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Rootstock Genetics & Improvement:

Tolerance to Abiotic Stress



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Rootstock Genetics & Improvement - Tolerance to Abiotic Stress

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Front cover: *V. berlandieri* x *V. vinifera* BC_1F_2 seedlings undergoing a screen for Cl^- exclusion

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1. Abstract

Australia's major grape-growing regions will face increased salinity problems in the future resulting from a warmer drier climate. Chloride exclusion is a major trait for salt tolerance and this study has led to a world-first breakthrough in the quest to understand the molecular mechanism that regulates this trait in grapevine. We utilised a combination of segregating mapping populations and transgenic vines with altered gene expression to identify not only the first locus for chloride exclusion but also the underlying causal gene. This knowledge will enable the development of a genetic marker to predict chloride exclusion in the rootstock breeding program.

2. Executive Summary

Climate change predictions suggest that the major wine regions of Southern Australia are likely to face reduced rainfall, combined with increased temperatures and more frequent heatwave events, thus increased evaporation. Coupled with the likelihood of reduced availability and affordability of quality irrigation water, there is a high chance that the issue of irrigation-related salinity in vineyards will increase.

Salinity stress can lead to vines accumulating chloride and sodium ions at levels that become toxic and disrupt cellular and metabolic processes. In the leaves, this can manifest as visual marginal leaf burn symptoms resulting in impaired photosynthetic activity and reduced yields. Accumulation of high concentrations of chloride and sodium ions in the berries can also have detrimental impacts on wine quality and marketability.

Ion-excluding rootstocks have the ability to limit the uptake of chloride and/or sodium into the shoot and are therefore a key tool for mitigating the negative effects of salinity on the vine. Guided by studies in other plant species, we previously identified the major gene, *HKT1;1*, that controls sodium exclusion in grapevine rootstocks and we developed a robust genetic marker for predicting this trait in the rootstock breeding program.

The primary aim of this project was to identify the major gene controlling chloride exclusion in grapevine rootstocks in order to develop a reliable genetic marker to this key trait. Unlike the case for sodium, there has been comparatively little research on chloride exclusion in other crop species and hence the major genes controlling chloride exclusion in plants are yet to be identified. This study has led to a world-first breakthrough in the quest to understand the molecular mechanism that regulates chloride exclusion in plants.

We first identified a locus, named *CLEX* for $\underline{Cl}^-$ exclusion, that accounts for 85-89% of the variation in Cl^- exclusion in a self-pollinated population derived from a (*V. berlandieri* x *V. vinifera* cv. Sultana) x *V. vinifera cv.* Biancone genotype. We showed that this segregating trait, first observed in juvenile seedlings, is carried through to pot grown vines propagated from cuttings, as well as mature vines in the field. We also showed that the variation attributed to the *CLEX* locus is dependent on the root system and is fully conferred to a grafted scion, making it an excellent target for rootstock improvement. In this population, the dominant (stronger) *CLEX* allele for exclusion is derived from *V. berlandieri* and a recessive (weaker) allele is derived from *V. vinifera*.

In a K51-40 (*V. champinii* 'Dogridge' x *V. riparia* 'Gloire de Montpellier') x 140 Ruggeri (*V. berlandieri* 'Boutin B' x *V. rupestris* 'du Lot') F_2 population, the *CLEX* locus was found to explain 72% of the variation in Cl^- exclusion. This result not only confirmed that this locus is likely to be the major locus for Cl^- exclusion across different grapevine species, but also led to the identification of single nucleotide polymorphisms to act as the basis for genetic markers to predict this trait in the breeding program.

Of the 72 predicted genes located in the *CLEX* locus, two stood out as potential candidate genes underlying this trait. *CHX20.1* and *CHX20.2* belong to a large family of genes known as *$\underline{C}$ation/$\underline{H}^+$ e$\underline{x}$changers*, the roles of which are poorly characterised in plants. Recent evidence in soybean, however, suggests that a homologue of *CHX20.1*, known as *GmSALT3,* encodes a shoot-based Cl^- exclusion mechanism in that species. To determine whether *CHX20.1* or *CHX20.2* contribute to Cl^- exclusion in grapevine, we genetically transformed Shiraz with

constructs designed to reduce their expression. Analysis of these transformed lines revealed that *CHX20.1* plays a major role in Cl^- exclusion, occurring primarily within the root stele, being active at both low and high salt treatments (Fig. 2.1) and potentially functioning in restricting the entry of Cl^- into, or its retrieval from, the xylem. *CHX20.2* on the other hand, is expressed in the petiole and appears to have a minor influence on the regulation of Cl^- concentration in the laminae, but only in low salt treatments. These results suggest that *CHX20.1* is the major gene underlying the variation in Cl^- exclusion in the *V. berlandieri* x *V. vinifera* and K51-40 x 140 Ruggeri populations. *CHX20.1* is also highly expressed in the bunch rachis, and therefore may play a role in regulating the Cl^- concentration in the berry. This hypothesis was not able to be tested in this study but will be important in the future to determine the importance of this gene to scion breeding and improvement.

The *CHX20.1* alleles derived from *V. berlandieri* and *V. vinifera* differ by 19 amino acids, one or more of which are likely to cause functional differences in the transport properties of the encoded proteins. In order to identify the key amino acid differences underlying this trait, our collaborators at the University of Adelaide, Dr Yue Qu and Professor Matthew Gilliham, sought to develop an assay to characterise the function of CHX20.1 proteins. While the *E.coli* and *Arabidopsis*-based systems that were tested both showed promise for characterising *CHX20.1* function, further work is required to perfect one or both of these assay systems. This will be required in order to identify the causal amino acids underlying functional differences if a perfect genetic marker for Cl^- exclusion is to be designed and targeted to the exact genetic mutations underlying variation in Cl^- exclusion in different rootstock genotypes.

Strategic crosses using 140 Ruggeri were performed to begin converting the scientific knowledge gained here into increased capacity for salt tolerance in the rootstock breeding program for durable and broad-spectrum resistance to the major soil pests, phylloxera and nematodes. We also investigated other germplasm as potential novel sources of Cl^- exclusion, which led to the identification of *V. longii* as a potentially superior source of Cl^- exclusion over 140 Ruggeri and *V. berlandieri*. Future work is needed to 1) determine the suitability of *V. longii* in rootstock breeding, 2) to confirm the presence of, and determine the role of, *CHX20.1* in *V. longii*, and 3) to begin the introgression of Cl^- exclusion from *V. longii* into the rootstock breeding program.

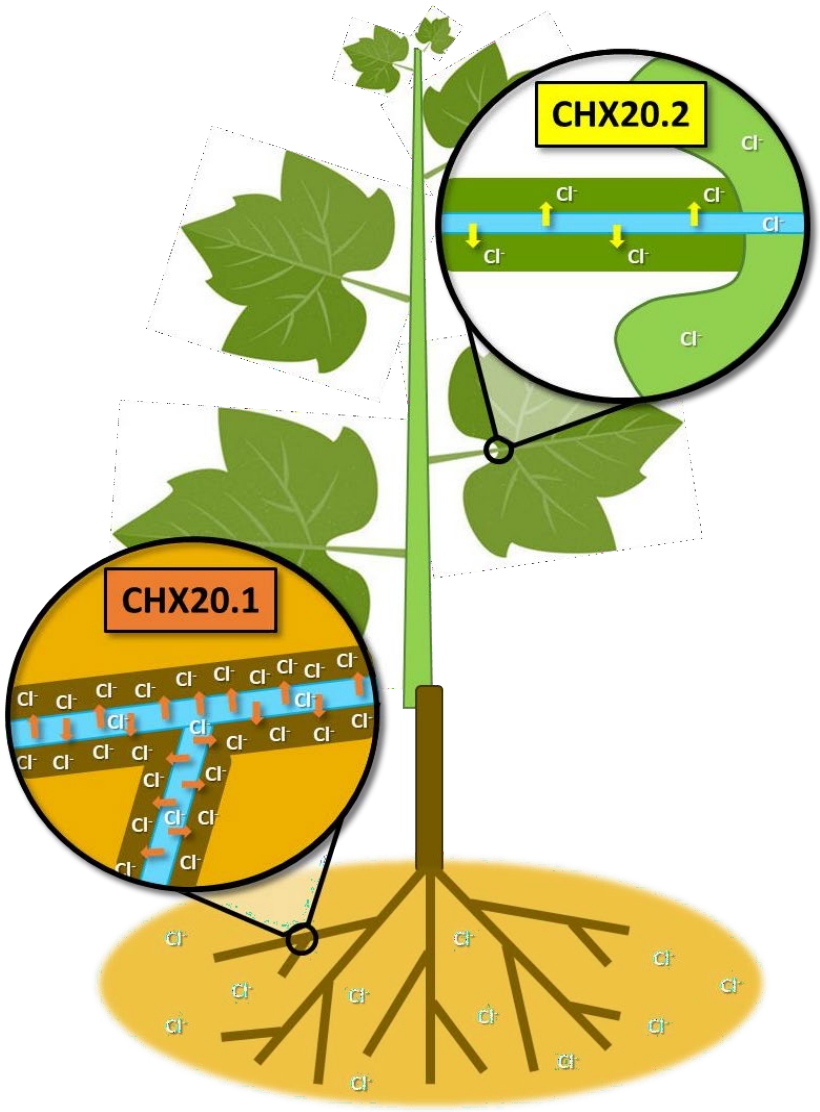


Figure 2.1 – Schematic of the proposed roles of *CHX20.1* and *CHX20.2* in Cl^- transport. *CHX20.1* is highly expressed in the root stele where it plays a major role in restricting Cl^- translocation to the shoot under both low and high salt concentrations, likely via removal of Cl^- from the xylem transpiration stream, or via phloem re-translocation. *CHX20.2* is expressed in the petiole where, under control conditions, it appears to play a role in sequestering of Cl^- into the petiole, thereby limiting the entry and accumulation of Cl^- in the laminae.

3. Background

The Millennium drought that impacted southern Australia during the late 1990s and early 2000s resulted in harsh water restrictions in large areas of winegrape production in Australia. In many areas of the Murray Darling Basin, such restrictions led to soil water deficit and insufficient leaching fractions with a consequent build-up of salts within the soil. In other regions, e.g. Langhorne Creek, this was exacerbated by irrigation water becoming highly saline, moreover, lower than usual winter rainfall led to reduced winter flushing of salt from the soil. Such conditions intensified the negative impacts of heatwaves on vines.

Australian grape growers are expected to face an increased frequency of such drought events in the future, resulting from a warming climate (Webb et al. 2013). Climate modelling predicts that the weather of Southern Australia will become more unstable and extreme, with a high probability of increased average temperatures, increased frequency and severity of heatwaves and reductions in winter rainfall (IPCC report 2013 http://www.ipcc.ch/report/ar5/wg1/). As was experienced in the Millennium drought, the combination of reduced rainfall, increased evaporation and reduced availability of quality water sources is expected to increase the occurrence of soil salinity in irrigated vineyards. A recent study suggests that without adaptation, the average quality of Australia's winegrapes may be reduced by 7% (lower warming prediction) to 39% (higher warming prediction) by 2030, and from 9-76% by 2050 (Webb et al. 2008). Resilient rootstocks, capable of dealing with these climate extremes and protecting the scion and fruit from the associated abiotic stresses, are therefore needed to help mitigate these future projections, as identified in the Wine Australia Strategic Plan 2020-2025.

CSIRO has had a longstanding rootstock breeding program, which until recently has relied on traditional phenotypic selection and evaluation approaches, which are both slow and land intensive. Advancements in genetic technologies have now enabled the identification and use of genetic markers to screen and select for key traits, dramatically improving the efficiency of the breeding process (Collard & Mackill 2008). CSIRO's current rootstock breeding strategy is primarily centred upon providing broad spectrum and durable disease resistance to safeguard against the devastating impacts of soil-borne pests, phylloxera (*Daktulosphaira vitifoliae*) and root-knot nematodes (*Meloidogyne javanica*). This is to be achieved using recently identified genetic markers to select new rootstock genotypes that possess multiple dominant loci conferring resistance to phylloxera, *RDV1* (Zhang et al. 2009) and *RDV2* (Smith et al., 2018a), and multiple loci for resistance to nematodes, *MJR1* (Smith et al., 2018b) and *MJR2* (for further information see CSA 1701-1.3). The aim of the current project was to provide new knowledge, tools and germplasm to feed into the breeding strategy for resistance to abiotic stresses, to ensure CSIRO's future rootstocks also possess adequate salt tolerance traits needed to withstand a hotter drier climate. Building on outputs from the previous project CSP 1302, this project sought to achieve this in three major ways: 1) to develop a predictive genetic marker for the key salt tolerance trait of chloride (Cl^-)

exclusion to aid in marker-assisted selection, 2) to develop new screening tools to rapidly assess rootstocks for salt tolerance characteristics that correlate to field responses, and 3) to explore novel germplasm for superior salt tolerance traits to introduce into the breeding strategy.

4. Project Aims

The major objective of this project was to develop tools for selecting and breeding grapevine rootstocks with better resilience to abiotic stresses. New germplasm from wild species such as *V. girdiana* will be assessed for ability to exclude ions. Planned experiments include utilising the Plant Accelerator facility for early screening of rootstocks, identifying and characterising genes involved in exclusion of ions and water use efficiency without excess vigour. Genes identified as potential candidates for conferring desirable resilience traits, will be further characterised in collaboration with Professor Matt Gilliham (University of Adelaide) using grapevine transformation (Project 1.7) as a tool to test for gene function.

In addition to conventional breeding, a new breeding strategy will be developed using 140 Ruggeri as a parent. To facilitate this process, 140 Ruggeri will be transformed (in project CSA 1701-1.7) with a construct to mimic the GAI mutation and provide a fast generation rootstock for rapid breeding, with similar characteristics to the microvine but without introducing the *V. vinifera* genetic background. In the final generation, only tall plants would be selected and the transgene footprint would therefore be removed (this work is reported on in the Final Report for project 1701-1.7).

Another aspect of the project is to complete analysis of two Padthaway trials containing salt-excluding rootstock line C7 (identified in previous projects) in Year 1 through plant and berry measurements, small-scale winemaking and subsequent sensory evaluation in the following year. This investigation will inform the choice on whether to make this rootstock C7 available to industry by the end of the second year.

Year 3 outputs:

- Decision made about commercial release of C7. If it is to be released, PBR completed and material released to nurseries.

- 140 Ruggeri with 'Microvine' characteristics available for rapid breeding of rootstocks, without introducing *V. vinifera* into the breeding strategy.

- The best available alleles for salt exclusion and salt tolerance identified from germplasm assessments including the newly imported *V. girdiana*. Genetic markers developed for salt exclusion.

Year 5 outputs:

- Information on loci and chromosomal regions available for heat, drought and water use efficiency, for input to breeding program. In addition, smart screening methods using the Plant Accelerator utilised to select the best material for input into the breeding program.

- Potential of Families 86 and 88 (probably *V. cinerea* X (Ramsey X 1103 Paulsen) and *V. cinerea* X *rupestris,* respectively) explored from material in plantings at Nuriootpa

(ungrafted) and Irymple (grafted) for possible integration into the breeding program or further trials from an abiotic stress perspective.

Year 10 aspirational outcomes:

- Trials underway to evaluate the performance of second-generation marker-assisted rootstock selections, bred under Australian conditions with improved resistance to biotic and abiotic stresses.

- If C7 is deemed suitable for release, increasing industry adoption of this CSIRO-bred rootstock.

Year 1

Output	Target	Activities
New germplasm with putative enhanced chloride exclusion ability available in quarantine for experimental analysis	30/06/18	• Import canes of wild Vitis species including *V. girdiana, V. doaniana* and *V. arizonica* and establish in quarantine glasshouse. Import seeds of *V. girdiana* x *V. arizonica* crosses and establish seedlings in quarantine glasshouse • Undertake marker screening for putative chloride exclusion genes (for example CHA, SLAH1.2, SLAH1.3, NRT and POT genes) in imported material and initiate experiments for phenotypic screening
Data from molecular experiments on sodium exclusion genes (HKT1) available to verify role	30/06/18	• Complete investigation of the roles of 5 similar HKT1 putative sodium exclusion genes using functional assays eg. Yeast, bacteria and oocytes in collaboration with Matt Gilliham (U of A). • Carry out transcriptional analysis of these genes to verify their location of activity in different tissues in grapevines in collaboration with Matt Gilliham (U of A). • Undertake analysis of transgenic grapevines with altered HKT1 genes to further verify the roles of these genes in planta. Constructs include knockouts of HKT1.1, HKT1.3 and a third construct which knocks out HKT1.6, 1.7 and 1.8. • Selected transgenic lines grown in containment glasshouse to confirm results obtained in tissue culture plantlets. Tissues to be sampled and analysed include leaf, petiole, stem and root.
Data from molecular experiments on chloride exclusion genes (CHA) available to confirm role	30/06/18	• Investigate the role of 2 CHA putative chloride exclusion genes using functional assays eg. Yeast, bacteria and oocytes in collaboration with Matt Gilliham (U of A). • Undertake analysis of transgenic grapevines with altered CHA1 or CHA2 to further verify the roles of these genes. • Carry out glasshouse experiments with selected transgenic lines to confirm results obtained in tissue culture plantlets. Tissues to be sampled and analysed include leaf, petiole, stem and root.
Data from benchmarking the chloride exclusion phenotypes of berlandieri seedlings (MU27-38 selfed) with 140 Ruggeri and K51-40 available	30/06/18	• Conduct glasshouse experiments to determine how the berlandieri seedlings from selfed MU27-38 compare with 140 Ruggeri, K51-40 and C7 in ability to exclude chloride • Initiate investigation to confirm whether ion exclusion traits in seedlings are similar to that of hardwood cuttings
Data from field trials testing performance of salt-excluding rootstock C7 collated and analysed	30/06/18	• Collect data from 2 rootstock field trials containing C7 for yield components, berry composition and dormant vine pruning weight and determine ability of C7 to exclude salt in berries compared to 140 Ruggeri. • Prepare wines for evaluation from 2 trials containing C7 rootstock (7 lines X 3 reps)

Year 2

Output	Target	Activities
Phenotypic testing of new germplasm in quarantine with putative enhanced chloride exclusion ability	30/06/2019	• Maintain imported canes of wild Vitis species including *V. girdiana, V. doaniana* and *V. arizonica* in quarantine glasshouse. • Complete phenotypic screening for imported canes listed above and compared to 140 Ruggeri for ability to exclude chloride. Germinate, grow and test about 80 imported seeds of *V. girdiana* x *V. arizonica* in quarantine glasshouse. • Collect leaf samples for DNA marker analysis as new markers become available. • Import new material including canes and seeds as they become available through collaboration with Andy Walker (UC Davis).
Data from molecular experiments on putative sodium exclusion genes (HKT1) available to verify role	30/06/2019	• Complete investigation of the roles of 5 similar HKT1 putative sodium exclusion genes and rootstock alleles using rapid functional assays e.g. Yeast, bacteria and oocytes in collaboration with Matt Gilliham (U of A). • Draft and submit publication on the role of other HKT genes, in collaboration with Matt Gilliham (U of A). • Complete analysis of transgenic grapevines with altered HKT1 genes to further verify the roles of these genes in planta. Constructs include knockout of HKT1.3 and a construct which knocks out HKT1.6, 1.7 and 1.8 including experiment to determine Na^+ in roots as well as leaves from tissue culture. • Grow selected transgenic lines in containment glasshouse with high sodium treatments to confirm results obtained in tissue culture plantlets. Conduct ion analysis of plant material.
Data from molecular experiments on chloride exclusion genes (CHX [previously referred to as CHA]) available to confirm role	30/06/2019	• Investigate the role of 2 CHX putative chloride exclusion genes using new bacterial functional assays in collaboration with Matt Gilliham (U of A). • Complete analysis of available transgenic grapevines with altered CHX1 or CHX2 to further verify the roles of these genes. • Complete glasshouse experiments with selected transgenic lines to confirm results obtained in tissue culture plantlets. Conduct ion analysis on collected plant material.
Data on the influence of the CHX locus on the chloride exclusion phenotype available for diverse genomic backgrounds	30/06/2019	• Conduct a Plant Accelerator experiment to determine the chloride exclusion patterns of at least 60 *V. berlandieri* x *V. berlandieri* genotypes belonging to four half sibling populations. • Undertake Genotype By Sequencing analysis to identify SNP markers between the 60 *V. berlandieri* x *V. berlandieri* genotypes. • Germinate approx 80 K51-40 x 140 Ruggeri F2 seedlings and use KASP markers to screen and select individuals that are homozygous for each of the four CHA alleles in that family. Determine chloride exclusion phenotype of these seedlings using facilities at the Plant Accelerator to gauge growth and provide an even growing environment.
Data from field trials testing performance of salt-excluding rootstock C7 to inform a decision on release of the rootstock in 2020	30/06/2019	• Complete analysis from 2 rootstock field trials containing C7 for yield components, berry composition and dormant vine pruning weight as required for decision about release. • Assess Padthaway Pinot Gris trial containing C7 rootstock (7 lines X 3 reps) for suitability for winemaking. • Perform sensory analysis of wines from Padthaway trials in conjunction with AWRI.

Year 3

Outputs	Target	Activities
Phenotypic testing of new germplasm in quarantine with putative enhanced chloride exclusion ability	30/06/2020	• Maintain imported canes of wild Vitis species including *V. girdiana, V. doaniana* and *V. arizonica* in quarantine approved glasshouse. • Complete phenotypic screening for imported canes listed above and compared to 140 Ruggeri for ability to exclude chloride. • Collect leaf samples for DNA marker analysis as new markers become available. • Import new material including canes and seeds as they become available through collaboration with Andy Walker (UC Davis).
Markers for sodium exclusion phenotype deployed across rootstock breeding germplasm	30/06/2020	• Complete investigation of transgenic plants expressing altered HKT1 genes and draft manuscript. • Initiate HKT1.1 marker screening of germplasm with potential for introduction into breeding program together with near-to-release cultivars for homozygous dominant positive alleles able to exclude sodium.
Data from molecular experiments on chloride exclusion genes (CHX [previously referred to as CHA]) available to assess validity of marker for breeding for chloride exclusion phenotype	30/06/2020	• Identify and isolate CHX2 alleles from *V. berlandieri* and *V. vinifera* homozygous BCS_1 lines and CHX1 alleles from the rootstocks K51-40 and 140 Ruggeri for functional characterisation. • Investigate the role of 2 CHX putative chloride exclusion genes and their range of alleles specified above using functional assays in Xenopus oocytes and bacterial systems. Transform *Arabidopsis* with a construct to express grapevine CHX1 alleles in stelar cells to further characterise the role in Cl^- exclusion. Carry out phenotypic and molecular analysis of transgenic Arabidopsis (collaboration with Matt Gilliham (U of A)). • Complete glasshouse experiments on transgenic 140 Ruggeri with altered CHX1 expression to further verify the role of this gene in Cl^- exclusion in a rootstock. • Complete glasshouse experiments on transgenic Shiraz with altered CHX2 expression to investigate the role of this gene in Cl^- partitioning using ion analysis and investigate a possible role in stomatal opening using Licor and/or porometer. • Score 107 K51-40 x 140 Ruggeri seedlings for their inheritance of the chloride exclusion (CLX) locus, using KASP markers. Undertake marker trait analysis to determine the influence of the CLX locus on chloride exclusion in this seedling family. Undertake Genotype By Sequencing analysis to identify SNP markers to be used for QTL analysis of other potential loci associated with Cl^- exclusion. • Conduct a reciprocal grafted experiment in the Plant Accelerator using homozygous *V. berlandieri* X *V. vinifera* BCS_1 lines with Shiraz to determine if the CLX locus contributes to Cl^- exclusion in the scion.
Data from growth analysis experiments available to determine the correlation between growth rates in the short-term plant accelerator experiments and growth measures in previous long-term salinity field studies.	30/06/2020	• Perform a plant accelerator experiment with 15 rootstocks, ungrafted and grafted with a Shiraz scion, to assess growth rates under various Cl^- treatments and in mild and hot temperatures. • Compare data obtained with the pruning weights and yield data from the field experiments conducted previously in the Riverland.
Data from field trials testing performance of salt-excluding rootstock C7 to inform a decision on release of the rootstock in 2020	30/06/2020	• Complete analysis from 2 rootstock field trials containing C7 for yield components, berry composition and dormant vine pruning weights as required for decision about release. • Perform sensory analysis of 2019 wines from Padthaway trials in conjunction with AWRI. • Collate all available information regarding C7 and hold discussions about potential for release

Year 4

Outputs	Target	Activities
Markers for sodium exclusion phenotype deployed across rootstock breeding germplasm	30/06/2021	• Complete investigation of transgenic plants with altered expression of HKT1 genes and submit manuscript. • Continue with HKT1.1 marker screening of germplasm with potential for introduction into breeding program together with near-to-release cultivars for homozygous dominant positive alleles able to exclude sodium. • Assess transgenic Shiraz and 140 Ruggeri with extra copies of HKT1.1 gene to determine if plants homozygous for the better alleles are better excluders of sodium.
Data from molecular experiments on chloride exclusion genes (CHX [previously referred to as CHA]) available to assess validity of marker for breeding for chloride exclusion phenotype A tentative growth phenotype conferred by the chloride exclusion (CLE) locus confirmed. Involvement of CHX20.1 and CHX20.2 in the tentative growth phenotype determined. Additional K51-40 x 140 Ruggeri seed generated for mapping other potential chloride loci Genetics of chloride exclusion investigated in V. longii (syn. V. acerifolia), a super excluder	30/06/2021	• Identify and isolate CHX2 alleles from *V. berlandieri* and *V. vinifera* homozygous BCS_1 lines and CHX1 alleles from the rootstocks K51-40 and 140 Ruggeri for functional characterisation. • Investigate the role of 2 CHX putative chloride exclusion genes and their range of alleles specified above using functional assays in Xenopus oocytes and bacterial systems. Transform Arabidopsis with a construct to express grapevine CHX1 alleles in stelar cells to further characterise the role in Cl^- exclusion. Carry out phenotypic and molecular analysis of transgenic Arabidopsis (collaboration with Matt Gilliham (U of A)). • Complete glasshouse experiments on transgenic 140 Ruggeri with altered CHX1 expression to further verify the role of this gene in Cl^- exclusion in a rootstock. • Complete glasshouse experiments on transgenic Shiraz with altered CHX2 expression to investigate the role of this gene in Cl^- partitioning using ion analysis and investigate a possible role in stomatal opening using Licor and/or porometer. • Perform a plant accelerator experiment to capture growth of 30+ *V. berlandieri* x *V. vinifera* BCS_1 genotypes both ungrafted and grafted with a Shiraz scion. • Analyse growth rate data to investigate the influence of the CLE locus on the conferred growth of a Shiraz scion. • Perform an experiment to investigate the growth of transgenic Shiraz vines with altered expression of CHX20.1 and CHX20.2. • Perform crosses of K51-40 X 140 Ruggeri and *V. longii* with *V. vinifera*
Data from growth analysis experiments available to determine the correlation between growth rates in the short-term plant accelerator experiments and growth measures in previous long-term salinity field studies.	30/06/2021	• Compare data obtained from last year's Plant Accelerator experiment on 15 rootstocks ungrafted and grafted with a Shiraz scion to compare growth rates with the pruning weights and yield data from the field experiments conducted previously in the Riverland. • Collate and mine information on families 86 and 88 used in long term trials at Padthaway and Irymple in regard to yield and abiotic stress tolerance
Smart breeding tool available for rootstock breeding program.	30/06/2021	• Characterise transformants and select best lines for propagation in glasshouse
Data from field trials testing performance of salt-excluding rootstock C7 to inform a decision on release of the rootstock in 2020	30/06/2021	• Collate all available information regarding C7 • Determine if more experiments or data are required. • Hold discussions about potential for release

Year 5

Outputs	Target	Activities
Markers for sodium exclusion phenotype deployed across rootstock breeding germplasm	30/06/2022	• Perform a Heat x Salt experiment on 25 additional K51-40 x 140 Ruggeri progeny to determine the involvement of a new locus potentially associated with Na^+ accumulation response to elevated temperature.
Involvement of CHX20.1 and CHX20.2 in Cl^- exclusion and other processes determined. Additional K51-40 x 140 Ruggeri progeny assessed for mapping other potential chloride loci Genetics of chloride exclusion investigated in V. longii (syn. V. acerifolia), a super excluder	30/06/2022	• Perform glasshouse experiments on selected Shiraz with altered CHX1 and CHX2 expression reciprocally grafted to investigate the role of this gene in Cl^- partitioning using ion analysis and investigate a possible role in stomatal opening using Licor and/or porometer. • Perform a plant accelerator experiment to potentially discover new loci involved in chloride exclusion using K51-40 X140R progeny • Perform glasshouse experiments with seedlings from crosses of K51-40 X 140 Ruggeri and *V. longii* with *V. vinifera*
Data from growth analysis experiments available to determine the correlation between growth rates in the short-term plant accelerator experiments and growth measures in previous long-term salinity field studies.	30/06/2022	• Compare data obtained from last year's Plant Accelerator experiment on 15 rootstocks ungrafted and grafted with a Shiraz scion to compare growth rates with the pruning weights and yield data from the field experiments conducted previously in the Riverland. • Collate and mine information on families 86 and 88 used in long term trials at Padthaway and Irymple in regard to yield and abiotic stress tolerance
Smart breeding tool available for rootstock breeding program.	30/06/2022	• Characterise transformants for growth phenotype and flowering time
Data from field trials testing performance of salt-excluding rootstock C7 to inform a decision on possible commercial release of the rootstock to nurseries	30/06/2022	• Collate all available information regarding C7 • Commission rooting trial • Hold discussions about potential for release
Submission of final project report. Communication of results as applicable.	30/06/2022	• Prepare final report • Prepare papers for publication • Disseminate results contained in final project report to industry where possible (e.g. AWRI Roadshow, industry seminars)

5. General Methods

5.1 Plant Propagation and screening

5.1.1 Dormant hardwood cuttings

Dormant canes were collected from mothervines located at the CSIRO Irymple Farm, Victoria and subjected to hot water treatment before import to SA. Canes were callused and grafted by Yalumba Nursery. In short, ungrafted cuttings were dipped in rooting hormone before being covered in vermiculite, laid horizontally, and stored at 28^{o}C for 14-18 days. Grafted cuttings were callused in a similar manner, except that an omega bench graft was applied prior to dipping in rooting hormone and the graft site was sealed by dipping into melted wax immediately after the callusing period. Callused canes were then planted in a 1:1 perlite:sand mixture in 0.11 L square tubes and maintained in a glasshouse with misting with rainwater for four weeks. Rooted cuttings were then potted into Van Schaik's commercial potting mix (Biogro, Mount Gambier), hereto referred to as Biogro, in 2 L pots. Potted cuttings were then allowed to establish for two weeks in a humidified glasshouse and a further two weeks with humidifiers turned off.

5.1.2 Deflasked tissue culture plantlets

Transgenic grapevine material was generated and described within project CSA 1701-1.7. Sterile plantlets were propagated and grown in rooting media (SM macronutrients (160 mg/L NH_4NO_3, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_2O$, 2.325 g/L KNO_3, 340 mg/L KH_2PO_4), MS micronutrients (6.2 mg/L H_3BO_3, 0.025 mg/L $CoCl_2.6H_2O$, 0.025 mg/L $CuSO_4.5H_2O$, 16.9 mg/L $MnSO_4.H_2O$, 0.25 mg/L $Na_2MoO_4.2H_2O$, 0.83 mg/L KI, 8.6 mg/L $ZnSO_4.2H_2O$), B5 vitamins (100 mg/L myo-inositol, 10 mg/L thiamine-HCl, 1 mg/L Nicotinic acid, 1 mg/L Pyridoxine HCl), Fe EDTA, 1.5% sucrose, 0.7% tissue culture agar, pH 5.7) in 250 mL polycarbonate tissue culture vessels. Plantlets were deflasked from tissue culture vessels by first removing all rooting media agar from the roots and transferring to a 0.6 L round pot containing a Grape Soil Mix (18 L composted Pine Bark, 9 L river sand, 30 g $FeSO_4$, 40 g dolomite, 20 g garden lime, 20 g gypsum, 140 g slow-release fertiliser (Osmocote Exact Standard)), misted with water and covered in a plastic bag to maintain humidity. Bagged pots were placed in the glasshouse under shade cloth and hardened-off over a three week period by gradually decreasing the humidity by opening bags and increasing light by removal of shade cloth. During this hardening off period plants were watered with 0.2 g/L Aquasol (Yates). After another 2-3 weeks of establishment under glasshouse conditions watering was changed to ¼ strength Hoagland's solution for plants growing in perlite:sand, or rainwater for plants growing in Biogro potting mix.

5.1.3 Germinated seedlings

Harvested seeds were cleaned of pulp and then sterilised overnight in 0.5% H_2O_2 in a 50 mL falcon tube with mild agitation (90 rpm). Sterilised seeds were washed with sterile water four times before being stored on moist cotton wool at 4^{o}C for a minimum of eight weeks. Seeds were germinated by first cutting longitudinally down the seeds with a scalpel to crack the seed coat and then placing the cracked seed onto 90 mm sterile plates containing either

four layers of wetted sterile filter paper (Whatman no.1) or MS/2 media (half MS macronutrients (825 mg/L NH_4NO_3, 220 mg/L $CaCl_2.2H_2O$, 185 mg/L $MgSO_4.7H_2O$, 985 mg/L KNO_3, 85 mg/L KH_2PO_4), MS micronutrients s (6.2 mg/L H_3BO_3, 0.025 mg/L $CoCl_2.6H_2O$, 0.025 mg/L $CuSO_4.5H_2O$, 16.9 mg/L $MnSO_4.H_2O$, 0.25 mg/L $Na_2MoO_4.2H_2O$, 0.83 mg/L KI, 8.6 mg/L $ZnSO_4.2H_2O$), C1 vitamins and amino acids (100 mg/L myo-inositol, 10 mg/L thiamine HCl, 10 mg/L Nicotinic acid, 1 mg/L Pyridoxine HCl, 1 mg/L D-pantothenic acid, 0.01 mg/L Biotin; 100 mg/L L-glutamic acid, 10 mg/L L-phenylalanine, 2 mg/L Glycine), Fe EDTA, 1.5% sucrose, pH 5.7, 0.7% tissue culture agar). Plates were stored at 27^{o}C for 2-4 weeks at which time germinating seedlings were transferred to Biogro potting mix in a humid box (an enclosed plastic tray with a clear Perspex lid). Over a 2-3 week period the humidity of the box was decreased by progressively opening and then removing the lid. Once acclimatised to glasshouse conditions, seedlings were potted into Biogro potting mix in 10 cm square pots.

5.1.4 Screening conditions

Plants were assessed for their ion exclusion abilities across many experiments that were conducted in either CSIRO glasshouse facilities or the Smarthouses of the Plant Accelerator (University of Adelaide). In all instances, growing spaces were set to standard conditions of 18-20^o C nights / 25-30^o C days.

Salt treatments were applied as mixed cation salts of NaCl : $MgCl_2$: $CaCl_2$ in a ratio of 6:1:1, which equates to a 10:6:1:1 ratio of Cl^- : Na^+ : Mg^{2+} : Ca^{2+} ions. The concentration given for mixed Cl^- treatments refers to the concentration of Cl^-. Rainwater (approximately 0.1 mM Cl^-) was applied for all control treatments. It should be noted that the Biogro potting mix is also a source of Cl^-, contributing approximately 1.0-1.5 mM of additional Cl^- to both control and salt treated plants.

5.2 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.

5.2.1 Chloride concentration

Dried samples were examined in duplicate for Cl^- 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 5 mL glass vials and 4 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.

5.2.2 Cation concentration

Dried samples were examined 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°C/min initially to 60°C, with a holding period at 60°C for 20 min, then at 85°C for 270 min. Ninety-six vessels were heated per batch. The tubes were allowed to cool, then made up to 15 mL with Millipore® 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.

5.3 Molecular analysis

5.3.1 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 was 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 chosen 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.

5.3.2 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.

5.3.3 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.

5.3.4 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.

5.3.5 Southern analysis

Two micrograms of genomic DNA 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.

6. Influence of the Cl^- exclusion (*CLEX*) locus in the *V. berlandieri* x *V. vinifera* BC_1F_2 population

6.1 Objectives

In the prior project (CSP 1302), we reported on the identification of a novel locus for Cl^- exclusion, *CLEX*, which was genetically mapped in a juvenile seedling population derived from the back crossing of *V. berlandieri* to *V. vinifera* (BC_1F_2). The objectives of the experiments described here were to confirm and gain a deep understanding of the influence of this locus on Cl^- exclusion in mature vines of this BC_1F_2 population, including ungrafted field-grown vines and both ungrafted and grafted vines in pots under low and high salinity treatments for short (14 days) and extended periods (100 days).

6.2 Background

The ability of a plant to tolerate growth on saline soils is a highly complex trait that can be divided into three sub-traits, namely, osmotic tolerance, ionic or tissue tolerance, and ion exclusion (Munns & Tester 2008). Of the three sub-traits, ion exclusion is arguably the most pertinent to grapevine rootstocks, as some rootstocks are better ion excluders compared to own-rooted *V. vinifera* cultivars, which are generally poor excluders. Thus, the use of ion excluding rootstocks reduces the amount of Na^+ and Cl^- ions that accumulate in the leaves of a grafted scion thereby avoiding ionic toxicity and leaf burn symptoms in the laminae (Downton 1977, Prior et al. 1992), as well as avoiding undesirable levels of these ions in the grapes and resulting wine (Walker et al. 2003, De Loryn et al. 2014, Walker et al. 2019). Ion exclusion traits are also the easiest of the three sub-traits to phenotype, requiring a simple petiole or laminae ion analysis, compared to tissue or osmotic tolerance which require more detailed analysis of reductions in growth between control and salt treatments. Hence, ion exclusion traits have received the majority of attention from previous studies aiming to untangle the genetics of salt tolerance in grapevines. In terms of genetic control, ion exclusion traits are also likely to be the least complex of the three-sub traits and therefore are the obvious first target for developing and deploying predictive genetic markers to breed for improved salt tolerance. Indeed, we have previously identified a single major QTL that explains up to 70% of the variation of Na^+ exclusion in a K51-40 x 140 Ruggeri population (Henderson et al. 2018). This led to the identification of the causal gene *HKT1;1* and two causative SNPs that alter the transport properties of HKT1;1 proteins to serve as the basis of a robust genetic marker for genetic screening for Na^+ exclusion in rootstock breeding programs (Henderson et al. 2018, Dunlevy et al. 2019). However, the major genes controlling Cl^- exclusion have remained elusive, despite the bulk of prior research being focused on this ion.

The majority of past studies that have investigated the inheritance of Cl^- exclusion in *Vitis* populations have concluded that the trait is likely multigenic (Gong et al. 2011, Fort et al. 2015), and this has hindered the identification of loci or genes contributing to this trait. A few

studies, however, have observed bimodal patterns of variation within populations indicating the influence of a major locus (Newman & Antcliff 1983, Sykes 1987). In the final report for the previous project CSP 1302, we described the generation of an interspecific population derived from a selfed (*V. berlandieri* x *V. vinifera* cv. L. Sultana) x *V. vinifera* cv. L. Biancone BC_1 individual, MU27-38. The resulting population of 217 BC_1F_2 individuals displayed a clear 3:1 segregation of Cl^- exclusion vs accumulation when screened as young unreplicated seedlings. This enabled the identification of the first locus, named *CLEX,* associated with Cl^- exclusion in grapevines. The *CLEX* allele derived from *V. berlandieri* is dominant for Cl^- exclusion, consistent with that species being a source of strong Cl^- exclusion, with the recessive allele being derived from the poor Cl^- excluder, *V. vinifera*. Genetic markers at this locus explained 89% of the variation in Cl^- exclusion in the unreplicated seedling screens. The aim of this chapter was to confirm the major influence of the *CLEX* locus in this population when screened as replicated, own-rooted cuttings under a range of different salt and graft treatments.

6.3 Materials and Methods

6.3.1 Seedling population phenotypes

As described in the final report for CSP 1302 (Section 6.9), BC_1F_2 seedlings were previously germinated and assessed in two batches, an initial screen of 69 individuals and a larger follow-up screen of 148 individuals. The follow-up screen consisted of a 25 mM mixed Cl^- treatment for 48 days and was divided into two blocks based on plant size, which were one week apart in timing. In the current report, we assessed the cation concentrations in the laminae of the larger 86 BC_1F_2 individuals that were part of block A of the follow-up screen.

We also assessed the laminae Cl^- concentration of 112 BC_1F_2 individuals that had been growing in the CSIRO vineyard at Irymple, Victoria, for 2.5 years. Seedling vines were planted over four rows, at two vines per panel and 3 m spacing between vines. Genotypes belonging to the three possible *CLEX* inheritance groups (*Vb Vb*, *Vb Vv*, or *Vv Vv*) were distributed randomly across the field. Vines were irrigated with Murray River water (approx. 3 mM Cl^-). Ten laminae were harvested from each vine in February of 2017.

6.3.2 Hardwood cutting benchmarking screen

Hardwood cuttings were collected from 16 BC_1F_2 mothervines that were large enough to provide sufficient cutting material at that time. Of these, six were homozygous for the *V. berlandieri CLEX* allele (*Vb/Vb*), five heterozygous (*Vb/Vv*) and five homozygous for the *V. vinifera CLEX* allele (*Vv/Vv*). Cuttings of *V. longii*, 140 Ruggeri and MI09-07 were also collected for referencing BC_1F_2 lines against strong Cl^- excluder genotypes. Similarly, K51-40 cuttings were collected for reference to a poor Cl^- excluder. Dormant cuttings were propagated as described in section 5.1.1 and potted as own-rooted cuttings into Biogro in 2.4 L pots and grown under glasshouse conditions. After seven weeks of growth, the eight most uniform cuttings of each genotype were selected and assigned to two salt treatments, four replicate cuttings per treatment, and arranged in a nested (treatment) randomised block (replicate) design. Single shoot cuttings were exposed to daily applications (250-450

mL) of either low (25 mM) or high (100 mM) mixed Cl^- treatments for 15 days. To keep track of the actual salt concentrations accumulating in the pots, the flow through was regularly captured and analysed from the two largest and two smallest vines in each salt treatment. Figure 6.1 shows the gradual build-up in actual Cl^- concentration in the pot water over the course of the experiment. At harvest, the Cl^- concentrations of soil run through water ranged from 36-46 mM in the low salt treatment to 125-148 mM in the high salt treatment. At the end of the experiment the five most mature leaves were harvested from all vines and dried for subsequent Cl^- analysis.

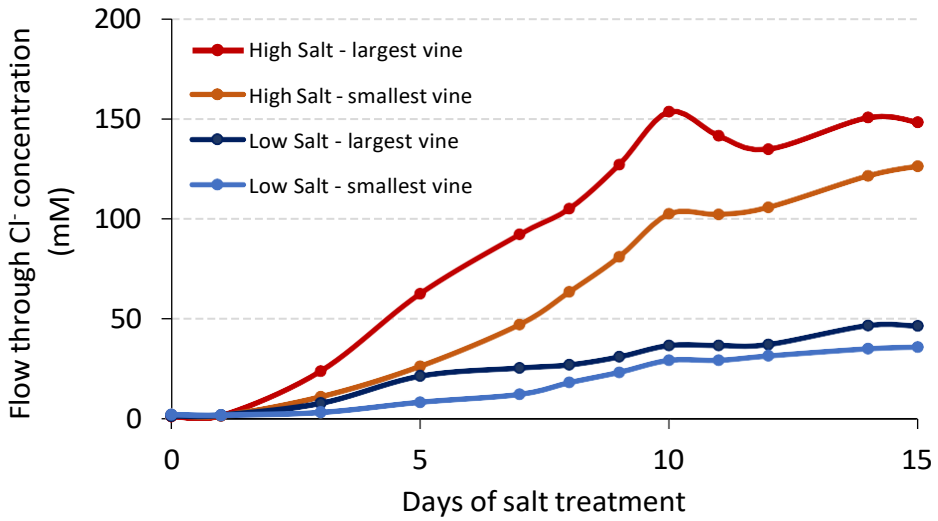


Figure 6.1 – Successive build-up of Cl^- concentration of flow through after watering with either 25 mM (low salt treatment) or 100 mM (high salt treatment) applied as mixed Cl^-. Flow through was only collected from the smallest and largest vines of each treatment with the assumption that transpiration differences resulting from plant size were the main factor leading to differences in Cl^- concentration of soil run through water, therefore the smallest and largest vines are the best estimate of the minimum and maximum pot Cl^- concentrations, respectively.

6.3.3 2019 TPA reciprocal grafting experiment (exp0502)

The major aim of this grafting experiment was to determine whether the Cl^- exclusion attributed to the *CLEX* locus was conferred to a grafted scion. For this experiment we chose five *Vb/Vb*, five *Vb/Vv* and five *Vv/Vv* genotypes based on mothervines that were large enough to provide sufficient material for a grafting experiment (pencil thickness at minimum). All genotypes were assessed ungrafted, as the rootstock grafted with a Shiraz scion, or reciprocally grafted as the scion onto Shiraz roots. The reciprocal graft treatment was performed to see if the *CLEX* locus plays any role in the shoot/scion. Ungrafted and self-grafted Shiraz were also included as a grafting control to ensure the act of grafting did not influence Cl^- accumulation into the laminae. This experiment was conducted in the SE

Smarthouse of the Plant Accelerator, where plants were automatically watered to a set weight daily, ensuring accurate and consistent pot salt concentrations. On day 1 and day 3, mixed Cl^- solution was manually applied to salt treatment plants equivalent to pot concentrations of 33 mM and 67 mM Cl^-, respectively, for a total pot concentration of 100 mM Cl^- maintained from day 3 to day 19 by automated watering to set weight with rainwater. Control plants received daily applications of rainwater to set weight. On completion, all shoot material was harvested for determination of dry weights and the Cl^- concentration of laminae.

6.3.4 2020 TPA reciprocal grafting experiment (exp0563)

This follow up reciprocal grafting experiment was essentially performed the same as the prior experiment except that it included 30 *Vb/Vb*, 32 *Vv/Vv* and five heterozygous Vb/Vv BC_1F_2 genotypes, together with *V. longii*, the parent MU27-38, 140 Ruggeri, 1103 Paulsen, Shiraz and K51-40. All plants received a 100 mM mixed Cl^- salt treatment, again achieved with 33 mM and 66 mM applications occurring on days 1 and 5. On all other days, each vine was automatically watered to a set weight. Vine growth was captured using a LemnaTec 3D Scanalyzer digital imaging system (LemnaTec, Aachen, Germany) on each day except for days of salt application and harvest. On day 22 the mature fully expanded leaves (roughly 6-8) were harvested from each vine for determining Cl^- concentration.

Relative growth rates were calculated from digital images as described in Dunlevy et al. 2022.

6.3.5 Longer term glasshouse experiment

This experiment utilised spare Shiraz-grafted BC_1F_2 material that was propagated for the 2019 reciprocal grafting experiment. Genotypes included five *Vb/Vb* and five *Vv/Vv* lines, which ranged between one and three replicates per treatment (control and salt) for a total of nine vines per salt treatment x inheritance group. The same number of replicates of each line was used across both salt treatments. Vines were propagated and established as described in section 6.3.3 before being potted into Biogro in 2.4 L pots and grown in a randomised block layout under glasshouse conditions (30^{o}C days / 18^{o}C nights). Each vine was hand watered with between 250-500 mL of rainwater (control) or 33 mM mixed Cl^- (salt treatment) for the first 31 days, after which time 1,000 mL of rainwater or 33 mM mixed Cl^- was applied daily per pot. The flow through was collected and its Cl^- concentration determined for a subset of vines throughout the experiment, which revealed Cl^- concentrations of soil run through water of approximately 0.5 mM in the control treatment and 50 mM in the salt treatment.

Measurements of photosynthetic capacity were made on days 92-94 using a Photosynthesis Yield Analyzer Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany). Sunlight was eliminated from leaves by covering with a foil pouch at least 30 min before measurements were taken. Four ‘dark-adapted’ leaves were measured per vine to calculate Fv/Fm - a measure of maximum quantum efficiency of photosystem II (Murchie & Lawson 2013). Four light exposed leaves per vine were also measured to determine ΔF/Fm’ - a measure of energy used in photochemistry of photosystem II under steady state conditions.

6.4 Results

6.4.1 Seedling population phenotypes

As described in the final report for project CSP 1302, the BC_1F_2 population segregates in a clear 3:1 ratio of excluders to accumulators when screened as juvenile 4–10-week-old seedlings. Furthermore, this segregation was found to be associated with a single dominant locus, *CLEX*, which explained 88.9% of the variation in Cl^- accumulation in that population. *Vb/Vb* genotypes on average accumulate significantly ($p<0.05$) less Cl^- in the laminae than *Vb/Vv* genotypes, while *Vv/Vv* genotypes on average accumulate significantly ($p<0.0001$) more Cl^- in the laminae than either of the other two genotypes (Figure 6.2A). To confirm that these results are not just attributed to screening the population as juvenile seedlings, we measured the laminae Cl^- concentration of 112 BC_1F_2 vines after 2.5 years of growth in the CSIRO vineyard at Irymple. This revealed that even as mature vines the *CLEX* locus accounted for 85.1% of variation in laminae Cl^- accumulation. Again, *Vv/Vv* genotypes accumulated significantly more Cl^- than either *Vb/Vb* or *Vb/Vv* genotypes, however there was no significant difference between the average of *Vb/Vb* or *Vb/Vv* genotypes. This may be attributed to the low salt stress in the vineyard, approximately 3 mM Cl^- in irrigation water. At this low salt concentration it is likely that only one copy of the dominant *Vb* allele is sufficient to exclude the low Cl^- load at the same rate as having two copies, whereas at higher Cl^- loads, possessing two copies of the *Vb* allele allows a greater rate of exclusion of Cl^- than having one copy allows.

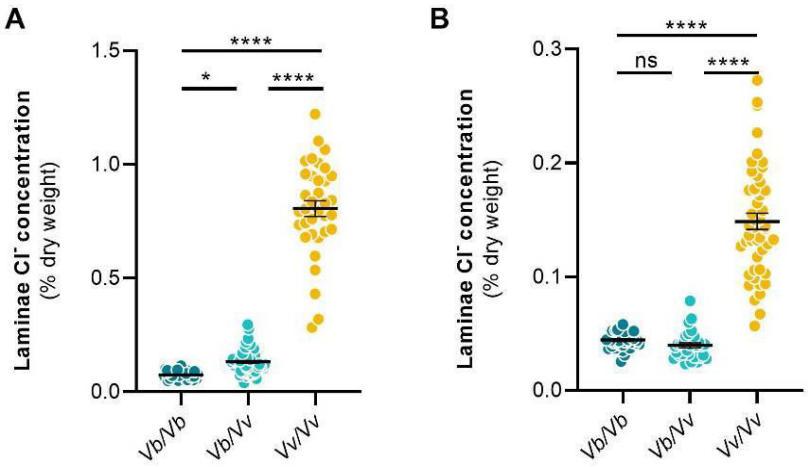


Figure 6.2 – Cl^- accumulation in the laminae of *V. berlandieri* x *V. vinifera* BC_1F_2 individuals when screened as (A) 4–10-week-old seedlings exposed to 25 mM mixed Cl^- treatment and (B) as 2.5-year-old vines grown at Irymple under River Murray water irrigation (approx. 3 mM Cl^-). Progeny are grouped by their inheritance of the *CLEX* locus (*Vb/Vb* - homozygous *V. berlandieri*, *Vv/Vv* homozygous - *V. vinifera*, *Vb/Vv* - heterozygous). Data points indicate the value of a single replicate of a BC_1F_2 individual, horizontal lines represent the mean of each group, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where differences between *CLEX* inheritance groups are significant as follows: ns = not significant, * = $p<0.05$, **** = $p<0.0001$.

We also examined the accumulation of major cations, Na^+, Mg^{2+}, Ca^{2+} and K^+, in the laminae of 86 BC_1F_2 genotypes from the second juvenile seedling screen. This subset of genotypes was chosen as they comprised block A (project CSP1302) which provided the cleanest segregation of Cl^- exclusion with the *CLEX* locus and therefore deemed the most appropriate to search for possible effects on other ions. The concentration of Na^+ in laminae was low (<0.04 % d.w.) relative to that for Cl^- but even at such low concentrations there were significant differences in mean Na^+ concentration between *Vb/Vb* genotypes and either *Vb/Vv* or *Vv/Vv* genotypes, but not between *Vb/Vv* and *Vv/Vv* genotypes (Fig. 6.3A). These differences were minor, with single marker analysis revealing that this locus explains only 12.1% of total genotypic variation in laminae Na^+. The *CLEX* locus also explained 14.5% of the variation in laminae Mg^{2+} concentration, with the only statistically significant difference occurring between *Vb/Vv* and *Vv/Vv* genotypes (Figure. 6.3B). Similarly, the only significant difference in laminae Ca^{2+} was between the *Vb/Vv* and *Vv/Vv* genotypes (Fig. 6.3C), with only 6.4% of the total variation attributed to the *CLEX* locus. For K^+, the CLEX locus only explained 4.7% of the total variation with no significant differences between the means of any of the inheritance groups (Fig. 6.3D)

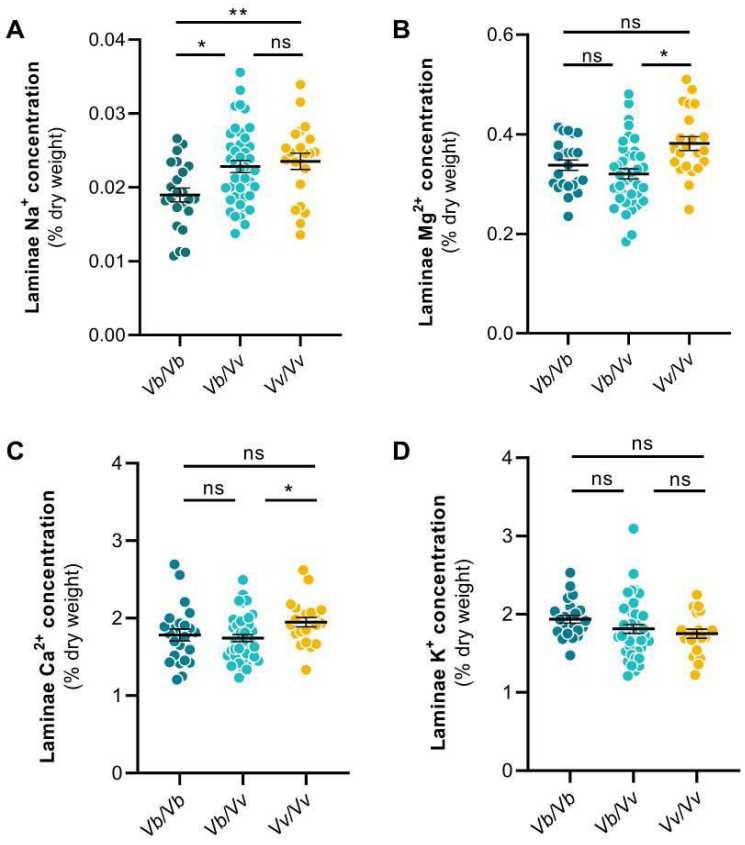


Figure 6.3 – Major cation concentrations in the laminae of *V. berlandieri* x *V. vinifera* BC_1F_2 individuals when screened as 4-10-week-old seedlings exposed to 25 mM mixed Cl^- treatment, (A) Na^+, (B) Mg^{2+}, (C) Ca^{2+} and (D) K^+. Progeny are grouped by their inheritance of the *CLEX* locus (*Vb/Vb* - homozygous *V. berlandieri*, *Vv/Vv* homozygous - *V. vinifera*, *Vb/Vv* - heterozygous). Data points indicate the value of a single replicate of a BC_1F_2

individual, horizontal lines represent the mean of each group, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where differences between *CLEX* inheritance groups are significant as follows: ns = not significant, * = $p<0.05$, ** = $p<0.01$.

6.4.2 2017 hardwood cutting benchmarking experiment

We next wanted to confirm that the increased Cl^- exclusion attributed to the *Vb* allele of the *CLEX* locus was carried through from seedling mothervines to clonally propagated cuttings, as practiced in industry. At the time, sufficient cutting material could only be collected from 16 BC_1F_2 mothervines (six *Vb/Vb*, five *Vb/Vv* and five *Vv/Vv*). We also wanted to benchmark the Cl^- exclusion of BC_1F_2 genotypes against existing rootstocks with strong Cl^- exclusion abilities; 140 Ruggeri, the most commonly used rootstock for salt tolerance; MI09-07, a CSIRO rootstock selected for good Cl^- exclusion and salt tolerance; and *V. longii*, a species recently identified for superior Cl^- exclusion. The poor Cl^- excluding rootstock K51-40 was also included. All genotypes were exposed to low (25 mM) or high (100 mM) mixed Cl^- applications for 15 days. This resulted in a continual build-up of Cl^- concentrations in soil run through water of between 36-46 mM in the low salt treatment and 125-148 mM in the high salt treatment (Fig. 6.1). Consistent with the results of the unreplicated juvenile seedling screen, *Vv/Vv* genotypes on average accumulated significantly more Cl^- in the laminae than *Vb/Vb* and *Vb/Vv* genotypes in both the low and high salt treatments (Fig. 6.4). Unlike the results of the seedling screen, there were no significant differences between *Vb/Vb* and *Vb/Vv* genotypes in either the low or high salt treatments. In both salt treatments *V. longii* and 140 Ruggeri accumulated lower Cl^- concentrations in laminae than the *Vb/Vb* or *Vb/Vv* BC_1F_2 genotypes, which were similar to that of MI09-07. The *Vv/Vv* genotypes accumulated Cl^- concentrations in laminae similar to that of K51-40. We caution that these comparisons should only be used a rough guide as there were major differences in the age and thickness of cutting material used to propagate reference genotypes compared to the younger BC_1F_2 mothervine cuttings, and this could have influenced the extent of Cl^- accumulation in the laminae. There were also differences between replicate numbers among rootstocks at both the low and high salt treatments, ranging from three to six.

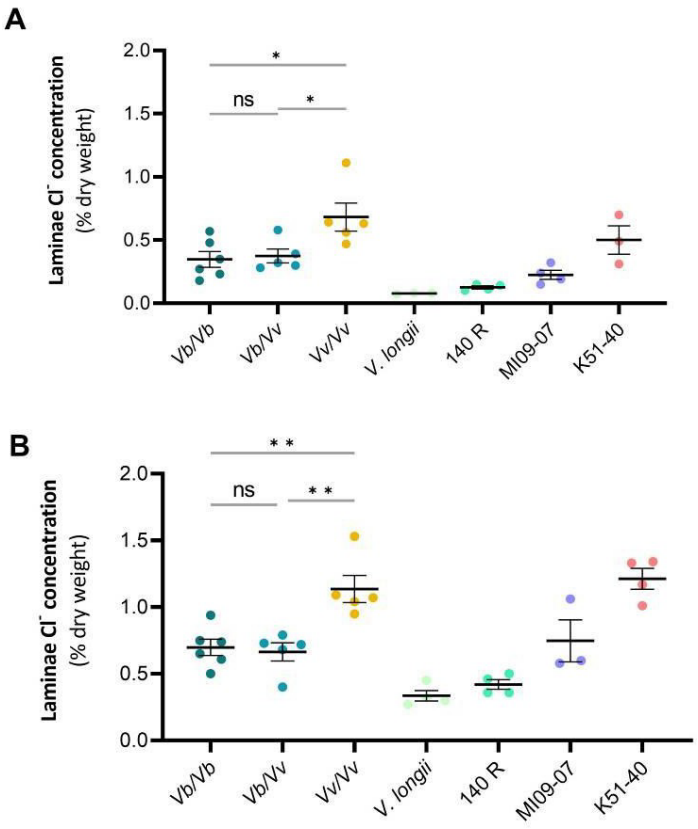


Figure 6.4 – Accumulation of Cl^- in the laminae of *V. berlandieri* x *V. vinifera* BC_1F_2 individuals and four reference lines grown from hardwood cuttings exposed to (A) a 25 mM or (B) 100 mM mixed Cl^- treatment. BC_1F_2 progeny are grouped by their inheritance of the *CLEX* locus (*Vb/Vb* - homozygous *V. berlandieri*, *Vv/Vv* homozygous - *V. vinifera*, *Vb/Vv* - heterozygous). For BC_1F_2 progeny data points indicate the mean of four biological replicates and the horizontal line indicates the mean of each group. For reference lines, each data point represents a replicate plant with horizontal lines indicating the mean for that genotype. Error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where differences between *CLEX* inheritance groups are significant as follows: ns = not significant, * = $p<0.05$, ** = $p<0.01$.

6.4.3 2019 TPA reciprocal grafting experiment (exp0502)

We next sought to determine if the differences in Cl^- exclusion linked with the *CLEX* locus were conferred to a grafted scion. Five genotypes of each inheritance group, *Vb/Vb*, *Vb/Vv* and *Vv/Vv,* were assessed ungrafted, as the rootstock grafted with a Shiraz scion, or reciprocally grafted as the scion onto Shiraz roots. This last graft treatment was performed to see if the *CLEX* locus plays any role in the shoot/scion. Again, *Vv/Vv* genotypes on average accumulated significantly more Cl^- in the laminae than *Vb/Vb* and *Vb/Vv* genotypes in both the 0 mM Cl^- (rainwater treatment) and 100 mM mixed Cl^- salt treatment and in both the ungrafted and grafted treatments (Fig. 6.5). The only significant difference between *Vb/Vb* and *Vb/Vv* genotypes was in the ungrafted 100 mM Cl^- treatment (Fig. 6.5B). When reciprocally grafted, there were no significant differences in laminae Cl^- concentration between the three *CLEX* inheritance groups in either the 0 mM or 100 mM Cl^- treatments. There was also no significant difference in laminae Cl^- concentration between ungrafted and self-grafted Shiraz, confirming

that the grafting process did not influence Cl^- exclusion. Taken together, these results confirm that the Cl^- exclusion mechanism attributed to the *CLEX* locus is active in the root-system and is fully conferred to a grafted scion. This is consistent with the finding of others (Storey et al. 2003, Tregeagle et al. 2010, Gong et al. 2011, Abbaspour et al. 2013) who have found that the major Cl^- exclusion mechanism in grapevines is present within the root vasculature.

At the end of the experiment, the dry weight of all shoot material from each vine was measured. Consistent with results from the seedling screen (Final Report CSP 1302), there were no significant differences in average shoot dry weight between any of the three *CLEX* inheritance groups when ungrafted (Figure 6.6). When grafted with a Shiraz scion, however, there was a consistent trend in both 0 mM Cl^- and 100 mM mixed Cl^- treatments of higher average shoot biomass in *Vb/Vb* lines with less in *Vb/Vv* genotypes and least in the *Vv/Vv* genotypes (Figure 6.6). However, these differences were only significantly different between the *Vb/Vb* and *Vv/Vv* genotypes under the 100 mM Cl^- treatment. Conversely, in the reciprocally grafted treatment, *Vv/Vv* genotypes were on average significantly larger in shoot biomass than *Vb/Vb* genotypes in both the 0 mM Cl^- and 100 mM mixed Cl^- treatments. However, the heterozygous *Vb/Vv* genotypes had the highest average shoot biomass when reciprocally grafted, which was significantly higher than *Vb/Vb* genotypes under both treatments, and higher than the *Vv/Vv* genotypes in only the 0 mM Cl^- treatment (Fig. 6.6). These results hint at the possibility that the *CLEX* locus not only controls Cl^- exclusion, but either directly or indirectly influences shoot growth, which appears to be in an inverse way in the root system versus the shoot. Given that these results are based on just a small number of genotypes (five for each *CLEX* inheritance group), the differences seen could simply be the chance result of a small sample size. In the following experiment we repeated this experiment with an increased number of BC_1F_2 genotypes to confirm whether the *CLEX* locus influences shoot biomass when grafted or reciprocally grafted.

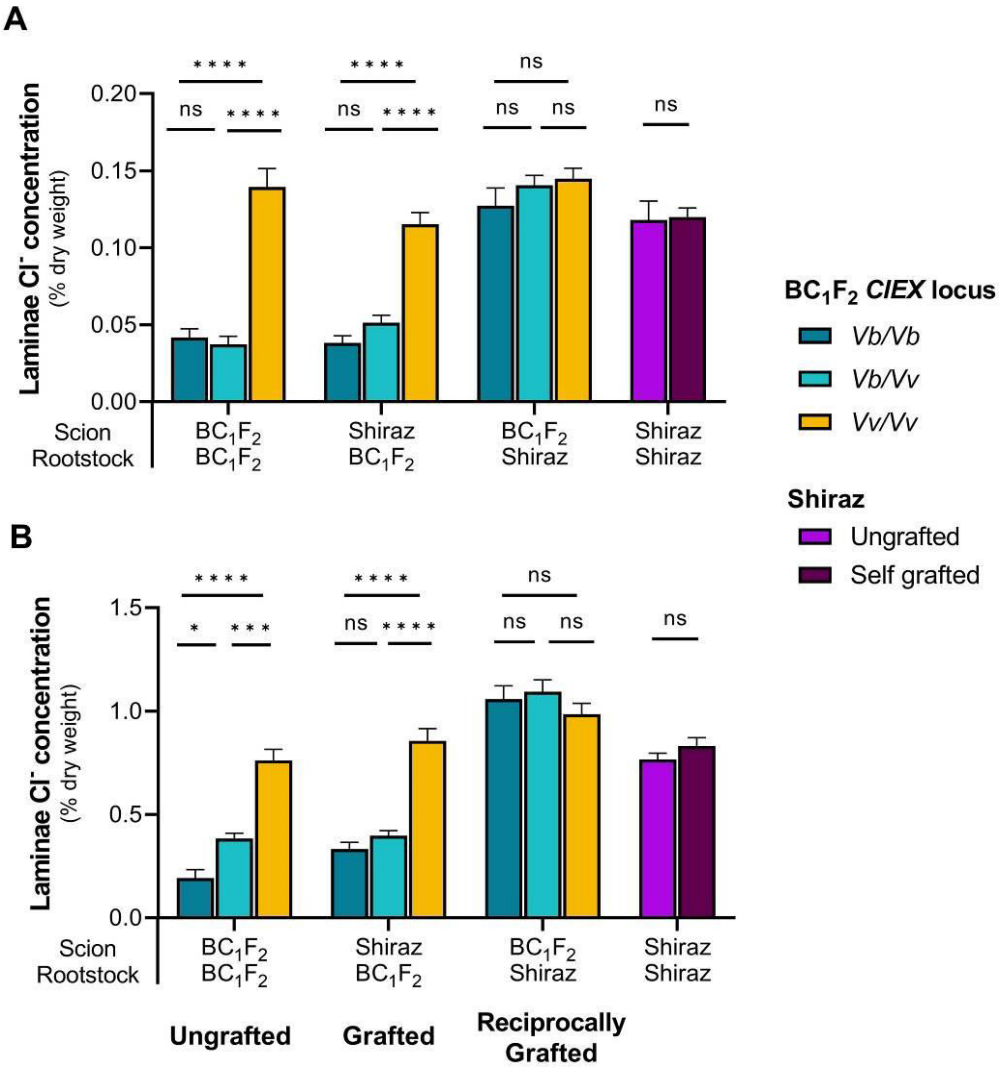


Figure 6.5 – Effect of *CLEX* locus on the laminae Cl^- accumulation of BC_1F_2 lines exposed to (A) 0 mM Cl^- and (B) 100 mM mixed Cl^- treatment and grown as either, ungrafted, grafted with a Shiraz scion, or reciprocally grafted onto Shiraz roots. Ungrafted and self-grafted Shiraz were included as a grafting control. For BC_1F_2 lines, each *CLEX* inheritance group consists of five genotypes x three biological replicates, with error bars indicating the standard error of the mean across genotypes. Tukey's multiple comparison tests indicate the levels of significance of difference between *CLEX* inheritance groups for each graft treatment as follows: ns = not significant, * = $p<0.05$,
*** = $p<0.001$, **** = $p<0.0001$.

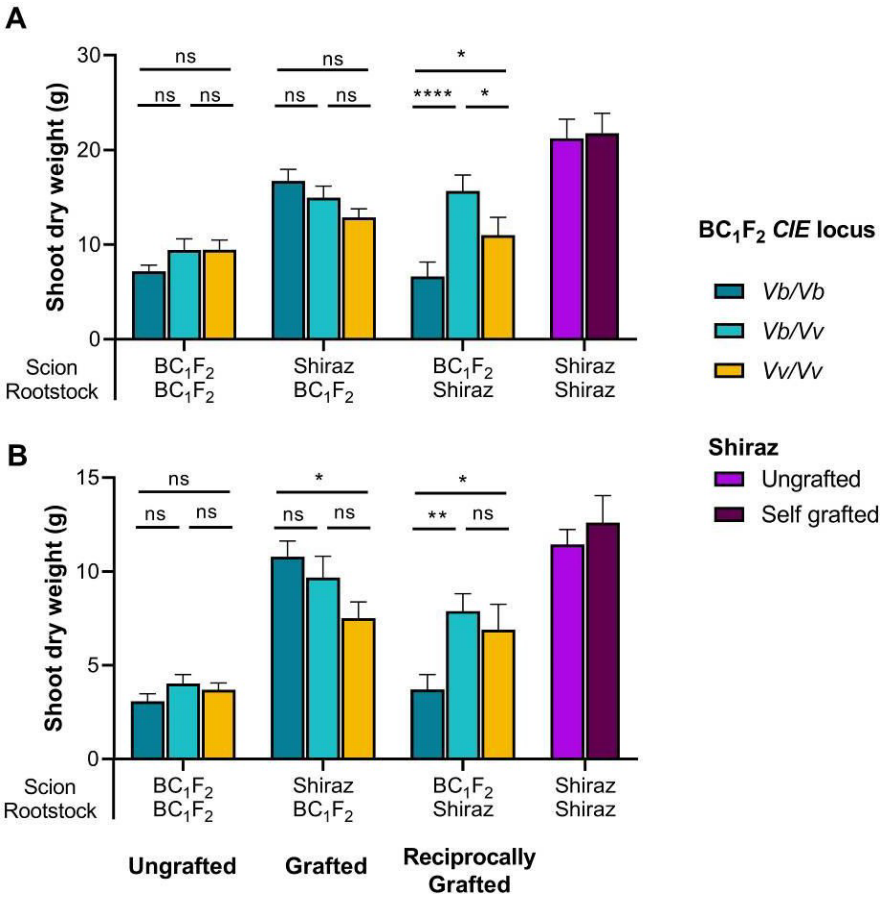


Figure 6.6 – Effect of *CLEX* locus on the shoot biomass of BC_1F_2 lines exposed to (A) 0 mM Cl^- and (B) 100 mM mixed Cl^- treatment and grown as either, ungrafted, grafted with a Shiraz scion, or reciprocally grafted onto Shiraz roots. Ungrafted and self-grafted Shiraz were included as a grafting control. For BC_1F_2 lines, each *CLEX* inheritance group consists of five genotypes x three biological replicates, with error bars indicating the standard error of the mean across genotypes. Tukey's multiple comparison tests indicate the levels of significance of difference between *CLEX* inheritance groups for each graft treatment as follows: ns = not significant, * = $p<0.05$, ** = $p<0.01$, **** = $p<0.0001$.

6.4.4 2020 TPA reciprocal grafting experiment (exp0563)

In this follow up grafting experiment we chose to focus primarily on homozygous *Vb/Vb* (n=30) and homozygous *Vv/Vv* (n=32) genotypes, with only five heterozygous *Vb/Vv* genotypes included. *V. longii*, the parent BC_1 genotype MU27-38, 140 Ruggeri, 1103 Paulsen, Shiraz and K51-40 were also included as reference genotypes. As with the previous experiment, all genotypes were screened ungrafted, grafted with a Shiraz scion, or reciprocally grafted onto Shiraz roots.

To further explore whether the *CLEX* locus has an influence on growth we looked at both total biomass, measured as final projected shoot area (sPSA), as well as relative growth rate following the second salt application from days 5 to 22. There were no significant differences between *Vb/Vb* and *Vv/Vv* genotypes for either measure of growth whether ungrafted, grafted or reciprocally grafted (Fig. 6.7). A large variation was apparent in both final biomass and relative growth rate across all genotypes. Thus, it appears that the influence of the *CLEX* locus on biomass seen in the previous experiment was likely an artifact of this large variation

and the small number of genotypes used in that experiment.

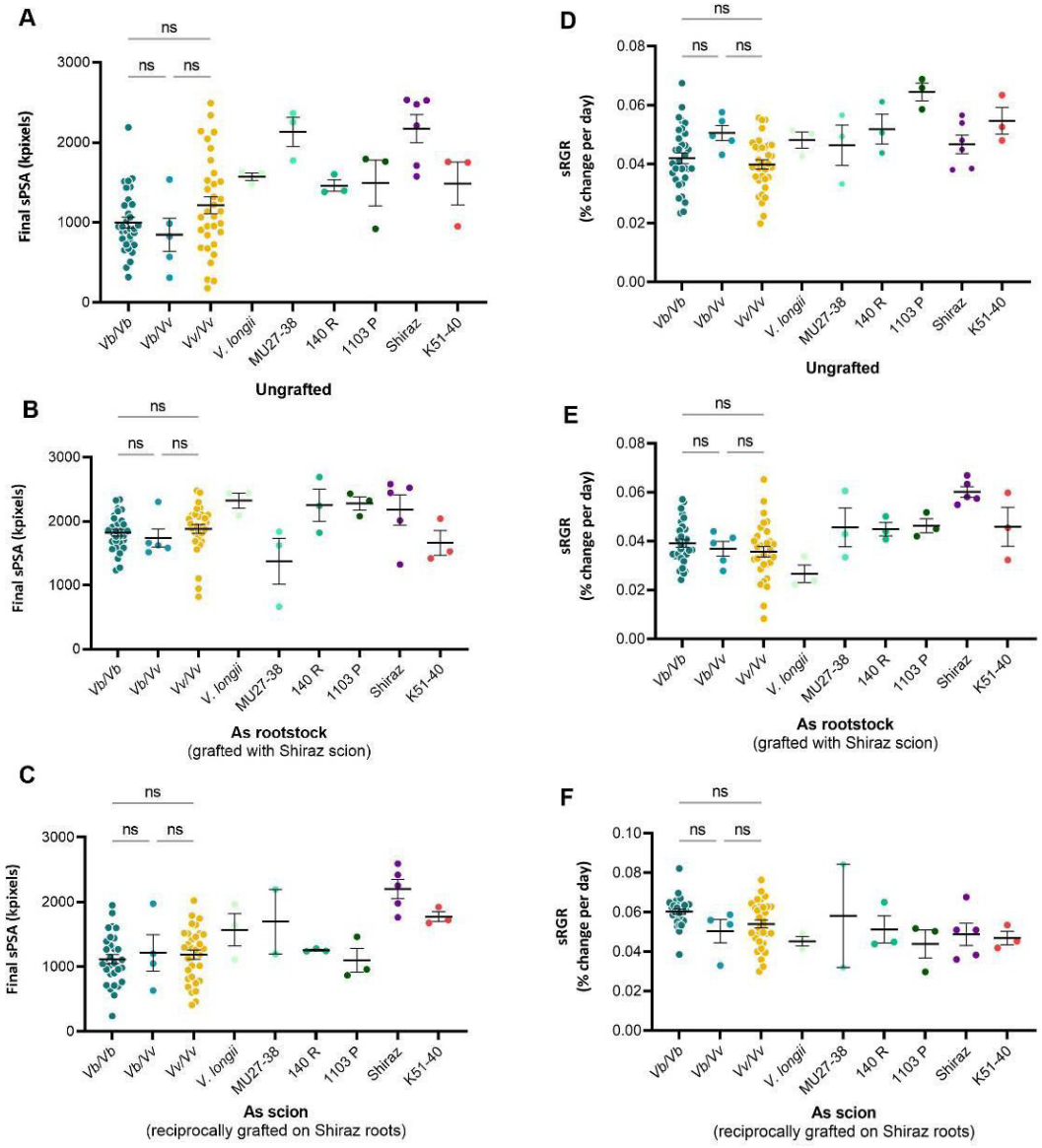


Figure 6.7 – Final projected shoot area (left) and relative growth rate (right) of BC_1F_2 lines exposed to a 100 mM mixed Cl^- treatment and grown as either (A & D) ungrafted, (B & E) as the rootstock grafted with a Shiraz scion, or (C & F) as the scion reciprocally grafted onto Shiraz roots. *V. longii*, the parent MU27-38, 140 Ruggeri, 1103 Paulsen, Shiraz and K51-40 were included as reference genotypes. For BC_1F_2 lines, data points represent the mean of three biological replicates, with n=30 *Vb/Vb* genotypes, n=5 *Vb/Vv* genotypes and n= 32 *Vv/Vv* genotypes. Error bars indicate the standard error of the mean across genotypes. Tukey's multiple comparison tests indicate no significant difference (ns) between *CLEX* inheritance groups. For reference lines, data points represent individual biological replicates with no statistical analysis applied.

Despite the lack of effect of the *CLEX* locus on growth and biomass, there were some interesting observations regarding the influence of this locus on Cl^- exclusion. Consistent with the previous experiment, *Vv/Vv* genotypes on average accumulated significantly more Cl^- in the laminae than *Vb/Vb* or *Vb/Vv* genotypes when ungrafted or grafted (Fig. 6.8 A&B). Surprisingly the inverse trend was seen when BC_1F_2 genotypes were used as the scion reciprocally grafted onto Shiraz roots, with *Vv/Vv* genotypes on average accumulating less Cl^- in the laminae than *Vb/Vb* genotypes (Fig. 6.8C). This result was not evident on the smaller number of genotypes in the previous experiment. Interestingly, this same trend was also evident in most of the reference genotypes with the strongest excluders as rootstocks, *V. longii* and MU27-38, showing the highest Cl^- accumulation when used as the scion. K51-40 was the only reference genotype that did not fit this trend. We found that K51-40 lacks expression of the candidate gene underlying the *CLEX* locus, *CHX20.1* (described in Chapters 7&8), which would explain why K51-40 does not display this inverse trend and results in high accumulation of Cl^- when used as the rootstock or the scion.

To explore this apparent inverse relationship of Cl^- accumulation between the rootstock and scion, we next correlated the laminae Cl^- accumulation of individual *Vb/Vb* and *Vv/Vv* BC_1F_2 genotypes when ungrafted, grafted or reciprocally grafted. When comparing ungrafted with grafted (as rootstock) results, across all genotypes there was a highly significant and strong (R^2=0.86) positive correlation, consistent with the major Cl^- exclusion mechanism being predominantly active in the root system (Fig. 6.9A). This correlation was also highly significant within *Vv/Vv* genotypes but with a moderate R^2 of 0.42, whereas there was no significant correlation within the *Vb/Vb* genotypes.

When looking between ungrafted and reciprocally grafted BC_1F_2 genotypes the only significant correlation was within the *Vv/Vv* genotypes (R^2=0.19), with insignificant correlations within the *Vb/Vb* genotypes (Fig. 6.9B). The weak negative correlation (R^2=0.04) across all genotypes was also not significant. This suggests that the inverse relationship in Cl^- exclusion conferred within the root system as seen in reference genotypes and the averages of BC_1F_2 inheritance groups does not hold up within individual BC_1F_2 genotypes. Similarly, no significant correlations were evident between grafted and reciprocally grafted laminae Cl^- concentrations across any genotype groups (Fig. 6.9C). Despite the inverse relationship in Cl^- exclusion with roots and shoots not being evident across individual BC_1F_2 genotypes, evidence presented in the next chapter shows that candidate genes within the *CLEX* locus, *CHX20.1* and *CHX20.2*, each contribute to Cl^- exclusion in the root and shoot respectively. Further discussion on root vs shoot Cl^- exclusion and how it may relate to the results presented here is provided in that chapter.

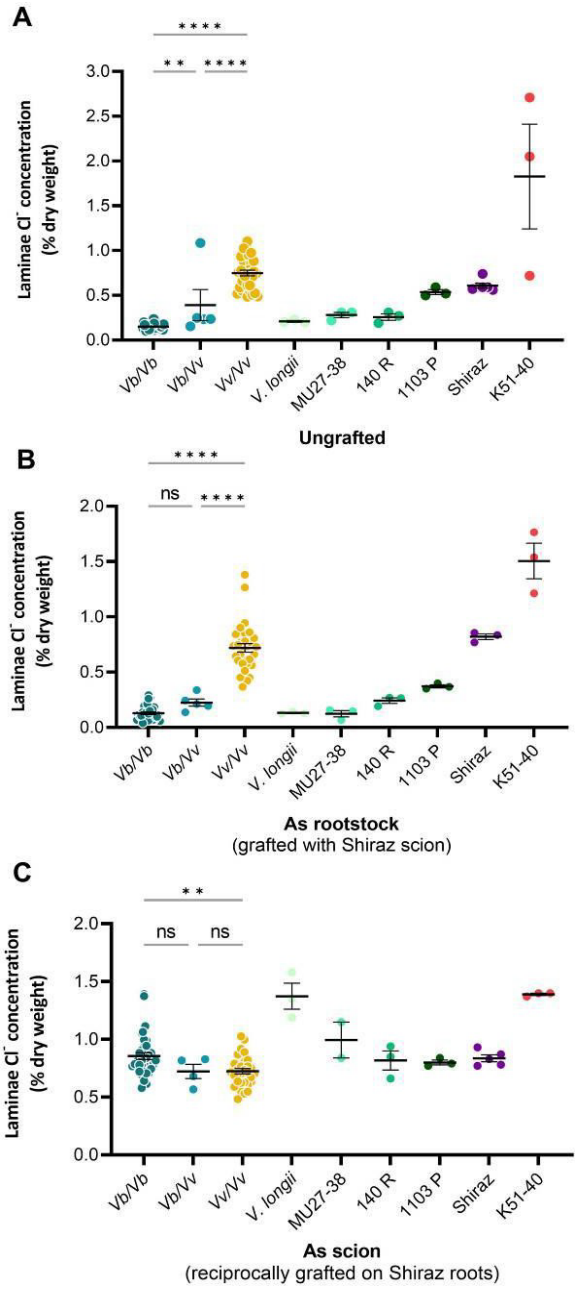


Figure 6.8 – Effect of *CLEX* locus on the laminae Cl^- concentration of BC_1F_2 lines exposed to a 100 mM mixed Cl^- treatment and grown as either (A) ungrafted, (B) as the rootstock grafted with a Shiraz scion, or (C) as the scion reciprocally grafted onto Shiraz roots. *V. longii*, the parent MU27-38, 140 Ruggeri, 1103 Paulsen, Shiraz and K51-40 were included as reference genotypes. For BC_1F_2 lines, data points represent the mean of three biological replicates, with n=30 *Vb/Vb* genotypes, n=5 *Vb/Vv* genotypes and n= 32 *Vv/Vv* genotypes. Error bars indicate the standard error of the mean across genotypes. Tukey's multiple comparison tests indicate the levels of significance of difference between *CLEX* inheritance groups as follows: ns = not significant, ** = $p<0.01$, **** = $p<0.0001$. For reference lines, data points represent individual biological replicates with no statistical analysis applied.

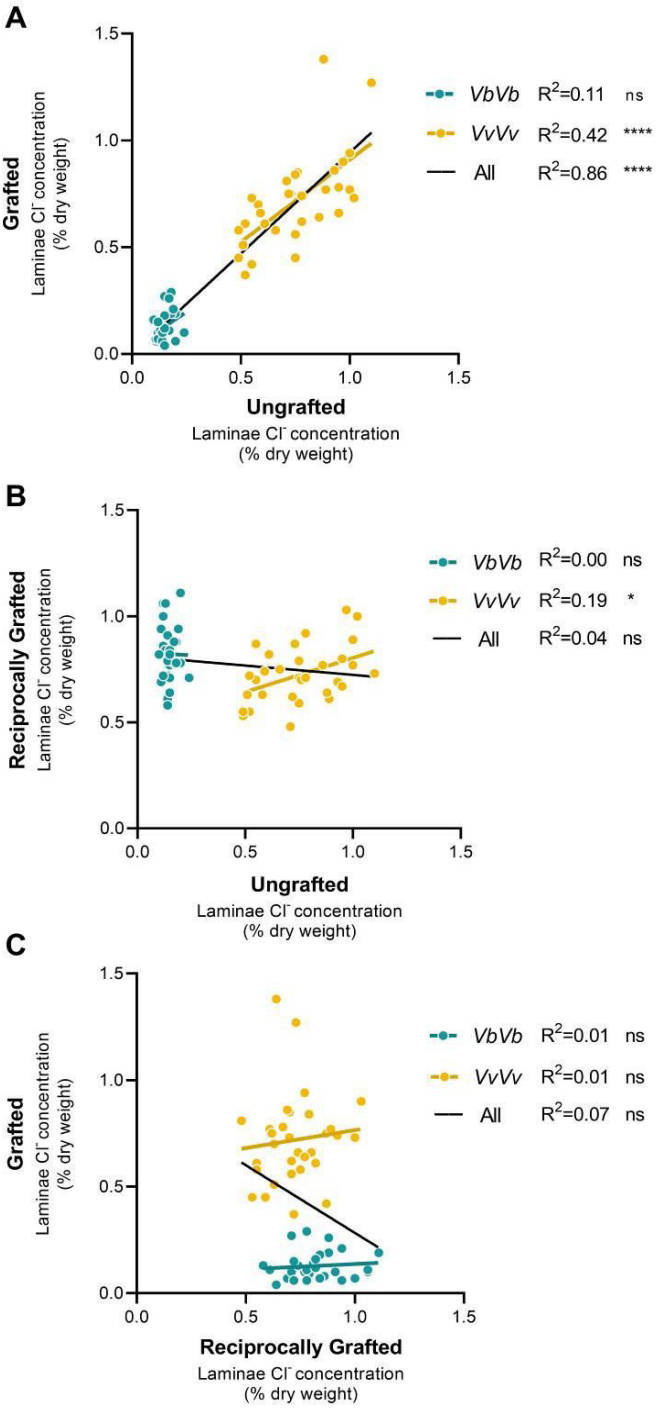


Figure 6.9 – Correlations, based on laminae Cl^- concentration, between(A) BC_1F_2 genotypes grafted (onto Shiraz roots) and ungrafted, (B) BC_1F_2 genotypes reciprocally grafted (with Shiraz as scion) and ungrafted and (C) BC_1F_2 genotypes grafted (onto Shiraz roots) and BC_1F_2 genotypes reciprocally grafted (with Shiraz as scion). Data points represent the mean of three biological replicates. Linear regressions were made across all data points as well as individually within *Vb/Vb* and *Vv/Vv* genotypes. ns = not significant, * = $p<0.05$, **** = $p<0.0001$.

6.4.5 Longer term glasshouse experiment (11C)

Previous studies have suggested that the concentration threshold at which Cl^- ions become toxic to *V. vinifera* cv. Sultana is approximately 1.5% dry weight (Walker et al., 1981). The build-up of Cl^- within the shoot and laminae is gradual, thus the symptoms of Cl^- toxicity – marginal leaf burn, impaired photosynthetic activity and reduced growth/yield – are slow to manifest. Each of the prior experiments performed on the BC_1F_2 population involved relatively short periods of salt treatment (between 15 and 24 days). As such, laminae Cl^- concentrations did not reach this toxicity threshold of 1.5%, meaning that differences in Cl^- exclusion between *Vb/Vb* and *Vv/Vv* genotypes did not translate into differences in biomass or visual marginal leaf burn symptoms. In the next experiment we aimed to obtain laminae Cl^- concentrations above this toxic threshold by exposing a small subset of Shiraz grafted *Vb/Vb* (n=5) and *Vv/Vv* (n=5) lines to a longer term (100 days) salt (approx. 50 mM mixed Cl^-) and control (0 mM) treatment.

After 100 days of treatment, the average Cl^- concentration in the laminae of Shiraz grafted to *Vb/Vb* lines was less than for *Vv/Vv* lines under both control and salt treatments as expected, yet this was only statistically significant in the control treatment (Fig. 6.10A). It should be noted that the Cl^- concentration measured for salt treated *Vv/Vv* lines is likely to be underestimated, as two vines suffered severe leaf loss which meant that the leaves likely to have the highest Cl^- concentrations could not be measured. Despite these leaves being excluded from the analysis, the average laminae Cl^- concentration of salt treated *Vv/Vv* lines was 2.59%, which is well above the toxicity threshold of 1.5%. The average Cl^- concentration of *Vb/Vb* lines was right on this threshold at 1.55% (Fig. 6.10B).

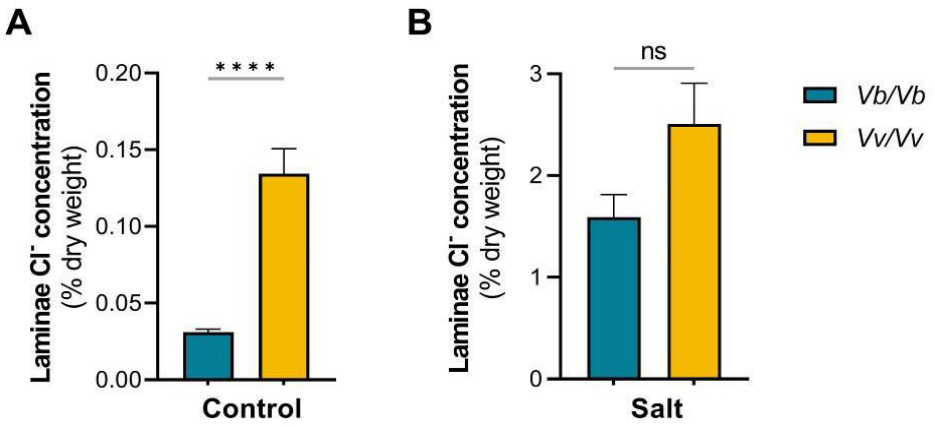


Figure 6.10 – Effect of the CLEX locus on laminae Cl^- concentration under 100-day exposure to (A) control or (B) 50 mM mixed Cl^- treatment. Each bar represents the mean of nine vines across five genotypes. ns = not significant,
**** = $p<0.0001$

In the control treatment, there were no visible differences in leaf symptoms between *Vb/Vb* and *Vv/Vv* lines, yet under salt treatment there were clearly visible differences between the two groups (Fig. 6.11A). None of the salt treated *Vb/Vb* lines showed significant marginal leaf burn whereas three of the *Vv/Vv* lines had severe leaf burn, or even senescence, while the remaining two lines displayed curled leaves, which are an early symptom of ionic toxicity. The average shoot biomass of *Vb*/*Vb* lines was 32% higher ($p<0.05$) than the average of *Vv/Vv* lines in the control treatment and 47% higher ($p<0.01$) in the salt treatment (Fig. 6.11B). Similarly, under salt treatment, higher Cl^- concentrations in *Vv/Vv* lines were associated with reduced photosynthetic efficiency when measured in both dark-adapted and exposed leaves (Fig. 6.11 C&D). Under control treatments and low Cl^- concentrations there were no significant differences in chlorophyll fluorescence between *Vb/Vb* and *Vv/Vv* lines.

Taken together, these results show that the enhanced Cl^- exclusion encoded by the *CLEX* locus translates to improved vine health and biomass of a grafted scion under only 100 days exposure to salt treatment. It is therefore reasonable to assume that in a salinity affected vineyard, rootstock genotypes possessing one or two copies of the *Vb CLEX* allele would confer improved vine health, biomass and yield over *Vv/Vv* genotypes. Nevertheless, such a trial should be conducted in the future to quantify the long-term benefit of the *Vb* allele in a vineyard over multiple seasons.

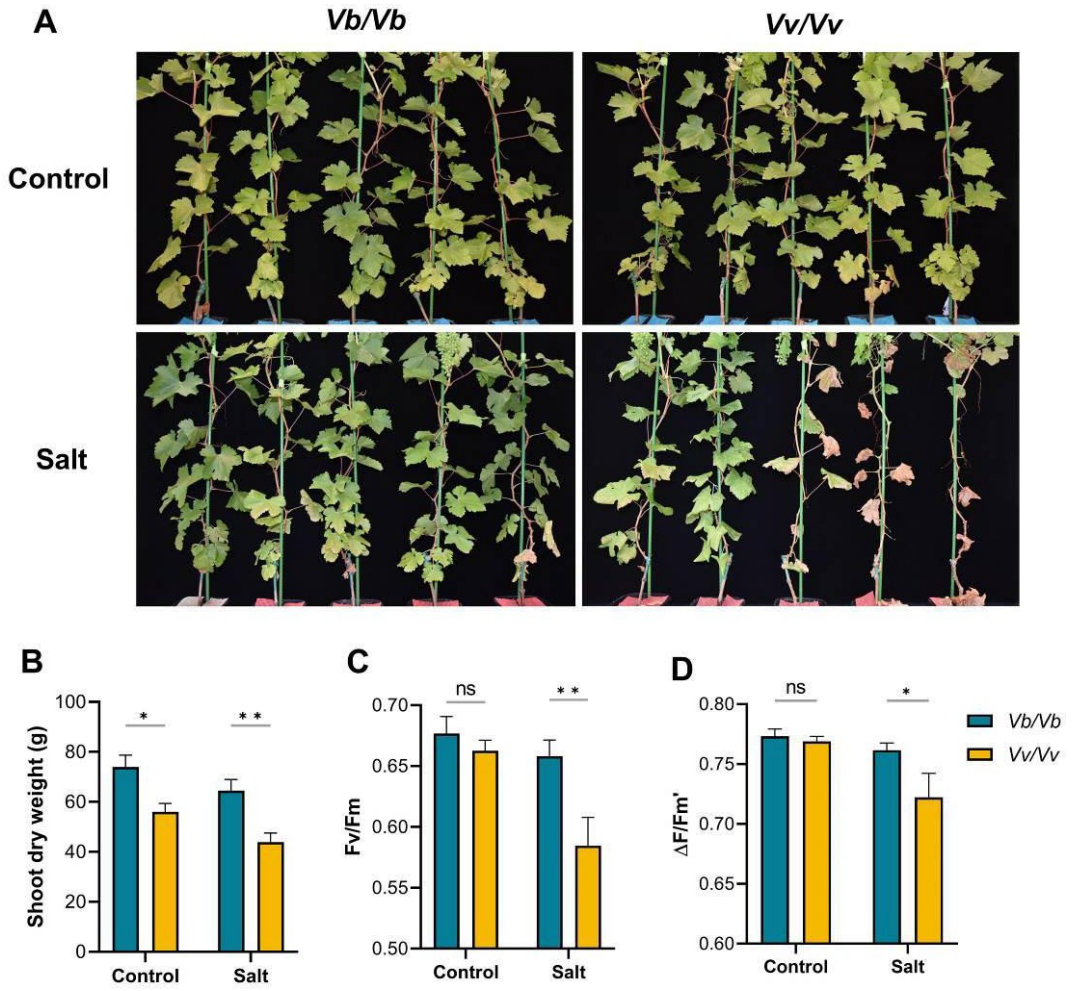


Figure 6.11 – Effect of the *CLEX* locus on long-term exposure of BC_1F_2 lines to either 0 mM Cl^- control or 50 mM mixed Cl^- treatment. (A) Images of five *Vb/Vb* genotypes and five *Vv/Vv* genotypes following 100 days of control or 50 mM mixed Cl^- salt treatment. (B) Shoot dry weight, and photosynthetic performance of (C) dark adapted and
(D) exposed leaves. ns = not significant, * = $p<0.05$, ** = $p<0.01$.

6.5 Discussion

An analysis of four separate studies of the K51-40 x 140 Ruggeri F_1 population, each performed under different soil or temperature conditions, revealed that the trait of Cl^- exclusion has a low heritability (0.22) and is highly influenced by environmental conditions (Dunlevy et al. 2022, Gong et al. 2011)). We previously reported on the identification of the *CLEX* locus associated with the segregation of Cl^- exclusion in the *V. berlandieri* x *V. vinifera* BC_1F_2 population when screened as juvenile unreplicated seedlings. Given the low heritability of this trait in the K51-40 x 140 Ruggeri F_1 population, it was important to confirm that the influence of the *CLEX* locus was heritable and reproducible under different conditions. The results presented in this chapter consistently show that the *CLEX* locus remains the major controlling genetic factor for Cl^- exclusion when the BC_1F_2 population is screened as replicated cuttings under either low or high salt concentrations, or as mature vines in the field. Thus, it appears that the heritability of the exclusion phenotype encoded by the *CLEX* locus is very high, which suggests that it is distinct from the unknown minor genetic elements contributing to the variation in the K51-40 x 140 Ruggeri F_1 population.

Under both control (0 mM Cl^-) and salt treatments (25 mM or 100 mM mixed Cl^-), the *Vb/Vb* genotypes display significantly lower Cl^- accumulation than *Vv/Vv* genotypes. This suggests that the gene/mechanisms encoded by the *CLEX* locus is likely to be constitutively expressed and not dependent on a salt treatment. However, when comparing *Vb/Vb* and *Vb/Vv* genotypes, there does appear to be an effect of salt treatment. Results from the original seedling screen, performed at 25 mM Cl^-, suggest that the *CLEX* locus is semi-dominant, that is, possession of two copies of the dominant *Vb* allele results in stronger exclusion than possession of just a single copy. This trend was also present when ungrafted cuttings were exposed to a 100 mM treatment. Yet when exposed to low salt conditions, whether that be seedling vines in the field, or ungrafted cuttings in the glasshouse, the trait appears to revert to being fully dominant, with no significant difference between *Vb/Vb* or *Vb/Vv* genotypes. Thus, under low salt loads, just one copy of the dominant *Vb* allele provides sufficient exclusion capacity to exclude Cl^- to the degree or rate equal to that of two copies. At higher external salt loads having two copies of the dominant *Vb* allele results in greater exclusion than a single copy, implying that at higher salt concentrations the activity of the encoded mechanism becomes saturated (working at maximum capacity). It would be interesting to perform a series of screens at increasing salt concentrations to determine the 'kinetics' of when BC_1F_2 genotypes with a single copy of the *Vb* encoded mechanisms becomes saturated, that is, the concentration at which they start to accumulate more Cl^- compared to genotypes possessing two copies. This information could be important for targeting future rootstocks to particular growing regions or vineyards that face different levels of salinity.

The grafting experiments clearly show that the enhanced Cl^- exclusion encoded by the dominant *Vb* allele of the *CLEX* locus is localised to the root system and fully conferred to a grafted scion. We also showed that when exposed to a 50 mM mixed Cl^- treatment for 100 days, *Vb/Vb* genotypes accumulated less Cl^- than *Vv/Vv* genotypes, and that this translated into higher biomass, increased photosynthetic capacity and reduced stress symptoms. Taken together these results suggest that in a long-term vineyard situation, rootstocks possessing

the dominant *Vb* allele would be expected to confer lower Cl^- accumulation in the scion, thus maintaining vine health and productivity, over those with the *Vv* allele. While we have not been able to collect any data on the effect of the *CLEX* locus on berry Cl^- concentration, other studies have established a positive linear correlation between Cl^- concentrations in the laminae and berries (Walker et al. 2010, Walker et al. 2014), therefore we expect the *Vb* allele to also confer exclusion from the berries.

Surprisingly, the *VbVb* genotypes on average accumulated more Cl^- in the laminae than the *VvVv* genotypes, reciprocally grafted as the scion on Shiraz roots. This inverse trend was also apparent for all but one of the reference genotypes. It is not clear what is driving this inverse trend, but it could be that the underlying *CLEX* mechanism is operating in different directions in the different organs, i.e. the dominant *Vb* allele may be actively excluding Cl^- in the roots but actively including Cl^- in the shoot. Alternatively, the *Vv* allele may be better at excluding Cl^- in the shoot than the *Vb* allele. A potential complicating factor is phloem re-translocation of Cl^- from laminae, which is known to occur in leaves of vines moved from a salt-stress to a non-salt stress situation (Walker et al. 1981). Whether or not this occurs in laminae of vines subjected to high salt treatment is difficult to determine due to different ionic gradients. Findings in the next chapter indeed suggest that the two major candidate genes, *CHX20.1* and *CHX20.2*, in the *CLEX* locus have differing roles in the roots and shoots, which is likely to be contributing to the opposing effect of the *CLEX* locus in roots and shoots seen here. K51-40 appears to lack expression of *CHX20.1*, the major gene controlling Cl^- exclusion, which may explain why it does not fit this inverse trend seen for the other reference genotypes. Results in Chapter 7 also show that *CHX20.2* is involved in sequestering Cl^- into the petiole of control vines, which under low salt loads leads to less Cl^- accumulating in the laminae. Thus, it is possible that the inverse trend seen in reciprocally grafted BC_1F_2 genotypes is the result of differences in compartmentation of Cl^- within different shoot tissues, rather than difference in total exclusion from the shoot. Further studies are required to fully understand the influence of this locus on Cl^- exclusion mechanisms occurring in the shoot as if one or more candidate genes are found to be actively including Cl^- into the laminae then they may make useful targets for gene editing to improve the Cl^- exclusion and thus salt tolerance of scion varieties.

The *CLEX* locus was first mapped using the rapid screening of unreplicated juvenile four-week- old BC_1F_2 seedlings. At that time it was unclear if the unreplicated data of such a novel approach for screening populations was valid. Furthermore, it was doubtful that results in four-week-old vines that contain perfect tap roots would be indicative of how clonally propagated cuttings would behave, let alone a mature grafted vine. Yet the consistent major influence of the *CLEX* locus on Cl^- exclusion of clonally propagated and grafted cuttings validates the use of the seedling screen to assess new genotypes for their ability to exclude Cl^-. Screening seedlings in this way is both fast and efficient as it allows a population to be assessed before deciding whether or not to undertake the costly exercise of planting it out into a vineyard. This is opposed to the traditional approach of screening replicated cuttings in a glasshouse environment which first requires the establishment of source vines of the new genotypes in a vineyard and then growing them to maturity before sufficient cuttings can be taken for a replicated screen. Thus, the seedling screen enables a far larger number of genotypes to be assessed, which is critical if the data are to be used for

mapping underlying genetic loci. In subsequent chapters we have utilised the rapid seedling screen to assess other populations for segregation of the Cl^- exclusion trait.

6.6 Summary

The major influence of the *CLEX* locus on the Cl^- exclusion of *V. berlandieri* x *V. vinifera* BC_1F_2 population previously observed in young seedlings was shown to be carried through to clonally propagated and grafted vines and is independent of the level of salt treatment applied. Furthermore, the enhanced Cl^- exclusion encoded by the dominant *Vb* allele led to improved scion biomass and photosynthetic capacity over a 100 day period. The *CLEX* locus consistently explains the majority of the Cl^- exclusion in the BC_1F_2 population and therefore makes an excellent target for developing a predictive genetic marker for use in breeding. In the next chapter we investigate candidate genes within the *CLEX* locus with the aim of developing a 'perfect' genetic marker based on the causal gene and or SNPs underlying this trait.

7. *In planta* characterisation of *CLEX* candidate genes, *CHX20.1* and *CHX20.2*

7.1 Objectives

The objective of the work presented in this chapter was to determine whether either of the two lead candidate genes that reside within the *CLEX* locus, *CHX20.1* and *CHX20.2,* have a role in Cl^- exclusion. This was achieved by investigating their expression across various genotypes, tissues and treatments as well as assessing the phenotypes of transgenic lines engineered to display reduced expression of these genes.

7.2 Background

In most crop plants, Na^+ is the ion that causes cellular toxicity resulting from salinity, therefore understanding the genetic control of Na^+ exclusion in plants has been a major focus of the salinity research in recent decades. This has led to the identification of a number of key gene families involved in the transport of Na^+ within plants, such as *HKT*, *SOS*, *NHX*-type genes (Hussain et al. 2021). Cl^- on the other hand, is the more destructive ion to only a few crops, including grape, avocado, citrus, almonds and soybean. Comparably little research attention has been invested into understanding the genetic control of Cl^- exclusion and key genes for this trait remain largely unknown.

In the previous chapter and the previous project report (CSP 1302), we describe the identification of the first major locus controlling Cl^- exclusion, not only in grapevine, but in any horticultural crop. The *CLEX* locus maps to a 685 kb region of chromosome 8 in which 74 genes reside on the PN40024 reference genome. Two neighbouring genes, both annotated as cation H^+ exchangers, *CHX20.1* (*VIT_08s0007g00020*) and *CHX20.2* (*VIT_08s0007g00030*), stand out as the most likely candidates to be underpinning the variation in Cl^- exclusion associated with this locus. The two *CHX20* genes belong to a large family of related genes called Cation Proton Antiporters (CPA). In *Arabidopsis,* there are reported to be 56 CPA genes which fall into three main clades, CPA1, CPA2 and CaCA (Maser 2002). CaCA members have been characterised as Ca^{2+} Cation Antiporters but may also transport other divalent cations such as Mn^{2+}, Cd^{2+} and Zn^{2+} (Cheng et al. 2002). A number of CPA1 clade members have been well characterised and include *AtNHX1*, an Na^+/H^+ exchanger that sequesters Na^+ in vacuoles (Bassil et al. 2019) and *AtNHX7* (SOS1), another Na^+/H^+ exchanger which is localised to the plasma membrane and primarily expressed in the xylem parenchyma (Shi et al. 2002). *AtCHX20* belongs to the CPA2 clade and has been characterised as a K^+/H^+ exchanger with a role in osmoregulation of guard cells, where it is predominantly expressed (Padmanaban et al. 2007). *AtCHX16-19* are also reported to be a K^+/H^+ exchangers; their biological roles in plants are unclear (Chanroj et al.2011). *AtCHX23*, another predicted K^+/H^+ exchanger appears to have a role in pollen tube development (Lu et al. 2011).

The published characterisations of *CHX* homologues from *Arabidopsis* do not indicate a role

in Cl^- exclusion or transport, however recent studies in soybean (*Glycine max*), suggest that a homologue of *CHX20.1*, called *GmSALT3 or Ncl*, contributes to the exclusion of both Na^+ and Cl^- (Qu et al., 2021). Two separate large mapping studies found *GmSALT3/Ncl* to be the single gene associated with salt-tolerance in soybeans. Despite Cl^- being the major ion responsible for salt stress in soybeans (Abel 1969) and that a QTL controlling Cl^- exclusion had previously been identified on the same chromosome as *GmSALT3/Ncl* (Lee et al. 2004), neither of the two mapping studies initially reported on the effect of this locus on Cl^- accumulation, but instead, looked at Na^+ accumulation (Guan et al. 2014, Qi et al. 2014). A couple of years later Liu et al. (2016) reported that *GmSALT3* regulates the shoot concentrations of both Cl^- and Na^+ and in the same year Do et al. (2016) reported that *Ncl* regulates the concentration in shoots of Cl^-, Na^+ and K^+. More recently Qu et al., (2021) reported that *GmSALT3* contributes to Na^+ exclusion in the roots, and to Cl^- exclusion in the shoot, likely via phloem re-translocation.

In situ PCR performed on soybean roots revealed that *GmSALT3* is localised in the phloem and xylem of primary root cells (Guan et al. 2014). This localisation of *GmSALT*3 is in agreement with the theory of the Cl^- exclusion mechanism, which is thought to occur in the xylem and/or phloem of the root vascular tissue. Sub-cellular localisation experiments in tobacco protoplasts suggest that GmSALT3 localises to the intracellular membrane of the endoplasmic reticulum (Guan et al. 2014), and not the plasma membrane, which is the case for HKT1 transporters. Thus, it is still not clear how the function of *GmSALT3* contributes to salt exclusion. This is partly due to the lack of a reliable heterologous system for the functional characterisation of CHX proteins.

Unlike grapevine and soybean, *Arabidopsis* only contains the single *AtCHX20* gene, which has greater similarity with *VvCHX20.2* than *VvCHX20.1*. *AtCHX20* also encodes an endosomal localised protein and is highly expressed in leaf guard cells (Padmanaban et al. 2007). *Atchx20* mutants show impaired stomatal opening upon light induction, suggesting a role in the osmoregulation of guard cells. *AtCHX20* is thought to be a K^+ transporter, although whether it has a role in Cl^- transport is not known.

To determine whether either *CHX20.1* or *CHX20.2* play a direct role in Cl^- exclusion, we assessed their expression profiles as well as the effect of reduced expression on shoot Cl^- concentration in transgenic Shiraz and 140 Ruggeri lines.

7.3 Materials and Methods

7.3.2 Gene expression experiments

The expression of *CHX20.1* and *CHX20.2* within different tissues and salt treatments was determined in two separate experiments. Experiment 1 was conducted on material harvested from the genotype MU27-38. This genotype was chosen as it is the parent of the BC_1F_2 mapping population in which the *CLEX* locus was identified. MU27-38 is heterozygous for the *Vb* and *Vv* alleles of the *CLEX* locus. Cuttings of MU27-38 were grown in the SE Smarthouse of the Plant Accelerator and exposed to either a 0 mM Cl^- control or a 100 mM mixed Cl^- salt treatment as described in section 6.3.3. After 22 days, tissue samples were harvested and snap frozen in liquid nitrogen. Samples included young and mature stems, young and mature petioles, and young and mature laminae. Whole root, root cortex and root stele samples were also taken.

Experiment 2 utilised the genotype *V. vinifera* cv. Carignan, as this cultivar flowers readily in the glasshouse and thus expression in berries and rachis could also be examined. Berry and rachis tissue were harvested from approximately eight-year-old potted vines that were watered with either rainwater (control) or a 100 mM mixed Cl^- solution for two weeks. Mature and young stems, petioles and laminae were harvested from nine-week-old cuttings that were watered with either rainwater (control), or a 25 mM or 100 mM mixed Cl^- solution for two weeks. Longitudinal segments of individual lateral roots were also taken from these vines, corresponding to approximately 0-3 cm, 3-6 cm and 6-12 cm away from the root tip.

All tissues were immediately snap frozen in liquid nitrogen and stored at -80°C. RNA extraction and cDNA synthesis were performed as described in section 5.3.1. Quantitative real-time PCR was performed to determine gene expression as described in section 5.3.2. Primers used for real-time PCR are shown in Table 6.1.

Table 7.1 – Primers used for quantification of *CHX20.1*, *CXH20.2* and housekeeping genes *GAPDH*, *Ubiquitin* and *Elongation Factor 1*.

Primer name	**Sequence 5'-3'**
CHX20.1 F	GTAGCAATAGCACAACTGTCGG
CHX20.1 R	GGATACACGAAGTTTCAGCTGC
CHX20.2 F	CGACAATGGTAGCGGAACTG
CHX20.2 R	GGGTCACACACAGTCATTTGTG
GAPDH F	CTGGAGAGGTGGAAGAGCTG
GAPDH R	TTGACCTCACTGTCAGGCTG
Ubiq F	AACCTCCAATCCAGTCATCTAC
Ubiq R	GTGGTATTATTGAGCCATCCTT
EF1 F	TTGAGGTCCCTTGGATGTTT
EF1 R	CCGCCTGCTGAAATCTGTAT

7.3.2 Gene silencing experiments

The *in planta* functions of *CHX20.1* and *CHX20*.2 were investigated using transgenic Shiraz and 140 Ruggeri lines harbouring a transgene containing a hairpin sequence targeted to either *CHX20.1* or *CHX20.2*. Expression of the hairpin sequence generally results in reduced expression of the endogenous target gene via the RNA interference pathway (Fusaro et al. 2006). Transgenic lines were developed within CSA 1701-1.7 – Enabling transformations. Southern blot analysis (described in 4.3.5) was performed on all lines to confirm that they derived from independent transformation events, i.e. produced a unique southern blot pattern. Independent lines with only one, or in a small number of cases two or three, insertions of the transgene were selected to minimise the occurrence of random off-target phenotypes. Due to limited space in PC2 certified glasshouses required for growing transgenic material, the analysis of selected *CHX20*.1 and *CHX20*.2-silenced lines was performed over multiple experiments, as detailed below.

In all instances (except Experiment 7) the starting plant material was propagated in tissue culture and deflasked into a 1:1 sand:perlite mix (Experiments 3 & 4) or into Biogro potting mix (Experiments 5-8) in 10 cm diameter 0.4 L pots, and hardened-off as described in section 5.1.2. Experiments 3-5 were performed in 10 cm diameter pots grown in CSIRO PC2 glasshouse facilities.

Experiment 3 consisted of seven Shiraz lines positively transformed with the *CHX20.1* silencing construct (C1, C4, C6, C7, C10, C11 and C13) and two negative transformant control lines (C12-N and C15-N), which had undergone the transformation process but lack the hairpin transgene. This experiment consisted of 0 mM, 10 mM and 50 mM mixed Cl^- treatments applied to each line for 14 days.

Experiment 4 consisted of four 140 Ruggeri lines positively transformed with the *CHX20*.1 silencing construct and an untransformed 140 Ruggeri control line. This experiment consisted of 0 mM, 50 mM and 100 mM mixed Cl^- treatments applied daily for 16 days.

Experiment 5 was conducted on nine Shiraz lines positively transformed with the *CHX20.2* silencing construct (CH1, CH4, CH5, CH7, CH10, CH12, CH13, CH15, CH16) and two negatively transformed lines (CH12-N and CH13-N). Treatments included 0 mM Cl^- and 50 mM mixed Cl^- applied for 22 days.

At the end of each experiment, either a root (experiments 3 & 5) or petiole (experiment 4) subsample was taken and immediately snap frozen for RNA extraction and analysis of *CHX20*.1 and *CHX20*.2 expression (described in sections 5.3.1 and 5.3.2). Laminae were harvested to determine Cl^- and cation (Experiment 3 only) concentrations. A combined stem and petiole sample was also taken for ion analysis as the plants had insufficient biomass to allow a petiole only sample to be taken.

Experiments 6-8 were performed in 2 L pots grown in the Plant Accelerator PC2 facilities, (Smarthouse for Experiments 6 & 7, glasshouse for Experiment 8), which allowed for a longer period of growth enabling collection of a larger petiole sample to better define Cl^- accumulation phenotypes in that tissue.

Experiment 6 consisted of a subset of both Shiraz *CHX20*.1-silenced lines and *CXH20*.2-silenced lines to allow a direct comparison of the resulting phenotypes. For *CHX20.1*-silenced lines, C6, C7, C37 and C38 were chosen together with the negative transformant control C12-N. *CHX20*.2-silenced lines were CH5, CH10, CH15 and CH16 and the negative transformant control CH-3. This experiment was performed under 0 mM Cl^- control treatment with vines harvested at approximately 12 weeks after deflasking. In addition to laminae, petiole and stem samples, a root sample was also taken to determine ion content.

Experiment 7 also consisted of a subset of both Shiraz *CHX20*.1-silenced lines (C1, C6, C7 and C12-N) and *CXH20*.2-silenced lines (CH5, CH10, CH15 and CH3-N) that were either ungrafted, grafted with a wild-type Shiraz scion or reciprocally grafted onto wild-type Shiraz roots. Material for this experiment was propagated from hardwood cuttings taken from 2two year-old transgenic vines grown in the glasshouse and wild-type Shiraz harvested from the PIRSA Research Centre, Nuriootpa. Grafting, callusing and propagation of cuttings was performed in CSIRO PC2 facilities following the procedure described in section 5.1.1. Ungrafted plants received both a 0 mM Cl^- control and 50 mM mixed Cl^- treatment. Due to low strike rate of grafted material, the grafted and reciprocally grafted plants only received a 0 mM Cl^- treatment. After three weeks, laminae, petioles and stems were harvested for Cl^- analysis.

Experiment 8 consisted of ungrafted *CHX20.2*-silenced Shiraz lines (CH5, CH10, CH15 and CH3-N) grown under moderate salt treatments of 10 mM and 25 mM mixed Cl^-. Plants were exposed to salt treatments for four weeks before laminae and petiole samples were taken.

7.6 Results

7.4.1 Expression analysis

Experiment 1 - Expression of *CHX20.1* in the BC_1F_2 parent genotype, MU27-38, was highest in the roots, with the majority of this expression occurring in the root stele, with comparably little expression in the root cortex (Fig. 7.1A). The major Cl^- exclusion mechanism in grapevines occurs within the xylem/phloem of the root stele (Storey et al. 2003, Tregeagle et al. 2010, Gong et al. 2011, Abbaspour et al. 2013), thus high expression of *CHX20.1* in this tissue supports a possible role of *CHX20.1* in Cl^- exclusion. Expression of *CHX20.1* was also evident in shoot tissues, being higher in the mature laminae, mature petiole and mature stem than the younger samples of these tissues, yet these were orders of magnitude lower than expression in the roots. There were no obvious effects of salt treatment on the expression of *CHX20.1*, except in the stem samples in which expression was significantly higher in the salt-treated samples.

Expression of *CHX20.2* in MU27-38 was also highest in the roots (Fig. 7.1B), yet this expression was predominantly attributed to the root cortex as opposed to the root stele as is the case for *CHX20.1*. Expression of *CHX20.2* in the root cortex and mature and young stem was significantly higher under the salt treatment than the control. Expression of *CHX20.2* was also apparent in the mature petioles and young laminae.

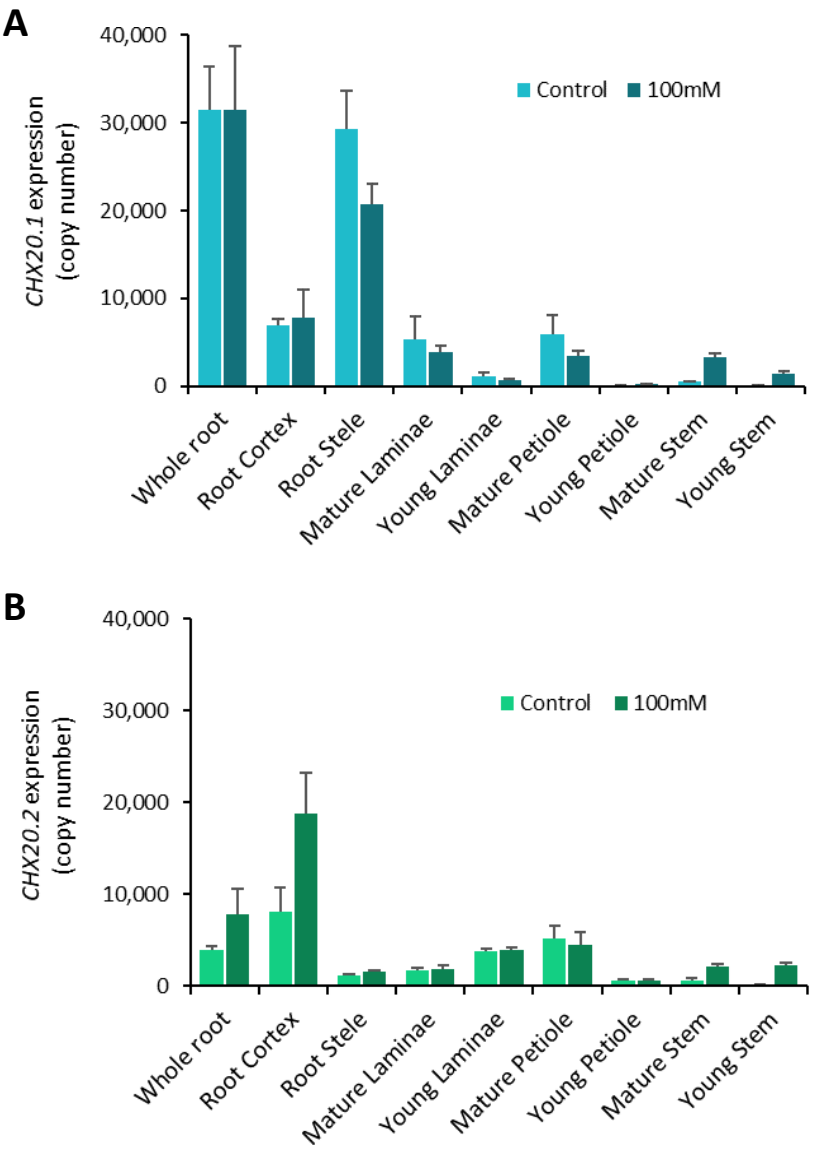


Figure 7.1 – Expression of *CHX20.1* (A) and *CHX20.2* (B) in different tissues of the parent genotype MU27-38 (*V. vinifera* cv. Biancone x (*V. vinifera* cv. Sultana x *V. berlandieri*). Gene expression is in copy number per 2.5 uL of cDNA and is normalised to the average expression of Ubiquitin, Actin and GAPDH housekeeping genes. Error bars indicate the standard error of the mean of three biological replicates.

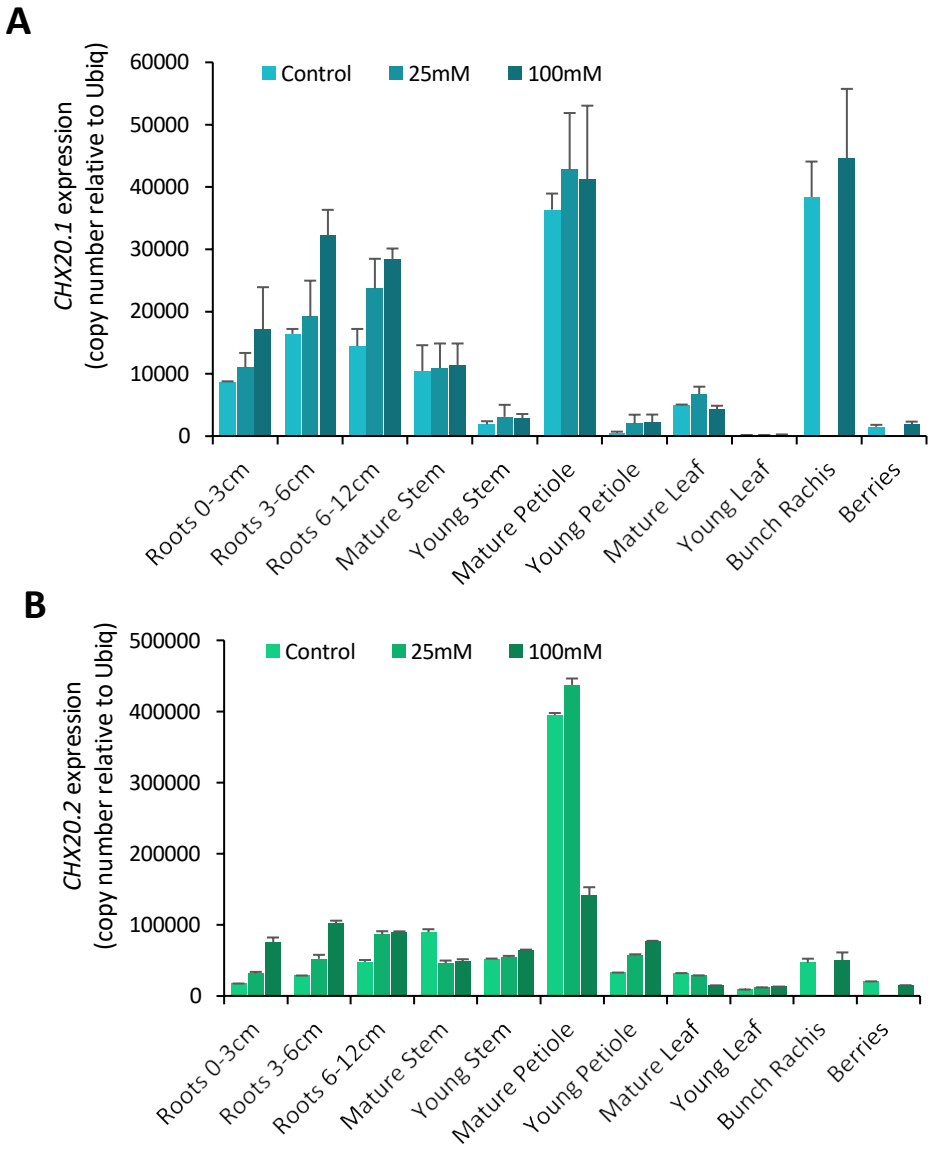


Figure 7.2 – Expression of *CHX20.1* (A) and *CHX20.2* (B) in different tissues of *V. vinifera* cv. Carignan. Expression values are expressed as copy number made relative to the expression of Ubiquitin. Error bars indicate the standard error of the mean of three biological replicates. Note there were no Bunch Rachis or Berries samples for the 25 mM treatment.

Experiment 2 - In the Carignan tissue series, expression of *CHX20.1* was highest in the mature petiole and bunch rachis (Fig. 7.2A). Expression of *CHX20.1* was again evident in the roots, which appeared to be higher in the older samples (3-6 cm and 6-12 cm from the tip) than that of young roots (0-3 cm from the tip).

Expression of *CHX20.2* was also highest in the mature petiole, but not in the bunch rachis (Fig. 7.2B). Neither *CHX20.1* nor *CHX20.2* were expressed at high levels in the berries. Salt treatment resulted in significantly higher expression of *CHX20.2* in the root cortex and mature and young stem (Fig. 7.2B).

7.4.2 Analysis of *CHX20.1*- and *CHX20.2*-silenced lines

Experiment 3 consisted of seven Shiraz lines positively transformed with a hairpin construct designed to knock-down the expression of *CHX20.1,* together with two negatively transformed control lines, which were screened under three treatments, 0, 10 and 50 mM mixed Cl^-. Analysis of *CHX20.1* expression in roots revealed reduced expression of *CHX20.1* in the positively transformed lines compared to control lines (Fig. 7.3). A two-way ANOVA revealed that there was no significant interaction (p=0.147) between salt treatment and transformant line, therefore the mean expression of *CHX20.1* across all three salt treatments was calculated and used as the measure of *CHX20.1* expression for subsequent analysis. Compared with the controls, the mean expression of *CHX20.1* was reduced in all positively transformed lines, ranging from 88% (C13) to 54% (C1) of that of the lowest control line, C15-N (Fig. 7.3).

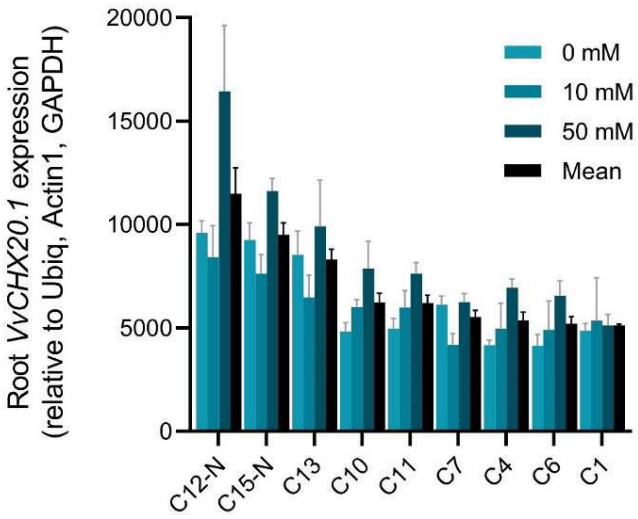


Figure 7.3 – Expression of *CHX20.1* in roots of Shiraz lines transformed with a hairpin construct targeted to reduce expression of *CHX20.1*. C12-N and C15-N are negative transformants that lack the hairpin construct and serve as controls. Four biological replicates of each line were exposed to treatments of 0 mM, 10 mM and 50 mM mixed Cl^-, with the mean across all 12 replicates shown in black. Transformant lines are ranked in decreasing order of mean *CHX20.1* expression. Error bars indicate standard error.

Analysis of Cl^- concentration in the laminae revealed that all lines with reduced expression of *CHX20.1* accumulated higher concentrations of Cl^- than in the control lines under each of the three salt treatments (Fig 7.4). In the 0 mM Cl^- treatment these differences were minimal but became more evident in the 10 mM and 50 mM mixed Cl^- treatments where the lines with reduced expression of *CHX20.1* accumulated between 24-52% more Cl^- than control lines. Linear regression analysis of root *CHX20.1* expression against laminae Cl^-

concentration revealed negative correlations that were moderate (R^2=0.39) and not quite significant (p=0.072) in the 0 mM Cl^- treatment, to strong (R^2=0.74) and very strong (R^2=0.85) significant correlations (p=0.003 and p=0.0004) in the 10 mM and 50 mM Cl^- treatments, respectively. These findings provide the first conclusive evidence of a role for *CHX20.1* in the exclusion of Cl^- from the laminae of grapevines. The Cl^- concentration of the combined stem and petiole sample was also examined in the 50 mM mixed Cl^- treatment (Fig. 7.4G). Again, a significant negative correlation (R^2=0.72, p=0.004) between mean *CHX20.1* expression in roots and Cl^- concentration of stem and petiole was evident (Fig. 7.4H). This fits with the expression of *CHX20.1* predominantly occurring in the root stele, thus its action affects the Cl^- concentration of all tissues beyond the roots.

The four major cations, Na^+, Mg^{2+}, Ca^{2+}, and K^+ were also determined in the laminae sample of the 50 mM mixed Cl^- treatment (Fig. 7.5). Significant negative correlations were evident between *CHX20.1* expression and laminae Na^+ (R^2=0.62, p=0.012) and Mg^{2+} (R^2=0.46, p=0.045) concentrations, while correlations with Ca^{2+} and K^+ concentrations were also negative (R^2=0.41 and 0.29, respectively), but not quite significant (p= 0.062 and 0.13, respectively). This suggests, that in addition to exclusion of Cl^-, *CHX20.1* is also involved in the exclusion of Na^+ and Mg^{2+}, and possibly Ca^{2+}. However, it is not clear if this is the result of direct transport activity of the CHX20.1 protein, or possibly the indirect result of the plant's need to maintain an ionic homeostasis through the action of other transporters/channels.

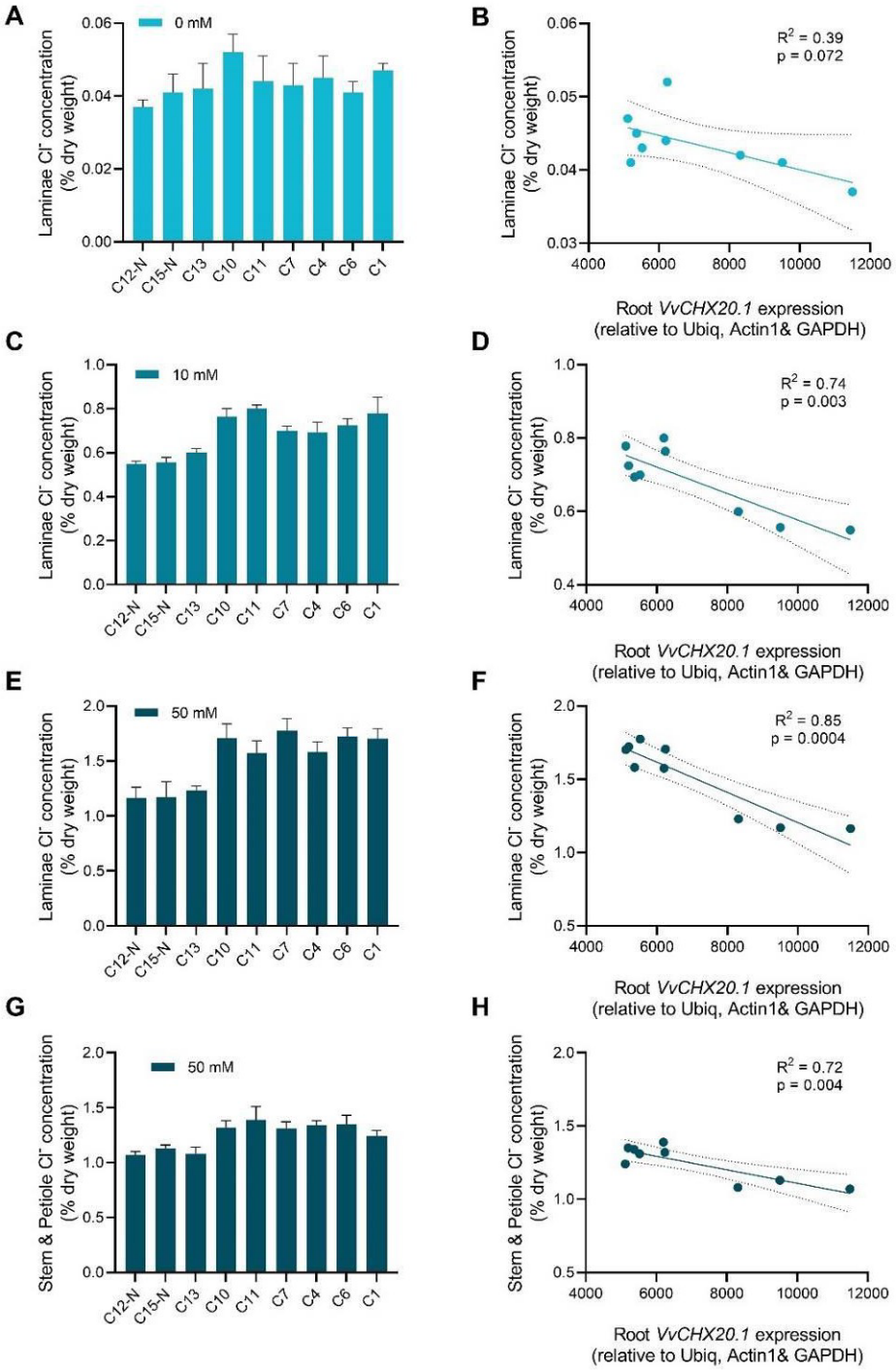


Figure 7.4 – Accumulation of Cl^- in the A, C & E) laminae of transformed Shiraz lines displaying reduced expression of *CHX20.1* under treatments of 0 mM, 10 mM and 50 mM mixed Cl^-, respectively, and G) in the combined stem & petiole tissues under 50 mM mixed Cl^- treatment. C12-N and C15-N are negative transformants that serve as controls. The remainder are positively transformed lines. Error bars indicate the standard error of the mean of four biological replicates. B, D & F) Linear regressions between the mean *CHX20.1* expression in roots of Shiraz transformants across three salt treatments (shown in Figure 7.3) and resulting laminae Cl^- concentrations under 0 mM, 10 mM and 50 mM mixed Cl^- treatment, respectively, and H) in the combined stem & petiole tissues under 50 mM mixed Cl^- treatment. Dotted lines indicate the upper and lower 95% confidence limits.

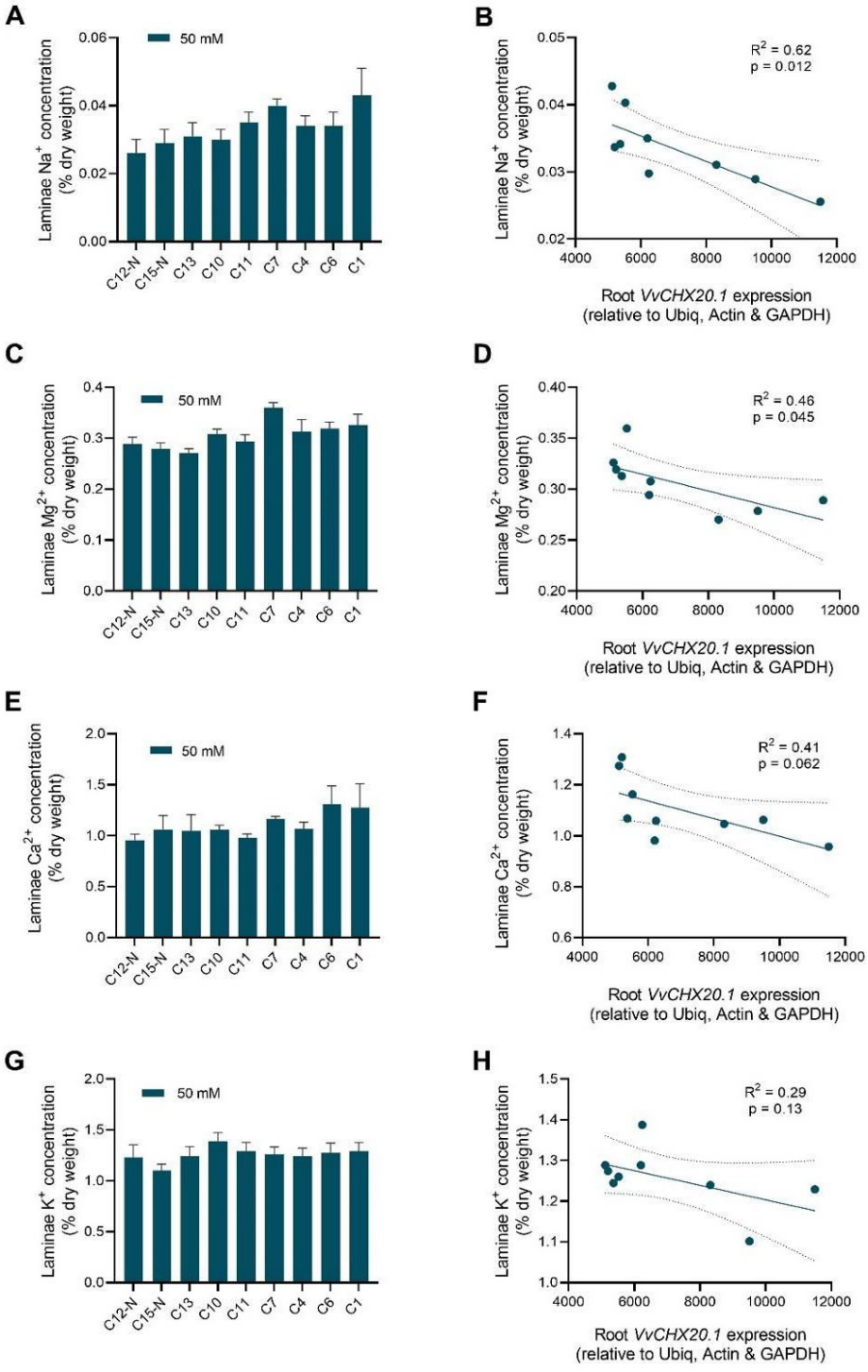


Figure 7.5 - Accumulation of A) Na^+, C) Mg^{2+}, E) Ca^{2+} and G) K^+ in the laminae of transformed Shiraz lines displaying reduced expression of *CHX20.1* under 50 mM mixed Cl^- treatment. C12-N and C15-N are negative transformants that serve as controls. The remainder are positively transformed lines. Error bars indicate the standard error of the mean of four biological replicates. B, D, F & H) Linear regressions between the mean *CHX20.1* expression in roots of Shiraz transformants across three salt treatments (shown in Figure 7.3) and respective laminae accumulation of Na^+, Mg^+, Ca^{2+} and K^+ under 50 mM mixed Cl^- treatment. Dotted lines indicate the upper and lower 95% confidence limits.

In Experiment 4, the effect of silencing *CHX20.1* in the 140 Ruggeri rootstock background was investigated. As the highly precise grapevine transformation method has been optimised for Shiraz, application of this method to 140 Ruggeri resulted in fewer successfully transformed lines. As such, only four positively transformed lines, RC48, RC49, RC50 and RC55 were screened under 0 mM, 50 mM and 100 mM mixed Cl^- treatments. The higher 100 mM Cl^- treatment was chosen because it was hypothesised that the CHX20.1 protein of 140 Ruggeri may have increased activity compared to Shiraz, which could mean that higher salt loads would be required to detect a phenotype if only partial silencing was achieved.

Expression analysis revealed moderately reduced expression in three of the four lines, RC55, RC49 and RC50, compared with controls, but not in RC48 (Fig. 7.6). A two-way ANOVA analysis revealed a significant interaction between salt treatment and line, therefore it was not appropriate to use the mean expression of each line across treatments for correlation against the resulting phenotype. Furthermore, there was not a significant effect of line on the mean expression levels, despite the slight reduction in RC55, RC49 and RC50.

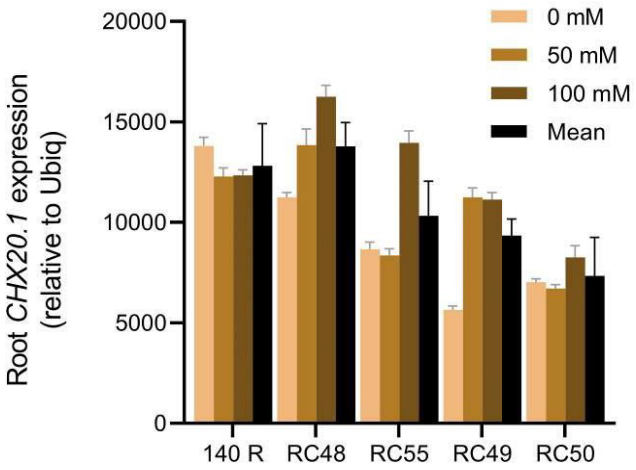


Figure 7.6 – Expression of *CHX20.1* in roots of 140 Ruggeri lines transformed with a hairpin construct targeted to reduce expression of *CHX20.1*. 140 R is un-transformed 140 Ruggeri that lacks the hairpin construct and serves as a control. The remainder are positively transformed lines. Four biological replicates of each line were exposed to treatments of 0 mM, 50 mM and 100 mM mixed Cl^-, with the mean across all 12 replicates shown in black. Error bars indicate standard error of the mean.

Analysis of the Cl^- accumulation in the laminae and combined stem and petiole showed that the two lines with the lowest expression of *CHX20.1* (RC49 and RC50) had the highest concentration of Cl^- in both tissues under the 0 mM Cl^- and 50 mM mixed Cl^- treatments, as well as in the laminae of the 100 mM mixed Cl^- treatment (Fig. 7.7). Surprisingly, the differences between RC49 and RC50 and control lines were greatest in the 0 mM Cl^- treatment (182-233% increase) followed by the 50 mM mixed Cl^-treatment (55-65% increase) and smallest in the 100 mM mixed Cl^- treatment (16-32% increase). Despite the small number of lines examined, the trend of reduced *CHX20.1* expression leading to increased accumulation

of Cl^- in shoot tissues is clearly evident, consistent with that seen in Shiraz *CHX20.1* silenced lines.

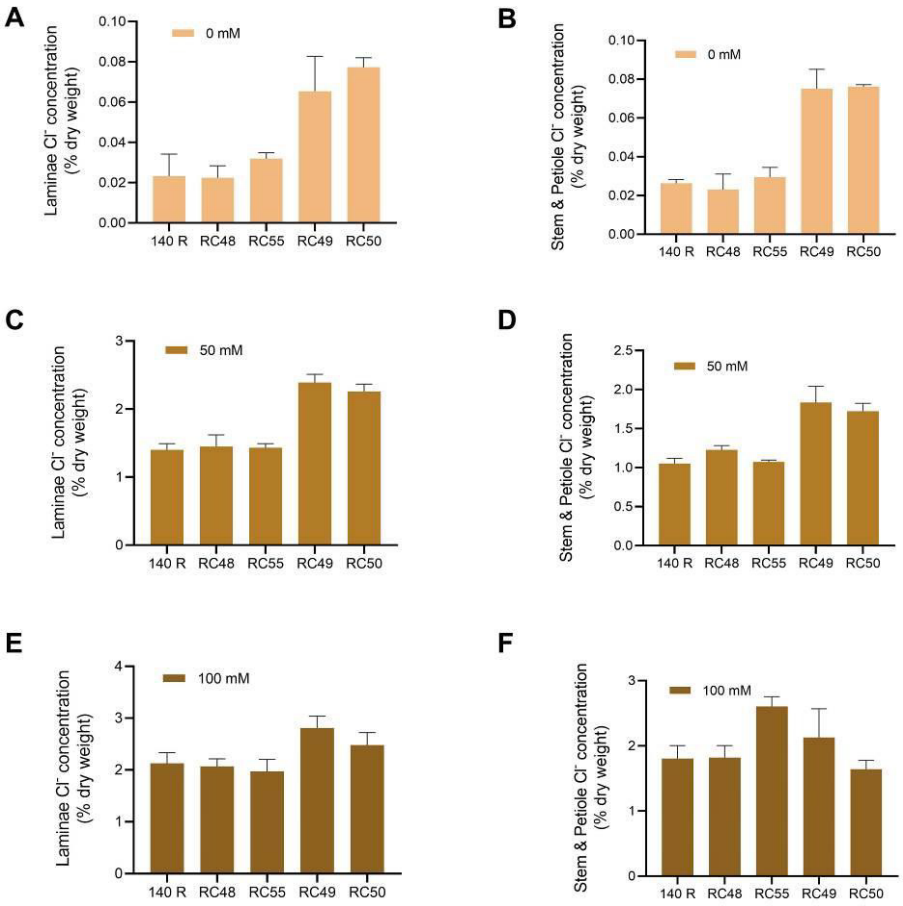


Figure 7.7. - Accumulation of Cl^- in the (A, C & E) laminae and (B, D & F) stem and petiole of 140 Ruggeri lines displaying reduced expression of *CHX20.1* under treatments of 0 mM, 50 mM and 100 mM mixed Cl^-, respectively. 140 R is untransformed and serves as a control. The remainder are positively transformed lines. Error bars indicate the standard error of the mean of four biological replicates.

In Experiment 5, nine Shiraz lines positively transformed with a hairpin construct targeted to *CHX20.2* were assessed alongside two negative transformant control lines. Limited glasshouse space restricted this experiment to just two salt treatments, a 0 mM Cl^- control and a 50 mM mixed Cl^- treatment. Analysis of *CHX20.2* expression in root samples revealed inconsistent data with a large degree of error between replicates (data not shown). This was most likely a result of the low expression level of *CHX20.2* in this tissue, as seen in *V. vinifera* cv. Carignan in Figure 7.2B. In contrast, analysis of *CHX20.2* expression in petioles, the tissue where this gene is most highly expressed (Fig. 7.2B), gave more consistent results and was therefore used to assess the degree of silencing within these lines. All nine positively transformed lines contained significantly less expression of *CHX20.2* in petioles compared with the two negative control lines (Fig. 7.8). A two-way ANOVA revealed no significant interaction between salt treatment and line, therefore the mean expression across the two treatments was used as the measure of gene silencing for subsequent analysis. The mean *CHX20.2* expression of

positive lines ranged from 35% (CH4) to 10% (CH5) that of the lowest negative control, CH3-N (Fig. 7.8).

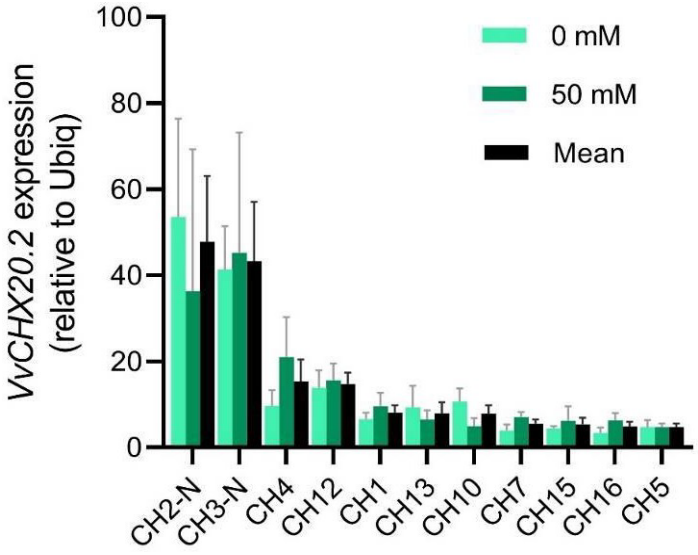


Figure 7.8 - Expression of *CHX20.2* in the petioles of Shiraz lines transformed with a hairpin construct targeted to reduce expression of *CHX20.2*. CH2-N and CH3-N are negative transformants that lack the hairpin construct and serve as controls. The remainder are positively transformed lines. Four biological replicates of each line were exposed to 0 mM Cl^- and 50 mM mixed Cl^- treatments, with the mean across all eight replicates shown in black. Transformant lines are ranked in decreasing order of mean *CHX20.2* expression. Error bars indicate standard error of the mean.

The Cl^- concentration in the laminae and combined stem and petiole revealed no significant difference between lines in either the 0 mM Cl^- or 50 mM mixed Cl^- treatments (Fig. 7.9). There was, however, a noticeable trend of marginally higher laminae Cl^- concentration in lines with the highest degree of silencing under the 0 mM Cl^- treatment only and this slight trend was significantly negatively correlated with *CHX20.2* expression (R^2=0.67, p=0.002) (Fig. 7.9B). Correlations between *CHX20.2* expression and Cl^- concentration in the combined stem and petiole were not significant in either the 0 mM Cl^- and 50 mM mixed Cl^- treatment (Fig 7.9 D&H), or in the laminae of the 50 mM treatment (Fig. 7.9F). These results hint towards a possible role of *CHX20.2* in exclusion of Cl^- from the laminae at low salt concentrations, for example, by retrieval of Cl^- from the xylem in the petiole, but any influence of this gene on Cl^- concentration of the laminae is not nearly as apparent as that of *CHX20.1*.

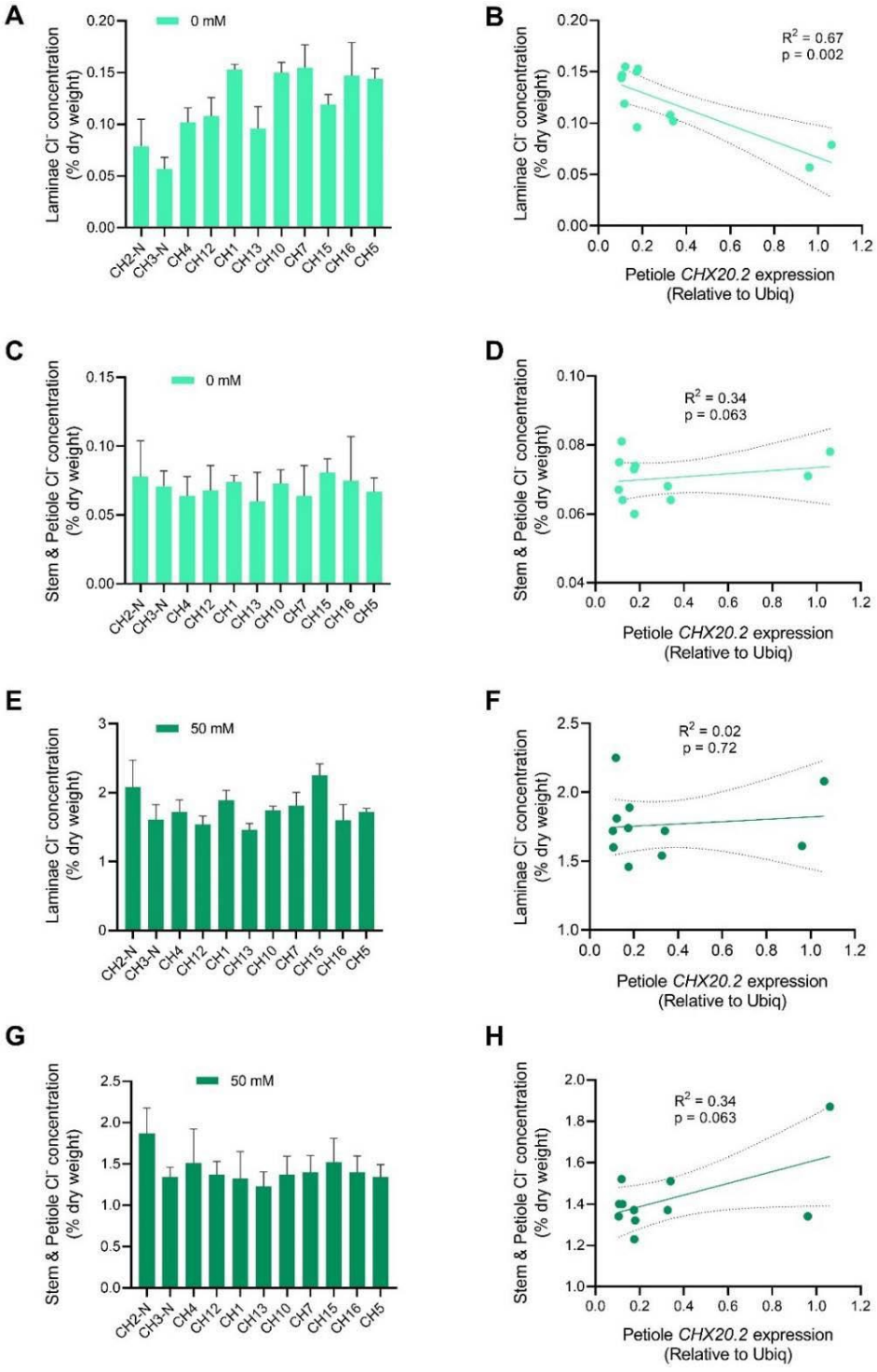


Figure 7.9 - Accumulation of Cl^- in the A & E) laminae and C & G) combined stem and petiole of Shiraz lines displaying reduced expression of *CHX20.2* in petioles under treatments of 0 mM Cl^- and 50 mM mixed Cl^-, respectively. CH2-N and CH3-N are negative transformants and serve as controls. The remainder are positively transformed lines. Error bars indicate the standard error of the mean of four biological replicates. Linear regressions are shown between the mean *CHX20.2* expression in petioles of Shiraz transformants across both salt treatments (shown in Figure 7.6) and resulting laminae Cl^- (A & F) and (D & H) stem and petiole Cl^- concentrations under the 0 mM Cl^- and 50 mM mixed Cl^- treatments. Dotted lines indicate the upper and lower 95% confidence limits.

The aim of experiment 6 was to gain a deeper understanding on the effect of reduced expression of *CHX20.1* and *CHX20.2* on Cl^- concentration of laminae, petiole, stem and root tissues. To obtain the biomass required for analysis of individual tissues, these transgenic plants were deflasked and grown in the Plant Accelerator for the first time. The deflasking process in the Plant Accelerator yielded a lower survival rate than earlier experiments, and consequently there were only enough replicates to perform a single salt treatment. We chose to apply a 0 mM Cl^- control treatment as this was the only treatment in which a phenotype for *CHX20.2* was seen in the previous experiment, while the *CHX20.1* phenotype is also evident under a control treatment.

Four *CHX20.1*-silenced (C) lines and four *CHX20.2*-silenced (CH) lines were assessed together with two respective negative transformants serving as controls. All lines had previously been assessed for degree of silencing in experiments 3 and 5 respectively, with the exception of C37 and C38. These two lines were included because they were slow growing lines in tissue culture and were hypothesised to display a more pronounced degree of silencing of *CHX20.1* expression than other lines already assessed. Unfortunately, however, due to time constraints, the degree of *CHX20.1* expression in these two lines was not determined.

For Cl^- accumulation in the laminae, C38 had similar concentrations to that of the negative control C12-N, whereas C7, C37 and C6 all accumulated higher concentrations, which were significantly different from the control for C37 and C6 (Fig. 7.10A). For *CHX20.2* silenced lines, despite accumulating marginally higher concentrations of Cl^- in the laminae than the control CH3-N, none of these differences were statistically significant (Fig. 7.8A). For *CHX20*.1 silenced lines, a similar trend of Cl^- accumulation as seen in the laminae was also evident in the petiole, with C7, C37 and C6 all showing significantly higher concentrations than the control (Fig. 7.10B). This is consistent with the expression of *CHX20.1* predominantly occurring in the roots. Intriguingly, *CHX20.2* silenced lines all displayed significantly lower Cl^- concentration in the petiole compared to the control (Fig. 7.10B). Consistent with the petiole being the tissue with highest *CHX20.2* expression, this result implies that *CHX20.2* is involved with inclusion/sequestration of Cl^- into the petiole, as lower expression results in lower accumulation. In the stem sample, a similar trend in Cl^- accumulation was seen as in the petiole, except that the differences between silenced lines and controls were not as pronounced and were not statistically significant (Fig. 7.10C). Interestingly, C38, which did not show a significant difference in laminae or petiole Cl^- concentration from the control, was the only line to show a significant difference in stem Cl^- concentration. This suggests that C38, like other *CHX20.1* lines, has likely increased Cl^- uptake into the shoot resulting from reduced expression of *CHX20.1*, but the compartmentalisation of Cl^- within different shoot tissues appears to be altered due to an unknown reason. C38 did display a noticeably reduced shoot biomass compared to other lines at the end of the experiment (Fig. 7.11), which supports the possibility that the random insertion of the transgene may have impaired some critical process in that line. No significant differences were seen between the root Cl^- concentration of control and *CHX20.1* or *CHX20.2* silenced lines (Fig 7.10D).

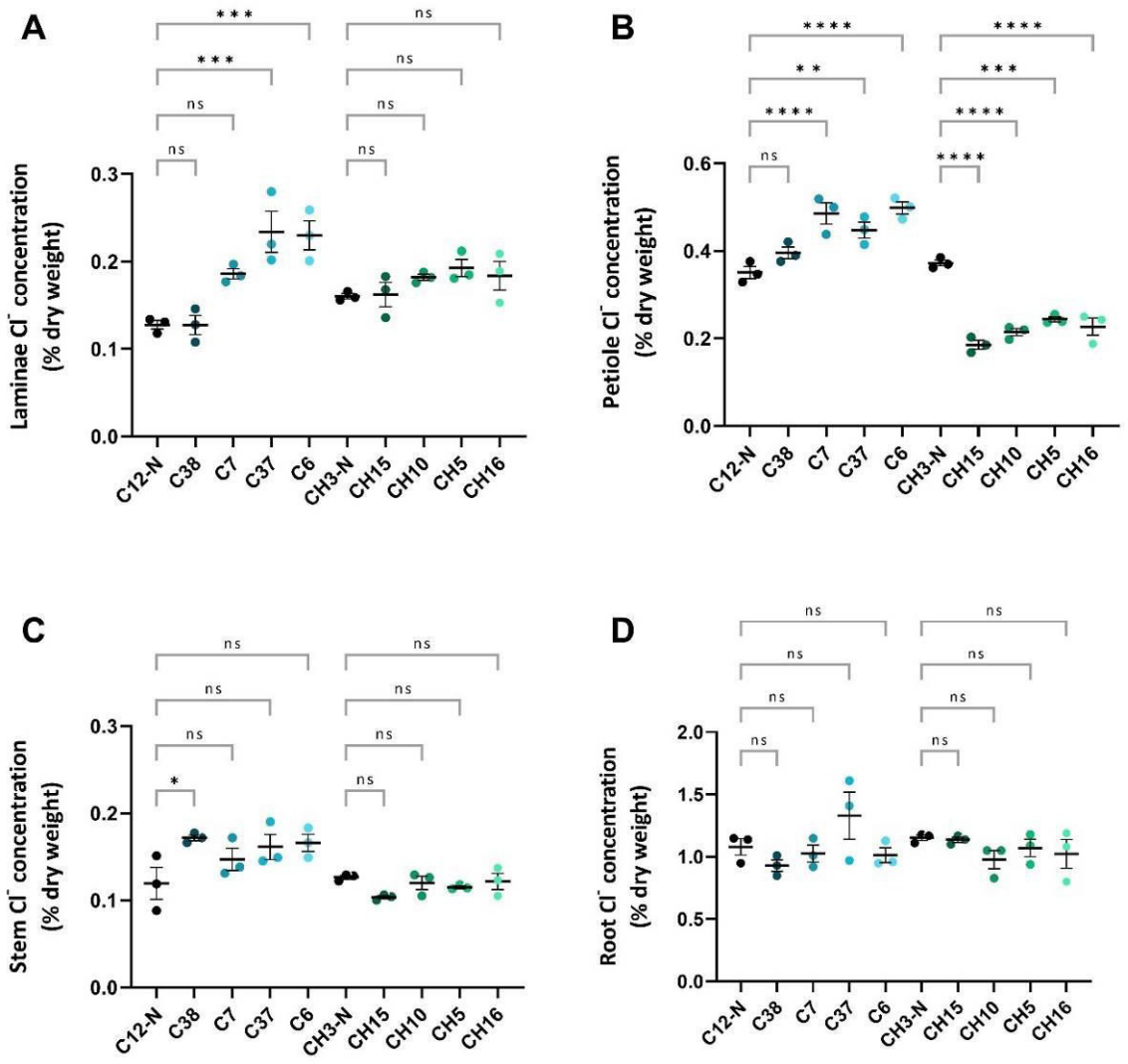


Figure 7.10 – Effect of reduced expression of *CHX20.1* (C lines) and *CHX20.2* (CH lines) on Cl^- accumulation in the (A) laminae, (B) petiole, (C) stem and (D) roots of Shiraz transgenic lines following a 0 mM Cl^- (rainwater) treatment. C12-N and CH3-N are negative transformant controls that have been regenerated through the transformation process but do not harbour the silencing transgene. All other lines are positive transformants. The reduction in *CHX20.1* expression of positively transformed C lines and of *CHX20.2* expression in positively transformed CH lines compared to controls is shown in Fig. 7.3 and Fig. 7.8, respectively. Data points indicate the value of a single biological replicate, horizontal lines represent the mean of each transformant, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where the difference between control and transformed lines is significant as follows: ns = not significant, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

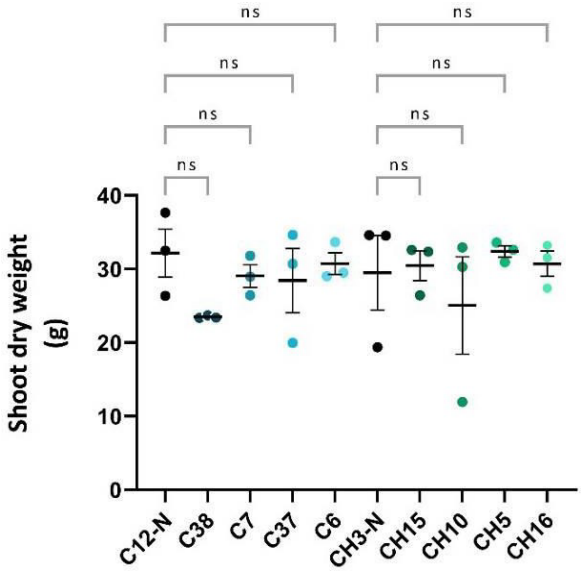


Figure 7.11 – Shoot dry weight of lines with reduced expression of *CHX20.1* (C lines) and *CHX20.2* (CH lines) following a 0 mM Cl^- (rainwater) treatment. C12-N and CH3-N are negative transformant controls that have been regenerated through the transformation process but do not harbour the silencing transgene. All other lines are positive transformants. The reduction in *CHX20.1* expression of C lines and of *CHX20.2* expression in CH lines is shown in Fig. 7.3 and Fig. 7.8, respectively. Data points indicate the value of a single biological replicate, horizontal lines represent the mean of each transformant, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests showed no significant difference (ns) between the control and transformed lines.

From the results of Experiment 6, a clear case can be made for a role of *CHX20.2* in determining Cl^- concentration in the petiole under control conditions. Yet it is unclear whether *CHX20.1* also plays a role in Cl^- transport within the petiole or shoot, as the major *CHX20.1* activity in the roots potentially masks any smaller differences that may occur within the shoot. Hence, the main objective of Experiment 7 was to determine whether *CHX20.1* contributes to Cl^- exclusion or inclusion in the shoot by isolating the reduced expression of *CHX20.1* to the shoot by reciprocally grafting silenced lines as the scion onto wild-type Shiraz roots. In total, three *CHX20.1*-silenced lines (C1, C6 and C7) and C12-N control, and four *CHX20.2*-silenced lines (C5, C10 and CH15) and CH3-N control, were assessed under a 0 mM Cl^- control treatment and three graft treatments as follows; 1) ungrafted; 2) as the rootstock grafted with a wild- type Shiraz scion, and 3) as the scion grafted onto wild-type Shiraz roots.

For ungrafted *CHX20.1*-silenced lines there was an unexpectedly small difference in laminae or petiole Cl^- concentration between silenced and control lines (Fig. 7.12A&B). These differences were only statistically significant for C1, but not for C6 or C7 which had both shown highly statistically significant differences in laminae and petiole Cl^- concentration under the same conditions of Experiment 6. For ungrafted *CHX20.2*-silenced lines, however, all three lines (CH5, CH10 and CH15) showed significantly higher concentrations of Cl^- in the laminae (Fig. 7.12A), and significantly lower concentrations of Cl^- in the petiole than in the control line CH3-N (Fig. 7.12B). This was consistent with the trends seen in Experiment 6, again suggesting a role of *CHX20.2* in retrieval and sequestering of Cl^- in the petiole as a means

of limiting Cl^- accumulation in laminae, although noting that the effect is only statistically significant for the control treatment.

When silenced lines were used as the rootstock grafted with a wild-type Shiraz scion there were, again unexpectedly, no significant differences in laminae Cl^- concentration between *CHX20.1*-silenced and control lines (Fig. 7.12C). When grafted with a wild-type Shiraz scion all three *CHX20.2*-silenced lines accumulated more Cl^- in the laminae than the control, yet these differences were not significant (Fig. 7.12C). When looking at petiole Cl^- concentration, *CHX20.2*-silenced lines actually had higher concentrations of Cl^- in the petiole than the control line, which was statistically significant for CH10 and CH15 (Fig. 7.12D). The increased Cl^- concentration in the petiole (and possibly the laminae) of *CHX20.2*-silenced lines when used as the rootstock grafted with a wild-type Shiraz scion implies that *CHX20.2* may play a role in Cl^- exclusion within the root system, where it is expressed in the root cortex. There is some evidence for this from studies with rooted leaves where a better Cl^- excluding rootstock (140 Ruggeri) was shown to accumulate higher concentrations of Cl^- in roots than a poor Cl^- excluding rootstock (K51-40) (Walker et al. 2018), implicating greater accumulation of Cl^- in root cortex cells of the better Cl^- excluder.

When silenced lines were used as the scion grafted onto wild-type Shiraz roots, there was no significant effect of *CHX20.1*-silenced lines on laminae or petiole Cl^- concentration (Fig. 7.12 E&F). Thus, it appears that *CHX20.1* does not have an influence on partitioning Cl^- within the shoot, at least under 0 mM Cl^- control conditions. Conversely, when used as the scion, *CHX20.2*-silenced lines displayed significantly lower Cl^- concentrations in the petiole than control line (Fig. 7.12F), as in the ungrafted treatment. Consistent with the results of the ungrafted treatment, when used as the scion, *CHX20.2*-silenced lines accumulated higher concentrations of Cl^- in the laminae than the control, however these differences were not statistically significant (Fig 7.12E). Thus, it seems that the reduced expression of *CHX20.2* in either the root or the shoot alone, in grafted or reciprocally grafted plants respectively, is not strong enough to provide a statistically significant effect on laminae Cl^- concentration, but in ungrafted plants the combined action in the two organs is compounded and becomes significant. To investigate if the effect of *CHX20.2* on laminae Cl^- concentration also holds true under high salt treatments, we exposed ungrafted plants to a 50 mM mixed Cl^- treatment. Under this high salt load there were no significant differences in laminae Cl^- concentrations between *CXH20.2*-silenced lines and the control (Fig. 7.13B). One can only speculate as to why the *CXH20.2* effect on petiole and laminae Cl^- concentrations is evident only in the control treatment. At higher applied salt treatments, with higher internal concentrations of ions, osmotic gradients are likely to be different, with a range of other membrane ion transport processes likely to be involved.

In the final Experiment, number 8, we exposed the same CH lines to a 10 mM and 25 mM mixed Cl^- treatment in an attempt to determine the salt exposure level at which the *CHX20.2* mechanism breaks down. For this experiment, material was propagated and deflasked from tissue culture plants and therefore is not directly comparable to the results of Experiment 7.

Again, no significant differences were seen between *CHX20.2*-silenced lines and the control at these moderate salt treatments (Fig. 7.14A&B). Despite the lack of significance, all *CHX20.2*-silenced lines contained higher mean laminae Cl^- concentration than the control.

The *CHX20.2* phenotype is more pronounced in the petiole than the laminae, therefore looking at the petiole Cl^- concentration of CH lines exposed to 10, 25 and 50 mM mixed Cl^- treatments may give a better indication of the concentration at which the *CHX20.2* mechanism becomes saturated and breaks down. Time restraints did not permit this investigation.

7.6 Discussion

The results presented in this chapter indicate that *CHX20.1* is the gene most likely to be responsible for the variation in Cl^- exclusion of the BC_1F_2 population (Chapter 6). *CHX20.1* expression in the BC_1F_2 parent MU27-38 was greatest in the root stele, the tissue in which the major mechanism of Cl^- exclusion occurs, resulting in reduced Cl^- concentration in the xylem (Storey et al. 2003, Tregeagle et al. 2010, Gong et al. 2011, Abbaspour et al. 2013). Moreover, the reduced expression of *CHX20.1* in Shiraz transformants resulted in a significant increase in laminae Cl^- accumulation, consistent with a role in Cl^- exclusion. This phenotype was most evident under 50 mM mixed Cl^- treatment, followed by the 10 mM Cl^- treatment and least evident under the 0 mM Cl^- control. On the other hand, reduced expression of *CHX20.2* does not impact laminae Cl^- concentration under a 50 mM salt treatment, yet does have a minor influence under the control treatment. *CHX20.2,* which is highly expressed in the root cortex as well as the petioles, contributes to this minor Cl^- phenotype in both the roots and the shoot. Reciprocal grafting experiments indicate that *CHX20.2* contributes to Cl^- exclusion in the roots, potentially in the root cortex, while in the shoot its action appears to be the result of retrieval and sequestration of Cl^- into the petiole. It is unclear why *CHX20.2* does not appear to impact shoot Cl^- concentrations at high external salt loads, but this may be due to altered ionic gradients and/or the kinetics of the encoded protein's transport properties.

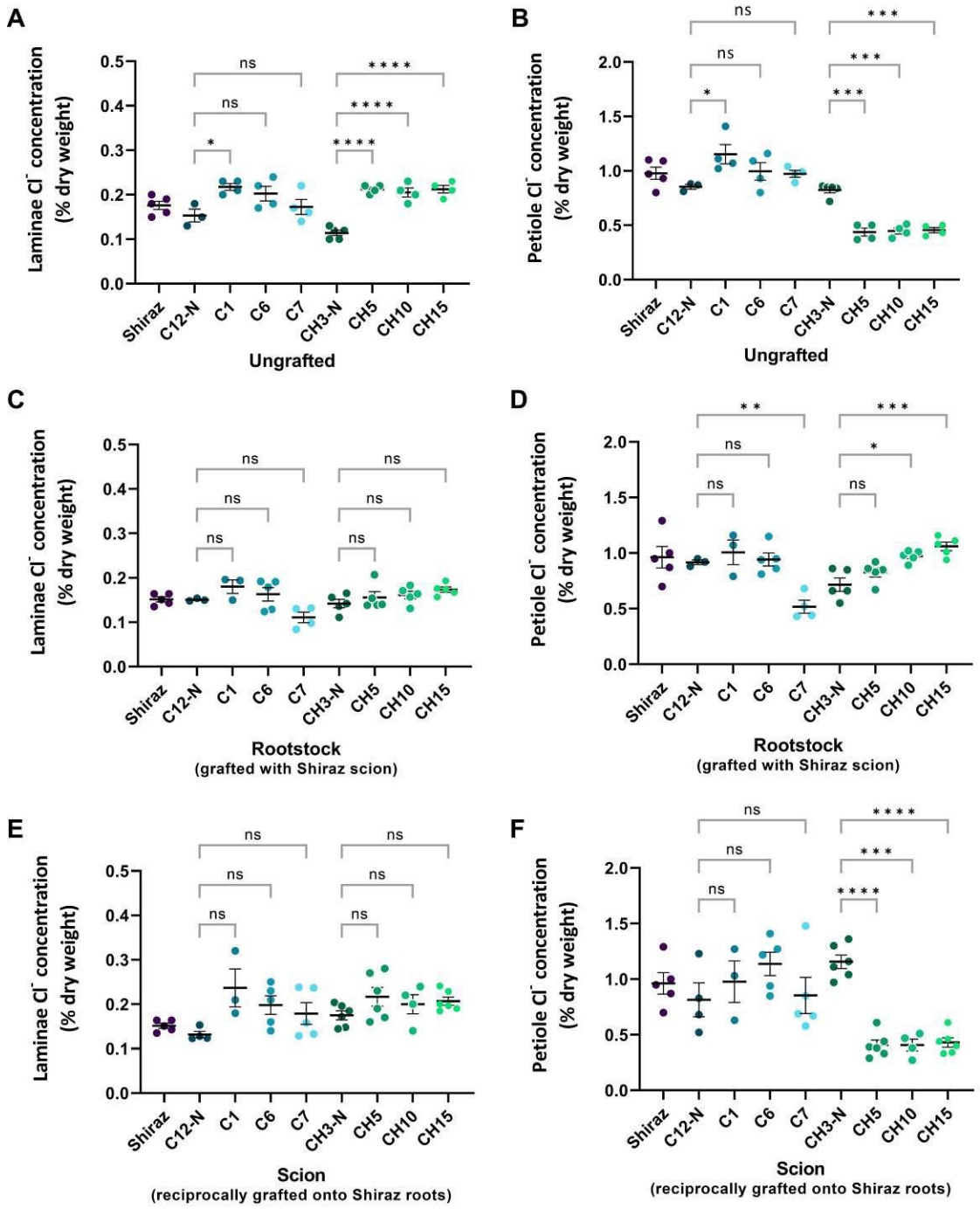


Figure 7.12 – Differences in laminae (A, C, E) and petiole (B, D, F) Cl^- accumulation due to reduced expression of *CHX20.1* (C-lines) and *CHX20.2* (CH-lines) in transgenic lines when used as ungrafted (A & B), the rootstock grafted with wild-type Shiraz scion (C & D), or as the scion grafted onto wild-type Shiraz roots (E & F). C12-N and CH3-N are untransformed negative controls. All other lines are positive transformants. The treatment applied was 0 mM Cl^- (rainwater). The self-grafted wild-type Shiraz data presented in C & D is the same as that presented in E & F. Data points indicate the value of a single biological replicate, horizontal lines represent the mean of each transformant, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where differences between the control and transformed lines are significant as follows: ns = not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

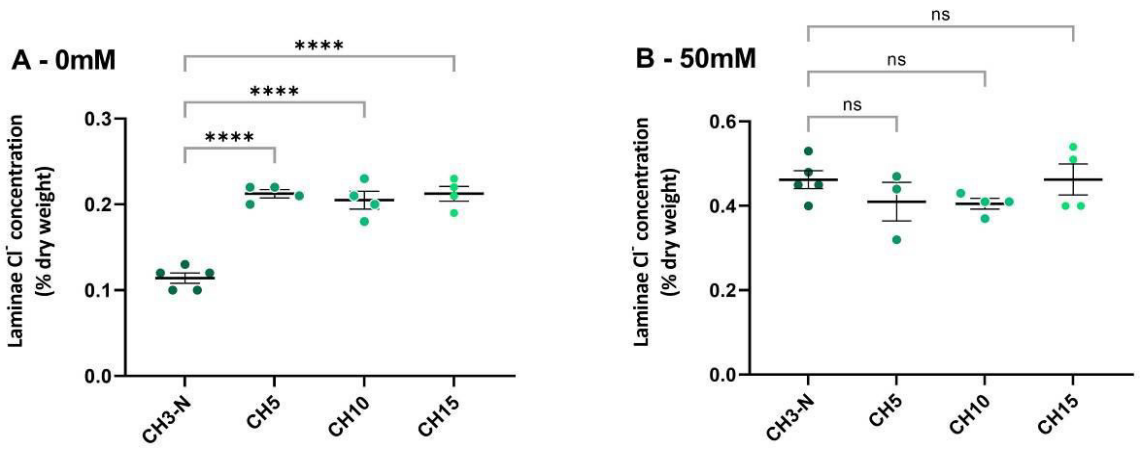


Figure 7.13 – Effect of (A) 0 mM Cl^- and (B) 50 mM mixed Cl^- treatments on Cl^- accumulation in the laminae of ungrafted transgenic Shiraz lines with reduced *CHX20.2* expression (CH5, CH10 and CH15) and a negative control (CH3-N). Plants were propagated from hardwood cuttings. Data points indicate the value of a single biological replicate, horizontal lines represent the mean of each transformant, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show where the difference between control and transformed lines is significant as follow: ns = not significant, **** = p<0.0001.

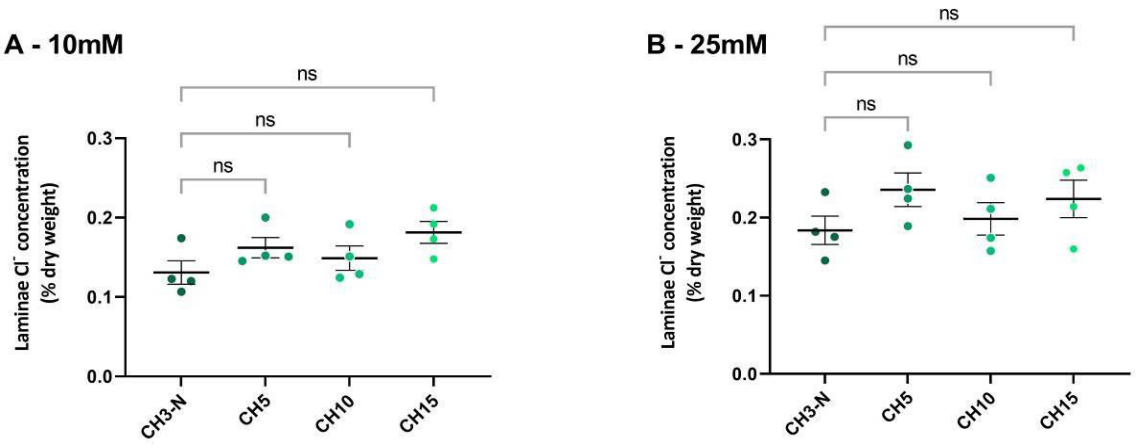


Figure 7.14– Effect of (A) 10 mM and (B) 25 mM mixed Cl^- treatments on Cl^- accumulation in the laminae of ungrafted transgenic Shiraz lines with reduced *CHX20.2* expression (CH5, CH10 and CH15) and a negative control (CH3-N). Plants were propagated and deflasked from tissue culture. Data points indicate the value of a single biological replicate, horizontal lines represent the mean of each transformant, and error bars indicate the standard error of the mean. Tukey's multiple comparison tests show no significant difference (ns) between control and transformed lines.

CHX20.1 is also highly expressed in mature petioles, yet no clear phenotype was seen when transgenic Shiraz lines with reduced expression of *CHX20.1* were used as the scion. Surprisingly, no clear phenotype was seen when lines with reduced expression of *CHX20.1* were used as the rootstock also. This may be because in that reciprocal grafting experiment only a 0 mM Cl^- treatment was applied, and a higher salt treatment is potentially needed to observe the *CHX20.1* phenotype. While there is unequivocal evidence for a role for *CHX20.1* at the root level, it is still unclear whether *CHX20.1* contributes to Cl^- exclusion at the shoot level. Hence, the reciprocal grafting experiment should be repeated in the future under a 50 mM salt treatment.

Unlike *CHX20.2,* expression of *CHX20.1* was also very high in the rachis (bunch stem), consistent with publicly available micro array data in the *V. vinifera* cultivar Corvina (Fasoli et al. 2012). Given that we have established a role for *CHX20.1* in Cl^- transport, its activity in the rachis may be involved in regulating Cl^- accumulation within the berry. Whether this is a role in Cl^- exclusion or inclusion into the berry is yet to be determined. Given the large influence of *CHX20.1* in the roots, to investigate the role of *CHX20.1* in the rachis, *CHX20.1*-silenced lines and control lines would need to be used as the scion grafted onto the same rootstock, similar to what we performed here for investigating its influence in the petiole. A number of years' growth would be required before such plants bear fruit, which is why this could not be assessed within this project.

The soybean homologue of *CHX20.1*, *GmSALT3*, has been shown to be a major gene conferring salt tolerance in soybean in multiple large mapping populations derived from salt-tolerant and salt-sensitive Chinese cultivars (Guan et al. 2014, Qi et al. 2014). To determine how *GmSALT3* contributes to salt tolerance, a recent study looked at three pairs of near-isogenic-lines, each pair containing a salt-tolerant and salt-sensitive line that share 96-99% similarity across the genome, aside from the *GmSALT3* locus at which they differ. The three salt-tolerant lines all share a common 1Mb region surrounding a functional version of *GmSALT3*, whereas the three sensitive lines share the same region associated with a non-functional truncated version of *GmSALT3* (Liu et al. 2016). Analysis of these near-isogenic lines has been interpreted to suggest that *GmSALT3* contributes to Na^+ exclusion via xylem retrieval in the roots, and to Cl^- exclusion via phloem recirculation in the shoot (Qu et al. 2021). Interestingly, a homologue of the grapevine *CHX20.2, Glyma03g32890*, is located adjacent to *GmSALT3* in the soybean genome, similar to *CHX20.1* and *CHX20.2* in the grape genome. Given that the near-isogenic lines share a 1Mb region, which likely contains 100+ additional genes, it is possible that the *CHX20.2* homologue is also contributing to the phenotype attributed to this locus.

In another Soybean study utilising a salt tolerant Brazilian cultivar, the activity of *GmSALT3* (referred to as *Ncl* in that study) was shown to be solely attributed to the roots, and not the shoot (Do et al. 2016). Interestingly, *Ncl* was found to control the exclusion of Na^+ and K^+, as well as Cl^- (Do et al. 2016). Our results on *CHX20.1*-silenced Shiraz lines suggests that *CHX20.1* also has an influence on laminae Na^+ accumulation, but not on K^+. This is consistent with observations seen in the BC_1F_2 population in which *VbVb* genotypes on average

accumulated significantly less Na^+ in the laminae than *VvVv* genotypes, but no differences in K^+ (Figure 6.3).

The *Arabidopsis AtCHX20* gene is expressed in guard cells and is involved in the osmoregulation of stomatal opening (Padmanaban et al. 2007). It is thought to function as a K^+/H^+ antiporter, however this supposed function is not drawn from observations in plants, but rather from the complementation of a yeast mutant deficient in a K^+/H^+ antiporter *Kha1*, which is far from conclusive. Given the clear roles of Cl^- transport for the grapevine and soybean homologues, it is possible that *AtCHX20* may have a similar affinity for Cl^- transport, for which it has likely not been tested. Cl^- fluxes are known to influence stomatal regulation (Demes et al. 2020), therefore it would fit that *AtCHX20* may also be acting as a Cl^- transporter to regulate guard cell opening. It is possible that either or both *CHX20.1* and *CHX20.2* also play a role in the osmoregulation of guard cells in grapevine. Neither *CHX20.1* or *CHX20.2* were found to be highly expressed in grapevine leaves, however, this does not rule out the possibility that either is highly expressed in the guard cells, which make up only a small fraction of total leaf tissue. This is something that would be worth investigating in the future. The partial degree of silencing achieved for *CHX20.1*, compared to stronger degree of silencing for *CHX20.2*, hints that the function of *CHX20.1* may be critical to plant growth and survival, whereas the function of *CHX20.2* is not. Indeed, *CHX20* has been shown to be the ancestral gene of the CPA2 family in plants, which is consistent with it having a significant function crucial to plant growth (Chanroj et al. 2012). An analysis of the genomes of 40 different plant species across all taxa revealed that they all contained at least one *CHX20* homologue (data not shown), supporting a critical role of this gene in plants.

A recent bioinformatic study in grapevine identified seven additional CHX-type genes in the grapevine genome. None of these grape CHXs have yet been characterised. They share homology with *AtCHX14*, *AtCHX16*, *AtCHX18*, *AtCHX19*, *AtCHX25*, *AtCHX26* and *AtCHX28* from *Arabidposis. AtCHX18* and *AtCHX19* are K^+/H^+ anitporters expressed in sperm cells and pollen tubes, respectively, and play a role in seed fertilisation (Cheung et al. 2017), while the role of other genes remains unknown.

7.6 Summary

Here we investigated two lead candidate genes residing within the *CLEX* locus for their potential roles in Cl^- exclusion. Although annotated as cation/H^+ exchangers, both *CHX20.1* and *CHX20.2* were found to contribute to the exclusion of the anion Cl^-. *CHX20.1* is primarily expressed in the root stele and has a major influence on the exclusion of Cl^-, and possibly Na^+, under high salt treatment. *CHX20.2* is expressed in the petiole and root cortex and has only a minor influence on the exclusion of Cl^- under control treatment, but not under high salt treatment. Taken together, *CHX20.1* is likely to be the major gene responsible for the variation in Cl^- exclusion associated with *CLEX* locus in the BC_1F_2 population and hence the main target for developing a perfect genetic marker for this trait.

8. Functional characterisation of *VbCHX20.1* and *VvCHX20.1* allelic variants

The work presented in this chapter was undertaken and prepared by Y. Qu and M. Gilliham with edits by J. Dunlevy and R. Walker.

8.1 Objectives

The aims of the experiments presented in this chapter were to better understand the function of the *CHX20.1* protein and to develop a functional assay to quantify the activity of proteins encoded by the *Vb* and *Vv* alleles. Ultimately this could enable the identification of key SNPs and amino acid differences underlying the *CLEX* locus to serve as the basis of a perfect genetic marker.

8.2 Background

The results presented in the previous two chapters suggest that the gene *CHX20.1* is responsible for the variation in Cl^- exclusion attributed to the *CLEX* locus in the BC_1F_2 population. In the Final Report for the previous project (CSP 1302) we showed that the *Vb* and *Vv* alleles of *CHX20.1* encode proteins predicted to differ by 19 amino acids. Our hypothesis is that one or more of these amino acid differences are likely to lead to a reduction or change in the functional activity of the *CHX20.1* protein. A good example of such a scenario is the gene *HKT1;1,* which is responsible for the majority of the variation in Na^+ exclusion in the K51- 40 x 140 Ruggeri population. The Gilliham lab was successful in the functional characterisation of the protein variants encoded by the four alleles present in that population. This revealed that each parent contained one dominant allele encoding an active protein and one recessive allele encoding a protein with reduced Na^+ transport activity (Henderson et al. 2018). F_1 hybrids that inherited both recessive alleles accumulated significantly more Na^+ in the laminae than those with one or more dominant alleles. Furthermore, through site directed mutagenesis of amino acid differences two key amino acids were identified that underlie these functional differences and this information enabled the development of a perfect genetic marker targeted to the causal SNPs encoding these amino acid differences (Henderson et al. 2018). This detailed and specific understanding of the key SNPs underlying this trait was only achievable because reliable yeast and *Xenopus* oocyte-based assays for quantifying the activity of HKT1;1 transport proteins were already available in Professor Matthew Gilliham's lab.

Understanding which of the 19 amino acids underlies the hypothesised functional differences between the CHX20.1 proteins encoded by the *Vb* and *Vv* alleles would enable the development of a perfect genetic marker targeted to the causal SNP or SNPs. However first a

reliable assay for assessing *CHX20.1* activity is needed. Unlike the case for *HKT1.1,* for which homologues had been well characterised in other plant species prior to its identification in the K51-40 x 140 Ruggeri population, the function of *CHX20* homologues remains poorly understood. Here we tested a range of techniques for suitability of characterising the function of CHX20.1 proteins.

8.3 Materials and Methods

8.3.1 Functional characterisation in *E.coli* strain TK2463

The *V. berlandieri* (Vb) and *V. vinifera* (Vv) derived alleles of *CHX20.1* were amplified and cloned from root cDNA of homozygous *VbVb* and *VvVv* BC_1F_2 individuals (described in CSP 1302). To investigate the transport function of the encoded CHX20.1 proteins, genes were expressed in *E. coli* strain TK2463 (mutant in K^+ uptake, lacking the native transporters Trk, Kup, and Kdp). Gateway entry vectors containing *VvCHX20.1*, *VbCH20.1*, CBL1n (non-functional amino acids as a negative control) and *AtKAT1* (inward-rectifying *Arabidopsis* K^+ channel as a positive control for K^+ transport), or ClC-0 (Torpedo voltage-gated Cl^- channel as a positive control for Cl^- transport) and were recombined into the expression vector pET-DEST42 (Life Technologies) using LR Clonase II (Life Technologies). The T7 promoter of the subsequent expression vectors was replaced with the TAC promoter by mutagenesis with InFusion HD (Clontech Laboratories Inc).

Cultures (5 mL) of TK2463 *E. coli* harbouring the different vectors, were grown overnight in KML medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L KCl) at 37 °C with shaking at 220 RPM. Cultures were pelleted for 5 min at 5000 x *g* and washed twice in low K^+ (approx. 2 mM) medium (10 g/L tryptone, 2 g/L yeast extract, 100 mM D-mannitol). Washed cells were added to 1 mL (final volume) of 'low K^+ medium' containing 500 mM IPTG to induce expression of target genes and to an OD_{600} of 0.05 in a 24-well microplate (Corvair). Plates were covered with sterile SealPlate® film (Sigma). Growth of *E. coli* was monitored by measuring OD_{600} every 15 minutes on a FLUOstar Omega plate reader (BMG Labtech) at 37 °C with continuous shaking at 200 RPM. Carbenicillin (100 µg/ml) was used in all media to maintain the plasmids. Complementation of the mutant phenotype was investigated in low K^+ medium supplemented with altered external concentration of KCl (0, 10, 30 or 50 mM) and NaCl (0, 50, 100, 150, 200 mM) and at three different pH levels (pH 6.5, 7.0 and 7.5). All treatment combinations were performed in triplicate.

8.3.2 Expression of *Vb* and *Vv CHX20.1* alleles in Arabidopsis root stelar cells

VbCHX20.1 and *VvCHX20.1* were cloned into pTOOL5::UAS_{GAL4} containing a promoter that drives expression specifically in root stelar cells, and this was confirmed by PCR and sequencing. The constructs were transformed into *Arabidopsis* line E2568 (ecotype Col-0), as described by Møller et al. (2009). Two independent transformations were performed for each construct. Successful transformants were selected using BASTA (120 mg/L BASTA for 10 days

sprayed onto the leaves every 2-3 days). The T1 plant genomic DNA was extracted, including two samples for each construct from a tray of plants.

T2 plants from lines E2586 *UAS_{GAL4}:VvCHX20.1* and *UAS_{GAL4}:VbCHX20.1* were screened by PCR. Successful transformants, null segregants and untransformed E2586 were grown in hydroponics for five weeks and subjected to five days of salt stress consisting of 25 mM increases in NaCl every 12 h until 100 mM NaCl was reached. $CaCl_2$ was supplemented at every addition of NaCl to maintain constant Ca^{2+} activity. After five days, plants were harvested for determining biomass and leaf ion accumulation.

8.3.3 Expression *of Vb* and *Vv CHX20.1* alleles in *Arabidopsis* guard cells

VbCHX20.1, *VvCHX20.1* and *GmSALT3* (homolog in soybean) were cloned into pMDC32::GC1 containing guard cell specific expressing promoter (GC1). The constructs were transformed into *Arabidopsis Atchx20* mutant plants. Two independent transformations were performed for each construct. Successful transformants were selected using hygromycin; about 400 plants were screened on ½ MS agarose plates with hygromycin. T2 plants were genotyped by PCR, and the confirmed transformants were used to investigate guard cell regulation as follows.

Five-week-old *Arabidopsis* plants were kept in the dark overnight, and epidermal strips were excised into a solution containing 5 mM KCl and 10 mM MES-KOH at pH 6.15. Epidermal strips were then either kept in the dark for a further 3 h or exposed to light (150 µE m^{-2} s^{-1}) for 3 h. The dimensions of stomatal aperture (width and length) were then measured under a microscope.

8.4 Results

8.4.1 Functional characterisation of *CXH20.1* in *E.coli* strain TK2463

The K^+ uptake deficient *E. coli* cells grow poorly in media containing no additional K^+ above the approximately 2 mM basal K^+ levels when at pH 6.5 (Fig. 8.1A). However, with additional 10 mM K^+, the cells grew better and *VbCHX20.1*, *VvCHX20.1* and *AKAT1* positive control were all able to rescue the mutant phenotype compared to the negative control *CBL1n*. The *AKAT1* improved growth the most, followed by *VvCHX20.1* which was significantly higher than *VbCHX20.1* (Fig. 8.1A). There were no significant differences between any construct when grown in media containing 30 mM K^+, but at 50 mM K^+ *VvCHX20.1* significantly improved the growth rate compared to the control, whereas *VbCHX20.1* and the positive control *AKAT1* did not (Fig. 8.1A).

At pH 7.0 with no additional K^+, the *VvCHX20.1* allele complements the *E. coli* phenotype to a greater degree than the *VbCHX20.1* allele (Fig. 8.1B); with additional K^+ (10 mM, 30 mM, and

50 mM), there were no significant differences in the growth rate of *E. coli* cells harbouring *VbCHX20.1* and *VvCHX20.1*.

At pH 7.5 and with no additional K^+, both *VbCHX20.1* and *VvCHX20.1* improved the growth rate compared to the negative control, but there was no significant difference between the two alleles (Fig. 8.1C). No significant differences in growth rates were present at K^+ concentration of 10, 30 and 50 mM (Fig. 8.1C).

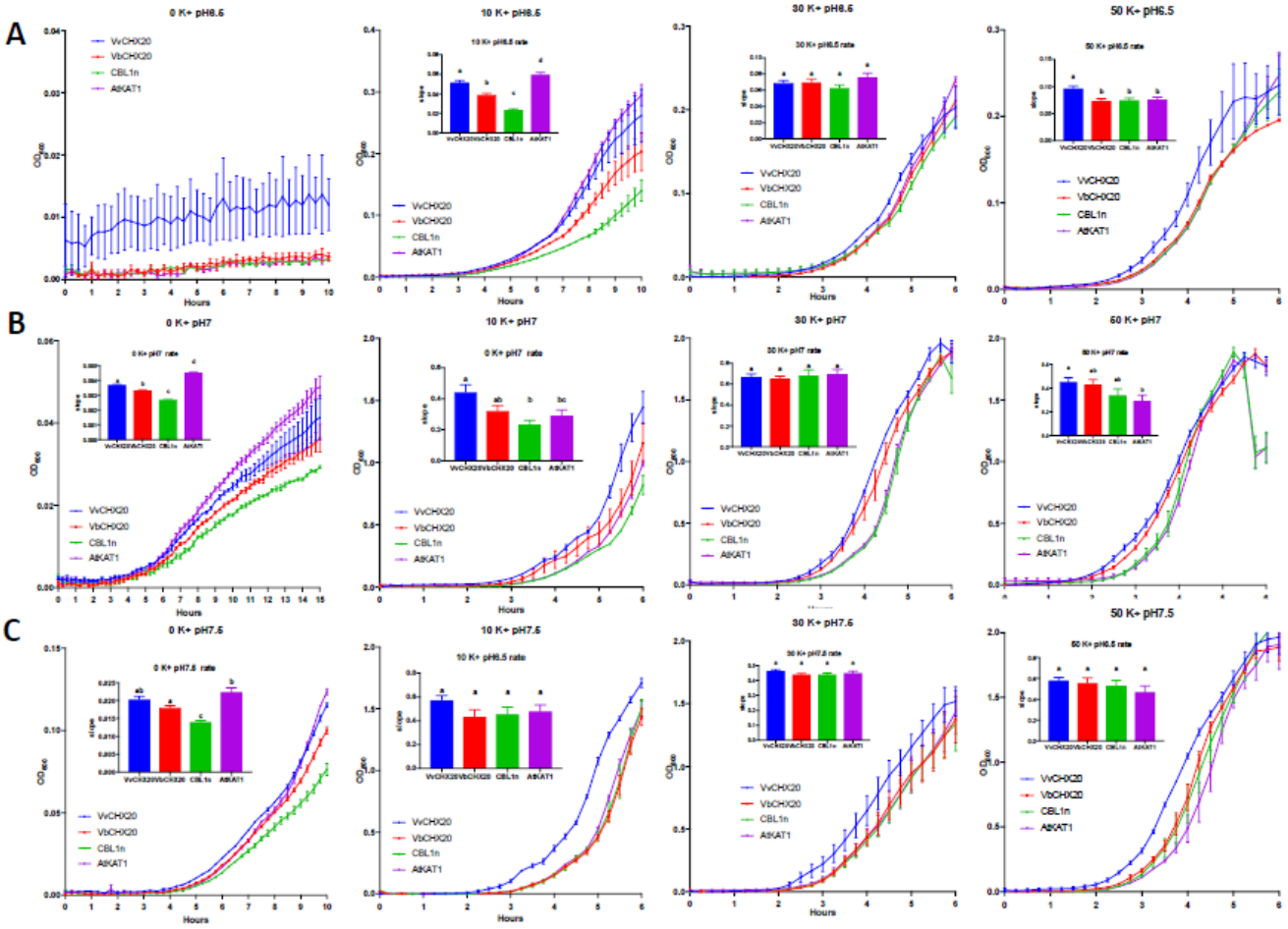


Figure 8.1. Both *VvCHX20.1* and *VbCHX20.1* complement *E. coli* (mutant in K^+ uptake) growth. *E. coli* strain TK2463 (defective in potassium uptake genes, *Trk*, *Kup*, and *Kdp*) harbouring a modified pET-DEST42 vector with *VvCHX20.1* (blue), *VbCHX20.1* (red), *CBL1n* (negative control – green), and *AtKAT1* (positive control – purple) were grown in low potassium medium with nil or additional external concentration of KCl to give 0 mM, 10 mM, 30 mM and 50 mM KCl at different pH levels. A, pH 6.5. B, pH 7. C, pH 7.5. Growth rates (OD600 per hour) of E.coli cells within the log phase are indicated between dotted lines, and their slopes are shown in the small bar graphs in each figure. Bars are mean ± standard error of the mean of three replicates. Different letters indicate statistically significant differences between *E. coli* cells harbouring different constructs (one-way ANOVA followed by Fisher's LSD test, $p < 0.05$).

A second experiment was performed to explore the effect of NaCl in the low K^+ media at pH 7.0. In the absence of any additional KCl or NaCl, the growth rate of cells harbouring *VvCHX20.1* was again higher than those harbouring *VbCHX20.1* (Fig. 8.2A). The addition of 50 mM, 100 mM, 150 mM, or 200 mM NaCl, caused *E. coli* cells to grow poorly and did not reach

log phase (Fig. 8.2B-E). When media was supplemented with 30 mM KCl and 200 mM NaCl the *VvCHX20.1* allele is able to complement the phenotype to a greater degree than *VbCHX20.1* (Fig. 8.2F).

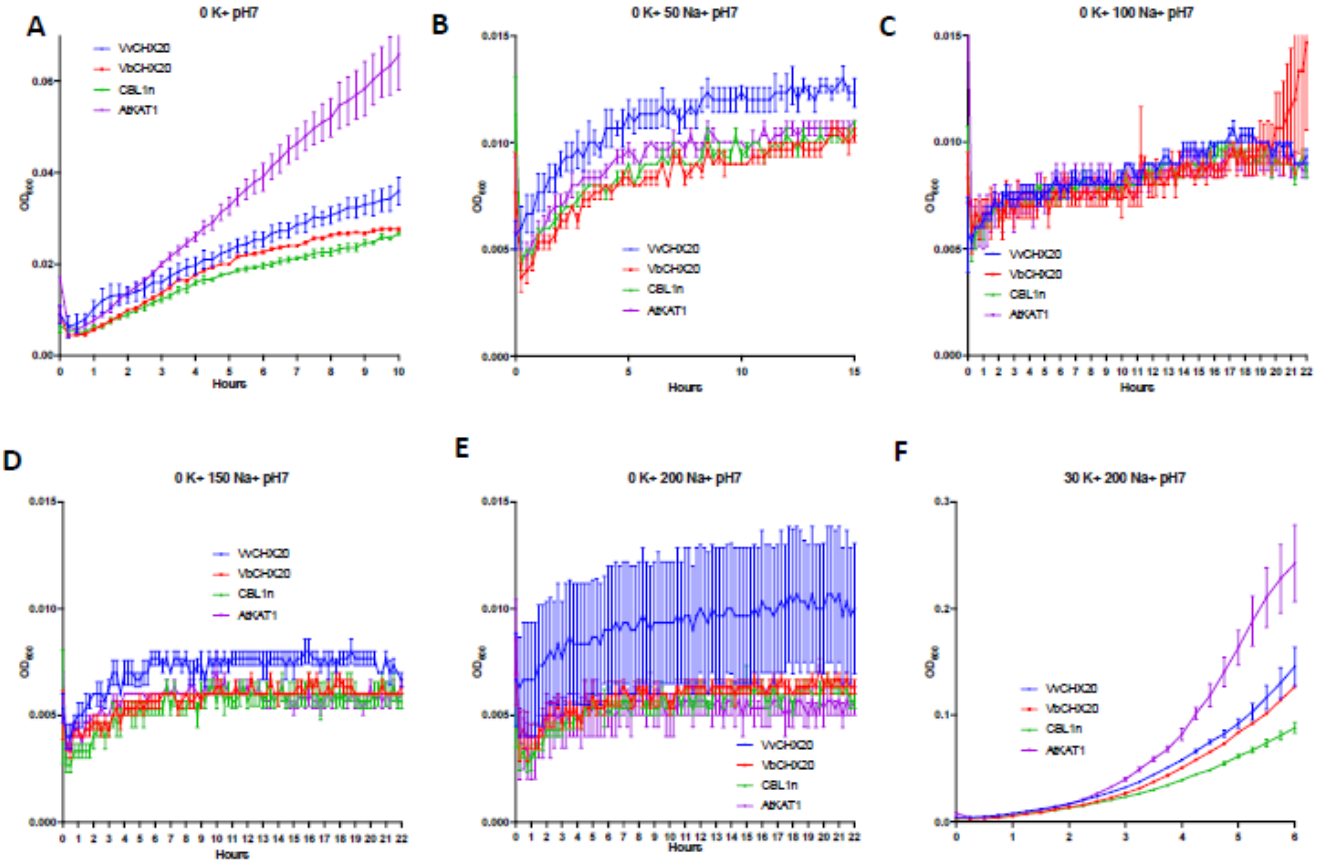


Figure 8.2. Complementation assay of *E. coli* growth in various saline conditions at pH 7. *E. coli* strain TK2463 (defective in potassium uptake, Trk, Kup, and Kdp) harbouring a modified pET-DEST42 vector with *VvCHX20.1, VbCHX20.1, CBL1n*, and *AtKAT1* were grown in low potassium medium (~2 mM K^+) with nil or additional external concentration of NaCl to give 0 mM (A), 50 mM (B), 100 mM (C), 150 mM (D), and 200 mM (E) NaCl. F, low potassium medium with additional 30 mM KCl and 200 mM NaCl. All the test media were supplemented with 0.5 mM Isopropyl β- D-1- thiogalactopyranoside (IPTG) and 100 µg/ml carbenicillin. OD600 was monitored every 15 min in 96-well microplate reader. Bars are mean ± standard error of the mean of three replicates.

We next measured the ion concentrations in *E. coli* cells grown under low K^+ at pH 7.0 to confirm the complementation is because of K^+ uptake. For this experiment the Cl^- permeable channel, *ClC-0*, was included as a control to investigate if it is able to complement the K^+ uptake deficient *E. coli*.

The OD_{600} of cultures show that the *VbCHX20.1* allele complements the *E. coli* phenotype to a lesser degree than *VvCHX20.1* allele in Low K^+ media (Fig 8.3A), which is consistent with previous experiments. Yet the *ClC-0* was able to rescue growth to a far greater degree than either *VvCHX20.1* or the positive control *AtKAT1* (Fig. 8.3A). There were no significant differences in Na^+ concentration between cells harbouring *VvCHX20.1*, *VbCHX20.1*, *AtKAT1* or *ClC-0*, which were all significantly higher than the negative control, *CBL-1n* (Fig. 8.3B).

Similarly, there were no significant differences in K^+ concentration between cells with *VvCHX20.1*, *VbCHX20.1*, *AtKAT1*, but the presence of *ClC-0* resulted in significantly lower concentration of K^+ even less than, but not significantly different from the control (Fig. 8.3C). There were no significant differences in Cl^- concentration among the different constructs (Fig. 7.3D). There were no significant differences detected in concentration of these ions in the external growth media between constructs (Fig. 8.4).

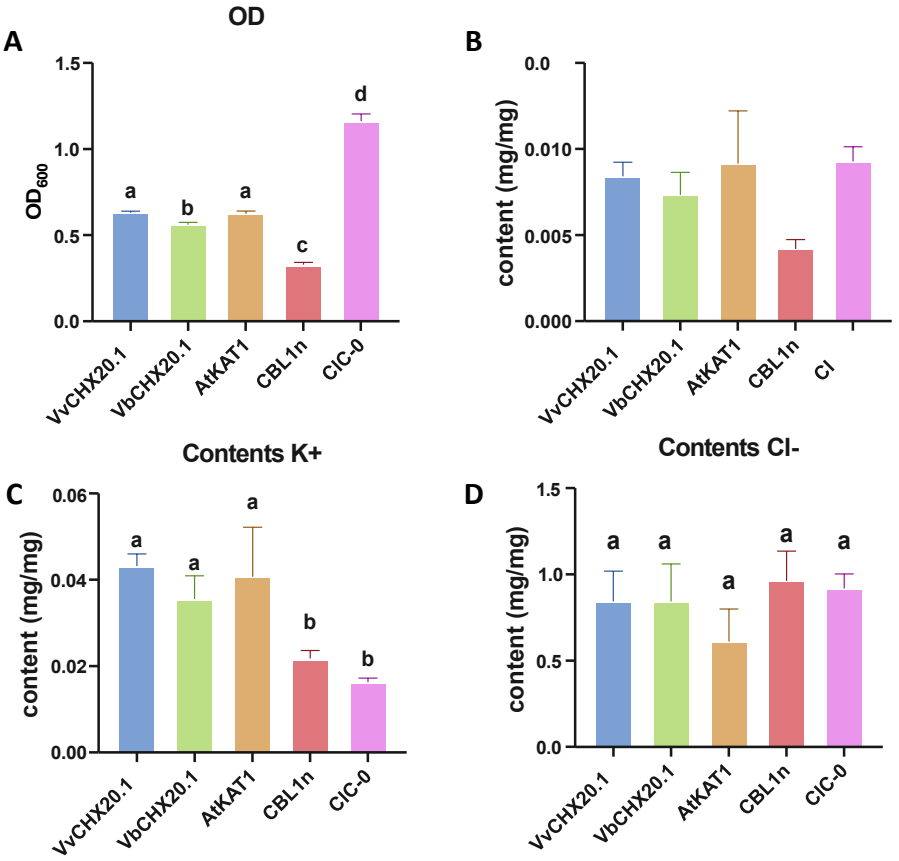


Figure 8.3. *VvCHX20.1* and *VbCHX20.1* facilitate *E. coli* to accumulate Na^+ and K^+ after growth in Low K^+ media. *E. coli* strain TK2463 (defective in potassium uptake, *Trk*, *Kup*, and *Kdp*) harbouring a modified pET-DEST42 vector with *VvCHX20, VbCHX20, AtKAT1* (inward-rectifying *Arabidopsis* K^+ channel as positive control), *CBL1n* (non-functional amino acids as negative control) or *ClC-0* (Torpedo voltage-gated chloride channel) were grown in a 10 ml tube with Low K^+ media; cells were harvested at 5 h, and cells were digested in 1% Nitric Acid at 65°C overnight. A, OD_{600} of cultures; B, Na^+; C, K^+; and D, Cl^- concentration. Data are means of six replicates ± standard error. Statistical significance was tested by one-way ANOVA followed by Fisher's LSD test. These experiments were repeated twice with the same results. Note only one experiment is shown.

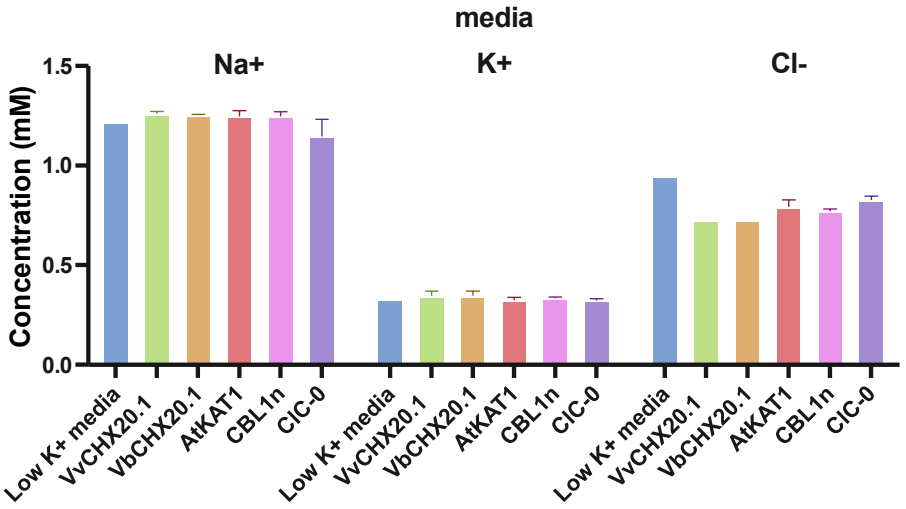


Figure 8.4. No significant differences in ion concentrations (growth media) among constructs. *E. coli* strain TK2463 (defective in potassium uptake, Trk, Kup, and Kdp) harbouring a modified pET-DEST42 vector with *VvCHX20, VbCHX20, AtKAT1, CBL1n* or *ClC-0* were grown in a 10 ml tube with Low K^+ media (~2 mM K^+) with additional 0.5 mM Isopropyl β- D-1-thiogalactopyranoside (IPTG); growth media were harvested after 5h growth. Data are means of six replicates ± standard error. Statistical significance was tested by one-way ANOVA followed by Fisher's LSD test.

The increased K^+ concentration of *E.coli* cells expressing *VvCHX20.1* and *KAT1*, compared to *VbCHX20.1* and the negative control *CBL-1n*, would suggest that the complementation of the reduced growth phenotype is the result of K^+ uptake. This makes sense given this mutant strain is deficient in three K^+ uptake genes. Yet the results for the Cl^- channel *ClC-0* do not agree with this, as *ClC-0* was able to complement the growth phenotype to the greatest degree, yet accumulated the least amount of K^+. Therefore, it seems that other factors could be contributing to the rescue of this phenotype. Indeed, it has previously been reported that genes of diverse function, and not encoding K^+ transporters, can complement the growth phenotype of K^+ uptake-deficient *E. coli* (Ito et al. 1997). It is therefore not possible to conclude the function of *VvCHX20.1* and *VbCHX20.2* proteins in this system. Furthermore, the difference in ability of *VvCHX20.1* and *VbCHX20.2* to complement the growth phenotype of TK2463 *E.coli* is not pronounced enough to serve as the basis of an assay to investigate key amino acid differences between these allelic variants.

8.4.2 Expression of *Vb* and *Vv CHX20.1* alleles in *Arabidopsis* root stelar cells

Next we investigated the potential of assaying *CHX20.1* activity in *Arabidopsis* plants. As *CHX20.1* is hypothesised to function within the root stele (based on its high expression in this tissue), *Arabidopsis* plants were transformed with constructs designed to express the two *CHX20.1* allelic variants specifically within the stele. The negative controls for this experiment were untransformed *Arabidopsis* (E2586) and null segregants, T2 generation derived from transformed plants but do not possess the transgene. Plants were grown in hydroponics for five weeks and subjected to a five day salt treatment of 100 mM NaCl (Fig. 8.5A).

At the end of the experiment there were no significant differences in dry weight between lines transformed with *VvCHX20.1*, *VbCHX20.1* or the two negative controls (Fig. 8.5B). Looking at leaf ion accumulation, *VvCHX20.1* significantly increased the concentration of Na^+, K^+ and Cl^- compared to E2586 and null segregants, whereas *VbCHX20.1* did not increase shoot Na^+, K^+ and Cl^- concentration compared to null segregants, but significantly increased ion accumulation compared to untransformed E2586 (Figure 8.5C-E). Differences in ion accumulation between *VvCHX20.1* and *VbCHX20.1* transformed lines were not significantly different, however, suggesting that heterologous expression in *Arabidopsis* stelar cells was not a viable technique for assaying *CHX20.1* activity or testing amino acid differences.

8.4.3 Expression *of Vb* and *Vv CHX20.1* alleles in *Arabidopsis* guard cells

AtCHX20, the Arabidopsis homolog closest to *VbCHX20.1* and *VvCHX20.1*, has previously been shown to participate in osmoregulation of guard cells (Padmanaban et al. 2007). This provides us with another possible pathway of assaying the function of *VbCHX20.1* and *VvCHX20.1* proteins. In this experiment, an *Atchx20* knockout mutant lacking *AtCHX20* activity and with impaired stomatal opening, was transformed with *VbCHX20.1*, *VvCHX20.1*, *GmSALT3* (homolog in soybean), all under the control of a guard cell specific promoter, to see if they could complement the *Atchx20* mutant phenotype. Wild type *Arabidopsis* was also included as a positive reference. To screen stomatal response, five-week-old plants were kept in the dark overnight, epidermal strips taken and exposed to either three hours of light or three hours of dark before the aperture dimensions (width and length) of stomata were measured under a microscope.

After 3 h dark, there were no significant differences in stomatal aperture, stomatal length or aperture/length ratio between the wild type, untransformed *Atchx20* mutant or *Atchx20* possessing *VvCHX20.1*, *VbCHX20.1* or *GmSALT3*, except that *VvCHX20.1* stomatal length was significantly longer than the wild type plants (Fig. 8.6B). After 3 h light, the stomatal aperture and aperture/length ratio of the *Atchx20* mutant was significantly reduced compared to WT, consistent with the findings of Padmanaban et al., (2007). A similar trend was seen for stomatal length, although not significantly different to WT. Stomatal aperture and aperture/length were therefore used to investigate the influence of the *Vv* and *Vb CHX20.1* constructs on this mutant phenotype. After exposure to light, the presence of *VvCHX20.1* significantly increased both the stomatal aperture and aperture/length ratio compared to the untransformed *Atchx20* mutant (Fig. 8.6C). This complementation by the *VvCHX20.1* resulted in stomatal aperture that was not significantly different from the wild-type control. Conversely neither *VbCHX20.1* or *GmSALT3* were able to significantly improve stomatal opening over the *Atchx20.1* mutant (Fig. 8.6C).

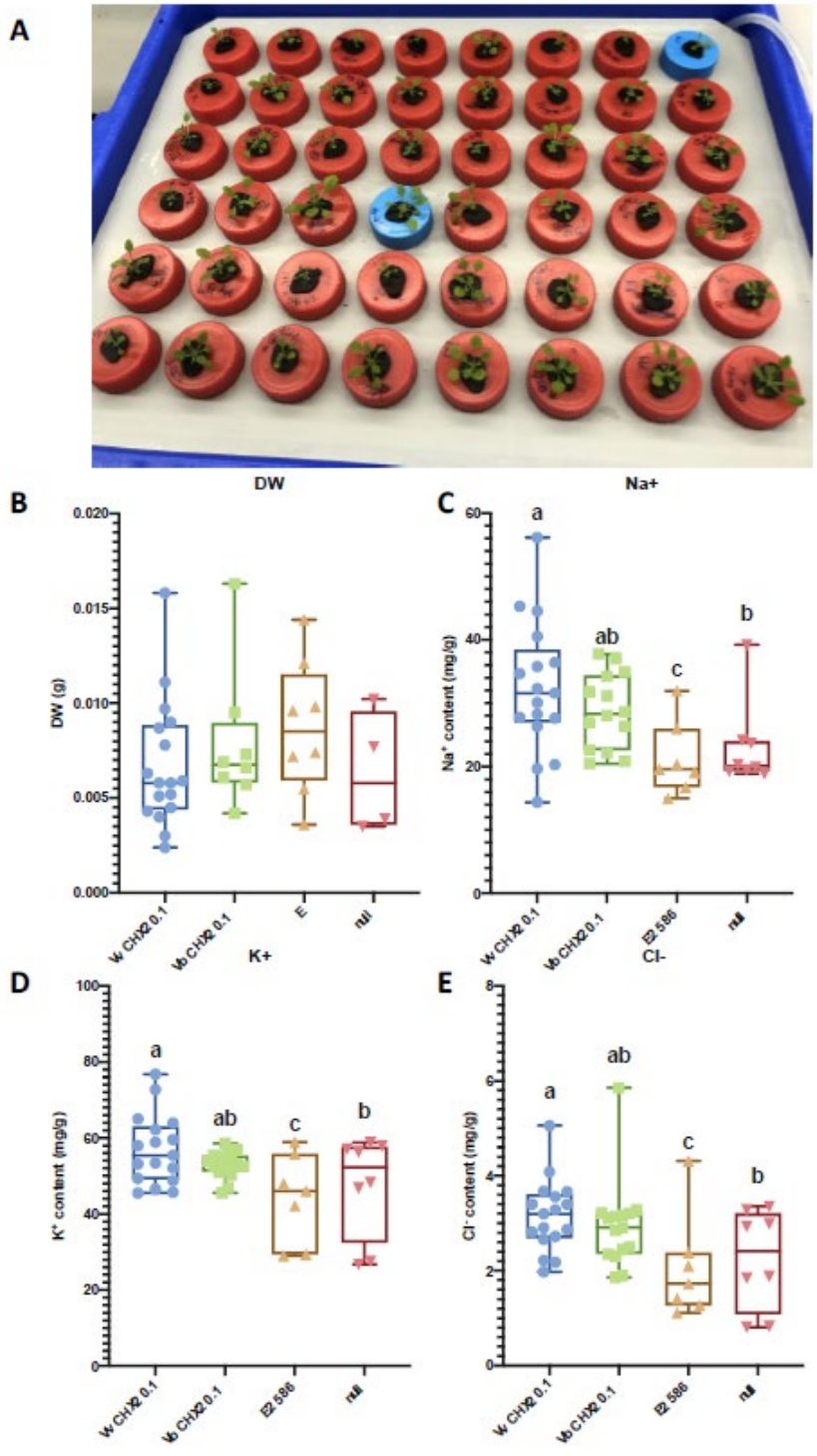


Figure 8.5. Stelar-specific expression of *VvCHX20.1* and *VbCHX20.1* in *Arabidopsis* E2586 significantly increased shoot Na^+, K^+ and Cl^- accumulation. T2 plants from lines E2586 *UAS_{GAL4}:VvCHX20.1* and *UAS_{GAL4}:VbCHX20.1* were screened by PCR and successful transformants were grown in hydroponics for 5 weeks and subjected to 5 d of salt stress consisting of 25 mM increases in NaCl every 12 h until 100 mM NaCl was reached. $CaCl_2$ was supplemented at every addition of NaCl to maintain constant Ca^{2+} activity. A, the plants were grown in hydroponics in a random design layout. B, the total dry mass of plants subjected to salt stress. Na^+ (C) and K^+ (D) concentrations were measured by flame photometer, Cl^- (E) concentrations were measured by chloride analyser. Data are means of 8 – 17 replicates ± standard error. Statistical significance was tested by one-way ANOVA followed by Fisher's LSD test.

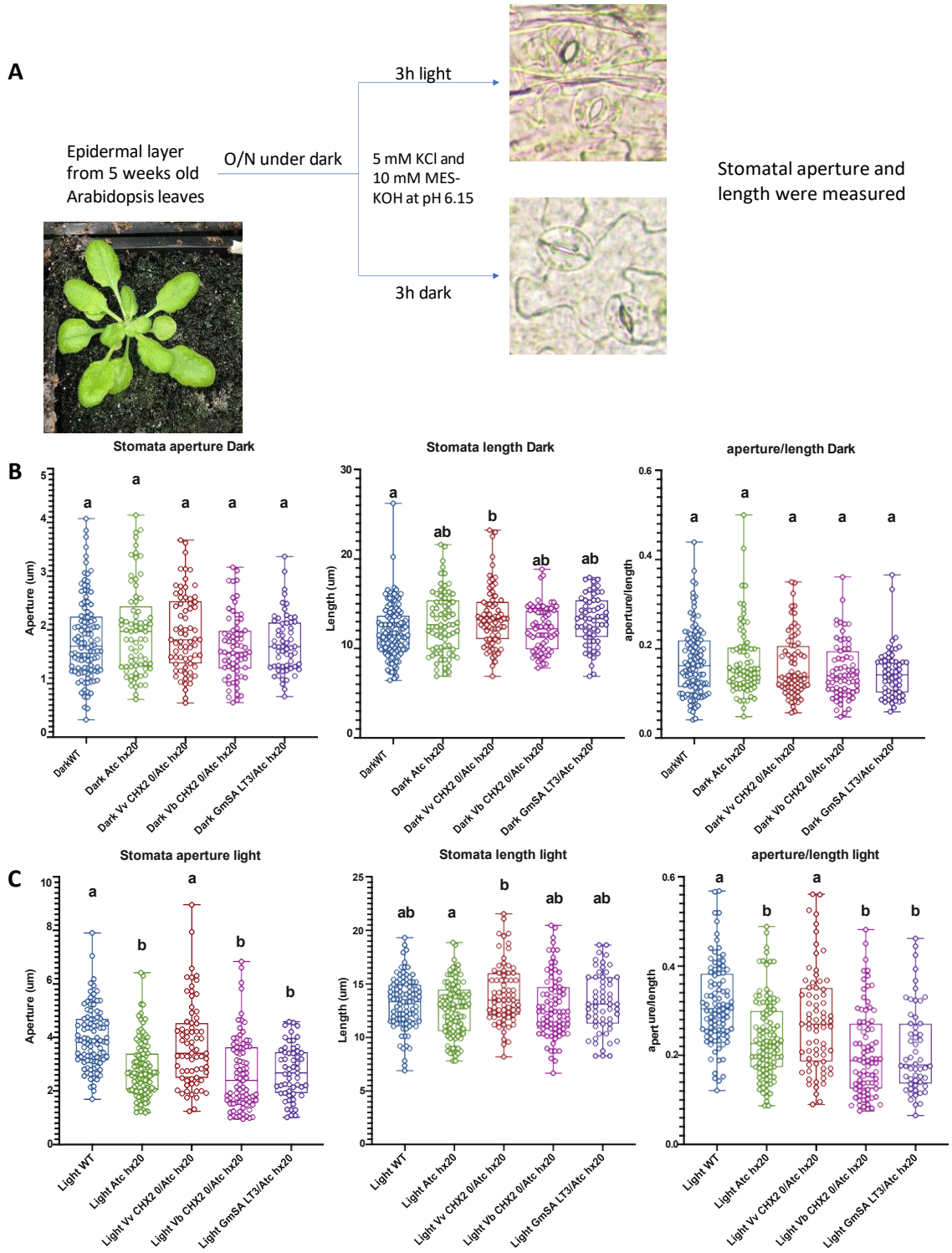


Figure 8.6. *VvCHX20.1* complemented *Atchx20* mutants' phenotype in reduced light-induced stomatal aperture. Arabidopsis plants at five-weeks-old were kept in the dark overnight, and epidermal strips were excised into a solution containing 5 mM KCl and 10 mM MES-KOH at pH 6.15. Samples were given 150 µE m^{-2} s^{-1} light or dark for 3 h. Stomatal aperture and length were measured after dark (B) and light (C) for WT (col-0; positive control), *Atchx20* (mutant), *VvCHX20.1/Atchx20*, *VbCHX20.1/Atchx20,* and *GmSALT3/Atchx20.* Data are means ± standard error. Statistical significance was tested by one-way ANOVA followed by Fisher's LSD test. n = 57 – 62.

8.6 Summary

A range of different methodologies for assaying the functional activity of CHX20.1 proteins was tested. The *VvCHX20.1* allele was able to complement the growth phenotype of a K^+ uptake deficient *E. coli* mutant significantly more than that of the *VbCHX20.1* allele. Complementation of the K^+ uptake deficient *E. coli* mutant by a known Cl^- channel *ClC-0* throws doubt over the transport function of genes able to rescue this phenotype. *VvCHX20.1* was also able to complement the reduced stomatal opening phenotype of the *chx20 Arabidopsis* mutant. With some further optimisation, each of these systems could be used to investigate the key amino acids underlying functional differences between the protein variants encoded by the *Vb* and *Vv CHX20.1* alleles.

9. Influence of the chloride exclusion (*CLEX*) locus in the K51-40 x 140 Ruggeri population

9.1 Objectives

The objective of the work presented in this chapter was to better understand the influence of the *CLEX* locus on Cl^- exclusion in the genetically diverse K51-40 x 140 Ruggeri population. This information will inform the utility of SNPs at this locus as the basis of a predictive genetic marker for Cl^- exclusion in rootstock breeding.

9.2 Background

In Chapter 6, we showed that inheritance of the novel *CLEX* locus accounts for up to 91% of the variation in Cl^- exclusion in the *V. berlandieri* x *V. vinifera* BC_1F_2 population. As *V. vinifera* is not suitable for rootstock breeding (for concerns it may introduce undesirable genetic factors that confer susceptibility to phylloxera and nematodes), the SNPs that distinguish between the dominant *Vb* and recessive *Vv CLEX* alleles cannot be used as the basis for a predictive genetic marker for breeding. In order to develop a robust genetic marker at this locus, we must first understand its involvement across diverse genomic backgrounds of the *Vitis* species that are used in rootstock breeding, as well as identify informative SNPs within those backgrounds. A recent study of 36 common rootstocks found that only three accessions namely *V. berlandieri* 'Resseguier 2', *V. rupestris* 'du Lot' and *V. riparia* 'Gloire de Montpellier' make up 39% of the genetic background of the 36 most common rootstocks. Including other accessions of these three *Vitis* species accounts for a massive 76% of these 36 rootstocks' genetic makeup (Riaz et al. 2019). Hence testing the influence of the *CLEX* locus in rootstocks of these backgrounds is an ideal starting point for developing a robust genetic marker.

140 Ruggeri (*V. berlandieri* 'Boutin B' x *V. rupestris* 'du Lot') is considered to be the industry benchmark for Cl^- exclusion and is therefore a highly salt tolerant rootstock. On the other hand, K51-40 (*V. champinii* 'Dogridge' x *V. riparia* 'Gloire de Montpellier') is one of the worst excluders of Cl^- and is highly sensitive to salinity. In 1985, an experimental cross between these two contrasting genotypes was initiated through the CSIRO rootstock breeding program and subsequent crosses made in the mid-90s to bulk up the population. In the early 2000s, the population was screened for capacity for Cl^- exclusion. The resulting F_1 progeny revealed a continuous variation in Cl^- exclusion when screened under a potting mix (50 mM Cl^-) or aerated solution culture (25 mM Cl^-) setup (Gong et al. 2011). This finding led the authors to suggest that Cl^- exclusion is controlled by multiple genes in this family.

In the previous project CSP 1302, we reassessed this F_1 population under both cool and hot temperatures in the Smarthouses of the Plant Accelerator, each under a 100 mM mixed Cl^- treatment. Similar to that of Gong et al., we observed continuous variation under both temperature regimes, indicative of control by multiple genes, with the *CLEX* locus explaining only 2% of the variation. An estimation of heritability across the data generated from the two temperature treatments and the two growing media experiments of Gong et al., revealed that only 21% of the variation in Cl^- exclusion could be attributed to genetic sources (Dunlevy et al., 2022). That is, in this F_1 population the trait is predominantly influenced by environmental variables.

The low heritability of the Cl^- exclusion in this population is in stark contrast to the high heritability of this trait in the *V. berlandieri* x *V. vinifera* BC_1F_2 population in which the *CLEX* locus consistently explains 85-89% of the variation. One explanation for why Cl^- exclusion appears to be under such different genetic control in the two populations is that the K51-40 x 140 Ruggeri F_1 individuals are all heterozygous at the *CLEX* locus. If this hypothesis is true then K51-40 must contain two 'poor' alleles and 140 Ruggeri must contain two 'good' alleles at this locus. Indeed, the contrasting phenotypes of the parents do fit with this theory.

To test this hypothesis further, we performed target crosses of selected F_1 progeny to generate two F_2 populations that would segregate for the four *CLEX* alleles, two derived from K51-40 and two from 140 Ruggeri. We then assessed the Cl^- exclusion phenotypes of these F_2 populations to determine whether the *CLEX* is indeed the primary locus for Cl^- exclusion across the major *Vitis* species (accounts for most of the variation), or if it is just one of a number of key loci for this trait (accounts for minimal variation). Furthermore, a better understanding of the influence of this locus in such a highly genetically diverse population will assist the development of a robust genetic marker for predicting Cl^- exclusion in rootstock breeding programs.

9.3 Materials and Methods

9.3.1 Genetic markers to K51-40 and 140 Ruggeri *CLEX* alleles

The original 40 K51-40 x 140 Ruggeri F_1s had previously been scored for the inheritance of the *CLEX* locus using genotype by sequencing data generated by DArTseq (Diversity Arrays Technology)(Final report CSP 1302). Using these scores of random SNPs across the CLEX locus, alleles were designated as R1 and R2 from 140 Ruggeri and K1 and K2 from K51-40. Here we sought to identify SNPs specifically within the *CHX20.1* gene to potentially serve as the basis of a more precise in-house genetic marker that does not rely on costly Genotype by Sequencing. To identify SNPs unique to each of the four *CLEX* alleles we chose to directly amplify and Sanger sequence across exons 3 and 4 of both parents. Primers used to amplify and sequence across exons 3 and 4 are listed in Table 8.1. This approach enabled us to score at least 25 SNPs across the four alleles that serve as the basis of our genetic marker. SNPs unique to the R1, R2, K1 and K2 alleles are shown in Table 8.2.

We next scored inheritance in the F_1 progeny, which agreed with that of the previous Genotype by Sequencing results at this locus. Two genotypes possessing the K2 and R1 alleles (MC 215-39 and MC 215-40) and two genotypes that contained the K1 and R2 alleles (MC 215-76 and MI 07-49), were identified and selected for crossing in order to generate F_2 progeny that are homozygous for each of the four alleles.

Table 9.1 – Primers used for the amplification and sequencing of *CHX20.1* from genomic DNA

Name	Purpose	Sequence 5'-3'
CHX20.1 F2	amplification & sequencing	ACAGGTACTTAACGATGAGGTG
CHX20.1 R2	of exons 3 & 4	GGATACACGAAGTTTCAGCTGC

Table 9.2 – SNPs present in exons 3 and 4 of *CHX20.1* distinguish the four alleles in this population. SNP positions are from the start of the *CHX20.1* F2 primer site (top row) and from the start of the *CHX20.1* gene ID sequence LOC100246026 (bottom row).

	pos. from *CHX20.1* F2	154	267	378	412	455	491	503	514	618	630	636	684	817	858	882	916	957	981	1051	1089	1105	1159	1192	1239	1294
	pos. on LOC100246026	5274	5390	5500	5534	5577	5613	5625	5636	5742	5754	5760	5808	5941	5982	6006	6040	6081	6105	6153	6213	6229	6282	6316	6363	6418
	R1	T	T	G	C	T	T	T	G	A	C	G	G	G	T	C	A	A	A	A	G	G	G	A	C	A
CHX20.1	**R2**	T	C	G	C	C	G	A	A	A	C	A	G	A	C	C	A	A	C	G	G	G	A	G	A	A
alleles	**K1**	C	C	G	C	C	G	A	A	C	C	A	G	A	C	T	C	C	A	A	G	C	G	G	C	G
	K2	T	C	A	G	C	G	A	A	C	G	A	C	A	C	T	A	C	A	A	C	G	G	G	C	G

9.3.2 K51-40 x 140 Ruggeri F_1 and F_2 crosses

All crosses were performed at the SARDI research vineyard at Nuriootpa. Between 15-20 bagged flowers of female F_1s, MC215-39 and MC215-76, were pollinated with fresh pollen from the males, MC215-40 and MI07-49, respectively, resulting in the segregation of *CLEX* allele combinations shown in Fig 9.1. Bagged flowers of K51-40 were also pollinated with dried pollen of 140 Ruggeri collected from the previous season and stored at -80°C. Crosses yielded the resulting seed numbers; K51-40 x 140 Ruggeri - 213 seeds, MC215-39 x MC215-40 -270 seeds, MC215-76 x MI07-49 - 450 seeds.

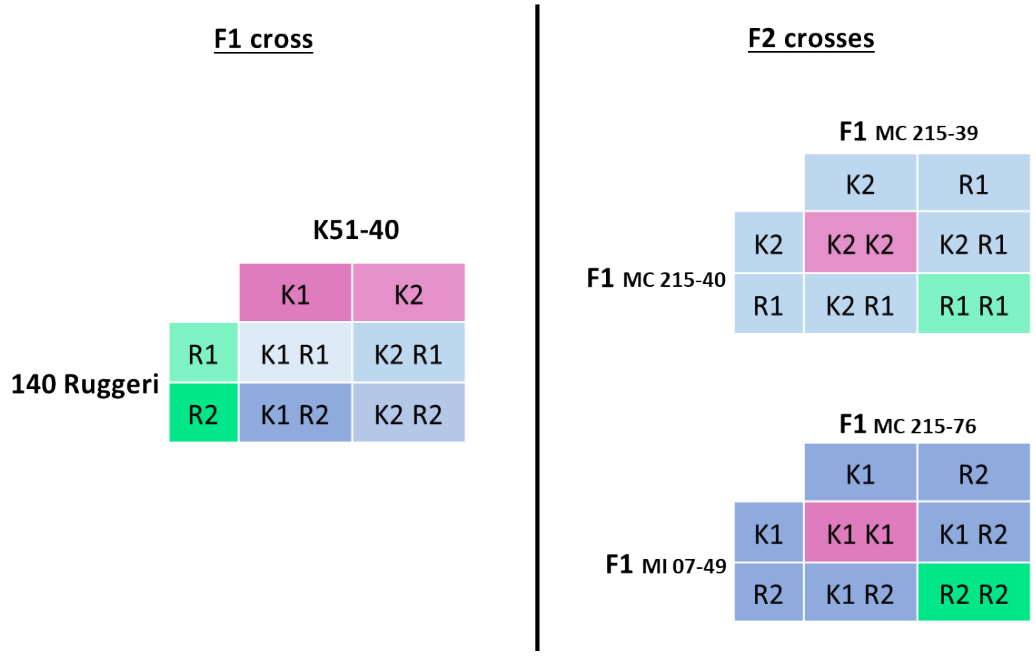


Figure 9.1 – Schematic representation of *CLEX* alleles in K51-40 and 140 Ruggeri parents and the resulting F_1 and F_2 populations.

9.3.3 Seedlings screens for Cl^- exclusion

F_1 and F_2 seeds were sterilised, germinated and raised as described in section 5.1.3. Unfortunately, fungal contamination of many plates resulted in only 33 of K51-40 x 140 Ruggeri, 38 of MC215-39 x MC215-40 and 19 of MI07-49 x MC215-76 seedlings establishing successfully. Approximately 5-6 weeks after germination, a single emerging leaf was taken from each seedling for DNA extraction and genotyping by direct sequencing across exons 3 and 4 of *CHX20.1* (described in 8.3.1). Salt treatment commenced one week later. All seedlings were watered with 50 mL of 25 mM mixed Cl^- for five consecutive days, equating to an estimated pot concentration of approximately 25 mM Cl^- based on a pot soil weight of 500 g (approximately 250 g soil + 250 g water). This was held for nine days by watering with 50 mL of rainwater daily. Due to variable size of seedlings, differences in transpiration would lead to slight differences in pot water content and therefore salt concentrations. To minimise this variability, on day 17 salts were flushed from the pot with three consecutive days of 150 ml rainwater, followed by a further five days of 50 mL of 25 mM mixed Cl^- to effectively 'reset' the salt concentration of all pots to 25 mM Cl^-. From days 24-29 seedlings were watered with between 50-75 mL of rainwater to maintain pot weights and treatment concentration at approximately 25 mM Cl^-. On day 29, the lower four leaves of each seedling were harvested for Cl^- analysis as described in section 5.2.1.

9.4 Results

9.4.1 Cl^- exclusion in the parents of K51-40 and 140 Ruggeri

Before embarking on the generation of F_2 populations to test the hypothesis that the entire F_1 population is heterozygous for the *CLEX* locus, we first examined existing data on the Cl^- exclusion of the respective parents of K51-40 and 140 Ruggeri. These data were originally generated from the screening of 55 genetically diverse genotypes as replicated cuttings in the Plant Accelerator under a 100 mM mixed Cl^- treatment (described in CSP1302 section 6.2). Encouragingly, 140 Ruggeri and both of its parent species, *V. rupestris* 'du Lot' and *V. berlandieri* 'MG54-47' (note 'MG54-47' was used as a surrogate *V. berlandieri* as the exact parent accession 'Boutin B' is not in the CSIRO collection) displayed relatively low concentrations of Cl^- in the laminae compared to that of K51-40 and its two parents *V. champinii* 'Dogridge' and *V. riparia* 'Gloire de Montpellier' (Fig. 9.2). Although not conclusive, these results support the theory that 140 Ruggeri could be homozygous for two 'good' alleles of the *CLEX* locus and K51-40 homozygous for two 'poor' alleles.

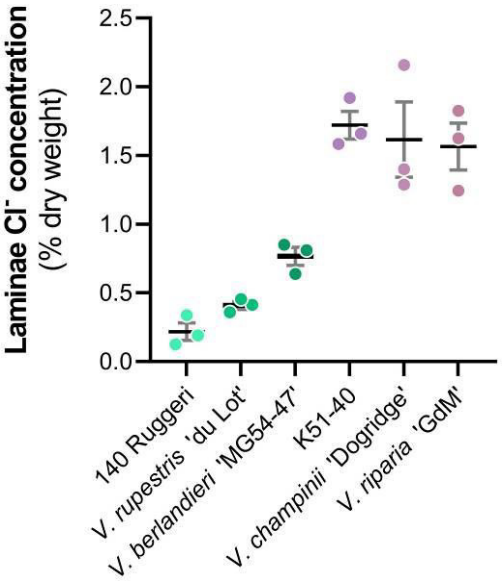


Figure 9.2 - Laminae Cl^- accumulation in 140 Ruggeri and K51-40 and their respective parents *V. rupestris* 'du Lot', *V. berlandieri* 'MG54-47', and *V. champinii* 'Dogridge', *V. riparia* 'Gloire de Montpellier', respectively exposed to a 100mM mixed Cl^- treatment. Note that the actual *V. berlandieri* parent of 140 Ruggeri is the accession 'Boutin B' (Riaz et al 2019), which was not available in the CSIRO collection. In its place we used the related *V. berlandieri* accession MG54-47 ('Resseguier 1' x 'Mazade'). Each data point represents an individual replicate vine with the mean and standard error of each genotype given.

9.4.2 K51-40 x 140 Ruggeri F_1 seedling population

Scoring of the 33 newly germinated K51-40 x 140 Ruggeri F_1 seedlings with SNP markers within *CHX20.1* revealed that six contained the K1 R1 alleles, 12 contained the K1 R2 alleles, five contained the K2 R1 alleles and 11 contained the K2 R2 alleles. There were no significant differences in the average laminae Cl^- concentration across these four inheritance groups (Fig. 9.3A). This is consistent with the results previously seen in the 40 existing F_1 genotypes (Fig. 9.3B) that had been screened as replicated hardwood cuttings as part of CSP1302. In both of these data sets the *CLEX* locus explains less than 5% of the variation in Cl^- exclusion of this F_1 population which is in keeping with the theory that they are each heterozygous at this locus.

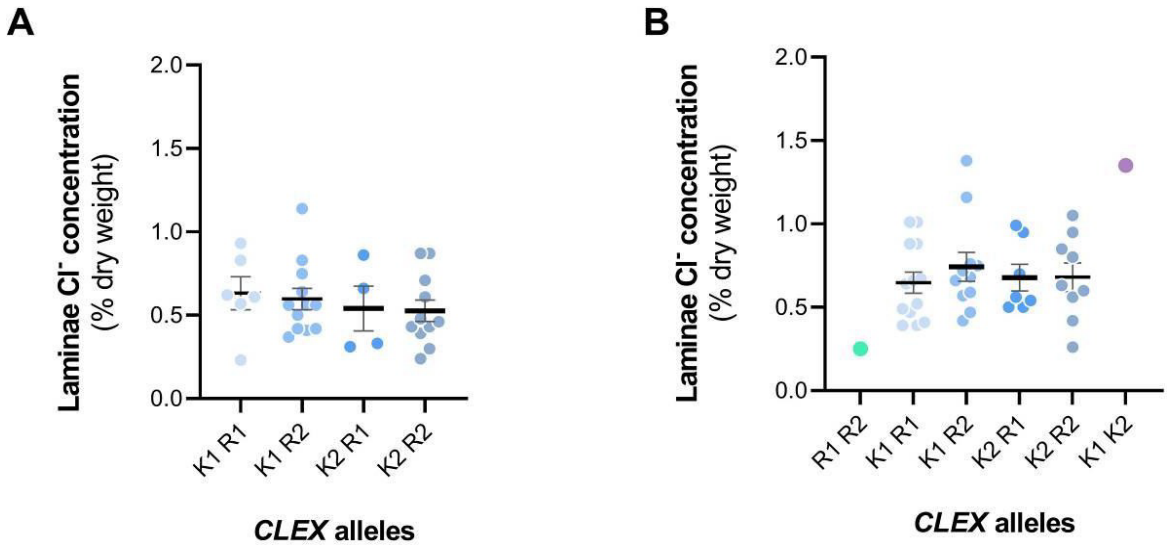


Figure 9.3 - Cl^- exclusion in K51-40 x 140 Ruggeri F_1 progeny grouped by inheritance of the *CLEX* locus. (A) Laminae Cl^- concentration of new F_1 progeny when screened as juvenile seedlings under 25 mM mixed Cl^- for 28 days. Each data point represents a single unreplicated seedling. (B) Laminae Cl^- concentrations of K51-40 (purple), 140 Ruggeri (green) and existing F_1 progeny when screened as hardwood rooted cuttings under 100 mM mixed Cl^- for 13 days (CSP 1302). Each data point represents the mean of three replicate rooted cuttings. Horizontal line and error bars represent the means and standard error of each inheritance group. No significant differences were present between the means of any allelic group.

9.4.3 K51-40 x 140 Ruggeri F_2 seedling populations

Scoring of the MC215-39 x MC215-40 F_2 population for SNPs within *CHX20.1* revealed that eight were homozygous for the R1 allele, seven homozygous for the K2 allele and 23 heterozygous for the R1 K2 alleles. Analysis of the laminae Cl^- concentration of this F_2 population revealed that the R1 R1 genotypes on average accumulated significantly less ($p<0.05$) Cl^- than the heterozygous R1 K2 genotypes, which in turn accumulated significantly less ($p<0.0001$) than the K2 K2 genotypes (Fig. 9.4A). These results clearly demonstrate that the R1 allele derived from 140 Ruggeri is dominant for exclusion over the K2 allele of K51-40.

For the MC215-76 x MI07-49 F_2 population, eight genotypes were found to be homozygous for the R2 allele, five genotypes homozygous for the K1 allele and six genotypes heterozygous R2 K1. Analysis of their laminae Cl^- concentration revealed a similar trend to that of the MC215-39 x MC215-40 F_2 population. The average Cl^- concentration of genotypes homozygous for the R2 allele was significantly less than heterozygous R2 K1 genotypes, which was significantly less than homozygous K1 genotypes (Fig. 9.4B). Again, these results confirm that the R2 allele of 140 Ruggeri is dominant for exclusion over the K1 allele derived from K51-40.

Across both F_2 populations the *CLEX* locus accounts for 72% of the variation in Cl^- accumulation. This is conclusive evidence that the *CLEX* locus is indeed the major locus for Cl^-

exclusion in K51-40 x 140 Ruggeri derived material, with the parents being homozygous recessive and homozygous dominant, respectively.

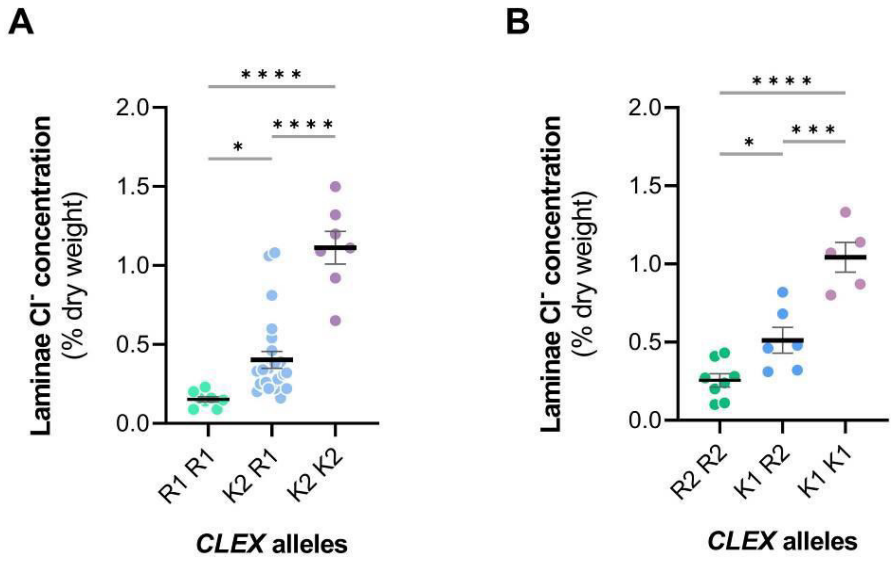


Figure 9.4 – Laminae Cl^- concentration of K51-40 x 140 Ruggeri F_2 progeny grouped by inheritance of the *CLEX* locus. (A) F_2 progeny derived from crossing the F_1 individuals, MC 215-39 x MC 215-40 (both K2 R1) and (B) F_2 progeny derived from crossing the F_1 individuals, MC 215-76 x MI 07-49 (both K1 R2). In both cases, F_2 progeny were screened as juvenile seedlings exposed to a 25 mM mixed Cl^- treatment for 28 days. Data points represent a single unreplicated seedling. Horizontal line and error bars represent the means and standard error of each inheritance group. Tukey's multiple comparison tests show where the difference between allelic group means is significant as follows: ns = not significant, * = $p<0.05$, *** = $p<0.001$, **** = $p<0.0001$.

When comparing the R1 R1 homozygous lines with that of the R2 R2 homozygous lines, the R1 lines on average accumulated significantly less Cl^- in the laminae than that of the R2 lines (Fig. 9.5A). This suggests that the R1 allele may confer stronger Cl^- exclusion than the R2 allele. There was no significant difference between the two heterozygous groups, although the average of K2 R1 lines was noticeably lower than that of K1 R2 lines (Fig. 9.5B), which is consistent with R1 being the stronger allele. There were no significant differences in laminae Cl^- accumulation between K1 K1 and K2 K2 lines (Fig. 9.5C).

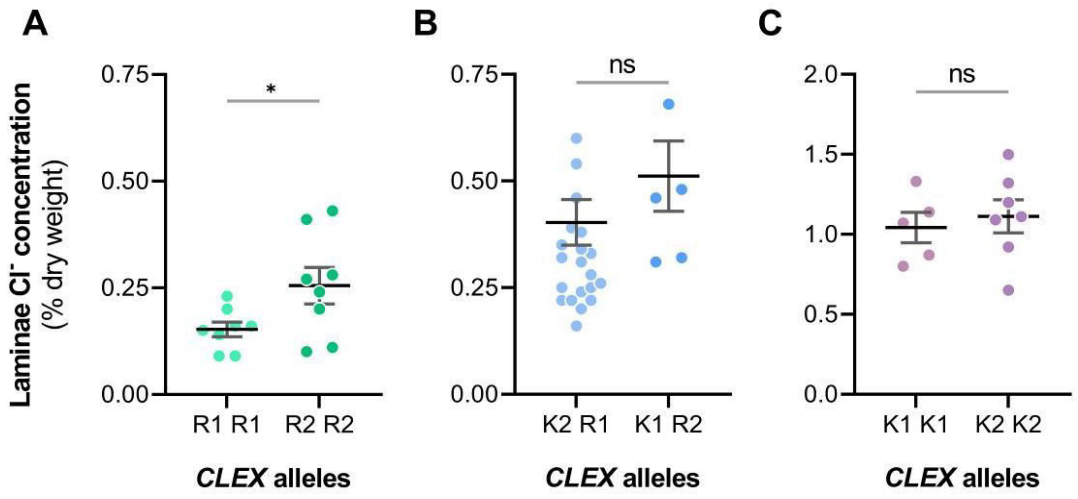


Figure 9.5 – Comparison of laminae Cl^- accumulation in K51-40 x 140 Ruggeri F_2 genotypes (A) homozygous for either the 140 Ruggeri derived alleles R1 or R2, (B) heterozygous, or (C) homozygous for either of the K51-40 derived alleles K1 or K2. F_2 progeny were screened as juvenile seedlings exposed to a 25 mM mixed Cl^- treatment for 28 days. Data points represent a single unreplicated seedling and are the same as presented in Figure 9.4, yet in a different order. Horizontal line and error bars represent the mean and standard error of each inheritance group. Tukey's multiple comparison tests show where the difference between allelic group means is significant as follows: ns = not significant, * = $p<0.05$.

9.5 Discussion

The findings of this chapter have significant implications for understanding the genetic control of Cl^- exclusion in grapevine rootstocks. Across four previous studies, the K51-40 x 140 Ruggeri F_1 population was seen to display a continual variation in Cl^- exclusion, with the *CLEX* locus explaining only 2% of this variation. Previously, the leading explanation for these observations was that the trait is multigenic, being under the control of multiple genetic loci. However, the results observed here drastically change this interpretation. The fact that the *CLEX* locus accounts for 72% of Cl^- exclusion in the K51-40 x 140 Ruggeri F_2 population makes it the single major locus for Cl^- exclusion in this family. While it is likely that other loci do influence the Cl^- exclusion to a small degree, evident by the variation seen in the F_1 population, the influence of these other loci is minimal compared to the *CLEX* locus. The heritability analysis performed across the F_1 population when screened under four different conditions indicates that environmental variables account for 79% of the variation in the F_1 population, while genetic factors only account for 21% of the variability (Dunlevy et al., 2022). As the other unknown loci contribute such a small percentage to the overall variability in Cl^- exclusion it is likely not worth attempting to identify these individual genetic loci.

Segregation in the F_2 populations show that each of the R1 and R2 alleles are dominant over the K1 and K2 alleles. Similar to that seen in the *V. berlandieri* x *V. vinifera* BC_1F_2 population, the exclusion trait is semi-dominant – that is two copies of the dominant allele confers increased exclusion over just one copy. Furthermore, the R1 allele appears to provide better exclusion over the R2 allele, although this is not a conclusive result. As these alleles segregate

in two separate F_2 populations it is possible that differences in the Cl^- accumulation is attributed to other unknown genetic loci that differ between the two populations. Yet given there is no noticeable difference in the means of the K1 K1 and K2 K2 lines it could be argued that differences in other unknown loci between the two F_2 populations are likely to be negligible, especially considering *CLEX* is the major determining factor.

Due to time constraints and other priorities, we were not able to determine the origin of the various alleles to the four grandparent species *V. berlandieri*, *V. rupestris*, *V. champinii* and *V. riparia*. This is something that should be followed up on, at least for the R1 allele, which appears to be of most use to rootstock breeding for improved Cl^- exclusion and salt tolerance.

Knowledge of the dominant and recessive nature of the respective 140 Ruggeri and K51-40 derived alleles of *CHX20.1* will aid in the identification of the one or more casual SNPs that underpin differences in the function of the proteins encoded by each allele. This is what was achieved for the major gene for Na^+ exclusion, *HKT1;1*, identified within the F_1 population. Two amino acid differences at the C-terminal end of the protein underpin different rates of Na^+ transport and the underlying SNPs serve as the basis of a perfect genetic marker (Henderson et al., 2018). For Cl^- exclusion, SNPs that differ between the R and K CHX20.1 alleles, but homozygous within each parent, are most likely to underlie the different phenotype. Before the causal SNPs can be interrogated by site-directed mutagenesis and functional testing, a reliable assay for quantifying the transport function of encoded proteins is still required.

9.6 Summary

The *CLEX* locus accounts for 72% of the variation in Cl^- exclusion in K51-40 x 140 Ruggeri F_2 populations. 140 Ruggeri is homozygous for two dominant alleles R1 and R2, with R1 significantly stronger than R2, while K51-40 is homozygous for two recessive alleles K1 and K2. SNPs between these alleles could be used as the basis of a potential perfect genetic marker for predicting Cl^- exclusion in rootstock breeding.

10. Investigating Cl^- exclusion in other genetic backgrounds

10.1 Objectives

The main objective of the work presented in this chapter was to examine Cl^- segregation patterns in populations derived from other genetic backgrounds for understanding the influence of novel *CLEX* alleles in these populations and/or to map new loci for Cl^- exclusion. Three recently imported North American species were also assessed for their Cl^- exclusion abilities and potential for incorporating them into the rootstock breeding program.

10.2 Background

Chloride exclusion is a key rootstock-conferred trait contributing to salt tolerance in the vineyard. When confronted with saline soils, the ability to limit the accumulation of Cl^- in the leaves helps maintain photosynthetic capacity and thus vine health, while reduced accumulation in the berries improves grape and wine quality. As such, numerous previous studies have investigated the genetic control of Cl^- exclusion in grapevine rootstocks (Newman & Antcliff 1983, Sykes 1987, Gong et al. 2011, Fort et al. 2015, Dunlevy et al. 2022).

In the previous chapter we showed that the *CLEX* locus is the major genetic determinant for Cl^- exclusion in the F_2 populations of the genetically diverse cross between K51-40 (*V. champinii* 'Dogridge' x *V. riparia* 'Gloire de Montpellier') x 140 Ruggeri. Before these results came to light, the continual variation observed previously within the K51-40 x 140 Ruggeri F_1 population had been interpreted to suggest that other genetic loci were likely to control Cl^- exclusion (Gong et al., 2011, Dunlevy et al., 2022). We now know that these other unknown loci are heavily influenced by environmental factors and therefore likely to only have a minor influence on the Cl^- exclusion phenotype. Yet at the time that the work presented in this chapter was conducted, we were working under the theory that other major loci for Cl^- exclusion were yet to be identified. This indeed may be the case in *Vitis* species or accessions other than those present in the K51-40 x 140 Ruggeri background.

10.2.1 *V. doaniana*, *V. girdiana* and *V. arizonica*

A recent survey of 325 wild accessions belonging to 14 different North-American *Vitis* species has identified a number of accessions that out-perform 140 Ruggeri in terms of their ability to exclude Cl^- from the laminae (Heinitz et al., 2020; Prof. Andy Walker, pers. comm.). In particular, some accessions of *V. doaniana*, *V. girdiana* and *V. arizonica* displayed the highest degree of Cl^- exclusion. Here, we sought to confirm these findings in accessions of these three species imported into the country. An F_1 population derived from accessions of *V. girdiana* x *V. arizonica* was also investigated for segregation of Cl^- exclusion with the aim of mapping novel loci for this trait.

10.2.2 *V. berlandieri* x *V. berlandieri* populations

V. berlandieri is widely considered the major source of salt tolerance in rootstocks such as 140 Ruggeri and 110 Richter. Furthermore, the *CLEX* allele dominant for exclusion of Cl^- in the *V. berlandieri* x *V. vinifera* BC_1F_2 population is derived from *V. berlandieri*, as is one of the dominant alleles present in 140 Ruggeri.

The CSIRO germplasm collection contains four inter-related populations of *V. berlandieri* x V. *berlandieri* F_1 genotypes that were germinated in 1968 from seeds imported from the US. The four populations are derived from factorial crosses between the two female accessions 'Resseguier #1' and 'Resseguier #2' and two male accessions 'Mazade' and '7651'. In November 1987, the petiole Cl^- concentration was assessed from individuals of these four *V. berlandieri* x *V. berlandieri* populations growing in an irrigated vineyard at the former CSIRO site at Merbein, Victoria. These legacy data indicate that the inheritance of Cl^- exclusion varies dramatically between these four populations, with one population displaying what appears to be 3:1 segregation (Fig. 10.1A), one a possible 1:1 segregation (Fig. 10.1B), one with minimal variation (Fig. 10.1C) and one with an extremely skewed pattern of variation (Fig. 10.1D). Whilst segregation patterns apparent within these data are extremely promising for determining the underlying genetic control, the methods in which these data were generated as well as the condition and position of the plants within the vineyard are all unknown to us. Therefore, we cannot rely on the accuracy of those data for further analysis and can only consider them as a guide to the potentials of these populations. Many of these *V. berlandieri* x *V. berlandieri* F1s were replanted in the Irymple collection. Here we sought to re-screen these populations as pot grown cuttings in the controlled conditions of the Plant Accelerator, with the aim of mapping the major locus responsible for the apparent segregation in Cl^- exclusion that was previously seen when grown at Merbein.

10.2.3 *V. longii* x Chardonnay seedlings

Findings presented in Chapter 6 suggest that *V. longii* (syn. *V. acerifolia*) displays superior exclusion of Cl^- compared to 140 Ruggeri, the benchmark excluding rootstock. A recent study from Prof. Andrew Walker's lab at UC Davis found that accessions of *V. longii* are superior excluders to *V. berlandieri* (Chen, PhD thesis, 2021). Furthermore, an apparent 1:1 segregation of Cl^- exclusion to accumulation in a population derived from *V. longii* '9018' (excluder) x GRN3 (accumulator) (Chen, PhD thesis, 2021) suggests that *V. longii* may be heterozygous for a dominant exclusion allele. Here we aimed to investigate the genetics of Cl^- exclusion in the single unknown accession of *V. longii* present in the CSIRO collection, by crossing it with the accumulator genotype *V. vinifera* 'Chardonnay'.

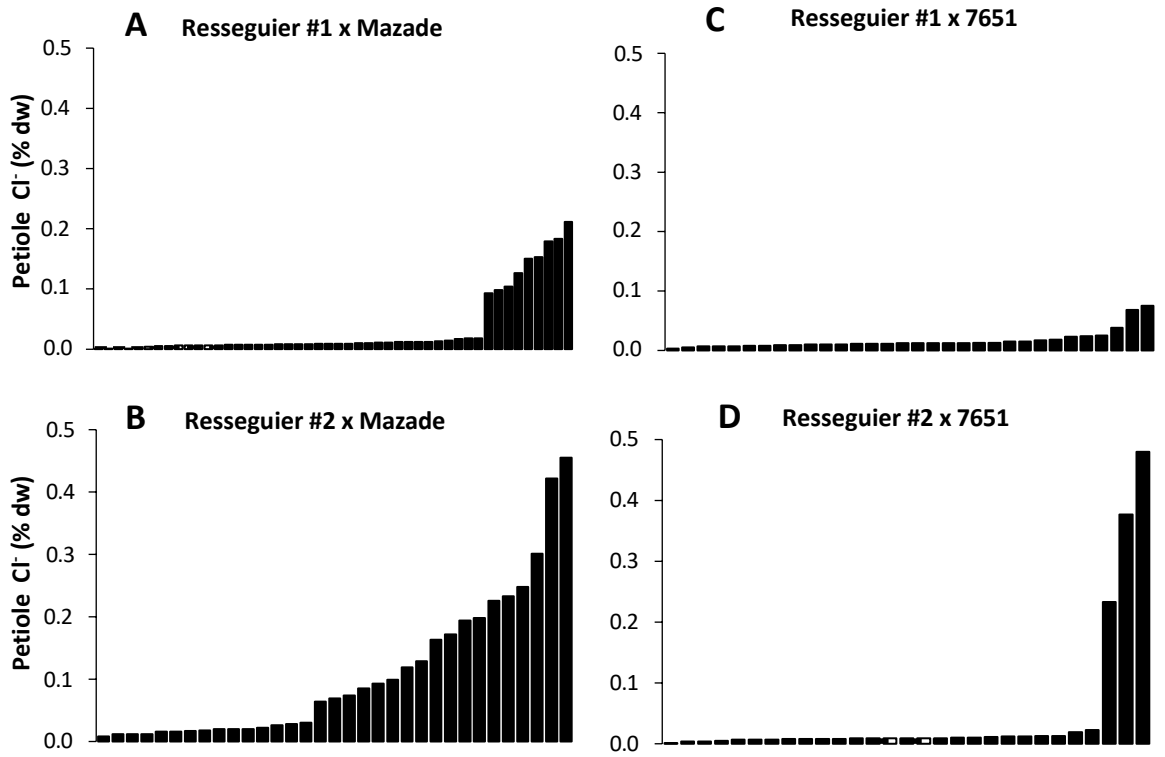


Figure 10.1 – Petiole Cl^- concentration of four inter-related *V. berlandieri* x *V. berlandieri* populations derived from the accessions (A) Resseguier #1 x Mazade, (B) Resseguier #2 x Mazade, (C) Resseguier #1 x 7651 and (D) Resseguier #2 x 7651. Data was obtained from field grown mothervines at the former CSIRO site at Merbein, Victoria, with petioles sampled in November 1987.

10.2.4 Ramsey x 140 Ruggeri seedlings

A previous CSIRO study reported that a *V. champinii* 'Ramsey' x *V. vinifera* 'Sultana' F_1 population displayed a bimodal distribution of petiole Cl^- accumulation, suggestive of control by a major locus (Sykes, 1985) and thus providing an ideal population to investigate genetic control of this trait. Ramsey is considered a moderate to good excluder of Cl^- and is therefore not considered a priority genotype to develop genetic markers for this trait. Ramsey is, however, an excellent source of nematode resistance, encoded by a single dominant locus *MJR2* (*MELOIDOGYNE JAVANICA RESISTANCE2*) and is central to the next step of the rootstock breeding strategy for durable disease resistance (Fig. 10.2). The breeding strategy for durable disease resistance also relies on resistant genotypes derived from *V. cinerea* and *V. riparia*, neither of which are considered to be strong sources of Cl^- exclusion or salt tolerance. Here we crossed *V. champinii* 'Ramsey' with 140 Ruggeri (which we have shown to be homozygous for dominant alleles of the *CLEX* locus) to introgress the superior Cl^- exclusion of 140 Ruggeri with the nematode resistance of Ramsey. We considered this to be the most efficient approach for incorporating increased salt tolerance into the central breeding strategy for durable disease resistance. This also provided an opportunity to investigate the genetic control of Cl^- exclusion within this family.

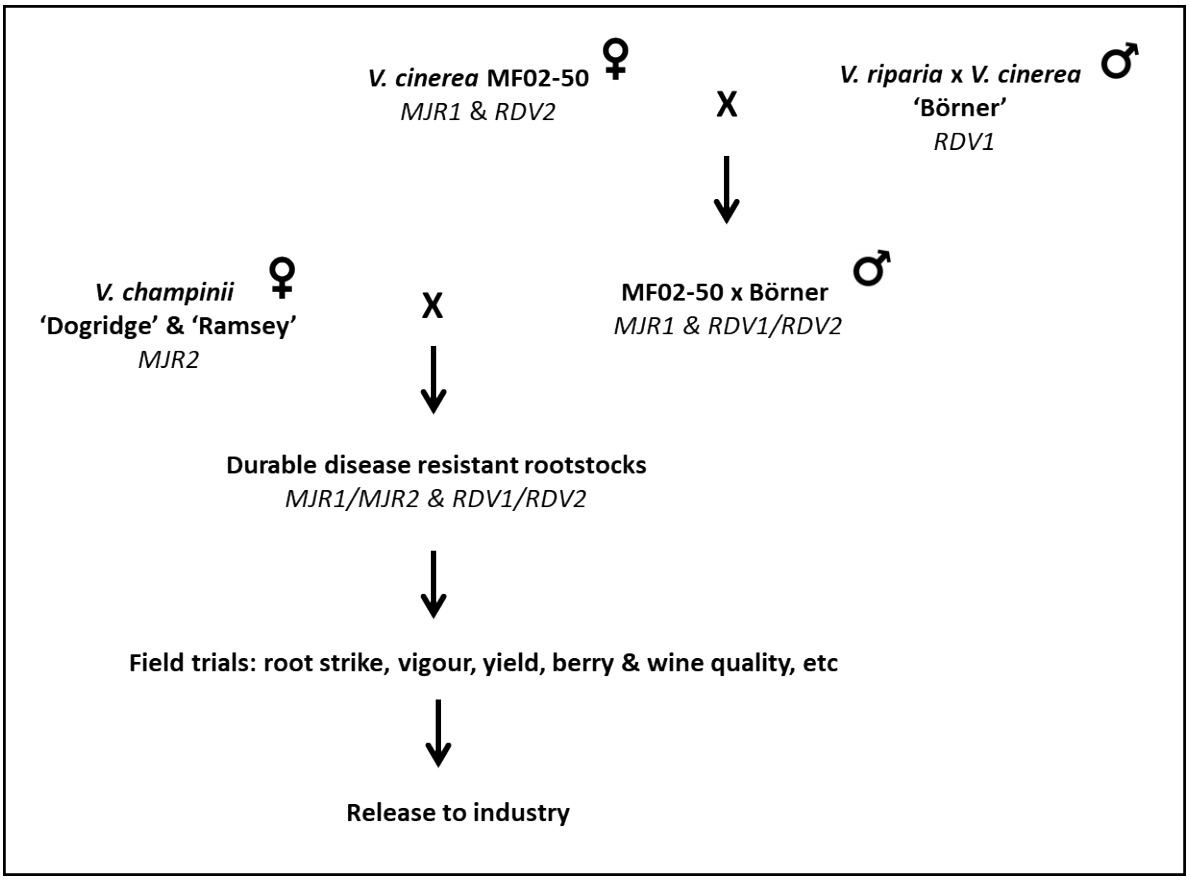


Figure 10.2 Rootstock breeding strategy for durable disease resistance by combining multiple nematode resistance loci, *MJR1* and *MJR2* and multiple phylloxera resistance loci *RDV1* and *RDV2* (described in detail in CSA 1701-1.3 Rootstock genetics and improvement: tolerance to biotic stress). ♀ - denotes females genotypes and ♂ denotes male genotypes.

10.3 Materials and Methods

10.3.1 *V. doaniana, V. girdiana and V. arizonica*

In 2017, hardwood cuttings of *V. arizonica* Engelmann (accession 5311 03-01-011), *V. doaniana* Munson (accession 5311 03-01-011) and *V. girdiana* (accession 5314 03-01-014) were imported from the Institute of Grapevine Breeding Geilweilerhof (Federal Research Centre for Cultivated Plants, Germany) under AQIS import permit number IP0000358129. In November 2018, a benchmarking screen was performed under quarantine glasshouse conditions to compare the Cl^- exclusion of these three species with that of 140 Ruggeri and K51-40. Replicated cuttings were propagated via green cuttings dipped in rooting hormone (Clonex) and rooted in 1:1 sand:perlite in a mist tunnel. After four weeks, rooted cuttings were transferred to 2.4 L pots containing sand:perlite and hardened-off to glasshouse conditions (18^{o}C nights 28^{o}C days) with daily watering with ¼ Strength Special Hoagland's solution. After 18 weeks of establishment, cuttings were transplanted into Biogro potting mix in 4.4 L pots, thinned back to a single shoot, and watered daily with ¼ Strength Special Hoagland's solution containing either 0 mM Cl^- or 50 mM mixed Cl^- for 28 days. On day 10, an unexpected fault occurred with the glasshouse air conditioner unit and the

glasshouse over-heated for a number of days, which may have compromised results, particularly for *V. doaniana,* which showed the most stress symptoms due to the elevated temperature. Therefore, in March 2020 a second benchmarking experiment was performed, as described for the first screen, except that *V. girdiana* was not included and the salt treatment consisted of 100 mM mixed Cl^-.

A cross between *V. girdiana* x *V. arizonica* was kindly performed by Professor Andy Walker's lab at University of California, Davis. The resulting F_1 seeds were imported under the AQIS permit no IP15016228. Seeds were sterilised, germinated and established under quarantine conditions as described in section 5.1.3. At eight weeks of age, 46 young seedlings were exposed to either a 0 mM Cl^- (n=5) or 50 mM (n=41) mixed Cl^- treatment for 35 days. At harvest, plant height and leaf number were recorded as a measure of plant size and the two most mature laminae sampled for Cl^- analysis as described in section 5.2.1.

10.3.2 *V. berlandieri* x *V. berlandieri* populations

Cuttings from *V. berlandieri* x *V. berlandieri* genotypes were taken from mothervines growing in block 5B of the CSIRO Irymple vineyard and propagated and established as described in section 5.1.1. The rate of successful callusing and root-strike of the *V. berlandieri* genotypes was relatively poor compared to other genotypes that were used as references, including 140 Ruggeri, *V. longii*, Shiraz and Cabernet Sauvignon.

Cuttings were screened for their Cl^- accumulation in the SE Smarthouse of the Plant Accelerator under control conditions (20^{o}C nights / 25^{o}C days). Eight weeks after establishment, cuttings were exposed to a 100 mM mixed Cl^- treatment, applied as 33 mM Cl^- on day 1 and 67 mM Cl^- on day 4. On all other days, cuttings were automatically watered to a set weight with rainwater. On day 16 the mature laminae were harvested for determination of Cl^- concentration.

10.3.3 *V. longii* x Chardonnay seedlings

Bagged flowers of *V. longii* (CSIRO Irymple vineyard Block 5B) were pollinated with fresh flowers of *V. vinifera cv.* Chardonnay (Irymple vineyard Block 2A). Chardonnay was chosen as the parent because it was the only *V. vinifera* cultivar flowering as early in the season as *V. longii*. Once matured, seeds were collected in Irymple, transported to Adelaide under quarantine import permit, and then sterilised, stored and germinated as described in section 5.1.3.

The resulting seedlings (n=109) were screened for Cl^- exclusion in the Plant Accelerator under control conditions. At approximately four weeks of age seedlings were exposed to a single application of mixed Cl^- solution, equivalent to a pot concentration of 25 mM Cl^-, and watered daily with rainwater to a set weight. On day 19 the lower five laminae were harvested for determining Cl^- concentration as described in section 5.2.1.

10.3.4 Ramsey x 140 Ruggeri seedlings

Bagged flowers of *V. champinii* 'Ramsey' (SARDI vineyard, Nuriootpa, Block 23) were pollinated with dried pollen collected from 140 Ruggeri (also SARDI Block 23) in the season prior. Seeds were sterilised and germinated as described in section 5.1.3, and the resulting seedlings (n=63) screened for Cl^- exclusion in the Plant Accelerator as described in 9.3.3 above.

10.4 Results

10.4.1 *V. doaniana, V. girdiana and V. arizonica*

Single accessions of *V. doaniana*, *V. girdiana* and *V. arizonica* were imported into the country on reports that these three species display excellent Cl^- exclusion. We sought to reference these accessions against the benchmark excluder 140 Ruggeri, as well as K51-40 as an accumulator. Under the 0 mM Cl^- control treatment 140 Ruggeri showed the lowest laminae Cl^- concentration, although this was not significantly different from any of the other four genotypes (Fig. 10.3A). The difference between 140 Ruggeri and *V. doaniana* ($p=0.054$) and that between 140 Ruggeri and K51-40 ($p=0.056$) was only marginally above the statistically significant threshold of $p=0.05$. Under the 50 mM mixed Cl^- treatment, 140 Ruggeri was again not significantly different from any of the three species under investigation, but was significantly different from the accumulator genotype, K51-40 (Fig. 10.3B). Unfortunately, a failure of the glasshouse air-conditioner occurred 10 days into this experiment which may have impacted results, particularly for *V. doaniana* and *V. arizonica* which displayed the most severe symptoms of heat stress. As such, a repeat experiment was performed to confirm these results.

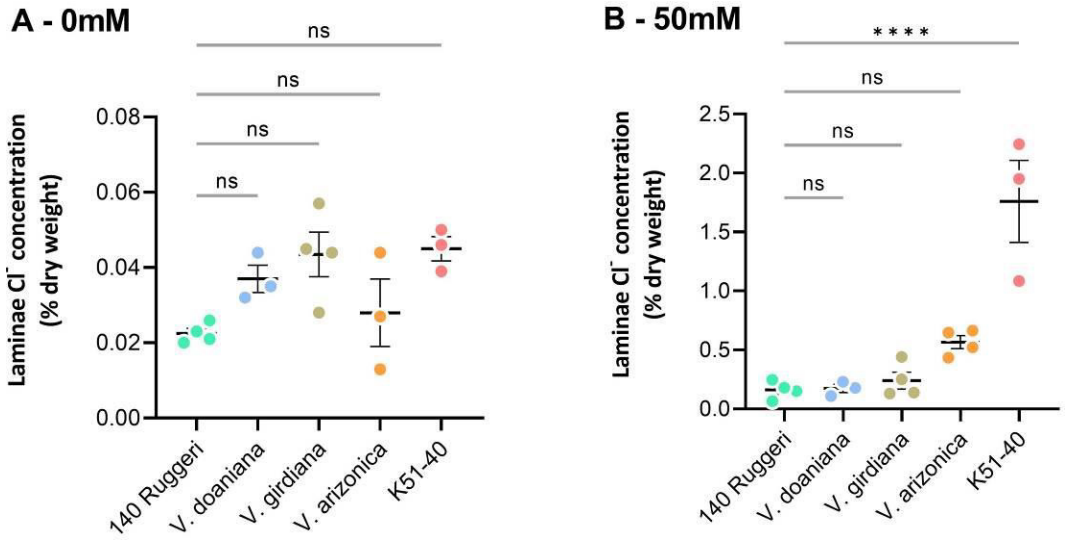


Figure 10.3 – Comparison of laminae Cl^- accumulation of three imported *Vitis* species, *V. doaniana*, *V. girdiana* and *V. arizonica*, against reference rootstocks 140 Ruggeri and K51-40 under (A) 0 mM Cl^- control and (B) 50 mM mixed Cl^- treatments. Data points indicate the value of a single replicate own-rooted cutting with horizontal lines representing the mean of each genotype, and error bars indicating the standard error of the mean. Tukey's multiple comparison tests show where differences between the benchmark genotype 140 Ruggeri and other genotypes are significant as follows: ns = not significant, **** = $p<0.0001$.

In the repeat experiment, neither *V. doaniana* nor *V. arizonica* accumulated significantly different concentrations of Cl^- compared to 140 Ruggeri under the control treatment (Fig. 10.4A), consistent with the first experiment. Unlike at 0 mM Cl^- in the first experiment, at 0 mM Cl^- in the second experiment 140 Ruggeri accumulated significantly less Cl^- than K51-40. This is what would be expected, suggesting that the data from this second experiment are more reliable than the first. In the second experiment the salt treatment was applied at 100 mM mixed Cl^-. Under this treatment the laminae Cl^- accumulation of *V. doaniana* (0.74% d.w.) was less than half that of 140 Ruggeri (1.85% d.w.), a difference that was highly significantly different ($p<0.001$). The Cl^- concentration of *V. arizonica* (2.26% d.w.) was marginally higher than 140 Ruggeri, but not statistically different.

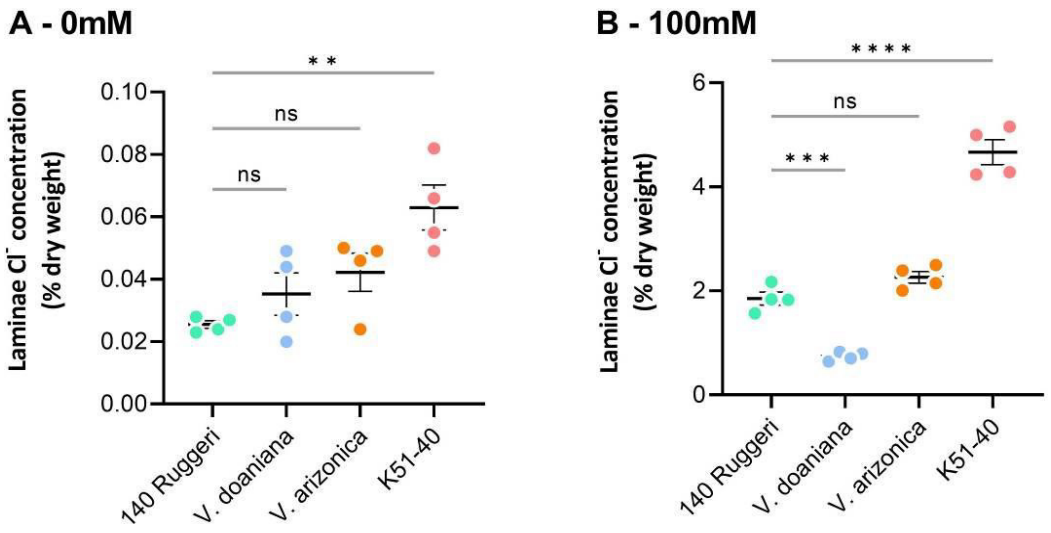


Figure 10.4 – Repeat comparison of laminae Cl^- accumulation of two imported *Vitis* species, *V. doaniana* and *V. arizonica*, against reference rootstocks 140 Ruggeri and K51-40 under (A) 0 mM Cl^- control and (B) 100 mM mixed Cl^- treatments. Data points indicate the value of a single replicate own-rooted cutting with horizontal lines representing the mean of each genotype, and error bars indicating the standard error of the mean. Tukey's multiple comparison tests show where differences between the benchmark genotype 140 Ruggeri and other genotypes are significant as follows: ns = not significant, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

A cross between an accession of *V. girdiana* and an accession of *V. arizonica* was made at UC Davis, California by Professor Andy Walkers group, with seeds collected and kindly provided to our group. We germinated the seeds and assessed the pattern of Cl^- exclusion in 41 F_1 individuals of this population as young seedlings for the purpose of investigating the genetic control of Cl^- exclusion in these exotic genotypes. A large variation in laminae Cl^- concentration was seen in this population, with individual results ranging from 0.60% d.w. to 6.26% d.w, when exposed to a 50 mM mixed Cl^- treatment for 35 days (Fig. 10.5). There was no apparent segregation of the Cl^- accumulation within this population, with the variation being mostly continuous suggesting control by multiple minor loci, rather than by one major locus as indicated by the skewed trend in the K51-40 x 140 Ruggeri F_2 population shown as an example in Fig. 10.6. As such, genetic analysis was not pursued in this population and the population was abandoned. This decision was also influenced by the drawbacks of working with quarantined material and the limited quarantine glasshouse space available to do so.

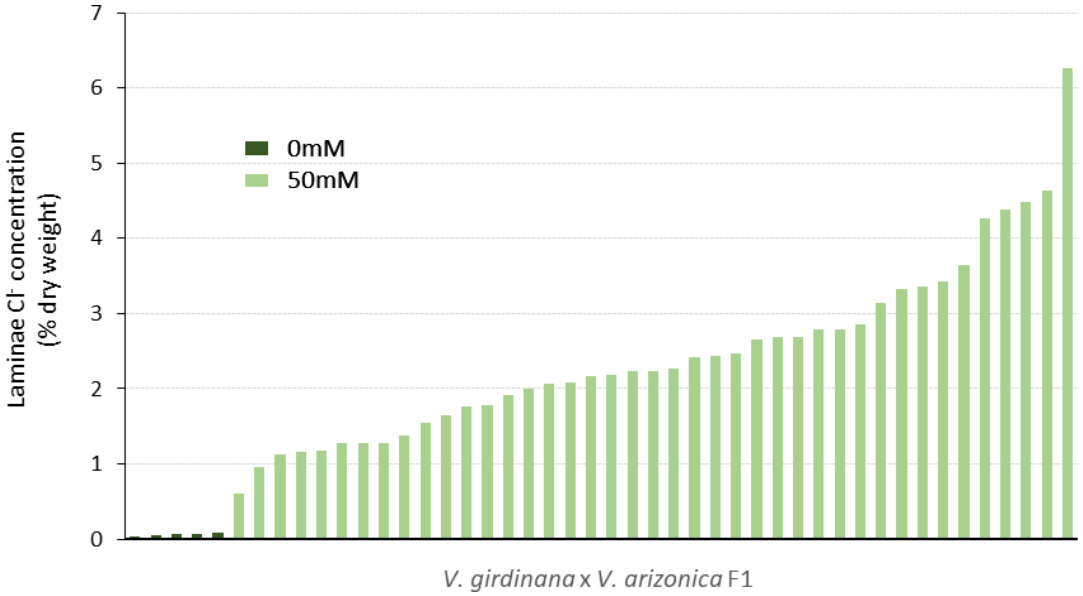


Figure 10.5 – Laminae Cl^- accumulation in a *V. girdiana* x *V. arizonica* F_1 population exposed to either a 0 mM Cl^- (dark green) or 50 mM (light green) mixed Cl^- treatment for 35 days. Each data point represents a single replicate.

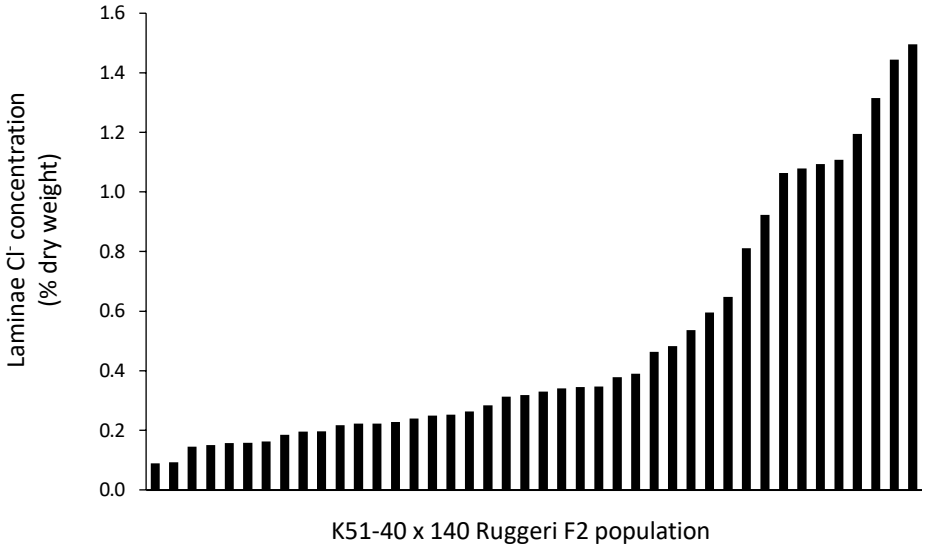


Figure 10.6 – Laminae Cl^- concentration in the K51-40 x 140 Ruggeri F_2 population (see Chapter 9) as an example of a population with a skewed phenotype, typical of control by a major locus.

10.4.2 *V. berlandieri* x *V. berlandieri* populations

V. berlandieri is considered to be an excellent source of Cl^- exclusion but is notoriously poor in terms of its rooting ability (Smith et al., 2013), as is evident for 140 Ruggeri. Unfortunately, we discovered first-hand how difficult it is to propagate *V. berlandieri* material while undertaking this experiment. Originally this experiment was intended to have two salt treatments and two temperature treatments, with three biological reps per treatment combination, for a total of 12 replicates per genotypes. As such, 25 cuttings of 90 different *V. berlandieri* genotypes were collected and underwent the callusing and rooting procedure described in Section 5.1.1. Upon transplanting cuttings to soil after the rooting period it was evident that the strike rate of many genotypes was far below what was anticipated. This eventuated in only 65 of the 90 genotypes with enough successfully rooted cuttings to

achieve four healthy replicates and a further six genotypes with only three healthy replicates. We chose to screen these 71 genotypes under a 100 mM mixed Cl^- treatment. *V. longii*, 140 Ruggeri, Shiraz, and Sauvignon Blanc were included as reference genotypes.

For the *V. berlandieri* Resseguier #1 x *V. berlandieri* Mazade F_1s, a continuous variation in laminae Cl^- concentration was seen between the 31 genotypes of this population, ranging from 0.40 to 2.13% d.w. (Fig. 10.7A). A continuous variation in laminae Cl^- concentration was also seen within the 24 genotypes of the *V. berlandieri* Resseguier #2 x *V. berlandieri* Mazade population, which ranged from 0.38 to 2.10% d.w. (Fig. 10.7B). For the *V. berlandieri* Resseguier #1 x *V. berlandieri* 7651 and *V. berlandieri* Resseguier #2 x *V. berlandieri* 7651 populations there were only 11 and four genotypes respectively, hindering any meaningful analysis of trends in these populations (Fig. 10.7C&D). There were no significant differences in the mean Cl^- concentration across the four populations (data not shown). The laminae Cl^- concentration of all *V. berlandieri* x *V. berlandieri* F_1 genotypes was higher than that of the excluder reference genotypes *V. longii* and 140 Ruggeri (Fig. 10.7E), which suggests these genotypes are not as superior at Cl^- exclusion as originally expected.

There was also a very low degree of consistency between the laminae Cl^- concentrations obtained for these vines in the Plant Accelerator experiments involving treatment with 100 mM mixed Cl^-, and those of the petiole Cl^- concentrations of mothervines at Merbein in 1987, irrigated with River Murray water (approximately 3 mM Cl^-), shown in black in Figure 10.7. It is unclear if this discrepancy between data sets is the result of differences in variables such as salt treatment, temperature or propagation type, or possibly the result of the poor and variable root strike of the genotypes included in this experiment. It may also be the result of the Merbein data being unreplicated and therefore the variability seen across that data set being due to the influence of vineyard position. Irrespective of the reason, we chose not to pursue this population for genetic analysis as the degree of Cl^- exclusion was not as strong as anticipated and the continuous variation did not imply control by a major locus.

Two CSIRO rootstocks, MG 54-89 and MG55-12, were included in this study as they are both of pure *V. berlandieri* origin. Neither of these rootstocks excluded Cl^- as well as the reference excluder 140 Ruggeri (Fig. 10.7).

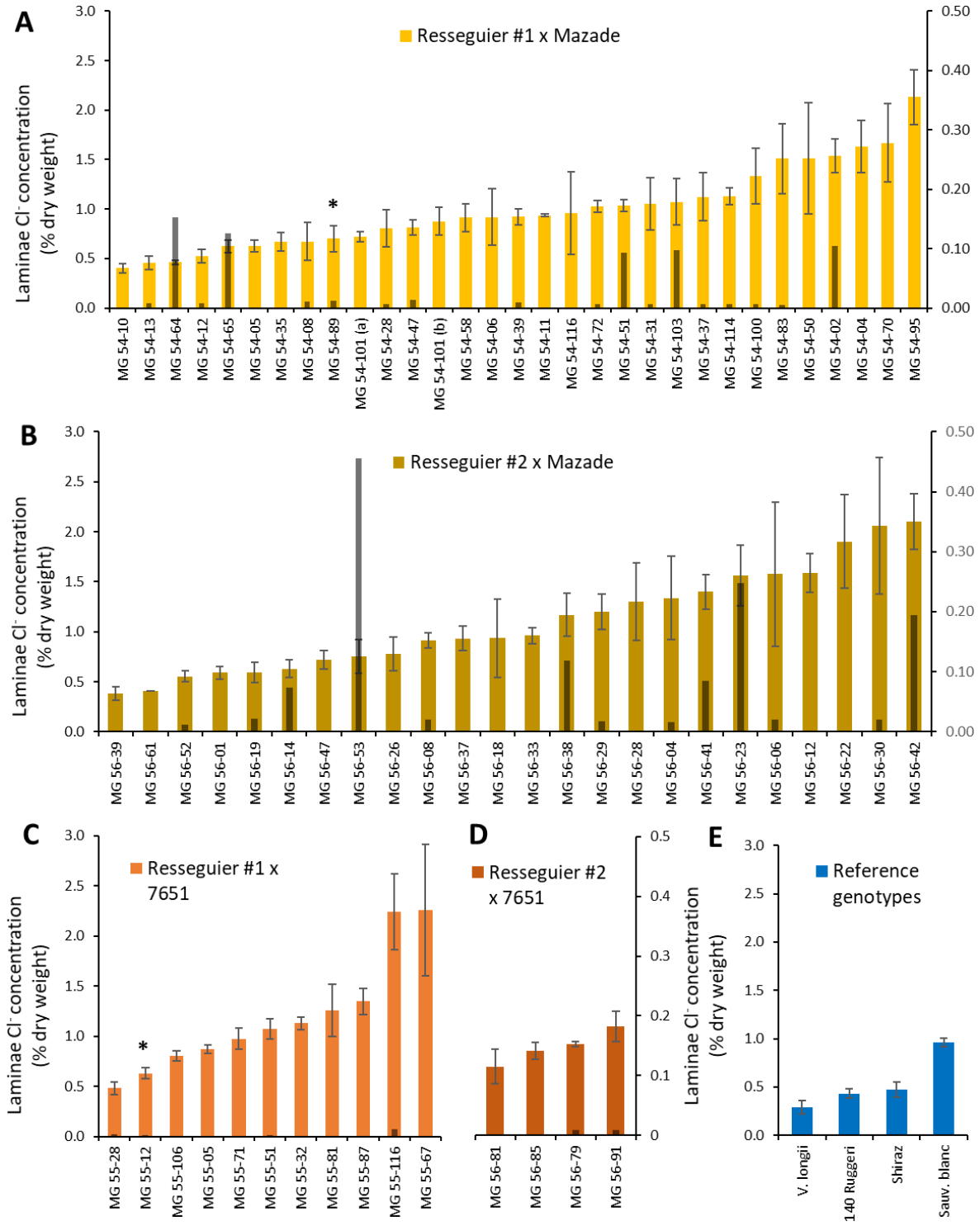


Figure 10.7 - Cl^- accumulation in *V. berlandieri* x *V. berlandieri* F_1 individuals belonging to four populations (A, B, C and D), as well as six reference genotypes (E) exposed to a 100mM Mixed Cl^- treatment. Coloured bars show laminae Cl^- concentration of genotypes screened in the Plant Accelerator as replicated rooted cuttings and are on the left axis. Grey bars indicate the petiole Cl^- concentrations of mothervines planted at Merbein and collected in 1987, where available, and are on the right axis. * indicates two of the CSIRO rootstocks that have been released, MG54-89 and MG55-12. Each data point represents the mean of three biological replicates, with error bars indicating standard error of the mean.

10.4.3 *V. longii* x Chardonnay seedlings

Our previous observations indicate that *V. longii* is superior to 140 Ruggeri in terms of Cl^- exclusion. A test-cross between *V. longii* x Chardonnay was performed with the aim of identifying potential loci responsible for the excellent Cl^- exclusion of *V. longii*. When screened under a 25 mM mixed Cl^- treatment the resulting 109 F_1 seedlings revealed a large range of laminae Cl^- concentrations, ranging from 0.09% to 2.13% d.w and with an average of 0.42% d.w (Fig. 10.8). This range was largely continuous suggesting either control by multiple genes or heterozygosity of the major CLEX locus. There was some degree of skewness towards the higher end of accumulators, but this only represented seven out of a total of 109 individuals, which does not fit a typical ratio that would otherwise indicate influence of a major locus. For this reason, genetic analysis was not pursued in this population.

10.4.4 Ramsey x 140 Ruggeri seedlings

A cross between Ramsey and 140 Ruggeri was performed for the purpose of introgressing the excellent Cl^- exclusion of 140 Ruggeri with the nematode resistance of Ramsey. We exposed the resulting 61 F_1 seedlings to a 25 mM mixed Cl^- treatment in the Plant Accelerator for 19 days. As with other populations investigated in this chapter, the Ramsey x 140 Ruggeri F_1s displayed continuous variation in laminae Cl^- concentration (Fig. 10.9). This is not unexpected as we know that 140 Ruggeri is homozygous at the *CLEX* locus, all F_1 individuals would contain one of these alleles. The laminae Cl^- concentration of this population ranged from 0.18 to 2.31% d.w, with an average of 0.62% d.w.

As seen in most populations, the vigour or fitness of seedling vines of this population was highly variable. We selected eighteen individuals that displayed strong growth as well as strong Cl^- exclusion to be used as potential parents in the next step of the breeding strategy (Fig. 10.10), which was unable to be pursued during the life of the project. These individuals will first need to be scored for the presence of the *MJR2* locus derived from Ramsey to ensure that they possess this key trait for nematode resistance.

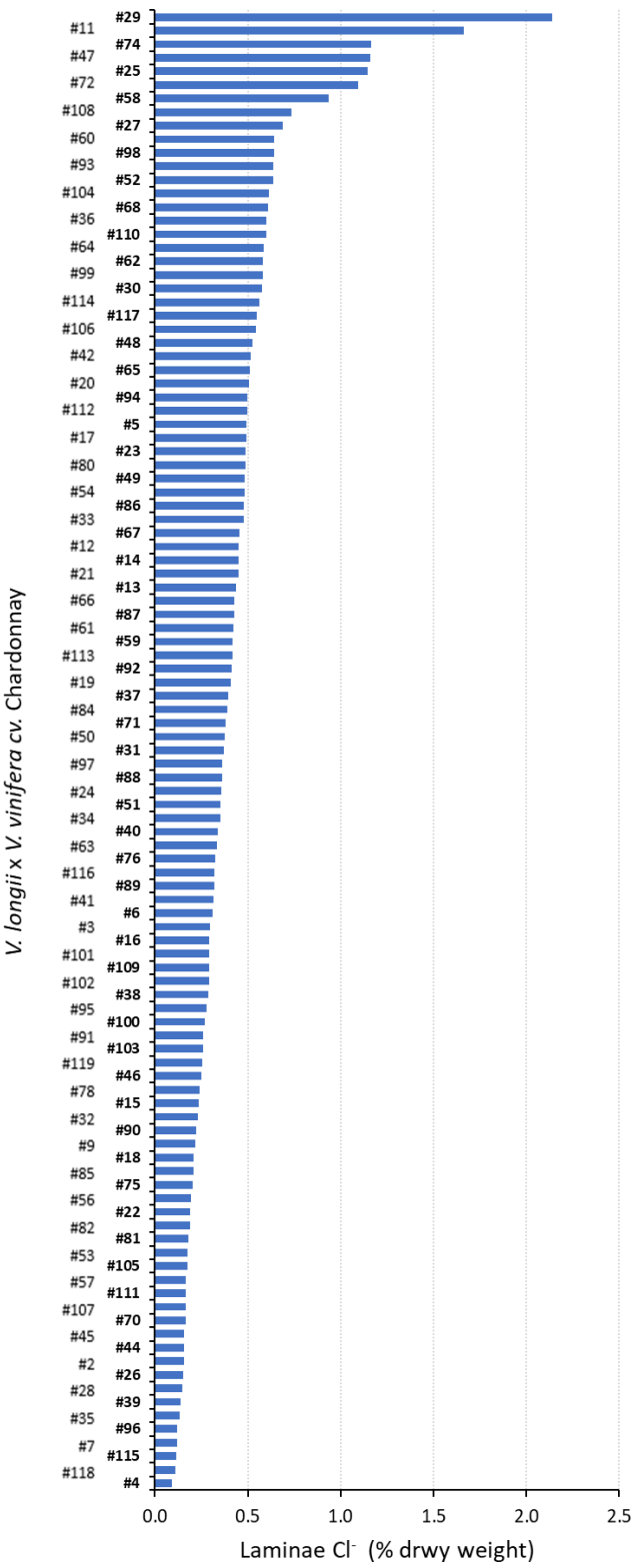


Figure 10.8 - Continual variation in laminae Cl^- concentration of a population of F_1 seedlings from the cross *V. longii* x *V. vinifera* cv. Chardonnay exposed to 25 mM mixed Cl^- for 19 days. Each bar represents a single replicate seedling.

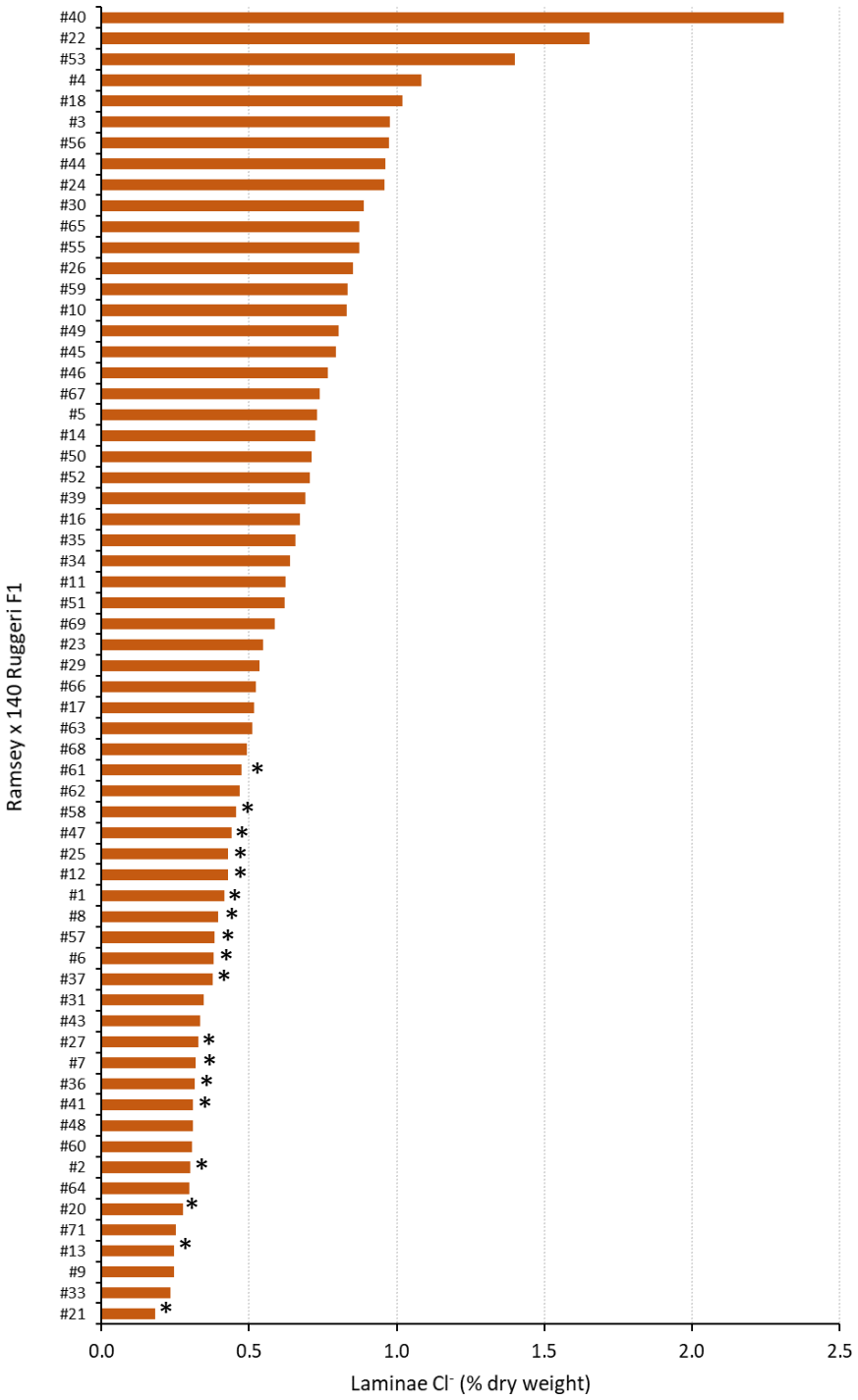


Figure 10.9 - Laminae Cl^- concentration of a population of F_1 seedlings from the cross Ramsey x 140 Ruggeri exposed to 25 mM mixed Cl^- for 19 days. Each bar represents a single replicate seedling. * indicates genotypes with both strong Cl^- exclusion and vigorous growth. These individuals were retained as potential breeding parents for incorporating strong Cl^- exclusion into the breeding program.

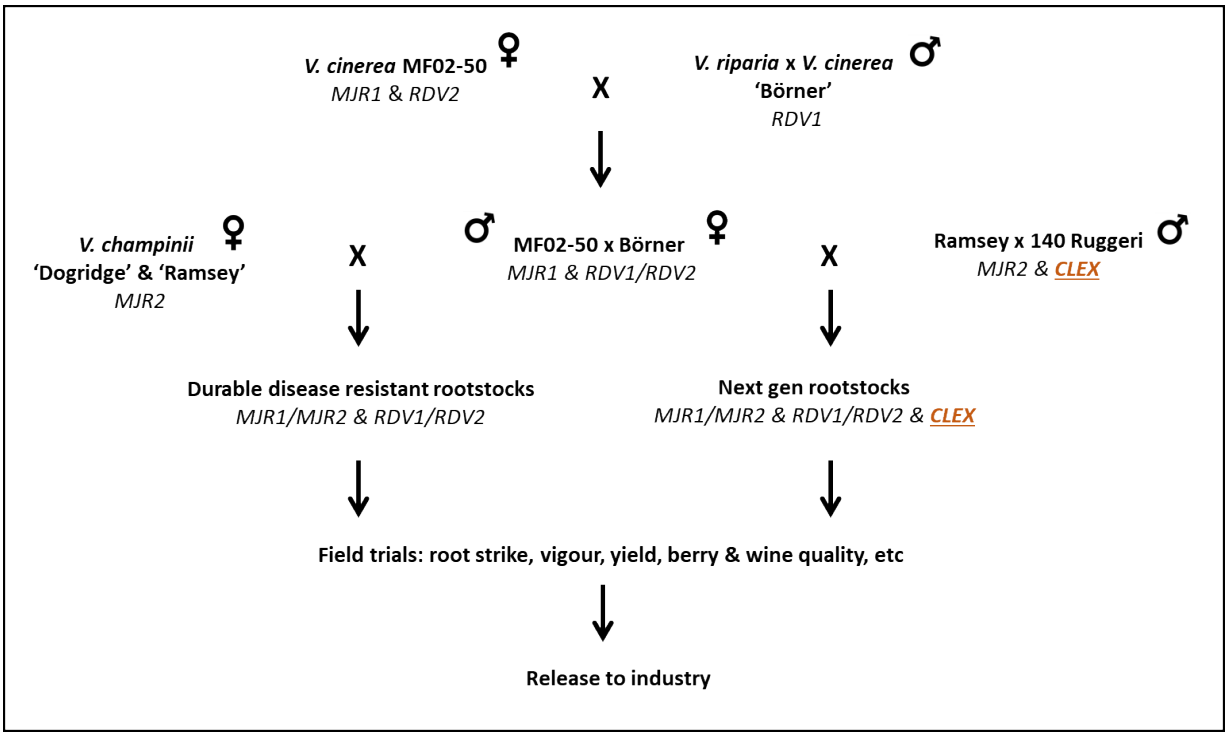


Figure 10.10 - Rootstock breeding strategy for combining Cl^- exclusion, encoded by the *CLEX* locus, with mulitple nematode resistance loci, *MJR1* and *MJR2* and multiple phylloxera resistance loci *RDV1* and *RDV2* (described in detail in CSA 1701-1.3 Rootstock genetics and improvement: tolerance to biotic stress). ♀ - denotes females genotypes and ♂ denotes male genotypes.

10.5 Discussion

None of the populations examined in this chapter displayed obvious segregation patterns in their Cl^- exclusion phenotype that is otherwise typical of control by a major locus. This is in contrast to what we have shown for the *V. berlandieri* x *V. vinifera* BC_1F_2 population (Chapter 6) and for the two K51-40 x 140 Ruggeri F_2 populations (Chapter 9). It is important to note that in each of these segregating populations there are only two alleles of the *CLEX* locus present. Being derived from a selfed parent, the BC_1F_2 population possesses one allele derived from *V. berlandieri* and one from *V. vinifera*. The K51-40 x 140 Ruggeri F_2 populations also each contain only two CLEX alleles, not because they are selfed, but because they were targeted crosses performed to create F_2 individuals homozygous for a given allele at the *CLEX* locus. In these populations in which only two *CLEX* alleles are present, the variation in Cl^- exclusion is most evident between homozygous individuals, but less clear between homozygous and heterozygous individuals, especially between heterozygous and those homozygous for the dominant allele. Our previous assumption was that a continual variation in Cl^- exclusion was indicative of control by multiple loci, however given our observations across five different populations, it now seems likely that clear segregation of the Cl^- exclusion phenotype may only be apparent when the population segregates for homozygous states of a major locus.

For any standard test cross there are likely to be four alleles of a given locus (as the genomes of *Vitis* species are highly heterozygous), resulting in four different allelic combinations in the F_1 progeny. Each one of these allelic combinations would be in a heterozygous state, such as the case for the K51-40 x 140 Ruggeri F_1 population (Chapter 9). It is therefore not surprising that all F_1 populations examined to date display a continuous variation in Cl^- exclusion. It fits that this may not be due to lack of influence of a major locus, but rather because none of the progeny have inherited that locus in a homozygous state necessary to see a segregation of the phenotype. Hence, in future attempts to map genes for Cl^- exclusion in segregating populations it is likely that either back cross, selfing, or targeted crossing strategies based on marker information are required. Furthermore, in order to identify these potentially unknown loci, it will be important to look at future populations in which the *CLEX* does not segregate, i.e. both parents are homozygous for the same allele. Alternatively, quantitative trait loci analysis could be undertaken in larger populations (200+ genotypes) with the influence of the *CLEX* locus accounted for as a co-variate.

140 Ruggeri is considered the benchmark rootstock for Cl^- exclusion and thus salt tolerance. Studies from Andy Walker's lab suggest that some *Vitis* species not widely utilised in rootstock breeding can exclude Cl^- at levels superior to 140 Ruggeri (Heinitz et al. 2020). We imported three *Vitis* species, *V. doaniana*, *V. girdiana* and *V. arizonica*, to assess their potential to improve Cl^- exclusion in the breeding program. In our studies *V. doaniana* was the only species to outperform 140 Ruggeri, while *V. girdiana* and *V. arizonica* did not perform as well as expected. This is likely because there is large intra-species variation for Cl^- exclusion (Heinitz et al. 2020), i.e. not all accessions of a species behave the same way. Although the single *V. doaniana* accession shows promise for improving Cl^- exclusion in the breeding program, relative to other genotypes it performed extremely poorly when inadvertently exposed to high temperatures arising from a faulty glasshouse air-conditioner. As such, and in addition to the drawbacks of working with quarantine material, we concluded that it would not be a priority for introduction into the breeding program.

V. longii shows the most promise for improving the Cl^- exclusion ability of future rootstocks beyond that of 140 Ruggeri (Chapter 6). Furthermore, the continual distribution within the *V. longii* x *V. vinifera* cv. Chardonnay population implies that *V. longii* is either homozygous for a major locus, or that the Cl^- exclusion trait is influenced by multiple loci. Results of a recent PhD study indicate that the superior Cl^- exclusion of *V. longii* may be in part due to its ability to exclude Cl^- from the roots (Chen 2021). Thus, it would fit that *V. longii* may have superior alleles of other unknown loci that control Cl^- exclusion from the root perhaps through efflux out of the root. Indeed, a nitrate/peptide transporter, *NPF2.2*, was recently identified and found to be expressed in grapevine roots, and when specifically expressed in the root epidermis and cortex of transgenic *Arabidopsis* it conferred reduced shoot Cl^- concentrations (Wu et al. 2022). The authors suggest that *NPF2.2* functions in the passive efflux of ions out of root cells, thereby reducing the symplasmic Cl^- available for root-to-shoot translocation and resulting shoot Cl^- concentrations (Wu et al. 2022). Despite its apparent superiority for Cl^- exclusion, *V. longii* has not previously been utilised for rootstock breeding and therefore requires further research to ensure that it does not come with any unwanted negative agronomic traits.

As a more prudent approach to improve the Cl^- exclusion and salt tolerance of the future durable disease resistant rootstocks, we chose to utilise 140 Ruggeri itself. Despite a lack of nematode resistance and average root-strike, it is otherwise an all-round excellent performing rootstock. Furthermore, results in Chapter 9 show that 140 Ruggeri possesses two dominant alleles of the *CLEX* locus, one from *V. berlandieri* and one from *V. rupestris*. To incorporate these alleles encoding strong Cl^- exclusion we crossed 140 Ruggeri with Ramsey, which is central to the breeding strategy for durable nematode resistance. Sykes (1985) previously showed that an F_1 population of Ramsey x *V. vinifera* cv. Sultana displayed a bimodal distribution of Cl^- exclusion phenotypes, suggesting that Ramsey is heterozygous for a dominant and a recessive allele of a major locus. Assuming that locus is the *CLEX* locus, then we would expect 50% of the Ramsey x 140 Ruggeri F_1s to possess two dominant copies, and to be the strongest excluders of Cl^-. Unfortunately, the genetic markers for the *CLEX* locus have not yet been sought or validated in these genomic backgrounds, thus we cannot yet determine which of the F_1s possess two dominant alleles. Instead, we simply selected 18 of the strongest excluding F_1s based on their Cl^- exclusion phenotype as well as for strong growth/biomass. In the near future these will also be screened for the presence of nematode resistance encoded by *MJR2* from Ramsey and for the *HKT1;1* alleles as 140 Ruggeri is heterozygous for one dominant Na^+ exclusion allele and one recessive allele which is associated with higher Na^+ accumulation. Ramsey is homozygous for the dominant allele (data not shown). Once the selected Ramsey x 140 Ruggeri vines reach flowering maturity, they will be crossed with MF02-50 x Börner F_1s and the resulting progeny selected with genetic markers for *MJR1*, *MJR2*, *RDV1* and *RDV2*. It is anticipated that the resulting progeny will possess stronger Cl^- exclusion, and thus salt tolerance, than those derived from Ramsey x (MF02-50 x Börner), which is the core breeding strategy. This hypothesis will be experimentally tested in the future once MF02-50 x Börner and Ramsey x 140 Ruggeri F_1s flower and the next steps of the strategy can be undertaken.

10.6 Summary

Within a given population, segregation of the Cl^- exclusion phenotype appears to be dependent on individuals segregating for homozygous states of a major locus. Simple F_1 test crosses, which predominantly result in individuals in a heterozygous state, are unlikely to result in segregation of the Cl^- phenotype. Segregating populations are more likely to come from a back cross, selfed, or marker-informed cross.

140 Ruggeri was crossed with Ramsey to combine the excellent Cl^- exclusion of 140 Ruggeri and the nematode resistance of Ramsey. The fittest progeny were selected for incorporating into the breeding program to ultimately generate new rootstocks that possess both durable disease resistance and strong salt tolerance traits.

11. Investigating the potential of C7 rootstock

11.1 Objectives

The aim of the work presented in this chapter was to reach an informed decision about a potential Plant Breeders Rights application for C7, and if so, to initiate plans for its commercial release. The intention was to build-on information obtained for C7 in previous Shiraz and Chardonnay rootstock trials at Merbein (Walker et al. 2010 and 2014) and on information reported for C7 from the Padthaway Pinot Gris and Shiraz rootstock trials (Clingeleffer et al. 2017). Results are presented from a Pinot Gris rootstock trial at Padthaway, SA, involving Pinot Gris on rootstocks Ramsey, 140 Ruggeri, C7 and own roots, and difference testing of wines made from Shiraz on 140 Ruggeri, C7 and own roots, also from a trial at Padthaway.

11.2 Background

C7 is a complex hybrid derived from a cross involving American *Vitis* species. The key species in its pedigree include *V. champinii*, *V. rupestris* and *V.berlandieri*. C7 was one of eight CSIRO rootstock hybrids and eight commercially available rootstocks chosen for inclusion in a trial planted in 1992 at two sites, the CSIRO Merbein and Irymple Farms situated near Mildura in north-western Victoria, as a component of Wine Australia Project CSH 8GW. Two scions were used in the study, Shiraz and Chardonnay, on their own roots, and grafted to the CSIRO rootstock hybrids and commercially available rootstocks.

Across the years evaluated until 2009, C7 impressed with an ability to maintain acceptable yield and good Cl^- and Na^+ exclusion capacity. Since performance data for C7 in the trials conducted in seasons 1996 to 1998 have not previously been reported, the data are presented here along with the key data from seasons 2008 and 2009 to enable a more complete picture of the potential of C7.

Following the promising data obtained from the earlier trials, it was decided to submit C7 for testing against a virulent strain of root knot nematode (RKN) and against two strains of phylloxera. Those results are also reported here.

Furthermore, it was decided to include C7 in rootstock trials in a different wine region, in this case, the Padthaway region of SA. Two trials were established, one comparing Pinot Gris on own roots and on three rootstocks, and one comparing Shiraz on own roots and on two rootstocks. The initial results of those trials were reported by Clingeleffer (2017). Here we report yield and grape juice Cl^- concentration from the Pinot Gris trial for seasons 2016 to 2019, and the results of difference testing for Shiraz wines from own roots, 140 Ruggeri and C7 made in 2016, 2017 and 2018.

11.3 Materials and Methods

11.3.1 Rootstock Trials

Merbein

The Merbein trial was irrigated with River Murray water supplemented with sodium chloride plus smaller amounts of calcium and magnesium chlorides to achieve an electrical conductivity of 2.1 dS/m. The Irymple trial was irrigated with River Murray water without salt addition and with an electrical conductivity of 0.4 dS/m. Further details regarding experimental set-up at both sites and methodology used are available in Walker et al. (2010). The Merbein trial was maintained at the same level of salinity treatment until 2009, providing an opportunity to investigate the longer-term responses to salinity (from 1996 and 1997 to 2008 and 2009) of the Shiraz and Chardonnay vines on their own roots and on the various rootstocks. The site was monitored in detail for the final two seasons, 2008 and 2009, with results reported in Walker et al. (2014).

Padthaway

Two rootstock trials were established adjacent to each other on a commercial vineyard at Padthaway, South Australia.

- Pinot Gris on own roots and grafted to 140 Ruggeri, Ramsey and C7. The trial was planted in 2012, with replacements planted in 2013.
- Shiraz on own roots and grafted to 140 Ruggeri and C7. The trial was planted in 2010, with replacements planted in 2011.

Both trials were trained to develop bilateral cordons on a single fruiting wire at 100 cm from the ground with provision for vertical shoot positioning. Row and vine spacing was 3.5 m and 1.8 m, respectively. GroGuards aided establishment. Soil was a uniform shallow brownish gravelly calcareous mottled clay. Irrigation was by drippers with water having an electrical conductivity of approximately 2.8 dS/m. Irrigation scheduling was managed by the vineyard owner at rates comparable with those applied for commercial vines of the varieties on the property. Drippers were spaced 50 cm apart with flow rates of 2.67 L/h. The drip line was situated 25 cm above ground level.

11.3.2 Yield and grape juice Cl^-

Procedures for yield determination, berry sampling, processing of sampled berries to obtain a grape juice sample, and the procedures for grape juice Cl^- determination were described by Walker et al. (2010).

11.3.3 Winemaking and wine assessment

Wines were made from two biological fruit replicates, each of approximately 10 kg, from Pinot Gris on own roots, Ramsey, 140 Ruggeri and C7 in 2019, and from Shiraz on own roots, 140 Ruggeri and C7 in 2016, 2017 and 2018. Harvested fruit was delivered to the winery on the

day of harvest and stored in cool rooms overnight prior to crushing and processing on the following day. Winemaking was done by the Australian Wine Research Institute at the Hickinbotham Winery of the University of Adelaide, Waite Campus, SA, using procedures described by Walker et al. (2019).

Descriptive assessment of Pinot Gris wines from vintage 2019 was conducted by skilled wine tasters from CSIRO. Shiraz wines were formally assessed by the Australian Wine Research Institute using the balanced reference triangle test. The triangle test is a forced choice test and is not valid for determining preferences, nor the character or degree of any perceived difference. At least 30 experienced, screened and qualified assessors were asked to evaluate the samples for aroma and palate, selecting the sample in the set that was different from the other two.

11.4 Results

11.4.1 Potential of C7 demonstrated from initial trials

Yield data, as the mean for each scion on each of own roots, 140 Ruggeri, 1103 Paulsen and C7 across the first three seasons (1996, 1997 and 1998) of the trials at Merbein and Irymple are shown Table 10.4.1. C7 yield for both Shiraz and Chardonnay at Merbein was comparable with 1103 Paulsen and higher by approximately 20% (Shiraz) and 32% (Chardonnay) than for own roots and 140 Ruggeri, which had similar yield. C7 yield for both Shiraz and Chardonnay at Irymple was again comparable with 1103 Paulsen but lower by approximately 14% (Shiraz) and higher by approximately 66% (Chardonnay) than for own roots and 140 Ruggeri, which had similar yield (Table 10.4.1).

Table 11.4.1 – Mean yield across three seasons (1996, 1997 and 1998) for Shiraz and Chardonnay on own roots, 140 Ruggeri, 1103 Paulsen and C7 rootstocks at the Merbein and Irymple sites. The Shiraz and Chardonnay at Merbein were irrigated at the rate of approximately 6.7 ML/ha with water of EC_{iw} 2.1 dS/m and at Irymple at the rate of approximately 10.2 ML/ha with water of EC 0.4 dS/m. Further details of the trials are provided in Walker et al. (2010). s.e.d. = standard errors of the differences of means.

Variety/Rootstock	Mean Yield '96-'98 (kg)			
	Merbein Shiraz	Merbein Chardonnay	Irymple Shiraz	Irymple Chardonnay
Own roots	13.74	8.54	12.45	6.19
140 Ruggeri	13.86	8.82	11.98	5.11
1103 Paulsen	16.36	11.73	11.58	9.51
C7	16.99	11.27	10.48	9.27
s.e.d.	1.38	1.24	1.25	1.16

Grape juice Cl^- concentration for Shiraz was not significantly different among C7, 1103 Paulsen and 140 Ruggeri, with the mean concentration for the three rootstocks (46.8 mg/L) approximately eight-fold lower than that for own roots (Table 10.4.2). Grape juice Cl^- concentration for Chardonnay was also not significantly different among C7, 1103 Paulsen and 140 Ruggeri, with the mean concentration for the three rootstocks (14.6 mg/L) approximately 13-fold lower than that for own roots (Table 10.4.2).

Grape juice Na^+ concentration for Shiraz on C7, 140 Ruggeri and 1103 Paulsen was in the range 28.0 – 39.1 mg/L, with the mean concentration (33.6 mg/L) 2.4-fold lower than that for Shiraz on own roots (Table 10.4.2). Grape juice Na^+ concentration for Chardonnay on C7, 140 Ruggeri and 1103 Paulsen was in the range 12.1 – 21.3 mg/L, with the mean concentration (16.7 mg/L) four-fold lower than that for Shiraz on own roots (Table 10.4.2).

Grape juice K^+ concentration was highest for C7 for both Shiraz and Chardonnay, exceeding that for Shiraz on own roots by approximately 19% and that for Chardonnay on own roots by approximately 14% (Table 10.4.2). There was, however, little difference in grape juice K^+ concentration between C7 and 1103 Paulsen, within approximately 10% of each other, for both Shiraz and Chardonnay (Table 10.4.2).

Table 11.4.2 – Mean grape juice Cl^-, Na^+ and K^+ concentration across two seasons (1996 and 1997) for Shiraz and Chardonnay at Merbein on own roots, 140 Ruggeri, 1103 Paulsen and C7 rootstocks. The Shiraz and Chardonnay at Merbein were irrigated at the rate of 6.7 ML/ha with water of ECiw 2.1 dS/m. Further details of the trials are provided in Walker et al. (2010).

Variety/Rootstock	Shiraz			Chardonnay		
	Grape juice ion concentration (mg/L)					
	Cl^-	Na^+	K^+	Cl^-	Na^+	K^+
Own roots	377.5	80.9	2243	193.9	66.1	1732
140 Ruggeri	47.5	39.1	2215	12.9	18.8	1591
1103 Paulsen	49.3	28.0	2426	13.6	12.1	1845
C7	43.6	35.1	2673	17.5	21.3	1967
s.e.d	49.4	11.4	183	16.0	9.6	139

The same trial site was assessed approximately 10 years later (seasons 2008 and 2009), coinciding with the millennium drought. Like every other property in the Mildura region, the

Merbein site was subject to water restrictions. The total amount of irrigation applied was reduced in accordance with the restrictions. The salt content of the irrigation water was also reduced due to concerns that reduced irrigation and consequent insufficient leaching would place the vines under severe stress. Across the two seasons, the vines were irrigated with mean EC_{iw} 1.65 dS/m and an application of 3.04 ML/ha. This compares with mean EC_{iw} 2.1 dS/m and an application rate of 6.7 ML/ha for the seasons 1996 and 1997.

Under the elevated stress conditions, the Cl^- and Na^+ exclusion capacity of Shiraz on 1103 Paulsen deteriorated, shown by the increase in grape juice Cl^- and Na^+ concentration to 588 and 472 mg/L, respectively, close to that for own roots, 668 and 535 mg/L, respectively (Table 10.4.3). That for C7 and 140 Ruggeri remained comparable (mean 78 mM for Cl^- and 93.5 mM for Na^+) and approximately 7.5-fold and five-fold lower than that for 1103 Paulsen for Cl^- and Na^+, respectively. For Chardonnay, on the other hand, the concentration of both Cl^- and Na^+ in grape juice was not significantly different among C7, 1103 Paulsen and 140 Ruggeri, however the mean concentration across the three rootstocks (127 mg/L for Cl^- and 153 mg/L for Na^+) was respectively 8.6-fold and 6.6-fold lower than the concentration for own roots (Table 10.4.3).

Table 11.4.3 – Two-year means (for seasons 2008 and 2009) for grape juice Cl^- and Na^+ concentration at harvest for Shiraz and Chardonnay at Merbein on own roots, 140 Ruggeri, 1103 Paulsen and C7 rootstocks. The Shiraz and Chardonnay at Merbein were irrigated with mean ECiw 1.65 dS/m and an application rate of 3.04 ML/ha. Further details of the trials are provided in Walker et al. (2014). Different letters indicate significant difference between means (P = 0.05).

Variety/Rootstock	Cl^- Shiraz	Cl^- Chardonnay	Na^+ Shiraz	Na^+ Chardonnay
	Concentration (mg/L)			
Own roots	668b	1096b	535c	1013b
140 Ruggeri	111a	81a	138ab	168a
1103Paulsen	588b	233a	472bc	109a
C7	45a	67a	49a	183a

11.4.2 Padthaway Pinot Gris

Yield of C7 in the initial years, 2016 and 2017, was marginally less than that for own roots, with Ramsey and 140 Ruggeri having lower but comparable yields (Figure 11.4.1). By 2018 and 2019, when vine establishment had stabilised, there were no 'stand out' differences in

yield among the varieties, with 140 Ruggeri being marginally better than own roots, Ramsey and C7 in 2018, and own roots and 140 Ruggeri marginally better than Ramsey and C7 in 2019 (Figure 11.4.1).

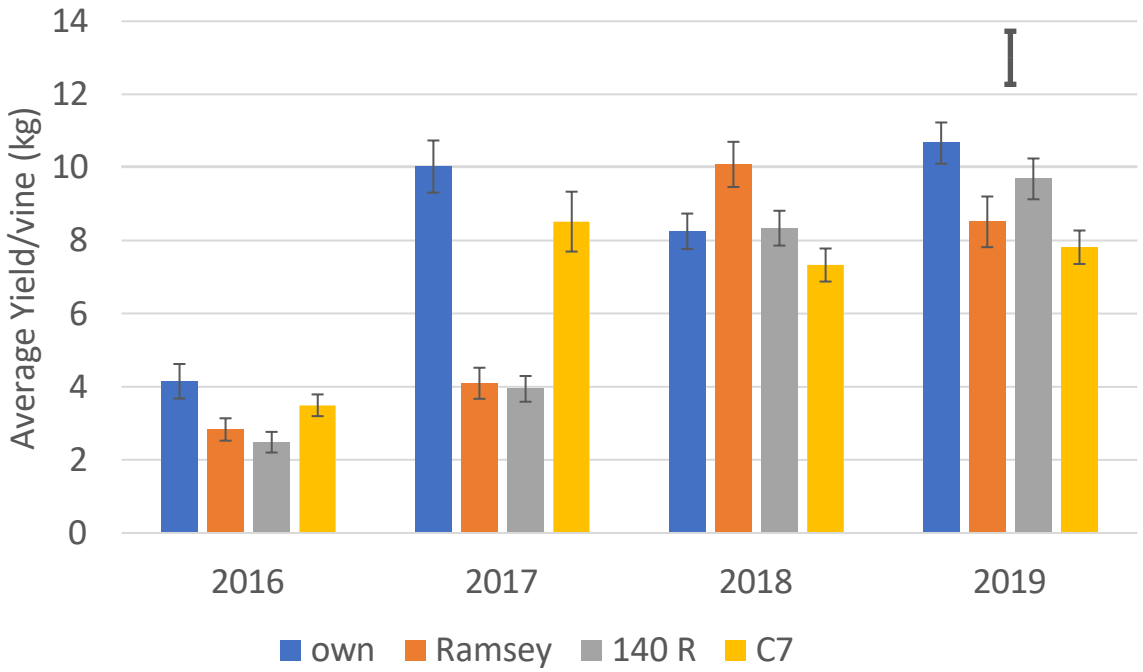


Figure 11.4.1 – Average yield of Pinot Gris on own roots and on Ramsey, 140 Ruggeri and C7 rootstocks in 2016, 2017, 2018 and 2019 at Padthaway, SA. Bars represent ± standard error. The vertical bar in bold type at the top right-hand corner represents the least significant difference (LSD) between means (P = 0.05).

Grape juice Cl^- concentration was highest for Pinot Gris on own roots in seasons 2016, 2017 and 2018, with no major difference among that for Ramsey, 140 Ruggeri and C7 (Figure 11.4.2). This result was not unexpected, as *Vitis vinifera* vines on own roots, in this case Pinot Gris, typically have higher Cl^- concentration in grape juice than grafted vines (Walker et al. 2010). In 2019 however, Ramsey had the highest grape juice Cl^- concentration, with no difference in concentration among that for own roots, 140 Ruggeri and C7, which was somewhat unexpected (Figure 11.4.2) and worthy of further investigation.

11.4.3 Evaluation of Pinot Gris wines from 2019

Wines made from two biological fruit replicates obtained from own roots, Ramsey, 140 Ruggeri and C7 were assessed. Both replicates from own roots were considered to lack aroma and have a somewhat bitter taste. Both replicates from Ramsey rootstock were also considered to lack aroma and have an acidic taste, which was less pronounced in one replicate. Replicate wines of 140 Ruggeri had a slight to slightly stronger aroma. Both were assessed as 'flat' on the palate. The aroma and palate of C7 wines were comparable with that of 140 Ruggeri wines, with one replicate assessed as having an oily texture.

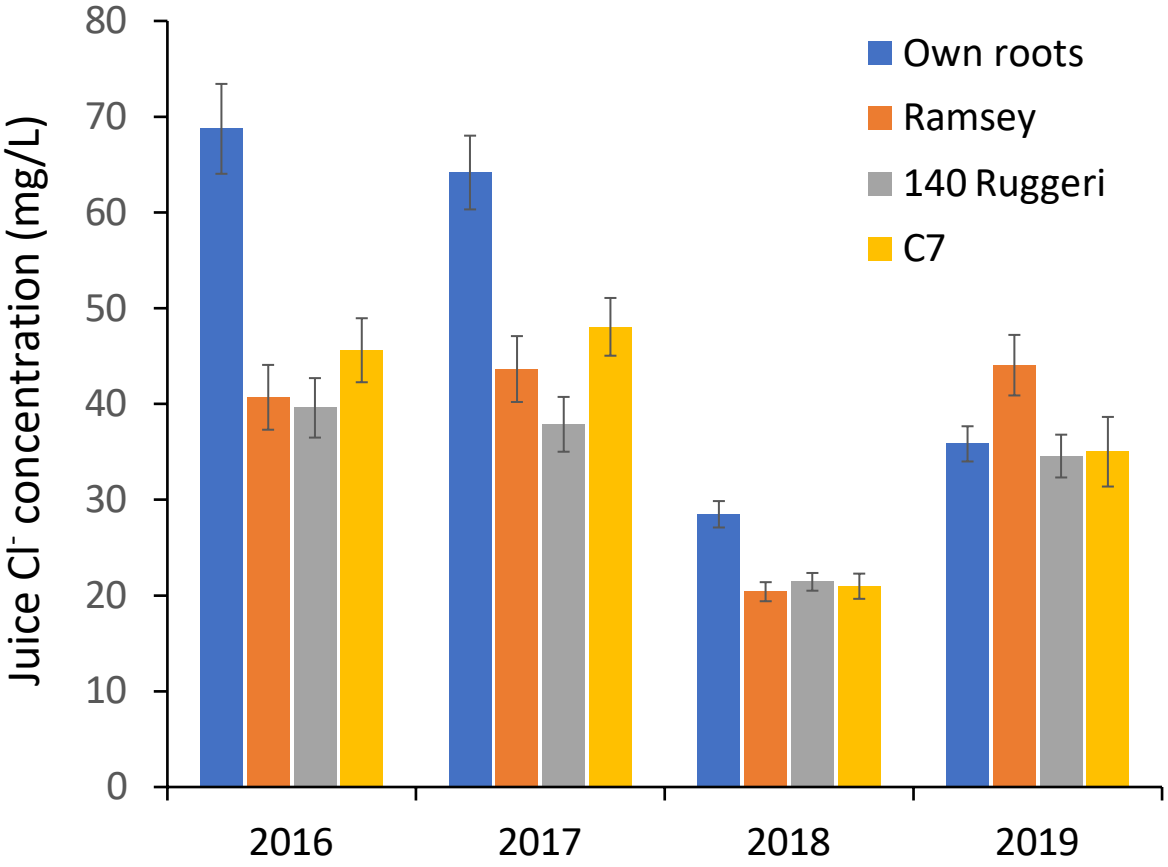


Figure 11.4.2 – Average grape juice Cl^- concentration of Pinot Gris on own roots and on Ramsey, 140 Ruggeri and C7 rootstocks in 2016, 2017, 2018 and 2019 at Padthaway, SA. Bars represent ± standard error.

11.4.3 Difference testing of Shiraz wines from three vintages

There was a significant difference ($P<0.05$) between wines of 140 Ruggeri and C7 across all vintages, showing perceptible sensory effects due to the different rootstocks (Table 10.4.4). A significant difference was also seen between the wines from own roots and 140 Ruggeri in the 2016 and 2017 vintages. The wines from own roots and C7 were significantly different in the 2017 vintage only (Table 10.4.4). Unfortunately, where significant differences between wines from the different rootstocks were detected, the triangle test did not permit determination of preferences. There were no informative comments from the judges along these lines.

Of interest was that there was no perceived difference in the wines from own roots and C7 in two of the three seasons (Table 10.4.4), noting that wines from own roots are typically perceived as the benchmark in comparisons of sensory characteristics of wines from rootstock trials.

Table 11.4.4 – Results of triangle (difference) tests. ns = not significant at P = 0.05, * P<0.05, ** P<0.01, *** P<0.001.

Test	Vintage	Comparison	Responses	Correct answers	Significance
1	2016	Own roots vs 140 Ruggeri	30	18	0.0025**
2	2016	Own roots vs C7	30	12	ns
3	2016	140 Ruggeri vs C7	30	15	0.044*
4	2017	Own roots vs 140 Ruggeri	31	22	<0.0001***
5	2017	Own roots vs C7	31	20	0.0004***
6	2017	140 Ruggeri vs C7	31	21	<0.0001***
7	2018	Own roots vs 140 Ruggeri	30	12	ns
8	2018	Own roots vs C7	30	11	ns
9	2018	140 Ruggeri vs C7	30	20	0.0002***

11.4.4 Testing for resistance to root knot nematode

Testing prior to this project (in 2011), using procedures described in Smith et al. (2017), demonstrated resistance of C7 against the virulent RKN strain *M. javanica '*pt1103' (Table 10.4.5). Follow-up work undertaken in 2021, also conducted using procedures described in Smith et al. (2017), sought confirmation of the earlier test result for C7 against RKN strain *M. javanica '*pt 1103P' strain. Results confirmed the resistance of C7 against this strain (Table 10.4.6).

Table 11.4.5 – Summary of the susceptibility/resistance phenotype of C7 when tested against root knot nematode *M. javanica* strain 'pt1103'.

Rootstock	Vine Replicate	Inoc. date	Score date	Egg masses	Root weight	Phenotype
C7	1	6/5/2011	14/6/2011	0	0.49	Resistant
C7	2	25/5/2011	5/7/2011	0	0.13	Resistant
C7	3	1/6/2011	11/7/2011	0	0.85	Resistant

Table 11.4.6 – Summary of the susceptibility/resistance phenotype of Ramsey, Shiraz and C7 when tested against root knot nematode *M. javanica* strain 'pt 1103P'.

Rootstock/Variety	Egg mass			Root weight			Phenotype
Ramsey	0	0	0	3.18	5.21	1.79	Resistant
Shiraz	65	25	57	15.38	8.72	10.15	Susceptible
C7	0	0	0	5.23	8.09	8.87	Resistant

11.4.5 Testing for resistance to phylloxera

Testing by DPI Victoria (K.S. Powell, A. Burns and G.A. Korosi) prior to this project, comparing the response of C7 with *V. vinifera* cv. Sultana to two phylloxera genetic strains, G1 and G4, revealed a relatively high level of resistance of C7 to both strains (data not shown). The cultivar Sultana was susceptible to both strains, as expected. The G1 and G4 strains of phylloxera were selected because they represented the two most geographically widespread and virulent genetic strains known to occur in Australia at the time of testing (2011).

That work was followed-up by additional testing during the life of this project, in this case comparing the response of C7 with *V. vinifera* cv. Riesling and rootstock Dogridge to phylloxera genetic strain G4. Testing confirmed the relatively high level of resistance of C7 to the G4 strain, while cultivar Riesling and rootstock Dogridge were susceptible (Table 10.4.7).

Table 11.4.7 – Summary of the susceptibility/resistance phenotype of Riesling, Dogridge and C7 when tested against G4 phylloxera strain (biotypeA).

Rootstock/Variety	Galls			Insects			Phenotype
V. vinifera cv. Riesling	10	34	147	8	302	455	Susceptible
Dogridge	18	161	318	75	412	1,007	Susceptible
C7	0	0	0	0	0	0	Resistant

11.4.6 Assessment of nursery characteristics

A qualitative assessment of nursery characteristics was conducted by a commercial nursery. C7 exhibited no tip burning characteristics under conditions of 40°C or higher. Grafting take (bench grafting) was assessed with two scions, Pinot Gris and Cabernet Sauvignon, with

satisfactory results (> 50% take) comparable with 101-14, Ramsey and 1103 Paulsen, and better than 140 Ruggeri and 110 Richter. Growth was ranked to three categories (slow, medium or fast) with C7 considered to have fast growth. For resistance to Crown Gall, C7 was ranked comparable to Ramsey, Richter 110, Richter 99 and 140 Ruggeri, all considered to have low resistance to Crown Gall. As mothervines established to produce a permanent cordon across a flat horizontal trellis system, C7 exhibited moderate vigour producing ideal cutting size (i.e. pencil thick) for bench grafting.

11.5 Discussion

Comparisons based on yield performance and the capacity for Cl^- and Na^+ exclusion revealed that C7 was in general comparable with that for 140 Ruggeri and 1103 Paulsen. It should be noted however, that due to the strong influence of environment and management, variability in yield performance should be expected and that results comparable with those for 140 Ruggeri and 1103 Paulsen should not be anticipated in all situations.

In Chapter 6 of this report, comparison of the Cl^- exclusion capacity of C7 with genotypes belonging to the three possible CLEX inheritance groups (*Vb/Vb*, *Vb/Vv* or *Vv/Vv*), under treatment with low (25 mM) or high (100 mM) mixed Cl^-, revealed that C7 accumulated Cl^- concentrations in laminae comparable with that for *Vb/Vb* and *Vb/Vv* genotypes. In contrast, the *Vv/Vv* genotypes accumulated concentrations comparable with that of the Cl^- accumulator K51-40.

Furthermore, as shown by Walker et al. (2014), C7 appeared to exhibit a better capacity for Cl^- exclusion than 1103 Paulsen when challenged with the combination of salinity and reduced irrigation.

A further potential advantage of C7 over 1103 Paulsen was its resistance to the root knot nematode strain *M. javanica* 'pt1103'. This aggressive root-knot nematode was linked to the poor performance of 1103 Paulsen in a McLaren Vale vineyard in South Australia. Vines grafted on 1103 Paulsen on which the strain was found to be reproducing exhibited stunted growth and in some cases vine death (Smith et al. 2017).

While phylloxera is present in Australia, it is confined to phylloxera exclusion zones in some, not all, states. Resistance to phylloxera is, however, a highly desirable trait for rootstocks in all production regions. Importantly, C7 demonstrated resistance to two virulent strains of phylloxera, G1 and G4, with repeat testing against G4 providing further confirmation of resistance.

C7 achieved a better success rate for bench grafting than its comparator 140 Ruggeri under qualitative assessment. Higher success rates translate to greater efficiency of nursery production.

11.6 Summary

C7 has demonstrated similar growth, production and fruit quality traits to 1103 Paulsen and 140 Ruggeri. It maintained good capacity for Cl^- exclusion in a long-term trial involving saline irrigation. In the final two years of that trial, under the combination of salinity and reduced irrigation, it demonstrated better capacity for Cl^- exclusion than 1103 Paulsen. Unlike 1103 Paulsen, C7 demonstrated resistance to root knot nematode strain 'pt1103'. C7 also demonstrated resistance to phylloxera strains G1 and G4. Overall, based on the traits assessed, C7 was considered suitable to be recommended for PBR protection and potential commercial release.

12. Outcomes/conclusions

Numerous past publications over several decades have described aspects of the Cl^- exclusion process. Yet untangling the genetic control of this trait had remained elusive, with multiple studies implying the trait is controlled by multiple unknown loci. This is the first study to unequivocally show the major gene responsible. Identification of *CHX20.1* as the primary gene controlling Cl^- exclusion, and the *V. berlandieri* derived allele as the dominant excluding allele, will enable the development and deployment of a genetic marker for this trait in the rootstock breeding program, and hence should be a high priority for future work. Ultimately this should lead to new rootstocks with improved Cl^- exclusion and thus better ability to maintain growth and minimise the accumulation of Cl^- in berries and resultant wines when grown on saline soils, thus reducing the potential for salty taste in wine. *CHX20.1* has been shown to account for around 72% of the variability in Cl^- exclusion in the populations used in this study. The Cl^- exclusion process is also strongly influenced by environmental variables such as temperature (Walker et al. 2022) and water-logging (West and Taylor 1984), with soil type having an indirect effect mediated by soil texture, where heavier textures having higher % silt plus clay lead to higher juice Cl^- concentration (Prior et al. 1992). Knowledge of the major gene responsible for this trait will enable future researchers to gain a deeper understanding of how the mechanism of Cl^- exclusion is influenced by various environmental factors. Such understanding could lead to better management practices in the vineyard, which combined with improved rootstocks would lead to a more resilient viticultural industry that is less reliant on the availability and affordability of high-quality water sources, thus mitigating some of the negative impacts anticipated with a changing climate.

Given that the soybean homologue of *CHX20.1* has also been implicated in Cl^- exclusion in that species, there is a high probability that the same gene performs a similar role in other plant species. Therefore, the identification of *CHX20.1* as the primary gene for Cl^- exclusion across multiple *Vitis* species may also lead to improvements in the salt tolerance of these other Cl^- sensitive crops such as citrus, almonds, avocado, mangoes and melons, to name a few.

CHX20.1 is also highly expressed in the bunch rachis, and therefore may play a role in regulating Cl^- concentration in the berry. Little information is available on Cl^- and Na^+ concentrations in bunch rachis. If the concentrations are high, bunch rachis could be a potential contributor to Cl^- and Na^+ concentrations in wine, if the ions are released from any rachis material included with the harvested grapes during crushing and processing.

CHX20.2 is expressed in the petiole and appears to have a minor influence on the exclusion of Cl^- from the laminae, but only at low salt treatments. At high salt treatments, other factors, potentially related to altered ionic gradients and ion transport processes, may mask this factor. *CHX20.2* may also play a minor role within the root system, where it is expressed in the root cortex and may be implicated in facilitating retention of Cl^- in root cortical cells, thereby limiting the amount of Cl^- transported to the stele and released into the xylem.

Access to alternative *Vitis* species (*V. doaniana, V. girdiana and V. arizonica*), previously unavailable in Australia, together with *V. longii* (syn. *acerifolia*), enabled comparative assessment of their capacity for Cl^- exclusion relative to that of 140 Ruggeri, the industry benchmark for Cl^- exclusion. *V. longii* exhibited superior Cl^- exclusion to that of 140 Ruggeri, however, further work would be required to determine if this is due to increased activity or expression of *CHX20.1* in *V. longii,* or the influence of other unknown loci that control Cl^- exclusion from the root, for example, through efflux out of the root.

13. Recommendations

We recommend a number of areas for further research to maximise the impact of this project's findings.

- As a high priority, we recommend continuing the development of a perfect marker for Cl^- exclusion targeted to causal SNPs underlying the differences between dominant *Vb* and recessive *Vv CHX20.1* alleles. The identification of causal SNPs will require a reliable functional assay to test the effect of amino acid substitutions between CHX20.1 proteins encoded by dominant and recessive alleles. Therefore, it is recommended that the development of an assay for quantifying the transport properties of CHX20.1 proteins is continued. One unexplored option for a quantitative assay could include the introduction of *CHX20.1* alleles into the poor chloride excluder K51-40 by stable genetic transformation. In the absence of a functional assay, further data on the influence of novel *CHX20.1* alleles on Cl^- exclusion could be readily gathered using the rapid seedling screen on populations derived from potential breeding material such as *V. cinerea*, *V. champinii* 'Ramsey', *V. aestivalis*, *V. longii* and *M. rotundifolia*. This would enable the identification of SNPs associated with dominant or recessive alleles over multiple populations, which may establish the causal SNPs underlying this trait.

- Confirm the influence of the *CLEX* locus on Cl^- exclusion from the shoot (laminae and berries) in grafted vines grown to maturity in the field. This could be achieved by setting up a randomised and replicated field trial with 10+ *VbVb* and 10+ *VvVv* BC_1F_2 genotypes grafted with a *V. vinifera* scion. It would be highly valuable to perform such a study in a saline effected vineyard to also quantify how the inheritance of the *CLEX* locus impacts overall salt tolerance, i.e. pruning weight and yield differences between *VbVb* and *VvVv* genotypes.

- Work from the previous project CSP 1302 established that the effect of temperature on the Cl^- exclusion ability of different genotypes is highly variable. This may be due to differential regulation or activity of *CHX20.1*, thus we recommend the influence of temperature on *CHX20.1* expression/activity be investigated. Similarly in the previous project we showed that Cl^- accumulation in the laminae was negatively correlated with ABA levels in Shiraz grafted onto K51-40 x 140 Ruggeri F1 hybrids. It would be worth determining whether ABA regulates the expression of *CHX20.1* as this knowledge could inform irrigation strategies to maximise *CHX20.1* expression in the vineyard.

- We found that the expression of *CHX20.1* is as high in the bunch rachis as it is in the root stele, where it appears to be active in the exclusion of Cl^- from the root xylem. Previous studies have shown that Cl^- accumulates to 2-10-fold higher concentrations than Na^+ in grapevine laminae, yet in the berry, these two ions are in similar concentrations in the pulp, whereas in the skin, the concentration of Cl^- is several-fold higher than that of Na^+ (Gong et al. 2010). We were unable to locate information on concentrations of Cl^- and Na^+ in rachis, an area recommended for further work. It is possible that in the rachis *CHX20.1* is active in the exclusion of Cl^- from the berry. It is important to determine whether *CHX20.1* does indeed restrict accumulation of Cl^- from the berry as there may be natural variation in this trait among scion cultivars resulting in some cultivars being better suited to berry production on saline soils. If it is shown that *CHX20.1* is involved in regulating entry of Cl^- into the berry, then it may make a suitable target for improving scion varieties via gene editing.

- Determine whether *CHX20.2* contributes to Cl^- exclusion in the root cortex. The role of *CHX20.2* in the root cortex could not be established with certainty, hence further work is required. We have shown that *CHX20.2* has a clear role in partitioning Cl^- into the petiole under control conditions. While we were unable to demonstrate a similar role under salt treatment conditions, it is well known that petioles accumulate significantly higher concentrations of Cl^- than laminae under salt treatment. Further work is required to confirm and understand the mechanism for possible Cl^- retrieval and retention in petioles under salt treatment conditions. The role of *CHX20.1* in the shoot under salt treatment should also be investigated in future studies.

- *V. longii* shows great potential as a source of Cl^- exclusion superior to that of *V. berlandieri*. Crosses should be initiated to introgress the Cl^- exclusion capability of *V. longii* into the rootstock breeding strategy. Furthermore, the influence of CHX20.1 alleles derived from V. longii should be investigated to determine if the strong Cl^- exclusion of this species is the result of a high gene expression or activity of *CHX20.1*, or due to other possible mechanisms such as efflux from the roots.

- Rootstock C7 is recommended for PBR protection and potential commercial release.

Appendix 1: Communication

Presentations

Jake Dunlevy, 12th International conference on grapevine Breeding and Genetics. Bordeaux, France, July 2018.
Title - Identification of loci and genes responsible for sodium and chloride exclusion in rootstocks for use in marker assisted selection.

Jake Dunlevy, Australian Wine Industry Technical Conference Rootstock Workshop. Adelaide, September 2019.
Title – Breeding for enhanced tolerance to abiotic stress: a focus on salinity.

Rob Walker, Australian Wine Industry Technical Conference Rootstock Workshop. Adelaide, September 2019.
Title – Guide to rootstock selection – rootstock selector tool.

Jake Dunlevy, Wine Australia Breeding Review. Adelaide, December 2019.
Title – Development of new rootstocks for Australian conditions with improved tolerance to abiotic stress.

Publications

Dunlevy, J.D., Blackmore, D.H., Betts, A., Jewell, N., Brien, C., Berger, B., Walker, R.R., Edwards, E.J. and Walker, A.R., (2022) Investigating the effects of elevated temperature on salinity tolerance traits in grapevine rootstocks using high-throughput phenotyping. *Aust. J. Grape Wine Res.* **28**, 276–291.

Dunlevy, J.D., Blackmore, D.H., Watkins, J.L., Edwards, E.J., Walker, R.R. and Walker, A.R., (2019) Short sequence repeat (SSR) genotyping and sodium exclusion phenotyping of a *Vitis* hybrid population (K51-40 × Schwarzmann), in: *Acta Horticulturae. International Society for Horticultural Science* (ISHS), Leuven, Belgium, pp. 513–520.

Walker, R.R., Blackmore, D.H., Dunlevy, J.D., Edwards, E.J. and Walker, A.R., (2022) Rootstock type influences salt exclusion response of grafted Shiraz under salt treatment at elevated root zone temperature. *Aust. J. Grape Wine Res.* **28**, 292–303.

Wu, Y., Henderson, S.W., Wege, S., Zheng, F., Walker, A.R., Walker, R.R. and Gilliham, M., (2020) The grapevine *NaE* sodium exclusion locus encodes sodium transporters with diverse transport properties and localisation. *J. Plant Physiol.* **246**–**247**, 153113.

Industry articles

Jake Dunlevy, Deidre Blackmore, Everard Edwards, Rob Walker, Bettina Berger and Mandy Walker, Rootstocks: Developing a smart, rapid screening method for desirable grapevine rootstock traits. Wine & Viticulture Journal 34 (4), 55-57

Rob Walker, Deidre Blackmore, Everard Edwards, Jake Dunlevy and Mandy Walker Rootstock salt exclusion at elevated root zone temperature. Wine & Viticulture Journal 34 (1), 54, 56-58

Appendix 2: Intellectual Property

The intellectual property involving the gene that was identified in this project and shown to play a role in the chloride exclusion process will be assessed by CSIRO for possible protection through a patent application. Hence, until a decision is made by CSIRO and Wine Australia on patentability of the gene, it is requested that this report be confidential to CSIRO and Wine Australia.

Populations developed during the project constitute project intellectual property.

A Plant Breeders Rights application has been submitted to IP Australia for the rootstock C7.

Other data obtained in this project are already in the public domain (see project publications) or will form part of future publications in internationally recognised, peer-reviewed journals.

Appendix 3: References

Abbaspour, N., Kaiser, B. and Tyerman, S., (2013) Chloride transport and compartmentation within main and lateral roots of two grapevine rootstocks differing in salt tolerance. *Trees - Struct. Funct.* **27**, 1317–1325.

Abel, G.H., (1969) Inheritance of the capacity for chloride inclusion and chloride exclusion by Soybeans 1 . *Crop Sci.* **9**, 697–698.

Bassil, E., Zhang, S., Gong, H., Tajima, H. and Blumwald, E., (2019) Cation specificity of vacuolar NHX-type cation/H^+ Antiporters 1. *Plant Physiol.* **179**, 616–629.

Chanroj, S., Lu, Y., Padmanaban, S., Nanatani, K., Uozumi, N., Rao, R. and Sze, H., (2011) Plant-specific cation/H^+ exchanger 17 and its homologs are endomembrane K^+ transporters with roles in protein sorting. *J. Biol. Chem.* **286**, 33931–33941.

Chanroj, S., Wang, G., Venema, K., Zhang, M.W., Delwiche, C.F. and Sze, H., (2012) Conserved and diversified gene families of monovalent cation/H^+ antiporters from algae to flowering plants. *Front. Plant Sci.* **3**, 1–18.

Chen, C.C.L., (2021) Exploration of grapevine rootstocks to combat chloride-induced toxicity. PhD Thesis, University of California - Davis.

Cheng, N.H., Pittman, J.K., Shigaki, T. and Hirschi, K.D., (2002) Characterization of *CAX4*, an Arabidopsis H^+/Cation antiporter. *Plant Physiol.* **128**, 1245–1254.

Cheung, A.Y., Maugel, T.K., Czerny, D.D., Leydon, A.R., Zou, Y., Su, R.T., Johnson, M.A., Sze, H., Chanroj, S., Padmanaban, S. and Levin, K.A., (2017) Transporters involved in pH and K^+ homeostasis affect pollen wall formation, male fertility, and embryo development. *J. Exp. Bot.* **68**, 3165–3178.

Clingeleffer, P.R. (2017) New rootstocks for Australian conditions. Final Report to Wine Australia Project CSP 1304.

Collard, B.C.Y. and Mackill, D.J., (2008) Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. *Philos. Trans. R. Soc. B Biol. Sci.* **363**, 557–572.

Demes, E., Besse, L., Cubero-Font, P., Satiat-Jeunemaitre, B., Thomine, S. and Angeli, A. De, (2020) Dynamic measurement of cytosolic pH and [NO^3] uncovers the role of the vacuolar transporter *AtCLCa* in cytosolic pH homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 15343–15353.

Do, T.D., Chen, H., Hien, V.T.T., Hamwieh, A., Yamada, T., Sato, T., Yan, Y., Cong, H., Shono, M., Suenaga, K. and Xu, D., (2016) *Ncl* synchronously regulates Na^+, K^+, and Cl^- in Soybean and greatly increases the grain yield in saline field conditions. *Sci. Rep.* **6**, 1–10.

Downton, W.J.S., (1977) Influence of rootstocks on the accumulation of chloride, sodium and potassium in grapevines. *Aust. J. Agric. Res.* **28**, 879–889.

Dunlevy, J.D., Blackmore, D.H., Betts, A., Jewell, N., Brien, C., Berger, B., Walker, R.R., Edwards, E.J. and Walker, A.R., (2022) Investigating the effects of elevated temperature on salinity tolerance traits in grapevine rootstocks using high-throughput phenotyping. *Aust. J. Grape Wine Res.* **28**, 276–291.

Dunlevy, J.D., Blackmore, D.H., Watkins, J.L., Edwards, E.J., Walker, R.R. and Walker, A.R., (2019) Short sequence repeat (SSR) genotyping and sodium exclusion phenotyping of a *Vitis* hybrid population (K51-40 × Schwarzmann), in: *Acta Horticulturae. International Society for Horticultural Science* (ISHS), Leuven, Belgium, pp. 513–520.

Fasoli, M., Dal Santo, S., Zenoni, S., Torniell, G.B., Farina, L., Zamboni, A., Porceddu, A., Venturini, L., Bicego, M., Murino, V., Ferrarini, A., Delledonne, M. and Pezzotti, M., (2012) The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *Plant Cell* **24**, 3489–3505.

Fort, K.P., Heinitz, C.C. and Walker, M.A., (2015) Chloride exclusion patterns in six grapevine populations. *Aust. J. Grape Wine Res*. **21**, 147–155.

Fusaro, A.F., Matthew, L., Smith, N.A., Curtin, S.J., Dedic-Hagan, J., Ellacott, G.A., Watson, J.M., Wang, M.B., Brosnan, C., Carroll, B.J. and Waterhouse, P.M., (2006) RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep.* **7**, 1168–1175.

Gong, H., Blackmore, D., Clingeleffer, P., Sykes, S., Jha, D., Tester, M. and Walker, R., (2011) Contrast in chloride exclusion between two grapevine genotypes and its variation in their hybrid progeny. *J. Exp. Bot.* **62**, 989–999.

Guan, R., Qu, Y., Guo, Y., Yu, L., Liu, Y., Jiang, J., Chen, J., Ren, Y., Liu, G., Tian, L., Jin, L., Liu, Z., Hong, H., Chang, R., Gilliham, M. and Qiu, L., (2014) Salinity tolerance in soybean is modulated by natural variation in *GmSALT3*. *Plant J.* **80**, 937–950.

Heinitz, C.C., Riaz, S., Tenscher, A.C., Romero, N. and Walker, M.A., (2020) Survey of Chloride Exclusion in Grape Germplasm from the Southwestern United States and Mexico. *Crop Sci.* 1–17.

Henderson, S.W., Dunlevy, J.D., Wu, Y., Blackmore, D.H., Walker, R.R., Edwards, E.J., Gilliham, M. and Walker, A.R., (2018) Functional differences in transport properties of natural HKT1;1 variants influence shoot Na^+ exclusion in grapevine rootstocks. *New Phytol.* **217**, 1113–1127.

Hussain, S., Hussain, S., Ali, B., Ren, X., Chen, X., Li, Q., Saqib, M. and Ahmad, N., (2021) Recent progress in understanding salinity tolerance in plants: Story of Na^+/K^+ balance and beyond. *Plant Physiol. Biochem.* **160**, 239–256.

Ito, M., Cooperberg, B. and Krulwich, T.A., (1997) Diverse genes of alkaliphilic *Bacillus firmus* OF4 that complement K^+-uptake-deficient *Escherichia coli* include an ftsH homologue.

Extremophiles **1**, 22–28.

Lee, G.J., Boerma, H.R., Villagarcia, M.R., Zhou, X., Carter, T.E., Li, Z. and Gibbs, M.O., (2004) A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. *Theor. Appl. Genet*. **109**, 1610–1619.

Liu, Y., Yu, L., Qu, Y., Chen, J., Liu, X., Hong, H., Liu, Z., Chang, R., Gilliham, M., Qiu, L. and Guan, R., (2016) *GmSALT3*, which confers improved soybean salt tolerance in the field, increases leaf Cl^- exclusion prior to Na+ exclusion but does not improve early vigor under salinity. *Front. Plant Sci.* **7**, 1–14.

De Loryn, L.C., Petrie, P.R., Hasted, A.M., Johnson, T.E., Collins, C. and Bastian, S.E.P., (2014) Evaluation of sensory thresholds and perception of sodium chloride in grape juice and wine. *Am. J. Enol. Vitic.* **65**, 124–133.

Lu, Y., Chanroj, S., Uozumi, N., Cheung, A., Zulkifli, L., Sze, H. and Johnson, M.A., (2011) Pollen tubes lacking a pair of K^+ transporters fail to target ovules in Arabidopsis. *Plant Cell* **23**, 81–93.

Maser, P., (2002) Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiol.* **126**, 1646–1667.

Møller, I.S., Gilliham, M., Jha, D., Mayo, G.M., Roy, S.J., Coates, J.C., Haseloff, J. and Tester, M., (2009) Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type - Specific alteration of Na^+ transport in Arabidopsis. Plant Cell **21**, 2163–2178.

Munns, R. and Tester, M., (2008) Mechanisms of Salinity Tolerance. *Annu. Rev. Plant Biol.* **59**, 651–681.

Murchie, E.H. and Lawson, T., (2013) Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *J. Exp. Bot.* **64**, 3983–3998.

Newman, H. and Antcliff, A., (1983) Cl^- accumulation in some hybrids of *V. berlandieri*. *Vitis* **23**, 106–112.

Padmanaban, S., Kwak, J.M., Chanroj, S., Li, X., Ward, J.M. and Sze, H., (2007) Participation of Endomembrane Cation/H^+ Exchanger *AtCHX20* in Osmoregulation of Guard Cells. *Plant Physiol.* **144**, 82–93.

Prior, L.D., Grieve, A.M. and Cullis, B.R., (1992) Sodium chloride and soil texture interactions in irrigated field grown sultana grapevines. I. Yield and fruit quality. *Aust. J. Agric. Res.* **43**, 1051–1066.

Qi, X., Li, M.W., Xie, M., Liu, X., Ni, M., Shao, G., Song, C., Kay-Yuen Yim, A., Tao, Y., Wong, F.L., Isobe, S., Wong, C.F., Wong, K. Sen, Xu, C., Li, C., Wang, Y., Guan, R., Sun, F., Fan, G., Xiao, Z., Zhou, F., Phang, T.H., Liu, X., Tong, S.W., Chan, T.F., Yiu, S.M., Tabata, S., Wang, J., Xu, X. and Lam, H.M., (2014) Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. *Nat. Commun*. **5**, 1–11.

Qu, Y., Guan, R., Bose, J., Henderson, S.W., Wege, S., Qiu, L. and Gilliham, M., (2021) Soybean CHX-type ion transport protein GmSALT3 confers leaf Na^+ exclusion via a root derived mechanism, and Cl^- exclusion via a shoot derived process. *Plant Cell Environ*. **44**, 856–869.

Riaz, S., Pap, D., Uretsky, J., Laucou, V., Boursiquot, J.M., Kocsis, L. and Andrew Walker, M., (2019) Genetic diversity and parentage analysis of grape rootstocks. *Theor. Appl. Genet.* **132**, 1847-1860.

Shi, H., Quintero, F.J., Pardo, J.M. and Zhu, J.K., (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *Plant Cell* **14**, 465-477.

Smith, B.P., Wheal, M.S., Jones, T.H., Morales, N.B. and Clingeleffer, P.R., (2013) Heritability of adventitious rooting of grapevine dormant canes. *Tree Genet. Genomes* **9**, 467–474.

Smith, B.P., Morales, N.B., Thomas, M.R., Smith, H.M. and Clingeleffer, P.R. (2017) Grapevine rootstocks resistant to the root-knot nematode *Meloidogyne javanica*. *Australian Journal of Grape and Wine Research* **23**, 125-131.

Smith, H.M., Clarke, C.W., Smith, B.P., Carmody, B.M., Thomas, M.R., Clingeleffer, P.R. and Powell, K.S., (2018) Genetic identification of SNP markers linked to a new grape phylloxera resistant locus in *Vitis cinerea* for marker-assisted selection. *BMC Plant Biol.* **18**, 1–13.

Smith, H.M., Smith, B.P., Morales, N.B., Moskwa, S., Clingeleffer, P.R. and Thomas, M.R., (2018) SNP markers tightly linked to root knot nematode resistance in grapevine (*Vitis cinerea*) identified by a genotyping-by-sequencing approach followed by Sequenom MassARRAY validation. *PLoS One* **13**, 1–27.

Storey, R., Schachtman, D.P. and Thomas, M.R., (2003) Root structure and cellular chloride, sodium and potassium distribution in salinized grapevines. *Plant, Cell Environ*. **26**, 789–800.

Sykes, S., (1987) Variation in chloride accumulation in hybrids and backcrosses of Vitis berlandieri and *Vitis vinifera* under glasshouse conditions. *Am. J. Enol. Vitic.* **38**, 313–320.

Sykes, S.R., (1985) Variation in chloride accumulation by hybrid vines from crosses involving the cultivars Ramsey, Villard blanc, and Sultana. *Am. J. Enol. Vitic.* **36**, 30–37.

Tregeagle, J.M., Tisdall, J.M., Tang, C. and Walker, R.R. (2010) Cl^- uptake, transport and accumulation in grapevine rootstocks of different capacity for Cl^- exclusion. Functional Plant Biology **37**, 665-673.

Walker, A.R., Lee, E., Robinson, S.P., (2006) Two new grape cultivars, bud sports of Cabernet Sauvignon bearing pale-coloured beries, are a result of deletion of two regulatory genes of the berry colour locus. *Plant Molecular Biology* **62**, 623-635.

Walker, R.R., Torokfalvy, E., Scott, N.S. and Kriedemann, P.E. (1981) An analysis of photosynthetic response to salt treatment in *Vitis vinifera*. Australian Journal of Plant Physiology **8**, 359-374.

Walker, R.R., Blackmore, D.H., Clingeleffer, P.R., Godden, P. and Francis, L., (2003) Salinity effects on vines and wines, in: *Bulletin de L'O.I.V.* pp. 200–227.

Walker, R.R., Blackmore, D.H., and Clingeleffer, P.R., (2010) Impact of rootstock on yield and ion concentrations in petioles, juice and wine of Shiraz and Chardonnay in different viticultural environments with different irrigation water salinity. *Aust. J. Grape Wine Res.* **16**, 243-257.

Walker, R.R., Blackmore, D.H., Clingeleffer, P.R., and Emanuelli, D., (2014) Rootstock type determines tolerance of Chardonnay and Shiraz to long-term saline irrigation. *Aust. J. Grape Wine Res.* **20**, 496-506.

Walker, R.R., Blackmore, D.H., Gong, H., Henderson, S.W., Gilliham, M. and Walker, A.R. (2018) An analysis of the salt exclusion phenotype in rooted leaves of grapevine (Vitis spp.). *Australian Journal of Grape and Wine Res.* **24**, 317-326.

Walker, R.R., Blackmore, D.H., Clingeleffer, P.R., Holt, H., Pearson, W., and Francis, I.L. (2019) Effect of rootstock on yield, grape composition and wine sensory attributes of Shiraz grown in a moderately saline environment. *Aust. J. Grape Wine Res.* **25**, 414-429.

Walker, R.R., Blackmore, D.H., Dunlevy, J.D., Edwards, E.J. and Walker, A.R., (2022) Rootstock type influences salt exclusion response of grafted Shiraz under salt treatment at elevated root zone temperature. *Aust. J. Grape Wine Res.* **28**, 292–303.

Webb, L.B., Watterson, I., Bhend, J., Whetton, P.H. and Barlow, E.W.R., (2013) Global climate analogues for winegrowing regions in future periods: Projections of temperature and precipitation. *Aust. J. Grape Wine Res.* **19**, 331–341.

Webb, L.B., Whetton, P.H. and Barlow, E.W.R., (2008) Climate change and winegrape quality in *Australia. Clim. Res.* **36**, 99–111.

West, D.W. and Taylor, J.A. (1984) Response of six grape cultivars to the combined effects of high salinity and rootzone waterlogging. *J. Amer. Soc. Hort. Sci*. **109**, 844-851.

Wu, Y., Henderson, S.W., Walker, R.R. and Gilliham, M., (2022) Root-Specific Expression of *Vitis vinifera VviNPF2.2* Modulates Shoot Anion Concentration in Transgenic Arabidopsis. *Front. Plant Sci.* **13**, 1–13.

Zhang, J., Hausmann, L., Eibach, R., Welter, L.J., Töpfer, R. and Zyprian, E.M., (2009) A framework map from grapevine V3125 (*Vitis vinifera* "Schiava grossa" × 'Riesling') × rootstock cultivar "Börner" (*Vitis riparia* × Vitis cinerea) to localize genetic determinants of phylloxera root resistance. *Theor. Appl. Genet.* **119**, 1039–1051.

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Deidre Blackmore contributed to the preparation and editing of this final report.

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Appendix 5: Budget reconciliation

Provided by CSIRO in a separate document.

Appendix 6: Germplasm

CLEX alleles, laminae Cl^- concentration and vineyard position data on individual K51-40 x 140 Ruggeri F_1 and F_2 individuals assessed within Chapter 9.

Original K51-40 x 140 Ruggeri F1s

CLEX alle	Name	Vineyard	Position field_row_panel_vin	Laminae Cl (%dw) from TPA 2013 Cool -Sal	S.E.
R1 R2	140 Ruggeri	Nuriootpa	23_7_26_1-3	0.25	0.03
K1 R1	MC 215-01	Irymple	5B_30_6_1	1.01	0.12
K1 R1	MC 215-09	Nuriootpa	23_6_11_2&3	0.39	0.11
K1 R1	MC 215-13	Irymple	5B_30_5_4	0.67	0.06
K1 R1	MC 215-19	Irymple	5B_30_5_2	0.47	0.05
K1 R1	MC 215-25	Irymple	5B_30_5_3	0.52	0.11
K1 R1	MC 215-26	Nuriootpa	23_6_10_1-3	0.39	0.03
K1 R1	MC 215-28	Irymple	5B_30_9_2	0.41	0.05
K1 R1	MC 215-49	Irymple	5B_30_4_4	1.01	0.16
K1 R1	MC 215-60	Irymple	5B_30_8_4	0.88	0.10
K1 R1	MC 215-71	Irymple	5B_30_3_1	0.88	0.24
K1 R1	MI 07-23	Irymple	5B_31_16_1	0.64	0.07
K1 R1	MI 07-27	Irymple	5B_30_14_1	0.66	0.06
K1 R1	MI 07-47	Irymple	5B_30_11_1	0.49	0.06
K1 R2	MC 215-23	Nuriootpa	23_7_10_1-3	0.68	0.10
K1 R2	MC 215-27	Irymple	5B_30_7_3	0.57	0.08
K1 R2	MC 215-33	Irymple	5B_30_9_4	0.47	0.10
K1 R2	MC 215-55	Irymple	5B_30_3_2	0.42	0.06
K1 R2	MC 215-69	Irymple	5B_30_4_2	0.75	0.17
K1 R2	MC 215-76	Irymple	5B_30_3_3	1.16	0.26
K1 R2	MC 215-78	Irymple	5B_31_17_4	0.72	0.05
K1 R2	MI 07-33	Irymple	5B_30_12_1	1.38	0.29
K1 R2	MI 07-34	Irymple	5B_30_13_3	0.66	0.11
K1 R2	MI 07-36	Irymple	5B_30_13_2	0.76	0.07
K1 R2	MI 07-49	Irymple	5B_30_14_2	0.59	0.03
K2 R1	MC 215-10	Irymple	5B_31_18_3	0.95	0.22
K2 R1	MC 215-29	Irymple	5B_30_9_1	0.50	0.06
K2 R1	MC 215-37	Irymple	5B_30_16_4	0.50	0.12
K2 R1	MC 215-39	Irymple	5B_30_16_1	0.70	0.03
K2 R1	MC 215-40	Irymple	5B_30_15_4	0.99	0.17
K2 R1	MC 215-64	Irymple	5B_30_11_3	0.56	0.08
K2 R1	MI 07-29	Irymple	5B_30_13_4	0.54	0.09
K2 R2	MC 215-04	Irymple	5B_30_1_4	0.60	0.09
K2 R2	MC 215-30	Irymple	5B_30_8_2	1.05	0.21
K2 R2	MC 215-43	Nuriootpa	23_7_10_1-3	0.63	0.05
K2 R2	MC 215-45	Irymple	5B_30_6_2	0.85	0.11
K2 R2	MC 215-54	Irymple	5B_30_4_1	0.56	0.12
K2 R2	MC 215-63	Irymple	5B_30_10_3	0.26	0.05
K2 R2	MI 07-39	Irymple	5B_30_14_3	0.95	0.11
K2 R2	MI 07-44	Irymple	5B_30_12_4	0.80	0.06
K2 R2	MI 07-46	Irymple	5B_30_13_2	0.42	0.03
K1 K2	K51-40	Nuriootpa	23_7_27_1-3	1.35	0.18

New K51-40 x 140 Ruggeri F1 seedlings

CLEX allele	Name	Vineyard	Position field_row_panel_vine	Laminae Cl (%dw)
K1 R1	102	Irymple	4B_14_11_01	0.62
K1 R1	114	Irymple	died	0.23
K1 R1	127	Irymple	died	0.57
K1 R1	149	Irymple	4B_15_04_03	0.93
K1 R1	159	Irymple	died	0.83
K1 R1	188	Irymple	4B_15_05_02	0.61
K1 R2	103	Irymple	4B_14_01_02	0.56
K1 R2	129	Irymple	4B_14_06_02	0.50
K1 R2	132	Irymple	4B_14_03_01	0.42
K1 R2	134	Irymple	died	0.42
K1 R2	141	Irymple	4B_14_16_02	0.41
K1 R2	151	Irymple	4B_15_02_02	1.14
K1 R2	161	Irymple	4B_14_14_02	0.37
K1 R2	180	Irymple	4B_15_05_03	0.75
K1 R2	181	Irymple	4B_14_07_02	0.56
K1 R2	197	Irymple	4B_14_12_02	0.83
K1 R2	200	Irymple	4B_14_10_01	0.56
K1 R2	201	Irymple	died	0.64
K2 R1	160	Irymple	4B_14_06_01	0.86
K2 R1	195	Irymple	4B_14_12_01	0.31
K2 R1	202	Irymple	4B_14_08_01	0.66
K2 R1	211	Irymple	4B_14_13_02	0.33
K2 R2	104	Irymple	4B_14_17_02	0.24
K2 R2	112	Irymple	4B_14_15_02	0.48
K2 R2	124	Irymple	4B_15_01_01	0.71
K2 R2	128	Irymple	4B_15_06_03	0.43
K2 R2	147	Irymple	4B_14_01_03	0.30
K2 R2	157	Irymple	4B_15_02_01	0.43
K2 R2	162	Irymple	4B_15_03_01	0.87
K2 R2	163	Irymple	4B_14_14_01	0.87
K2 R2	175	Irymple	4B_15_01_03	0.61
K2 R2	176	Irymple	4B_15_04_01	0.46
K2 R2	187	Irymple	4B_14_10_03	0.39

K51-40 x 140 Ruggeri F2 (MC215-76 x MI07-49)

CLEX allele	Name	Vineyard	Position field_row_panel_vine	Laminae Cl (%dw)
R2 R2	47	Irymple	4B_15_19_03	0.10
R2 R2	63	Irymple	4B_16_02_03	0.11
R2 R2	49	Irymple	4B_16_03_03	0.20
R2 R2	64	Irymple	4B_16_02_02	0.24
R2 R2	53	Irymple	4B_16_03_02	0.27
R2 R2	48	Irymple	4B_15_18_02	0.28
R2 R2	59	Irymple	4B_16_01_01	0.41
R2 R2	58	Irymple	4B_16_01_02	0.43
K1 R2	60	Irymple	4B_16_01_03	0.31
K1 R2	55	Irymple	4B_16_03_01	0.32
K1 R2	61	Irymple	4B_15_19_01	0.46
K1 R2	52	Irymple	4B_15_18_01	0.48
K1 R2	54	Irymple	4B_15_18_03	0.68
K1 R2	56	Irymple	died	0.82
K1 K1	51	Irymple	4B_15_20_02	0.80
K1 K1	62	Irymple	4B_15_19_02	0.87
K1 K1	50	Irymple	4B_15_17_03	1.07
K1 K1	57	Irymple	4B_15_20_01	1.14
K1 K1	46	Irymple	4B_15_20_03	1.33

K51-40 x 140 Ruggeri F2 (MC215-39 x MC215-40)

CLEX allele	Name	Vineyard	Position field_row_panel_vine	Laminae Cl (%dw)
R1 R1	12	Irymple	4B_15_08_03	0.09
R1 R1	41	Irymple	4B_15_09_02	0.09
R1 R1	7	Irymple	4B_15_16_03	0.14
R1 R1	26	Irymple	died	0.15
R1 R1	17	Irymple	died	0.16
R1 R1	27	Irymple	4B_15_12_02	0.16
R1 R1	13	Irymple	4B_15_17_01	0.20
R1 R1	35	Irymple	4B_15_08_01	0.23
K2 R1	37	Irymple	4B_15_11_03	0.16
K2 R1	42	Irymple	4B_15_07_03	0.20
K2 R1	28	Irymple	4B_15_09_03	0.22
K2 R1	40	Irymple	4B_15_17_02	0.22
K2 R1	24	Irymple	4B_15_13_01	0.22
K2 R1	43	Irymple	4B_15_10_02	0.24
K2 R1	10	Irymple	4B_15_12_03	0.25
K2 R1	18	Irymple	4B_15_11_01	0.25
K2 R1	25	Irymple	4B_15_14_02	0.26
K2 R1	38	Irymple	4B_15_15_01	0.28
K2 R1	5	Irymple	died	0.31
K2 R1	36	Irymple	died	0.32
K2 R1	2	Irymple	died	0.33
K2 R1	19	Irymple	4B_15_16_02	0.34
K2 R1	23	Irymple	4B_15_15_03	0.35
K2 R1	16	Irymple	4B_15_13_03	0.38
K2 R1	11	Irymple	4B_15_07_02	0.39
K2 R1	3	Irymple	4B_15_16_01	0.46
K2 R1	1	Irymple	4B_15_15_02	0.54
K2 R1	6	Irymple	4B_15_14_03	0.60
K2 R1	39	Irymple	4B_15_11_02	0.81
K2 R1	30	Irymple	4B_15_08_02	1.06
K2 R1	34	Irymple	died	1.08
K2 K2	20	Irymple	died	0.65
K2 K2	9	Irymple	4B_15_09_01	0.92
K2 K2	22	Irymple	4B_15_10_03	1.09
K2 K2	15	Irymple	died	1.11
K2 K2	32	Irymple	died	1.20
K2 K2	4	Irymple	died	1.32
K2 K2	31	Irymple	4B_15_12_01	1.50