shiraz on salt-treated hybrids MC 215-09 and MI 07-36 behaved similarly to Shiraz on 140 Ruggeri, showing lower concentrations of Cl^- in laminae and petioles at the hot root zone treatment, while Shiraz on salt-treated hybrid genotype MI 07-33 again exhibited a similar response to Shiraz on K51-40 with higher concentrations of Cl^- in laminae and petioles at the hot root zone treatment.

6.3.4.3 Stomatal conductance

Stomatal conductance for laminae of Shiraz on 140 Ruggeri, K51-40 and six hybrid rootstocks for control and salt-treated plants at hot and cool root zone temperature treatments were measured at two time points, the first on 31 March 2015 (day 12 of the experiment) and the second on 10 April 2015 (day 23 of the experiment).

At the initial set of measurements (day 12), stomatal conductance for Shiraz laminae on salt-treated 140 Ruggeri, K51-40 and all six hybrid rootstocks was higher for the hot root zone treatment than for the cool root zone treatment. For the controls, there was no difference between the hot and cool treatments for Shiraz on 140 Ruggeri, MC 215-09, MC 215-19, MC 215-30, MI 07-33 and MI 07-36. For the Shiraz grafted on K51-40 in the control, stomatal conductance for laminae of the hot root zone treatment was higher than that of the cool root zone treatment, whereas for the Shiraz on MC 215-37 control, the reverse occurred, with a lower stomatal conductance in the hot root zone treatment.

At the second set of measurements (day 23), stomatal conductance for Shiraz laminae of both control and salt-treated K51-40 and all six hybrid rootstocks was similar between the hot and cool root zone treatments. For control plants of Shiraz on 140 Ruggeri, stomatal conductance was similar for the hot and cool treatments, but for salt-treated Shiraz on 140 Ruggeri plants, it was slightly higher for the hot root zone treatment relative to the cool root zone treatment.

6.3.5 Discussion

The hot irrigated regions of Riverland, Sunraysia and Murrumbidgee Irrigation Area produce around 60% of Australia's wine grapes. They and most other wine grape regions in Australia are subject to periodic heatwaves that can have a warming effect on soils, with the effect most pronounced in the hotter regions.

Soil temperature at 20-40 cm depth is variable depending on season and prevailing weather conditions. Changes in soil temperature in the top 40 cm are highly correlated with changes in air temperature but lag behind changes in air temperature by around two days (R. R. Walker and D.H. Blackmore, unpublished data).

The observation that Cl^- exclusion capacity of 140 Ruggeri was enhanced at elevated root zone temperature while that of K51-40 was reduced, has significant implications for rootstock choice, particularly for warm to hot regions where irrigation water may be saline and where there is a relatively high likelihood of heatwaves during summer.

Reasons for the contrasting responses by the two rootstocks are not clear but would appear to be linked to the underlying mechanism for Cl^- exclusion that is present in 140 Ruggeri but not in K51-40. When measured earlier during the treatment period, grafted plants of salt-treated 140 Ruggeri, K51-40 and all six hybrid rootstocks had higher stomatal conductance at the hot root zone treatment than at the cool root zone treatment; however with the exception of Shiraz on 140 Ruggeri, where higher stomatal conductance was recorded at the

hot relative to the cool root zone treatment, there was no difference in stomatal conductance between the root zone temperature treatments when measured later in the treatment period.

Laminae and total dry mass were similar between hot and cool treatments for salt-treated Shiraz on 140 Ruggeri and K51-40. In instances of higher stomatal conductance, which normally involves higher water flux through the plant, one might anticipate that without strong regulation of ion transport at root level, a higher flux of ions, e.g. of Cl^-, may occur. This may have been the case in grafted, salt-treated K51-40 plants, at least during the initial part of the treatment period. However, the opposite occurred in grafted, salt-treated 140 Ruggeri plants at the hot root zone temperature treatment, indicating regulation of ion transport at root level, which has previously been hypothesised and thought to involve membrane-located ion transporters (Tregeagle et al., 2010; Gong et al., 2011; Wege et al., 2017). These results suggest that the Cl^- exclusion mechanism in 140 Ruggeri operates even better at higher root temperatures.

6.3.6 Summary

When root zone temperature was increased above standard glasshouse air temperature, the impact was different between rootstocks. A rootstock with poor capacity for salt exclusion, i.e. K51-40, showed even poorer capacity for salt exclusion at high root zone temperature, while a rootstock with good capacity for salt exclusion, i.e. 140 Ruggeri, showed better capacity for salt exclusion.

The results demonstrate the importance of choosing rootstocks with good salt exclusion capacity for regions with elevated irrigation water and soil salinity, particularly given future climate scenarios. Persisting with rootstocks with poor salt exclusion capacity could increase the potential for elevated leaf and shoot Cl^- concentrations and by implication elevated grape juice and wine Cl^- concentrations with the associated risk of salty taste in wines.

6.4 Functional differences in transport properties of natural HKT1;1 variants influence shoot Na^+ exclusion in grapevine rootstocks

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This paper details a large body of work regarding the identification of HKT1;1 from grapevine rootstocks with a role in sodium exclusion in the root and was the result of collaboration between CSIRO and University of Adelaide and conducted as part of this project.

A copy is included as Appendix 5 with permission from New Phytologist for reporting purposes.

6.5 Whole plant ion analysis using K51-40, 140 Ruggeri and selected hybrids

6.5.1 Objective

To determine the biomass and concentrations of chloride and sodium in the main organs and also the whole plant of one year old potted vines of K51-40, 140 Ruggeri and selected hybrids when challenged with mixed chlorides.

6.5.2 Background

In many instances, screens for chloride and sodium exclusion involve subjecting plants to salt treatment at specific concentrations for specific durations and sampling laminae and/or petioles or part or whole shoots followed by ion analysis. While this enables a ranking of genotypes for chloride and/or sodium exclusion capacity, it only provides an indication of root to shoot transport of these ions and does not provide an indication of whole plant capacity for ion exclusion.

We have previously demonstrated that the exclusion capability for chloride resides in the root and is not dependent on the scion. This suggests that the chloride ion exclusion mechanism is probably confined to a particular cell type in a specific tissue of the root. However, the genetics of this trait are complex hinting at a number of different components to the mechanism.

We have also demonstrated that chloride and sodium exclusion are regulated by different mechanisms. For sodium exclusion from laminae, we have demonstrated the important role that HKT plays in this process. While sodium exclusion is primarily located in the root and lower plant, it appears to involve retrieval of sodium from xylem and storage of retrieved sodium in roots, woody stem and shoots.

Here, we assessed the capacity of K51-40 and 140 Ruggeri and six hybrids selected from the cross between them for chloride and sodium exclusion capacity on a whole plant basis. The biomass of plants was measured and the capacity of the key organs to accumulate chloride and sodium was compared between the genotypes on a concentration basis.

6.5.3 Materials and Methods

Six hybrid lines selected for their range in capacity to exclude chloride and sodium, together with the parents K51-40 and 140 Ruggeri, were chosen for this experiment for having a range of ability of chloride exclusion. One line, MC215-59, was subsequently shown to have a different pollen parent from the others and the results from this line are not presented here. They were established in pots for 12 months prior to the experiment (details, see Experiment 4, Figure 3). Mixed chloride (25 mM) treatment (described in previous sections of this report) was used to hand-water on day one of the experiment, followed by 50 mM mixed chloride on subsequent days. All tissues of the plants were harvested after three weeks, dried, weighed and ground for ion analysis, also as previously described in this report.

6.5.4 Results

In experiment 4, conducted in 2014, five K51-40 x 140 Ruggeri progeny lines plus the two parents were screened for detailed ion concentration in each of the vines major tissues, roots, old stem, shoot, petiole and laminae. Dry biomass of each tissue was determined and also on the whole plant basis and demonstrates that the plants of the parental lines were fairly similar in size to each other and in both treatments. Some tissues might be slightly different for example, the roots of 140 Ruggeri appear to be larger than those of K51-40 in salt treatment.

For the salt treated plants, the data show much higher concentration of chloride in laminae, shoot and petioles of K51-40 than in comparable organs of 140 Ruggeri, as observed in previous studies. This confirms the capacity of 140 Ruggeri to restrict root to shoot transport of chloride relative to that of K51-40. Whole plant concentration of chloride is significantly lower in 140 Ruggeri than in K51-40, suggesting that net uptake of chloride is lower in 140 Ruggeri than in K51-40. This indicates that there might be an additional mechanism in 140 Ruggeri for chloride exclusion as well as a way of stopping chloride entering the transpiration stream. This second mechanism could act to limit influx and/or expel chloride from the root. While the growth of most of the hybrids was less than that of the parents, particularly in the salt treatment, chloride concentration was higher in petioles and lamina of all the hybrids relative to that in 140 Ruggeri.

However, for sodium, while the concentration of sodium in laminae of both 140 Ruggeri and K51-40 is relatively low and similar, there is a higher concentration of sodium in roots, old stem, and shoots. K51-40 has much higher concentrations of sodium particularly in petioles compared with 140 Ruggeri. This suggests evaluating sodium exclusion capacity on the basis of laminae concentrations may obscure other important information about the sodium exclusion process for these rootstocks. For example, the data appear to indicate that the overall uptake of sodium into 140 Ruggeri plants is less than that of K51-40. Furthermore, while K51-40 may accumulate more sodium, it appears to have the capacity to retrieve it from xylem and store it in roots, old stem, shoots and petioles to maintain a lower concentration in laminae. The progeny with the exception of MC 215-76 also had higher levels of sodium in the laminae than the parents, which is consistent with the result in the Plant Accelerator Experiment 1. The hybrid MC 215-76 generally has low concentrations of sodium in all tissues except roots where the sodium concentration is on the higher end of the range particularly compared to 140 Ruggeri.

6.5.5 Discussion

The significantly lower whole plant chloride concentration in 140 Ruggeri than that of K51-40 has increased our understanding of the chloride exclusion process, as we previously thought the chloride exclusion mechanism was primarily regulating root to shoot transport and occurring in the root stele, but we now know that there is likely to also be differences in the efflux of chloride out of the roots between the two parents. Using radioactive $^{36}Cl^-$, Abbaspour

et al. (2013) have previously shown that K51-40 has reduced efflux of Cl^- from roots to the external medium in comparison to 1103 Paulsen, a Cl^- excluder. Thus, it appears likely that 140 Ruggeri, which is also a Cl^- excluder like 1103 Paulsen, has a greater capacity for Cl^- efflux out of the root than K51-40. This hypothesis agrees with the continual variation in lamina chloride accumulation among the progeny, implying control by multiple genes. This new knowledge may assist in identifying genes responsible for this genetically complex trait that is likely to be occurring at two sites in the roots and controlled by different genes.

This experiment has also raised questions about the mechanism of sodium exclusion. In line with previous observations (Gong et al., 2011), there was little difference in sodium concentration in the laminae between 140 Ruggeri and K51-40, while four out of five of the progeny had increased sodium concentration. This is similar to observations made in experiment 1, Section 6.1 where the data were then used to identify *HKT1;1* as the gene responsible for this difference.

However, on a whole plant concentration basis, 140 Ruggeri had a significantly lower concentration of sodium than K51-40, indicating lower net uptake. While the uptake of sodium by K51-40 is higher than that of 140 Ruggeri, it has the ability to compartment the accumulated sodium in other organs while keeping laminae concentrations low.

6.5.6 Summary

This experiment has provided important information about the chloride and sodium exclusion processes in grapevine rootstocks. In this case, 140 Ruggeri not only restricts root to shoot transport of chloride, but it also has lower net uptake into the plant which could be due to lower root influx, higher efflux or both. Multiple genes are implicated. Similarly, while lamina sodium concentrations indicated little difference in sodium exclusion capacity between 140 Ruggeri and K51-40, this experiment has shown that 140 Ruggeri has a better capacity to limit uptake of sodium to the whole plant, and that while K51-40 takes up more sodium, it is able to retrieve it from xylem, store it in roots, old stem, shoots and petiole and keep laminae concentrations low. These results also provide the opportunity to investigate sodium exclusion in much more detail in different tissues.

6.6 Elucidating the role of HKT1;1 in planta

6.6.1 Objective

The role of HKT1;1 was studied in a number of ways documented in the publication and section Appendix 5. In this experiment we generated transgenic Shiraz plantlets with reduced expression of the *HKT1;1* gene to determine whether this gene plays a major role in sodium exclusion in the roots of grapevines.

6.6.2 Background

A number of experiments were conducted as part of this project to determine the role of *HKT1;1* isolated from grapevines; some of these are summarised in the publication documented in Appendix 5. Our lab has developed and refined transformation of grapevine over the last two decades, enabling the direct testing of gene function. In project CSP 1306, Shiraz was transformed with a number of antisense constructs with the aim of testing the role of specific genes in salinity tolerance. The *HKT1* family was targeted as a part of that project.

6.6.3 Materials and Methods

The transformations were performed as a part of project CSP 1306 and reported in the final report. Essentially, antisense constructs were developed in the pHANNIBAL vector (Wesley et al., 2001), transferred to pART27UbiGFP and used to transform *Agrobacterium*. Pre-embryogenic callus was then transformed with the *Agrobacterium* and cultured on kanamycin selection for several transfers. GFP positive embryos were germinated and grown into plantlets. Plantlets were selected for further analysis based on the probability they resulted from separate transformation events. Twenty eight plants were analysed by Southern blot and shown to contain at least one band with the two end probes and to be derived from separate transformations (see final report CSP 1306).

6.6.3.1 Tissue culture salt assay

This method is considered to be commercial-in-confidence at this time.

6.6.3.2 Glasshouse salt assay

This method is considered to be commercial-in-confidence at this time.

6.6.3.3 Transcript analysis

RNA was extracted and gene expression measured as detailed in the Methods 5.6.1 and 5.6.2 using primers listed in Table 1.

Table 1. Sequence of primers used for qPCR of *HKT1;1*, the *HKT1;1* antisense construct and ubiquitin housekeeping gene

Gene	Primer	Sequence
HKT1;1	HKT1.1_SHqPCRF	GACCCCCTAAACTTCAGTGTGT
HKT1;1	HKT1.1_SHqPCRR	ATCTAGTTTCCAGGCTCTGCC
35S promoter	LH35SF	TTCGCAAGACCCTTCCTCTA
For HKT1;1 antisense	HKT1a-Vv-A-HJR1	TGGTACCACCAAAAATATGTGCAGGCTACA
Ubiquitin	Vv Ubiq F1	AACCTCCAATCCAGTCATCTAC
Ubiquitin	VvUbiq 1R	GTGGTATTATTGAGCCATCCTT

6.6.4 Results

Shiraz grapevine embryogenic callus was transformed with Agrobacterium containing a DNA vector designed to decrease expression of the *VvHKTI;1* gene using a culture established in 2014. Based on the visual presence of GFP, 257 germinated embryos were selected and transferred to shoot production medium. Seventy-six percent of the transferred embryos developed shoots. Of those plantlets produced, 70 were designated as KA lines and 28 lines were tested for the presence of the transgene using Southern blot analysis. All of these were confirmed as positive for *VvHKTI;1* transgene construct. The copy number of inserts was determined.

Of embryos that germinated and grew strongly, the first 28 lines that had arisen from independent transformation events, as determined by Southern blot analysis, were propagated for further assays. Plants in batches 6 and 7 tissue culture assays were harvested 20 days after exposure to the salt solution in vitro and analysed for sodium content in order of ability to exclude sodium. By statistical analysis (Tukey test) nine lines, KA6, KA20, KA21, KA9, KA17, KA31, KA5, KA11 and KA4, all took up more sodium than the control Shiraz plants.

Plants grown in tissue culture are in an enclosed system with little photosynthesis or transpiration. To test whether the differences in sodium concentration between Shiraz and the nine lines with higher sodium content would still be observable in glasshouse conditions, 13 lines were selected for a range of phenotypes. Although the overall concentration of sodium is much lower, the trend is still the same with the lines such as KA21 and KA17 having high concentrations of sodium compared to KA43 and Shiraz controls.

Transcript analysis was performed using RNA extracted from roots of plants from the tissue culture assay batches 6 and 7. Results were obtained only from plants subjected to salt applications as water control plants gave the same results indicating that *HKT1;1* expression was not induced by salt. The transcript analysis shown in Figures 36-38 confirmed that those plants for which there was no change in sodium exclusion compared to Shiraz controls, also showed no apparent silencing of the *HKT1;1* transcript (KA2 and KA3 in Batch 6, KA43 in Batch 7). By comparison KA5, KA11, KA4, and KA9 from Batch 6 and KA17, KA21 and KA31 from Batch 7 all had lower levels of *HKT1;1* expression and took up significantly more sodium. The expression of the transgene appears to be quite low compared to ubiquitin and the endogenous *HKT1;1* gene, KA17 had the highest level of transcript, which also correlates with the increased sodium uptake.

6.6.5 Discussion

In previous experiments (Henderson, Dunlevy et al., 2018) we had shown that HKT1;1 was likely to be involved in sodium exclusion in grapevine roots. Unlike other inbred short life cycle plants such as Arabidopsis, maize and tomato, it is difficult to generate conclusive evidence about the role of a gene in grapevines. Transformation of grapevine, targeting just a single

gene, is one of the few tools available, despite the time and effort required to generate and test transgenic plants.

6.6.6 Summary

In this experiment we were able to demonstrate conclusively that in grapevine *HKT1;1* plays a key role in excluding sodium from the shoot and the generation of transgenic plants provides an opportunity to further characterise this gene.

6.7 Further characterisation of HKT1;1 by Sam Henderson and Matt Gilliham

This section (6.7) has been summarised to provide the conclusions of experiments that remain commercial-in-confidence.

6.7.1 Grapevine HKT1s

Sodium exclusion from the shoot in plants, and subsequent salt tolerance phenotypes, are often mediated by high affinity potassium (K) transporters (HKT). Therefore, at the outset of this project we decided to investigate HKT proteins within the grapevine genome with homology to characterised HKTs from other species, as these are likely candidates for controlling sodium exclusion in grapevine. Accession numbers for six *Vitis vinifera* HKT genes have been published in the supplementary files of Ali et al., (2012) (Table 2).

Table 2. *Vitis* HKT transporters annotated in the supplementary files of Ali et al., (2012)

Gene ID	Species	Protein ID
VvHKT1;1	*Vitis vinifera*	CAO64083
VvHKT1;2	*Vitis vinifera*	CAO64075
VvHKT1;3	*Vitis vinifera*	CAO64081
VvHKT1;6	*Vitis vinifera*	CAO64069
VvHKT1;7	*Vitis vinifera*	CAO64070
VvHKT1;8	*Vitis vinifera*	CAO64071

Our search of the *V. vinifera* genome database originally yielded eight HKTs. Blast searches revealed two of these have no homology with the plant HKT transporters (Table 3).

Table 3. Predicted HKTs from grapevine. Proteins with no homology to HKTs from other species, and therefore unlikely gene candidates, are displayed in strikethrough font.

Gene ID	Functional annotation
~~VIT_07s0005g06410~~	~~High-affinity K+ transporter 1 (HKT1)~~
~~VIT_11s0103g00040~~	~~High-affinity K+ transporter 1 (HKT1)~~
VIT_11s0103g00050	High-affinity K+ transporter 1 (HKT1)
VIT_11s0103g00090	High-affinity K+ transporter 1 (HKT1)
VIT_11s0103g00130	Potassium-sodium symporter HKT1
VIT_11s0103g00140	Potassium-sodium symporter HKT1
VIT_11s0103g00150	Potassium-sodium symporter HKT1
VIT_11s0103g00010	Potassium-sodium symporter HKT2

The predicted amino acid sequences of the remaining six HKTs from Table 2, most likely to encode functional plant HKTs, were analysed phylogenetically. The results are consistent with published grapevine HKTs. VvHKT1;3 and 1;2 appear to have the closest homology to HKT proteins from Arabidopsis, wheat and rice although the bootstrap values are quite low.

Expression difference in HKT genes (and other putative Na^+ transporters) between root tissues of the Cl^- excluding rootstocks 140 Ruggeri and K51-40 has been examined. Out of the six HKTs present in the grapevine genome, three were expressed differently in roots of the contrasting rootstocks. *HKT1;1* and *HKT1;3* were further studied as candidates as detailed in Henderson, Dunlevy et al., (2018). The remaining part of this section details data not provided in that paper and the preliminary characterisation of the other HKTs in grapevine that was performed.

6.7.2 Characterisation of HKT1;1 and HKT1;3

Initial characterisation was carried out using a yeast toxicity assay. *Saccharomyces cerevisiae* strain INVSc2 was transformed with pYES-DEST52 plasmids harbouring either VvHKT1;1, VvHKT1;3 (both isolated from Cabernet Sauvignon), Arabidopsis AtHKT1.1 (positive control), empty vector (negative control), or TmHKT1.5a (non-functional protein control). This expression vector contains the GAL1 promoter for galactose-inducible expression. Yeast serial dilutions were spotted onto solid medium (containing a basal level of 8.91 mM Na^+) and grown for four days. Equal growth was observed for all yeast strains when expression was repressed on 2 % glucose, demonstrating that equal numbers of cells were spotted in the assay. When expression was induced in the presence of 2 % galactose, growth of yeast transformed with VvHKT1;1 and AtHKT1.1 (positive control) was inhibited. Growth of yeast transformed with VvHKT1;3, or the empty vector (negative control) or TmHKT1.5a was not

inhibited. Addition of 100 mM Na^+ from three different Na^+ salts (Cl^-, SO_4^- or gluconate) further inhibited the growth of yeast expressing the known Na^+ transporter AtHKT1.1, but had no effect on yeast expressing VvHKT1;3. These data indicate that expression of VvHKT1;1 is inhibitory to yeast when grown on low Na^+, which is likely due to Na^+ transport into the yeast at toxic levels. Conversely, expression of VvHKT1;3 has no inhibitory effect on yeast when grown on low (< 9 mM) or high (100 mM) Na^+, suggesting that VvHKT1;3 may not transport this cation.

To further investigate the ion selectivity properties of VvHKT1;1 and VvHKT1;3, these proteins were expressed in *Xenopus laevis* oocytes and two electrode voltage clamp experiments were performed. Oocytes injected with cRNA encoding *VvHKT1;3* showed no conductances above that of water-injected control oocytes for either Na^+ or K^+. Possible explanations for these findings are that VvHKT1;3 is either non-functional, or more likely is selective for other ions, or alternatively VvHKT1;3 is dependent on activation by a mechanism not present in this experimental system.

6.7.3 Subcellular localisation of HKT1;1

We showed that VvHKT1;1 with an N-terminal GFP tag was detected on the plasma membrane of tobacco cells, but the signal was weak. To further investigate this, we generated a C-terminal GFP fusion, VvHKT1;1-GFP, downstream from the constitutive 35S promoter and transiently expressed this fusion protein in tobacco leaves. Expression of VvHKT1;1 with a C-terminal GFP tag in tobacco epidermis showed a strong signal that co-localised with the plasma membrane marker FM4-64. Cytoplasmic GFP showed a different signal to HKT1;1-GFP, and did not co-localise with FM4-64, suggesting that the HKT1;1 signal was not cytoplasmic. Furthermore, HKT1;1-GFP did not co-localise with the endoplasmic reticulum (ER) marker HDEL-mCherry. These results demonstrate that VvHKT1;1 is a plasma membrane protein. This work is reported in Henderson, Dunlevy et al., 2018.

6.7.4 Tagging HKT1;1 for localisation in oocytes

We attempted to quantify the amount of HKT1 protein expressed in *X. laevis* oocytes using a fluorescent tag in case protein expression levels were the reason that we say differences in *HKT1* allele activity. Unfortunately, we were unable to image the fluorescent protein on the oocyte membrane despite seeing it on the plasma membrane in tobacco, which is not the case for other HKTs from other plants that we have studied. However, HKT1 tagged with fluorescent protein was functional and behaved in a similar way to the untagged protein.

For methods and additional data on HKT1;1 please see Henderson, Dunlevy et al., (2018).

6.7.5 Structure-function relationship

In collaboration with Maria Hrmova (ACPFG, University of Adelaide), we investigated the structural basis that underlies the functional differences between K51-40 allele A and K51-40 allele B of *HKT1;1*. It was envisaged that this would give us the answer as to why the different

proteins had different properties. To achieve this, we constructed the 3D models of these proteins. These models are based on the structural similarity between the HKT class of proteins and a chain I of the solved KtrB K^+ transporter (4J7C:I) from *Bacillus subtilus* (Vieira-Pires et al., 2013).

6.7.6 HKT1;1 structural homology model validation

The sequence identity and similarity between the KtrB template (accession number 4J7C:I) and, for example, wild-type B^k are 18% and 34% respectively. Due to these relatively low values, the structural homology model required experimental validation. To achieve this, we performed site directed mutagenesis of amino acid residues that are predicted by the models to induce structural changes that result in observable functional variations for Na^+ transport. We investigated mutant variants of Vitis B^k, VvHKT1;3 (Cabernet Sauvignon), and OsHKT1;3 from *Oryza sativa*. The latter protein from rice was chosen because it displays a strong inward rectification for Na^+ transport in Xenopus oocytes – a functional property that it shares with B^k – as described by Jabnoune *et al*., (2009). A range of mutations were made; however these mutations are unable to functionally validate the homology model of the B^k protein.

In addition to single and double point mutations, we generated an insertional mutant of B^k with a nine amino acid HA tag at a position 322 that was predicted not to affect the permeation of Na^+ through the pore, and therefore enable Western-blotting and voltage clamp measurements to be performed on single oocytes. However, this insertion abolished the function of the B^k protein when expressed in oocytes, which added a further element of doubt as to the validity of the homology model.

With the aim of confirming the homology model in a different plant species, we performed parallel mutations in rice OsHT1;3, and tested whether these mutations affected inward rectification. The wild-type OsHKT1;3 strongly inward rectifies. Mutating the equivalent 'rectification residue' (R534 in B^k) to serine (N215S) did not release rectification as predicted. Mutating this N residue into an arginine did not increase rectification as predicted, however it would be difficult to increase the already extreme rectification in this protein. Finally, the model predicted that K518S mutation would release rectification, but did not.

6.7.7 Summary of HKT1;1 structural homology model validation

We tested 12 mutations as a functional test of a homology model that we hoped could explain the structural basis of rectification and conductance in the grapevine HKT allele B^k. None of these mutations gave the result that was predicted by the homology model. This suggests that this particular homology model is a poor indicator of plant HKT structure and should not be used for predicting the functional properties of this class of transporter.

6.7.8 Summary of other HKT1;1 mutations tested in yeast and Xenopus oocytes

Other residues were mutated, and the outcome of the mutations on protein function in yeast and oocytes did not support the model.

6.7.9 Information on other HKT proteins in grapevine

Previous reports also acknowledged that other HKT proteins may be expressed in roots of different grapevine cultivars and were simply not detectable in Corvina (Fasoli et al., 2012). To investigate this, RTPCR was carried to measure expression of *VvHKT1.2, VvHKT1.6, VvHKT1.7* and *VvHKT1.8*. *VvHKT1.6* was expressed at a low level in roots, while *VvHKT1.7* and *VvHKT1.8* expression was not detected.

In conclusion, the role of HKTs other than HKT1;1 is not known. They are expressed and are likely to contribute to Na^+ and K^+ movement in the plant and contribute to salt tolerance. As HKT1;1 only contributes ~70% to shoot Na^+ exclusion then there will be additional genetic factors important in shoot Na^+ exclusion. They are likely to protect other parts of the plant from salt such as flowers. It will be important to characterise these further to know what role they have in the plant and how they might be able to be used to improve salt tolerance of grapevines.

6.8 Role of *HKT1;1* alleles in Na^+ exclusion in different rootstocks

6.8.1 Objective

To determine the extent to which variation in Na^+ exclusion is predicted by *HKT1;1* markers in diverse *Vitis* genotypes and hence develop the most useful markers for predicting the Na^+ exclusion trait.

6.8.2 Background

In our paper published in *New Phytologist* (Henderson, Dunlevy et al., 2018) we showed that the inheritance of the *HKT1;1* locus accounts for 70% of the genetic variation in Na^+ exclusion in the K51-40 x 140 Ruggeri F1 population, with the remaining 30% of the variation due to unknown genetic differences. The mutagenesis studies on *HKT1;1* allelic variants presented in this paper (Appendix 5) suggest that the amino acids present at the key residues 534 and 537 dictate the dominant (active) or recessive (mutant) nature of *HKT1;1* alleles in this family and is therefore a good candidate as a genetic marker for Na^+ exclusion. The aim of the experiments described here was to investigate whether the composition of these key residues in the *HKT1;1* gene predicts Na^+ exclusion in genotypes other than the hybrids derived from K51-40 and 140 Ruggeri. This information will inform its utility as a genetic marker for Na^+ exclusion in populations of rootstocks with broader genetic makeup.

6.8.3 Results

This section is commercial-in-confidence at this time.

6.8.4 Summary

At present we have identified at least six different *HKT1;1* alleles. The information we have obtained from the 3' sequence of the *HKT1;1* gene has helped identify and predict the genetic nature of these six alleles. Together with previous findings we can tentatively rank these alleles for ability to exclude Na^+ as $E^{K/R} > E^V > e^K > E^D > e^R$.

The initial disparity between the original genetic marker score and Na^+ exclusion for two K51-40 x Schwarzmann hybrids highlights the importance of testing genetic markers in any potential parental genotypes before using them in marker-assisted selection of new populations. From conducting this analysis, markers are now available to predict sodium exclusion in a broad range of genotypes.

6.9 A locus associated with Cl^- exclusion

This section (6.9) detailing the experiments and findings about a locus with a role in chloride exclusion is commercial-in-confidence and not available at this time.

6.9.1 Objective

The objective of these experiments was to identify loci and/or genes involved in Cl^- exclusion.

6.9.2 Background

The majority of past studies that have investigated the inheritance of Cl^- exclusion in *Vitis* populations have concluded that the trait is multigenic (Gong et al., 2011; Fort et al., 2015). We also found evidence for this in the K51-40 x 140 Ruggeri F1 population, in which the continuous variation in Cl^- exclusion was not conducive to mapping the underlying genes (Section 6.1).

6.9.3 Summary

Using a novel screen, we have identified a locus associated with Cl^- exclusion in a segregating population. Markers at this locus may be useful for predicting the Cl^- exclusion ability of new material; however further work is required to determine their utility in more diverse genetic backgrounds.

6.10 Further characterisation of the candidate chloride excluder gene by Sam Henderson and Matt Gilliham

Details of this section (6.10) are commercial-in-confidence and not available at this time.

6.10.1 Methods

We used techniques to characterise the candidate gene from grapevine that had been developed for use in other plants, and also some novel techniques that were specific to this project. This work included subcellular localisation using GFP tagged protein and functional characterisation in heterologous systems including oocytes, yeast and bacteria.

6.10.2 Summary

In conclusion, we suggest that the candidate gene from grapevine is an ER protein. However, we cannot confirm how it functions at present. Further characterisation will be needed to confirm how it works and if it confers improved salt tolerance to grapevine, prior to its development as a perfect marker for salt tolerance.

7 Combined effects of heat and water deficit on young vines

7.1 Use of the Plant Accelerator to screen for water deficit tolerance

7.1.1 Objective

The aim of this experiment was to investigate the effectiveness of utilising the Plant Accelerator as a novel tool to screen for water deficit tolerance in a family of related rootstock genotypes, progeny of a *V. cinerea* x *V. vinifera* cv. Riesling cross.

7.1.2 Background

Rootstocks are generally considered to differ in their ability to tolerate water stress, with the choice of rootstock influencing vigour and yield of grafted grapevines under water limited conditions (McCarthy *et al.,* 1997, Edwards *et al.,* 2012, Serra *et al.,* 2014). Although the vast majority of Australian vineyards are irrigated (ABS 2009), for many of these irrigation is considered supplemental and with reduced rainfall in south-eastern Australia predicted in most climate change scenarios (IPCC 2015), breeding new rootstocks with a high capacity to tolerate water deficit remains of high importance.

Tolerance of water deficit may be due to root systems accessing a water supply not available to drought susceptible genotypes, or due to an ability to extract more water from a given volume of soil than susceptible genotypes. Utilising the Plant Accelerator can only test the latter trait, but nevertheless, the automated watering system of the Plant Accelerator provides a controlled and equal water stress to be imposed on many genotypes at once, allowing a direct comparison between genotypes to be made. The physiological response to water deficit cannot currently be assessed using the Plant Accelerator imaging platform, but plant production is the ultimate measure of stress tolerance and could be assessed as previously described.

In 2014 a second large Plant Accelerator experiment was undertaken to determine the effects of water deficit stress in a population of rootstock genotypes. This experiment also included a warming treatment, allowing the interaction between drought and heat to be examined. These traits are likely to be much more complex than salt tolerance, so the aim of the work was not only to map potential QTLs responsible for these traits, but to examine the potential of the Plant Accelerator as a rapid tool to screen new rootstock material for water deficit tolerance.

7.1.3 Materials and methods

For this study we utilised 84 hybrid progeny derived from a *V. cinerea* x *V. vinifera* cv. Riesling cross, which previous field observations suggested varied in their ability to tolerate a water deficit. Other genotypes, Riesling, Shiraz, 140 Ruggeri, K51-40, *V. longii* and *V. candicans* were

also included for comparison with standard rootstocks, *V. vinifera* scion genotypes and wild *Vitis* species.

Dormant canes were collected, established and potted up as described in section 5.2.5. In this experiment we employed factorial treatments of cool and hot temperatures combined with control and water deficit irrigations. Three replicate cuttings of each genotype were included for each of the four treatment combinations, totalling 12 replicates per genotype.

The two temperature treatments were applied by using the two Smart Houses, which were adjacent to each other and thus received very similar lighting conditions. The cool Smart House was set to a maximum day-time temperature of 25°C and a minimum night temperature of 20°C. The hot Smart House was set to the same initial conditions, but the maximum day-time temperature was ramped up to 40°C on day 17 and the minimum night temperature to 23°C on day 3 and to 30°C on day 17. Unfortunately, these set temperatures were not reached for most of the experimental period and only a small differential in temperature between the two Smart Houses was achieved.

Control irrigated vines were watered daily to a set target weight equivalent to 100 % soil capacity. Deficit treated vines were watered to capacity on day 1 and then to a set target weight equivalent to 62% capacity each day afterwards. Due to variability in daily water use of the different genotypes, the rate at which the deficit vines dried down to this target weight varied. Figure 8 shows the proportion of deficit vines that had reached the target weight on each day of the experiment. By day 9 over 50 % of deficit treated vines had reached their target weight in both the cool and hot Smart Houses. This day was therefore chosen as the beginning of the period to analyse the vines relative growth rates and water use efficiency. Projected shoot areas were calculated from the three digital images captured daily for each vine as described in section 5.3.1. Water use and RGRp were calculated as described previously.

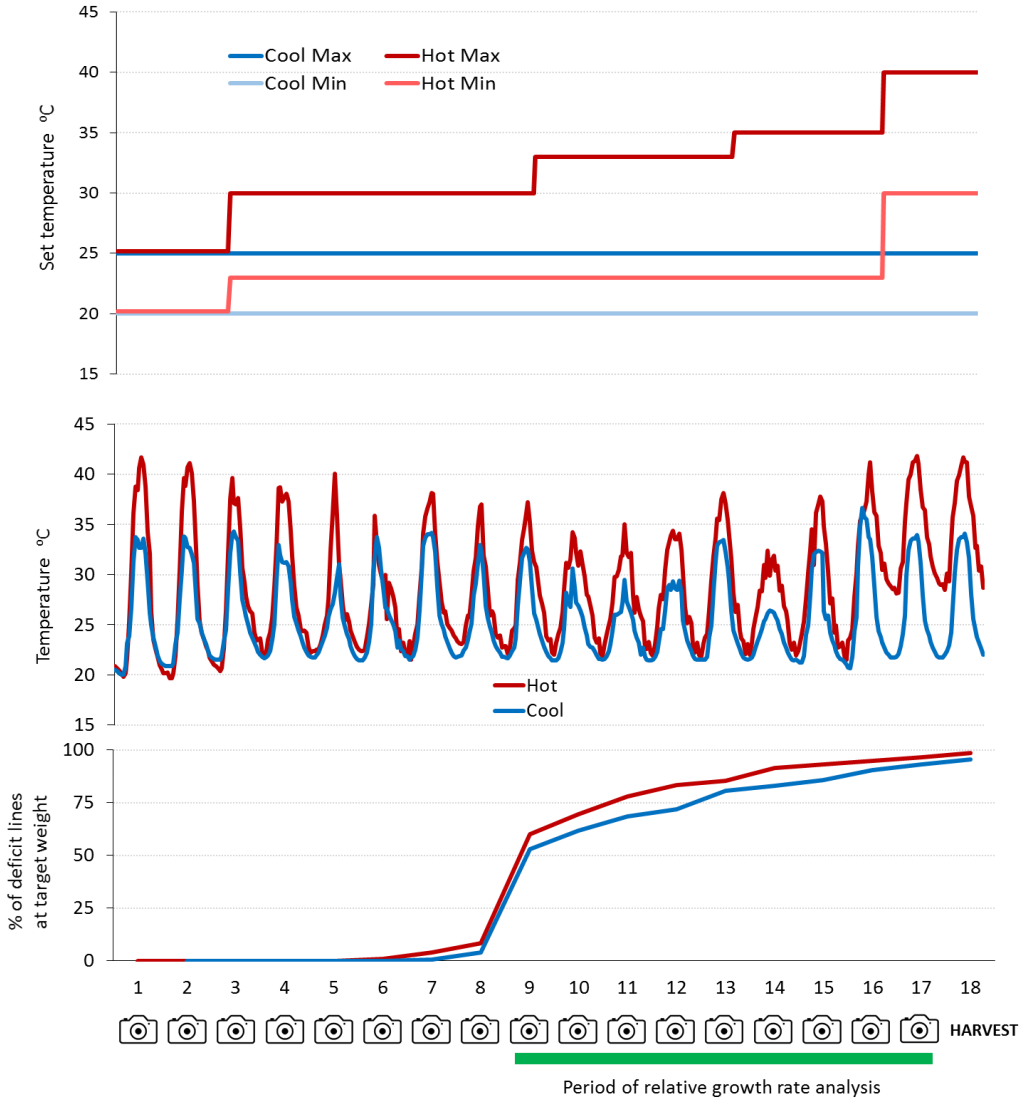


Figure 8. Set temperatures (Top) and actual recorded temperatures (Middle) of the cool and hot Smart Houses in 2014. The percentage of the deficit treated vines that had reached the target weight equivalent to a soil water content 62 % that of the field capacity controls in each Smart House (Bottom). The green bar indicates the period over which relative growth rates and water use efficiency were determined which coincides with when over 50 % of deficit treatment vines had reached the deficit target weight.

7.1.4 Results and discussion

As in the 2013 experiment 1, 144 vines were destructively harvested and the leaves passed through a leaf area meter to confirm the accuracy of projected shoot area values calculated from the digital images. A strong linear correlation was measured between projected shoot area and measured leaf area for all 144 vines. The temperature and water treatments did not appear to influence this correlation or the slope of the trend line.

7.1.4.1 Vine performance under drought and/or heat stress

As in the earlier sections, rootstock tolerance to abiotic stress was assessed using growth. As not all genotypes or vines were of identical size, and their storage reserves were unknown, growth was assessed as RGRp. Indeed, plant size at the start of the treatments was the largest single driver of plant size at the end of the experiment so any difference in size between genotypes resulting from field conditions of the mother vines (which were not all from the same region, let alone the same vineyard) would be interpreted as genetically derived if plant size or absolute growth was used for assessment. The RGRp of individual vines were calculated from the slope of the natural log of projected shoot areas from day 9 to 17. An analysis of variance showed that both water deficit and heat treatments had a highly significant effect on relative growth rates. Genotype also had a significant effect, suggesting that the genetic range included in the experiment was driving a difference in growth rate (vigour). However, post-hoc testing only found significant differences between the most extreme results.

There was no significant interaction between genotype and water, indicating that all the genotypes were responding to water deficit in a similar way. Despite that, it appears that, although RGRp was almost universally reduced by water deficit, the reduction was greater in the higher vigour genotypes. A difference in the *extent* of the water deficit response between genotypes was tested by calculating the relative change in RGRp for each plant (vine RGRp / mean RGRp for genotype controls). Analysing these data using ANOVA did indeed result in a significant genotype effect ($p = 0.002$). However, the lower vigour vines took longer to dry down under the water deficit treatment and some were still drying down during the period used for the RGRp calculation. As a result, more soil moisture would have been available to these vines and a lesser effect of the deficit treatment on RGRp might be expected.

Comparing the trendlines suggests that there is no relationship between RGRp under control water and RGRp under deficit. Thus, it seems likely that significant variation observed in the *extent* of the water deficit response between genotypes was not simply driven by vigour and may be amenable to further investigation for genetic markers.

Across the data set as a whole, there was a significant *increase* in RGRp with heat, likely because the air temperature only reached levels that were likely to be a stress on the final two of the 10 days over which RGRp was calculated (Figure 8 middle). In contrast to the ANOVA result for water deficit, there was a significant interaction between heat and genotype. The increase in RGRp with heat was primarily in the low vigour genotypes and very few of the high vigour genotypes responded to the treatment. There was also an interaction with water deficit, due to the heat effect occurring in well-watered vines, but not water deficit vines.

It seems that water deficit under heat was a limiting factor on growth rate for all genotypes, whereas water deficit under the cool treatment did not limit growth rate in the lower vigour genotypes. One explanation would be a greater water use under heat than under cool

conditions (thus faster dry down of the water deficit treated low vigour genotypes, Figure 8), consequently, limiting soil moisture availability would be reached in all genotypes under heat, but not under cool conditions.

7.1.4.2 Vine water use under drought and/or heat stress

Daily vine water use was recorded by the Plant Accelerator as loss of weight per pot. This was used to calculated water use on a leaf area basis (g per 100,000 pixels projected shoot area) for each plant on each day over the period for which RGRp was calculated. As this value varied from day to day due to atmospheric conditions in the Smart House, an average value across the ten day period was calculated.

An analysis of variance demonstrated that water use per unit leaf area (WUp) was significantly affected by all three main effects, with the only significant interaction being between temperature and water. This suggested that, although genotype was driving differences in WUp, the changes in WUp in response to heat or drought, did not vary between genotypes. There was little evidence that the response of WUp to heat or drought stress was likely to be amenable to QTL analysis or similar.

7.1.4.3 Vine water use under drought and/or heat stress

In irrigated viticulture high water use efficiency (WUE) is often a more valuable trait than water deficit tolerance *per se*. WUE based on whole plant transpiration, as measured in the Plant Accelerator, will vary by day due to differences in sunlight and environment, and by plant size due to differences in vine structure. Consequently, WUEp was calculated using RGRp (which is based on projected shoot area) and water use per pixel of projected shoot area. Both calculations were already on a daily time step and represented the same period of growth. Thus, WUEp was defined as RGRp / water use per pixel and expressed as: pixels PSA g water^{-1} d^{-1}. An ANOVA using the full data set demonstrated a significant effect of genotype, water and heat, but no significant interactions, although genotype by heat and genotype by water were marginally significant.

Water deficit resulted in a small improvement in WUEp across all genotypes and irrespective of temperature, whereas heat resulted in a reduced WUEp, irrespective of water status. The lack of an interaction with genotype in all cases offered little scope for QTL or similar analysis in regard to stress response, though WUEp itself may have potential.

7.1.4.4 Mapping QTLs for relative growth rate and water use efficiency traits

The family of *V. cinerea* x *V. vinifera* cv. Riesling hybrids had previously had genotype by sequencing analysis performed on them for the purpose of mapping loci linked with the traits of nematode and phylloxera resistance as part of CSP 1304. We planned to use these available SNP data in an attempt to identify QTLs linked with growth and water use traits measured in the Plant Accelerator. Of the 80 hybrids screen in the Plant Accelerator water deficit experiment, only 63 of these were included in the genotype by sequencing analysis and

mapping of pest resistance traits. A linkage map was created based on 2891 SNPs in these 63 genotypes using the program JoinMap® Version 4.1, as described in the New Phytologist paper, Appendix 5. The software MapQTL® was then used to search for SNPs associated with the two traits. Mean genotype values in the cool and hot Smart Houses and for control and deficit irrigation were used for the interval mapping as well as overall genotype means. However, no significant ($p<0.05$) QTLs could be detected for either relative growth rate or water use efficiency.

In a comparable study undertaken in France, significant QTLs for water use traits were detected in a family of 138 F1 genotypes from a *V. vinifera* Cabernet Sauvignon x *V. riparia* cross (Marguerit *et al.* 2012). The relatively small size (n=63) of the *V. cinerea* x *V. vinifera* cv. Riesling population used in our study is the likely reason why we did not detect any significant QTLs for growth or water use efficiency traits.

7.1.5 Summary

Water deficit and heat stress both affect many aspects of plant physiology, development and production, most of which are not measured in the Plant Accelerator and/or may be driven by shoot, rather than root, effects. Using vine performance (RGRp) as a measure of stress tolerance with ungrafted rootstocks meant that only tolerance mechanisms driven by root traits (e.g. morphology, osmotic adjustment, etc) would be relevant to use of those rootstocks in grafted vines. However, the experimental system was unable to separate genotype performance due to root traits or shoot traits (e.g. stomatal responsiveness to water deficit).

RGRp, WU and WUEp were all strongly impacted by genotype, but the primary aim of the work was to determine differences between genotype in their response to abiotic stress. The only parameter where a significant interaction between genotype and stress was observed was for the temperature response of RGRp and it is possible that this was an artefact due to the slower dry-down of the soil in lower vigour genotypes when not heated (Figure 78 bottom). Consequently, while the Plant Accelerator has clear potential to assess these traits (and genotype differences), either the response of these traits to abiotic stress does vary between *Vitis* species, or the cross used was inadequate in this respect.

7.2 Reliability of the use of the Plant Accelerator as a rapid screen to assess vine growth

7.2.1 Objective

To understand the extent to which results from high-throughput phenotyping of ungrafted rootstock rooted cuttings can be used to predict field performance.

7.2.2 Background

High throughput phenotyping, offered by facilities such as the Plant Accelerator, provides the opportunity to characterise the abiotic stress response of large numbers of genotypes within a relatively short period of time. However, for a perennial species like grapevine, the size limitation for plants used in the system necessitates the use of very young plants (rooted cuttings). Furthermore, while grafted plants can be (and were) used in the facility, utilising ungrafted plants normally allows smaller cuttings to be grown, reduces the cost associated with grafting large numbers of plants and simplifies the preparation required.

Using ungrafted plants to assess rootstock material restricts the traits of value to those that are within the root system itself. For example, a response such as stomatal closure at high vapour pressure deficit (VPD), which is likely to be shoot driven, would not be passed on to a grafted scion. In contrast, a root driven response, such as a drought signal to the shoot at low soil water content, will probably still occur in a grafted plant. Consequently, a number of scenarios in regard to the applicability of results from ungrafted plants can be envisaged, ranging from field performance precisely matching phenotyping results through to no relationship between phenotyping. The intention of the Plant Accelerator experiments developed through this project was to target traits that are likely to be root driven and present whether the rootstock is grafted or not.

To determine the applicability of Plant Accelerator results with ungrafted rootstocks to field grown, commercially managed, vineyards, testing was undertaken at a variety of 'stages'. Two experiments with grafted vines in the Plant Accelerator, under different stress conditions, were compared with ungrafted vines of the same genotype. Two high density field 'nursery' experiments with young grafted vines were undertaken, representing a sub-set of the genotypes used in the Plant Accelerator and, finally, data from two existing field trials with mature vines were compared to the phenotyping data.

7.2.3 Materials and Methods

7.2.3.1 Grafted vs ungrafted young vines

In 2015 a follow up to the large heat x drought experiment described in 7.1, a second smaller experiment was performed in the Plant Accelerator to determine the extent to which traits are conferred to a grafted scion. This experiment comprised of a subset of six *V. cinerea* x *V. vinifera* cv. Riesling hybrids, together with Riesling and Shiraz as rootstocks. The choice of hybrids was based on their responses in the 2014 ungrafted screen and the availability of grafting.

Twenty four cuttings of each genotype were grafted with Shiraz by Yalumba Nursery and established in a mist house for one month. The callused cuttings were transferred to the Waite Campus, planted in sand/perlite for four weeks and then the 20 most uniform cuttings transplanted into Biogro medium. Following another month of establishment, the 16 most

uniform plants were selected for the experiment, providing four replicates per treatment. For one line, MR 11-39, there was only enough healthy material to be included in one Smart House, with the control temperature chosen.

As previously, the cuttings were exposed to a heat x drought factorial stress regime, with the hot Smart House ramped up to a set air temperature of 40°C day, 30°C night, over a period of ten days. The cool Smart House was maintained at 25°C day, 20°C night. A target weight equivalent to a 62% water capacity was again applied to the water deficit treated vines. Twelve days after the onset of the heat and water deficit treatments the vines were harvested as previously described. This provided an 11 day period of imaging, from which the increase in green pixels was calculated, relative to the green pixels already present (RGRp), also as described previously.

7.2.3.2 Smart House grown young vines vs field grown grafted vines

In section 6.2.4 we showed that there was a significant and strong correlation between the relative growth rate of ungrafted vines in the Plant Accelerator and growth of mature grafted vines in the CSIRO Irymple vineyard, measured as pruning weights. This correlation was only significant for hot salt treated vines in the Plant Accelerator which best align to the conditions of the salt-treated field trial. In the experiments described below we set out to test the extent to which relative growth rates of genotypes exposed to a water deficit treatment in the Plant Accelerator are correlated with field responses to water deficit conditions.

Two 'vine nursery' style plantings were established at the CSIRO Irymple Farm in Victoria, using grafted vines grown over an 18 month period. The first, planted in November 2014 (Nursery Experiment 1), included nine rootstock lines used in the high-throughput phenotyping experiments, five lines from the previously described *V. cinerea* x *V. vinifera* cv. Riesling hybrids, Riesling, Shiraz, K51-40 and 140 Ruggeri. All were used as rootstocks, including the two *V. vinifera* varieties and grafted with Shiraz, clone 1654. Eight replicate experimental blocks were established in the vineyard, four per row in two adjacent vineyard rows. Row spacing was 3 m and vine spacing was 0.5 m. Vines were trained to a 0.4 m cordon along a single wire. Each block consisted of two vines from each line with low or standard irrigation applied. The lines were randomised within each irrigation treatment in each block. Two buffer vines were planted at the start and end of each row and between irrigation treatments. The vines were allowed three months to establish before the low irrigation treatment was implemented (19 February 2015). The irrigation treatments were imposed by using drippers with different outputs, 1.6 L/hr for the standard irrigation and 0.7 L/hr for the low irrigation. However, as the two rows were part of a larger block, timing and duration of irrigation was determined for the block as a whole, not specifically for the nursery rows.

Soil moisture was monitored during the implementation of the irrigation treatments (12 to 28 February 2015) using two SMM soil moisture monitoring systems, each with ten 15 cm sensors (ICT International, Armidale, NSW, Australia). Stomatal conductance was measured

at two times (morning and afternoon) on 25 March 2015, using a Delta-T AP4 Porometer (MEA, Magill, SA, Australia) to determine the impact of the irrigation treatments. Pruning weights were measured at leaf fall, in June 2015.

Irrigation treatments were maintained during the 2015/16 season, with spot measurements of soil moisture made on two occasions (30 November 2015 and 18 January 2016) using a Trase TDR system (ICT International, Armidale, NSW, Australia). Further stomatal conductance measurements were made on 1 December 2015 and 18 February 2016.

On 14 March 2016, six replicates of the eight lines were harvested to determine aboveground biomass and canopy size. For each vine, all the leaves were removed and a fresh weight obtained. A sub-sample of leaves was then taken, weighed and leaf area determined using a LiCor Li3500 Leaf Area Meter (LiCor, Nebraska, USA). Whole canopy area was determined using the ratio between the sub-sample and total leaf fresh weight. The leaf sub-sample was subsequently oven-dried at 60^{o}C. Cane length was estimated and cane and stem fresh weights determined. The cane and stem material were then air-dried on site. A subsample of air-dried stem material was then oven-dried to determine the remaining moisture content. All dried samples were weighed and used to determine the total aboveground dry weight of the vines.

The second nursery experiment was established in November 2015 in a different section of the CSIRO Irymple field site, also across two rows (Nursery Experiment 2). Vine and row spacing and vine training were as previously described. For this planting, seven of the *V. cinerea* x *V. vinifera* cv. Riesling hybrids were used, together with Riesling and Shiraz. As previously, all lines were used as rootstocks and grafted with Shiraz, clone EN TAV470. Irrigation was also set up as previously and eight replicate blocks used (four per row). No measurements were made for this experiment during the 2015/16 season. A spot measurement of soil moisture using the Trase system was made on 21 February 2017, prior to the heating system being established.

In March 2017, a passive heating system was put in place to provide a heat stress to the vines, consisting of a 20x6x3 m polytunnel built over the central section of the two rows (Figure 9). The tunnel encompassed four of the eight blocks. The tunnel covering was put in place 21 March and the tunnel ends were closed or raised manually to provide some control of the internal temperature. The intention was to generate a daily maximum air temperature in excess of 40°C, over a four week period.



Figure 9. The heat tent erected across two rows of the second nursery trial planted at the Irymple vineyard.

The physiological status of the vines was assessed on the first and third day of heating by taking morning and afternoon readings of stomatal conductance and mid-day measurements of stem water potential. A selection of growing tips was also tagged in order to assess subsequent growth.

The heat treatment remained in place for 29 days, after which the polytunnel was removed and the vines harvested as for the previous experiment.

7.2.3.3 Smart House grown young vines vs field grown young vines

A field nursery planting of 75 *V. cinerea* x *V. vinifera* cv. Riesling F1 genotypes was planted at the CSIRO Irymple Farm in 2014 as part of project CSP 1304 as described in that project's final report. Six replicates of each genotype were planted, three received a control irrigation and three received deficit irrigation. Pruning weights were collected in 2017.

7.2.3.4 Smart House grown young vines vs field grown mature grafted vines

A field trial of various rootstock genotypes grafted with a Shiraz scion under both control and deficit irrigation (n=2 per treatment) was previously established at the CSIRO Irymple Farm in 2010. When fully mature as fruit-bearing vines, these genotypes were used to determine how well relative growth rates observed in the Plant Accelerator reflect the growth of mature vines in the vineyard. Thirteen of these genotypes were screened in the Plant Accelerator in 2015 under both control and deficit irrigation as described above.

7.2.4 Results and discussion

7.2.4.1 Grafted vs non-grafted vines

The grafted lines exposed to a factorial heat x drought stress were grown with the same set conditions as the ungrafted heat x drought stressed vines, however, the actual air temperatures were somewhat different between the experiments due to differences in conditions outside the Smart Houses and limitations of the Smart House air-conditioning. Gaps indicate missing data due to sensor failure. The temperature differential between the treatments was, therefore, greater for the grafted line experiment (Figure 10). Differences in the rate of soil drying in the deficit vines between the heat and control treatments were accentuated in the experiment with the grafted lines relative to the ungrafted experiment, probably as a result of the heat treatment driving a 61% increase in plant transpiration rate under water deficit in the grafted experiment, vs a 23% increase in the ungrafted experiment (Figure 10). On average, vines in the hot Smart House reached the target weight 1-3 days sooner than vines in the cool Smart House. On day 16 three vines in the cool Smart House were mistakenly watered to capacity and as a result did not reach the target deficit weight.

The simplest and most comprehensive assessment of the potential effect of grafting on rootstock response to stress in these experiments is given by a comparison of the calculated RGRp values between the two data sets (grafted and ungrafted) for the eight genotypes in each of the four treatments. Although this relationship was significant, the proportion of the variability in the data explained by the relationship was low. This suggests that grafting has greatly altered the rootstock effect on plant production under stress. However, as the experimental conditions were not identical, these may have affected the result.

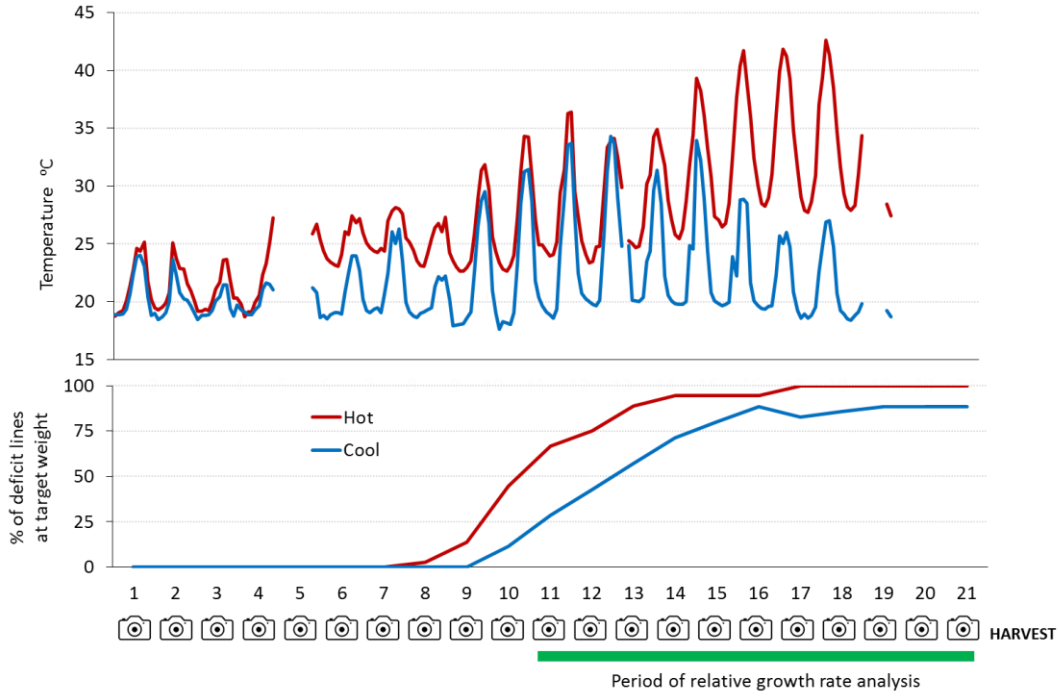


Figure 10. Temperature and water use in the Plant Accelerator 2015 (Top) Temperatures of cool and hot Smart Houses (Bottom) Percentage of vines that had dried down to the set target weight equivalent to 62% water capacity, in cool and hot Smart Houses. The Green bar indicated the period over which relative growth rates were calculated.

The overall response to the treatments and their interaction were tested using an analysis of variance across the whole data set. Whilst the main effect of water deficit was, unsurprisingly, a significant reduction in both experiments, the main effect of heating was only significant in the grafted experiment. Heating was a significant effect when the whole ungrafted dataset was examined and there was no genotype x temperature interaction (Section 7.1.4), so it is likely that the lack of effect in the genotypes matching the grafted experiment was due to the difference in heating conditions between the two experiments, which drove a much greater effect overall in the grafted experiment. Further, the result suggests that a better comparison between grafted and ungrafted plants than the one above might be to compare the data excluding the temperature effect. In both experiments there was a significant interaction between water deficit and heating. A result of a greater reduction in RGRp under the combination of deficit and heat, than the reduction driven by deficit alone.

Although the effect of genotype itself was significant in both experiments, without which it would be impossible to draw any useful conclusions, the significance of that effect was greater in the ungrafted dataset. The cause of this was probably the greater RGRp in the ungrafted experiment and the greater range between genotypes within a treatment, although that does not inform as to whether the reduced variability between genotypes was an experimental artefact of the result of grafting with a single scion. It is certainly the case that grafting with Shiraz will remove any shoot derived differences in overall vigour (e.g. any effect due to differences in photosynthetic capacity) and emphasises the need to carefully

consider or determine whether specific traits are actually root or shoot driven when doing pre-breeding screens for rootstock development.

For the current work, the most important aspect is whether any differences in genotype response to stress are maintained when grafted. This should be seen in a similar genotype x treatment interaction between the experiments. If the main effect of temperature is disregarded (for the reasons given above), two interactions can be considered, genotype x water deficit and the three-way interaction genotype x deficit x heating. The latter was not significant in either dataset, suggesting that all genotypes had a similar response to the combined treatment, but the genotype x deficit effect was only significant for the ungrafted experiment. Again, this suggests that grafting has removed variability between the genotypes, potentially as a result of removing genotype differences in the shoot response, such as differences in stomatal response to VPD, as seen between Shiraz and Grenache (Soar et al., 2006). Certainly, the effect of water deficit across genotypes was much stronger in the grafted experiment. Again, this emphasises the highly multi-genic nature of the trait and the care needed to separate root and shoot responses to this stress.

Splitting out the treatments to undertake correlations between the experiments again resulted in results with poor explanatory power, whether by major effect, or when considering all four factorial combinations separately. Thus, despite a significant effect of genotype on relative growth rates, when grafted these differences in growth rates are not correlated with or predicted by the growth of an ungrafted vine.

The extent to which differences in water use of ungrafted rootstocks were passed onto a grafted scion under these treatments was also examined. Due to the potential confounding effects of plant size, water use was calculated per 100,000 pixel of projected shoot area, equating to transpiration per unit leaf area.

Comparing grafted vs ungrafted vines, using the mean per genotype per treatment for the eight genotypes provided a significant relationship. However, water use per unit leaf area was strongly affected by water deficit (see section 7.1.4.2); splitting the data set into well-watered or water deficit groups, resulted in regressions with different slopes for well-watered and water deficit respectively. Note that the difference between experiments on a per pixel basis was due to a change in camera resolution between the experiments and thus the number of pixels per unit leaf area, not due to a large difference in water use per unit leaf area between experiments.

As water use per unit leaf area was also affected by warming, each treatment combination was also examined individually. Within a treatment combination the relationship between the water use of grafted and ungrafted genotypes was relatively weak, except for the well-watered, heated vines.

Clearly, grafting did not alter the overall effect of the treatments on vine physiology, but the continued presence of a significant relationship in the well-watered, heated treatment

suggests that in the absence of other limiting variables (e.g. water supply) the rootstocks that have high, or low water use per unit leaf area, confer that same high or low water use on the scion grafted to them.

Interestingly, for both RGRp and for water use the variation in the results was lower for the grafted vines (a single scion genotype) than for the ungrafted vines (shoot genotype the same as the root), suggesting that even where the root genotype has altered the shoot response in the grafted vines, the extent of the effect was muted.

Rootstock differences in water use per se are of limited value as a breeding trait unless they generate some difference in water use efficiency (WUE). WUEp was calculated as efficiency of projected shoot area production over the period for which RGRp was calculated as RGRp/WUp (expressed as pixels per g). The four treatment combinations clearly separated for the grafted vines, but not for the ungrafted vines, where only temperature was significant. Again, the variation between genotypes was less for the grafted than ungrafted vines. It is unclear whether the lack of correlation between grafted and ungrafted vines was due to the unintended differences in environmental conditions between experiments or the grafting itself. However, this is consistent with the findings of Virgona et al. (2003), who used carbon isotope discriminations to study transpiration efficiency in six different rootstocks and Shiraz. When ungrafted there was significant variation in transpiration efficiency; however when grafted with Shiraz there was no significant variation (Virgona et al., 2003). This suggests that differences in the water use efficiency of ungrafted rootstocks is largely controlled by mechanisms contained in leaves or shoots and this variability is largely lost when grafted with a common scion.

7.2.4.2 Smart House grown young vines vs field grown grafted vines

For the Plant Accelerator to be a useful tool for rapidly screening rootstock genotypes, it must first be proven to produce reliable data that is consistent with the growth of genotypes in the field. Two field trials with young vines were established in an attempt to determine how well plant responses in the Plant Accelerator aligned with plant responses in the field. In addition, limited data were collected from mature field grown vines in existing trials.

The Nursery Experiment 1 intended to compare field grown responses with water deficit to the water deficit responses in the Smart House. However, although the deficit irrigation application was at half the rate of the control irrigation application, the irrigation system was on the same valve in the field as a mature block and thus received long duration irrigation applications. As a result, although the deficit irrigation treatment did significantly reduce soil moisture during the experiment it was only by 2% volumetric soil water. Consequently, it was likely that the young vines, with a lower requirement for water than mature vines due to their smaller canopies, were not subjected to a major water stress; specifically, not to the extent that the vines in the Plant Accelerator experiment were.

This conclusion was supported by the stomatal conductance measurements made on those vines, with no significant difference at any of the measurement time-points. Stomatal conductance is generally viewed as a good integrating indicator of plant water stress (Bartlett et al., 2016). Based on these results, comparison of water deficit response between these, field-grown, vines and the response of vines in Plant Accelerator experiments was not possible. However, significant genotype differences were observed on both of the measurement dates where they were assessed. Therefore, the field and Smart House experiments could still be compared in terms of rootstock conferred vigour, an important trait (Walker and Clingeleffer, 2009), even if not the primary intention of the work.

Unfortunately, it was not possible to assess growth rate in the nursery experiments, but total above-ground dry mass was measured and represented the cumulative growth from planting. There was a significant effect of genotype, with the greatest growth exhibited in vines grafted to 140 Ruggeri rootstocks, the lowest growth when grafted to K51-40. However, regressions between these nine genotypes, grafted and grown in the field, and the same genotypes, ungrafted in the Plant Accelerator, were not significant. This was the case, whether specific treatments were regressed against each other, or the mean data per genotype.

Although utilising RGRp for comparisons across rootstocks was preferable to using projected shoot harvest at any given time point, the canopy size measured in Nursery Experiment 1 was directly comparable to projected shoot area at the end of the Plant Accelerator experiment in concept. In the field, canopy size was very closely related to total above-ground biomass, despite the canopy representing only a third of that biomass. However, regressions of canopy size of grafted plants in the field (directly measured) and ungrafted plants in the Plant Accelerator (projected shoot area) were also not significant.

The grafted heat x deficit experiment in the Plant Accelerator (section 7.2) was designed to be compared back to the ungrafted Plant Accelerator experiment, rather than the nursery experiment, but four lines were used in both. A regression of RGRp from the Plant Accelerator and total above-ground biomass in Nursery experiment 1 in these four lines suggested that the lack of a single grafted scion may explain the lack of correspondence between the ungrafted plant accelerator experiment and the field Nursery experiment 1. However, the very low number of genotypes (four) and the low variability between those lines in both experiments, limit any confidence in this conclusion. Furthermore, comparing canopy sizes in the same way resulted in a no correlation.

In summary, the results from the first Nursery experiment 1 suggested that, for vine vigour at least, the data from ungrafted vines in the Plant Accelerator were not predictive of field performance of grafted plants.

As with the first field Nursery experiment 1, Nursery Experiment 2 did not effectively implement a water stress on the vines, despite a large difference in application rate from the drippers. Neither soil moisture, nor stomatal conductance differed between irrigation treatments, although there were small differences in stem water potential and leaf

temperature. Again, it was assumed that the duration of irrigation required for mature vines on the same valve was enough to provide excess soil water, irrespective of application rate.

The imposition of heat stress was effective, with the differential between the control and heat treatments reaching 16°C on the initial three days of treatment, during which physiological measurements were taken. Air temperatures in the heat treatment exceeded 50°C on several days during the one month heat stress imposed and night-time temperatures were generally also raised, despite the system relying on solar heating. This was presumably due to soil and infrastructure continuing to radiate heat during the night. Vapour pressure deficit (VPD) was also highly affected, exceeding 8 kPa in the heat treatment on some days and the daily maximum VPD was generally more than double that of the outside air.

The impact of the treatments on vine physiology was assessed during the first three days of the heat stress. Midday stem water potential (Ψ_{stem}) was measured on the first day, with air temperature being an average of 36 and 49°C during the measurement period for the ambient and heated treatments respectively. Despite this difference there was no significant effect of the heating on Ψ_{stem}, although there was a slight effect of irrigation. In contrast, there was a significant reduction in stomatal conductance (gs) under heat stress, but no effect of irrigation. Leaf temperature was, not surprisingly, significantly higher under the heat stress regime, but there was also a significant interaction between heat and irrigation, with mean leaf temperature under ambient conditions unaffected by irrigation, but the low irrigation vines under heat stress being 0.8°C hotter than the high irrigation vines. There was no significant difference between genotypes for any of these parameters.

After four weeks of heat stress the whole vine was harvested and vine biomass and canopy area determined. Remarkably, there was no treatment effect on either parameter, although there were significant rootstock genotype differences in both vine fresh weight and canopy area.

As with the first Nursery experiment, the lack of a treatment impact on the field grown vines limited the comparison with the Plant Accelerator data (whether grafted or ungrafted) to rootstock conferred vigour. Again, regressions of RGRp vs above-ground biomass or of canopy size did not suggest there was any significant relationship between rootstock effects in the Plant Accelerator and rootstock effects in the field.

The lack of a relationship across the various experiments, Plant Accelerator ungrafted, Plant Accelerator grafted and the two field nursery experiments, is not surprising as they were not designed to look at rootstock conferred vigour itself and comparing vigour of grafted and ungrafted plants is also unlikely to be useful, as the shoot is likely to be the most significant driver of genotype differences in non-stressed, ungrafted plants.

7.2.4.3 Smart House grown young vines vs field grown young vines

A third approach for comparing relative growth rates in the Plant Accelerator with growth in the field utilised the available pruning weight data from a large planting of ungrafted *V. cinerea* X *V. vinifera* cv. Riesling F1 genotypes established by Brady Smith and Peter Clingeleffer as part of project CSP 1304. In this trial 75 genotypes were planted at the Irymple vineyard as ungrafted vines under both control and deficit irrigation, three replicates per treatment. Pruning weights were recorded the second year after planting. In the control treated vines there was a significant albeit weak positive correlation between field pruning weights and relative growth rates of all control treated vines in the Plant Accelerator. No such correlation was seen between the deficit treated vines of each experiment. A significant correlation was also seen between the relative growth rates of control treated vines and the mean pruning weights of both control and deficit treatments in the field.

7.2.4.4 Smart House grown young vines vs grafted mature vines in the field

A final comparison between relative growth rates in the Plant Accelerator and growth in the field was investigated utilising 13 genotypes planted at the CSIRO Irymple vineyard as mature Shiraz-grafted fruit bearing vines. Established in 2010, each genotype consisted of two replicate vines under control irrigation and two replicate vines under deficit irrigation. Winter pruning weights were collected in 2014 as part of CSP 1304.

The 13 genotypes were screened as ungrafted vines in the Plant Accelerator in 2015 under control and deficit water conditions, in the cool Smart House. A significant, but surprisingly negative correlation was seen between relative growth rate of ungrafted vines and pruning weights of grafted vines in the field under deficit conditions. A similar negative correlation was present under control conditions however this was not statistically significant. The correlation between the overall means of both treatments was significant with a slightly stronger correlation than for drought treated vines alone. The negative relationship between these two growth measures of the same genotypes is illogical and therefore seems likely to have arisen by chance. This negative correlation appears to be largely driven by two genotypes with low relative growth rates in the Plant Accelerator and when these two genotypes are removed from the analysis the negative relationship disappears.

7.2.5 Summary

As noted above, complex traits, which are influenced by both shoot and root, are likely to show only limited correlation between young ungrafted plants in controlled conditions and field grown grafted vines, as only the root derived component of that trait can be conferred to the scion. For the genotypes and experiments described here the lack of, or very limited, interaction between genotype and treatment meant that there was little genotype driven variation to be correlated with the field and so it is unsurprising that strong relationships between field and Plant Accelerator were not observed. Differences in traits such as above-ground vigour and, particularly, water use per unit leaf area among ungrafted grapevine

genotypes appear to be largely attributable to shoot processes. Therefore, future studies or selection of rootstock material for these traits in the Plant Accelerator would best be conducted as grafted vines, although the project was not designed to study these traits directly, rather their response to abiotic stresses.

7.3 Effect of graft union on stress tolerance

7.3.1 Objective

The aim of this experiment was to determine whether anatomical properties of the graft union correlate with stress tolerance traits.

7.3.2 Background

Many studies have linked aspects of rootstock hydraulics with both conferred vigour and drought tolerance (e.g. Zhang et al., 2016 and references therein). It was anticipated that this project would provide a platform to link these ideas to the wide range of experiments to be undertaken with the same genotypes across multiple environments and stresses. Examination of the graft unions together with measurements of the hydraulics from a small number of genotypes, as young field grown plants, could be linked to the response of those plants to abiotic stresses and then compared with the much wider range of genotypes utilised in the Plant Accelerator experiments. Inference from these results would enable more detailed future studies beyond the scope of the current project.

To achieve this, four of the genotypes in the Nursery Experiment 1 (see above), underwent additional measurements and their graft unions were removed at harvest for further study.

7.3.3 Materials and methods

The 140 Ruggeri, K51-40, Riesling and Shiraz rootstocks in Nursery Experiment 1 were assessed for whole vine and root system hydraulic conductivity prior to harvesting. Stem water potential and leaf water potential, using a Plant Water Status Console (ICT International, Armidale, NSW, Australia) and leaf transpiration, using a LiCor 6400 XT Portable Photosynthesis System (LiCor, Nebraska, USA) were measured on three to four replicates for each irrigation treatment, immediately prior to the trunk being severed below the graft union and whole root system hydraulic conductivity being measured with a High-Pressure Flow-Meter (ICT International, Armidale, NSW, Australia). Following this, the trunk was cut again, above the graft union, the graft union was removed and stored in ethanol until further use.

Cross-sections of the stem from the top (above) and bottom (below) were cut from the stored graft union using a sharp blade and photographed through a light microscope. The resulting digital images were thresholded and the area of xylem vessel and total cross-section

determined using ImageJ software. Two whole graft-unions were imaged using a Micro-CT scanner by the Adelaide University Microscopy Unit.

7.3.4 Results and discussion

As discussed earlier, the irrigation treatments were not effective in generating any water stress in the vines and, as a result, there was no significant difference in either stem water potential or leaf water potential.

Similarly, there was no difference in root system hydraulic conductivity, whether calculated on a canopy size basis or a stem cross-section basis. When uncorrected, or corrected on a trunk cross-section size basis, there was also no effect of genotype, but when corrected on a canopy size basis, there was a significant difference between K51-40 and 140 Ruggeri.

The higher root hydraulic conductivity per unit canopy area of K51-40, could indicate a greater capacity of this rootstock to provide water to that canopy, but as the soil moisture was not limiting, K51-40 had the smallest canopy size and there was no difference when expressed on a trunk cross-section basis it is difficult to draw any firm conclusion.

The lack of a difference in any measured parameter with irrigation and the lack of a genotype x water deficit interaction in the Plant Accelerator experiment meant that the primary aim of linking rootstock hydraulics, graft union structure and vine performance could not be achieved. Nevertheless, two graft unions were imaged using the micro-CT facility to look for genotype differences. Unfortunately, whilst the system was able to determine structure in the graft union, the resolution was inadequate to fully visualise the vessels and their joining between rootstock and scion.

As the vessel sizes could not be determined using the micro-CT images, they were determined for each scion and rootstock using light microscopy. There was a marginal difference between the vessel area of the scion side of the graft union and the rootstock side, but this was mainly due to a bigger difference in the Riesling grafted vines than the other genotypes. Trunk cross-section size was similar in all genotypes, so calculating vessel area as a fraction of total cross-sectional area did not alter the results.

7.3.5 Summary

In summary, the supporting data (genotype x stress treatment interaction of the ungrafted Plant Accelerator vines, irrigation effect in the Nursery Experiment vines) did not allow the original intention of this work to be achieved, but the trial data collected demonstrated the potential for these techniques to be used in grafted vines and the small effects observed ruled out any very large differences in hydraulics between these rootstock genotypes when grafted.

7.4 Combination of all three abiotic stresses: Heat x Salt x Water Deficit in the Plant Accelerator

7.4.1 Objective

The objective of this experiment was to investigate the effect of factorial heat x salt x water deficit treatments on the relative growth rate, water use efficiency and ion exclusion traits utilising the Plant Accelerator.

7.4.2 Background

Climate change modelling suggests that many Australian grape growers face a likely scenario of reduced water quality and reduced water availability in the future.

In the previous experiments we have investigated the individual effects of salt stress and water deficit stress under two temperatures on the growth, water use and ion accumulation of rootstock genotypes. The aim of this experiment was to examine the combined effect of salt and water stress under both cool and hot conditions, to better reflect what some vineyards may experience in a climate change worse-case scenario.

7.4.3 Materials and methods

In this experiment, factorial treatments of high and low temperature, capacity and deficit watering, and control and salt treatments were applied. Four genotypes were chosen, 140 Ruggeri, K51-40, MC 215-09 and MI 07-33, which display contrasting phenotypes for the four traits measured when screened in the 2013 heat x salt experiment. Three replicate ungrafted cuttings per genotype were included for each of the eight treatment combinations.

This experiment was run in parallel with the diverse genotype screen thus the cool and hot Smart House temperatures were the same as shown in Figure 6. Capacity and deficit irrigation treatments were the same as described in section 7.1.3, equating to 100 % and 62 % soil water capacity respectively. Control and salt treatments were the same as described in section 6.2.3 which consisted of a control rainwater application or three successive applications of a concentrated salt solution resulting in an estimated 100 mM mixed Cl^- concentration (60 mM Na^+: 10 mM Mg^{2+}: 10 mM Ca^{2+}) in the pot when at full soil capacity. For water deficit + salt treated vines the reduced soil water compounds the intensity of the salt treatment which equates to an estimated 161 mM mixed Cl^- concentration when at the 62 % soil water target weight.

Relative growth rates and relative water use were calculated over days 12-26 as for the diverse genotype screen described in section 5.3.2 and 5.3.3 respectively.

By the end of this experiment all three rooted cuttings of K51-40 and two of MI 07-33 which received the combined hot / water deficit / salt treatment had died and could not be used in the analysis.

7.4.4 Results

7.4.4.1 Relative growth rate and water use

Genotype did not have a significant effect on relative growth rates of the four lines included in this experiment with overall mean values of 0.042, 0.041, 0.045 and 0.048 for 140 Ruggeri, MC 215-09, MI 07-33 and K51-40 respectively. Temperature had the smallest effect on relative growth rate with 0.048 and 0.040 in the cool and hot Smart Houses respectively. Remarkably, salt treatment and water deficit treatment had an equal overall effect on relative growth rate, with 0.056 in both the overall mean of the control treatments and capacity treatments, and 0.032 in the salt treatments and deficit treatments. Water deficit and salt treatments had an additive effect with the lowest mean growth rates under the salt/deficit treatment (0.025) compared with control/deficit or salt/capacity (both 0.039). The most severe effects of combined salt and deficit treatment were seen under the hot conditions, however this could not be fully quantified due to all three K51-40 cuttings dying in this treatment.

Genotype, temperature, salt and water deficit treatments all had highly significant individual effects on relative water use. The magnitude of the genotype effect was quite small however, with overall means of 13.3, 11.2, 10.9 and 10.7 for 140 Ruggeri, K51-40, MC 215-09 and MI 07-33 respectively. Salt treatment had the most severe effect on relative water use with overall means of 15.6 for control treated vines and 7.5 for salt treated vines. As with other experiment, higher temperature resulted in increased relative water use. In the cool Smart House overall mean relative water use was 9.3 compared with 13.8 in the hot Smart House. Interestingly water deficit treatment had a similar but opposite effect as temperature with overall mean relative water use values of 13.7 in the field capacity watered vines and 9.3 in the deficit treated vines.

7.4.4.2 Cl^- and Na^+ exclusion

Genotype, temperature, salt and water deficit treatments all had highly significant individual effects on both Cl^- and Na^+ exclusion. The interaction of genotype x temperature treatments was not significant on either Cl^- or Na^+ concentration in lamina in this experiment. However, there were significant interactions of genotype x salt and genotype x water deficit for both ions. Genotype x temperature x salt interactions were significant for Na^+ but not for Cl^-, whereas the interaction of genotype x temperature x water deficit was significant for Cl^- but not for Na^+. For both ions the interaction of genotype x salt x water deficit was highly significant, whereas the interaction of all four treatments was not significant.

For 140 Ruggeri and MC 215-09 there was no apparent effect of the deficit treatment under cool conditions, whereas both MI 07-33 and K51-40 accumulated more Cl^- under deficit treatment than control. In all genotypes hot temperature had a larger effect on Cl^- accumulation than did the deficit treatment, yet the combination of both hot temperature and deficit resulted in the highest levels of Cl^- accumulation.

7.4.5 Summary

The combination of heat, water deficit and salt treatments had a combined negative effect on each of the traits of relative growth rate, Cl^- exclusion and Na^+ exclusion. Water use efficiency did not follow the same trend as demonstrated by increases in vine growth per mL of water transpired by salt treated plants in these glasshouse experiments.

8 Outcomes and conclusions

The most significant outcome of this project is the identification and development of a genetic marker for sodium exclusion to assist future efforts to breed rootstocks with an ability to withstand saline conditions. This marker distinguishes between alleles of the *HKT1;1* gene, which encode transport proteins with differing functional activity, contributing to the vines ability to exclude sodium. As with any genetic marker, to be a really effective tool for the CSIRO rootstock breeding program the efficacy of this marker would need to be tested in potential parents of crosses and shown to align with the expected phenotype of the trait.

There is also potential to discover whether the other *HKT1* genes contribute to sodium exclusion in different tissues. This could lead to a suite of markers for sodium exclusion.

Chloride exclusion appears to be a more complex trait which is probably influenced by multiple genes and therefore has proved a more difficult trait to study. In this project we have identified a candidate gene for this trait and are working to characterise the gene function and provide a marker for future breeding opportunities.

Results from the salinity screens conducted in the Plant Accelerator were verified using a number of different experiments using grafted plants and comparing to previous results in field and pot experiments. Information from our screen of genetically diverse rootstocks and *V. vinifera* cultivars in the Plant Accelerator could be used to provide a ranking of the ability of each genotype to exclude sodium and chloride. This ranking could provide extra data for inclusion in the Rootstock Selector Tool.

9 Recommendations

Substantial progress was made in this project to develop markers for the exclusion of sodium from the lamina; however, results from glasshouse experiments suggest that there may be tissue specific differences in sodium uptake/sequestration that require elucidation before a conclusive suite of markers can be implemented in the breeding program.

Tantalising results from a rapid seedling screen were used to map a single genetic locus for chloride exclusion in a *V. berlandieri* x *V. vinifera* family. However, in the more complex K51-40 x 140 Ruggeri population this locus was not linked with the variation in chloride exclusion. Therefore, to develop this information into a robust genetic marker, a greater understanding of the influence of this locus in broader genotypes as well as characterisation of the function of potential gene candidates and verification of gene function using a transgenic approach is required. Some of this characterisation needs the specialist skills of our colleagues at the University of Adelaide in Prof Matt Gilliham's lab. The potential influence of ABA and root temperature on the complex trait of chloride exclusion warrants further investigation. Utilisation of the rapid seedling screen to identify new segregating populations may help identify other genetic loci linked with this multigenic trait.

The Plant Accelerator has proven to be a very powerful tool particularly in screens for ion exclusion, which is thought to be a root-driven trait. The large numbers of plants that can be grown at the same time under very similar conditions is not achievable in other growth facilities. Further experiments should be conducted using the Plant Accelerator to elucidate the complexities of ion exclusion for development of robust markers for breeding of rootstocks. Positive correlations between the relative growth rates of young salt treated vines in the Plant Accelerator and pruning weights of mature salt treated vines in the field shows the promise of the Plant Accelerator as a valuable tool for screening for salt tolerance.

Drought tolerance and water use efficiency traits are complex and will, necessarily, involve both the rootstock and scion. Consequently, only aspects that can be specifically assigned to the root system can be studied using ungrafted material, such as root interactions with the rhizosphere or differences in root morphology and structure. If the Australian Plant Phenomics Centre provides a facility to image the root system *in situ*, this could prove very useful for developing markers to these traits.

Heat tolerance was expected to be largely shoot driven, with the most likely influence of the rootstock to be via differences in ability to greatly upregulate water supply over a short time period (hours). This was only partially tested in the current project, but from the results obtained, it is recommended that future work in this area be undertaken in well-established grafted plants, preferably outdoors, in order to maximise the chance of observing rootstock conferred differences in whole vine hydraulics.

In line with the recommendations from CSP 1304, CSIRO selections that have shown to have good salt exclusion phenotypes were included in many of our experiments and should be further assessed for potential release.

10 Appendix 1: Communication

10.1 Presentations

Table: List of conference presentations that included some of the data that is described in this report.

Date	Event/Conference	Location	Presentation title	Type	Presenter
24 November 2013	CSIRO/Industry Rootstock Reference Group Meeting	CSIRO, Waite Campus	Genetic and mechanistic characterisation of rootstock traits conferring abiotic stress tolerance to grapevines	Oral	Mandy Walker
25 November 2015	CSIRO/Industry Rootstock Reference Group Meeting	CSIRO, Waite Campus	Developing genetic markers for Na^+ and Cl^- exclusion traits	Oral	Jake Dunlevy, Ev Edwards & Mandy Walker
3 May 2016	Treasury Wine Estate, Viticulturist Meeting	Penfolds Magill Estate Cellar Door, Adelaide, Australia	Improving the abiotic stress tolerance of grapevine rootstocks: A focus on salinity	Oral	Jake Dunlevy
10 May 2016	ARC Centre of Excellence in Plant Energy Biology annual retreat	La Trobe University, Melbourne, Australia	Allelic variation in gating and conductance of HKT1;1 controls grapevine sodium exclusion	Oral	Sam Henderson
29 May – 3 June 2016	Salt & Water Stress in Plants (Gordon Research Conference)	Les Diablerets Conference Center, Les Diablerets, Switzerland	Allelic variation in a grapevine HKT contributes to differential leaf sodium exclusion	Poster	Sam Henderson
5-10 June 2016	17th International Workshop on Plant Membrane Biology	Loew's Annapolis Hotel, Annapolis, Maryland, USA	Allelic variation in a grapevine HKT contributes to differential leaf sodium exclusion	Poster	Sam Henderson
29 July 29 2016	AWITC Rootstock Workshop 2016	Adelaide Convention Centre	New screening techniques for abiotic stress tolerance	Oral	Mandy Walker
25 August 2016	Waite Campus Conference	Waite Campus	How can we help stressed-out grapevines?	Oral	Mandy Walker
3-7 October 2016	ComBio	Brisbane Convention Centre, Brisbane, Australia	Allelic variation in a grapevine HKT contributes to differential leaf sodium exclusion	Poster	Sam Henderson

Date	Event/Conference	Location	Presentation title	Type	Presenter
4 December 2016	CSIRO Waite Campus Seminar	CSIRO, Waite Campus	Improving the abiotic stress tolerance of grapevine rootstocks: A focus on salinity	Oral	Jake Dunlevy
8-11 May 2017	ARC Centre of Excellence in Plant Energy Biology annual retreat	Adelaide Hills Convention Centre. Hahndorf, Australia	Natural variation in Na+ exclusion from grapevine shoots is determined by functional differences in HKT1;1	Oral	Sam Henderson
11 August 2017	University of Adelaide, Future Leaders Seminar	ACPFG, Waite Campus	Grapevine rootstock breeding: Developing markers to capture beneficial genetic diversity of wild *Vitis* species	Oral	Jake Dunlevy
2-5 October 2017	ComBio	Adelaide Convention Centre, Adelaide, Australia	Using Heterologous Expression to Functionally Characterise Proteins Involved in Grapevine Salt Exclusion	Oral	Sam Henderson
2-5 October 2017	ComBio	Adelaide Convention Centre, Adelaide, Australia	Unravelling the genetics of sodium exclusion in North American *Vitis* species to improve grapevine rootstock breeding strategies	Oral	Jake Dunlevy

10.2 Publications

Henderson SW*, Dunlevy JD*, Wu Y, Blackmore DH, Walker RR, Edwards EJ, Gilliham M, Walker AR (2018) Functional differences in transport properties of natural HKT1;1 variants influence shoot Na+ exclusion in grapevine rootstocks. New Phytologist **217:** 1113-1127

Walker RR, Blackmore DH, Gong H, Henderson SW, Gilliham M, Walker AR (2018) An analysis of the salt exclusion phenotype in rooted leaves of grapevine (Vitis spp.). Australian Journal of Grape and Wine Research **24**(3), 317-326.

***** Authors contributed equally

10.3 Industry articles (ready for submission with Wine Australia)

1. **Jake Dunlevy, Deidre Blackmore, Everard Edwards, Rob Walker, Bettina Berger and Mandy Walker** Developing a smart, rapid screening method for desirable grapevine rootstock traits

2. **Rob Walker, Deidre Blackmore, Everard Edwards, Jake Dunlevy and Mandy Walker**
   Rootstock salt exclusion at elevated root zone temperature

10.4 Media exposure

Henderson, Dunlevy *et al* (2018) gained media exposure through the following outlets:

1. http://www.barossaherald.com.au/story/5077325/new-discovery-to-accelerate-development-of-salt-tolerant-grapevines/
2. http://www.weeklytimesnow.com.au/agribusiness/horticulture/saline-tolerant-gene-found-in-grapevines/news-story/d1ab4888ebf9e437ddaa6ae274b03b05
3. http://www.newphytologist.org/blog/keeping-the-salt-out-of-grapes/
4. https://phys.org/news/2017-11-discovery-salt-tolerant-grapevines.html
5. https://www.adelaide.edu.au/news/news96722.html
6. Findings were also discussed live on air at 'Noosa FM' with Pete Dillon 24 Nov 2017.

10.5 Discussions with collaborators

Meetings were held between INRA Bordeaux and CSIRO staff

26-30 August 2013

11-13 April 2016

4-7 September 2017

12-17 November 2017

Meetings held with UC Davis and CSIRO staff

5 December 2016

12-21 November 2017

11 Appendix 2: Intellectual Property

The role of a gene identified in this project as a candidate encoding a protein involved in chloride exclusion will be considered for the basis of a patent application, if this role can be verified. This work is ongoing as part of the Bilateral Agreement between CSIRO and Wine Australia, Project 1.4.

Other data obtained in this project are already in the public domain or will form part of publications in internationally-recognised peer-reviewed journals. Some of the material has been published in New Phytologist (2018) or presented at industry meetings and will continue to be extended as opportunities arise.

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13 Appendix 4: Dunlevy: Final Report for 2016 Science Award for Young People in Agriculture, Forestry and Fisheries Summary

Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry
2016 Round
12-MONTH PROGRESS AND FINAL REPORT

Australian Government
Department of Agriculture and Water Resources



Wine Australia

1. **Description of your project** Please provide a short paragraph describing your project, including any improvements or variations. If your project changed from your original application please explain how and why.

Sodium (Na^+) and chloride (Cl^-) accumulation in grapevine leaves and berries is both damaging to vine health and detrimental to berry/wine quality. The aim of this project was to map genes underlying key salt tolerance traits of Na^+ and Cl^- exclusion in a sibling family of *Vitis* interspecific hybrids (n=40) derived from a cross between the rootstock genotypes K51-40 and 140 Ruggeri, and in a genetically diverse *Vitis* population (n=55). Funding permitted genotype by sequencing to be performed on these genotypes by Diversity Arrays Technology Pty Ltd. (DArT, Canberra). The data generated from this analysis enabled the identification of 80,000+ single nucleotide polymorphism (SNP) genetic markers across the genome. Association mapping was also performed by DArT, using Na^+ and Cl^- exclusion data already generated as part of another Wine Australia-CSIRO funded project (CSP 1302), revealing no strong marker-trait associations for Cl^- exclusion in either population. In the sibling family, the Na^+ exclusion trait was strongly associated with a large region on chromosome 11. Subsequent linkage map construction and quantitative trait loci (QTL) analysis delineated the borders of this locus, designated *NaE* for $\underline{Na}^+$ Exclusion, and revealed poor Na^+ exclusion is associated with the inheritance of two recessive alleles of this locus. However, the strong association of this locus with Na^+ exclusion was not observed in the genetically diverse population. This may be due to one of the recessive alleles being rarely represented in the diverse population, which could explain why the range in Na^+ accumulation concentration measured across the diverse *Vitis* population was lower than that observed in the sibling family.

14 Appendix 5: New Phytologist paper

New Phytologist

Research

Functional differences in transport properties of natural HKT1;1 variants influence shoot Na^+ exclusion in grapevine rootstocks

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Key words: 140 Ruggeri, hybrids, K51-40, North American rootstocks, salinity, site-directed mutagenesis, *Xenopus laevis* oocytes, yeast.

Summary

- Under salinity, *Vitis* spp. rootstocks can mediate salt (NaCl) exclusion from grafted *V. vinifera* scions enabling higher grapevine yields and production of superior wines with lower salt content. Until now, the genetic and mechanistic elements controlling sodium (Na^+) exclusion in grapevine were unknown.
- Using a cross between two *Vitis* interspecific hybrid rootstocks, we mapped a dominant quantitative trait locus (QTL) associated with leaf Na^+ exclusion (*NaE*) under salinity stress. The *NaE* locus encodes six high-affinity potassium transporters (HKT). Transcript profiling and functional characterization in heterologous systems identified *VisHKT1;1* as the best candidate gene for controlling leaf Na^+ exclusion.
- We characterized four proteins encoded by unique *VisHKT1;1* alleles from the parents, and revealed that the dominant HKT variants exhibit greater Na^+ conductance with less rectification than the recessive variants. Mutagenesis of VisHKT1;1 and TaHKT1.5-D from bread wheat, demonstrated that charged amino acid residues in the eighth predicted transmembrane domain of HKT proteins reduces inward Na^+ conductance, and causes inward rectification of Na^+ transport.
- The origin of the recessive *VisHKT1;1* alleles was traced to *V. champinii* and *V. rupestris*. We propose that the genetic and functional data presented here will assist with breeding Na^+-tolerant grapevine rootstocks.

Introduction

Saline soils and irrigation water reduce growth and productivity of crop species worldwide, which has negative economic, environmental and social impacts (Pannell, 2001; Munns & Gilliham, 2015). Grapevine (*Vitis vinifera* L.) is moderately sensitive to irrigation water and soil salinity (Maas & Hoffman, 1977), suffering decreased growth and yield (Prior *et al.*, 1992; Walker *et al.*, 2002; Stevens *et al.*, 2011). In addition, fruit and wine quality can be reduced due to the accumulation of sodium (Na^+) and chloride (Cl^-) ions in berries (Li *et al.*, 2013). Unfavourable salty and soapy attributes in wine are associated with excessive wine Na^+ and Cl^- concentrations (Walker *et al.*, 2003; de Loryn *et al.*, 2014), whereas high salt concentrations in grape juice reduce fermentation efficiency by decreasing the viability of wine yeasts, and result in undesirable increases in acetic acid content of wine (Donkin *et al.*, 2010). Some countries impose legal limits for Na^+ and Cl^- concentrations permissible in wines (Leske *et al.*, 1997; de Loryn *et al.*, 2014). The Organisation Internationale de la Vigne et du Vin (OIV) recommends a maximum free Na^+ concentration of 60 mg l^{-1} in wine (Stockley & Lloyd-Davies, 2001). Wineries often reject fruit with Na^+ concentrations above this level. Growers may attempt to negate salinity in vineyards by flushing salts from soils with large volumes of irrigation water. However, this practice is not economically viable or practical in regions with limited quality water – a problem expected to worsen with climate change (Elliott *et al.*, 2014). A more cost-effective and efficient approach to limit the effects of salinity on grapevines is by grafting onto rootstocks that can restrict root-to-shoot transport of Na^+ and Cl^-, a trait referred to as shoot ion exclusion. Because wineries use Na^+ concentrations in berries and grape juice as a basis for rejection, finding a genetic solution for shoot Na^+ accumulation in grapevine would be advantageous to the wine industry so it can maintain quality wine production in salt-affected regions.

Grapevine rootstocks derived from wild North American *Vitis* species were first used in viticulture in the late 19th Century to provide resistance to the soil-born parasite phylloxera, which destroyed vineyards throughout Europe – an episode called the Great French Wine Blight (Stevenson, 1980). An estimated 80% of vines in vineyards worldwide are now grafted onto interspecific

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rootstocks (Ollat *et al.*, 2016). Rootstocks are selected for beneficial traits including phylloxera resistance (Benheim *et al.*, 2012), nematode resistance (Ferris *et al.*, 2012), controlled vigour and yield (Walker *et al.*, 2002), drought resistance (Serra *et al.*, 2014) and ion exclusion (Tregeagle *et al.*, 2010). Rootstock breeding programmes can utilize marker-assisted selection to hasten the pyramiding of key rootstock traits into new elite genotypes (Ollat *et al.*, 2016), yet little is currently known about the genetic mechanisms controlling ion exclusion in grapevines.

Most grapevine studies have concentrated on Cl^- toxicity (Ehlig, 1960; Downton, 1977a; Walker *et al.*, 2004) and the mechanistic basis of Cl^- exclusion from shoots (Gong *et al.*, 2011; Henderson *et al.*, 2014, 2015; Fort *et al.*, 2015). In other crops, shoot Na^+ exclusion is more widely studied and it is acknowledged that the major component of both Na^+ and Cl^- exclusion from plant shoots occurs via root-based mechanisms (Munns & Gilliham, 2015; Li *et al.*, 2017). As a result, Na^+ exclusion from leaves correlates well with exclusion from berries (Supporting Information Fig. S1; Walker *et al.*, 2004), and can be used as a proxy measure of whole shoot Na^+ exclusion *per se*.

Quantitative trait loci (QTLs) for shoot Na^+ exclusion in bread wheat (*Kna1*), durum wheat (*Nax1* and *Nax2*), rice (*SKC1*) and tomato are underpinned by genes encoding high-affinity potassium (K^+) transporter (HKT) proteins (Ren *et al.*, 2005; Byrt *et al.*, 2007; James *et al.*, 2011; Asins *et al.*, 2013). HKT proteins belong to the Ktr/TrK/HKT family of monovalent cation transporters present in plants, bacteria and fungi, but not in animals (Corratgé-Faillie *et al.*, 2010). Proteins from the HKT1 subgroup selectively transport Na^+ over other cations (Mäser *et al.*, 2002; Horie *et al.*, 2009; Waters *et al.*, 2013), and confer Na^+ exclusion from shoots through retrieval of Na^+ from root xylem vessels into surrounding xylem parenchyma cells (Sunarpi *et al.*, 2005; Davenport *et al.*, 2007; Møller *et al.*, 2009; Byrt *et al.*, 2014). Emerging evidence indicates that molecular mechanisms of HKT1-mediated salinity tolerance between plants may depend upon not only expression differences, but also, in some cases, subtle differences in functional properties that may be attributed to single amino acid substitutions and possible allelic variations (Ren *et al.*, 2005; Rus *et al.*, 2006; Baxter *et al.*, 2010; Cotsaftis *et al.*, 2012; Ali *et al.*, 2016; Tounsi *et al.*, 2016). The benefits of identifying natural variants of HKT has been demonstrated with HKT1.5 from salt-tolerant wheat *Triticum monococcum* which, when introgressed into salt-sensitive *T. durum*, increased grain yield on saline soil by 25% in the field (Munns *et al.*, 2012).

Here, we examine the genetic basis of Na^+ exclusion in grapevine. We take advantage of a family of heterozygous hybrid vines derived from a cross between two interspecific rootstock hybrids K51-40 (*V. champinii* × *V. riparia*) and 140 Ruggeri (*V. berlandieri* syn. *V. cinerea* var. Helleri × *V. rupestris*) that was previously shown to exhibit variation in leaf Na^+ by 30-fold (Gong *et al.*, 2014). We identified a QTL, named *NaE* for Na^+ exclusion, containing six closely located *HKT1* genes, of which one was expressed in roots and encoded a functional Na^+ transporter. Differences in *HKT1;1* alleles from the parent rootstocks were identified and used to investigate the importance of key amino acid residues contributing to differences in Na^+ transport properties and variation in shoot Na^+ exclusion in the hybrid progeny. The identified single nucleotide polymorphisms are being utilized by breeding programmes for generating Na^+-excluding rootstock germplasm via marker-assisted selection.

Materials and Methods

Grapevine material and Na^+ screens

A family of 40 hybrid rootstocks from a cross between K51-40 (*Vitus champinii* × *V. riparia*) and 140 Ruggeri (*V. berlandieri* × *V. rupestris*) (Gong *et al.*, 2011), together with both parents, were screened for leaf sodium (Na^+) exclusion ability in The Plant Accelerator Phenomics Facility, Adelaide, Australia in December 2013. Dormant cuttings were callused for 2 wk at 28°C (Yalumba Nursery, Nuriootpa, SA, Australia) and transferred to a 50 : 50 sand : perlite mix under regular misting for 4 wk to initiate root development. Rooted cuttings were transplanted into 1.8 kg of potting mix (6 mm Premium Mix Van Schaik's; Biogro, Mt Gambier, SA, Australia) in 2.5-l pots, established for 4 wk, then loaded onto The Plant Accelerator's conveyor belt. Before loading, vines were trimmed to a single shoot and axillary growth was continually removed. Each genotype consisted of three replicate vines, in three randomized blocks. Salt applications, consisting of 10 : 6 : 1 : 1 ratio of Cl^- : Na^+ : Mg^{2+} : Ca^{2+}, were applied to pots in containers (to avoid run through) on days 1, 3 and 7, equating to soil Na^+ concentrations ramping from 21 to 42 to a final concentration of 60 mM. On all other days, the pots were automatically weighed and watered to a target weight to replace water lost through evapotranspiration, ensuring all pots maintained equivalent salt concentrations at full water capacity. On Day 13, laminae of fully matured leaves of each vine were harvested and dried at 65°C for 2 d. For determining Na^+ content, powdered sample (100 mg) was digested in 2 ml concentrated HNO_3 at 95°C, diluted to 12 ml with deionized water, and analysed by CSIRO Analytical Services Unit (Adelaide) using inductively coupled plasma optical emission spectrophotometry (ThermoFisher, Cambridge, UK).

In a subsequent experiment in 2014, five selected hybrid progeny, the parents, accessions of the grandparent species, *V. champinii* 'Dogridge', *V. riparia* 'Gloire', *V. berlandieri* 'R1xMazade' and *V. rupestris* 'du Lot', and 10 common *V. vinifera* cultivars, were screened to compare Na^+ exclusion abilities. Establishment of cutting material and experimental conditions were similar to the first hybrid Na^+ screen, except final salt application occurred on Day 8, and leaves were harvested on Day 19.

Linkage mapping and QTL analysis

Genome-wide single nucleiotide polymorphism (SNP) data were generated by Diversity Arrays Technology Pty Ltd (Canberra, Australia) using DArTSeq™ genotype by sequencing (GBS). This uses *PstI*/*TaqI* restriction digestion of genomic DNA followed by PstI-specific adapter targeted short-read sequencing using HiSeq2500 (Illumina, San Diego, CA, USA). The DArTSeq

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methods were performed as described in Courtois *et al.* (2013), except that the sequence reads were aligned to the grapevine reference genome (Jaillon *et al.*, 2007). SNP markers aligned to the genome and those with call rates of 1 were used to construct a consensus linkage map, in JoinMap® v.4.1 software using maximum-likelihood mapping (Van Ooijen, 2006). A consensus framework of linkage groups was obtained using a logarithm of odds (LOD) likelihood score between 3.0 and 5.0, and the Haldane mapping function to calculate the genetic distance between markers. Chromosome assignment of linkage groups was determined from the alignment of DArTSeq SNP reads to the grapevine reference genome (Jaillon *et al.*, 2007). Quantitative trail loci (QTLs) for Na^+ exclusion were investigated using interval mapping analysis performed in MapQTL® v.6 (Van Ooijen & Kyazma, 2009). The permutation test was used to estimate the genome wide empirical threshold for QTL detection based on LOD values of $P < 0.01$. Interval mapping resulted in a single significant QTL. To identify potential small modifying QTLs, multiple QTL mapping (MQM) analysis was undertaken with the strongest marker from this QTL used as a cofactor. No other significant QTLs were detected.

Sequencing and cloning

VviHKT1;1 and *VviHKT1;3* coding sequences (HKT, high-affinity potassium transporters) were PCR-amplified from *V. vinifera* (cv Cabernet Sauvignon) root cDNA using Phusion Polymerase (ThermoFisher Scientific) and primers designed to these gene sequences (Table S1). Purified fragments were ligated to pCR8/GW/TOPO. To identify polymorphisms in each genotype, the *VisHKT1;1* gene was PCR-amplified from K51-40 and 140 Ruggeri genomic DNA, and sequenced (accession numbers in Table S2). Using polymorphisms as a reference, coding sequences of *VisHKT1;1* allelic variants were PCR-amplified from root cDNA of parent rootstocks using PfuUltra II Fusion HS DNA Polymerase (Agilent, Santa Clara, CA, USA), and ligated to pENTR/D-TOPO. *VisHKT1;1-E*^{K} and *VisHKT1;1-e*^{K} were isolated from K51-40, and *VisHKT1;1-E*^{R} and *VisHKT1;1-e*^{R} from 140 Ruggeri. Cleavage-amplified polymorphic sequence (CAPS) markers designed to score the inheritance of each allele are given in Table S3.

VviHKT1;1 sequences from *V. champinii*, *V. riparia*, *V. berlandieri*, *V. rupestris*, and *V. vinifera* cultivars Grenache, Merlot, Pinot noir, Shiraz, Chardonnay, Pinot gris, Riesling, Sauvignon blanc and Semillon, were obtained by sequencing PCR products amplified from genomic DNA.

Subcellular localization

Vitis vinifera (cv Cabernet Sauvignon) *VviHKT1;1* without a stop codon was recombined with pMDC83 (Curtis & Grossniklaus, 2003) using LR Clonase II (Life Technologies, Carlsbad, CA, USA) to generate 2x35S:*VviHKT1;1-GFP*. Plasmid ER-rk containing HDEL-mCherry (Nelson *et al.*, 2007) was used for co-localization. Plasmid pUBN-GFP-DEST (Grefen *et al.*, 2010) was used to express cytosolic green fluorescent protein (GFP). Plasmid pEAQ-HT-DEST harbouring p19 gene-silencing suppressor was used to maximize expression (Sainsbury *et al.*, 2009). Vectors were incorporated into *Agrobacterium tumefaciens* (Agl-1) via freeze-thawing. Overnight cultures of *A. tumefaciens* harbouring pMDC83-VviHKT1;1, ER-rk, pUBN-GFP-DEST or pEAQ-HT-DEST were resuspended in 10 mM $MgCl_2$, 150 μM acetosyringone and 10 mM MES pH 5.6. Cultures were combined as follows with final OD_{600} shown in brackets: pMDC83-VviHKT1;1 (0.5) and pEAQ-HT-DEST (0.2); pMDC83-VviHKT1;1 (0.5), ER-rk (0.2) and pEAQ-HT-DEST (0.2); pUBN-GFP-DEST (0.5) and pEAQ-HT-DEST (0.2). Bacterial suspensions were infiltrated into the abaxial side of fully expanded leaves of 5-wk-old *Nicotiana benthamiana* with a 1-ml syringe. Leaf sections were imaged after 2 d using a Nikon A1R confocal laser scanning microscope with ×63 water objective lens and NIS-Elements C software (Nikon Corp., Tokyo, Japan). FM4-64 (Sigma, St Louis, MO, USA) was used to infiltrate tobacco leaves and imaged after 10 min at room temperature. Under these conditions, the plasma membrane (not endomembrane) is predominantly stained. Excitation/emission conditions were GFP (488 nm/500–550 nm), FM4-64 and mCherry (561 nm/570–620 nm).

Site-directed mutagenesis

Site-directed mutants of *VisHKT1;1* allelic variants were generated in yeast and *Xenopus laevis* expression vectors. Mutagenesis was performed by inverse PCR of expression vectors using primers (see Table S1) with 15-bp overlaps at their 5′ ends, and mutations incorporated within. Reactions contained 0.2 ng plasmid, 500 nM forward and reverse primers and 0.25 units Phusion Polymerase (ThermoFisher Scientific) in 25 μl. Reactions were treated with Cloning Enhancer (Clontech Laboratories Inc., Mountain View, CA, USA), and re-circularized using In-Fusion HD (Clontech).

Oocyte expression assays

cDNAs encoding *VisHKT1;1* variants were recombined into the *X. laevis* expression vector pGEMHE-DEST, using LR Clonase II (Life Technologies). Plasmids were linearized with *Nhe*I or *Sbf* I (New England Biolabs, Ipswich, MA, USA). Capped RNA (cRNA) was synthesized *in vitro* with mMessage mMachine T7 kit (Ambion, Carlsbad, CA, USA) using linear plasmids as templates. cRNA was purified by phenol/chloroform extraction followed by ethanol precipitation and elution in water. Stage V and VI oocytes were injected with 42 nl cRNA (21 ng), or sterile water. Injected oocytes were incubated in calcium Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl, 0.6 mM $CaCl_2$, 5 mM Hepes, 5% (v/v) horse serum, 500 μg ml^{-1} tetracycline and 1× penicillin-streptomycin (Sigma P4333)). Electrophysiology was performed 1 d after injection.

Electrophysiology

Whole-cell currents were recorded using two-electrode voltage clamping (TEVC) on a Roboocyte with integrated ClampAmp

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amplifier (Multichannel Systems, Reutlingen, BW, Germany). Electrodes were filled with 3 M KCl. Oocytes were perfused with solutions containing 1.8 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM MES, variable Na^+-gluconate, pH 5.5 (adjusted with Tris) and osmolality of 230 mOsm kg^{-1} (adjusted with D-mannitol). From a holding potential of −40 mV, oocytes were clamped stepwise from +60 mV to −140 mV in 20 mV decrements for 550 ms. Currents mediated by grapevine or wheat HKT1;1 were determined by subtracting mean currents of water-injected controls from the same batch of oocytes in the same solutions.

Yeast assays

VisHKT1;1 variants and VviHKT1;3 (Cabernet Sauvignon) were recombined into pYES-DEST52 using LR Clonase II (Life Technologies). *Saccharomyces cerevisiae* strain INVSc2 (MATa, *his3Δ-1*, *ura3-52*) was transformed with plasmids using the lithium acetate procedure. Transformants were selected on yeast nitrogen base (YNB) (Difco) without uracil, with 2% (w/v) D-glucose. For Na^+ toxicity assays, yeast was grown overnight in YNB without phosphates or NaCl (MP Biomedicals, Santa Ana, CA, USA), supplemented with 1 g l^{-1} KH_2PO_4, 95 mg l^{-1} L-histidine-HCl, and 2% (w/v) D-glucose. Yeast was diluted to an OD_{600} of 1.0 in sterile water. Ten-fold serial dilutions were prepared in sterile water, and 5 µl spotted onto plates containing YNB without phosphates and without NaCl (MP Biomedicals), supplemented with 1 g l^{-1} KH_2PO_4, 95 mg l^{-1} L-histidine-HCl, 1.5% (w/v) D-raffinose, 0.001–0.005% (w/v) D-galactose for induction and 0.25% phytagel (Sigma). The basal Na^+ concentration was *c.* 500 µM from phytagel. For Na^+ toxicity screening, growth was monitored on media containing 50 mM NaCl. Control plates to demonstrate equal dilutions contained 2% (w/v) D-glucose. Plates were incubated at 28°C for 3 d.

Quantitative real time PCR (qPCR)

RNA from root stele and root epidermis/cortex enriched tissue was prepared from grapevine rooted leaves as described previously (Henderson *et al.*, 2014). cDNA was synthesized from 0.5 µg total RNA using Superscript III (Life Technologies) following the manufacturers' procedures. qPCR was performed on a QuantStudio 12K Flex Real-Time PCR machine (Thermo Fisher Scientific). Ten microlitre qPCR reactions contained 250 nM forward and reverse primer, 1× KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Charlestown, MA, USA) and 1 µl cDNA (diluted 1 : 4). Triplicate reactions were performed using 40 cycles of the following: 95°C 1 s, 55°C 15 s, 72°C 5 s. Melt curve analysis was performed to ensure a single band was amplified. Relative expression levels were calculated using primer pair efficiencies (*E*) and normalized to *VvElongation-factor-1-α* using the formula ($E_{HKT1;1}^{\Delta ct}/E_{EF1\text{-}\alpha}^{\Delta ct}$).

VisHKT1;1 allele-specific qPCR reactions were performed using forward and reverse primers (Table S1) containing allele-specific SNPs in their 3′ terminal base. Specificity was achieved by performing reactions as described above, except annealing at 63°C. Allele copy numbers were estimated by interpolation from standard curves made from serial dilutions of linearized pGEM-HE plasmids quantified by UV-spectrophotometry. Data were normalized to cDNA volume. The sum of two allele copy numbers was compared to total copies of *VisHKT1;1* transcript using nonspecific primers in the same samples.

Statistical analysis

Data were analysing GraphPad Prism v.7.00 for Windows (GraphPad, La Jolla, CA, USA). All data are presented as mean ± SE. Means were compared using the different statistical tests described in the figure legends.

Results

Mapping a major QTL for Na^+ exclusion in a hybrid grapevine population

We aimed to detect QTL for leaf Na^+ exclusion in a population of hybrid rootstocks derived from a cross between K51-40 and 140 Ruggeri (Gong *et al.*, 2014). Forty hybrids were exposed to a mixed cation treatment for 13 d, and the lower leaves (lamina only) were harvested for analysis of Na^+ accumulation. A >40-fold difference in Na^+ accumulation was seen within the hybrid progeny with the lowest, 0.005% DW, and the highest, 0.213% DW, whereas two parents K51-40 and 140 Ruggeri had similar leaf Na^+ concentrations of 0.016 and 0.015% DW, respectively (Fig. 1a). The transgressive variation seen in this near-Mendelian trait indicated that it was probably heterozygous in each parent. A broad sense heritability score of 0.91 was estimated for Na^+ exclusion in this population, suggesting a high degree of genetic control.

GBS was performed on the 40 hybrids and parents. The resulting SNP data were used to construct a consensus linkage map consisting of 514 SNP markers distributed over 19 linkage groups, at a mean interval of 5.3 cM (Fig. S2). A single major QTL explaining up to 72% of the genetic variation in Na^+ exclusion was identified on chromosome 11, spanning a 42-cM region of *c.* 14 Mb (Fig. 1b). We named this locus *NaE*, for Na^+ exclusion. SNP markers underlying the peak of the *NaE* QTL were used to group the 40 hybrids based on their inheritance of this locus, and confirmed that the associated alleles were likely to be heterozygous in both parents (Fig. 1a). This analysis revealed that the Na^+ exclusion trait is dominant over Na^+ accumulation, because hybrids with low Na^+ accumulation generally were associated with the inheritance of one or more dominant loci, designated as NaE^K and NaE^R for excluder locus derived from K51-40 and 140 Ruggeri. By contrast, high Na^+ accumulation generally was associated with the inheritance of two recessive loci, designated Nae^K and Nae^R (Fig. 1a).

Based on the grapevine reference genome (Jaillon *et al.*, 2007), the *NaE* locus contains 583 genes (Table S4). Six *HKT1* genes were clustered within a 320-Kb region located close to the peak LOD score of the *NaE* locus (Fig. 1b,c) and knowledge of Na^+ exclusion from other plant species suggested that members of the *HKT1* family were strong candidate genes. Phylogenetic analysis

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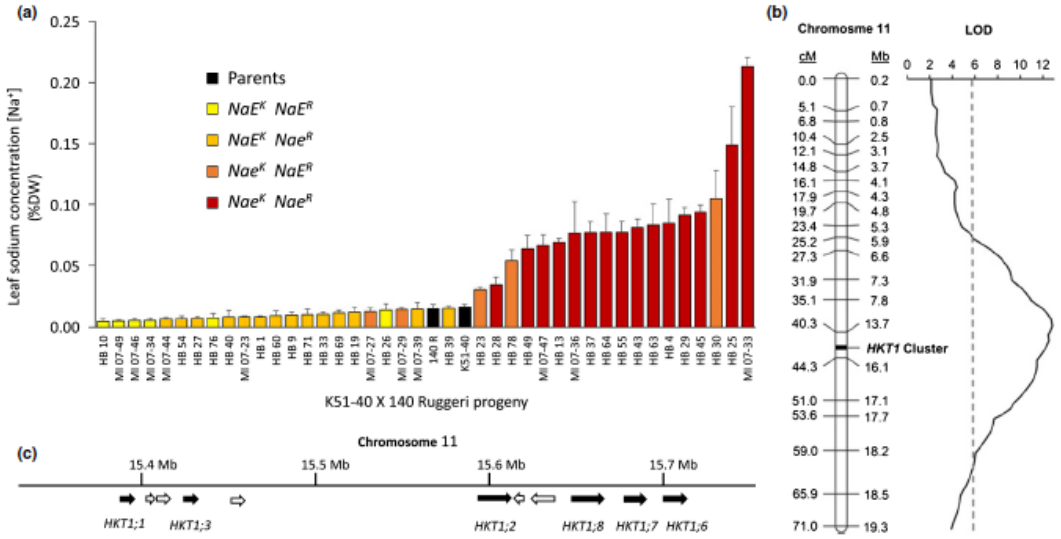


Fig. 1 Sodium (Na^+) exclusion is associated with a major quantitative trait locus (QTL), *NaE*, on chromosome 11 which contains a cluster of six *HKT1* genes. (a) Leaf Na^+ concentration in a population of *Vitis* interspecific rootstock hybrids. The parents K51-40 and 140 Ruggeri are indicated in black. Hybrid progeny are coloured according to their inheritance of single nucleotide polymorphism (SNP) markers at 13.7 Mb within the *Na^+ exclusion* (*NaE*) locus. Hybrids homozygous for the dominant *NaE* locus are indicated in yellow and progeny homozygous for recessive *nae* locus are indicated in red. Progeny heterozygous for a dominant NaE^K locus from K51-40 and a recessive nae^R locus from 140 Ruggeri are indicated in mustard, whereas progeny heterozygous for a recessive nae^K locus from K51-40 and dominant NaE^R locus from 140 Ruggeri are indicated in orange. Bars are mean + SE of three biological replicates. (b) Schematic of chromosome 11 Na^+ exclusion QTL mapping interval, shows logarithm of odds (LOD) score against map units (in cM) (left) and the physical position of SNPs (in Mb) corresponding to the grapevine reference sequence (right). Vertical dashed line represents the genome-wide significance threshold *P*-value of 0.01. (c) Relative positions of *HKT1* genes (black arrows) on part of chromosome 11 based on the grapevine reference genome. *VviHKT1;1* (VIT_211s0103g00010), *VviHKT1;3* (VIT_211s0103g00050), *VviHKT1;2* (VIT_211s0103g00090), *VviHKT1;8* (VIT_211s0103g00130), *VviHKT1;7* (VIT_211s0103g00140), *VviHKT1;6* (VIT_211s0103g00150). White arrows signify unrelated genes. Not to scale.

of these grapevine HKT1s against HKTs from other plant species has been reported previously (Ali *et al.*, 2012). This showed that, of the well-characterized HKTs, all grapevine HKT1s are most similar to rice OsHKT1;1. The grapevine HKT1s fall into two subclades, whereby VviHKT1;1, VviHKT1;2 and VviHKT1;3 share a degree of homology (53–63% identity), and VviHKT1;6. VviHKT1;7 and VviHKT1;8 share higher sequence similarity (85–97% identity) (Fig. S3).

HKT1;1 is expressed in the root stele and encodes a Na^+ selective plasma membrane transporter

In wheat and Arabidopsis, HKT proteins in roots regulate Na^+ accumulation in shoots by controlling Na^+ retrieval from root xylem vessels (Davenport *et al.*, 2007; Munns *et al.*, 2012; Byrt *et al.*, 2014). We therefore mined genome-wide expression data to determine which grapevine *HKT1* transcripts were expressed in roots. Only two *HKT1* transcripts, *VviHKT1;1* and *VviHKT1;3* were highly abundant in whole roots of *V. vinifera*, and both transcripts were present at similar levels (Fig. 2a). To identify whether *VviHKT1;1* and *VviHKT1;3* encoded Na^+ transport proteins, full-length cDNAs corresponding to each protein were isolated and expressed in *X. laevis* oocytes. When oocytes were clamped at negative membrane potentials, VviHKT1;1 mediated large inward currents (2 μA at −140 mV) in the presence of 30 mM Na^+ (Fig. 2b), whereas VviHKT1;3 did not increase currents above the level of the water control (Fig. 2b). Furthermore, functional characterization in yeast indicated that *VviHKT1.3* did not function as a Na^+ transporter because its expression did not lead to inhibition of yeast growth on plates containing 50 mM Na^+ unlike *AtHKT1* (Fig. S4). These findings implicate *HKT1;1* as a gene important for Na^+ exclusion in grapevine, whereas the function of *HKT1;3* remains unknown.

Quantitative PCR of root epidermal/cortical and stelar fractions of K51-40 and 140 Ruggeri showed that *HKT1;1* transcripts were more abundant in the root stele of both genotypes (Fig. 2c). Expression of *VviHKT1;1* with a C-terminal GFP tag in tobacco epidermis showed a strong signal that co-localized with the lipohilic dye FM4-64 at the plasma membrane, but did not co-localize with free cytoplasmic GFP, or the endoplasmic reticulum (ER) marker HDEL-mCherry (Fig. 2d,e). These findings confirm that VviHKT1;1 protein is likely to be localized on the plasma membrane of grapevine root stelar cells.

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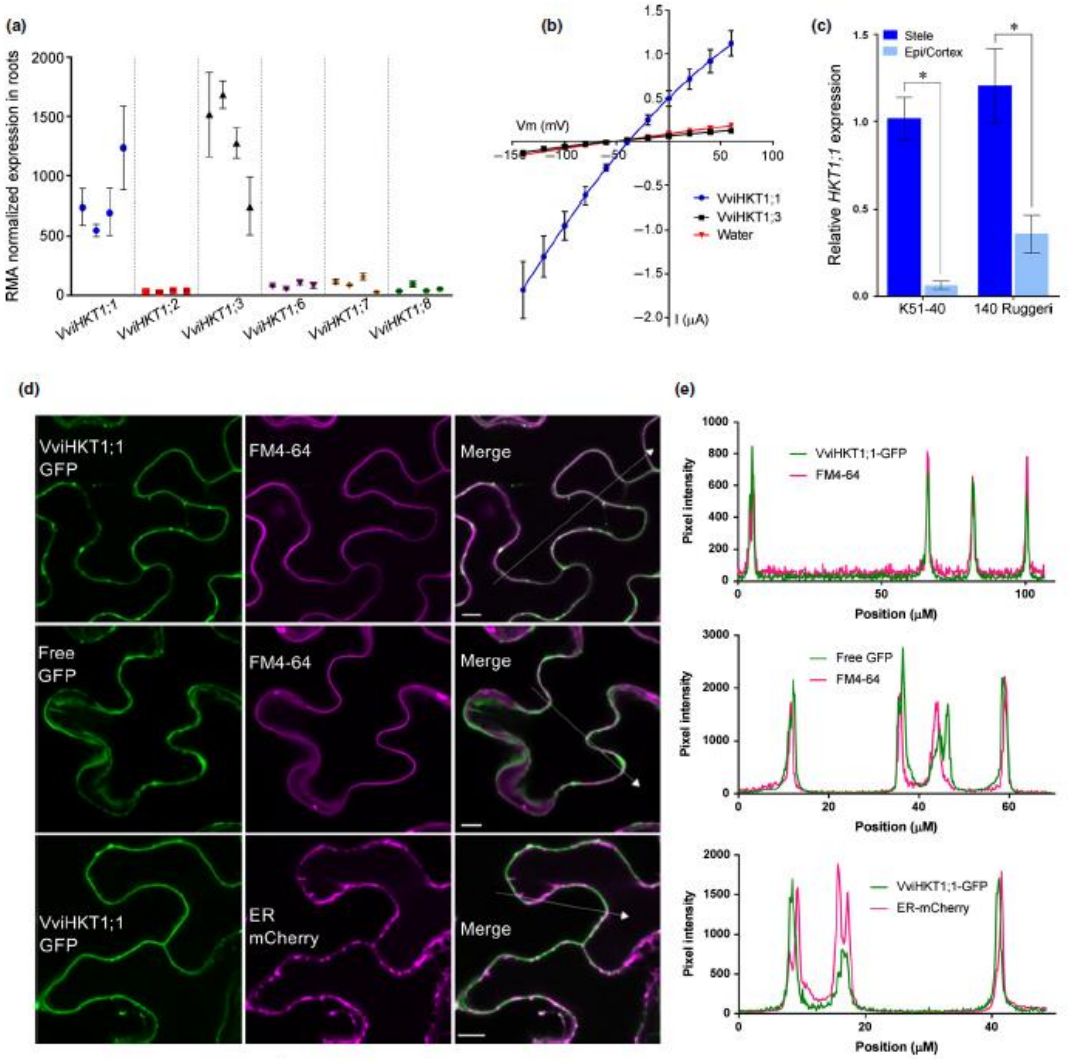


Fig. 2 HKT1;1 is a functional sodium (Na^+) transporter on the plasma membrane of grapevine root stelar cells. (a) Expression level of high-affinity potassium (K^+) transporter (*HKT*) transcripts in *Vitis vinifera* roots determined by microarray hybridization. Each point represents a unique probe from the microarray. Data are mean ± SE of three biological replicates. Data are from Fasoli *et al.* (2012). (b) Current–voltage relationship of *Xenopus laevis* oocytes injected with water (red diamonds) *VviHKT1;3* (black squares) or *VviHKT1;1* (blue circles). Oocytes were clamped from +60 to −140 mV in a solution containing 10 mM Na^+-gluconate, pH 5.5. Data are mean ± SE ($n = 3$ oocytes). (c) Relative *HKT1;1* transcript abundance in root tissue enriched in stelar (dark blue bars) or epidermal/cortical cells (light blue bars) from grapevine rootstocks K51-40 and 140 Ruggeri. Data are means ± SE of three biological replicates of pooled tissue from multiple plants. Data are relative to the K51-40 stelar replicate with the greatest transcript abundance. Asterisk denotes significant difference between tissue types ($P < 0.05$; Student's *t*-test). (d) Tobacco (*Nicotiana benthamina*) leaf epidermal cells expressing VviHKT1;1-GFP (upper panels), free GFP (middle panels), or VviHKT1;1-GFP plus the endoplasmic reticulum (ER) marker HDEL-mCherry (lower panels). Leaves in the upper and middle panels were imaged 10 min after infiltration with FM4-64, which predominantly stained the plasma membrane. Leaves were imaged by confocal microscopy 2 d after agroinfiltration. Bars, 10 μm. (e) Signal profiles of GFP (green) and FM4-64 or mCherry (magenta) corresponding to the arrow in the adjacent merged image from (d). Overlapping peaks indicate colocalization between the two signals.

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Four unique *HKT1;1* alleles from K51-40 and 140 Ruggeri display different functional properties

HKT1;1 coding sequences were isolated from the two parents of the mapping population, K51-40 and 140 Ruggeri, and given the prefix *Vis* for *Vitis interspecific*. Four unique *VisHKT1.1* alleles were identified, two from each parent (Figs 3a, S5). CAPS markers designed to the *VisHKT1;1* alleles were used to score hybrids for their allelic inheritance. Resulting marker scores were identical to that of SNPs mapped nearby (Fig. 1a); hence, we named the *VisHKT1.1* alleles as per the associated *NaE* locus (*VisHKT1;1-E*^{K} and *VisHKT1;1-e*^{K} from K51-40; *VisHKT1;1-E*^{R} and *VisHKT1;1-e*^{R} from 140 Ruggeri). Eighteen amino acid differences were observed between encoded proteins of the four alleles (Figs 3a, S5). Six polymorphic residues, at positions 106, 129, 163, 391, 534 and 537, were conserved between the two *VisHKT1;1-E* allelic variants and also between the two *VisHKT1;1-e* variants (Fig. 3a).

In order to assess whether allele-specific expression contributes to the variation in Na^+ exclusion in the hybrid population, absolute transcript abundance of these four alleles in root fractions was quantified in the parents using allele-specific primers. Expression of *HKT1;1* alleles in root epidermal/cortical and stelar fractions, assayed by qPCR, showed *VisHKT1;1-E* alleles to be approximately two-fold more abundant than *VisHKT1;1-e* alleles (Fig. 3b). There were no differences in expression of *VisHKT1;1-E*^{K} and *VisHKT1;1-E*^{R} or between *VisHKT1;1-e*^{K} and *VisHKT1;1-e*^{R} alleles (Fig. 3a). The sum of *VisHKT1;1-E* and *VisHKT1;1-e* transcript copy number was equivalent to the total *VisHKT1;1* copy number using non-allele-specific *HKT1;1* primers in each sample, demonstrating that the PCR primers were highly allele-specific (Fig. 3b).

For functional analysis, the *VisHKT1;1* alleles were first expressed in yeast. On control plates containing glucose, which represses gene expression, all strains grew at the same rate demonstrating equal dilution (Fig. 3c). When *VisHKT1;1* allelic variants were expressed by substituting glucose with raffinose and galactose (which induces gene expression), all strains grew at a similar rate on low (0.5 mM) Na^+ (Fig. 3c). Conversely, growth of yeast expressing *VisHKT1;1* allelic variants on induction medium was strongly inhibited by 50 mM Na^+, whereas growth of yeast containing an empty vector was not inhibited (Fig. 3c). This suggests that each grapevine VisHKT1;1 allelic variant transports Na^+ in yeast. Na^+-induced growth-inhibition was greater in yeast strains expressing *VisHKT1;1-E* variants compared to *VisHKT1;1-e* variants from both parents (Fig. 3c), implying that VisHKT1;1-E proteins have higher Na^+ transport activity than VisHKT1;1-e proteins.

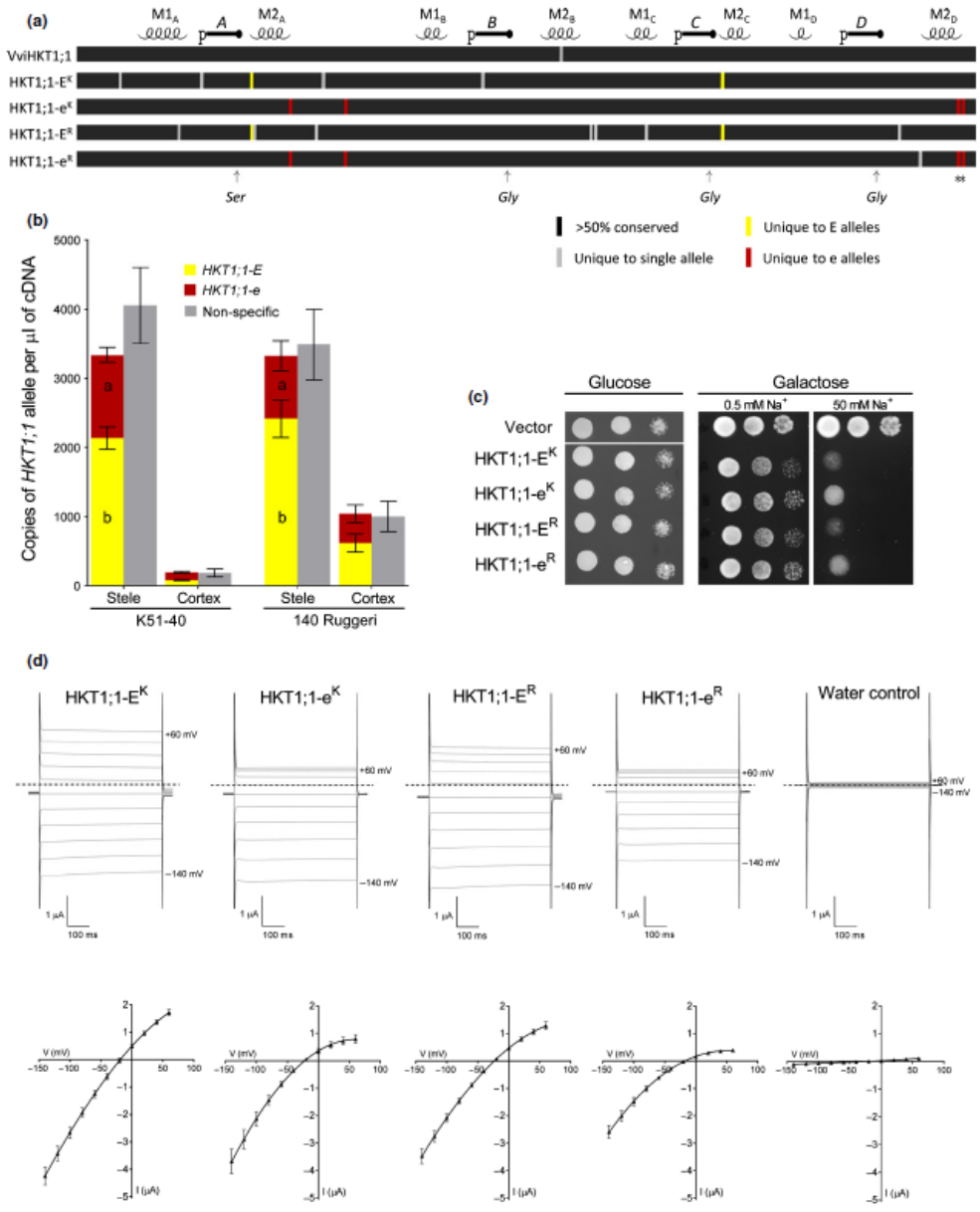
In order to confirm these differences, we performed TEVC of *X. laevis* oocytes injected with different VisHKT1;1 allelic variants. When perfused with 30 mM external Na^+ ($[Na^+]_{ext}$), VisHKT1;1-E variants mediated Na^+ transport in both directions across the oocyte membrane (Fig. 3d). Conversely, both VisHKT1;1-e variants displayed inward rectification – characterized by inward currents at negative voltages but restricted outward currents at positive voltages (Fig. 3d). We quantified inward rectification as the ratio between inward (−140 mV) and outward (+60 mV) chord conductance (G_{Na+}), where a ratio of 1 indicates equal G_{Na+} in both directions. From both rootstocks, VisHKT1;1-E variants had lower (*c.* 50%) inward rectification ratios than VisHKT1;1-e, irrespective of $[Na^+]_{ext}$ (Fig. S6). Inward rectification is an intrinsic property of grapevine VisHKT1;1-e variants, and not due to differences in $[Na^+]_{int}$, because increasing $[Na^+]_{ext}$ did not significantly alter the reversal potential (E_{rev}) between allelic variants, and E_{rev} followed a positive Nernstian shift (Fig. S7). In addition to differences in inward rectification, we also observed differences in the inward current at physiologically relevant negative membrane potentials in plants (−140 to −80 mV) between the VisHKT1;1 variants (Fig. 3d). To examine this in detail, we thoroughly investigated the difference between VisHKT1;1-E^K and VisHKT1;1-e^K, finding that the VisHKT1;1-E^K variant displayed *c.* 25% larger currents at −140 mV in 30 mM Na^+ solutions, and that this difference was consistent across different batches of oocytes (Fig. S8).

We examined monovalent cation selectivity of different VisHKT1;1 allelic variants in oocytes. All variants were highly selective for Na^+, mediating large inward currents in 10 mM NaCl, each with an E_{rev} close to the predicted Nernst potential for this cation (Fig. S9). When Na^+ was substituted for lithium (Li^+), potassium (K^+), rubidium (Rb^+) or caesium (Cs^+), all VisHKT1;1 allelic variants were unable to mediate inward currents (Fig. S9). This demonstrates that all VisHKT1;1 allelic variants characterized here were selective only for Na^+.

Two polymorphisms (Ser-534-Arg and Gly-537-Asp) are responsible for differences in Na^+ conductance and rectification

Of six polymorphic residues conserved between VisHKT1;1-E and VisHKT1;1-e allelic variants, two residues occur in the predicted $M2_D$ transmembrane region near the carboxy (C)-terminus, where charged residues are important for correct functioning of plant and bacterial Trk/Ktr/HKT proteins (Kato *et al.*, 2007). In VisHKT1;1-E variants, these residues are uncharged, Ser-534 and Gly-537, but differ in VisHKT1;1-e variants with oppositely charged Arg-534 (positive) and Asp-537 (negative) (Figs 3a, S5). To investigate whether these SNPs affected protein function, we performed mutagenesis of VisHKT1;1-e^K to generate two single mutant proteins, VisHKT1;1-e$^K_{D537G}$, VisHKT1;1-e$^K_{R534S}$, and a double mutant protein VisHKT1;1-e$^K_{R534S/D537G}$. Expression of mutant alleles in oocytes revealed that the D537G mutation alone had no effect on G_{Na+} or rectification, as this protein behaved like the original VisHKT1;1-e^K (Figs 4a, S10). Conversely, a single R534S mutation resulted in larger outward currents, reducing the inward rectification observed in VisHKT1;1-e^K, and also resulted in significantly higher inward slope G_{Na+} in oocytes compared to wild-type (WT) (Figs 4a, S10). When R534S mutation was combined with D537G mutation in e$^K_{R534S/D537G}$, to mimic residues present at the C-terminus of VisHKT1;1-E variants, rectification was abolished and the inward slope G_{Na+} was further increased compared to the e$^K_{R534S}$ single mutant (Fig. 4a), although this

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increase was not statistically significant compared to the R534S single mutant (Fig. S10). These mutations had no effect on cation selectivity of the e^K protein (Fig. S11). Comparing the chord conductance ratio of each protein confirmed that Arg-534 is the rectification residue in VisHKT1;1, as e^K-WT and e^K_{D537G} variants had significantly higher ratios compared to e^K_{R534S} mutant and $e^K_{R534S/D537G}$ (Fig. 4b). When expressed in yeast, both single mutant proteins inhibited growth on 50 mM Na^+ to

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Fig. 3 Grapevine rootstock *VisHKT1;1* allelic variants have different expression, and sodium (Na^+) transport properties. (a) Amino acid alignment of *Vitis vinifera* HKT1;1 from Cabernet Sauvignon with protein sequences predicted from unique alleles of *VisHKT1;1* present in K51-40 (E^K and e^K) and 140 Ruggeri (E^R and e^R). Residues conserved in three or more sequences are shown in black with unique residues encoded by a single allele shown in grey. Encoded residues conserved between E alleles are highlighted in yellow and those conserved between e alleles are highlighted in red. Predicted transmembrane regions ($M1_{A\text{-}D}$ and $M2_{A\text{-}D}$), and P-loop domains ($P_{A\text{-}D}$) are shown above. Arrows indicate the position of residues in the selectivity filter. Asterisks indicate residues selected for mutagenesis. (b) Copy number of mRNAs encoding different *VisHKT1;1* alleles in root tissue enriched stelar or epidermal/cortical cells from grapevine rootstocks K51-40 (left) and 140 Ruggeri (right). Data are mean ± SE of three biological replicates of pooled tissue from multiple plants. Significant differences are denoted by different letters ($P < 0.05$, one-way ANOVA with Tukey's post-hoc test). (c) VisHKT1;1 allelic variants differentially inhibit yeast (*Saccharomyces cerevisiae*) growth on high Na^+. Wild-type strain INVSc2 was transformed with pYES-DEST52 empty vector as a control, or the same vector containing allelic variants of VisHKT1;1. Diluted yeast strains were spotted (5 µl) onto plates containing D-glucose (spotting control), or D-raffinose and D-galactose (induction) ± 50 mM NaCl. Plates were incubated at 30°C for 3 d. (d) Typical currents observed from oocytes injected with cRNA encoding VisHKT1;1-E^K, VisHKT1;1-e^K, VisHKT1;1-E^R, VisHKT1;1-e^R, and water, in solutions containing 30 mM Na^+-gluconate. Dashed lines represent zero current levels. Data represent total oocyte currents without background subtraction. Data are mean ± SE ($n \geq 5$ oocytes).

the same degree as e^K-WT (Fig. 4c). Only $e^K_{R534S/D537G}$ inhibited yeast growth on 50 mM Na^+ to the same degree as E^K-WT (Fig. 4c). This suggests that variable yeast growth inhibition mediated by the VisHKT1;1 allelic variants is due to the larger inward Na^+ conductance of allele E variants and not due to differences in inward rectification. To further confirm the role of Arg-534 in rectification and reduced conductance of VisHKT1.1-e variants, we mutated the equivalent residue in wheat TaHKT1.5-D from serine to arginine. This mutation greatly reduced the inward Na^+ conductance, with TaHKT1.5-D_{S506R} showing currents half the magnitude of WT TaHKT1.5-D (Fig. 4d,e). Furthermore, TaHKT1.5-D showed almost no inward rectification, whereas TaHKT1.5-D_{S506R} was strongly inward rectifying (Fig. 4f).

Dominant *HKT1;1-E*^{K} and *HKT1;1-E*^{R} alleles associated with Na^+ exclusion are derived from *V. riparia* and *V. berlandieri*, respectively

In order to gain understanding of the origin of *VisHKT1;1* alleles, we sequenced *VisHKT1;1* from four *Vitis* species that make up this complex hybrid family (Fig. 5a). The dominant E^K and E^R alleles are likely to be derived from *V. riparia* and *V. berlandieri*, respectively. Both of these accessions were homozygous for *E* alleles and thus provide ideal material for breeding new grapevine rootstocks with strong Na^+ exclusion ability. Conversely, the recessive e^K allele was found to originate from *V. champinii*, which is heterozygous for the e^K allele and a unique *E* allele. The e^R allele of 140 Ruggeri was derived from *V. rupestris*, which is also heterozygous for the e^R allele and another unique *E* allele (Fig. S12).

The Na^+ exclusion ability of the four *Vitis* species was compared against the two parents and a selection of hybrids to determine if this trait behaved as predicted by their *HKT1;1* genotypes (Fig. 5b). Indeed, *V. riparia* and *V. berlandieri* both display strong Na^+ exclusion, with minimal Na^+ accumulation in leaves, which agrees with their homozygous *HKT1;1-E* allelic makeup. Furthermore, *V. champinii* and *V. rupestris*, which both carry a recessive *HKT1;1-e* allele, showed a moderate degree of Na^+ accumulation, which was lower than hybrids homozygous for *HKT1;1-e* alleles (HB55, HB25 and MI07-33) but greater than other heterozygous genotypes, K51-40 and 140 Ruggeri and hybrid HB76.

Ten *V. vinifera* wine grape cultivars commonly grown in Australia were assessed for their *HKT1;1* genetics by sequencing the SNPs underlying residues 534 and 537. All *V. vinifera* cultivars were homozygous for alleles encoding the Ser and Gly residues associated with dominant *VisHKT1;1-E* alleles. As predicted by their *HKT1;1* allelic makeup, these cultivars were strong Na^+-excluders, except for Shiraz which was a moderate Na^+-excluder (Fig. 5b). This result suggests that *HKT1;1* is the major gene contributing to Na^+ exclusion in grapevines, and other minor loci yet to be identified may also contribute.

Discussion

The *NaE* quantitative trait locus (QTL) for sodium (Na^+) exclusion in grapevine contained six tightly linked *HKT* genes, which is the entire complement of high-affinity potassium (K^+) transporter (*HKT*) genes in the grapevine reference genome. This contrasts with cereals, where single *HKT* genes have been found within QTLs for Na^+ exclusion (Ren *et al.*, 2005; Huang *et al.*, 2006; Byrt *et al.*, 2007), and tomato, where a QTL for Na^+ exclusion contained two closely linked *HKT* genes (Asins *et al.*, 2013). All six *HKT* genes within the *NaE* locus are predicted to encode class-1 HKT proteins containing Ser-Gly-Gly-Gly selectivity filters, suggesting that they may have evolved by gene duplication, and are likely to be Na^+ selective (Mäser *et al.*, 2002; Waters *et al.*, 2013). Genetic and functional analyses presented here strongly indicate that *VisHKT1;1* is the gene responsible for variation in Na^+ exclusion associated with the *NaE* locus. VviHKT1;1 localized to the plasma membrane and was transcribed in cells associated with the root vasculature, suggesting that the Na^+ exclusion mechanism in *Vitis* species involves retrieval of Na^+ from root xylem vessels into surrounding cells, which is consistent with the role of HKT proteins in other plants (Munns & Tester, 2008; Horie *et al.*, 2009; Byrt *et al.*, 2014). *HKT1;3* was expressed in roots, but its function could not be determined here. The functions of grapevine *HKT1;2*, *HKT1;6*, *HKT1;7* and *HKT1;8* remain to be characterized; however, microarray data suggest they are not expressed in *V. vinifera* roots and are therefore unlikely to influence direct transfer of Na^+ into the root xylem. Whether they transport Na^+ and the tissues in which they function was beyond the scope of this study.

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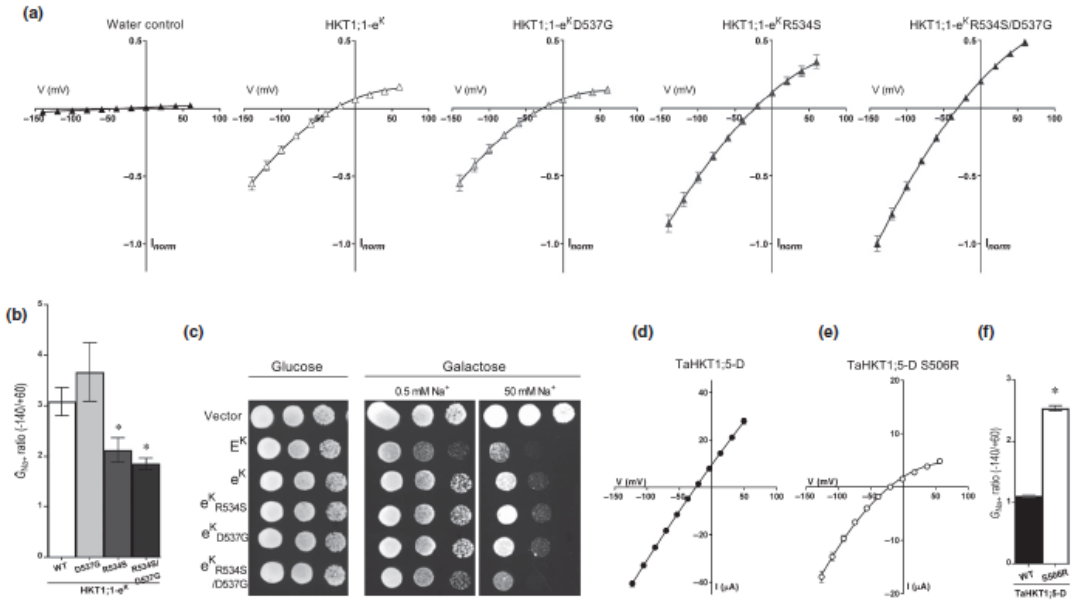


Fig. 4 Arg-534 and Asp-537 control the gating and sodium conductance of VisHKT1;1-e^K. (a) Normalized current–voltage relationships of oocytes injected with water or cRNA encoding VisHKT1;1-e^K, VisHKT1;1-e$^K_{D537G}$, VisHKT1;1-e$^K_{R534S}$, and VisHKT1;1-e$^K_{R534S/D537G}$ in solutions with 30 mM Na^+-gluconate. Data represent the HKT-mediated currents without background subtraction. Data are mean ± SE ($n \geq 9$ oocytes from two batches). (b) The inward chord conductance (G_{Na+}) ratio of *Xenopus laevis* oocytes expressing wild-type (WT) VisHKT1;1-e^K and mutated variants in solutions containing 30 mM Na^+-gluconate. The rectification ratio was determined by dividing G_{Na+} at −140 mV by G_{Na+} at +60 mV. Chord conductances were calculated from current–voltage relationships using the equation $G_{Na+} = I/(V_m - V_{rev})$. Data are mean ± SE ($n \geq 5$ oocytes) and calculations were performed after subtraction of mean background currents from water-injected control oocytes. Asterisks denote significant difference from WT (*t*-test, $P < 0.05$). (c) Comparison of Na^+-induced growth inhibition of yeast (*Saccharomyces cerevisiae*) strains expressing WT and mutant *VisHKT1;1* alleles from K51-40. Yeast were spotted onto plates containing D-glucose (control), or D-raffinose and D-galactose (induction) ± 50 mM NaCl. Plates were incubated at 30°C for 2 d (glucose) or 4 d (galactose). (d, e) Current–voltage relationships of oocytes injected with cRNA encoding (d) TaHKT1;5-D and (e) TaHKT1;5-D$_{S506R}$ in solutions with 30 mM Na^+-gluconate. Data represent the HKT-mediated currents after background subtraction. Data are mean ± SE ($n \geq 9$ oocytes). (f) The inward chord conductance (G_{Na+}) ratio of *Xenopus* oocytes expressing TaHKT1;5-D and TaHKT1;5-D$_{S506R}$ in solutions containing 30 mM Na^+-gluconate. Data are mean ± SE ($n \geq 9$ oocytes). Asterisk denotes significant difference from WT (*t*-test, $P < 0.01$).

In other species, QTL mapping has identified allelic variants encoding HKT proteins that confer greater Na^+ exclusion or greater K^+ : Na^+ ratios (Ren *et al.*, 2005; Asins *et al.*, 2013; Jaime-Pérez *et al.*, 2016). It has been suggested that single nucleotide polymorphisms (SNPs) within the coding region between tolerant and sensitive alleles of *OsHKT1;5* are responsible for the functional differences between HKT allelic variants (Cotsaftis *et al.*, 2011), but this has not been proven using mutagenesis and functional characterization of the encoded proteins. Thus, few specific amino acid residues have been identified within HKT proteins that are responsible for different Na^+-exclusion abilities of tolerant and sensitive lines. Here, we identified amino acid residues 534 and 537 in VisHKT1;1 allelic variants that impart strong and weak shoot Na^+ exclusion when uncharged (Ser-534, Gly-537) and charged (Arg-534, Asp-537), respectively.

Single point mutations in plant HKT proteins have been shown previously to have functional impacts. For example, a Ser or Gly residue in the first pore-loop domain (p-loop A) determines Na^+ or K^+ permeability in HKTs from Arabidopsis, wheat, rice and Venus flytrap (*Dionaea muscipula*) (Mäser *et al.*, 2002; Böhm *et al.*, 2016). Mutation of an Asp to Asn in the second p-loop domain of *Thellungiella salsuginea* TsHKT1;2 and the Arabidopsis orthologue (to create TsHKT1;2$_{D207N}$ and *AtHKT1*$_{N211D}$) altered their Na^+/K^+ selectivity, and expression of TsHKT1;2 or the mutant Arabidopsis protein conferred greater salt tolerance to Arabidopsis *hkt1-1* knockout plants due to increased K^+ accumulation in shoots (Ali *et al.*, 2012). In the present study, the VisHKT1;1-e proteins had identical cation selectivity to VisHKT1;1-E variants, suggesting that the increased Na^+ transport activity of VisHKT1;1-E variants confers the mechanism for improved Na^+ exclusion in *NaE* progeny.

HKT proteins from rice that display strong (OsHKT1;1) and weak (OsHKT1;3) inward rectification have been observed (Jabnoune *et al.*, 2009). We identified a single amino acid residue (Arg-534) that caused greater inward rectification of VisHKT1;1-e variants compared to VisHKT1;1-E variants. We

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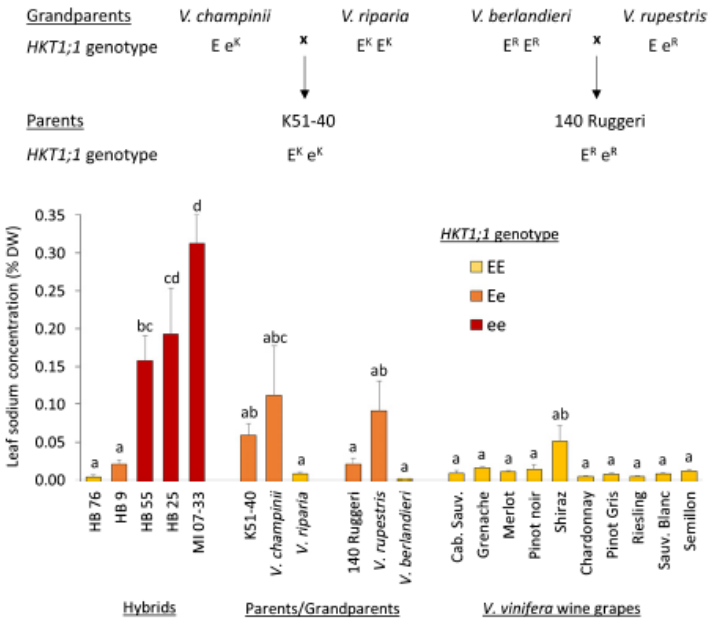


Fig. 5 Recessive alleles, *VisHKT1;1*-e^K and *VisHKT1;1*-e^R, are derived from *Vitis champinii* and *V. rupestris*, respectively. (a) Pedigree diagram of K51-40 and 140 Ruggeri illustrating the inheritance of *VisHKT1;1* alleles from four different grandparent *Vitis* species. *Vitis champinii* and *V. rupestris* are heterozygous carriers of the recessive *VisHKT1;1-e*^{K} and *VisHKT1;1-e*^{R} allele, respectively, whereas *V. riparia* and *V. berlandieri* appear to be homozygous for the dominant *VisHKT1;1*-E^K and *VisHKT1;1*-E^R alleles, respectively. (b) Sodium (Na^+) exclusion in selected progeny of K51-40 × 140 Ruggeri (from Fig. 1a), the parents and grandparents, and *V. vinifera* cultivars. Individuals are coloured according to their deduced amino acids at the key residues 534 and 537 of VisHKT1;1 as determined by direct genomic DNA sequencing (Supporting Information Fig. S12). Yellow bars indicate genotypes homozygous for the dominant *HKT1;1-E* alleles (residues Ser-534 and Gly-537). Red bars indicate genotypes homozygous for the recessive *HKT1;1-e* alleles (residues Arg-534 and Asp-537), and orange bars indicate heterozygous genotypes that contain one dominant *HKT1;1-E* allele and one recessive *HKT1;1-e* allele. Bars are mean + SE of three biological replicates. Significant differences are denoted by different letters ($P < 0.05$, one-way ANOVA with Tukey's post-hoc test).

found that mutating the corresponding residue in wheat TaHKT1.5-D also caused inward rectification and reduced Na^+ conductance. Both rice OsHKT1;1 and 1;3 appear to rectify more strongly than VisHKT1;1-e and wheat TaHKT1.5-D_{S506R}, and neither contain a residue equivalent to Arg-534. TsHKT1;2 and AtHKT1$_{N211D}$ showed inward rectification of Na^+ transport, but the reverse D207N mutation in TsHKT1;2 did not abrogate this (Ali *et al.*, 2016). These findings indicate that additional residues influence rectification in HKT proteins.

Ali *et al.* (2016) suggested that inward rectification of AtHKT1;1$_{N211D}$ would impose a greater Na^+ retention in root cells, preventing Na^+ efflux to the xylem apoplast. However, they found no difference in shoot Na^+ accumulation between Arabidopsis plants expressing either the rectifying or nonrectifying AtHKT1 variants. By contrast, in our study, inheritance of the noninwardly rectifying VisHKT1;1-E alleles correlated with greater shoot Na^+ exclusion. Therefore, the greater Na^+ conductance observed through the HKT1 variants at physiologically relevant membrane potentials is likely to be the major contributing factor to shoot Na^+ exclusion. This aligns with the suggestion by Ren *et al.* (2005) that the rice *SKC1* allelic variant from salt-tolerant variety Nona Bokra shows greater Na^+ conductance than the allele from salt-sensitive Koshihikari. It also agrees with thermodynamics of Na^+ transport in root xylem parenchyma cells, which is predicted to be inward (to the cytoplasm) through channel-like proteins such as HKT, and outward (towards to xylem apoplast) through secondary active Na^+/H^+ exchangers (Munns & Tester, 2008). The functional role of inward rectification of plant HKT proteins therefore remains unclear. However, it must be noted that a greater degree of rectification was correlated with a lower magnitude of Na^+ current at negative potentials in all the HKT variants and mutants studied here, so the two properties are likely to be linked.

In our study, the R534S mutation in VisHKT1;1-e^K reduced inward rectification and increased inward Na^+ conductance. Within physiological pH ranges, the side chain of Arg-534 in VisHKT1;1-e^K is expected to be positively charged. The importance of positively charged residues in the $M2_D$ region of Trk/Ktr/HKT proteins has previously been demonstrated for AtHKT1 and TaHKT2;1 – mutation of Arg-519 in TaHKT2;1 and the equivalent Arg-487 in AtHKT1 to uncharged Gln reduced the transport activity of both proteins (Kato *et al.*,

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2007). By contrast, we found that replacing the charged residues in the $M2_D$ region with uncharged residues increased the activity of VisHKT1;1-e^K. The additive effect of a D537G substitution on transport activity in the VisHKT1;1-$e^K_{R534S/D537G}$ double mutant protein in yeast, but no change in conductance of the VisHKT1;1-e^K_{D537G} single mutant, might be due to interactions between positive and negative side chains of the Arg-534 and Asp-537 residues in the original VisHKT1;1-e^K. Future structural investigations into plant HKT proteins through protein crystallization would help to explain these observations.

In the parental rootstocks, expression of recessive *VisHKT1;1-e* alleles was approximately two-fold less than the dominant *VisHKT1;1-E* alleles in the root stele. The F_1 progeny are likely to have similar expression levels of each allele. Arabidopsis double knockout mutants of two transcription factors *arr1/arr12* showed a six-fold increase in *AtHKT1* expression in roots, which correlated with a 50% reduction in shoot Na^+ concentration (Mason *et al.*, 2010). A two-fold increase in transcript abundance of allele *E* variants over allele *e* is therefore probably not large enough to explain the large difference in leaf Na^+ that we observed between the homozygous *NaE* and *Nae* hybrid progeny. Although expression differences may contribute to leaf Na^+ exclusion, functional differences in Na^+ transport are likely the major determining factor for the near-Mendelian inheritance of Na^+ exclusion seen in our study.

Ten *V. vinifera* cultivars examined here, were all homozygous for *VvHKT1;1* alleles encoding Ser and Gly residues at 534 and 537 – typical of dominant *VisHKT1;1-E* alleles. The majority of these cultivars showed strong Na^+ exclusion abilities consistent with their *HKT1;1* alleles. This finding highlights the importance of selecting for dominant *VisHKT1;1-E* alleles in new rootstock genotypes, as introducing recessive *VisHKT1;1-e* alleles could result in severely diminished Na^+ exclusion capability compared with own-rooted *V. vinifera*.

In plants, both Na^+ and Cl^- can be toxic in high enough concentrations (Tavakkoli *et al.*, 2011), and which ion is more damaging depends on both their relative accumulation and thresholds of toxicity (Munns & Tester, 2008). In grapevines, Cl^- has been attributed as the ion most associated with salinity related ion toxicity; however, early studies that established this link were limited to *V. vinifera* cultivars (Woodham, 1956; Ehlig, 1960; Downton, 1977b). In our study, all the *V. vinifera* cultivars were found to be relatively strong excluders of Na^+. Considering that Na^+ exclusion is much more variable in rootstock genotypes derived from other *Vitis* species and the prevalent use of rootstocks in viticulture, the impact of elevated Na^+ accumulation on vine health warrants further investigation. The homozygous recessive *Nae* hybrids identified here provide the ideal rootstock material for quantifying the effect of high Na^+ accumulation on vine health and yield in longer-term future studies.

In conclusion, we identified *VisHKT1;1* as a major gene controlling Na^+ exclusion in grapevine rootstocks. Transgressive variation of Na^+ exclusion in the progeny of a heterozygous rootstock cross is caused by the inheritance of *VisHKT1;1* alleles that encode proteins with differences in Na^+ conductance, voltage dependence, and have small differences in root expression levels. We propose that the mechanism for enhanced Na^+ exclusion in *NaE* progeny is the enhanced Na^+ conductance through VisHKT1;1-E variants, which also display limited rectification. These functional properties are conferred by a neutrally charged amino acid residue at the C-terminus of VisHKT1;1. Identification of dominant *VisHKT1;1-E* alleles provide a valuable genetic marker to select for strong Na^+ exclusion in rootstock breeding programmes, which will assist with quality grape and wine production from saline soils.

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Author contributions

S.W.H. performed yeast assays, qRT-PCR, cloning, confocal microscopy, site-directed mutagenesis and electrophysiology of grapevine HKT variants; J.D.D. performed linkage mapping, QTL analysis, primer design, cloning of *HKT1;1* allelic variants, and sequencing of *HKT1;1* from *Vitis* species; Y.W. performed mutagenesis and electrophysiology of TaHKT1;5-D; A.R.W., E.J.E., R.R.W., D.H.B. and J.D.D., devised and performed grapevine Na^+ screens and D.H.B. measured the leaf Na^+ content; S.W.H. and J.D.D. analysed data; M.G., R.R.W. and A.R.W. supervised research; S.W.H. and J.D.D. wrote the manuscript; M.G., R.R.W. and A.R.W. revised the manuscript; and all authors commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Correlation between juice and leaf Na^+ concentration.

Fig. S2 Consensus genomic map of K51-40 × 140 Ruggeri progeny.

Fig. S3 Amino acid sequence alignment of six VviHKT1 proteins present in the grapevine reference genome.

Fig. S4 VviHKT1;3 does not function as a sodium transporter in yeast.

Fig. S5 Amino acid alignment of VviHKT1;1 from Cabernet Sauvignon *VisHKT1;1* alleles from K51-40 and 140 Ruggeri.

Fig. S6 Inward and outward sodium chord conductance allelic variants of *VisHKT1;1*.

Fig. S7 Effect of $[Na^+]_{ext}$ on the reversal potential of VisHKT1;1 allelic variants.

Fig. S8 Functional differences in the transport properties between VisHKT1;1-e^K and VisHKT1;1-E^K.

Fig. S9 Allelic variants of VisHKT1;1 are each highly sodium-selective.

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Fig. S10 Comparing the inward sodium transport rate of VisHKT1;1-eK and mutant variants.

Fig. S11 C-terminal mutations of K51-40 VisHKT1;1 alleles do not affect sodium selectivity.

Fig. S12 Tracing the origin of the *VisHKT1;1* alleles.

Table S1 List of primers used in this study

Table S2 *HKT1* gene accession numbers

Table S3 CAPS markers designed to score K51-40 × 140 Rugeri hybrid progeny for their inheritance of *HKT1;1* alleles

Table S4 Genes located with the mapped *NaE* locus

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15 Appendix 6: Staff

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Research assistance was also provided by the following people:

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16 Budget reconciliation

This will be provided by CSIRO in a separate document.