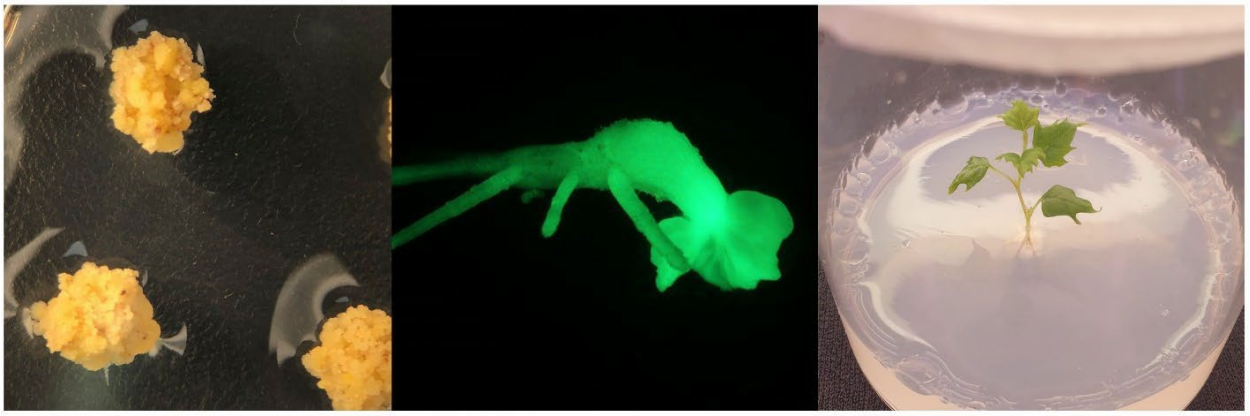
Australian Government
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CSIRO

# Enabling Technologies and Genetic Resources



FINAL REPORT TO WINE AUSTRALIA

Project Number: **CSA 1701 - 1.7**

Principal Investigator: **Mandy Walker**

Research Organisation: **CSIRO**

Date: **November 2023**

Australia's National
Science Agency

# CSA 1701 - 1.7 Enabling technologies and genetic resources

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CSA 1701 - 1.7

November 2023

Wine Australia Final Report

Australian Government
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# 1 Abstract

Genetic transformation of grapevines is an essential tool for understanding gene function, which builds our understanding of how traits are controlled. In Australia, the capability to transform grapevines is unique to CSIRO and this project served to maintain this enabling technology along with the germplasm collection at Irymple. Transformed plants were produced to assist the understanding of genes involved in salt tolerance and to test fast breeding systems involving rapid flowering and floral sex. The optimisation of exciting new methods to produce improved clones of existing varieties using DNA-free gene editing was also progressed.

# 2 Executive summary

Traits are controlled, in large part, by genes. Therefore, any fundamental understanding of important traits that will improve the profitability and/or the sustainability of growing grapes and making wine requires knowledge of the underlying genes. Genetic mutants are the best way to study the relationship between genes and traits. This is the reason why so much research has been conducted on the model plant *Arabidopsis* due to its short life cycle, small stature and ease of transformation. In non-model plants, especially perennial crops, the production of a large collection of mutated plants is not possible. A means to fill this void is to alter the expression of genes suspected of being involved in certain traits using genetic transformation methods. This is not an easy task and only a few laboratories around the world can currently transform winegrape cultivars. Researchers at CSIRO developed *Agrobacterium*-based grapevine transformation methods in the late 1990s (Franks *et al.* 1998; Iocco *et al.* 2001) and have been maintaining this capability in Australia ever since. We also have access to CSIRO-developed RNAi technology for the suppression of gene expression or can over-express genes using constitutive promoters (e.g. CaMV 35S). Both techniques assist in the characterisation of genes whose functions are unknown.

This grapevine transformation capability was used to assist other projects to characterise candidate genes for traits targeted in the various lines of research. For example, transporter genes predicted to be involved in salt tolerance, putatively identified through genetic mapping strategies, were tested in transgenic vines that were engineered to either over-express genes or have their expression suppressed by RNAi. This provided evidence that certain candidate genes did indeed play a role in salt exclusion. In addition, this project was involved in the production of plants with editing copies of a gene suspected of being involved in the production of six-carbon aldehyde and alcohols in grape berries, which contribute to the green character of wine. The assessment of these plants is ongoing.

Transformation technology was also used to demonstrate our ability to develop rapid flowering versions of scions and rootstocks. The original ‘microvine’ was derived from the outer cell layer of Pinot Meunier (Boss and Thomas 2002) and as such the genetic background of any rapid breeding strategy using this plant has to consider that Pinot-like traits may be predominant in the progeny. The mutated *Vvgai1* gene was introduced into Shiraz and 140 Ruggeri to test whether this is a viable strategy to reduce the stature of the vine and accelerate flowering. When expressed constitutively or under its own promoter, the *Vvgai1* gene was able to accelerate flowering in dwarfed Shiraz vines, but interestingly, only the endogenous promoter could produce the same effect in 140 Ruggeri. Such plants could be used in rapid breeding provided that the transgene is segregated away in the final selections.

Flower sex is also an important part of any breeding strategy, as female plants are much easier to use for pollinations. While the flower sex locus was mapped to a small region of grapevine chromosome 2 some years ago (Marguerit *et al.* 2009; Fechter *et al.* 2012; Battilana *et al.* 2013), the causal gene had not been identified. Understanding how this trait is controlled allows for greater efficiencies in a breeding program. Gene editing, using CRISPR/Cas9 technology introduced via *Agrobacterium* stable transformation, was used to demonstrate that the *VvPLATZ1* gene causes normal stamen production and when mutated, results in the production of flowers with reflex stamens (Iocco-Corena *et al.* 2021). This information allows the development of strategies to

produce clones of cultivars or breeding lines with female flowers to aid pollination and makes breeding more efficient. These experiments also aided the development of some fundamental tools required for the application of gene editing in grapevine.

While gene editing of grapevine had been demonstrated with the *VvPLATZ1* flower sex experiments, the plants produced were transgenic and would require the removal of the transgene through a sexual reproduction step if they were to be grown commercially. The genomes of winegrapes are highly heterozygous, meaning that any sexual reproduction step will mean that a new cultivar is formed which is unlikely to be similar to the parent(s). An exciting new methodology allowing the introduction of the gene editing machinery to plant protoplasts via ribonucleoproteins enables the production of edited plants that are not regarded as genetically modified organisms. This enables the production of new clones of existing premium varieties with improved traits. Experiments conducted as part of this project demonstrated that we could produce protoplasts from embryogenic callus and then regenerate plants from the isolated protoplasts. We also showed that we could transfect grape protoplasts with nucleic acid and maintain their viability under mock transfection conditions. These developments provide a sound basis for the future application of DNA-free gene editing in grapevine scions to generate improved clones of existing cultivars that are not regarded as genetically modified.

# 3 Background

Transformation technologies have been used for over 40 years to assess the roles of specific genes and pathways in plants and have revolutionised our understanding of gene function and plant physiology (Anami *et al.* 2013). Our ability to alter the expression or function of a gene *in planta* provides a technique to test the function of a gene. The information gained then provides a fast and reliable tool for crop improvement in many species, through targeted and smart breeding using marker-assisted breeding. It also has the potential to change the characteristics of a plant to make controlled improvements without changing other beneficial characters. In some cases, genetically modified crops have gained acceptance in the marketplace, particularly in the US and Canada, and in some non-food crops like cotton in Australia. However, in Europe, parts of Asia and Oceania, genetic modification of food crops using DNA insertions has been opposed by some of the population, which has resulted in tight controls over its use.

Traditionally, plant transformation has been carried out by the insertion of a constructed piece of DNA into the plant genome, often through to use of an unarmed strain of *Agrobacterium tumefaciens* (hereafter referred to as *Agrobacterium*), resulting in the modification of the expression of a gene (antisense or RNAi) or adding a new gene along with a selectable marker for easy identification of plants with the DNA construct present in the genome. The altered plant characters were then dependent on the maintenance of this piece of DNA integrated at a random place in the genome. Inferences were then made about the function of the gene depending on the change in phenotype caused by over-expression or a reduction in expression caused by antisense or RNAi suppression.

Methods to transform grapevine have developed over the last few decades and we have found that most reliable techniques include the use of pre-embryogenic callus established regularly from anther cultures and DNA vectors with antibiotic resistance genes and GFP (green fluorescent protein gene) as selectable markers for the integration of the construct in the genome (Iocco *et al.* 2001). The construct is delivered by co-cultivation of the callus with *Agrobacterium* that is carrying the appropriate DNA vector, followed by many rounds of transfer of callus on selection plates and then selection of GFP-positive embryos. The process is time consuming and labour intensive but other protocols have proven to be less successful. At the beginning of this project we could transform Shiraz, Chardonnay and certain genotypes of microvine reliably (Chaib *et al.* 2010) and were developing methods for grapevine rootstock transformation.

In the context of the Wine Australia-CSIRO Strategic Research Alliance, this project supported research leading to the development of the next generation of winegrape varieties and rootstocks as proposed in projects 1.1, 1.2, 2.1 and 2.2. At the outset of this project, genetic transformation remained the only way to verify the function of important genes identified as part of the CSIRO scion and rootstock breeding projects. Verification of function allows us to design perfect markers with a high level of confidence to the target genes for use in marker-assisted selection. We are the only facility in Australia able to routinely perform transformations and one of the very few in the world.

More recently, a genetic transformation technology that has become known as ‘gene editing’ has shown promise for increasing the precision of changes made to the genome of organisms (Kim 2016). We recognised during the project that we have the capability to develop new gene editing technologies that will allow the precise editing of genes and provide the opportunity to modify

genes in elite wine cultivars, without leaving any foreign DNA. This became a focus of the work as the project progressed. Gene editing relies on delivering an enzyme and a nucleic acid guide to target the cleavage of a specific gene in a cell, enabling the gene of interest to be altered or edited. This methodology was developed from a prokaryotic immune system designed to cleave foreign DNA (Wiedenheft *et al.* 2012). When using the wild-type bacterial nuclease, the type of changes to the DNA are not easy to control as the changes are based on the imprecise repair of the double-stranded break made in the DNA, but if they result in a deletion or insertion (that is not a multiple of three), the resulting frameshift mutation usually abolishes gene function. Currently in Australia, these so called 'SDN-1' (site directed nuclease -1) modified organisms are not regulated as GMOs, provided that any transgene that has been used to generate the SDN-1 event has been subsequently removed from the genome (see https://www.ogtr.gov.au/resources/publications/overview-status-organisms-modified-using-gene-editing-and-other-new-technologies). For inbred, fast generation plants like wheat, barley and rice, gene editing can be achieved by transforming a plant with a construct that introduces DNA into the genome encoding the site directed nuclease and associated guide RNA. Then, once the gene editing has occurred at the target gene, the construct can be removed by self-fertilising or backcrossing to the same original genotype and selecting for the plants with the gene-edit, but no transgene, resulting in a known cultivar with a single targeted change and no foreign DNA. However, grapevine is a very heterozygous, outbred plant, so self-fertilising a cultivar does not lead to offspring with similar characteristics and known varieties cannot be regained by going through sexual cycles of propagation. Hence this technique to remove the DNA construct after gene editing cannot be used to modify a single gene in an elite grapevine cultivar.

To overcome this barrier, techniques have been developed to deliver the site directed nuclease and guide RNA into a cell without the integration of a DNA construct into the genome (Tsanova *et al.* 2021). One advantage of this technique is that the enzyme and RNA guide degrade after they have edited the target gene and there is no foreign DNA introduced into the plant genome, hence any regenerated plants are not considered to be GMOs. Furthermore, the regenerated plants are genetically identical to the original cultivar except for the gene-edit and hence would behave like the original cultivar except for the trait controlled by the targeted gene. While this sounds simple in theory, these methodologies are more complicated and technically more difficult to accomplish than traditional genetic transformation methods. Nevertheless, the tremendous benefit of having such technology available to the Australian grape and wine sector warrants a concerted effort to establish this methodology in Australia and to demonstrate our ability to gene-edit varieties of particular importance to Australia. In the context of scientific discovery, this technique does not yet replace the older transformation techniques where we are able to add genes, enhance expression of endogenous genes or reduce the expression of a gene through RNAi technology. However, it is a great addition in the arsenal of tools to characterise the function of important genes. We also believe that the application of gene editing for germplasm improvement has tremendous potential to provide new solutions to issues facing the Australian grape and wine sector.

The other major resource that underpins the CSIRO Breeding Program is the grapevine germplasm collection at Irymple and this was maintained as part of this project. The collection includes: (a) CSIRO grapevine variety collection (b) CSIRO research germplasm collection and (c) scion and rootstock breeding populations that are undergoing phenotyping and mapping. A comprehensive germplasm collection is crucial to the success of our breeding program because it provides a source of new traits such as resistance genes to pests and diseases. The CSIRO collection has undergone DNA typing as part of Wine Australia project CSP 1501 and it is anticipated that the collection will be

re-opened to the sector in the future to provide DNA certified planting material that could assist regional nurseries as well as growers selling to wineries that require an audit trail.

# 4 Project Aims

There were three main objectives of this project. The first objective was to support the other Wine Australia - CSIRO bilateral projects 1.1, 1.2, 2.1 and 2.2 by providing the capability to enable gene function to be verified. This information aids the development of perfect markers for breeding. To achieve this outcome, embryogenic cultures have to be initiated from flowers (anthers) every year. The cultures are then maintained and prepared for genetic transformation with constructs that either alter endogenous gene activity or introduce genes from other wild *Vitis* species such as pathogen resistance genes. The resultant transgenic plants can then be assessed to determine the function of the modified/introduced grape gene.

The second objective was to develop gene editing technologies for use in grapevine systems, which do not leave a footprint other than the altered target gene. This type of technology could be used to pyramid desirable traits in existing premium varieties, which is not possible with traditional breeding techniques.

The third objective relates to the basic maintenance of the germplasm collections at the Irymple farm. This includes not only the scion and rootstock breeding populations used for phenotyping and mapping new traits but also the CSIRO Variety Collection. This maintenance will involve basic spraying, fertilising, irrigation, vineyard floor management and mechanical pruning. However, if the CSIRO Variety Collection is re-opened, we have budgeted to undertake hand-pruning to reduce the potential for crossover of canes between variety panels and the risk of human error when cuttings are collected. We will also remove bunches retained from the previous season and reduce sources of disease inoculum on cutting material.

3 year outputs:

- Embryogenic cultures initiated and maintained for gene function studies.
- Functional characterisation of salt exclusion genes completed.
- Evaluation of new gene editing vectors for grape completed.
- 140 Ruggeri rootstock transformed with GAI construct to develop rapid breeding lines (see Wine Australia-CSIRO bilateral project 1.4).
- Agreement developed between CSIRO and Wine Australia on the future of the CSIRO Grapevine Germplasm Collection in relation to the concept of an Australian National Grapevine Germplasm Collection.
- Subject to agreement by CSIRO and Wine Australia, development of processes and protocols to facilitate distribution of germplasm from the CSIRO collection to Vine Improvement Associations for propagation and distribution to registered nurseries and growers.

5 year outputs:

- Functional evaluation of candidate genes conferring resistance to nematodes and Phylloxera completed.
- Transgenic microvines generated with modified fruit characters using gene editing technology developed.
- CSIRO contributing to a functional Australian National Grapevine Germplasm Collection. Annual reports to Wine Australia regarding the identity and number of cuttings provided to Vine Improvement Associations

10 year aspirational outcomes:

- Ability to use transgenic technology to pyramid desirable traits in premium winegrape varieties without leaving a transgenic fingerprint.

The outputs designed in each annual operating plan are listed below.

Year 1:

| OUTPUT | TARGET | ACTIVITIES |
|---|---|---|
| Shiraz and rootstock callus available for transformation. | 30/06/18 | Establish fresh Shiraz and 140 Ruggeri pre-embryogenic callus each year.<br>Maintain callus from previous 2 years unless it becomes untransformable. |
| Microvine embryogenic cultures maintained for transgenic research. | 30/6/18 | Three microvine embryogenic culture lines established from anther culture.<br>Microvine embryogenic lines maintained by regular subculture for transformation studies. |
| Gene editing constructs evaluated and transformed into a microvine line. | 30/6/18 | Development of a transient or short-term assay to rapidly evaluate efficiency of gene editing constructs.<br>Two gene editing constructs transformed into a microvine line.<br>Transgenic microvine lines selected and plant regeneration initiated. |
| Transformed grapevines available for further analysis by other projects. | 30/6/18 | Perform at least 10 transformations with 5 constructs annually and supply up to 20 individual lines for each construct. |
| Callus and suspension cultures available when required. | 30/6/18 | Maintain grapevine and tobacco callus. Suspension cultures maintained when needed |

Year 2:

| OUTPUT | TARGET | ACTIVITIES |
|---|---|---|
| Shiraz and rootstock callus for transformation. | 30/6/19 | Establish fresh Shiraz, 140 Ruggeri and K51-40 pre-embryogenic callus each year.<br>Maintain callus from previous 2-3 years unless it becomes untransformable. |
| Microvine embryogenic cultures for transgenic research. | 30/6/19 | Establish two new microvine embryogenic culture lines (V3 and V6) from anther culture.<br>Maintain microvine embryogenic lines by regular subculture for transformation studies. |

| | | |
|---|---|---|
| Gene editing constructs evaluated in transient assays and transformed microvines. | 30/6/19 | Develop a transient or short-term assay to rapidly evaluate the efficiency of gene editing constructs.<br>Select transgenic microvine lines and initiate plant regeneration. Evaluate plantlets for effectiveness of gene editing. |
| Transformed grapevines available for further analysis by other projects. | 30/6/19 | Perform at least 10 transformations with 5 constructs annually.<br>Continue transformations from previous year and supply up to 20 independent lines for each construct. |
| Callus and suspension cultures available when required. | 30/6/19 | Maintain grapevine and tobacco callus.<br>Initiate suspension cultures to ensure their availability 4-6 weeks later when requested. |
| Maintained winegrape germplasm collections at Irymple including the CSIRO Variety collection and scion/rootstock breeding populations | 30/6/19 | Continue disease management, fertilisation, irrigation, vineyard floor management and pruning of grapevines at the Irymple site, as required. |

## Year 3:

| AIM (YEAR 3 / YEAR 5 DELIVERABLES) | OUTPUTS | ACTIVITIES | ACTIVITY TARGET DATE |
|---|---|---|---|
| **Yr 1 outputs ongoing:**<br>**Shiraz and rootstock callus available for transformation.**<br>**Microvine embryogenic cultures for transgenic research.**<br>**Ongoing Yr 3 output:**<br>**Embryogenic cultures initiated and maintained for gene function studies.** | Shiraz and rootstock and microvine embryogenic callus available for transformation | Establish fresh Shiraz, 140 Ruggeri and K51-40 pre-embryogenic callus.<br>Establish fresh microvine embryogenic culture lines (V3, V4 & V6).<br>Where possible maintain 2 embryogenic callus lines from each cultivar for transformation. | 30/6/2020 |
| **Yr 1 output ongoing:**<br>**Callus and suspension cultures available when required.** | *In vitro* plantlets, callus and suspension cultures available when required. | Maintain V. vinifera cultivars (Shiraz, Chardonnay, Cab Sauv & Riesling), rootstocks (140 Ruggeri, K51-40, Ramsey & Schwarzmann) and microvine genotypes (V3, V4, V6) as in vitro plantlets.<br>Maintain grapevine and tobacco suspension callus | 30/6/2020 |
| **Yr 1 output ongoing:**<br>**Winegrape germplasm collections maintained at Irymple including CSIRO Variety collection and scion/rootstock breeding populations.** | Maintained winegrape germplasm collections at Irymple including the CSIRO Variety collection and scion/rootstock breeding populations. | Continue disease management, fertilisation, irrigation, vineyard floor management and pruning of grapevines at the Irymple site, as required. | 30/6/2020 |
| **Yr 3 output: Transformations for functional characterisation of salt exclusion genes completed.** | Plantlets with extra HKT1.1 alleles available for phenotyping. | Complete selection of transformed Shiraz plantlets with altered HKT1.1 genomic constructs to determine if increasing the number of "good" sodium exclusion alleles can increase sodium exclusion from the shoot. | 30/6/2020 |

| | | | |
|---|---|---|---|
| **Yr 3 output: Evaluation of new gene-editing vectors for grape completed.** | Data obtained regarding efficiency of gene-editing constructs optimised for CRISPR/Cas9 expression and deletion in grapevine. | Perform microvine transformations with each of the new gene-editing constructs:<br>- pGCAS9INT_FS1 (35S promoter and terminator for Cas9 expression)<br>- pVCAS9_FS1_FS6 (dual sgRNA vectors for improving deletion efficiency & screening efficiency) | 30/6/2020 |
| **Yr 3 output: 140 Ruggeri rootstock transformed with GAI construct to develop rapid breeding lines (see Project 1.4).** | Transformed 140 Ruggeri ready for testing for dwarf phenotype | Perform at least 2 rootstock 140 Ruggeri transformations with the GAI construct with native promoter (construct already being tested in Shiraz). | 30/6/2020 |
| **Yr 3 outputs: Agreement developed between CSIRO and Wine Australia on the future of the CSIRO Grapevine Germplasm Collection in relation to the concept of an Australian National Grapevine Germplasm Collection.**<br>**Subject to agreement by CSIRO and Wine Australia, development of processes and protocols to facilitate distribution of germplasm from the CSIRO collection to Vine Improvement Associations for propagation and distribution to registered nurseries and growers.** | Agreement reached between CSIRO and Wine Australia about the future of the CSIRO Grapevine Germplasm Collection and germplasm distribution. | Hold discussions with Wine Australia about the future of the CSIRO Grapevine Germplasm Collection and the protocol for possible distribution of germplasm | 30/6/2020 |
| **Year 5 output: Transgenic microvines generated with modified fruit characters using gene-editing technology developed.** | Transformed microvines with modified expression of fruit character genes | Perform at least 2 transformations and select positive embryos/plants.<br>Continue selection of positive embryos/plants from transformations carried out in previous year and supply up to 20 independent lines for each construct. | 30/6/2020 |

Year 4:

| AIM (YEAR 3 / YEAR 5 DELIVERABLES) | OUTPUTS | ACTIVITIES | ACTIVITY TARGET DATE |
|---|---|---|---|
| **Ongoing Yr 1 output:** | Shiraz and rootstock and microvine embryogenic callus | Establish fresh Shiraz, pre-embryogenic callus. | 30/6/2021 |

| | | | |
|---|---|---|---|
| **Shiraz and rootstock callus available for transformation.**<br>**Microvine embryogenic cultures for transgenic research.**<br>**Callus and suspension cultures available when required.** | available for transformation. In vitro plantlets, callus and suspension cultures available when required. | Establish fresh microvine embryogenic culture lines (V3 & V4).<br>Where possible maintain 2 embryogenic callus lines from each cultivar above and the two rootstocks, K51-40 and 140 Ruggeri for transformation.<br>Maintain V. vinifera cultivars (Shiraz, Chardonnay, Cab Sauv & Riesling), rootstocks (140 Ruggeri, K51-40, Ramsey & Schwarzmann) and microvine genotypes (V3, V4, V6, V10) as in vitro plantlets.<br>Maintain grapevine and tobacco suspension callus | |
| **Ongoing Yr 3 output: Transformations for functional characterisation of salt exclusion genes completed.** | Plantlets with extra HKT1.1 alleles available for phenotyping. | Complete selection of transformed Shiraz plantlets with altered HKT1.1 genomic constructs to determine if increasing the number of "good" sodium exclusion alleles can increase sodium exclusion from the shoot. | 30/6/2021 |
| **Yr3 output cont'd: Evaluation of new gene-editing vectors for grape.** | Data obtained regarding efficiency of gene-editing constructs optimised for CRISPR/Cas9 expression and deletion in both microvine and shiraz.<br>Protocols for transient gene editing delivery systems for grapevine protoplasts evaluated. | Transfer synthesised constructs into agrobacterium in preparation for transformation.<br>Evaluate more rapid in-planta assay for CRISPR/Cas9 construct activity using hairy root assays on Shiraz and microvine. Perform at least 3 sets of hairy root transformations, sequence and analyse the results for editing efficiency.<br>Perform at least 2 transformations with each of the new gene-editing constructs:<br>- pGCAS9_MYB14 & pVCAS9_MYB15<br>- pVCAS9_GAI<br>Monitor literature for new information regarding the testing of efficacy of purified CRISPR/Cas9 ribonucleoproteins as a transient delivery system to target genes in grape protoplasts<br>Evaluate protocols for regeneration of grapevine plants from Shiraz protoplasts | 30/6/2021 |
| **Yr 3 output cont'd: 140 Ruggeri rootstock transformed with GAI construct to develop rapid breeding lines (see Project 1.4).** | Transformed 140 Ruggeri ready for testing for dwarf phenotype. | Repeat at least 2 rootstock 140 Ruggeri transformations with the GAI construct with native promoter | 30/6/2021 |
| **Year 5 output. Transgenic microvines generated with modified fruit characters using gene-editing technology developed.** | Transformed grapevines with modified expression of target genes (i.e. resistance genes, flavour genes, hormone biosynthesis genes etc) from other SRA projects available for analysis. | Perform at least 2 transformations and select positive embryos/plants.<br>Continue selection of positive embryos/plants from transformations carried out in previous year and supply up to 20 independent lines for each construct. | 30/6/2021 |
| **Ongoing Yr 1 output:**<br>**Winegrape germplasm collections maintained at Irymple including CSIRO Variety collection and scion/rootstock breeding populations.** | Winegrape germplasm collections maintained at Irymple including the CSIRO Variety collection and scion/rootstock breeding populations.<br>Agreement reached between CSIRO and Wine Australia about the future of the CSIRO | Continue disease management, fertilisation, irrigation, vineyard floor management and pruning of grapevines at the Irymple site, as required.<br>Discussions between CSIRO and Wine Australia about the future of the CSIRO Grapevine Germplasm Collection | 30/6/2021 |

| | |
|---|---|
| **Yr 3 output cont'd: Agreement developed between CSIRO and Wine Australia on the future of the CSIRO Grapevine Germplasm Collection in relation to the concept of an Australian National Grapevine Germplasm Collection.** | Grapevine Germplasm Collection and processes formulated to facilitate germplasm distribution. |

Year 5:

| AIM (YEAR 5 DELIVERABLES) | OUTPUTS | ACTIVITIES | ACTIVITY TARGET DATE |
|---|---|---|---|
| **Ongoing Yr 1 output:**<br>**Shiraz and rootstock callus available for transformation.**<br>**Microvine embryogenic cultures for transgenic research.**<br>**Callus and suspension cultures available when required.** | Shiraz and rootstock and microvine embryogenic callus available for transformation.<br>*In vitro* plantlets, callus and suspension cultures available when required. | Establish fresh Shiraz pre-embryogenic callus.<br>Establish fresh microvine embryogenic culture lines (V3 & V4).<br>Where possible maintain 2 embryogenic callus lines from each cultivar above and one culture each of the two rootstocks, K51-40 and 140 Ruggeri for transformation.<br>Maintain V. vinifera cultivars (Shiraz, Chardonnay, Cab Sauv & Riesling), rootstocks (140 Ruggeri, K51-40, Ramsey & Schwarzmann) and microvine genotypes (V3, V4, V6, V10) as in vitro plantlets.<br>Maintain grapevine and tobacco suspension callus | 30/6/2022 |
| **Yr3 output cont'd: Evaluation of new gene-editing vectors for grape** | Data obtained regarding efficiency of new gene-editing constructs: (a) with multiple guide RNAs (b) with promoters optimised expression in grape<br>Testing of new guide RNAs against new grapevine gene targets | Continue selection of positive embryos/plants from transformations carried out in previous year (pVCAS9_MYB14.6, pPC_FS_WRKY52) and supply up to 20 independent lines for each construct. Extract DNA, sequence and analyse the results for gene editing efficiency.<br>Design and evaluate new CRISPR/Cas9 constructs with in vitro cleavage assay and in-planta hairy root assays. Perform at least 4 sets of hairy root transformations, sequence and analyse the results for gene editing efficiency.<br>Perform at least 4 stable transformations with new gene-editing constructs. | 30/6/2022 |
| **New Yr5 output: Development of methods for gene editing without DNA vector integration** | Protocols for transient gene editing delivery systems for grapevine protoplasts evaluated. | Evaluate protocols for generation of viable protoplasts.<br>Develop methods for regeneration of plants from protoplasts.<br>Test protocols for transfection of protoplasts. | 30/6/2022 |

| | | | |
|---|---|---|---|
| | | Monitor literature for new information regarding maximising efficacy of delivery of purified CRISPR/Cas9 ribonucleoproteins into grapevine protoplasts as a transient delivery system. | |
| **Year 5 output: Transgenic microvines generated with modified fruit flavour and ripening characters using gene-editing technology developed.**<br>**Yr 3 output cont'd: Completion of rootstock 140 Ruggeri transformation with GAI construct to develop breeding lines (see SRA project 1.4).** | Transformed grapevines with modified expression of target genes (i.e. resistance genes, flavour genes, hormone biosynthesis genes etc) from other SRA projects available for analysis.<br>Transformed 140 Ruggeri ready for testing for dwarf phenotype. | Continue selection of positive embryos/plants transformed with hexenal isomerase gene editing construct and supply up to 20 independent lines for analysis in SRA project 1.2.<br>Perform at least 2 transformations with gene-editing constructs targeting auxin pathway gene (see SRA Project 2.2) Select positive embryos/plants.<br>Complete selection of 140Ru transformants with GAI constructs Analyse grape chemistry, GCMS and genotyping data from 1st generation mildew resistant varieties to identify consumer traits that can be exploited in future breeding, gene discovery and/or gene editing projects. | 30/6/2022 |
| **Ongoing Yr 1 output:**<br>**Winegrape germplasm collections maintained at Irymple including CSIRO Variety collection and scion/rootstock breeding populations.** | Winegrape germplasm collections maintained at Irymple including the CSIRO Variety collection and scion/rootstock breeding populations. | Continue disease management, fertilisation, irrigation, vineyard floor management and pruning of grapevines at the Irymple site, as required. | 30/6/2022 |
| **New Yr 5 output:**<br>**Communication of results of project.** | Submission of final project report. Communication of results as applicable. | Prepare final report | 30/7/2022 |

# 5 Method

## 5.1 Overview

The methods used in this project fell into five main categories. The first involved techniques to produce embryogenic callus from explants derived from anther filaments of immature grape flowers. The second involved the stable transformation of grapevine embryogenic callus using *Agrobacterium* and the associated tissue culturing of this material. The third consisted of methods to test the RNA guides designed to target the Cas9 nuclease to specific gene targets and these were both *in vitro* and *in vivo* techniques. The fourth category included methods to produce viable protoplasts from embryogenic callus and then regenerate plants from the protoplasts. Finally, methods for the delivery of ribonucleoprotein complexes into protoplasts were also used but are presented in sections 6.4. Underpinning all of this work was the use of molecular biology techniques such as cloning, nucleic acid extractions and PCR, but these are not described here as they are routine and used by all molecular biology laboratories.

## 5.2 Grapevine Stable transformation

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

Embryogenic callus was initiated and maintained using media and methods described previously (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 was 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 × 15 mm) containing PIV medium, which comprised 1 × 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 $KH_2PO_4$), 1 × Fe EDTA (37.2 mg/L $Na_2EDTA.2H_2O$, 9.3 mg/L $FeSO_4.7H_2O$); 1 × 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 $ZnSO_4.2H_2O$); 1 × 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 (1 × MS macronutrients (1,650 mg/L $NH_4NO_3$, 440 mg/L $CaCl_2.2H_2O$, 370 mg/L $MgSO_4.7H_2O$, 1,970 mg/L $KNO_3$, 170 mg/L $KH_2PO_4$); 1 × Fe-EDTA; 1 × MS micronutrients; 1 × 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 (90 × 20 mm) (Iocco *et al.* 2001).

Embryogenic callus was maintained on C1 by subculturing selected tissue to fresh medium every five weeks using a stereomicroscope. At all stages Petri dishes were sealed with Parafilm and incubated at 27 °C in the dark.

When preparing grape embryogenic callus for transformation, an improved method developed from those of Iocco *et al.* (2001) and Li *et al.* (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 1 × NN macronutrients, 1 × MS micronutrients, 1 × B5 vitamins, 1 × 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, Phytotechnology Laboratories) (Franks *et al.* 1998). Callus was grown on this medium for 14 days at 27 °C in the dark before infection.

### 5.2.2 Transformation of embryogenic callus of *Vitis vinifera* cv. Shiraz and Chardonnay

*Agrobacterium* was grown on LB agar plates with 25 μg/mL rifampicin and the selection antibiotic. 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 (5 g/L mannitol, 1 g/L L-glutamate, 0.15 g/L $KH_2PO_4$, 0.1 g/L $MgSO_4.7H_2O$, 1 × Fe-EDTA, 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, biotin 20 mg/L, pH 7.0) liquid medium (Iocco *et al.* 2001), containing selection antibiotic, 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 eight minutes. The supernatant was discarded, and 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 9 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 9-10 days, callus was transferred to plates containing GS1CA with timentin as before and the selection antibiotic. Four weeks post-infection, callus was transferred to Petri dishes containing NN medium with timentin and the selection antibiotic. 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 70 mL 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 rooting medium (SM medium – 1 × SM macronutrients (160 mg/L $NH_4NO_3$; 440 mg/L $CaCl_2.2H_2O$; 370 mg/L $MgSO_4.7H_2O$; 2,325 mg/L $KNO_3$; 340 mg/L $KH_2PO_4$), 1 × MS micronutrients, 1 × B5 vitamins, 1 × 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 ½ MS (0.5 × MS macronutrients, 1 × MS micronutrients, 1 × C1 vitamins and amino acids, 1 × 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 glossy leaf, partially to just expanded, 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 embedding the stem into rooting medium in 500 mL polycarbonate tissue culture containers.

### 5.2.3 Initiation of embryogenic callus of rootstock varieties 140 Ruggeri and K51-40

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 K51-40. 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 a number of weeks, any embryogenic callus produced on the initiation media 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 27 °C in the dark.

### 5.2.4 Transformation of embryogenic callus of 140 Ruggeri

*Agrobacterium* inoculum setup, co-cultivation and subculture onto NN medium supplemented with timentin and selection antibiotic are as described above in the Shiraz transformation protocol (Section 5.2.2).

Germinated embryos expressing GFP were collected from these plates. Hypocotyls were trimmed, with 5 mm remaining on the embryo. Trimmed cotyledons were transferred to Petri dishes containing SM medium with 0.8% agar supplemented with 10 µM BAP and incubated for 14 days in low light at 27 °C. Cotyledons were trimmed to expose the top of the stem. Small vertical cuts were made on cotyledons that did not have any visible bud to encourage bud development. Trimmed cotyledons were transferred to Petri dishes containing SM medium supplemented with 5 µM BAP and incubated for 4 weeks in low light at 27 °C. Embryos that produced primary shoots were transferred to 70 mL polycarbonate tubs containing 15 mL SM medium to encourage shoot growth and leaf expansion. Tubs were incubated for four weeks under cool white and Grolux lights (Sylvania) with a photoperiod of 16 hours light: 8 hours dark at 27 °C. Remaining cotyledons were trimmed to remove dying material if necessary and moved to fresh SM + 5 µM medium every four weeks to encourage further bud development. Explants on SM medium were transferred to rooting medium as described above and incubated in the same conditions as explants on SM medium. Leaf harvest to confirm the presence of the introduced gene in the plantlets is as described above.

### 5.2.5 Initiation of embryogenic callus of microvine varieties

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 microvines denoted as 04CO23V0004, 04CO23V0006 and 04C023V0019 which are progeny from the original Pinot Meunier L1 microvine crossed with Grenache as described in Chaib *et al.* (2010). The 04CO23V0004 and 04C023V0019 lines are black-berried and the 04CO23V0006 line produces white berries. Explants were placed into Petri dishes containing PIV initiation medium although it was found that the 04CO23V0006 microvine line preferred PIV medium supplemented with 150 mg/L citric acid. Each Petri dish contained approximately 70 explants. After 3 – 4 weeks developing callus was transferred to C1 medium and embryogenic callus would normally be observed after 9 – 10 weeks.

### 5.2.6 Transformation of embryogenic callus of microvine varieties

The *Agrobacterium* inocula for the microvine transformations were prepared as described above in the Shiraz transformation protocol (Section 5.2.2).

An *Agrobacterium* culture with an $OD_{550\ nm}$ of 0.8 - 1 was transferred to a 50 mL centrifuge tube and the cells were precipitated by centrifugation at 5000 rpm for 8 min at room temperature. The supernatant was discarded and the pellet resuspended in 25 mL induction medium (2 mM $NaH_2PO_4$, 1x AB salts, 40 mM MES pH 5.6, 0.5% glucose) supplemented with 100 µM acetosyringone. The culture was adjusted to an $OD_{500nm}$ of 0.5 – 0.7 and incubated for 2 h at 28 °C with shaking. The culture was transferred to a 50 mL centrifuge tube and the cells were precipitated by centrifugation at 5000 rpm for 8 min at 18°C. The supernatant was discarded and the pellet resuspended in LCM, and the $OD_{500nm}$ was adjusted to 0.5 – 0.7 prior to inoculation.

Embryogenic callus was picked from GS1CA plates and added to the *Agrobacterium* culture. Approximately 45 – 50 mL of the *Agrobacterium* culture was used per 1.5 – 2 g of embryogenic callus tissue. The Falcon tube containing the *Agrobacterium* and embryogenic tissue was placed on a gentle shaking platform at 28 °C for approximately 7 min (no more than 12 min). The tissue was recovered by filtering the material through a 100 µm mesh filter unit, and excess liquid was blotted off using sterile Whatman filter paper #1. The tissue was collected and placed onto charcoal GS1CA plates, in 0.5 – 1 cm clumps, and incubated in the dark for 45 - 48 h (no more than 48 h) at 22 °C. Following this incubation, callus tissue was collected and washed in a 50 mL Falcon tube with 45 – 50 mL of LCM supplemented with 1000 µg/ mL timentin. The tissue was collected by filtering the material through a 100 µm mesh filter unit, and excess liquid was blotted off using sterile Whatman filter paper #1. The inoculated callus was then transferred in 0.5 – 1cm clumps to GS1CA plates supplemented with 1000 µg/ mL timentin and incubated in the dark at 27 °C for 10 – 20 days from the date of inoculation.

Selection of transformed callus was achieved by then moving the material onto GS1CA medium supplemented with 500 µg/mL timentin and the appropriate selection antibiotic (7 µg/mL hygromycin or 15 µg/mL kanamycin) and incubated in the dark at 27 °C for three weeks. To promote germination the callus was then transferred to NN plates supplemented with timentin and hygromycin and incubated in the dark at 27 °C. Developing callus continued to be transferred to this medium at three-weekly intervals. Germinated embryos expressing green fluorescent protein (GFP) were collected from these plates.

Germinated embryos were transferred to Petri dishes containing SM media with 1.5% Bacto agar, supplemented with 10 µM BAP. Embryos were incubated under low light at 27 °C. Developing embryos and cotyledons from this step onwards were handled as described in the rootstock stable transformations protocol (Section 5.2.4).

### 5.2.7 GFP detection of transformed material

About 4 days after washing to remove *Agrobacterium*, transformed callus was examined under a Nikon SMZ25 stereoscope with GFP fluorescence imaging. A burst of transient expression of GFP was usually observed at this time. Callus that did not have good levels of GFP expression was discarded. About 10-12 weeks after transformation, callus was examined again for GFP fluorescence. Transformed embryos were then sub-cultured together with fluorescent callus. All germinated embryos were checked for the presence of GFP before being transferred to shoot development medium.

### 5.2.8 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 95 °C for 1 minute followed by 30 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 20 seconds. The transgene of interest was assessed for its presence in the extracted DNA using primers designed specifically for each transgene. 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').

### 5.2.9 Target genes and vectors used in stable transformation experiments

#### Abiotic stress gene targets

The following transformations were done in collaboration with the staff in Project SRA 1.3. Transformations were done in both Shiraz and/or 140 Ruggeri to test gene function of genes potentially involved in salt exclusion.

**Table 1 List of transformation experiments conducted to assess function of genes involved in abiotic stress. ST = Shiraz transformation and RT = 140 Ruggeri transformation.**

| TRANSFORMATION CODE(S) | GENE | CONSTRUCT | DESCRIPTION |
|---|---|---|---|
| **RT27, RT28** | *VvHKT1;1* | pVv::HKT1;1 | HKT1;1 gene under the control of the *Vitis vinifera* promoter from Shiraz |
| **ST181, RT25, RT26** | *VvHKT1;1* | pMG56-12::HKT1;1 | HKT1;1 gene under the control of the *Vitis vinifera* promoter from Shiraz |
| **RT21, RT22, RT23** | ClCG1 | p1CHAsi | RNAi silencing construct designed to reduce expression of a candidate gene for chloride exclusion. |
| **RT24** | ClCG2 | p2CHAsi | RNAi silencing construct designed to reduce expression of a candidate gene for chloride exclusion. |

#### CRISPR/Cas vectors

The series of stable transformations below was conducted to test the gene editing technology in grapevine and to test aspects of the methodology such as guide design.

**Table 2 List of transformation experiments conducted to test gene editing technology in grapevine. MiT = Microvine transformation and ST = Shiraz transformation.**

| TRANSFORMATION CODE(S) | GENE | CONSTRUCT(S) | DESCRIPTION |
|---|---|---|---|
| **MiT2** | *VviPLATZ1* | pVCAS9FS1, pVCAS9FS2, pVCAS9FS3, pVCAS9FS4 | Constructs designed for gene editing of *VviPLATZ1* using a single guide RNA. CAS9 was codon-optimised for grapevine. |
| **MiT3** | *VviPLATZ1* | pVCAS9FS1, pVCAS9Multi1 | Constructs designed for gene editing of *VviPLATZ1*. The pVCAS9Multi1 construct was testing a multi-guide delivery system. |
| **MiT4, 7** | *VviPLATZ1* | pGCas9INT_FS1, pGCas9INT_FS4 | Constructs designed for gene editing of *VviPLATZ1* using a single guide RNA. Differed from the previous constructs as it had a different promoter and intron. |
| **MiT5, ST182** | *VviPLATZ1* | pVCAS9Multi1 | Testing a multi-guide RNA delivery system. |
| **ST183, ST185** | *VviPLATZ1* | pVCAS9FS1 | Construct designed for gene-editing of *VviPLATZ1* using a single guide RNA. CAS9 was codon-optimised for grapevine. |

| | | | |
|---|---|---|---|
| **ST184, ST186** | *VviPLATZ1* | pGCas9INT_FS1 | Constructs designed for gene editing of *VviPLATZ1* using a single guide RNA. Differed from the previous constructs as it had a different promoter and intron. |
| **MiT15, MiT15, ST187** | *VvMyb14* | pVCAS9Myb14_6 | Construct designed for gene editing of *VvMyb14* using a single guide RNA. |
| **MiT17, ST188** | *VvWRKY52* | pPC_FS_WRKY52 | Polycistronic construct designed for gene editing of *VvWRKY52* and *VviPLATZ1*. |

### Grapevine dwarf vine vectors

Some vectors were designed to explore the possibility of producing microvine versions of other scions or rootstocks to use as rapid breeding lines. These could be used to pyramid traits into a breeding line with the transgene crossed out in a final cross to produce new superior scion or rootstock selections.

**Table 3 List of transformation experiments conducted to alter the stature and flowering of grapevines using the mutated *VvGAI1* gene. MiT = Microvine transformation and ST = Shiraz transformation.**

| TRANSFORMATION CODE(S) | GENE | CONSTRUCT | DESCRIPTION |
|---|---|---|---|
| **RT29, RT31, RT33, ST171, ST173, ST179** | *VvGAI1* | pVvGAI::GAI | A vector designed to express the mutant version of the *VvGAI1* gene under the control of its own promoter. |
| **RT30, RT32, RT34, ST172, ST174, ST180** | *VvGAI1* | p35S::GAI | A vector designed to express the mutant version of the *VvGAI1* gene under the control of the constitutive 35S CaMV promoter. |
| **RT35** | *VvGAI1* | pCAS9_GAImulti | A polycistronic gene editing construct targeting multiple sites in the *VvGAI1* gene. |
| **RT36** | *VvGAI1* | pCAS9_GAI_13D | A gene editing construct targeting the *VvGAI1* gene with a single RNA guide. |

### Grape composition vectors

In collaboration with SRA Project 1.2, some grapevine stable transformations were conducted in order to gene-edit *VvHI* genes to test gene function. These genes are predicted to be involved in determining the balance of six-carbon aldehydes and alcohols that are present in grapes, which influences the 'green' sensory characters in wine.

**Table 4 List of transformation experiments conducted to determine the function of genes suspected of being involved in the lipoxygenase pathway. MiT = Microvine transformation.**

| TRANSFORMATION CODE(S) | GENE | CONSTRUCT | DESCRIPTION |
|---|---|---|---|
| **MiT9, MiT13, MiT18** | *VvHI* | pVvHIguide1 | A gene editing construct targeting the *VvHI* genes with a single RNA guide. |
| **MiT8, MiT10, MiT14** | *VvHI* | pVvHIguide2 | A gene editing construct targeting the *VvHI* genes with a single RNA guide. |

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## 5.3 Abiotic stress assays of transgenic plants

The ability of certain genetic modified plants to exclude chloride or sodium from the shoot was assessed by salt assays using plants grown in tissue culture and the glasshouse.

### 5.3.1 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 5 mM, 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+:1Ca2+:1Mg2+ 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.

### 5.3.2 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 + 25 mM chlorides or RO water at 50 mL per plant. On day 2, plants were watered with ¼ strength Hoagland's + either 50 mM mixed chloride or RO water and from day 3 until the end of the experiment with ¼ strength Hoagland's + either 75 mM chlorides or RO water added at 50 mL/plant/day. Plants were harvested on day 28. Meristem, root and leaf samples were taken for molecular or ion analysis.

## 5.4 Analysis of gene expression

Expression of target genes was determined by real time PCR. 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. 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 5.

**Table 5 – qPCR primer sequences**

| PRIMER NAME | SEQUENCE | TARGET |
|---|---|---|
| **Ubiq_F** | AACCTCCAATCCAGTCATCTAC | Ubiquitin |
| **Ubiq_R** | GTGGTATTATTGAGCCATCCTT | Ubiquitin |
| **GAI_qPCR_WT_F** | CGGCATGGATGAGCTTCT | Wild-type allele (*VvGAI*) |
| **GAI_qPCR_M_F** | CGGCATGGATGAGCTTCA | Mutant allele (*Vvgai*) |
| **GAI_qPCR_R** | AAGCGAGATGAGAGAGGCC | Both |

## 5.5 Ion analysis

### 5.5.1 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.

### 5.5.2 Cation concentration

Dried samples were examined in duplicate for the concentration of the cations, $Na^+$, $K^+$, $Ca^{2+}$ 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.

## 5.6 Hairy Root Transformation

### 5.6.1 Growth and transformation of *Agrobacterium rhizogenes* A4

*Agrobacterium* A4 was grown on ABB plates, which comprised of 50 mL/L of 20 × Buffer (60 g/L $K_2HPO_4$, 23 g/L $NaH_2PO_4.2H_2O$, pH 7.3) and 20 × salts (20 g/L $NH_2Cl$, 12.3 g/L $MgSO_4.7H_2O$, 3 g/L KCl, 0.265 g/L $CaCl_2.2H_2O$, 0.5 g/L $FeSO_4.7H_2O$), 10 g/L D-glucose, 15 g/L bacto agar, with 2 µg/L biotin and selection antibiotics added after autoclaving. Plates were streaked and incubated at 28 °C until large colonies appeared.

Electroporation competent cells were prepared by first inoculating 10 mL of MG/L medium with a loop of *A. rhizogenes* A4 cells, either from a glycerol stock or single colony from a plate, which was

incubated overnight at 28 °C with shaking. The following day the 10 mL overnight culture was used to inoculate 300 mL of MG/L which was shaken at 28 °C until the $A_{600nm}$ was between 0.5 and 0.8 (usually after 4 h). When the culture reached the required optical density the culture was placed on ice to chill for 15 - 20 min. The cells were then pelleted by centrifugation at 4000 × g at 4 °C for 15 min and the liquid medium discarded. The pellet was resuspended in 10 mL of cold (4 °C) 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 and transferred to two 50 mL Falcon tubes. The pellet was then washed twice again in fresh 1 mM HEPES pH 7.0 following centrifugation. After the final spin, the pellet is then resuspended in 10 mL 10% (v/v) glycerol, pelleted by centrifugation at 5000 RPM at 4 C for 15 minutes and resuspended in 3 mL of cold (4 °C) 10% glycerol. The *A. rhizogenes* A4 cell suspension was then dispensed as 40 µL aliquots, snap frozen with liquid $N_2$ and stored in – 80 °C freezer.

Transformation of *A. rhizogenes* A4 competent cells was achieved by first thawing an aliquot on ice. The cells were then carefully pipetted into a 1.5 mL tube containing the plasmid to be transformed into the microbe, mixed gently by pipetting up and down before pipetting the mix into a electroporation cuvette that was pre-chilled on ice. The cuvette was then placed into the electroporator, which had been set at a capacitance of 250 µF, resistance of 2.5 Ω and a voltage of 2.0 V, and the electric pulse delivered to the cells in the cuvette. The cells are then removed from the cuvette after the addition of 250 µL of MG/L and incubated at 28 °C with shaking for 1 h. The *A. rhizogenes* A4 were then plated on ABB plates with the appropriate selection antibiotic and incubated at 28 °C for 2 d.

### 5.6.2 Transformation of leaf petioles with *A. rhizogenes* A4 to produce hairy roots

The day before infection, a single colony of *A. rhizogenes* A4 harbouring the desired binary vector was selected and used to inoculate 5 mL of MG/L liquid medium containing selection antibiotic. The suspension was incubated overnight at 28 °C with shaking at 200 rpm.

On the day of infection, healthy leaves were harvested from tissue culture grown plantlets of the desired variety. Plantlets had been prepared by first transferring propagated tips onto rooting medium until root nodules appear (approximately two weeks), followed by transfer of the tip and root nodule to SM medium. Leaves were ready for inoculation when fully expanded leaves had developed on the plantlets and they had reached the top of the 500 mL polycarbonate tubs. Excised leaves were placed on square Petri dishes containing SM agar medium, with the waxy surface of the leaf in contact with medium and the petiole pointing up. At least 50 leaves were used for each construct to ensure sufficient chance of detecting gene editing. The *A. rhizogenes* suspension was grown until the culture had an $OD_{600}$ of 1.2-1.8 and freshly cut petioles were infected with the *A. rhizogenes* suspension using a bacterial loop. Approximately four weeks later the petioles were then checked to ensure a callus of approximately 5 mm in diameter has formed. If the callus was present the leaves were transferred so that the petioles and callus were embedded into the media onto to square Petri dishes containing SM agar medium supplemented with 500 µg/mL timentin and incubated at 25 °C under full light. Every four weeks, leaves were transferred to fresh SM agar supplemented with 500 µg/mL timentin.

Plates were checked weekly for root growth, and developing roots were checked under the SMZ25 stereoscope with GFP fluorescence imaging to confirm the presence of GFP. Roots approximately 7 cm in length were harvested and placed into a 1.2 mL cluster tube for storage at -80 °C.

## 5.7 Gene editing methodologies

Gene editing technologies represent a breakthrough in the targeted altering of the DNA of an organism. This can potentially be used to improve crops by altering genes involved in determining important phenotypes such as yield, composition, stress tolerance and water use so long as appropriate target genes have been identified. Certain methodologies can also be used that result in an edited plant that is not regarded as a genetic modified organism.

### 5.7.1 Design and testing of guide RNAs

Guide RNAs to target genes were designed using the online tool on the Synthego web site (https://www.synthego.com/products/bioinformatics/crispr-design-tool). Further checks of the guide RNA characteristics were done using various online algorithms including WU-CRISPR (https://crisprdb.org/index.html) and Tuscan (https://gt-scan.csiro.au/tuscan). It was observed that the best guides tend to come from regions of target genes that are rich in protospacer adjacent motifs (PAMs), which are the sequences recognised by the Cas9 nuclease downstream of the guide RNA and are essential for cleavage. It was also found that G-rich guides give better results, perhaps as they have internal PAM sites.

Once designed, guide RNAs were first tested *in vitro* using the Guide-it sgRNA In Vitro Transcription and Screening kit (Takara Bio USA, Inc). If the *in vitro* assay showed a high percentage of cleavage of the target DNA, binary vectors were then constructed for stable transformation experiments. The Cas9 sequence was codon optimised for grapevine and contained both a nuclear localisation sequence to target the protein correctly and an intron to avoid issues of leaky Cas9 expression in bacteria during cloning (Li *et al.* 2013). This was inserted between the *Arabidopsis* UBIQUITIN promoter and terminator (Miao *et al.* 2013) and cloned into the pCLB1301 binary vector (Chaib *et al.* 2010) which also contains a hygromycin resistance gene the endoplasmic reticulum localised green fluorescent protein (GFP-5ER) reporter for the selection of transformed embryos. The guide RNAs were placed under the control of the UBIQUITIN6 promoter from *Arabidopsis* (Nekrasov *et al.* 2013). The guides were then testing *in planta* using the hairy root transformation system (Section 5.6). Following the transformation, GFP positive roots were selected, DNA-purified and the targeted gene amplified using appropriate primers. The amplified DNA was then sequenced and the data interpreted using the Inference of CRISPR Edits (ICE) software available online (https://www.synthego.com/products/bioinformatics/crispr-analysis; (Conant *et al.* 2022)) to determine those guides providing the greatest editing frequency.

### 5.7.2 Stable transformation

Binary vectors that were found to induce a high frequency of editing in the target gene were used to produce transgenic plants using the stable transformation protocol outlined in Section 5.2.

### 5.7.3 Suspension cultures for protoplast production

Pre-embryogenic calli suspension cultures were initiated by collecting 150 - 200 mg of friable callus (prepared as outlined in section 5.2.1) from C1 plates 5-6 weeks after being sub-cultured. The calli were placed in a sterile 125 mL flat bottom flask and 15 mL liquid C1 was added. The flask was covered with sterile foil, sealed with Parafilm and incubated in the dark at 27 °C with shaking at 120 rpm.

For slow growing calli, the liquid culture was supplemented weekly by removing half of the liquid C1 medium (7.5 mL) and replacing it with the same volume of fresh liquid C1. This was repeated weekly until the calli needed a greater volume of medium to remain healthy. For prolific calli, the entire contents in the flask were transferred to a 250 mL flat bottom flask after a week, and an additional 15 mL of liquid C1 was added for a final volume of 30 mL. This was repeated on a weekly basis until 120 mL of culture in a 500 mL flat bottom flask was established. If volumes of culture larger than 120 mL were required, the culture would be transferred to a 1 L flat bottom flask.

Once a suspension culture was established, it was subcultured weekly by placing the flask at a 45° angle on a metal holder to allow the callus to sink under gravity to the base of the flask. Half of the liquid medium was carefully pipetted out without disturbing the calli and disposed of. The flask was placed upright and the same volume of fresh C1 that was removed, was added. The fresh C1 medium was pipetted down the walls of the flask so that any calli that clung to the walls were washed down. The flask was covered again in foil, placed back on the shaker, and incubated in the same conditions as used for culture initiation.

When the callus cultures had produced a lot of mass, some of which was clumped, the culture was sieved to obtain small, uniform pieces of calli. This was done by placing the flask at a 45° angle on a metal holder to allow the calli to settle to the base of the flask. Then 50 mL of liquid C1 was removed from the flask and pipetted into a sterile Falcon tube. The remaining culture in the flask was poured through a tea-strainer sieve into a fresh, sterile flat bottom flask. The original flask was washed with the liquid C1 in the Falcon tube and subsequently poured through the same sieve into the new flask. The sieve containing the callus was placed aside and the new flask was placed on a 45° angle on the metal holder. Once the small grains of calli had settled, 80 – 90 mL of the liquid C1 was removed and pipetted back into the old flask. The new flask was placed upright and the sieve containing the callus material was placed on top of the new flask. The 80-90 mL of liquid C1 was used to wash the callus material to retrieve any remaining small clumps. This washing step was repeated twice to maximise the yield of small calli. Once completed, the new flask containing the small calli was placed back on the metal holder, and 60 mL of the liquid C1 was removed. The flask was placed upright and the sieve containing the callus material was placed back on. Then 60 mL of fresh C1 was added through the sieve, to achieve a final volume of 120 mL. The flask was covered again in foil, placed back on the shaker, and incubated in the same conditions as culture initiation.

When calli material began proliferating and all material needed to be retained, the material was divided into two flasks by placing the original flask at a 45° angle on the metal holder, allowing material to sink to the bottom, and removing 50 mL of liquid C1 into a sterile Falcon tube. The flask was then placed upright and the calli material was divided and pipetted into two sterile Falcon tubes. The calli material was allowed to settle into the bottom of the tubes, and the tubes were topped up with liquid C1 to a volume of known value. Each culture was poured into a new flask, and the Falcon tubes were rinsed with the reserved liquid C1 to obtain a volume that was half of the

volume of the original starting culture (i.e. 60 mL if original culture was 120 mL). Fresh liquid C1 was added to each flask to obtain the same volume of the original starting culture. The flasks were covered again in foil, placed back on the shaker, and incubated in the same conditions as for culture initiation.

### 5.7.4 Protoplast production and plant regeneration

The development of the methods for the protoplast production and regeneration is described in detail in section 6.4.

### 5.7.5 Transfection of protoplasts

The development of the methods for the transfection of protoplasts is described in detail in section 6.4.

### 5.7.6 Cell staining methodologies

#### Calcofluor

Calcofluor white is a non-specific fluorochrome that binds to cellulose and chitin present in cell walls. It is used to see if the cell wall enzyme digest has worked sufficiently and can also be used to see if the cell walls have grown back around the protoplasts after transfection.

To use, a 0.1% stock of Calcofluor white (C.I. Fluorescent Brightener 28) was prepared in water. This was then diluted 1:10 with water before the addition of 10 µL to 90 µL of a cell or protoplast suspension before mounting 30-40 µL onto a slide. The slide is then examined under UV light and cell walls should fluoresce bright green to blue, whereas other material is reddish-orange.

#### Fluorescein:Propidium iodide (plant cell viability assay)

The use of both fluorescein and propidium iodide acts as a dual colour fluorescent system of staining to allow the identification of both viable and non-viable cells simultaneously. Viable cells are assumed to have esterase activity that cleaves the fluorescein diacetate substrate, which is both membrane-permeable and non-fluorescent. Esterase activity removes the acetate groups which makes the molecule polar, stopping it from crossing the membrane again, and fluorescent. An intense green staining is indicative of cell viability. Conversely, propidium iodide can only enter cells with damaged cell membranes. Upon entry it then intercalates with double stranded DNA resulting in a bright red fluorescence in non-viable cells.

The staining was done following the manufacturer's instructions (Plant Cell Viability Assay Kit, Sigma). In brief, the 10 × staining solution was made by adding 1 µL of each of the fluorescein diacetate and propidium iodide solutions to 98 µL of water. Then 10 µL of the staining solution is added to 90 µL of the cell suspension. The cells are incubated for 1-2 min before mounting 30- 40 µL on a slide. The fluorescein is viewed under the fluorescence microscope with an excitation maximum of 494 nm and emission maximum at 518 nm, and propidium iodide has an excitation maximum at 536 nm and emission maximum at 617 nm. The ratio of green to red cells gives the viable to non-viable cell ratio.

### Trypan blue viability stain

Trypan blue is a large molecule that can only enter cells that have a damaged plasma membrane. Once it has entered the cell, trypan blue binds to proteins and turns the non-viable cells a bluish colour. This assay allows the identification of viable (unstained) and non-viable cells (blue) in a mixture of cells.

For the assay, a 0.4% (w/v) solution of Trypan blue was prepared in 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$; pH 7.4) and filter sterilised through a 0.22 µm filter membrane. The protoplasts were then diluted 1:100 in culture medium in a total volume of 100 µL and added to 100 µL of the 0.4% Trypan blue solution. An aliquot of the cells was then placed on a haemocytometer and visualised under a bright field microscope to allow the counting of viable and non-viable cells.

### Nuclei staining using DAPI

DAPI (4',6-diamidino-2-phenylindole) is a blue-fluorescent stain that binds to double-strained DNA which enhances the fluorescence 20-fold. The excitation wavelength for DAPI is 360 nm and the emission wavelength is 460 nm. It is cell permeable and is used for staining nuclei in intact cells.

For staining we used the 'DAPI ready-made solution' from Sigma. An aliquot of the DAPI solution was diluted 1:10 with sterile water and 10 µL of the dilution added to 90 µL of sample. This was then incubated for 3-5 min before 30-40 µL was placed on a slide and covered with a coverslip before visualisation on a microscope with DAPI filters.

# 6 Results/Discussion

## 6.1 Stable transformations assisting other projects in the Strategic Research Agreement

### 6.1.1 Introduction

Some rootstock genotypes such as 140 Ruggeri are capable of restricting the accumulation of sodium ($Na^+$) and/or chloride ($Cl^-$) ions in the shoot of grafted scions, thereby improving tolerance to saline soils. A better understanding of the genes underlying these important traits will ultimately help to breed new rootstocks with improved salt tolerance.

We previously identified a single major locus, *NaE*, that is associated with the root-based exclusion of $Na^+$ from the shoots of a K51-40 x 140 Ruggeri hybrid rootstock population (See CSP 1302 and (Henderson *et al.* 2018). Based on the PN40024 reference genome this locus contains six genes belonging to the high affinity $K^+$ transporter (*HKT1*) family, which have been shown to control sodium exclusion in other plant species (Ali *et al.* 2019). Of the six *HKT1* genes residing in the *NaE* locus only two, *HKT1;1* and *HKT1;3*, were found to be expressed in roots (Henderson *et al.* 2018), and thus deemed to be the likely candidate genes underlying this trait.

In the previous project CSP 1302, we also identified two lead candidate genes for chloride exclusion that each reside in a locus, *CLEX* (for $Cl^-$ exclusion), which is associated with this trait in a *V. berlandieri* × *V. vinifera* backcross F2 population.

To develop perfect genetic markers to allow early selection of $Na^+$ and $Cl^-$ exclusion traits in the rootstock breeding program it is vital to know the underlying causal genes. Here we assessed the phenotypes of transformed Shiraz vines possessing hairpin constructs designed to down-regulate these candidate $Na^+$ and $Cl^-$ exclusion genes, to conclusively determine which of them are involved in the exclusion of sodium and chloride ions and thus rootstock-conferred salt tolerance. We also investigated the potential to add additional copies of *HTK1;1* as a means to improve $Na^+$ exclusion in transgenic vines.

### 6.1.2 *HKT1* sodium exclusion genes

#### Down-regulation of a high affinity $K^+$ transporter, *HKT1;1* (KA lines)

In the Final Report of the previous project CSP 1306 we described the generation of 70 Shiraz KA lines in which the lead candidate gene for sodium exclusion, *HKT1;1,* was targeted for down-regulation via the introduction of a hairpin construct targeted to this gene. When grown in tissue culture, reduced expression of *HKT1;1* in the roots of most KA lines (Figure 1A) resulted in greater accumulation of $Na^+$ in shoots and leaves compared to control lines and KA lines not displaying a reduction in gene expression (Figure 1B). A significant negative correlation between the expression

of *HKT1;1* and shoot $Na^+$ concentrations confirms a direct role of this gene on $Na^+$ exclusion (Figure 1C). A similar result was seen when plants were screened in the glasshouse (data not shown).

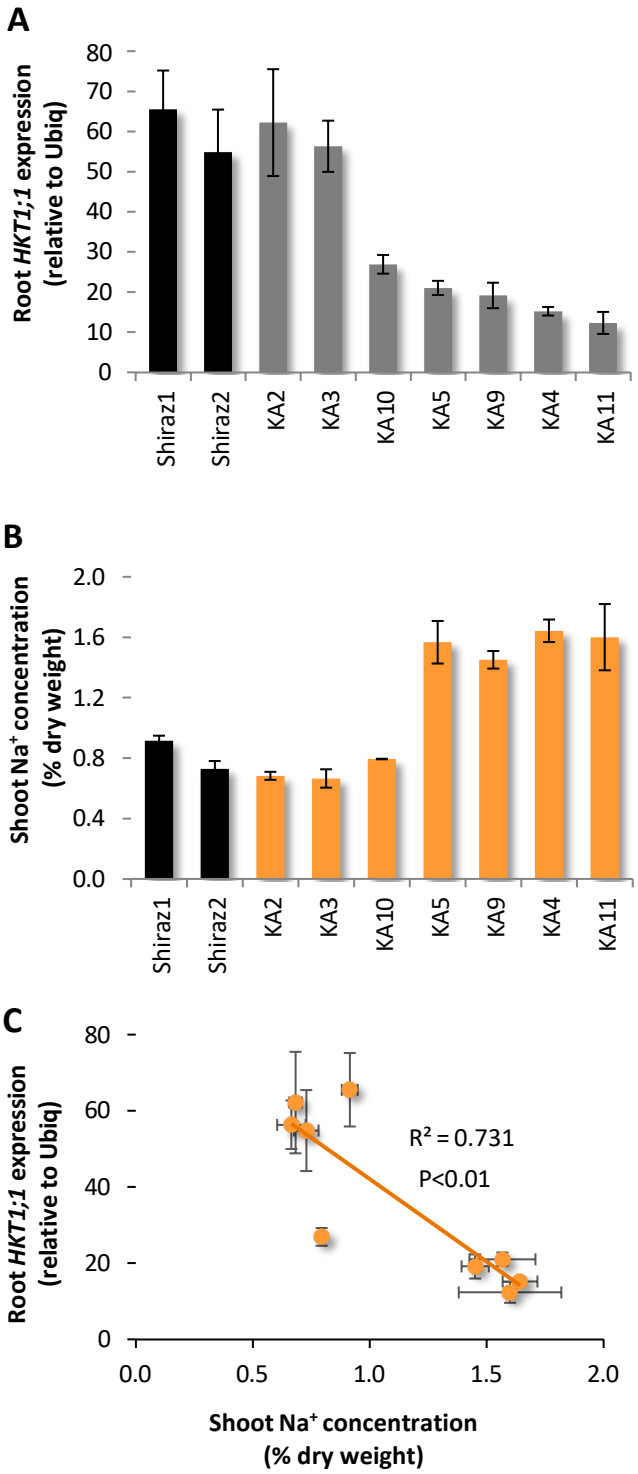



**Figure 1 – Analysis of KA lines harbouring a hairpin transgene targeting the down-regulation of HKT1;1 screened in tissue culture assay under 75 mM mixed $Cl^-$ (45 mM $Na^+$) for three weeks. (A) Expression of HKT1;1, relative to the reference gene Ubiquitin, and (B) Shoot $Na^+$ concentrations in KA lines and two untransformed Shiraz control lines. (C) Negative correlation between HKT1;1 expression and resulting shoot $Na^+$ concentrations. Error bars represent the standard error of the mean of four biological replicates.**

Although these results were previously reported in project CSP 1302, we present them again here for comparison with the following results on the down-regulation of *HKT1;3* and up-regulation/additional copy of *HKT1;1*.

### Down-regulation of a high affinity $K^+$ transporter *HKT1.3* (KB lines)

In the Final Report of the previous project CSP 1306 we described the generation of 34 Shiraz KB lines in which a second candidate gene residing within the *NaE* locus, *HKT1;3,* was targeted for down-regulation via the introduction of a hairpin construct. Here we assessed the $Na^+$ exclusion phenotypes of 33 of these lines. Firstly, 17 lines were screened in a tissue culture assay (described in 5.3.1) together with two lines of untransformed Shiraz controls. Plants received 75 mM mixed $Cl^-$ (45 mM $Na^+$, 7.5 mM $Ca^{2+}$, 7.5 mM $Mg^{2+}$) for three weeks before whole shoots (stem + petiole + laminae) were harvested and dried. Analysis of $Na^+$ concentration via ICP revealed 15 of the transgenic lines that possessed the hairpin transgene had shoot $Na^+$ concentrations lower than the untransformed controls, although differences were not statistically significant, while only two lines had concentrations higher than the controls (Figure 2A). This is the opposite trend to what is seen for KA lines in which reduced expression of *HKT1;1* results in increased $Na^+$ concentrations (Figure 1).

To further investigate this possible opposite trend of KB lines to KA lines, an additional 16 KB lines were assessed alongside the two Shiraz control lines. For this assay, plants were deflasked from tissue culture tubs and grown in potting mix in the glasshouse in an attempt to obtain a greater separation of control and transformant phenotypes. Plants received 25 mM mixed $Cl^-$ (15 mM $Na^+$, 2.5 mM $Ca^{2+}$, 2.5 mM $Mg^{2+}$) for three weeks before the laminae were harvested for determining $Na^+$ content. Again, the majority of KB lines (12 of 16) accumulated less $Na^+$ in the laminae than the control lines Figure 2B), however these were not significantly different from control lines.

The reduced $Na^+$ phenotype of transgenic lines harbouring a hairpin construct targeted to *HKT1;3* hints at the possibility that *HKT1;3* is involved in $Na^+$ inclusion as opposed to $Na^+$ exclusion. In other plants, the process of $Na^+$ inclusion, or loading into the xylem, has been largely attributed to other transporter genes such as *SOS1* (Shi *et al.* 2002) and *CCC* (Zhu *et al.* 2017), however there is also new evidence to suggest that HKT1 genes may also be contributing to sodium accumulation via xylem loading (Venkataraman *et al.* 2021).

As differences between KB positive transformants and control lines were not significantly different in either of the two screens it was decided not to undertake gene expression analysis and to focus resources in other areas of the project. In hindsight, being able to quantify the level of expression of *HKT1;3* in the KB lines could help determine whether the trend of reduced $Na^+$ accumulation is correlated with a reduction in gene expression, or if it is just a coincidence. Thus, further work is required to determine if *HKT1;3* has a role in $Na^+$ inclusion. If so it could make an interesting knock-out target via gene editing, which may result in vines accumulating less $Na^+$ in the shoots.

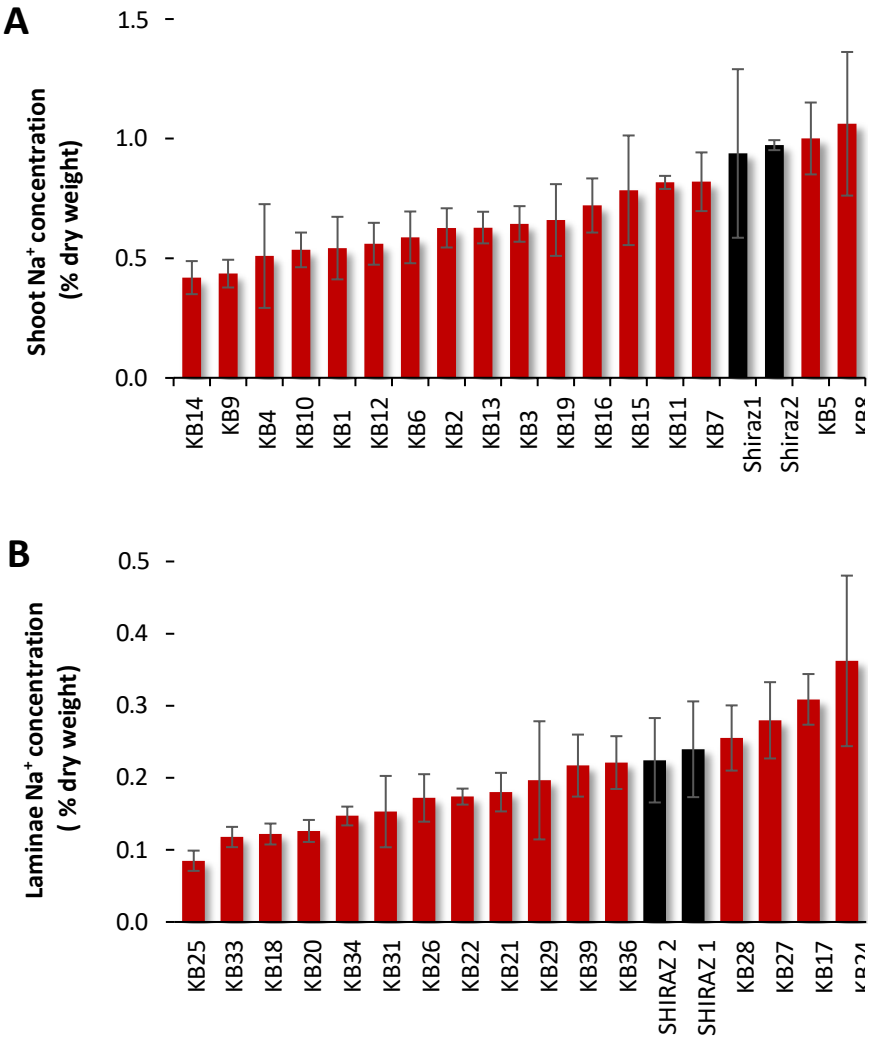



**Figure 2 – $Na^+$ exclusion of transgenic KB lines possessing a hairpin construct targeted to HKT1;3. A) Shoot $Na^+$ concentration of 17 lines assessed in a tissue culture screen exposed to 75 mM mixed $Cl^-$. B) Laminae $Na^+$ concentration of 16 lines grown in potting mix in the glasshouse and exposed to 25 mM mixed $Cl^-$. Untransformed Shiraz control lines are highlighted in black. Each line consisted of four biological replicates, with error bars representing the standard error of the mean.**

### Additional copies of the dominant HKT1;1-E allele (HV lines)

Another possible avenue to improve the $Na^+$ exclusion of rootstocks is to introduce additional copies of the dominant *HKT1;1*-E allele, which has been linked with increased exclusion of $Na^+$ from the shoot (Henderson *et al.* 2018). To test this approach two different *HKT1;1*-E alleles were amplified along with their native promoters from *V. vinifera* 'Shiraz' and *V. berlandieri* accession 'MG56-12'. These two genotypes were chosen as they both contain the dominant and highly functional HKT1;1-E alleles based on key amino acids 534 and 537. Amplified sequences were cloned into the pART27 vector.

*Agrobacterium*-mediated transformation of Shiraz embryogenic callus with the HKT1;1-MG56-12 construct resulted in 74 embryos and 22 plantlets. However, the number of lines positive for the transgene could not be properly determined as when screening for positive transformants by PCR it was discovered that the promoter was likely cloned into the construct in the wrong direction. As such this transformation was abandoned.

Transformations with the HKT1;1-VvShiraz construct resulted in 167 embryos, 52 plantlets and 45 positive for the transgene. Ten of the resulting HV lines were screened alongside a negative control line for their ability to exclude $Na^+$ from the laminae when grown in potting mix in the glasshouse. All lines were exposed to three treatments; a 0 mM rainwater control, a 60 mM NaCl, and a 100 mM mixed $Cl^-$ (60 mM $Na^+$, 10 mM $Ca^{2+}$, 10 mM $Mg^{2+}$), each for 28 days. Analysis of $Na^+$ in the laminae revealed that three HV lines (HV44, HV4, HV8) accumulated less $Na^+$ in the laminae than that of the negative control HV20, however these differences were not statistically significant (Figure 3). Unexpectedly, six of the HV lines (HV43, HV14, HV3, HV1, HV7, HV46) accumulated significantly higher amounts of laminae $Na^+$ that were up to 50 times greater than the control line. This hyper-accumulation of $Na^+$ was not only evident under the two salt treatments, but amazingly also under the rainwater control treatment. In this treatment the accumulated $Na^+$ must have originated in the potting mix. By the end of the experiment, lines HV3, HV1, HV7 and HV46 all showed severe leaf burn symptoms under the two salt treatments, consistent with the extremely higher accumulation of $Na^+$ in their leaves (Figure 4). Minor leaf burn symptoms were also present on a few leaves in the control treatment (Figure 4).

The reason for this hyper-accumulation of $Na^+$ is not clear and unfortunately time did not permit analysis of resulting gene expression levels. One plausible explanation is that the presence of an additional copy or copies of *HKT1;1* resulted in the co-suppression of the native *HTK1;1* alleles via natural RNAi pathways that serve as a defence against plant viruses (Napoli *et al.* 1990; El-Sappah *et al.* 2021). It is interesting to note that when the expression of *HKT1;1* was reduced in KA lines via the introduction of a hairpin construct, this only led to a 2x and 5x increase in $Na^+$ accumulation in tissue culture (Figure 1) or glasshouse screens respectively. This smaller phenotypic effect in KA lines could be because only a partial silencing of the gene was achieved with the hairpin construct (Figure 1A), whereas the hypothesised co-suppression in HV lines may be leading to a more complete silencing. Thus, gene silencing via the introduction of a full-length transgene may provide a more complete level of silencing, completely abolishing HKT1;1 activity, however this needs to be confirmed through analysis of gene expression.

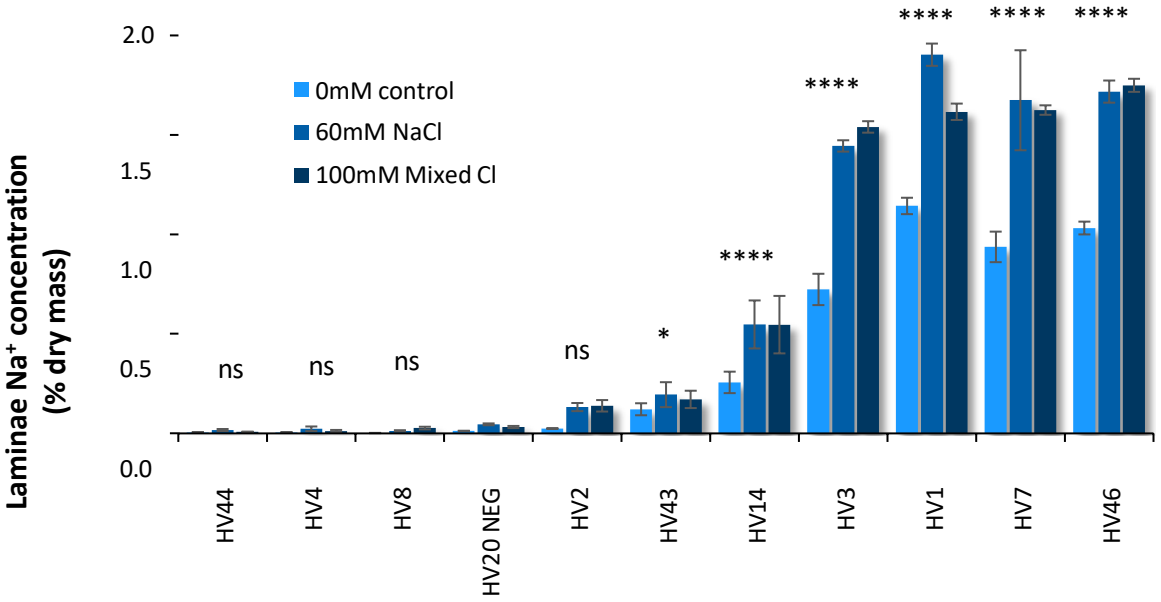



**Figure 3 – $Na^+$ exclusion of transgenic Shiraz HV lines possessing one or more additional copies of HKT1;1 driven by the native Shiraz promoter. HV lines were grown in the glasshouse and exposed to a rainwater control treatment, a 60 mM NaCl, and a 100 mM mixed $Cl^-$ treatment equivalent to 60 mM $Na^+$. A negative transformant lines HV20 serves as the control line. Each line consisted of four biological replicates, with error bars representing the standard error of the mean. The significance of differences between positive lines and the negative control under the 100 mM mixed $Cl^-$ treatment as determined by a one-way ANOVA are highlighted above bars with ns = not significant, * = p<0.05, **** = p<0.001.**

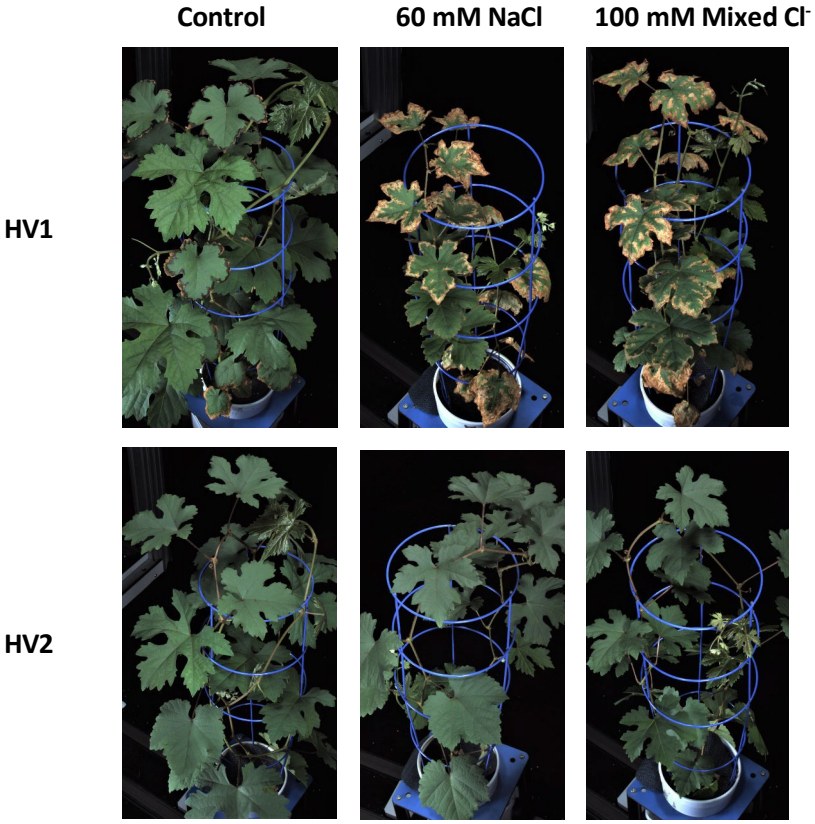



**Figure 4 – Images highlighting leaf burn symptoms on line HV1 under the three treatments. Similar symptoms were present on each of the other hyper-accumulating HV lines, whereas no leaf burn symptoms were present in lines with normal $Na^+$ concentrations, as shown for HV2.**

Despite the apparent co-suppression and resulting hyper-accumulation of $Na^+$ in some lines, three lines did accumulate less $Na^+$ in the leaves than the control line.

The rootstock 140 Ruggeri is heterozygous for the *HKT1;1* gene; that is it possesses one allele of the dominant E allele linked with increased exclusion, and one allele of the recessive e allele linked with poor exclusion or accumulation (Henderson *et al.* 2018). It was therefore hypothesised that introducing additional copies of the functional E allele (from Shiraz), might lead to a greater improvement of exclusion of $Na^+$ from the shoot than seen in the Shiraz background, which is homozygous for the E allele.

Transformation of 140 Ruggeri embryogenic callus with *Agrobacterium* housing the HKT1;1-Vv pART27 construct resulted in 135 embryos, 50 plantlets and 44 lines positive for the transgene. A glasshouse experiment was performed to assess the $Na^+$ exclusion phenotypes of 20 of these lines, two negative transformant lines and untransformed 140 Ruggeri as controls. All lines received a rainwater control treatment as well as a 100 mM mixed $Cl^-$ treatment for 28 days, after which laminae were harvested for ICP. Time did not permit analysis of this material.

### 6.1.3 Chloride exclusion genes

Two candidate genes potentially involved in the root-based exclusion of chloride from the shoot, leaves and berries were identified in the previous project CSP 1302 'Genetic and mechanistic characterisation of rootstock traits conferring abiotic stress tolerance to grapevines'. The name and identity of these two genes have not yet been disclosed due to a pending patent application. Initial transformations with a hairpin construct designed to reduce their expression via RNAi were carried out in project CSP 1306 'Genetic transformation of grapevine to test significant abiotic stress and pest resistance genes'. In the current project, additional transformations were carried out resulting in a further 23 C lines (candidate gene 1) and a further 16 CH lines (candidate gene 2). Phenotypic assays of the transgenic Shiraz lines as part of CSA 1701-1.4 revealed reduction in expression of chloride gene 1 and resulted in a significant increase in laminae chloride concentrations, indicating a role in the chloride exclusion process. Conversely, chloride gene 2 did not influence the root-based exclusion of chloride from the shoot but may play a minor role in the sequestration of chloride into the petiole under low salt treatments.

As chloride exclusion is primarily a root-based trait, we sought to test the importance of chloride gene 1 in the rootstock genotype 140 Ruggeri, a known excluder of chloride. Due to difficulties and ongoing method optimisation of this new genotype, only five transformant lines were generated from 783 embryos germinated. Phenotypic assays of the resulting transgenic lines supported a role of chloride gene 1 in chloride exclusion in the rootstock 140 Ruggeri (CSA 1701-1.4).

## 6.2 The use of the mutated *VvGAI1* gene to generate breeding lines with rapid flowering

### 6.2.1 Introduction

The Pinot Meunier L1 microvine exhibits a unique dwarfed stature and a precocious or rapid flowering phenotype resulting from a single point mutation within the DELLA domain of the Gibberellic Acid Insensitive (*VvGAI1*) gene (Boss and Thomas 2002). While these traits are not particularly desirable in a commercial vineyard, they do provide unique advantages to accelerate the breeding of new varieties. Their small stature allows more vines to be readily grown in glasshouses and in dense numbers, and their rapid and year-round flowering reduces the time between breeding cycles (Torregrosa *et al.* 2019). Indeed, these beneficial traits of the microvine have recently been exploited to accelerate the breeding of new disease resistant scion cultivars in projects CSP 0904, CSP 1301 and CSP 1701-1.1.

Unfortunately, the Pinot Meunier derived microvine cannot simply be employed to speed up the breeding process of rootstocks as it can for scions. This is due to concerns that its *V. vinifera* background could introduce undesirable susceptibility factors to Phylloxera – there is a widely held notion that to ensure robust resistance to Phylloxera, rootstocks must be purely derived from North American *Vitis* species. One avenue to overcome these unidentified susceptibility factors could be to successively backcross the mutant *Vvgai1* gene into different rootstock or North American *Vitis* genotypes until the *V. vinifera* genome is sufficiently diluted to assume no susceptibility factors remain. However, such an endeavour would be very time-consuming, requiring at least six backcrosses to obtain less than 2% genome derived from *V. vinifera*.

A potentially quicker approach to introducing the mutant *Vvgai1* gene from Pinot Meunier into a rootstock background is via genetic transformation. It is predicted that this approach would result in a rootstock with dwarfed and rapid flowering traits that is essentially 100% (except for the Vvgai transgene) derived from North American *Vitis* species, avoiding any issues of potential susceptibility factors. However, being transgenic vines, they would be considered and regulated as a Genetically Modified Organisms (GMOs).

The Gene Technology Act (2000) defines a GMO as;

(a) an organism that has been modified by gene technology; or

(b) an organism that has inherited particular traits from an organism (***the initial organism***), being traits that occurred in the initial organism because of gene technology.

Under the current legislation, any dwarfed transgenic lines carrying the *Vvgai1* gene would be regarded as GMOs and therefore restricted from release by the Office of the Gene Technology Regulator. Yet, any offspring of such lines, which do not possess the *Vvgai1* transgene or associated microvine traits would not be regulated as a GMO. As the microvine traits are not desirable in any final rootstocks for release, but are only beneficial to accelerating the breeding process, the *Vvgai1* transgene and associated microvine traits could simply be bred out of genotypes intended for field evaluation and commercial release. Thus, transgenic microvine rootstocks are a viable option to accelerate the breeding of new rootstocks genotypes. Furthermore, the benefit of having the ability

to accelerate breeding cycles is only going to grow in the future as more and more genetic markers and genes are identified for key traits derived from diverse *Vitis* species, which will require multiple rounds of crosses to stack them into new elite rootstock genotypes.

Here we aimed to generate transgenic microvine rootstocks, by introducing the mutant *Vvgai1* allele from Pinot Meunier into the genome of 140 Ruggeri via genetic transformation.

### 6.2.2 Shiraz transformations

Firstly, as a proof-of-concept step, the readily transformable Shiraz was used to test the effects of introducing the *Vvgai1* allele into a genome that already possesses two of the wild-type *VvGAI1* alleles. Two separate transformations of Shiraz embryogenic callus were made as described in section 5.2.2;

1) Using the pCLB1301NH DNA vector containing the native promoter of the *Vvgai1* allele to drive expression (GA lines).

2) Using the pART27 DNA vector containing the 35S constitutive promoter to drive expression of *Vvgai1* in all cell types (GC lines).

From the GA transformation, 185 embryos gave rise to 68 plantlets of which 29 tested positive for the transgene. The GC transformation resulted in 142 embryos, 61 plantlets of which 17 possessed the transgene. When growing in tissue culture as young plantlets, GA and GC transformant lines displayed a pronounced range of growth phenotypes, ranging from fully dwarfed like the picovine (*Vvgai1*/*Vvgai1*), to semi dwarfed like the heterozygous microvine (*VvGAI1*/*Vvgai1*) through to wild-type Shiraz (*VvGAI1*/*VvGAI1*), as shown in (Figure 5) for GA lines.




**Figure 5 - Shiraz transgenic lines (GAs) possessing one or more copies of the dwarf *Vvgai1* gene display a range of dwarfed phenotype ranging from the fully dwarfed picovine to the semi-dwarfed microvine and the 'tall' wild-type Shiraz.**

To investigate the phenotypes of transformed lines further, an experiment was undertaken in which a subset of 16 GA lines and 14 GC lines displaying a range of statures were assessed for various growth measures when grown as potted vines in the glasshouse. For each line, 10 replicate plantlets were propagated in tissue culture on the same day and following four weeks of growth were then deflasked into a 1:1 sand:perlite mix in 0.4 L pots enclosed in a clear plastic bag. Vines were acclimatised to glasshouse conditions over the course of a week by gradually opening and removing the plastic bag. Following a further four weeks of growth, vines were potted up into commercial Biogrow potting mix in 2 L pots. At this stage triplicate vines were selected from each line and grown for a further five weeks before being destructively harvested to measure internode length and stem and root biomass. Meristems were also taken and immediately snap frozen in liquid nitrogen for

RNA extraction and quantification of *Vvgai1* expression. Of the 16 GA lines possessing the *Vvgai1* gene under its native promoter, 12 lines had internode lengths that were significantly shorter (reduced by 39-92%) than those of the untransformed wild-type Shiraz control, while four lines had internode lengths similar to wild-type Shiraz (Figure 6). For GC lines possessing the *Vvgai1* gene driven by the 35S constitutive promoter, only six of 14 lines had internode lengths significantly shorter than wild-type Shiraz, with the remaining eight lines not significantly different from wild-type Shiraz (Figure 6). Typical of the microvine, a reduction in both stem and root biomass was also associated with reduced internode length in both GC and GA lines (Figure 6).

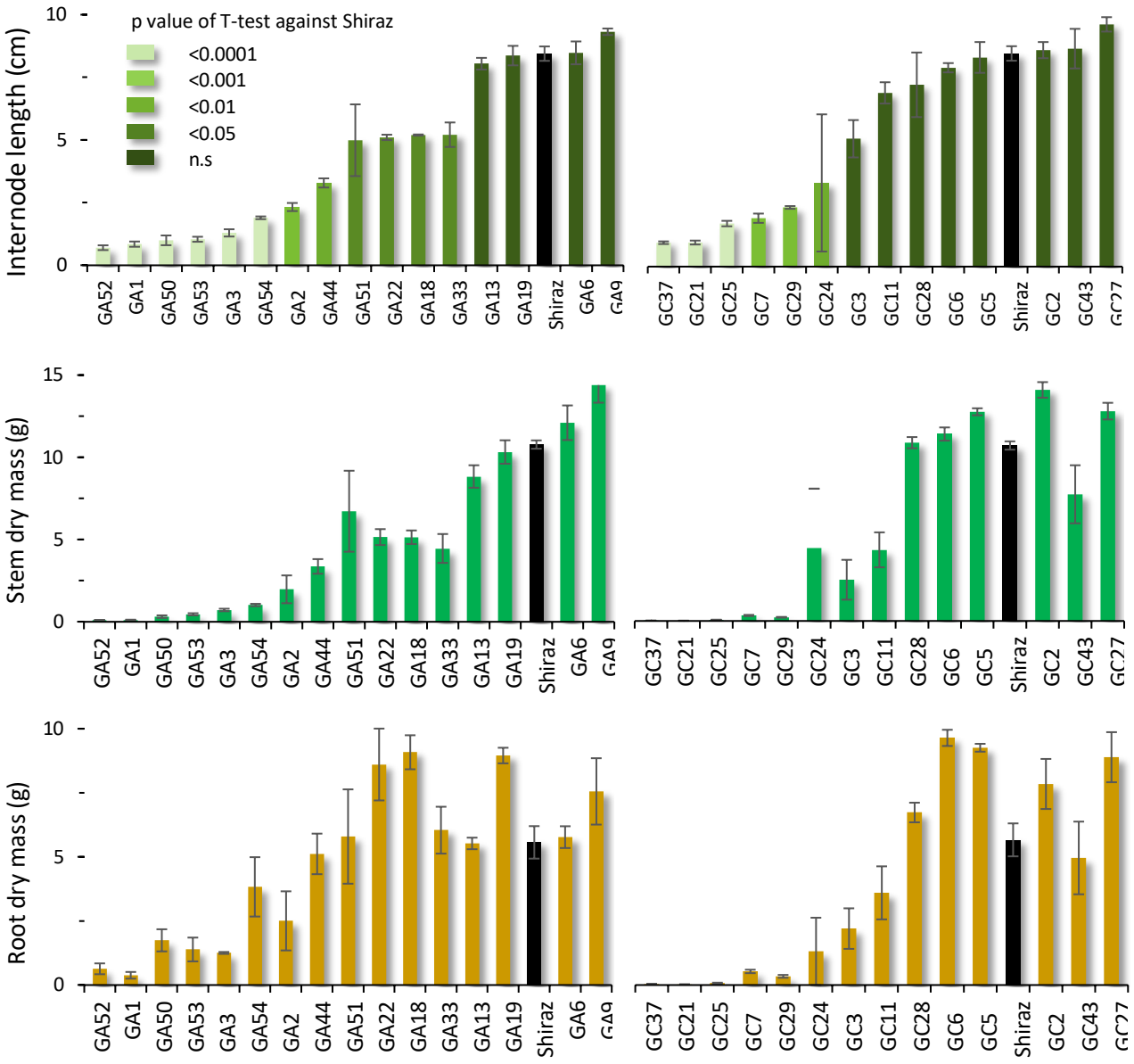
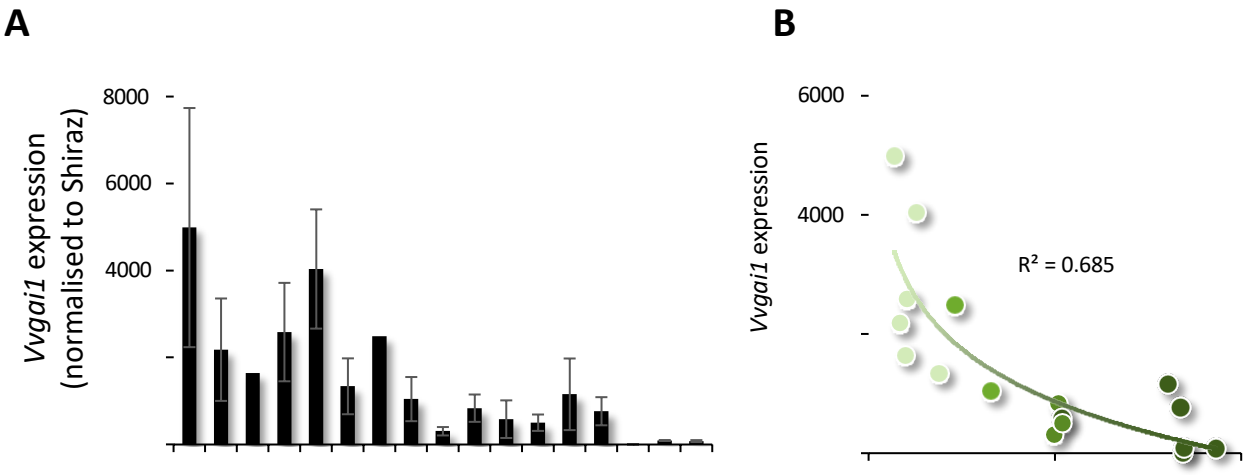



**Figure 6 - Internode length, stem and root dry mass of pot grown Shiraz transformant lines possessing the mutant *Vvgai1* allele driven by the native promoter (GA lines) or the 35S constitutive promoter (GC lines). An untransformed Shiraz line is included as a control, shown in black. Error bars indicate the standard error of the mean of four biological replicates.**

Next we sought to quantify the expression of the mutant *Vvgai1* allele. This was only undertaken in GA lines as they displayed a greater range of phenotypes compared to the GC lines. RNA was extracted from meristem samples and cDNA synthesised as described in section 5.4. Quantitative real-time PCR was performed using primers designed to amplify only the *Vvgai1* allele and not the wild-type *VvGAI1* allele (Primer sequences shown in Table 5). A large variation in expression of the mutant *Vvgai1* transgene was seen between GA transgenic lines as well as compared to wild-type Shiraz (Figure 7A). Although in reality Shiraz lacks the mutant allele, quantitative PCR resulted in background level amplification of *Vvgai1* from Shiraz (likely due to off-target amplification of the wild-type allele). Expression units are relative to Shiraz, which has an arbitrary unit of 1. As expected, GA lines displaying a dwarfed phenotype tend to show a higher level of *Vvgai1* expression. Indeed, a strong negative correlation ($R^2$=0.685) was seen between *Vvgai1* expression and internode length (Figure 7B). This relationship was not linear, but logarithmic, suggesting that there is a point at which higher expression of the *Vvgai1* allele no longer impacts the severity of the dwarfed phenotype.



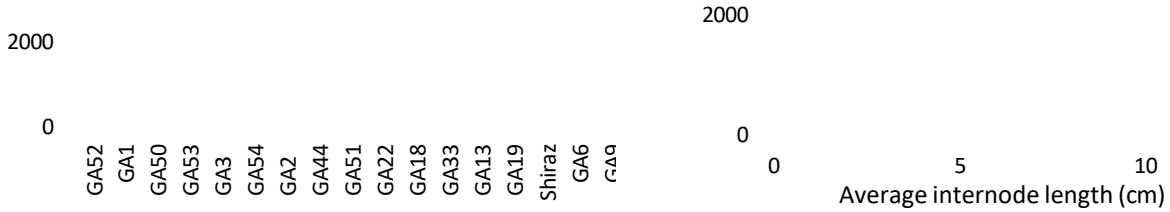



**Figure 7 – A) Expression analysis of the *Vvgai1* transgene in the meristems of GA transformants. Gene expression is relative to the expression Ubiquitin and normalised to Shiraz. Values are the mean of between 3-5 reps with error bars indicating standard error of the mean. B) A negative logarithmic relationship exists between expression of *Vvgai1* and resulting length of internodes.**

To assess whether the GA transformant lines displaying dwarfed phenotypes also possessed the ability to flower rapidly, 17 GA lines were grown in 15 cm diameter pots in the glasshouse, together with wild-type Shiraz as a control. After only seven months of growth, four lines (GA22, GA27, GA33 and GA54) had all flowered and set fruit (Figure 8). GA27 and GA54 produced sequential and full bunches along their entire growing stems typical of the microvine phenotype, whereas GA33 produced fewer flowers, and GA22 produced only couple of small bunches that ripened simultaneously, more typical of wild-type vines. Wild-type Shiraz did not flower in the 12 months. These results highlight that not all transgenic lines behave the same likely resulting from the *Vvgai1* transgene landing in different genomic regions, or possibly due to differences in copy number of the transgene. Nonetheless, these results demonstrate that the valuable precocious flowering and dwarf stature traits of the microvine can be successfully introduced into a wild-type genotype via the introduction of the mutant *Vvgai1* allele through stable transformation.

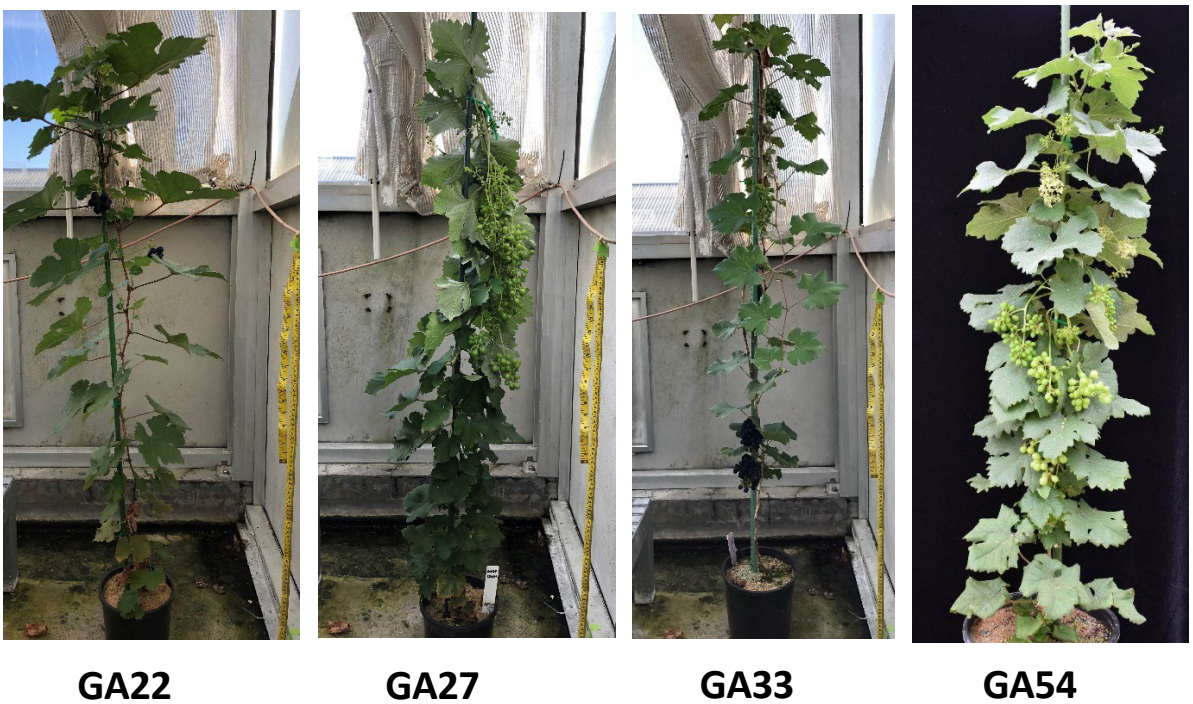



**Figure 8 - Transgenic Shiraz dwarf vines displaying rapid and successive flowering, characteristic of the microvine. Vines were imaged seven months after potting at which time wild-type Shiraz had not flowered (not shown).**

### 6.2.3 140 Ruggeri transformations

In order to introduce microvine traits into a rootstock background, we next transformed 140 Ruggeri embryogenic callus with the same two *Vvgai1* constructs used to transform Shiraz, as follows;

1) Using the pCLB1301NH DNA vector containing the native promoter of the *Vvgai1* allele to drive expression (**RGA lines**).

2) Using the pART27 DNA vector containing the 35S constitutive promoter to drive expression of *Vvgai1* in all cell types (**RGC lines**).

For RGA transformations, 325 embryos gave rise to 73 plantlets of which 62 tested positive for the transgene. The RGC transformation resulted in 145 embryos, 30 plantlets of which 26 possessed the transgene. As was the case for Shiraz lines, an obvious dwarfed phenotype was seen for many of the RGA lines, however unlike in Shiraz, no RGC lines appeared to have a dwarfed phenotype in the 140 Ruggeri background.

To examine the phenotypes of these transformants further, 19 RGA and 10 RGC lines were deflasked into potting mix and grown in the glasshouse for eight weeks. Two negatively transformed lines RGA9 NEG, and RGC2 NEG were included as controls. For RGA lines, all but one line had shorter internodes than the negative control, RGA9, however only 11 of these were significantly different (Figure 9). Conversely, none of the 10 RGC lines had significantly shorter internodes than the negative control RGC2 (Figure 9).

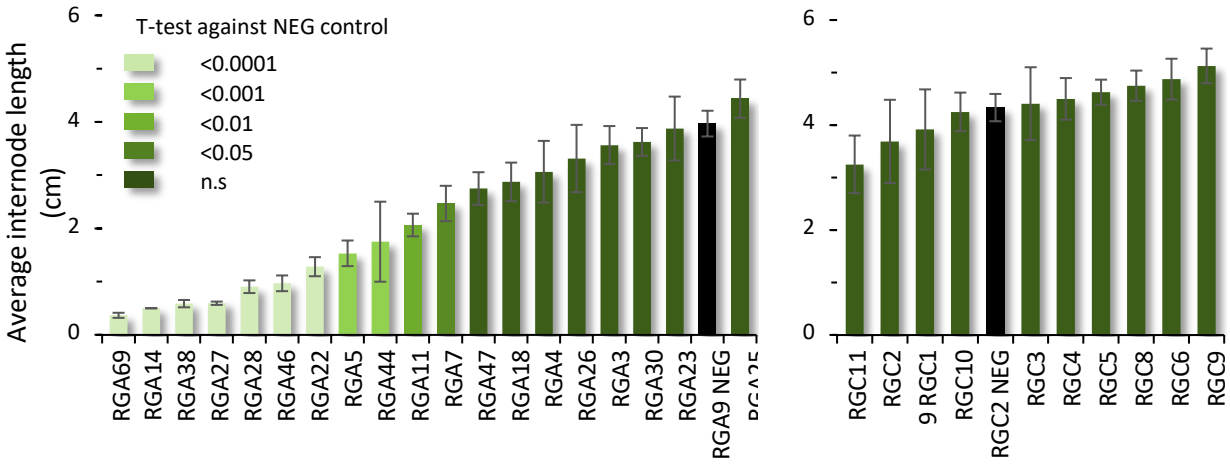



**Figure 9 – Internode length of pot grown 140 Ruggeri transformant lines possessing the mutant *Vvgai1* allele driven by the native promoter (RGA lines) or the 35S constitutive promoter (RGC lines). Negatively transformed lines as well as untransformed 140 Ruggeri are included as controls, shown in black. Error bars indicate the standard error of the mean of four biological replicates.**

Lastly, 22 RGA lines that displayed a strong or moderate dwarfed phenotype in tissue culture were deflasked into potting mix and established in the glasshouse to assess their propensity to flower quickly (Figure 10 – Example of the range in dwarfed phenotypes of 140 Ruggeri transformant lines possessing the mutant *Vvgai1* allele driven by the native promoter). Unfortunately, before the conclusion of this project, plants had not had sufficient time in the glasshouse (four weeks) to be able to properly assess their flowering phenotypes, however assessment is ongoing.

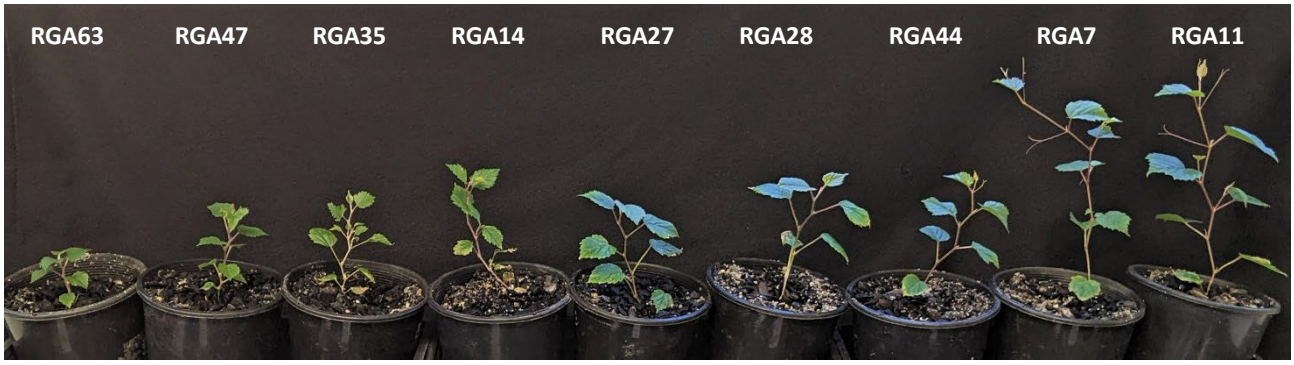



**Figure 10 – Example of the range in dwarfed phenotypes of 140 Ruggeri transformant lines possessing the mutant *Vvgai1* allele driven by the native promoter.**

### 6.2.4 Discussion

This work has shown that the introduction and expression of the mutant *Vvgai1* gene under its native promoter can confer a dwarfed phenotype in both Shiraz and 140 Ruggeri. Furthermore, a number of the dwarfed Shiraz lines also flowered rapidly in the glasshouse. While time did not permit the identification of which of the dwarfed 140 Ruggeri lines possesses the ability to flower rapidly, based on results in Shiraz it is likely that some 140 Ruggeri dwarf lines will show this trait. Such lines could be introduced into the rootstock breeding strategy for durable pest resistance and tolerance to abiotic stresses, which would reduce the time between successive crossing

generations. One minor limitation of using transgenic lines in the breeding strategy is that 140 Ruggeri has male flowers. As such, the pollen from these transgenic plants would not be permitted to leave a PC2 glasshouse, therefore crosses could not be performed on mature vines in the vineyard. Instead, the female vines would need to be propagated in a PC2 glasshouse, which would limit the number of inflorescences available compared to field grown vines. Although not a major limitation, the use of transgenic dwarfed breeding lines would be more restrictive than using wild-type genotypes.

## 6.3 Demonstration of gene editing technology in grapevine using stable transformation

### 6.3.1 Introduction

Gene editing is a relatively new technology that has exciting potential for precisely targeting genome modifications. The CRISPR-Cas system evolved in bacteria as a defence against foreign DNA (Barrangou *et al.* 2007), and consists of a guide RNA in complex with the Cas9 nuclease, which cleaves one strand of the target DNA. The Cas9 enzyme is directed to the target DNA by the complementary sequence of the guide RNA. This system has quickly been recognised as an important tool for the precise alteration of DNA in organisms and its potential for improving the traits of crops is widely recognised. Ten years ago, it was first demonstrated that the CRISPR-Cas9 technology could be used for genome editing in eukaryotic cells (Cong *et al.* 2013; Mali *et al.* 2013) and was reported to have been used in crop plants for the first time in the same year (Shan *et al.* 2013). Furthermore, the development of DNA-free gene editing (Woo *et al.* 2015) has opened the possibility that such techniques can be used in highly heterozygous perennial crops such as grapevine to improve traits in existing cultivars without having to produce a transgenic plant.

As the DNA-free technology was beginning to be established in grapevine in our laboratory, we wanted to demonstrate that the gene editing mechanism itself could be successfully applied in grapevine. It was decided to target a trait of interest for the breeding program, that of flower sex, which can segregate in some crosses due to it being a heterozygous phenotype in some cultivars. Having female breeding lines can greatly simplify a grapevine breeding program by dramatically increasing the number of crosses that can be conducted and reducing the number of self-pollinated plants that need to be identified in the offspring. While the flower sex locus has been mapped to Chromosome 2 of the grape genome by multiple research groups, the gene or genes responsible for determining flower sex had not been identified (Marguerit *et al.* 2009; Fechter *et al.* 2012; Battilana *et al.* 2013).

### 6.3.2 Identification of *VviPLATZ1* as a candidate flower sex gene

The Iocco-Corena *et al.* (2021) publication describes in detail the scientific evidence used to identify *VviPLATZ1* as a potential candidate gene for determining that flower sex would be female. In brief, it was thought that the flower sex locus on chromosome 2 contains genes that determine both male and female flower development (Figure 11). The hypothesis is that the female flower phenotype is caused by a recessive mutation that causes flowers to be females when the genotype is *f/f*, whereas male flower determination relies on the presence of a dominant male flower-promoting factor (M) that stops the development of the pistil. The perfect flowers in cultivated grapevines are due to the plants having an *H/H* or *H/f* genotype where the M-factor has low or no activity.

Previous linkage studies suggested that the M-factor is located towards the 5’ end of the sex determining region and the *f* mutation towards the 3’ end of the same region (Massonnet *et al.* 2020; Zou *et al.* 2021). The prime candidate for the M-factor is the *VviYABBY3* gene as its expression is associated with male flowers (Massonnet *et al.* 2020; Zou *et al.* 2021) and orthologues in other species are associated with carpel development (Bowman *et al.* 2002). While genetic studies suggested a role for the *VviINP1* gene in pollen sterility in female flowers (Zhou *et al.* 2019; Badouin

*et al.* 2020; Massonnet *et al.* 2020), there was no evidence that this gene would affect floral architecture.

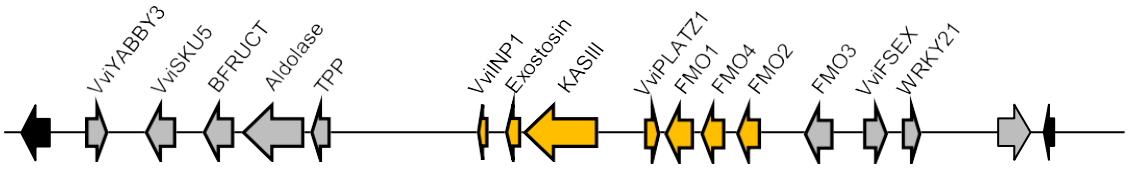



**Figure 11 – The genetic structure of the sex-determining region from grapevine chromosome 2. Fine mapping narrowed the female determining gene to the seven genes indicated in yellow. This figure is redrawn from (Iocco-Corena *et al.* 2021).**

Several pieces of evidence suggested that the *VviPLATZ1* gene in this locus was responsible for at least the reflex stamens typical of a female grapevine flower. First, previous studies had shown that the expression of *VviPLATZ1* was decreased when flowers had reflex stamen (Zhou *et al.* 2019; Badouin *et al.* 2020; Massonnet *et al.* 2020). This was confirmed by looking at the expression of this gene in flowers from hermaphrodite (H/H) and female (f/f) flowers from two progeny of a cross between Grenache and microvine (Figure 12). Expression of *VviPLATZ1* was higher in the perfect flowers in the early stages of flower development than in those with reflex stamen.

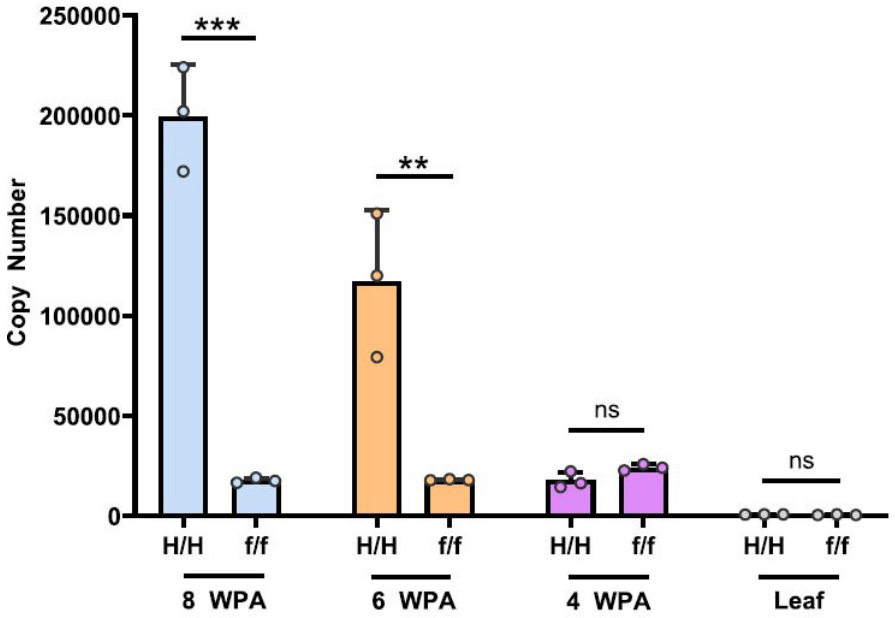



**Figure 12 – Expression of *VviPLATZ1* in flowers sampled at 8, 6 and 4 weeks before anthesis (WPA) and also in leaves of grapevines producing either hermaphrodite (H/H) or female (f/f) flowers. Data are presented as mean ± standard error. Student's t-test; *** $p < 0.001$, ** $p < 0.01$, ns = not significant. This figure is redrawn from (Iocco-Corena *et al.* 2021).**

Second, the other genes from this region (Figure 11) did not show a correlation between expression and reflex stamen production in the flowers in previous published data (Zhou *et al.* 2019; Badouin *et al.* 2020; Massonnet *et al.* 2020). Third, polymorphisms were identified in the *VviPLATZ1* gene

that correlated with the floral phenotype. Some of these were located in the 5'-untranslated region and in the start codon, thus moving the open reading frame slightly and eliminating two amino acids. There were also seven other mutations common to the genotypes that cause the production of female flowers that caused changes in the amino acid sequence of the VviPLATZ1 protein (Iocco-Corena *et al.* 2021). Fourth, *in situ* hybridisation of *VviPLATZ1* in immature flowers showed that transcript abundance was high in hermaphrodite flowers and was mainly localised in elongating filaments, anthers and microspores of those flowers. However, in female flowers expression was greatly reduced in these tissues (Iocco-Corena *et al.* 2021).

Gene function is best tested by the study of mutants where the coding region of the gene is disrupted or altered to examine the effect this has on the phenotype of the plant. Gene editing provides an excellent mechanism to produce targeted mutations in a specific gene while leaving the rest of the genome intact allowing for the functional analysis of that gene. This was applied to the *VviPLATZ1* gene to show the technology can be used to grapevine.

### 6.3.3 Gene editing of *VviPLATZ1* using stable transformation

Guide sequences were designed to the *VviPLATZ1* DNA sequence using the protocol described in section 5.7.1 and tested for their ability to cleave using the Guide-it sgRNA In Vitro Transcription and Screening kit (Takara Bio USA, Inc). Two guide sequences names FS1 and FS4 were selected and these both targeted exon 2 of the *VviPLATZ1* gene and edits were predicted to produce truncated proteins. Binary vectors were designed for stable transformation of grapevine with the various gene editing machinery and selectable markers. The Cas9 gene sequence was codon-optimised for *Vitis* and was controlled by the *UBIQUITIN* promoter and terminator from *Arabidopsis*. The sgRNAs based on the FS1 and FS4 guide sequences were driven by the *UBIQUITIN6* promoter and the selectable markers in the T-DNA were the hygromycin resistance gene and the green fluorescent protein localised to the endoplasmic reticulum. Each vector containing either FS1 or FS4 was transformed into the EHA105 strain of *Agrobacterium tumefaciens* and transgenic plants of the microvine known as 'V6' produced using the methods outlined in section 5.2.6. GFP positive and hygromycin resistant plantlets were then tested for gene editing by isolating DNA from immature leaves, amplifying the region surrounding the sequence targeted by the guide RNAs and sequencing the amplified sequence. Analysis of the chromatograms was used to identify insertions or deletions in the *VviPLATZ1* sequence in the $T_0$ plants and seven were chosen for self-pollination. After germination of the $T_1$ seed, root tissue was harvested from the seedlings and the guide region of the *VvPLATZ1* gene amplified using PCR and sequenced in order to identify plants homozygous for the mutated alleles.

### 6.3.4 Phenotypic characterisation of the gene editing microvines

The floral morphology was examined in all of the homozygous $T_1$ and compared to plant with wild type hermaphrodite and female flowers (Figure 13). In hermaphrodite flowers the stamens had an upright growth pattern and were elongated, averaging 3 mm in length (Iocco-Corena *et al.* 2021). Female flowers had reflex stamens that bent away and hung down from the pistil (Figure 13) and in a small number of cases were stunted, only growing to about half the length of the normal stamens.

The grapevines homozygous for the *Vviplatz1* mutations induced by the gene editing process all produced flowers that looked similar to female flowers (Figure 13). Also similar to female flowers, those stamens that were not reflex (1-5%) were stunted and grew to about half the length of the normal stamens.

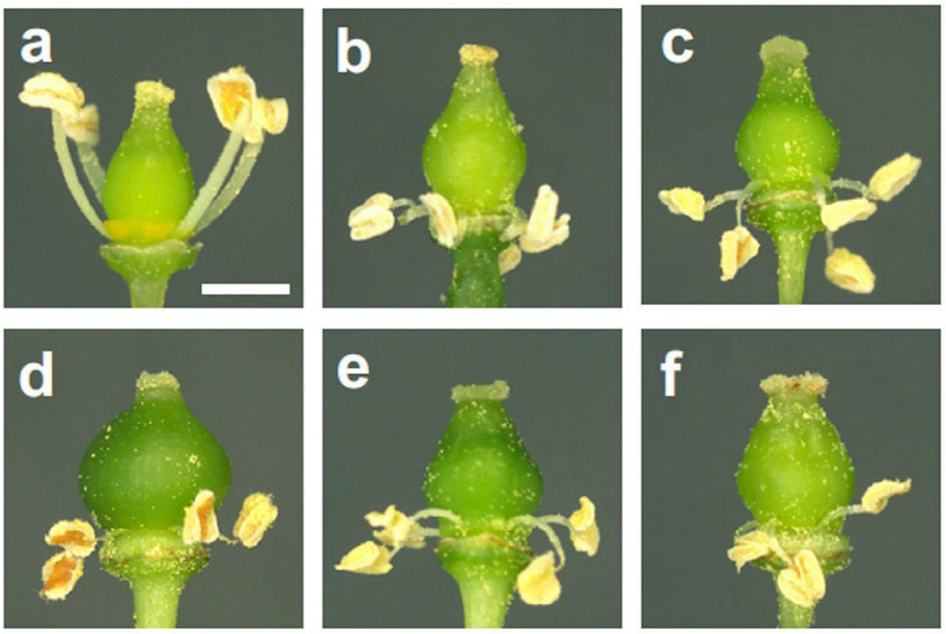



**Figure 13 Images of flowers from grapevines that have (a) the hermaphrodite genotype (H/H), (b) the female genotype (f/f), or (c-f) are homozygous for a mutation in the *Vviplatz1* gene. The reflex stamens in (b-f) are distinct from the upright growth pattern in (a). The scale bar is 1 mm long and applies to all images. This figure is redrawn from (Iocco-Corena *et al.* 2021).**

These sets of experiments were important and informative for two main reasons. Firstly, they showed that we could use stable transformation to induce gene edits in target genes of interest in grapevine. Furthermore, the use of the microvine makes this technique more powerful as we are able to rapidly obtain plants homozygous for the mutation, which gives us a means to test the function of genes of interest relevant to traits that will deliver industry impact. Secondly, it was shown that the *VviPLATZ1* gene is the key regulator for the formation of female flowers in grapevine and this may be able to be used to generate female versions of plants with important genotypes to enable more streamlined breeding for the production of novel winegrape varieties or rootstocks.

## 6.4 Initial development of DNA-free gene editing technologies

### 6.4.1 Introduction

Gene editing is an exciting new method of making targeted changes to the genome of a plant where the resulting plant is indistinguishable from one that might have been generated using a chemical or physical mutagen. Such plants are not regulated as genetically modified organisms. As has already be outlined in this report, the main issue with using stable transformation to generate gene-edited grapevines is that the transgene needs to be segregated away from the edited target gene in order for the final plants to be released as non-genetically modified organisms. This segregation requires the introduction of a sexual reproduction step, meaning that the highly heterozygous, common winegrape varieties used by the wine sector are lost during this process and there is no guarantee that the progeny will perform like the parent. However, a method for delivering the gene editing machinery into plant cells as a ribonucleoprotein, and hence without the use of foreign DNA, was first reported in 2015 (Luo *et al.* 2015; Woo *et al.* 2015) and since then some reports of the method being adapted to grapevine have been published (Malnoy *et al.* 2016; Bertini *et al.* 2019). Using these reports, and previous methodologies for the regeneration of grapevines from protoplasts (Zhu *et al.* 1997), efforts were made to establish a protocol for DNA-free gene editing of grapevines in the CSIRO laboratories in Adelaide. The following sections outline the progression of the method development, which was initially focus on the regeneration of whole plants from protoplasts derived from embryogenic callus and moved onto the use of ribonucleoproteins.

### 6.4.2 Early experiments focussing on regenerating plantlets from protoplasts

#### Protoplast regeneration trials 1 and 2

For the initial experiment, a suspension culture of Shiraz embryogenic callus (120 mL), initiated nine weeks previous and sub-cultured two days before use, was pelleted and the cells washed in a medium containing 10 mM $CaCl_2.2H_2O$, 0.5 M mannitol, 5 mM MES at pH 5.7 (hereafter known as cell wall enzyme buffer). A subsample of the callus was checked for viability by staining with fluorescein diacetate (FDA), which is converted into a green fluorescent compound in living cells and Calcofluor which binds to cell walls and is blue under UV light. Cell viability was excellent and cell walls were visible (Figure 14).

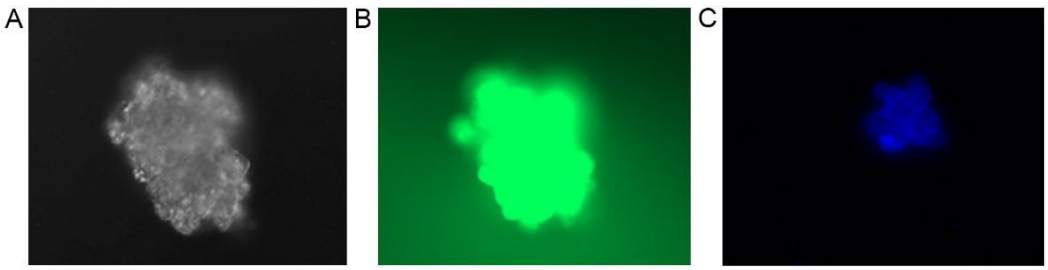



**Figure 14 - Shiraz embryogenic callus, after pelleting and washing in cell wall enzyme buffer, stained with FDA and Calcofluor. (A) bright field, (B) FDA fluorescence, (C) Calcofluor staining.**

After another wash in cell wall enzyme buffer, the supernatant was removed and the cells resuspended in a smaller volume of cell wall enzyme buffer so they could be transferred to a Petri dish and weighed. To each aliquot of cells (~1 g), 5 mL of cell wall enzyme solution was added (cell wall enzyme buffer + 2% w/v Cellulase, 1% w/v Macerozyme R-10, 0.05% w/v Pectolyase Y-23) and incubated at room temperature with gentle shaking at 30 rpm for 4 h before addition of another 5 mL of cell wall enzyme solution and further incubation for 1 h. During the incubation, FDA viability staining and Calcofluor staining showed that the protoplasts were viable and eventually most had no cell walls. Some protoplasts had died but the majority was alive and most lacked cell walls by three hours of incubation (Figure 15).

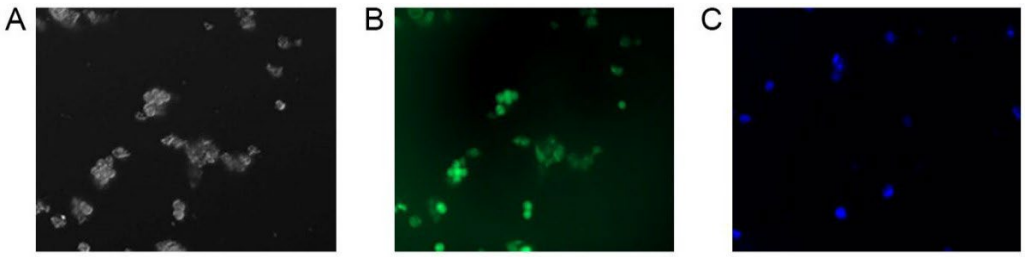



**Figure 15 - Shiraz embryogenic callus, after incubation with cell wall enzyme solution for 4 h, stained with FDA and Calcofluor. (A) bright field, (B) FDA fluorescence, (C) Calcofluor staining.**

Following the incubation, the protoplasts were collected and filtered under gravity through a 70 µm sterile filter and washed twice in 10 mM $CaCl_2.2H_2O$, 0.5 M mannitol with the cells pelleted at 120 × g for 3 min between washes. FDA-stained protoplasts were visualised and counted using a haemocytometer. From 0.8 g of starting material, $1 \times 10^7$ protoplasts were generated and from 1.34 g, $1.4 \times 10^7$ protoplasts were produced.

The next stage of the process would normally be to conduct the transfection of the protoplasts with the RNPs, but we initially wanted to ensure that we could regenerate plantlets from protoplasts, and so moved onto this part of the protocol. There have been different methods described for culturing the protoplasts so that they develop into embryos that then germinate and form plantlets. In this experiment two methods were trialled. The first involved culturing the protoplasts in an agar pool. These were washed with GS1CA (1/2 glucose) every week. The second used the technique of disc culturing. In this case an equal volume of 2 × phytagel medium (2 × NN macronutrients, 2 × MS micronutrients, 2 × B5 vitamins, 2 × Fe/EDTA, 21.5 µM NAA, 4.5 µM BAP,
0.6 M glucose, 0.18 M sucrose, pH 5.7 + 4 g/L phytagel) was added to the protoplasts and 800 µL aliquots were added as discs to a Petri dish (Figure 16). The discs were allowed to set and then overlaid with a liquid medium consisting of 1 × the phytagel medium described above without the phytagel and with the addition of 0.3% activated charcoal. Unfortunately, after three days the discs had mainly dissolved into the medium. After three weeks, some of the protoplasts from both the agar pools and disc cultures were stained with FDA for viability and Calcofluor for cell wall detection and showed that some of the protoplasts had regenerated cell walls and were still alive (Figure 17).

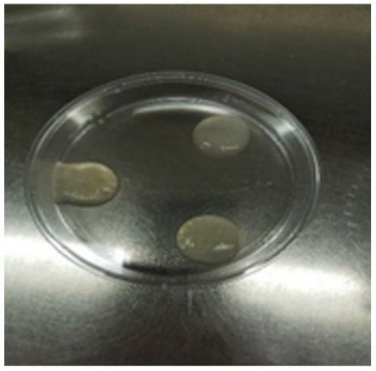


**Figure 16 – Disc culturing of protoplasts with phytagel.**

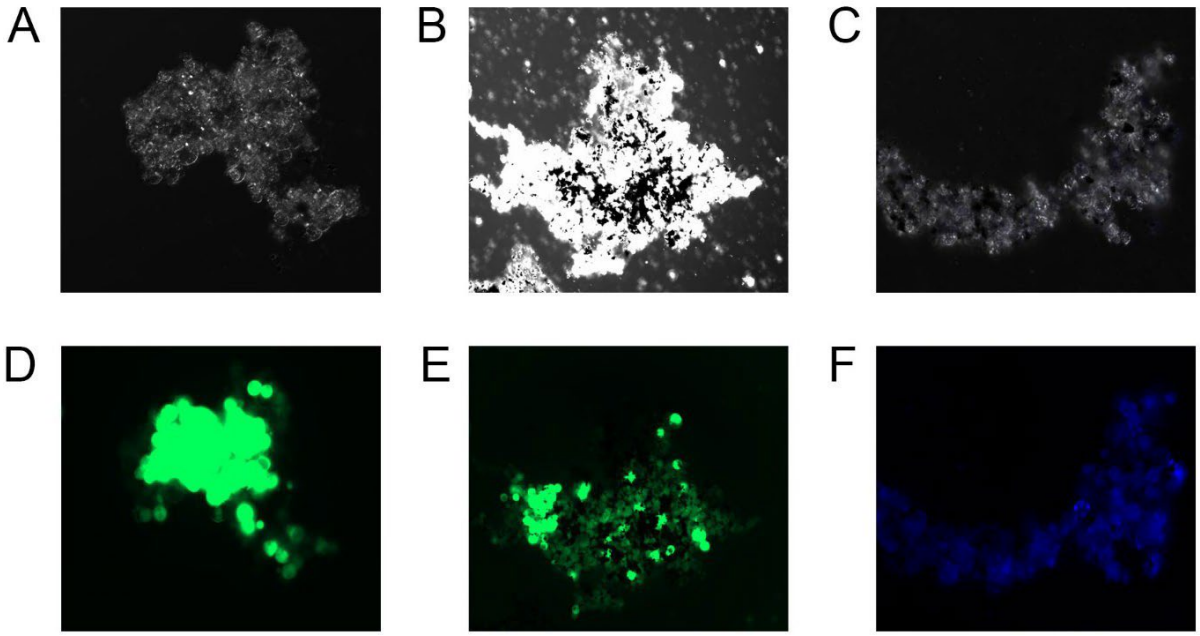



**Figure 17 – Protoplasts three weeks post-isolation from agar pools (A & D) or disc cultures (B, C, E, F) viewed using differential interference contrast (DIC) (A-C), showing FDA fluorescence (D,E) or Calcofluor staining of cell walls.**

### Protoplast regeneration trial 3

The previous trial revealed an issue with the phytagel discs dissolving in the liquid medium and so another protoplast experiment was initiated to test a different gelling agent (gelrite) and to also explore some variations in the reservoir media used.

The embryonic callus and cell wall digestion was conducted the same as described above, starting with ~2 g of callus prior to digestion (digestions were done on 1 g aliquots in 10 mL of digestion solution). The protoplasts were then pelleted and resuspended in either NN medium (1 × NN macronutrients, 1 × MS micronutrients, 1 × B5 vitamins, 1 × Fe/EDTA, 5.38 µM NAA, 1.13 µM BAP, 0.15 M glucose, 0.045 M sucrose, pH 5.7) or NN1969 medium (1 × NN macronutrients, 1 × NN micronutrients, 1 × NN vitamins, 1 × amino acid mix , 1 × Fe/EDTA, 6.45 µM NAA, 1.35 µM BAP, 0.18

M glucose, 0.054 M sucrose, pH 5.7) before the addition of an equal volume of 0.4% gelrite (Duchefa Biochemie, Netherlands). The protoplast concentration was adjusted to 1 × $10^5$ cells/mL and 2 mL aliquots mixed with 2 mL of 0.4% gelrite before 5 × 800 µL droplets of the mixture were added to shallow Petri dishes and allowed to set for 5 min. The discs were then overlaid with the corresponding culture medium used for the production of the discs with the addition of 0.3% activated charcoal. The protoplast disc cultures were incubated at 25 °C in the dark with the reservoir medium changed every two weeks to the same medium without glucose.

Six weeks post digestion, there were more microcalli visible in the plates with NN1969 medium than the NN medium (data not shown) and so this appeared to be the preferred reservoir medium for the Shiraz protoplasts. Some discs from the plates were transferred to NN solid agar and keep in the dark along with some of the disc cultures. These were examined a few months later for the presence of germinated embryos and 45 were found on one of the plates, indicating that the protoplasts still have the competency to produce embryos, however the methodology needed to be optimised to increase the numbers, as no embryos were recovered from any of the other discs.

### Protoplast regeneration trial 4

Method development continued with more variables tested in this experiment including the ratio of suspension culture callus to enzyme mix and the testing of another medium for the disc cultures following cell wall digestion. The size of the culture plates used was also found to have an effect on viability. In this experiment a new methodology for viability tests was tested using Trypan Blue (section 5.7.6).

Shiraz suspension callus cultures were harvested as outlined above and a pre-digestion count of cell viability indicated that ~64% of cells were viable. Cell wall digestion was conducted with the same enzyme mix used in previous experiments, but in this case either 1 g or 0.5 g of the callus was incubated with 10 mL of the cell wall digestion enzyme mix. Following digestion and filtration the cell viability was tested again and the 1 g/10 mL incubation had ~69% viable cells and the 0.5 g/10 mL incubation had ~65% viable cells indicating there was not much effect of the amount of starting material on cell viability immediately after digestion.

The cells from each digestion were then split into two 2 mL aliquots, one resuspended in NN1969 liquid medium and the other in MS (1 × MS macros, 1 × MS micros, 1 × Fe/EDTA, 1 × vitamin mix, 1 × amino acid mix, 6.45 µM NAA, 1.3 5µM BAP, 0.18 M glucose, 0.054 M sucrose, pH 5.7). Live cell counts were made again and the aliquots diluted with the appropriate amount of liquid culture to finish with approximately 2-4 × $10^5$ cells/mL. Disc cultures were then plated as described above using both 100 mm and 55 mm diameter plates and overlaid with the corresponding liquid culture with 0.3% activated charcoal before being stored in the dark at 25 °C. The liquid culture was replaced every two weeks with the same medium without glucose.

In this experiment, the progress of the protoplasts over time was followed more closely to examine viability, cell wall production, cell division and germination. After two weeks it was found that both the 1 g and 0.5 g digestions in the NN1969 medium on the smaller plates had the best viability and those on MS had very poor viability. No cell division was detected at this time. After four weeks cell aggregates were observed under the microscope and a dividing cell was observed using DAPI staining of DNA (data not shown). Thirteen weeks after cell wall digestion, a germinated embryo was observed in one of the discs on a 55 mm diameter plate with the NN1969 liquid medium (Figure 18).



**Figure 18 – Germinated Shiraz embryo pushing out of the solid NN1969 medium 14 weeks after cell wall digestion.**

### Protoplast regeneration trial 5

The main aim of this experiment was to test the agar-pool method of culturing the protoplasts with different media. Vital stains and cell wall stains were also tested as well as the ability to purify genomic DNA from the proliferating protoplasts.

Again, Shiraz suspension cultures were used as the starting material and these were harvested as already described above. Exact viability was not calculated, but FDA staining indicated that they were highly viable and the assay kit used gave excellent results using the manufacturer's instructions. Due to the success of the cell wall digestion when using 0.5 g of callus per 10 mL of enzyme solution in the previous experiment, this was adopted for this experiment. Five 0.5 g aliquots were digested, and 0.1% Calcofluor was added to one aliquot to stain cell walls (section 5.7.6). Following digestion, a new filtration method was tried in which the protoplasts were filtered first through a 100 µm filter and then a 70 µm filter, and this appeared to provide a cleaner preparation of cells. Viability counts indicated that the addition of the Calcoflour reduced viability by ~70%. The other four aliquots were incubated in one of four media: A, NN1969 medium plus 10.75 µM NAA and 2.25 µM BAP; B, NN1969 medium plus 10 µM NOA and 1 µM BAP; C, NN medium plus 10.75 µM NAA and 2.25 µM BAP; D, NN medium plus 10 µM NOA and 1 µM BAP. These media were present in the solid media and the liquid media within the agar-pools.

The protoplasts were examined under a microscope at regular intervals and it was found that the liquid in the pools reduced in volume more rapidly than expected, and so it was replaced every week. After four weeks, a vital stain indicated that the protoplasts were dead suggesting that this

that the culturing method using the agar-pools is not as good as those used previously. Agar with embedded protoplasts was sampled at two time points for genomic DNA purification using the protocol used by AGRF and both were successful.

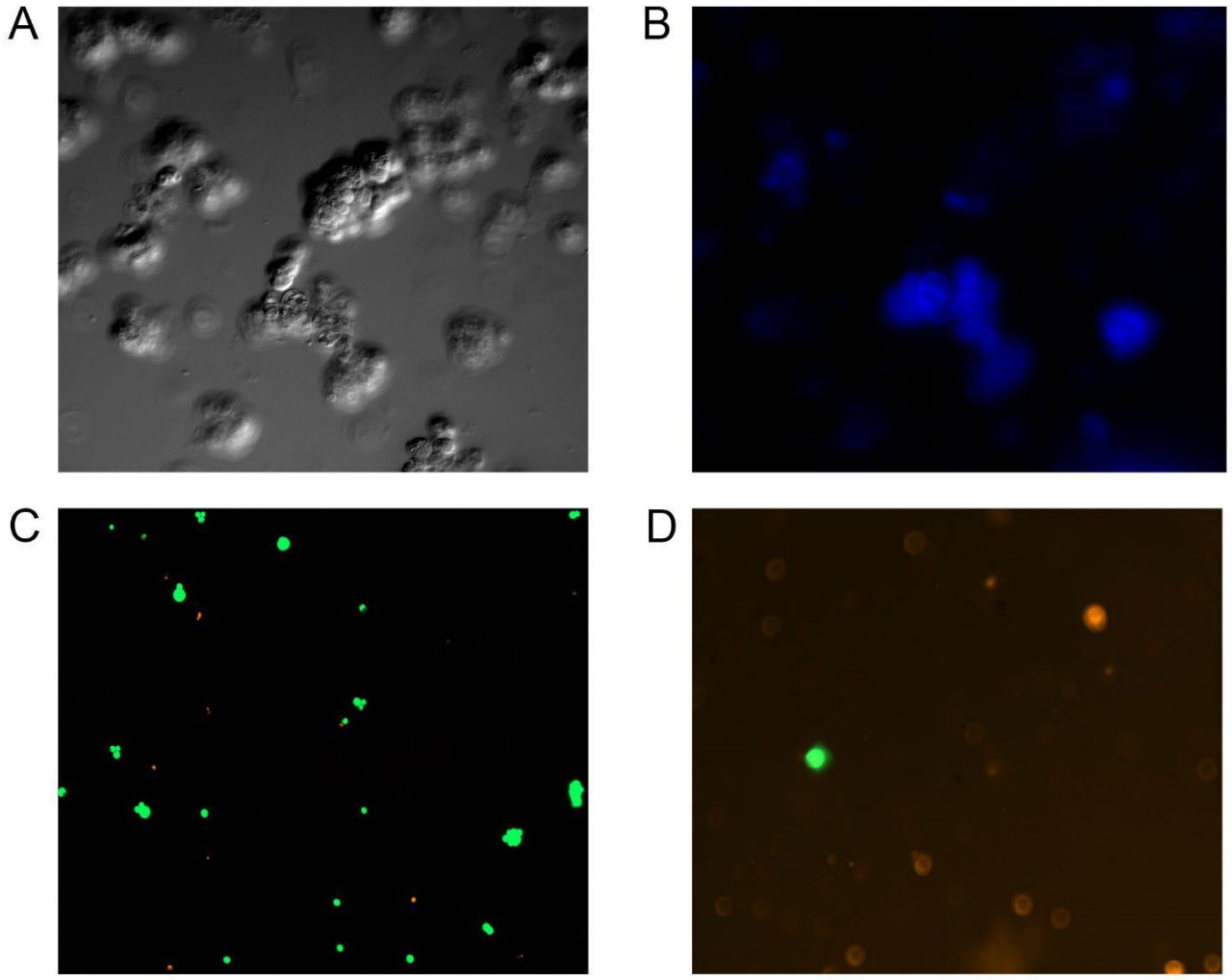



**Figure 19 – Images of protoplasts from various stages of the fifth protoplast regeneration trial. (A) DIC image of protoplasts 1 h into digestion and (B) same cells stained with Calcofluor showing the presence of cell walls (blue) in some cells. (C) FDA/propidium iodide staining of protoplasts after digestion showing high viability (i.e. more green than orange fluorescence) but after two weeks (D) few viable cells were left.**

### Protoplast regeneration trial 6

In this experiment, the source of embryogenic callus was changed to one of the transformable microvine genotypes known as 'V4' and the use of amino acids in the liquid culture media after digestion was tested. Glutamate was used due to its *in vivo* conversion to glutamine, a compound shown to be important for plant regeneration (Hu *et al.* 2015) and proline has also been implicated in cell wall synthesis in plants (Kavi Kishor *et al.* 2015) and was added to test its effect.

The same protocols that been established with the Shiraz calli (see above) was used in the harvesting, washing and digestion of the cell walls from the embryogenic suspension culture of microvine V4. Four aliquots of approximately 0.5 g were digested in 10 mL of cell wall enzyme

solution giving protoplasts with high viability (Figure 20) and these were pooled and diluted so that there were 5 × $10^6$ cells/mL. Two mL of the protoplasts were then mixed with 2 mL of gelrite liquid culture medium and discs prepared using 800 µL of the mixture for each one. Between 1-4 discs were prepared on a number of plates and four different reservoir culture media were tested (all with 0.3% activated charcoal): a) liquid culture medium (NN1965 + 10.75 µM NAA and 2.25 µM BAP), no amino acids; b) liquid culture medium, 3 g/L glutamic acid; c) liquid culture medium, 3 g/L glutamic acid, 3 g/L proline; d) liquid culture medium, 3 g/L proline. Eight days after protoplast isolation, one plate from each treatment had the reservoir culture medium replenished with the relevant amino acid treatment and with the addition of 200 mg/L ascorbic acid and 150 mg/L citric acid. These compounds were tested for their ability to enhance regeneration of plants from protoplasts due to their anti-oxidant properties (Masani *et al.* 2013; Forleo *et al.* 2021). The liquid media was then replaced every fortnight.

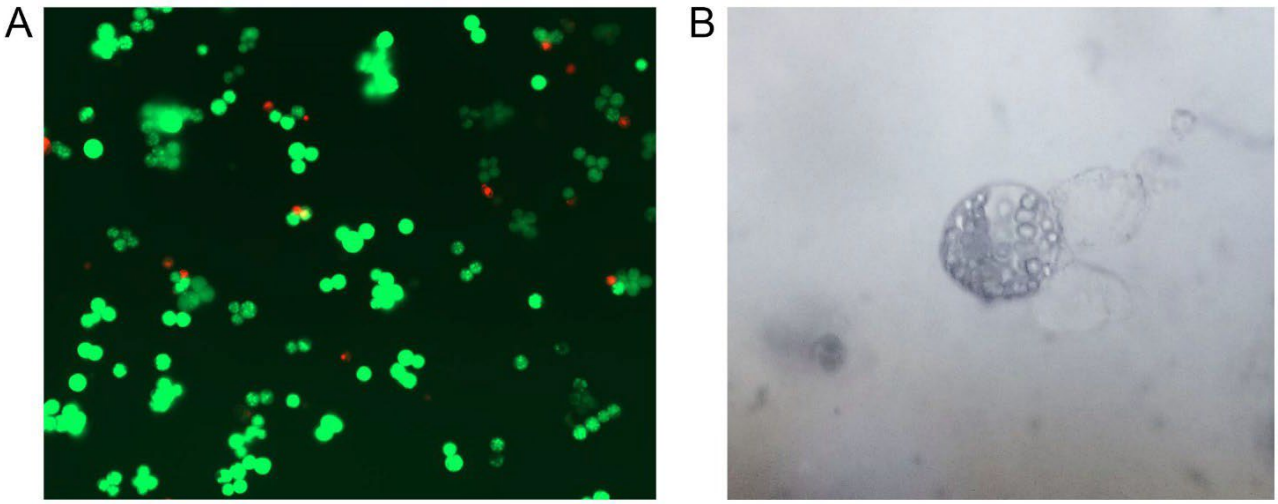



**Figure 20 – (A) FDA/propidium iodide staining of protoplasts after 5 h of digestion showing high viability and (B) a healthy protoplast embedded in a gelrite disc 8 days after protoplast isolation.**

Just over two months after cell wall digestion, embryos were observed emerging from the gelrite discs on three of the 'no-amino acid' treatment plates and one of the 'glutamic acid' treatment plates (Figure 21). It appeared that, overall, there was no advantage from adding the amino acids to the reservoir culture media. Eventually, five months after the start of the experiment, more than 500 embryos had been harvested from the discs with some taken through tissue culture to produce plantlets. This was a positive result that suggested we could regenerate large numbers of germinated embryos from grape protoplasts derived from embryogenic callus.

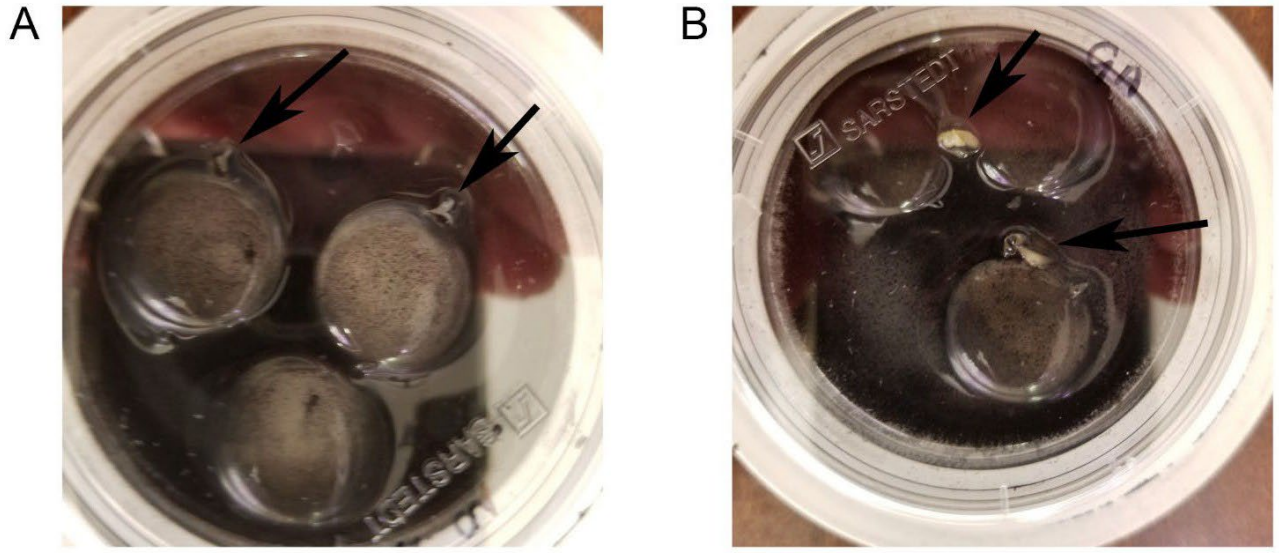



**Figure 21 – (A) Gelrite discs of protoplasts incubated with liquid culture medium without amino acids two months after isolation with two germinated embryos visible (arrowed) and (B) two germinated embryos also observed in a plate incubated with liquid culture medium with glutamic acid.**

### 6.4.3 Protoplast experiments including transfection

#### Protoplast regeneration plus transfection trial 1

The protoplast isolation phase of this experiment tested different cell wall digestion solutions, and the addition of antioxidants during digestion, on the viability of the protoplasts produced. Three different reservoir media were also tested. An initial attempt at transfection was conducted using a binary vector designed to be used for the stable transformation of grapevines. This vector contained GFP under control of the 35S CaMV promoter, which should be active if the plasmid enters the nucleus of the protoplast.

A Shiraz suspension culture derived from an embryogenic callus culture established in 2020 was used for this experiment. Approximately 0.8 g aliquots of callus were used in four cell digestion incubations. The four cell wall digestion solutions tested included: 1) the usual medium previously used made up of 5 mM MES, 10 mM $CaCl_2$, 0.5 M mannitol pH 5.8; 2) medium 1) with the addition of 200 µg/mL ascorbic acid and 50 µg/mL reduced L-glutathione; 3) 1 × NN macros, NN micros, NN vitamins, FeEDTA, 7 mM $CaCl_2.H_2O$, pH 5.8; 4) medium 3) with the addition of 200 µg/mL ascorbic acid and 50 µg/mL reduced L-glutathione. Prior to digestion, two aliquots of suspension culture cells were washed with digestion solution 1) and then used for digestion in media 1) and 2), and the other two aliquots were washed with medium 3) before digestion using media 3) and 4). After 16 h the protoplasts in digests 1) and 2) looked healthy, but those in 3) and 4) were deformed, probably due to the lack of mannitol in those digests and they were discarded.

The impact of the antioxidants on the viability of the protoplasts after digestion was tested by vital staining and cell counting using a haemocytometer. The aliquots examined from the protoplasts produced using medium 1) had on average 84.5% live cells and those produced using medium 2) with antioxidants included had on average 86.5% live cells, suggesting that the effect was not significant.

Gelrite discs of the protoplasts from both digestions were prepared and incubated with three different reservoir media: 1) Liquid culture medium (LCM: 1 × NN macros, micros, vitamins, FeEDTA 10.75 µM NAA, 2.25 µM BAP, 0.3 M glucose, 0.09 M sucrose) + 0.3 % activated charcoal; 2) LCM + 1% PVP-40; 3) LCM + 200 µg/mL ascorbic acid and 50 µg/mL reduced L-glutathione. These were replaced over time with lower concentrations of glucose until the solutions only contained sucrose as the carbon source. By the end of the project, protoplasts were still viable and appeared to be growing and dividing in the discs.

The first attempt at transfection used the pVCAS9FS1 plasmid vector which contains GFP driven by a constitutive promoter and followed a protocol published in Bertini *et al.* 2019. At the end of the protoplast washing after digestion, the cells were resuspended in MMG medium (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7) and divided into two aliquots, each containing approximately 2 × $10^6$ cells in 200 µL. To one of the aliquots was added 250 µL of water and 210 µL of a solution containing 40% polyethylene glycol (PEG) 4000, 0.2 M mannitol and 0.1 M $CaCl_2$ and to the other the water was replaced with 250 µL of 0.1 µg/µL pVCAS9FS1 in water. The protoplasts were then incubated for 20 min at 25 °C in the dark before the slow addition of 950 µL of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7). The cells were pelleted by centrifugation at 100 × g for 3 min at room temperature before being resuspended in 1 mL W1 solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7) and checked for viability. As viability looked fine, 1 mL W1 was added to the cells in a microtiter plate and incubated for three days at 25 °C in the dark with slow rotation before being checked for GFP fluorescence. Cells were observed with GFP fluorescence in the aliquot incubated with the pVCAS9FS1 vector indicating that the plasmid had entered the cell and GFP was being expressed from that vector, whereas the negative control did not have cells with the same amount of fluorescence (Figure 22). This was a very promising result that suggested we could use PEG to transfect grapevine protoplasts with macromolecules.

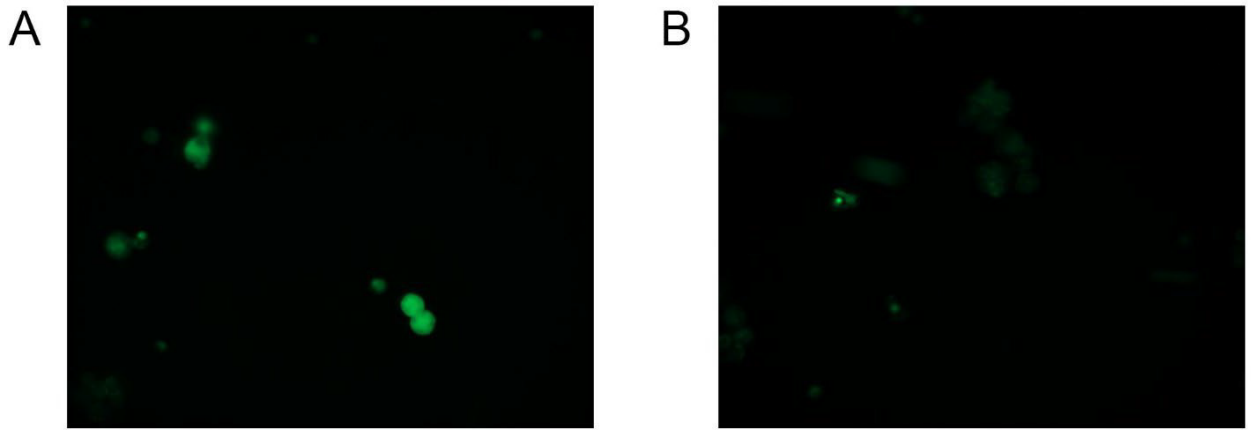



**Figure 22 – (A) GFP fluorescence observed in protoplasts three days after transfection with the pVCAS9FS1 plasmid and the (B) negative control.**

### Protoplast regeneration plus transfection trial 2

The final protoplast experiment of the project tested more protocol variables to get cleaner preparations free from cell debris and alginate beads were used as a medium for growing the protoplasts after digestion. Two transfection protocols were also examined (Woo *et al.* 2015;

Osakabe *et al.* 2018) in a mock experiment to test their effect on cell viability and the suitability of the media used for overnight incubations during the transfection process.

The embryogenic callus used was a suspension culture of a Shiraz culture established in 2020 and initiated into suspension culture four months before the experiment was conducted. The overnight digestion was conducted on two 1 g aliquots of the callus as described above. Following digestion, the cells were strained, centrifuged, washed twice and resuspended in 2 mL of wash solution (0.5 M mannitol, 10 mM $CaCl_2.2H_2O$, pH 5.6). One aliquot was further purified by layering the 2 mL of resuspended protoplasts onto 8 mL of a 16% (w/v) sucrose solution in a 15 mL centrifuge tube as described in (Scintilla *et al.* 2022). The protoplasts at the interface of the wash solution and the 16% sucrose solution after centrifugation were harvested using a Pasteur pipette and washed twice in 20 mL of wash solution before being resuspended in 2 mL of the same solution. Protoplast viability counts were conducted on the aliquots prepared with and without the sucrose gradient (Figure 23). There were $1.5 \times 10^8$ cells/mL in the aliquot not purified using the gradient, and cell viability was 76%. In contrast, there were fewer protoplasts in the sucrose-purified aliquot ($1.1 \times 10^6$ cells/mL), but the viability was considerably better at 89%.

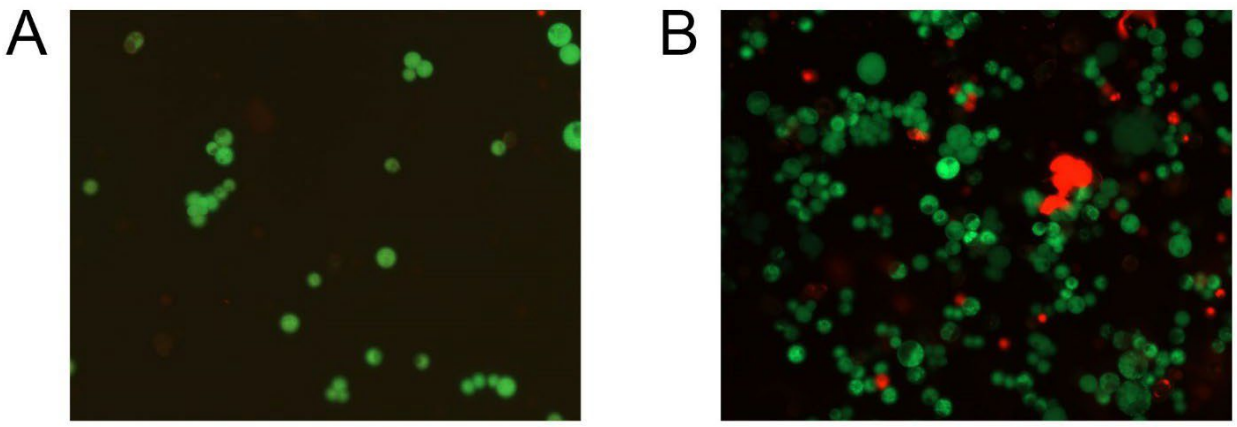



**Figure 23 - FDA/propidium iodide staining of protoplasts after purification using a 16% sucrose solution (A) or no clean up step (B).**

An aliquot of the sucrose purified protoplasts and one of the non-purified protoplasts were then re-washed twice using W1, before being finally resuspended in 1 mL W1. These were used to test the method of culturing the protoplasts in alginate beads rather than the gelrite discs. To produce the beads, the 1 mL aliquots of protoplasts were gently mixed with 1 mL of a sterile solution of 3.2% sodium alginate. This was then poured into a 5 mL syringe with a 27G needle attached and the mixture slowly expelled drop-wise into a solution containing 50 mM $CaCl_2.2H_2O$, 0.4 M mannitol, pH 5.8. The beads were incubated in this solution for 30 min before being collected in a sieve, rinsed with LCM and moved to a 55 mm Petri dish. The dish was then filled with LCM with 0.3 M glucose and 0.3% activated charcoal (Figure 24). At the end of the project the protoplasts had been embedded in the alginate beads for a week and at that time they looked healthy and had been dividing.

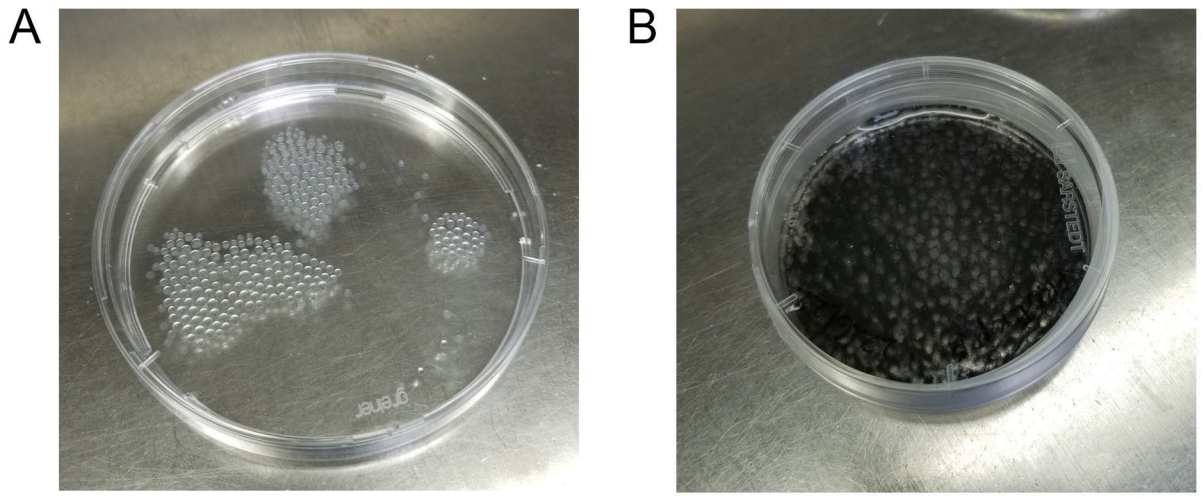



**Figure 24 – Protoplasts embedded in alginate beads: (A) in the original 50 mM $CaCl_2.2H_2O$, 0.4 M mannitol solution and (B) in LCM with activated charcoal.**

The mock transfection experiment used the protoplasts not purified using the sucrose gradient. When testing the Osakabe *et al.* (2018) method, five 20 µL aliquots of the protoplasts (equal to ~ 3 × $10^6$ cells) were resuspended in 200 µL of MMG medium, centrifuged at 100 × g for 3 min and the cells resuspended in another 200 µL MMG. To this was added 12 µL of 25% glycerol (to mimic the Cas9/sgRNA RNP) and 200 µL of PEG solution (PEG4000 40%, 0.2M mannitol, 0.1 M $CaCl_2$) before incubating the mixture at 25 °C for 20 min in darkness. Following the 20 min incubation, 400 µL of W5 solution was added to the protoplasts before incubating for a further 10 min at 25 °C after which a further 800 µL of W5 was added before another 10 min incubation at 25 °C. The protoplasts were then pelleted by centrifugation at 50 × g for 5 min. The pellets were then resuspended in 1 mL of one of five solutions: a) W1, b) LCM + 0.5M mannitol, c) LCM + 0.5M glucose, d) LCM + 0.3M glucose, e) W5 and incubated overnight at 25 °C in darkness. The Woo *et al.* (2015) method was trialled using aliquots of cells from the same purification as described above. After the incubation of the protoplasts with 25% glycerol and the PEG solution, 950 µL of W5 solution was added and the cells immediately pelleted at 100 × g for 3 min before being suspended in 1 mL of the five solutions listed above and incubated overnight at 25 °C. The following day, aliquots of all the incubated protoplasts were taken and checked for viability using FDA/propidium iodide staining. While the average number of cells was reduced in all of the treatments, it was found that those incubated overnight in either W1 or W5 had the highest level of viability regardless of which transfection procedure was used. The cells in the LCM media tended to clump together into aggregates. Therefore, in the future either transfection method seems appropriate as long as the protoplasts are resuspended in W1 or W5 solution after the PEG step of the protocol.

# 7 Outcome/Conclusion

This project has played an important role in the CSIRO/Wine Australia Strategic Research Agreement through the maintenance of research capability and grapevine germplasm unique to Australia. The capability maintained and established in this project has benefited other projects by assessing gene function through grapevine transformation methodologies. However, the team also recognised the importance of developing new capability in this area and sought to demonstrate how these could be used to assist the Australian wine sector through the development of improved germplasm. The outcomes listed below are seeds that have the potential to germinate into impacts that could greatly benefit the Australian wine sector by providing a solid basis for future work to establish technologies to provide the wine sector with improved germplasm and a better understanding of how important grapevine traits are controlled.

- An important germplasm resource based at Irymple in Victoria has been maintained as part of this project. The collection consists of 993 accessions from 681 varieties, 309 of which are unique to the CSIRO collection. The collection includes winegrape varieties, table and/or dried fruit varieties and rootstocks and represents a significant source of allelic variation for future studies designed to identify genes underlying important traits. The collection has been made even more valuable through the completion of the DNA typing of the accessions in project CSP 1501.
- *Agrobacterium*-mediated transformation of grapevine is essential for the characterisation of gene function and this capability is unique to CSIRO in Australia. This resource has been maintained as part of this project. The research team has continued to establish and maintain embryogenic callus cultures from Shiraz and 140 Ruggeri rootstocks using methodologies developed in previous Wine Australia funded projects. We have also established embryogenic callus cultures from three microvine genotypes which are the progeny of a microvine (original L1 cell layer of Pinot Meunier) × Grenache cross as described in Chaib *et al.* (2010). Co-inoculation of the cultures with *Agrobacterium* and subsequent selection procedures have been optimised as required for each unique embryogenic callus culture. This capability has been utilised by other projects to generate plants with altered gene expression to understand gene function.
- In collaboration with project CSA 1701-1.4, genes predicted to be involved in salt tolerance have been characterised through RNAi suppression and/or over-expression. The transgenic plants produced confirmed a direct role for the *HKT1;1* gene in $Na^+$ exclusion, but the results from other experiments hinted at the possibility that *HKT1;3* is involved in $Na^+$ inclusion as opposed to $Na^+$ exclusion. Expression of two genes predicted to be involved in chloride exclusion was also altered in transgenic plants. Results suggest that one of these genes appears to be involved in chloride exclusion in Shiraz and 140 Ruggeri rootstock.
- This project demonstrated that the mutated *Vvgai1* gene can be used to produce rapid flowering breeding lines that could be used to speed up traditional breeding programs. In both Shiraz and 140 Ruggeri, transgenic plants expressing *Vvgai1* under the control of its native promoter were shown to have a dwarf growth habit and in the Shiraz vines rapid

flowering and fruiting were also observed. Expression of the *Vvgai1* gene under the 35S constitutive promoter also promoted dwarfism in Shiraz, but this was not observed to be the case in 140 Ruggeri. Such transgenic plants have the potential to be used as breeding lines, especially if there are important traits that need to be rapidly introduced into a breeding strategy. Segregation of the transgene can be easily followed by observing the stature of the plant and the final selections will all need to have wild-type growth habits, thus removing the need to have the transgene present.

- Flower sex is also an important part of any breeding strategy, with the production of female breeding lines making subsequent crosses more efficient, as emasculation of flowers becomes unnecessary. While the genetic locus that determines flower sex in grapevine had previously been identified, there were a number of candidate genes in the region that had the potential to be responsible for the phenotype. Transgenic plants produced in collaboration with this project showed that the *VviPLATZ1* gene controlled the production of normal stamens and, when mutated, plants produced flowers with reflex stamens making them, in effect, female flowers. This finding has the potential to enhance traditional breeding programmes through transgenic strategies to control floral sex determination in breeding lines.
- After the commencement of this project it became clear that while RNAi and over-expression of genes are important tools, the ability to gene edit genomes using CRISPR/Cas technologies opens huge possibilities for research and wine sector impact. The experiments conducted on flower sex also represented a test case for our ability to harness the technology to create significant phenotypic change in a grapevine. This success showed us the power of gene editing and led to additional activities in this project to establish new capabilities to deliver improved germplasm for the wine sector.
- DNA-free gene editing was identified as the best use of the CRISPR/Cas technology to deliver impact for the Australian wine sector. The main benefit of this technology is that the resulting plants are not regarded as genetically modified organisms, meaning they can be treated as a new clone of an existing cultivar. However, the methodologies required to produce these plants are not straightforward. Nevertheless, a series of experiments was conducted to begin to optimise the two main technological challenges that need to be overcome to obtain DNA-free gene-edited plants: a) production of protoplasts and regeneration of plants from those protoplasts; b) transfection of CRISPR/Cas ribonucleoprotein complexes into the protoplasts leading to editing of the target gene(s). By the end of this project, we had demonstrated that we could produce protoplasts from embryogenic callus, embed these in solid media and recover germinated embryos and plants after a few months. We also demonstrated that we could transfect DNA into these protoplasts and maintain the viability of protoplasts after mock transfection experiments. This knowledge forms a solid basis for the future development of a full DNA-free gene editing protocol for winegrape cultivars important to Australia.

# 8 Recommendations

As the world's climate changes and the social licence for conducting business becomes more complex, agricultural sectors need to find cost-effective solutions to these issues. Plants, in general, have a broad range of adaptations to various macro- and micro-environmental challenges due to their sessile nature, which means they cannot remove themselves from the effects of stressors. The difficulty in introducing these useful adaptations into elite winegrape cultivars is many-fold and confounded by the heterozygous nature of winegrape varieties. The heterozygosity present in the genome of the most widely grown winegrape varieties means that any attempt to use traditional breeding to introduce novel traits into elite cultivars can only result in the development of a new variety. These new varieties may be superior to existing cultivars in many ways, but they are saddled with issues concerning name recognition and marketing that make it difficult for them to gain traction with wine companies and consumers, hence the lack of investment in this area by breeding companies. One solution involves the introduction of adaptive traits into elite cultivars using *Agrobacterium*-based transformation methods. Such technology has been available to the sector since the late 1990s but is hindered by two main problems. Firstly, the restriction and/or regulation of the use of genetically modified organisms for food products by many countries, especially those in Europe, has placed a barrier on their use. Secondly, it is often not known which genes underpin important adaptive traits, meaning that we have few targets to actually use for a transformation-based variety improvement strategy. Recent developments in the area of gene editing, especially the development of DNA-free methodologies that result in plants not considered to be genetically modified, go some way towards addressing the first problem associated with regulation. However, the methodology is restricted at the moment to producing gene knockouts and still does not solve the issue of having a limited number of gene targets for important traits. Nonetheless, the work described in this report has increased our capability in this area and provides the foundation for work going forward to realise the promise of new technologies for germplasm improvement and the discovery of genes associated with traits that can be improved in existing elite winegrape cultivars. Recommendations for four areas of future work are listed below:

1. Maintain capabilities/resources essential for grapevine gene discovery

As outlined above, one of the barriers to germplasm improvement, regardless of the strategy, is the identification of genes that control important traits, and how this control is regulated by the gene of interest. This initially relies on the presence of genetic material that contains allelic variation that results in major phenotypic differences in the trait of interest. The variety collection at Irymple represents the greatest source of winegrape genetic variation in Australia, which is even more valuable now that it has been DNA-typed to confirm the identification of the cultivars. It is important to maintain at least those varieties that are unique to the Irymple collection, even if they are not seen as being varieties that will be widely grown by the Australian wine sector, as there could be much value in the genes that they carry. Once important genetic variation is identified, gene mapping allows regions of the genome to be identified that are associated with traits of interest. Subsequent characterisation and confirmation that candidate genes confer certain traits on grapevines often requires transformation techniques to over-express genes of interest, as reported

herein with salt tolerance genes (section 6.1). Knocking out genes is also important in determining function, as shown in this project with the floral sex gene (section 6.3), and this technique will also be essential for the identification of future gene targets. CSIRO has a unique capability in Australia to be able to perform *Agrobacterium*-based genetic transformation of winegrapes, which we have extended to rootstocks in this project (section 6.2.3). It is essential to maintain this capability for any future germplasm improvement program in Australia.

2. Establish DNA-free gene editing methodologies

The development of CRISPR/Cas9 gene editing technology offers the possibility of genetically modifying specific traits in existing premium varieties without these plants having to be labelled as genetically modified organisms. This requires the use of 'DNA-free' gene editing which has two main components: the efficient delivery of CRISPR/Cas9 editing machinery into grape protoplasts so that gene editing can take place without integration of the CRISPR/Cas9 transgene into the plant genome and the regeneration of whole plants from gene-edited protoplasts. The preliminary experiments conducted in this project showed that it was possible to purify viable protoplasts from embryogenic callus digested with cell wall enzymes and to then culture these protoplasts in a mixture of solid and liquid media until they regenerated cell walls, formed embryos which germinated and developed into plants. It was also shown that DNA could be delivered into these protoplasts using a PEG-based transfection protocol. We are now confident that we can put these two components together to develop a reliable protocol to edit targeted genes in winegrapes. However, other complimentary areas of research will also be required. Firstly, gene targets need to be identified for certain traits as outlined above. Secondly, as we use embryogenic callus as the starting material for protoplast production, we will need to develop more efficient ways to establish these cultures from other premium varieties. Previous research has shown that different varieties require different hormone concentrations when establishing embryogenic callus from dissected anthers, a method which itself is labour intensive and may be improved by using other explants. Thirdly, there are still a number of variables related to the DNA-free gene editing process that will increase the efficiency of transfection and regeneration that will need to be explored but can be done in parallel to the development of improved cultivars. The ultimate aim of this work should be to establish a pipeline dedicated to producing improved versions of existing varieties to support the Australian wine sector into the future.

3. Consult with the Australian wine sector on research priorities

Initial consultation with the Australian wine sector occurred towards the end of this project where the concept of DNA-free gene editing of premium cultivars was presented to the Research Advisory Committee of Australian Grape & Wine. Subsequent positive feedback led to the drafting of a new research proposal to pursue this strategy of germplasm improvement. This consultation needs to be continuous to ensure that research resources are focused on traits that will deliver the best return on investment for the Australian wine sector and this will involve extensive consultation with all stakeholders. Maintaining this dialogue should also facilitate uptake of the improved cultivars as the awareness of the work among grape growers and winemakers should remain high. However, there is also the need to balance expectations for this technology with what can be achieved in the short term, given the currently limited knowledge of gene targets for important traits and the fact

that it is simpler to produce gene knockouts than other outcomes such as gene over-expression or altered protein function. This may change over time as the technology develops, hence there will be a need to update priorities as future projects mature. We would envisage that some form of priority list that is flexible and revisited regularly would be a great tool to promote ongoing consultation about target traits.

## 4. Australian wine sector impact

Sector impact can be achieved through two main pathways: 1) the creation of improved germplasm and 2) a greater understanding of the genetic basis for important traits which could inform strategies for managing existing vineyards.

Work in this project has progressed the establishment of DNA-free gene editing technology in Australia, which provides a mechanism to improve existing cultivars in a way that is not regulated as genetically modified in this country. There is also a possibility that grapevines produced through traditional transformation technologies might be differently regulated in the future, which means that the maintenance of this capability has the potential to also lead to improved versions of existing varieties. It should be remembered that gene editing and trait discovery methodologies do not exist in isolation and having integrated projects looking at both will give us the capacity to be agile and speed up delivery depending on the priorities of the wine sector and the maturity of the science for each trait.

Additional benefits accrue from determining the genetics of important traits, as knowledge of the genes that regulate traits will allow us to understand the biology behind how these are controlled in the vineyard. This understanding will require the use of transformation and gene editing technologies to alter genes to test function. This knowledge can then lead to the development of strategies to manipulate traits like berry composition, phenology or plant architecture in existing vineyards. It would be essential to have close partnerships with wine sector collaborators or other research providers to add value to existing experiments and increase wine sector awareness of research outcomes.

# 9 Appendix 1: Communication

*Publications arising from collaborations associated with this project:*

Iocco-Corena P, Chaïb J, Torregrosa L, Mackenzie D, Thomas M R, Smith H M (2021). *VviPLATZ1* is a major factor that controls female flower morphology determination in grapevine. Nature Communications 12: 6995.

Dry IB, Davies C, Dunlevy JD, Smith HM, Thomas MR, Walker AR, Walker RR, Clingeleffer PR (2022) Development of new wine-, dried-and tablegrape scions and rootstocks for Australian viticulture: past, present and future. Australian Journal of Grape and Wine Research. 28:177-95.

*Non-peer reviewed communications:*

Dunlevy J, Henderson S, Blackmore D, Walker R, Edwards E, Gilliham M, Walker A (2018) Identification of loci and genes responsible for sodium and chloride ion exclusion in grapevine rootstocks for use in marker assisted selection. 12th International Conference on Grapevine Breeding and Genetics, Bordeaux, France, 15-20th July.

Anon (2021) Colour the fabric of Mandy's life and career. RD&A News Researcher in Focus. https://www.wineaustralia.com/news/articles/colour-the-fabric-of-mandys-life-and-career.

# 10 Appendix 2: Intellectual Property

- Methodologies for the production of grapevine protoplasts from embryogenic callus cultures and the subsequent regeneration of plantlets from the protoplasts.
- Methods for grapevine transformation.
- Information about roles of genes to exclude chloride in rootstocks (with Wine Australia projects CSP 1302 and CSA 1701-1.4).
- Information about the *VviPLATZ1* gene and its role in determining flower sex.

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# 12 Appendix 4: Staff

All staff below spent periods of time on the project:

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