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Genetic transformation of grapevine to test significant abiotic stress and pest resistance genes



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Principal Investigator: **Mandy Walker**

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Genetic transformation of grapevine to test significant abiotic stress and pest resistance genes

Authors

Mandy Walker, Debra McDavid, Lauren Hooper, Deidre Blackmore

CSIRO Agriculture and Food

Waite Campus

Locked Bag 2, Glen Osmond, SA 5064

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Front cover: Shiraz plantlet transformed with *ALMT2* silencing construct to test role of *aluminium-activated malate transporter2* gene in chloride exclusion.

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1 Abstract

Grapevine transformation is a slow and tedious procedure, but it is an essential technique for establishing the roles of key genes in grapevine, which subsequently leads to better breeding outcomes through marker-assisted selection. In this project we successfully transformed Shiraz to test a range of sodium and chloride exclusion candidate genes, assayed several sets of altered lines for their ability to exclude salt and produced other lines for testing at a later date. Methods for rootstock transformation were also trialled. These techniques were partially successful and, with further refinement, will be useful to support CSIRO's rootstock breeding projects.

2 Executive summary

Rootstocks for winegrapes provide the scion with a number of important protective features, such as resistance to pests like phylloxera and nematodes, exclusion of unwanted salts, control of vigour, and better water use efficiency. Predictions for climate change suggest that stresses such as salt and reduced water quality and quantity will increase over the next few decades. To assist in the protection of the Australian winegrape industry into the future, CSIRO, in partnership with Wine Australia, is developing new rootstocks that can tolerate these stresses to a higher degree than rootstocks currently available. Marker-assisted selection is a large part of the breeding of new rootstocks. To develop perfect markers for a desired trait, the isolation and characterisation of genes that encode these important resistance traits is an essential step.

To assist with the development of markers we are utilising grapevine transformation techniques to test the function of key genes. This project focussed on genes that are candidates for major roles in exclusion of chloride and sodium. Putative chloride exclusion genes were selected at the start of the project, based on previous work conducted with our collaborators, Dr Sam Henderson and Prof Matt Gilliham at the University of Adelaide. Three putative chloride transporters were chosen because of their patterns of gene expression and their homology to genes of known function in other plants. Transgenic lines were subjected to mixed chlorides in both tissue culture and glasshouse experiments. The results suggest that there could be a role for these genes in the chloride exclusion process.

During the course of a related project (CSP 1302), two new genes for chloride exclusion and six new genes for sodium exclusion were identified using gene mapping techniques. These genes are now being tested in grapevines using transformation techniques. Transgenic lines were produced with reduced HKT1;1 expression and the characterisation of their phenotype supports an active role in sodium exclusion, as reported in the CSP 1302 Final Report, confirming conclusions reached in our publication (Henderson, Dunlevy et al., 2018, New Phytologist). These results assist in decision making regarding marker development for this locus.

While the development of the rootstock transformation methods was not completely successful, considerable progress has been made that will be continued in future projects.

Overall, the project developed capability in scion and rootstock transformation, further improving our world-class grapevine transformation facility and trained new people in these ever-improving techniques. It has paved the way for the investigation of new gene technologies such as CRISPR, which is designed just to make changes to a single endogenous gene and may speed up breeding of new rootstocks.

3 Background

Transformation of plants, animals or bacteria is the incorporation of usually a single genetic change in the genome of the organism, which is then heritable. The change could be insertion of a constructed piece of DNA, or as small as a single base change aimed at changing some aspect of the phenotype of the organism through changing the expression of a gene, modifying the function of a gene or adding a new gene.

Genetic transformation has been developed over the past 35 years to become an essential tool for understanding the roles of specific genes and pathways in plants. The information gained then provides a fast and reliable way forward for crop improvement in many species, through targeted and smart breeding using marker assisted breeding.

Fast generation inbred crops such as wheat and barley also provide more practical ways in which genes can be characterised through, for example, mutant plants that only differ from wildtypes by a single gene. However, heterozygous, long generation time plants like grapevines are not amenable to mutagenesis studies. Genetic modification through transformation technologies provides a mechanism to produce targeted mutants for phenotypic analysis.

Agrobacterium-mediated gene transfer is currently the most commonly used method of transformation. Ballistic methods (DNA-coated particle bombardment to deliver genes directly) and viral vectors have also been employed successfully. Grapevine transformation was initiated in the late 1980s (Nakano et al., 1994) though compared to methods in other species such as Arabidopsis (Clough and Bent, 1998) it is still a difficult and time-consuming process taking many months as it requires going through embryogenesis, where as other transformation systems such as those for tomato or tobacco utilise explants such as stems or leaves that can then produce a viable shoot.

Over the course of the previous project, we have improved the transformation process of Shiraz to make it a practicable method for characterisation of genes.

This project supports several others, particularly in the rootstock breeding area, providing the opportunity to test genes for their ability to provide resistance to stresses. Genes involved in salinity tolerance have been characterised in many species and some candidates have also been isolated from grapevine (Henderson et al., 2014).

Biotic stress genes are often very specific for the pest or pathogen. Their function is difficult to predict from sequence analysis so the best way to determine function is alter the expression of the target gene and then test with the pathogen.

Aims of the project:

1. To provide continuing capability to transform grapevine, entailing the training of staff and the initiation of new callus annually.
2. To test putative stress tolerance genes, identified in rootstock projects, for their ability to protect grapevines from abiotic or biotic stresses.
3. To develop the ability to transform selected rootstocks, providing a better platform for gene testing.

4 Project aims and performance targets

	Output	Date	Activity
1a	Two transformation ready vectors	31/12/2013	Two candidate genes identified from current chloride exclusion project CSP 1002, sequences isolated by PCR and ligated to vectors ready for grapevine transformation.
1b	Initiated Shiraz callus for 2013	30/06/2014	Flowers of Shiraz dissected, anthers collected and callus sub-cultured as required
1c	Small transformed plantlets containing chloride genes	30/06/2014	Two rounds of transformation completed for each construct, and at least X positively transformed material selected.
2a	Documented choice of biotic resistance genes for further evaluation	30/09/2015	Industry reference group meeting to decide on most promising biotic resistance genes to include in next round of transformations (activity 2d)
2b	Pre-embryogenic callus from three rootstock lines	30/06/2015	Rootstock lines selected for generation of transformation in conjunction with GWRDC funded rootstock projects, CSP 1302 and CSP 1304 Methods developed and callus established
2c	Data confirming role of putative chloride exclusion genes in Shiraz	30/06/2015	Transformed plantlets from 1c tested for ability to exclude chloride
2d	Two sets of Shiraz plantlets containing biotic resistance genes	30/06/2015	Generate DNA constructs for transformation Perform *Agrobacterium* transformation of Shiraz callus and select positive embryos Culture at least X plantlets from positive embryos
3a	Documented choice of genes for further evaluation	30/09/2016	Industry reference group meeting to decide on most promising genes to include in next round of transformations (activity 3d)
3b	Data demonstrating transformability of rootstock callus	31/12/2016	Test the new rootstock callus (output 2b) for transformability
3c	Transformed embryos with biotic stress gene available for selection	30/06/2016	Transform Shiraz with tomato nematode resistance gene Mi1 genes for ability to cope with applied stress

3d	Rootstock plantlets containing new resistance genes	30/06/2016	Perform *Agrobacterium* transformation of rootstock callus and select positive embryos Culture plantlets from positive embryos
4a	Callus material available to continue transformations	30/06/2017	Establish new callus cultures of Shiraz, 140 Ruggeri and one other rootstock Maintain current transformable callus cultures
4b	Shiraz plantlets transformed with new chloride gene available for assays to assess chloride exclusion	30/06/2017	Build constructs for the testing of newly identified chloride exclusion gene and transform Shiraz to test function
4c	Data available characterising the function of biotic resistance gene in Shiraz	30/06/2017	Test the Shiraz plantlets transformed with biotic stress gene for ability to cope with applied stress
4d	Information available regarding the role of HKT1 genes in sodium exclusion in grapevines	31/12/2016	Plantlets with altered HKT1.1, HKT1.2 and HKT1.3 tested in assay to determine role of this group of very similar genes
4e	Information disseminated by at least three extension mechanisms	30/06/2017	One industry article, one scientific publication and Final Report submitted

5 General methods

5.1 Development of DNA constructs to alter expression of target genes

To support the rootstock breeding projects, eleven grapevine genes were selected as candidates for manipulation of expression using plant transformation technology. Three genes (encoding ALMT2 (aluminium-activated malate transporter 2, a rapid-activating R-type channel), SLAH1.2 and 1.3 (slow-type anion channel-associated proteins 1.2 and 1.3)) were selected based on previous experiments (Henderson et al., 2014 and 2015). Three constructs targeted the HKT (high affinity potassium transporter) genes, which in our parallel project CSP 1302 (Henderson, Dunlevy et al., 2018) we showed that HKT1;1 was instrumental in sodium exclusion from the scion. The use of these three constructs to transform grapevine provided a way to test the role of the other *HKT* genes. Two putative chloride exclusion genes also identified in CSP 1302 were the basis for gene silencing constructs to test their role. One gene (*Mi1.2*) previously identified in tomato as playing a role in nematode resistance (Cortada et al., 2008) was also used.

Two constructs were made for native expression and constitutive expression respectively under own promoter and 35S (SLAH1.2, Mi1.2). The gene constructs (SLAH1.2si, SLAH1.3si, ALMT2si, HKT1Asi, HKT1Bsi, 3HKTsi, and two other putative chloride exclusion gene silencing constructs) were prepared using the pHANNIBAL vector (Wesley et al., 2001) to incorporate sense and antisense fragments of the target genes using the primers listed in Table 1. These fragments were generated by Polymerase Chain Reaction (PCR) amplification of grapevine DNA using oligonucleotide primers based on the reference genome (Jaillon et al., 2007). The purified PCR fragments were initially ligated to an entry vector for ease of cloning and sequencing. They were then generally transferred to another vector such as pART7 or pHANNIBAL. The details for the generation of each construct are given below.

Table 1. Sequences of primers used to generate constructs for transformation.

Construct	Gene targets	Forward and Reverse Primers	DNA template	Product size
SLAH1.2 overexpression	SLAH1.2	aaatttgcggccgcGGATCAAGAGGAACCGAACA aatttagcggccgcGACAGGTTTAGTTAAT	140 Ruggeri	3146 bp
SLAH1.2si sense fragment	SLAH1.2	TCTCGAGATTTCGCATGGTGGAGGCAG TGGTACCTCCGATGACCGACAACTGACTC	140 Ruggeri	255 bp
SLAH1.2si antisense fragment	SLAH1.2	TGGATCCGATTTCGCATGGTGGAGGCAGA TATCGATTCCGATGACCGACAACTGACTC	140 Ruggeri	255 bp
SLAH1.3si sense fragment	SLAH1.3	TCTCGAGATCCATTGTTGCAACCCCATCT TGGTACCACAAGCACCTTAGAATATAGAT	140 Ruggeri	280 bp
SLAH1.3si antisense fragment	SLAH1.3	TGGATCCATCCATTGTTGCAACCCCATCT TATCGATACAAGCACCTTAGAATATAGAT	140 Ruggeri	280 bp
ALMT2si sense fragment	ALMT2	CGGATCCCATTACAATTAGTGGGTCATTGTC CATCGATAAGCAAAGCACAAAGTCGCTCA	140 Ruggeri	249 bp
ALMT2si antisense fragment	ALMT2	ACTCGAGCATTACAATTAGTGGGTCATTGTC CGAATTCAAGCAAAGCACAAAGTCGCTCA	140 Ruggeri	249 bp
HKT1Asi sense fragment	HKT1;1	TCTCGAGTGTTCGTCATGTTCTTTGGAAGAC TGGTACCACCAAAAATATGTGCAGGCTACA	Shiraz	260 bp
HKT1Asi antisense fragment	HKT1;1	TGGATCCTGTTCGTCATGTTCTTTGGAAGAC TATCGATACCAAAAATATGTGCAGGCTACA	Shiraz	260 bp
HKT1Bsi sense fragment	HKT1;3	TCTCGAGATCAACTGGGTATCACTGTGCT TGGTACCAGTAGTCGAAAACCTTGCTCAC	K51-40	260 bp
HKT1Bsi antisense fragment	HKT1;3	TGGATCCATCAACTGGGTATCACTGTGCT TATCGATAGTAGTCGAAAACCTTGCTCAC	K51-40	260 bp
3HKTsi sense fragment	HKT1;6, HKT1;7, HKT1;8	TCTCGAGGCCTGCTTCTTATCATCATTC TGGTACCGGCACCGATGATTTTCTGATAAG	Shiraz	294 bp
3HKTsi antisense fragment	HKT1;6, HKT1;7, HKT1;8	TGGATCCGGCCTGCTTCTTATCATCATTC TATCGATGGCACCGATGATTTTCTGATAAG	Shiraz	294 bp
Mi1.2 constitutive expression	Addition of a tomato resistance gene	GGTACCTATGGAAAAACGAAAAGATATTGA GGATCCTACTTAAATAAGGGGATATTCTTC	Plasmid vector	3.85 kb

5.1.1 *SLAH1.2* expression (SLAH1.2)

Using genomic DNA isolated from 140 Ruggeri, PCR was performed with an annealing temperature of 55°C using Pfx polymerase to generate a product of 3,146 bp *SLAH1.2* encompassing the *SLAH1.2* gene sequence plus the promoter and terminator. The primers used in this reaction are listed in Table 1. The product was purified using the gel extraction kit (Qiagen) and then ligated to TOPO Zero Blunt vector (Thermo Fisher) and used to transform OneShot TOP10 electrocompetent *E. coli*. Over fifty colonies were checked for the presence of the *SLAH1.2* fragment via colony PCR and plasmid DNA was extracted from a positive clone (Bioline Miniprep Kit) and sequenced (AGRF). NotI enzyme was used to cut the TOPO:SLAH1.2 vector and the pART27uGFP binary vector (pART27 (Gleave, 1992) containing GFP5 driven by

the Arabidopsis *ubiquitin* promoter). The transformation vector pART27uGFP was ligated with the SLAH1.2 gene fragment and transferred to XL1-Blue (Stratagene) electrocompetent *E.coli* cells. Colonies were screened by PCR, followed by NotI digestion.

5.1.2 *SLAH1.2* silencing construct (SLAH1.2si)

PCR was performed with an annealing temperature of 55°C using Pfx polymerase and *SLAH1.2*-specific primers listed in Table 1 to generate sense and antisense products of 255 bp from 140 Ruggeri DNA. The products were purified using a QIAGEN gel extraction kit and ligated separately to the TOPO Zero Blunt vector and used to transform TOP10 electrocompetent cells. Colonies were screened via colony PCR and DNA was extracted from potential positives and sequenced. The TOPO:SLAH1.2 sense fragment was digested with Xho1 and Asp718. The antisense vector pHANNIBAL (Wesley et al., 2001) was also restricted with the same enzymes, and the sense fragment ligated to pHANNIBAL. The vector containing the sense fragment was used to transform XL1-Blue electrocompetent cells. Colonies were screened via PCR and six clones were selected for DNA extraction (Bioline). DNA from a positive clone was then cut with BamHI and ClaI as was the TOPO:SLAH1.2 antisense vector. The fragments were ligated and used to transform XL1-Blue cells. Four colonies were screened by digesting extracted DNA with HindIII and SpeI. NotI enzyme was used to cut the fragment from pHANNIBALSLAH1.2si and this was ligated to pART27uGFP (previously cut with NotI). Restriction digest confirmed the correct insertion of the cassette.

5.1.3 *SLAH1.3* silencing construct (SLAH1.3si)

An annealing temperature of 55°C and Pfx polymerase were used for PCRs to generate sense and antisense products of 280 bp of the *SLAH1.3* gene from 140 Ruggeri DNA. The products were purified using a QIAGEN gel extraction kit and sub-cloned separately into the TOPO Zero Blunt vector and used to transform TOP10 electrocompetent cells. Clones were screened using colony PCR and plasmid DNA from potential positives was sequenced. The TOPO:SLAH1.3 sense fragment was digested with Xho1 and Asp718. pHANNIBAL was also cut with Xho1 and Asp718, and the sense fragment ligated to pHANNIBAL. The vector containing the sense fragment was used to transform electrocompetent XL1-Blue competent cells. Colonies were screened via PCR and DNA was purified from six clones. DNA from a positive clone and the TOPO:SLAH1.3 antisense vector were then restricted with BamHI and ClaI. The fragments were ligated and used to transform electrocompetent XL1-Blue cells. Four colonies were screened by DNA extraction followed by restriction enzyme digestion with NotI enzyme to cut the fragment from pHANNIBALSLAH1.3si. The isolated fragment was ligated to the vector Not1-restricted pART27uGFP. Restriction digest confirmed the correct insertion of the gene silencing cassette.

5.1.4 *ALMT2* silencing construct (ALMT2si)

PCRs were performed with an annealing temperature of 46°C using MyTaq polymerase to generate sense and antisense products of 300 bp from a 140 Ruggeri genomic DNA template. The products were purified using a QIAGEN gel extraction kit, ligated separately to the QIAGEN pDrive vector and used to transform XL1-Blue cells by electroporation. Clones from the sense fragment cloning were screened via colony PCR, and DNA from potential positives was sequenced. The pDrive:ALMT2 sense construct was digested with BamHI and ClaI. pHANNIBAL was also cut with the same enzymes, and the sense fragment ligated to it and used to transform XL1-Blue cells. Colonies possibly containing the antisense fragment were screened by PCR and six clones were selected for DNA extraction. A positive clone was then cut with XhoI and EcoRI as was the pDrive:ALMT2 antisense vector. The fragments were ligated and used to transform XL1-Blue cells by electroporation. DNA from five clones was screened by digesting with XhoI and BamHI and sequencing. NotI enzyme was used to cut the fragment from pHANNIBALALMT2si and this was ligated to pART27uGFP (previously cut with NotI). Restriction digest confirmed the correct insertion of the ALMT2 cassette.

5.1.5 *HKT1;1* silencing construct (HKT1Asi)

Due to the similarity of the six *HKT* gene sequences, the design of this and the following two constructs was carefully planned to target only the desired genes. Using Shiraz genomic DNA as the template, PCRs were performed with an annealing temperature of 50°C and Pfx polymerase to generate sense and antisense products of 260 bp of the *HKT1;1* gene. The products were purified using a QIAGEN gel extraction kit and ligated separately to the TOPO Zero Blunt vector and used to transform TOP10 electrocompetent cells. Four clones were selected and plasmid DNA sequenced. The TOPO:HKT1A sense fragment and the vector pHANNIBAL were digested with Xho1 and Asp718 and ligated together before being used to transform XL1-Blue electrocompetent cells. The same procedure was followed for the antisense fragment except that the verified fragment was removed from the TOPO vector using BamHI and ClaI. This fragment was ligated pHANNIBAL which already contained the sense fragment and had been restricted with BamHI and ClaI enzymes. The fragments were ligated and used to transform XL1-Blue electrocompetent cells. DNA was isolated from four colonies and screened by restriction enzyme digestion. NotI enzyme was used to cut the fragment from pHANNIBALHKT1Asi and this fragment of DNA was ligated to NotI–restricted pART27uGFP. Restriction digest confirmed the correct insertion of sense-antisense cassette.

5.1.6 *HKT1;3* silencing construct (HKT1Bsi)

PCR was performed with an annealing temperature of 50°C using Pfx polymerase to generate sense and antisense products of the 260 bp of HKT1;3 gene from genomic DNA extracted from the rootstock K51-40. The cloning strategy was followed as above in 5.1.5 with the PCR fragments first being sequenced in the entry vector, TOPO before being ligated to pHANNIBAL which provides a cassette with the 35S constitutive promoter and an intron to help with

expression and stabilisation of the hairpin sequence required for this method of gene silencing. The cassette is then inserted in the plant transformation vector, pART27uGFP.

5.1.7 Construct to silence all three *HKT1;6*, *1;7* and *1;8* (3HKTsi)

The primers for this construct were carefully designed to generate a DNA fragment with sufficient homology to all three HKT gene targets, *HKT1;6*, *1;7* and *1;8* to silence these genes, while not silencing *HKT1;1* or *1;3*. PCRs were performed with an annealing temperature of 50°C using Pfx polymerase to generate sense and antisense products of 294 bp. The template for these PCRs was genomic DNA extracted from Shiraz.

5.1.8 Chloride gene 1 silencing construct

PCRs were performed with an annealing temperature of 58°C using MyTaq polymerase, Shiraz genomic DNA and gene specific primers and to generate sense and antisense products of 135 bp of the gene. The products were purified using a QIAGEN gel extraction kit and sub-cloned separately into the TOPO TA vector and used to transform TOP10 chemically competent cells by heat shock. Six clones were selected and plasmid DNA sequenced. The TOPO sense fragment was digested with Xho1 and EcoRI. pHANNIBAL was also cut with Xho1 and EcoR1, and the sense fragment ligated to the vector; this was used to transform XL1-Blue electrocompetent cells. Colonies were screened using PCR and four clones were selected for DNA extraction. A positive clone was then cut with BamHI and XbaI as was the TOPO: antisense vector. The fragments were ligated and used to transform XL1-Blue electrocompetent cells. DNA was extracted from six clones and screened by restriction enzyme digestion. NotI enzyme was used to cut the fragment from pHANNIBAL from the selected clone and this was ligated to pART27uGFP (previously cut with NotI). Restriction digest confirmed the correct insertion of the gene silencing cassette.

5.1.9 Chloride gene 2 silencing construct

PCRs were performed with an annealing temperature of 58°C using MyTaq polymerase to generate sense and antisense products of 138 bp. The products were purified using a QIAGEN gel extraction kit and ligated separately to the TOPO TA vector and used to transform TOP10 chemically competent cells. Plasmid DNA was extracted from four clones each containing the sense and the antisense fragments and sequenced. The TOPO sense construct was digested with EcoR1 and Asp718 to release the sense fragment. The vector pHANNIBAL was also restricted with EcoR1 and Asp718, and the sense fragment ligated to the vector. The vector containing the sense fragment was used to transform electrocompetent XL1Blue cells. Colonies were screened using PCR and four clones were selected for further investigation. Plasmid DNA from a positive clone was then cut with BamHI and ClaI as was the TOPO: antisense vector. The ligated fragments were used to transform XL1-Blue electrocompetent cells. Four clones were screened by restriction enzyme digestion of extracted plasmid DNA. NotI enzyme was used to cut the fragment from pHANNIBAL and used to ligate to

pART27uGFP (previously cut with NotI). Restriction digest confirmed the correct insertion of the gene silencing cassette. The construct was then used to transform *Agrobacterium,* ready for plant transformation.

5.1.10 *Mi1.2* constitutive expression

This construct proved very difficult to generate and it was found to be unstable in most *E.coli* strains that were tried. Using a plasmid DNA as the template PCR was performed with an annealing temperature of 50°C and a long extension time utilising Pfx polymerase to generate the coding region 3.85 kb. The product was purified using the QIAGEN gel extraction kit and then ligated with TOPO Zero Blunt vector and used to transform TOP10 electrocompetent cells. Ten colonies were checked for the presence of the *Mi1.2* fragment via colony PCR and DNA of a positive clone was sequenced. The TOPO: Mi1.2 vector was digested with Asp718 and BamHI, as was pART7 and these fragments were ligated, used to transform *E.coli* DH10B and colonies were screened. NotI enzyme was used to cut the TOPO:Mi1.2 vector and the pART27uGFP binary vector, which were then ligated together and used to transform DH10B Electromax competent cells (Thermo Fisher). Positive clones were screened by PCR and digesting with NotI.

5.1.11 *Agrobacterium* transformed with each construct

Transformation-ready constructs in the vector pART27uGFP4a were used to transform *Agrobacterium* strain EHA105 using the electroporation method and positive colonies were checked by PCR or back-transformation to *E.coli*, for further analysis. Stocks of the transformed *Agrobacterium* were then stored as 15 % glycerol solutions frozen at -40^{o}C. For plant transformation, a loop of the glycerol stock was dilution-streaked on a LB agar plate containing rifamcin and spectinomycin and incubated at 28^{o}C for two days.

5.2 Grapevine transformation

Transgenic grapevines were produced by *Agrobacterium* transformation of an embryogenic callus culture derived from anthers. Transformation methods are based on the methods of Iocco et al., (2001) with improvements based on discussions with Alain Bouquet, Montpellier, France and Dennis Gray and his lab, Tampa, Florida in 2008 (Li et al., 2008). These improvements were detailed in the Final Report for CSP 1001.

5.2.1 Initiation and culture of embryogenic callus of *Vitis vinifera* cv. Shiraz

Embryogenic callus was initiated and maintained using media and methods as described by Franks and Iocco (Franks et al. 1998; Iocco et al. 2001). Embryogenic callus was initiated in spring from immature flower buds (at the stage where anthers are colourless) collected from the Coombe Vineyard. Flower buds were sterilised for 40-50 seconds in 7% calcium hypochlorite that had been filtered through two sheets of Whatman 2V filter paper and to which two drops of Tween 20 were added per 100 mL. After sterilisation, flowers were rinsed

three times in sterile water. Filaments with anthers attached were dissected from flower buds and placed, concave-side down, into Petri dishes (100 x 15 mm) containing PIV medium, which comprised NN macronutrients (720 mg/L NH_4NO_3, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_2O$, 950 mg/L KNO_3, 170 mg/L KH_2PO_4), Fe EDTA (37.2 mg/L $Na_2EDTA.2H_2O$, 9.3 mg/L $FeSO_4.7H_2O$), 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), 4.5 μM 2,4–dichlorophenoxyacetic acid (2,4D), 8.9 μM benzylaminopurine (BAP), 3% sucrose, pH 5.7, solidified with 0.3% Phytagel (Sigma) (Franks et al., 1998). Each Petri dish contained about 70 anthers. After 6-8 weeks developing embryogenic callus was transferred to C1 medium (MS macronutrients (1.650 g/L NH_4NO_3, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_2O$, 1.970 g/L KNO_3, 170 mg/L KH_2PO_4), Fe EDTA, MS micronutrients, 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), 5.0 μM 2,4D, 1.0 μM BAP, 0.1% casein enzymatic hydrolysate, 3% sucrose, pH 5.8, 0.5% Phytagel) in deep Petri dishes (100 x 20 mm) (Iocco et al., 2001). Embryogenic callus was maintained on C1 by sub-culturing selected tissue to fresh medium every five weeks using a stereomicroscope. At all stages Petri dishes were sealed with Parafilm and incubated at 28°C in the dark.

For transformation of grape embryogenic callus, an improved method developed from those of Iocco et al. (2001) and Li et al. (2008) was used. Two weeks after transfer to C1, embryogenic callus selected for transformation, was transferred to a modified GS1CA medium comprising NN macronutrients, MS micronutrients, B5 vitamins, Fe EDTA, 10 μM naphthoxyacetic acid (NOA), 1.0 μM BAP, 6% sucrose, pH 6.2 with 0.25% activated charcoal and 0.7% tissue culture agar (A175, Phyto Technology Laboratories) (Franks et al., 1998). Callus was grown on this medium for 14 days at 28°C in the dark before infection.

5.2.2 Transformation of embryogenic callus of *Vitis vinifera* cv. Shiraz

Agrobacterium was grown on LB agar plates with 25 μg/mL rifampicin and 100 μg/mL spectinomycin. Plates were streaked five days prior to callus infection (Iocco et al., 2001) and incubated for 2-3 days at 28°C. Two days prior to infection, a single colony was selected and used to inoculate 3 mL of modified MG/L liquid medium (Iocco et al., 2001), containing spectinomycin, and incubated overnight shaken at 200 rpm and at 28°C. On the afternoon prior to infection, 25 mL cultures of MG/L plus spectinomycin in a 125 mL conical flask were inoculated with dilutions of *Agrobacterium* solution from the 3 mL culture to obtain a culture, with an OD at 600 nm of 0.8-1 after incubation of 16-18 h (Li et al., 2008). The selected culture was transferred to a 50 mL centrifuge tube and the cells precipitated by centrifugation at 5,000 rpm for 8 minutes. The supernatant was discarded and the pellet resuspended in 25 mL

LCM (GS1CA medium without hormones, charcoal or agar) with 2% sucrose. This culture was incubated for 3 h and then used for infection of grape callus.

Embryogenic callus was collected from GS1CA plates and placed into an empty sterile Petri dish. Immediately 3-5 mL of bacterial suspension was added to the callus and incubated for 10 minutes at room temperature. After infection the bacterial suspension was pipetted from the callus. Callus was then collected and placed in small clumps in 90 mm Petri dishes each containing three pieces filter paper (Whatman #1) saturated with GS1CA liquid medium modified by a reduction in sucrose to 3% and the omission of charcoal and agar (Li et al., 2008). Callus was incubated in the dark at 22°C for 48 hours.

Infected embryogenic callus was then collected and washed in LCM medium with 6% sucrose, and timentin (Glaxo Smith Kline, Australia) at 1000 μg/mL (Iocco et al., 2001). Callus was collected on a 100 μm nylon net filter (Millipore), blotted onto a double layer of filter paper (Whatman #1) and then transferred to deep Petri dishes containing GS1CA with 1000 μg/mL timentin and incubated at 28°C in the dark. After 10 days callus was transferred to plates containing GS1CA with timentin as before and the selection antibiotic kanamycin. Kanamycin was used at a concentration of 100 μg/mL until eight weeks post-infection after which it was reduced to 70 μg/mL or 50 μg/mL. Four weeks post-infection, callus was transferred to Petri dishes containing GS1CA medium without hormones and with timentin and kanamycin. Developing callus continued to be transferred to this medium at four weekly intervals. Germinated embryos expressing green fluorescent protein (GFP) were collected from these plates.

Intact embryos were transferred to 70mL polycarbonate tissue culture vessels containing 15 mL MG2 medium (GS1CA medium with 2 μM BAP and sucrose reduced to 3%) (Torregrosa et al., 2002) and incubated for 4-6 weeks. Throughout this period, as they developed, primary shoots were removed and transferred to 250 mL polycarbonate tissue culture vessels with RM (rooting medium, similar to SM medium - 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, B5 vitamins, Fe EDTA, 1.5% sucrose, pH 5.7, 0.7% tissue culture agar) supplemented with 0.5 μM naphthaleneacetic acid (NAA)). Roots were produced on explants within 2-3 weeks. Explants that did not produce primary shoots on MG2 were removed and trimmed, so that cotyledons measured 5 mm or less and were supported by about 5 mm of hypocotyl. These explants were transferred to 70 mL polycarbonate tissue culture vessels containing 15 mL of modified-Murashige and Skoog medium called MS/2 (half MS macronutrients, MS micronutrients, C1 vitamins and amino acids, Fe EDTA, 1.5% sucrose, pH 5.7, 0.7% tissue culture agar) with 2 μM BAP. When primary shoots developed they were placed on rooting medium as described above. All explants growing in tissue culture vessels were incubated under cool white and Grolux lights (Sylvania) with a photoperiod of 16 hours light: 8 hours dark at 28°C.

To confirm the presence of the introduced gene in plantlets, the youngest terminal, partially to just expanded, glossy leaf was removed using sterile technique, from a young rooted plantlet, frozen in liquid nitrogen and stored at -80°C prior to DNA extraction and PCR testing. Positive transgenic plantlets were propagated by taking tip and nodal cuttings and planting these in 500 mL polycarbonate tissue culture containers containing 75 mL of RM.

5.3 Initiation and culture of embryogenic callus of grapevine rootstock varieties

5.3.1 Initiation of embryogenic callus from anthers and ovaries

Inflorescences with immature flower buds were collected and sterilised using the method described for Shiraz in 5.2.1. Anthers were dissected from flower buds of 140 Ruggeri and both anthers and ovaries from flower buds of K51-40 and Ramsey. Explants were placed into Petri dishes containing initiation medium. Each Petri dish contained approximately 70 explants. Three different initiation media were tested PIV (see section 5.2.1), Harst (Harst - NN macronutrients, Fe EDTA, MS micronutrients, B5 vitamins, 10.0 μM 2,4D, 5.0 μM Thidiazuron (TDZ), 3% sucrose, pH 5.7, solidified with 0.3% Phytagel (Franks et al., 1998), and MS1 (MS macronutrients, micronutrients and vitamins, 4.5 μM BAP, 5.0 μM 2,4 D, 0.1g/L myo inositol, 2% sucrose, pH 6.0, 0.7% tissue culture agar) (Gray and Mortensen, 1987). After 7-8 weeks developing callus was transferred to C1.

5.3.2 Initiation of embryogenic callus from *in vitro* leaves

To provide a source of *in vitro* leaves from which to initiate embryogenic callus it was first necessary to obtain healthy shoots in sterile culture. Plantlets of rootstocks K51-40, 140 Ruggeri, Schwarzmann and Ramsey together with *V. vinifera* cv. Riesling were established in culture using a method routinely used successfully for *V. vinifera* cultivars. Young canes were collected and leaves removed at the point of attachment of the petiole. Canes were cut through internodes into sections about 8 cm long and containing one or more nodes, so that there was at least 1.5 cm of stem tissue remaining either side of each node. These nodal sections were sterilised in a solution of sodium dichloroisocyanurate (500 mg/L) containing Tween 20 (100 μL/L) overnight at room temperature. Sterilising solution was removed and tissue rinsed three times with sterile water. Stems were cut into single node sections, petioles trimmed to 2-3 mm and each node placed into a 120 mL polycarbonate container with 30 mL of SM medium. Nodes were incubated under cool white and Grolux lights (Sylvania) with a photoperiod of 16 hours light: 8 hours dark at 28°C.

Healthy growing tips were removed and placed on RM. A second batch was harvested from SM medium eight weeks after initiation and placed on SM. From SM, plantlets were transferred to and maintained on MS/2 medium to provide leaves for initiation of embryogenic callus. Long-term cultures were maintained on RM with regular subculturing of

tips and nodes. All shoot cultures were grown in 500 mL polycarbonate tubs under the same conditions as those used for initiation.

A method based on those of Dhekney et al. (2009) and Harst (1995) was used to initiate embryogenic callus. Unopened leaves 1.5 to 5 mm long or small expanding leaves were harvested from *in vitro*-grown shoot cultures growing on MS/2 medium. Whole unopened leaves were placed on to NB2 medium (NN basal salts (Phyto Technology Laboratories N613), MS vitamins (Phyto Technology Laboratories M553), 1000 μM 2,4D, 1000 μM BAP, 100 mg/L myo inositol, 2% sucrose, pH 6.0, 0.7% agar or NB3 medium (NN basal salts N613, MS vitamins M553, 1000 μM NOA, 1000 μM TDZ, 100 mg/L myo inositol, 2% sucrose, pH 6.0, 0.7% agar) in deep Petri dishes. The pedicel was removed from opened leaves and these were placed adaxial side down onto the same two media. Five leaves were placed on each Petri dish and these incubated in the dark at 27°C for 5-7 weeks for callus to develop. Pre-embryogenic callus was transferred to the light (65 $\mu m.m^{-2}.s^{-1}$) for five weeks and plates checked weekly for the development of embryogenic callus. Embryogenic callus was transferred to X6 medium which comprised X6 macronutrients (364 mg/L NH_4Cl_2, 3.030 g/L KNO_3, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_20$, 170 mg/L KH_2PO_4), MS micronutrients, Fe EDTA, X6 vitamins (0.01 mg/L Thiamine HCl, 0.05 mg/L Nicotinic Acid, 0.05 mg/L Pyridoxine HCl), 1 g/L myo inositol, 6% sucrose, pH 5.8, 0.5 g/L activated charcoal and 0.7% agar and was sub-cultured every 4-6 weeks.

5.4 GFP detection of transformed material

About four days after washing to remove *Agrobacterium*, transformed callus was examined under a Leica dissecting microscope with mercury source and appropriate filters for GFP fluorescence. A burst of transient expression of GFP is usually observed at this time. Callus that does not have good levels of GFP expression is discarded at this stage. At about 10-12 weeks after transformation, callus is examined again for GFP fluorescence. Usually around this stage, transformed embryos start to become visible and callus with fluorescence is sub-cultured. All embryos are checked for the presence of GFP before being transferred to shoot development medium.

5.5 Confirmation of transgenic vines

5.5.1 PCR test for the presence of the vector in *in vitro* plantlets

To screen the plantlets for the presence of the transgene, total grapevine genomic DNA was extracted from the youngest expanded glossy leaf using the Isolate II Plant DNA Mini Kit (Bioline) and subjected to PCR. PCR conditions were: initial denaturation at 94°C for three minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds, and a final extension of 72°C for 10 minutes. For the *Mi1.2* construct, the extension step of 72°C for 60 seconds was extended to 90 seconds. The transgene of interest was assessed for its presence in the extracted DNA using primers listed in Table 2. An endogenous

sequence originally developed as a marker for berry colour was used as a control for DNA quality and amplified using the primers 20D18F (5'-GATGACCAAACTGCCACTGA-3') and 20D18R (5'-ATGACCTTGTCCCACCAAAA-3').

Table 2. Sequences of primers used to test for presence of transgene.

Transgene	**Primer**	**Product size**
SLAH1.2 over expression	p274a874For (5'-ATGATTACGCCAAGCTATTT-3') SLAH1.2LHREV12 (5'-AACGTCGCTTTCTTAATCC-3')	1359bp
SLAH1.2silencing	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') SLAH1.2Asp718R1 (5'-TGGTACCTCCGATGACCGACAACTGACTC-3')	305bp
SLAH1.3silencing	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') SLAH1.3Asp718R1 (5'- TGGTACCACAAGCACCTTAGAATATAGAT-3')	340bp
ALMT2silencing	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') ALMT2EcoR1JWR1N (5'-CGAATTCAAGCAAAGCACAAAGTCGCTCA-3')	309bp
HKT1;1 silencing	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') HKT1A-Vv-A-HJR1 (5'-TGGTACCACCAAAAATATGTGCAGGCTACA-3')	320bp
HKT1;3 silencing	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') HKT1BsiAsp718JWR1 (5'-TGGTACCAGTAGTCGAAAACCTTGCTCAC-3')	320bp
3HKTsilencing HKT1;6, 1;7, 1;8	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') 3HKT1Asp718JWR1 (5'-TGGTACCGGCACCGATGATTTTCTGATAAG-3')	354bp
Mi1.2 expression	35SF3 (5'-TTCGCAAGACCCTTCCTCTA-3') JWM:1.2R3 (5'-CAGCATCCCCAAAGAATGAT-3')	1435bp

5.5.2 Southern blot analysis to assess copy number of vector inserts and determine independence of transformation events

Genomic DNA was extracted from 1 g of very young leaflets from glasshouse-grown plants as described in Walker et al. (2006) and 2 μg of 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.

5.6 Gene expression analysis

Expression of tannin pathway genes was determined by real time PCR. Leaves were collected from transgenic grapevines in October each year. The leaves were still expanding and were approximately 15 cm^2 in area (Stage 3, Bogs et al., 2005). Leaf laminae were frozen in liquid nitrogen and stored frozen in sealed plastic bags at -80°C. Samples of developing berries were collected two weeks after flowering (2 wpf) and at veraison (following first signs of colour change, around 9-11° Brix) in 2013/14 (RR Series). The berry samples were collected on ice then frozen in liquid nitrogen in the laboratory. The veraison samples were dissected into skin and seeds on ice prior to freezing in liquid nitrogen. The frozen tissue samples were ground to a fine powder while frozen using a mortar and pestle or an electric grinding mill (IKA Model A11) cooled with liquid nitrogen.

RNA was isolated from the frozen and finely ground tissues of leaf, stem and root from the transgenic vines and Shiraz control vines grown in the same glasshouse compartment using the Spectrum Total Plant RNA Kit (Sigma) including an on-column DNase digestion step using an On-Column DNase 1 Digest Set (Sigma Cat. DNASE70). For leaf, 50-70 mg of ground tissue was used for each preparation and 350-500 μL of binding buffer was used for loading onto the binding column. For stem and root tissues, low RNA contents made it necessary to modify the procedure slightly. The RNA was isolated as before but preparations were made using only 250 μL of binding buffer. cDNA was synthesised from 1 μg aliquots of sample of RNA using the Phusion-RT-PCR Kit (Thermo Scientific). Before use for qPCR, the cDNAs were diluted 1:10 with sterile water.

Quantitative Real Time PCR (qPCR) was undertaken on a Rotor-Gene 3000 machine (Corbett Research, NSW, Australia). Diluted template cDNA (5 μL) was added to each PCR reaction in a final volume of 15 μl containing 266 nM of each primer (listed in Table 3) and 1 x LightCycler 480 SYBR Green I Master mix (Roche Life Sciences). Thermocycling conditions were as follows: an initial enzyme activation of 15 min at 95°C, followed by 35-40 cycles of denaturation for 25 s at 95°C, annealing for 25 s at 58°C, and extension for 25 s at 72°C. After cycling, a 5 min hold step at 72°C was followed by a melt gradient starting from 72°C and heating to 95°C at a rate of 0.2°C s^{-1}. The fluorescence of reactions was measured with an excitation wavelength of 470 nm and a detection wavelength of 510 nm at the end of each extension step and at each 1°C increment of the melt profile. The specificity of the PCR product generated for each set of primers was tested in three ways: i) by the melt gradient in which fluorescence decreases at a single discrete temperature indicating separation of the two strands of a single DNA species, ii) by agarose gel electrophoresis showing a single PCR product of the correct size and iii) by obtaining the correct DNA sequence of the gene from a PCR product extracted from an agarose gel. All cDNA samples to be compared for gene expression levels were analysed in a single batch for each primer pair and each set of analyses was conducted in triplicate. *Ubiquitin* was used as an internal standard gene and runs analysing each target

gene were accompanied by analysis of *ubiquitin* expression. The sequences of the forward and reverse primers used for each of the target genes is shown in Table 3.

Table 3. Sequences of primers used for qPCR.

Target Gene	Forward Primer	Reverse Primer	Product (bp)
VvUbiquitin	UbiF1 AACCTCCAATCCAGTCATCTAC	UbiR1 GTGGTATTATTGAGCCATCCTT	182
ALMT2	qAMLT F1 ATTCTCTCGCATGGACGGAC	ALMTEcoR1-JWR1N CGAATTCAAGCAAAGCACAAAGTCGCTCA	265

5.7 Phenotypic assays of transgenic plants

Ability to uptake chloride or sodium was assessed by salt assays using plants grown in tissue culture and the glasshouse.

5.7.1 Tissue culture salt assay

Plantlets were grown on RM medium for 3-5 weeks. Tips, 1.5 cm in length, were taken and placed into the centre of a tub containing 70 mL RM medium solidified with tissue culture grade agar A175 (Phyto Technology Laboratories), eight for each line. RM media has no added chloride as part of the salts; however, it does contain about 5mM, probably from the agar.

Tubs were placed in low light for two days and then moved onto shelves with four lights and grown for 4-5 weeks. Tubs were rotated twice a week to ensure more even light and heat distribution during the experiment. At week 4, pairings (four per line) were made so that similarly growing plantlets were matched. One of the pair became the water control and the other became the plantlet treated with salt. On day 1 of salt application, 1.4 mL 1.25 M mixed chloride in the ratio of $6Na^{+}$:$1Ca^{2+}$:$1Mg^{2+}$ was added to opposite sides of the 'salt' tub using aseptic technique and allowed to absorb for two hours before moving the tub back into the growth chamber. On day 1, 4.2 mL of sterile Milli-Q water was added to the control tubs and placed back into the growth room. The agar in these tubs contained about 5 mM chloride as measured at the end of the experiments. On day 2, 1.4 mL of 1.25 M chloride was added to the 'salt' tubs and allowed to absorb for two hours, then another 1.4 mL of 1.25 M chloride was added and allowed to absorb for another two hours to a final concentration of 75 mM mixed chloride. Plants were placed back into the growth room and allowed to grow for 14-20 days. On the day of harvest, meristems and a root sub-sample were snap frozen in liquid nitrogen for molecular biology use. The number of leaves and laterals were counted and recorded. The tops of the plant were rinsed and dried then wrapped in foil and placed in drying oven. Roots were collected in the first two experiments but the amount of material recovered after drying was very small, so they were only collected for gene expression after this.

5.7.2 Glasshouse salt assay

Plants were de-flasked into 100 mm standard black pots containing a sand perlite mix and watered with 0. 2 g/L Aquasol (Yates). Plantlets were hardened-off over a three week period by decreasing the humidity and increasing light to standard glasshouse growing conditions (26°C day - 23°C night with a light-break 18:00-24:00h).

Plants were de-flasked and left to grow under normal glasshouse conditions. When the plants had been growing for about six weeks the watering changed to ¼ strength Hoagland's solution for about three weeks. On day 1 of the salt assay, plants were watered with ¼ strength Hoagland's + 25mM chlorides or RO water at 50 mL per plant. On day 2, plants were watered with ¼ strength Hoagland's + either 50mM mixed chloride or RO water and from day 3 until the end of the experiment with ¼ strength Hoagland's + either 75mM chlorides or RO water added at 50mL/plant/day. Plants were harvested on day 28. Meristem, root and leaf samples were taken for molecular or ion analysis.

5.8 Ion analysis

5.8.1 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 4 mL glass vials and 3 mL of acid reagent (100 mL glacial acetic acid and 6.4 mL of concentrated nitric acid per litre) added and allowed to extract for 30 min, then four drops of gelatin reagent (Labonco Buchler Instruments, Labonco Corporation, Kansas City, MO, USA) were added just prior to analysis (Walker et al., 2010).

5.8.2 Cation concentration

Dried samples were examined in duplicate for the concentration of the cations, K^+, Na^+, Ca^{2+} and Mg^{2+}. Extracts of approximately 100 mg powdered samples were weighed into 15 mL polypropylene digestion vessels (SC415) (Environmental Express, Mt Pleasant, SC, USA) and 2 mL of 15 mol/L nitric acid was added. They were held in a fume hood 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 60 min and subsequently at 85°C for 210 min. Ninety-six vessels were heated per batch. The tubes were allowed to cool, then made up to 15 mL with MilliporeR reverse osmosis water and then inverted to mix thoroughly. The solutions were then filtered through 125 mm Whatman No 42 filter paper. Aliquots of approximately 10 mL were transferred to polypropylene tubes for cation analyses.

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

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

6 Results

6.1 Analysis of Shiraz transformants

Ten constructs were successfully used to transform Shiraz callus in 28 different transformation events, eventually producing significant numbers of transformed embryos. Nine of the constructs were generated using sequences from genes associated with salt stress while one construct was designed to provide resistance to the biotic stress of nematodes.

6.1.1 Over-expression of a slow-type anion channel-associated gene (ST143 & ST149 SLAH1.2)

In two separate experiments Shiraz embryogenic callus (2012 & 2014) was transformed with *Agrobacterium* strain EHA105 containing the DNA vector designed to increase expression of the *VvSLAH1.2* gene. From the first transformation 29 embryos gave rise to six plantlets positive for the construct by PCR tests. However, these were weak plants and none was used for later characterisation. In the second experiment, 116 germinated embryos were selected based on GFP fluorescence and transferred to shoot production medium. Eighty-four percent of embryos transferred developed shoots. Of those plantlets produced, 59 were designated as SL lines and tested by PCR for the presence of the transgene. Three were shown to be negative for the *VvSLAH1.2* construct. Plantlets negative for the construct are very useful as negative controls in further experiments and are therefore propagated together with selected positive lines. Many of the lines had similar embryo numbers suggesting that they might be derived from the same transformation event and many were slow to come through the transformation process; ultimately only 24 lines were selected for analysis.

The selected lines were propagated to increase the number of plantlets of each line for salt assays and to provide young leaves for DNA extractions. Southern blots were performed as outlined in section 5.5.2. Interpretation of the Southern blots led to the following conclusions. Only one line, SL26, appears to be negative and the other lines negative by PCR testing are positive for both ends of the construct. SL3 is missing the left border and therefore does not contain a complete DNA insertion. SL20, SL25 and SL30 are all derived from the same insertion event and are just clones of each other as are pairs SL21 and SL23, and SL27 and SL31.

To ascertain whether SLAH1.2 could increase the ability of Shiraz to exclude chloride, propagated lines were placed in tubs and assayed in five batches, together with A lines and Shiraz controls. Conditions were kept as similar as possible for each batch so that the results of each could be compared. Each line consisted of four replicate cuttings in individual 500 mL tubs for salt and control treatments. When the results of all batches were compared, it was seen that the controls generally contained more salt than many of the transgenic lines.

For a glasshouse experiment, seven lines were selected that had a range of phenotypes together with the negative control SL26 and two A lines, A5 and A40, that were also negative

controls. The experiment was carried out following the methods given in section 5.7.2. We showed that SL32 took up more chloride than the controls while all the other transgenic lines had less chloride. This result suggests that an even better effect might be seen if the accumulator K51-40 was transformed with this construct.

6.1.2 Down-regulation of a slow-type anion channel-associated homologue (ST154 & ST166 SLAH1.2si)

Shiraz grapevine embryogenic callus, from cultures initiated in 2014 and 2015, was transformed with *Agrobacterium* containing a DNA silencing vector designed to decrease expression of the *VvSLAH1.2* gene. From the transformation using the Shiraz embryogenic culture initiated in 2014 and based on the visual presence of GFP, 61 germinated embryos were selected and transferred to shoot production medium. 77% of the transferred embryos developed shoots. Of those plantlets produced, 35 have been designated as SS lines and tested for the presence of the transgene. By PCR testing, 33 lines were confirmed as positive for the *VvSLAH1.2si* construct and two were negative.

From the second transformation using the Shiraz embryogenic culture initiated in 2015, 64 GFP-expressing germinated embryos were selected and transferred to shoot induction medium. Eighty six percent of these embryos produced a shoot. Seven plantlets from this transformation have been tested for the presence of the transgene, and all of these were confirmed positive for *VvSLAH1.2si*. PCR testing of additional plantlets from this transformation should be completed in August 2017.

This set of transgenic plants will be assayed in 2017/18.

6.1.3 Down-regulation of a slow-type anion channel-associated homologue (ST167 SLAH1.3si)

Shiraz grapevine embryogenic callus from cultures initiated in 2015, was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the *VvSLAH1.3* gene. Based on GFP fluorescence, 55 germinated embryos were selected and transferred to shoot production medium, 50 of which developed shoots. Plantlets produced from this transformation were designated as SI lines and tested for the presence of the transgene using PCR. So far, 12 plantlets have been tested and nine of these have been confirmed as positive for *VvSLAH1.3si*. Work is ongoing with this transformation and by the end of September 2017 we expect to have in excess of 40 plantlets produced and tested.

This set of transgenic plants will be assayed in 2017/18.

6.1.4 Down-regulation of an aluminium-activated malate transporter (ST148 ALMT2si)

Shiraz grapevine embryogenic callus (2014) was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the *VvALMT2* gene. Based on the visualisation of GFP, 163 germinated embryos were selected and transferred to shoot

production medium. Ninety percent of transferred embryos developed shoots. Of those plantlets produced, 67 were designated as A lines and tested for the presence of the transgene using PCR. Of these, 62 were presumed to be transformed with the *VvALMT2* silencing vector while five lines appeared to be negative for the construct.

Thirty-five lines were selected based on different embryo identification numbers and propagated by tip and node cuttings in RM media. Some young plant material was used for DNA extraction. When sufficient material had been generated, salt assay experiments Batch 1-5 included lines from this transformation.

Southern blot analysis was carried out with genomic DNA extracted from 35 A lines and probed with the ends of the DNA construct.

The line A5, A12 and A40, which had all been negative in PCR reactions, were confirmed to be negative by Southern blot analysis. Line A9, A10, A19 and A24 were all from the same insertion event as were A6 and A23. Some lines which appeared to have very similar embryo numbers were shown to be independent transformants and shows the importance of performing this analysis.

Salt assays were conducted in tissue culture across five batches and the chloride concentration in the shoots of the salt-treated plants was measured. Duplicate lines that were assayed and were shown to be very consistent for this transformation.

Some lines were selected for a glasshouse salt assay which was performed together with selected SL lines to have a range of phenotypes including two with the lowest chloride uptake and four with the highest in tissue culture assays. As ALMT2 was predicted to enhance chloride exclusion, the expected result was that the transformants would take up more chloride than the negative controls. However, most lines had very similar levels to Shiraz and the other negative controls. A7, which had the lowest concentration in the tissue culture experiments, had the highest in the glasshouse experiment.

Gene expression was examined in the roots of a few lines of the ALMT2si plants. Methods for RNA extraction are summarised in section 5.6. A reduction of ALMT endogenous gene expression of 2-3 fold in lines A9 and A11 relative to the negative control of A12 was determined by qPCR and this result is consistent with a silencing event.

These results suggest that ALMT2 may not play a critical role in chloride exclusion. Another possibility is that there is redundancy in this role so reducing the expression of ALMT2 does not have an impact on chloride exclusion when assayed in these young plants.

6.1.5 Down-regulation of a high affinity K^+ transporter HKT1;1 (ST150 HKT1Asi)

Shiraz grapevine embryogenic callus (2014) was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the *VvHKTI;1* gene. Based on the visual presence of GFP, 257 germinated embryos were selected and transferred to shoot production

medium. Seventy-six percent of transferred embryos developed shoots. Of those plantlets produced, 70 were designated as KA lines and tested for the presence of the transgene (as in 5.5.1). All of these were confirmed as positive for *VvHKTI;1*.

Analysis of these transgenic lines was reported in the CSP 1302 Final Report and showed that these experiments confirmed the essential role of HKT1;1 in sodium exclusion.

6.1.6 Down-regulation of a high affinity K^+ transporter HKT1;3 (ST153 & ST165 HKT1Bsi)

On two occasions Shiraz grapevine embryogenic callus was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the *VvHKTI;3* gene. The first one ST153 was carried out in September 2015 using callus established in 2014. However, only five embryos were transferred to shooting medium and only two produced very weak shoots (KB1 and KB2), despite the Shiraz 2014 callus proving to be easy to transform with other constructs. Hence the transformation was repeated in October 2016 using the culture established in 2015; 64 embryos were transferred to shooting medium. Shoots and roots were produced on 37 KB lines, 34 of which have been confirmed by PCR as positive for *VvHKTI;3*. These plants were not available for phenotypic assays until after the project end.

This set of transgenic plants will be assayed in 2017/18.

6.1.7 Down-regulation of high affinity K^+ transporters *VvHKT1.6*, *VvHKT1.7* and *VvHKT1.8* (ST151 3HKT1si)

Using a culture established in 2014, Shiraz grapevine embryogenic callus was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the three genes *VvHKT1.6*, *VvHKT1.7* and *VvHKT1.8* all at the same time. Based on the visual presence of GFP, 128 germinated embryos were selected and transferred to shoot production medium. Eighty-one percent of embryos transferred developed shoots. Of those plantlets produced, 59 were designated as Na lines and tested (as in 5.5.1) for the presence of the transgene. Of these 54 were confirmed as positive for the construct containing the three genes and designated *Vv3HKT1*.

Thirty-four lines were selected based on embryo numbers for further analysis. After propagation, young leaves and tips were collected for DNA extraction and Southern blot analysis was carried out. Three lines Na7, Na24 and Na34 were all negative for the construct. Every line was shown to be the result of an independent transformation event even though many of the lines had very similar numbers. 18D1 and 18D2 embryo numbers gave rise to four and two separate lines respectively, and were picked very early on in the transformation process. It is an important result as it suggests that early embryos even if they come from the same piece of material can be the result of separate transformation events and should be considered for analysis.

Two batches of salt assays (TC Batch 8 & 9) were conducted *in vitro*. Although the experimental conditions should have been similar, TC Batch 8 appeared to have lower levels of sodium compared to TC Batch 9 and subsequent chloride analysis of the plant media collected after the experiment end and frozen, suggests that the plantlets were given only 50 mM mixed chlorides instead of 75mM.

Potassium levels were also collated from these two batches but no correlation was seen between sodium or potassium uptake.

The transgenic lines generally appear to take up the same or a little more sodium than Shiraz or the negative controls, except Na34 (the negative control with higher level of sodium). While these results are not conclusive, it would be worthwhile to challenge some lines in a glasshouse experiment with a higher level of sodium.

6.1.8 Down-regulation of a potential chloride gene 1 (ST163 & ST168)

Shiraz grapevine embryogenic callus (2015) was transformed twice with *Agrobacterium* containing a DNA vector designed to decrease expression of the gene 1.

From the first transformation, based on the visual presence of GFP, 48 germinated embryos were selected and transferred to shoot production medium. Twenty-two of these embryos have developed shoots. Plantlets produced from transformations were designated as C lines. Fifteen plantlets have been tested for the presence of the transgene. Of these, 14 were confirmed as positive. Additional embryos and plantlets will be selected from this transformation in coming months.

The second inoculation was done in February 2017. The first GFP-expressing embryos have germinated and been transferred to shoot production medium. Work is ongoing with this transformation.

This set of transgenic plants will be assayed in 2017/18.

6.1.9 Down-regulation of a potential chloride gene 2 (ST164)

Shiraz grapevine embryogenic callus initiated and established in 2015 was transformed with *Agrobacterium* containing a DNA vector designed to decrease expression of the gene. Based on the visual presence of GFP, 24 germinated embryos were selected and transferred to shoot production medium. Fifteen of these embryos developed shoots. Of those plantlets produced, nine were designated as CH lines and tested for the presence of the transgene. Of these, seven were confirmed as positive for the gene. Work is continuing with this transformation.

This set of transgenic plants will be assayed in 2017/18.

6.1.10 Constitutive expression of the Mi1.2 resistance gene from tomato (ST158-162)

Six transformations were carried out with *Agrobacterium* containing the Mi1.2 construct designed to express the gene *Mi1.2* from tomato, previously shown to provide resistance to root-knot nematode. The first two transformations were discarded before embryos could be selected as only poor GFP fluorescence was detected. On the third occasion, callus from 2014 performed poorly with fewer than 20 embryos germinating and expressing GFP. Shoots produced on these embryos were discarded and are not included in further propagation and analysis. From the three transformations performed with the Shiraz callus initiated in 2015 222 germinated embryos were selected (based on the visual presence of GFP) and transferred to shoot production medium. Eighty-two percent of embryos transferred developed shoots. Of those plantlets produced, 61 were designated as M lines and tested for the presence of the transgene. Of these, 57 lines were confirmed as positive for Mi1.2. At each step additional healthy embryos or plantlets were available but not required and hence not propagated.

These lines were handed over to the Biotic stress team led by Harley Smith for further analysis.

6.1.11 General discussion

The transformation experiments using Shiraz were very successful in this project with many lines being generated for each construct. Analysis was started on three sets of lines (*ALMT2* silencing, *SLAH1.2* expression and silencing of three *HKT* genes) as reported here.

We also graphed chloride exclusion against final dry biomass of shoots in the *in vitro* batches 1-5 to determine whether there was a relationship. There was a weak correlation (R^2 value is 0.22) suggesting that smaller plants took up less chloride than those that were larger by the end of the experiment. All the cuttings were taken as tip cuttings from plantlets that had all been propagated on the same day and cutting size was as consistent as possible. However, some differences in size were noted. Other field and glasshouse experiments showed that there was little relationship between pruning weight and chloride uptake or between growth rates and final chloride concentrations in Plant Accelerator experiments as reported in the CSP 1302 Final Report.

If plant size does influence chloride uptake, this could be one of the variables in our tissue culture experiments that then tends to make the results less reliable. Plants growing in tissue culture are quite different from plants growing in the field. Transpiration rates are low and photosynthesis is not required for growth as the media is supplemented with sucrose. The glasshouse experiments are closer to field conditions; however, it is impossible to compare between them as day-length, light and temperature varies so much depending on the time of the year and the weather.

Anion channels such as SLAH and ALMTs are implicated in many processes such as regulation of stomatal opening/closing and are stimulated by ABA (Roelfsema et al., 2012) and the

transgenic plants generated in this project could prove to be useful for studying this interaction.

6.2 Transformation of grapevine rootstock varieties

This section is included in results rather than methods as development of the methods for rootstock transformation was an aim of this project.

6.2.1 Initiation of embryogenic callus from anthers and ovaries of rootstocks

In late September 2013, anthers alone or attached to the ovary for K51-40 and Ramsey and anthers of 140 Ruggeri were sterilised and placed on to PIV and Harst media. During the first two weeks after initiation, callus appeared to be developing structures likely to form pre-embryogenic callus. However most of this then developed into a very soft callus. Seven weeks after initiation, the most suitable tissue available was transferred to C1. Two more sub-cultures where the most suitable callus was selected and transferred to C1 medium were completed. Five weeks later, as the Ramsey and K51-40 cultures were not producing embryos or embryogenic callus and seemed unlikely to do so from this material, these cultures initiated in 2013 were discarded. The 140 Ruggeri culture was a mixture of developing embryos with embryogenic callus budding from these embryos and softer callus with firm cores. At this point, modified C1 (with the addition of 5 μm NOA and varying levels of BAP and 2,4D) and X6 was used for growth of the 140 Ruggeri callus. Sub-culture of this material to X6 continued and it was eventually used in 21 transformations. One transformation was performed with callus from C1.

In early October 2014, anthers of 140 Ruggeri were collected from the Coombe Vineyard (Waite Campus, Urrbrae) sterilised and placed on to PIV, Harst and MS1 media (section 5.3). Eight weeks later, tissue from all media types, considered to have potential to develop embryogenic callus, was transferred to C1. At this time, it was noted that some embryos had formed on the Harst and PIV media. Selected material continued to be sub-cultured every 5-6 weeks to C1. Material initiated on MS1 was selected for transformation in July 2015 and used in nine transformations.

In mid-late October 2016, anthers of 140 Ruggeri and K51-40 were harvested from the Coombe Vineyard (Waite Campus, Urrbrae), sterilised and placed on to PIV, Harst and MS1 media. Seven to eight weeks later, tissue from all media types, material thought to have potential to develop embryogenic callus was transferred to C1. Selected material continued to be sub-cultured every 5-6 weeks on C1. This callus is expected to be suitable for transformation within the next 2-3 months.

6.2.2 Initiation of embryogenic callus from *in vitro* leaves

Callus was initiated from plantlets that had been placed in tissue culture (section 5.3.2). Schwarzmann was the most successful of the rootstocks tried in this method and this embryogenic callus was eventually used for transformations RT12 and RT13.

6.2.3 Transformation of embryogenic callus of selected rootstock varieties

Dennis Gray's lab uses embryos for transformation, relying on the development of secondary embryos from single transformed cells on the hypocotyl/root boundary (Li et al., 2006 and 2008). We attempted a large number of experiments which included transfer from initiation medium to X6 (Li et al., 2006).

Embryogenic callus of 140 Ruggeri was used 16 times and Schwarzmann four times. Good initial GFP expression was observed for all embryogenic callus of 140 Ruggeri initiated in 2013 when infected with eight *Agrobacterium*-vector combinations. Six of the nine *Agrobacterium*-vector combinations used to transform 140 Ruggeri initiated in 2014 showed good initial GFP expression. GFP expression was observed following infection of callus with vectors encoding mybPA1si, ALMT2si, SLAH1.2si, SLAH1.3si, SLAH1.2si-SLAH1.3si co-transformation, HKT1Asi, HKTIBsi, 3HKTsi and chloride gene 1. For Schwarzmann, no GFP expressing callus was observed post-*Agrobacterium* infection for the first and second transformations and only a small amount of GFP-expressing callus was observed after infection for the third and fourth transformations. Positive and negative results occurred with HKT1Asi, a positive result with 3HKT1si and a negative result with HKT1Bsi.

6.2.3.1 Transformation 1 (RT1 VvMYBPA1si)

Agrobacterium containing a control vector for silencing the *VvMYBPA1* gene was prepared as in section 5.2.2 except that the bacterial pellet was resuspended in 25 mL of X2 medium (modified MS macros and MS vitamins, MS micros, Fe, 1g/L myo inositol, 2% sucrose; Li et al., 2006).

Embryos of 140 Ruggeri at the early-cotyledonary stage of development were used. These were selected on the morning of infection and briefly stored on MS/2 medium. For infection, embryos were either placed into an empty Petri dish and 3-5 mL of bacterial suspension was added, or the callus was added to bacterial suspension in the Petri dish. Embryos were incubated with the *Agrobacterium* solution for 10 minutes at room temperature. After infection, the bacterial suspension was removed from the embryos using a pipette. The embryos were collected and placed in 100 mm Petri dishes each containing three pieces filter paper (Whatman #1) saturated with liquid NB medium which contains MS macros and micros, MS vitamins, Fe EDTA, 0.1 g/L myo inositol, 5 μm NOA, 2.5 μm BAP, 2% sucrose (Dhekney et al., 2008). Embryos were incubated in the dark at 22°C for 84 hours. Embryos were transferred to a flask containing 30 mL of liquid NB medium with 1000 μg/mL timentin (T) and placed at 26°C on a shaker at 100 rpm for three days. Embryos were collected on a 100 μm

nylon net filter (Millipore), blotted onto a double layer of filter paper (Whatman #1) and then transferred to deep Petri dishes containing solid NB medium with timentin (1000 μg/mL) and kanamycin (100 μg/mL).

Embryos were incubated for 90 days on the same media transferred with frequent subcultures. Some sectors of GFP fluorescence could be seen. After this time the callus was transferred to X6 to induce embryogenesis. A second transfer on X6 with 70 μg/mL kanamycin (K) was carried out five weeks later. Eventually, 11 embryos were transferred to shooting medium but these never developed shoots or green meristems.

6.2.3.2 Transformation 2 (RT2 MYBPA1si and ALMT2si)

Agrobacterium was prepared as in 5.2.2 except that the bacterial pellet was resuspended in 25 mL of X2 medium (section 6.2.3.1). Two *Agrobacterium* cultures were used, one containing a vector designed to decrease expression of the *VvALMT2* gene and the other to decrease expression of the *VvMYBPA1* gene.

Embryos of 140 Ruggeri at the mid-cotyledonary stage of development were collected on the morning of infection and briefly stored on X6 medium (same as X2 except 6% sucrose). For infection embryos were added to 3-5 mL of bacterial suspension in a Petri dish. Embryos were incubated with the *Agrobacterium* for 10 minutes at room temperature. After infection, the bacterial suspension was pipetted from the embryos and then the embryos transferred to 100 mm Petri dishes each containing three pieces filter paper (Whatman #1) saturated with liquid NB medium. Embryos were incubated in the dark at 22°C for 72 hours. Infected embryos were collected and washed in NB medium with timentin at 1000 μg/mL. Callus was collected on a 100 μm nylon net filter (Millipore), blotted onto a double layer of filter paper (Whatman #1) and then transferred to deep Petri dishes containing NB medium with 1000 μg/mL timentin and incubated at 28°C in the dark for four days.

Embryos were then transferred to plates containing NB with timentin and kanamycin 100 μg/mL and incubated for 90 days with monthly transfers on NB with antibiotics. After this, the callus was transferred to X6 with cefotaxime and carbenicillin, each at a concentration of 200 μg/mL, and reduced kanamycin (70 μg/mL). The next transfer was to media without kanamycin. Despite good GFP fluorescence being observed during subculturing, no embryos were recovered from this transformation.

6.2.3.3 Transformation 3 (RT3 SLAH1.2si & SLAH1.3si)

The method for this transformation was similar to that of RT1 using embryos of 140 Ruggeri at the mid-cotyledonary stage. Two *Agrobacterium* cultures were used, one containing a vector designed to decrease expression of the *SLAH1.2* gene and the other to decrease expression of the *SLAH1.3* gene. A co-transformation using both of these *Agrobacterium* in the same inoculation was also done. Five mL of each culture was mixed together prior to use and the infection time extended to 20 minutes. Post-infection and co-cultivation embryos

were washed in liquid medium and selected as described for RT2, using a flask wash as in RT1. Subculturing continued on NBTK for a year before being discarded.

6.2.3.4 Transformation 4 (RT4 SLAH1.2si & SLAH1.3si & SLAH1.2si+SLAH1.3 & ALMT2si)

In this set of transformations, the methods used in RT2 were also used. Three *Agrobacterium* cultures were used, each contained a vector designed to decrease gene expression. The genes were *VvALMT2*, *SLAH1.2* and the other *SLAH1.3*. A co-transformation using *SLAH1.2si* and *SLAH1.3si* containing *Agrobacterium* mixed together was also performed. Infection time for the co-transformation was 10 minutes. Post-infection and co-cultivation embryos were washed in liquid medium and selected as described for T3 except that cefotaxime and carbenicillin each at a concentration of 200 μg/mL were used in place of timentin from the start of the transformation.

6.2.3.5 Transformation 5 (RT5 HKT1Asi & 3HKT1si)

Methods used were the same as for RT4. Two *Agrobacterium* cultures were used for inoculation, one containing a vector designed to decrease expression of the Vv*HKT1A (HKT1;1)* gene and the other to decrease expression of three genes *VvHKT1.6*, *VvHKT1.7* and *VvHKT1.8* (designated 3HKT1si).

6.2.3.6 Transformation 6 (RT6 HKT1Asi & 3HKT1si & HKT1Bsi)

As for RT4, three *Agrobacterium* cultures were used, the same as those used in T5 and also one containing a vector designed to decrease expression of the Vv*HKT1B (HKT1;3).*

6.2.3.7 Transformation 7 (RT7 ALMT2si & SLAH1.2si & 3HKT1si)

All the embryos from plates of 140 Ruggeri (2013) were transformed with three constructs. This was different from the previous transformations where only embryos of a specific size were selected and used. The constructs ALMT2si, SLAH1.2si and 3HKTsi were used for this transformation and the method modified with X2 media replacing NB during the transformation and then placed immediately on X6. Kanamycin (70 μg/mL) for selection was not introduced into the media until 48 days after the transformation inoculation. However, this did not increase the viability of transformed material.

6.2.3.8 Transformation 8 (RT8 SLAH1.2si & SLAH1.3si)

140 Ruggeri 2013 was transformed with two constructs using the same methods as in RT7 with kanamycin (70 μg/mL) introduced after two weeks. This transformation was maintained through several subcultures but did not produce secondary embryos.

6.2.3.9 Transformation 9 (RT9 HKT1Asi & HKT1Bsi)

The same methods were used as in RT7 using two HKT1 silencing constructs with kanamycin (70 μg/mL) introduced three weeks after inoculation. This transformation was maintained through several subcultures but did not produce secondary embryos.

6.2.3.10 Transformation 10 (RT10 HKT1Asi)

140 Ruggeri callus initiated on to MS1 in 2014 and prepared for transformation by a two week growth on C1 and then two weeks on GS1CA. All material (embryos and callus) from the plates was used in the transformation with *Agrobacterium* containing the HKT1Asi vector. Methods were modified again with the *Agrobacterium* pellet resuspended in LCM (NN macros, MS micros, B5 vitamins, Fe EDTA, 2% sucrose. Solid media used was GS1CA (similar LCM with 3% sucrose and added 10 μM NOA and 1 μM BAP) with timentin as antibiotic to kill *Agrobacterium*. The next subculture was also onto GS1CA media followed by transfers to NNTK100.

Forty-eight embryos were transferred to MG2 with 5 μM BAP beginning nearly four months after the transformation. Only three eventually produced a shoot and were transferred to SM and two finally produced plantlets that could be propagated and were labelled RKA1 and RKA2.

6.2.3.11 Transformation 11 (RT11 3HKT1si)

This transformation was carried out as in RT10 with a construct to silence *HKT1;3*. To trial a range of media options for germinated embryos, seven embryos were transferred to MS2 with 5 μM BAP, 39 to MG2 and nine directly to C2D4B (Chee and Poole Basal Medium (Phyto Technology Laboratories), 1000 μM BAP, 3% sucrose, pH 5.8, 0.7% agar). After two months those on MG2 were transferred to C2D4B, then finally to MSN (Murashige and Skoog Basal Salts (Phyto Technology Laboratories), Murashige and Skoog Vitamins (Phyto Technology Laboratories), 1000 μM NAA, 100 mg/L myo inositol, 3% sucrose, pH 5.8, 0.7% agar).

One embryo survived to produce a plantlet and this was called RNa1

6.2.3.12 Transformation 12 (RT12 HKT1Asi & HKT1Bsi)

The callus used in this transformation was derived from Schwarzmann. Apart from the starting material, the methods for this transformation were the same as RT10 with the addition of a second construct designed to silence *HKT1;3*; after one subculture on NNTK this material was discarded.

6.2.3.13 Transformation 13 (RT13 HKT1Asi & HKT1Bsi)

This transformation was precisely the same as RT12 using all the same material and methods. After one subculture on NNTK this material was discarded.

6.2.3.14 Transformation 14 (RT14 HKT1Bsi)

RT14 used 140 Ruggeri (2014) with the *HKT1;3* silencing construct. The methods were very similar to those of RT10. Kanamycin (100 μg/mL) was used for the first two subcultures then reduced to 70 μg/mL. This was one of the most successful transformations for generating embryos, with 95 being selected and most positive for GFP fluorescence. This allowed for trialling of different hormones in MG including BAP or NAA. However, none of the embryos ever produced a stable shoot.

6.2.3.15 Transformation 15 (RT15 SLAH1.2si)

RT15 was performed at the same time as RT14 using the same methods and *Agrobacterium* culture containing the *SLAH1.2* silencing construct. After two subcultures, this material was discarded.

6.2.3.16 Transformation 16 (RT16 SLAH1.3si)

RT16 and RT17 were performed at the same time and using the same 140 Ruggeri callus (2014) as RT10. The methods were similar, but the second subculture was onto NNTK100, followed by a subculture to NNTK70. No embryos were recovered from this material.

6.2.3.17 Transformation 17 (RT17 MYBPA1si)

As with RT16.

6.2.3.18 Transformation 18 (RT18 SLAH1.3si)

Transformations RT18 and RT19 were conducted at the same time and after the first subculture on GS1CA with TK100, the following sub-cultures were onto NNTK70, then NNTK50. 34 embryos were picked to MS2 with 2 μM BAP, and two eventually became plantlets with very similar embryo numbers and called RSI1 and RSI2.

6.2.3.19 Transformation 19 (RT19 Mi1.2)

This transformation was very similar to RT18 but using the tomato gene *Mi1.2* construct. Three subcultures on NNTK50 were carried out before material was discarded, as embryogenesis seemed very unlikely.

6.2.3.20 Transformation 20 (RT20 gene 1)

140 Ruggeri callus, initiated in 2014 and prepared for transformation by 7-8 weeks on C1 and then two weeks on GS1CA, was used in transformation with the silencing construct using the media and method as in 5.2.2 except that the concentration of kanamycin was reduced to 25 μg/mL and only used until 12 weeks post-infection.

No embryos were selected before the end of the project although the material is looking good and has GFP fluorescence.

6.2.4 Characterisation of the transformed rootstock plantlets

Southern blot analysis was preformed using genomic DNA extracted from young leaves and shoots of plantlets grown *in vitro*. RNa1 was the only plant with a single copy insert and RSI1 had two bands with the right border but no band with the left border probe (Figure 1).

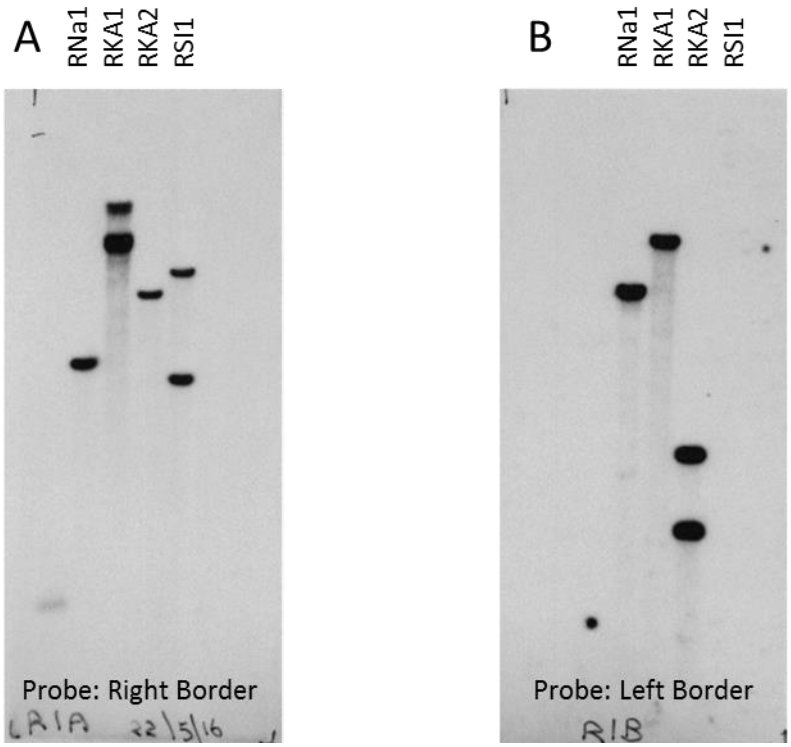


Figure 1. Southern blot of rootstock transformant DNA probed with the two ends of the construct.

These plants have yet to be analysed for salt uptake.

6.2.5 General discussion

The establishment of callus in a similar fashion to establishment of Shiraz pre-embryogenic callus proved to be a more successful method than those used by Dennis Gray's lab, where they use embryos for the transformation and rely on secondary embryogenesis to produce transformed plants. This method was tried for the first six transformations, but no embryos were recovered after transformation. This was in contrast to the untransformed material which continued to bud new embryos at a considerable rate.

The large number of transformations that were performed allowed us to trial a lot of different media and antibiotics. Some transformations such as RT14 produced a good number of embryos, whereas a parallel transformation did not develop any. It is not clear why this might be the case, but suggests perseverance with multiple transformations to achieve the desired results.

The major block in perfecting the rootstock transformation is the conversion of the embryo to a shoot. One possible way around this might be to try a vector with different antibiotic resistance, for example hygromycin rather than kanamycin, in case the rootstock material is just too sensitive to kanamycin.

Other methods might also be worth trying, such as those of Xie et al., 2016 where they used different hormones to regenerate what they refer to as meristematic bulks that do not go through an embryonic stage.

7 Outcomes and conclusions

During this reporting period the team carried out a large number of transformations of Shiraz, producing a lot of embryos for analysis by other teams, particularly the abiotic and the biotic stress teams. Here we presented some of the results from the transformations to test putative chloride and sodium exclusion genes. The SLAH1.2 gene experiments suggest it could play a role in chloride exclusion, but further experiments are needed to confirm this. This gene might become one of the major markers used for rootstock breeding to improve rootstocks for the Australian industry. On the other hand, lines transformed with the ALMT2 silencing showed little difference to the control, suggesting that this gene may not be so important in excluding chloride from the shoot.

Our routine Shiraz transformation techniques have been used for testing genes in support of several projects and the training of staff for maintenance of this capability. This capability remains unique in Australia and only a few laboratories around the world can perform transformations on a regular basis.

Secondly, advances in rootstock transformation could provide the first steps that would eventually lead to new rootstocks developed using CRISPR and related new technologies. Overcoming the difficulties of rootstock transformation has afforded the opportunity to try out a lot of different methods which could prove important for increasing transformation efficiency using any cultivar. For reliable use of the new technologies, high rates of transformation are essential.

8 Recommendations

Grapevine transformation is the only way to test the role of genes unless a mutant plant can be identified such as for white grapes. For many genes, clues can be accumulated from heterologous systems such as expression in yeast or tobacco, but direct evidence is the most convincing. This is a difficult and highly specialised method and maintaining the capability to perform transformations relies on having dedicated, well-trained staff. This capability also involves initiating anther cultures every year so that callus is available when required, rather than having to wait up to 18 months if callus is not available.

Rootstocks have proved to be more difficult to transform than we initially thought, but after making some progress with the techniques it is recommended that further techniques be explored to improve the outcome.

Further experimentation could include the use of the microvine, as it grows more quickly than other varieties (Chaib et al., 2010). If transformation for the microvine can be shown to be as efficient as Shiraz, it could provide a faster way to test ion exclusion genes.

The last recommendation is to investigate the use of CRISPR technologies to introduce changes in DNA as this could provide a shortcut for altering single target genes as part of a broader breeding strategy.

9 Appendix 1: Communication

Date	Event/Conference	Location	Presentation title	Type	Presenter
24 November 2013	CSIRO/Industry Rootstock Reference Group Meeting	CSIRO, Waite Campus	Genetic and mechanistic characterisation of rootstock traits conferring abiotic stress tolerance to grapevines	Oral	Mandy Walker
25 November 2015	CSIRO/Industry Rootstock Reference Group Meeting	CSIRO, Waite Campus	Developing genetic markers for Na^+ and Cl^- exclusion traits	Oral	Mandy Walker
29 July 2016	AWITC Rootstock Workshop 2016	Adelaide Convention Centre	New screening techniques for abiotic stress tolerance	Oral	Mandy Walker
25 August 2016	Waite Campus Conference	Waite Campus	How can we help stressed-out grapevines?	Oral	Mandy Walker

10 Appendix 2: Intellectual Property

Some of the work presented in this report will form part of publications in internationally-recognised peer-reviewed journals.

11 Appendix 3: References

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12 Appendix 4: Staff

Mandy Walker

Debra McDavid

Jacinta Watkins

Tanya Kovalenko

Casuals: Bharati Das, Mana Rezaei

Contributors, particularly for salt assays: Karin Sefton, Lauren Hooper, Deidre Blackmore

Occasional support: Inna Mazonka, Amy Smith, Adelle Craig, Kaitlin Tepper, Simon Robinson, Jim Speirs, Jake Dunlevy

Discussions: Pat Corena, Mark Thomas, Rob Walker, Peter Clingeleffer, Harley Smith, Jake Dunlevy

13 Budget reconciliation

This information will be provided by CSIRO in a separate document.