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Epi-breeding - Using the epigenetic memory of stress to prime Australian grapevines for a changing environment



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Epi-breeding – Using the epigenetic memory of stress to prime Australian grapevines for a changing environment

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1. Abstract

Epigenetic mechanisms are an interface between the environment and the plant genotype (the plant's collection of genes) that regulate gene expression in response to changing environments. When the epigenetic 'memory' of stress improves the response to subsequent stress, the plant is said to be epigenetically primed. We exposed Cabernet Sauvignon plants to drought, heat and combined drought and heat stresses and monitored their physiological responses and gene expression. These plants were propagated via different methods and subsequently re-challenged with combined drought and heat stress in the following year, together with the original plants. Environmental stresses had a significant impact on the plants' gene expression in both experiments and the profile of expression was different with each of the stresses. However, there was no effect of prior stress exposure on vine physiological response during vegetative growth.

2. Executive summary

Although drought and high temperature stress often occur together in the summer, and heatwave incidents will occur more frequently with climate change, the effect of the combination of these two stresses on grapevine has rarely been studied. We grew Cabernet Sauvignon plants in two years where plants were exposed to drought alone, heat stress alone, and the combination of the two stresses in year 1. Plants were then propagated, either from dormant buds or by layering, and exposed to the dual stress in the second year.

We found that plant growth was affected by each of the stresses and that the effect of heat on top of drought was more than just additive. Plant gene expression was more sensitive following combined stress treatment than in either stress alone i.e. more genes were up- and down-regulated.

We also found that plant gene expression was more sensitive to drought and heat stress in plants propagated by layering than from dormant buds. However, there were no significant differences in plant growth between the two propagation methods during the vegetative phase and there was no effect of the previous year's exposure on any of the stresses after propagation by either method.

We recommend that future research is directed to the combination of drought and heat stress on grapevine and that the effects of the changes in gene expression on final yield and berry quality are investigated.

The genes changing expression in common in each of the treatments are likely to be important for response and adaptation to the stresses. Genes in heat shock response pathways and sugars metabolism were over-represented in this category and any genetic variation in these genes could be useful for the breeding of improved varieties to cope with climate change.

Acknowledgements

The authors acknowledge and thank Wine Australia and the University of Adelaide for funding support for the project, and Yalumba Nursery (Nuriootpa, SA) for the provision of vine material.

3. Background

The Australian wine industry is currently under a range of significant pressures, which will be exacerbated by climate change. Australia produced 1.7 million tonnes of wine grapes in 2012-13 but grape production is highly sensitive to climate. Climate change will expose this industry to an unprecedented challenge to maintain production due to the changes in availability and price of water, higher degrees of salinity in soil/water, heatwaves and a predicted reduction of suitable land with adequate water by 2050 (Anderson *et al*., 2008). The industry needs to be able to adapt quickly to the pressure imposed by climatic irregularities. To address the impact of climate change we need to understand and exploit grapevine plasticity to make it a more adaptable crop.

When grown in the field, grapevines are subject to a variety of abiotic stresses, such as soil water deficit, salinity, high temperature, high vapour pressure deficit (VPD) and high light stress. Moreover, these stresses often occur in combination. Despite this, abiotic stresses are typically considered in isolation, with factorial studies of the effects multiple stresses on grapevine performance being uncommon, perhaps due to the additional complexity and resource requirements involved.

Soil water deficit is probably the abiotic stress most commonly faced in viticulture and can occur during periods of low rainfall in rain-fed vineyards, during the application of regulated deficit irrigation, or due to the lack of available irrigation water. As the effects of climate change start to be seen, heatwaves have become more common in Australia and often occur at times when there is a higher probability of soil water deficit (i.e. summer). Furthermore, anecdotally, the impacts of heatwaves on grapevine performance are more pronounced during periods of water stress (Webb *et al*., 2009) and studies on potted vines have demonstrated an interactive effect of the two stresses (Edwards *et al*., 2011). This interaction has also been observed in the transcriptome (Rocheta *et al*., 2014).

Epigenetic marks superimposed on the DNA provide plasticity in response to environmental changes. Agronomical traits such as yield, water use efficiency, disease resistance, seed content and secondary metabolite production have been shown to be epigenetically regulated in some plants. Furthermore, epigenetic modifications have been shown to prime genomes following stress, enabling a faster and stronger response to future environmental cues thanks to a better regulation of genes associated with stress response. Heat and drought stress in combination make a desirable target for the study of potential epigenetic changes in grapevine. The experiments described here were designed to 1) generate and phenotype plants with a complex epigenetic signal as a result of factorially combining two stresses, 2) test whether past history of vine and vine parent has an impact on performance during future exposure to that combination of stresses and 3) collect samples to allow the epigenome and transcriptome of these plants to be investigated.

Epigenetic memory could be used to enhance plant resilience while maintaining a level of plasticity that tends to be lost by lengthy, conventional, genetic breeding programmes. For seed-propagated plants, the maintenance of epigenetic memory of stress has been shown to improve the offspring's tolerance to stress. However, grapevines are propagated vegetatively, and little is known about how vegetative propagation affects the epigenetic memory and long-term maintenance of stress-induced epigenetic modifications.

Currently we have very little scientific understanding of how long living plants such as grapevines use epigenetic mechanisms to adapt to changing environments. Combining genomic and physiological data gives us the opportunity to achieve a better understanding of which are the molecular mechanisms driving plant adaptation to the environment. When applied during plant propagation, this understanding can be used to enhance planting material, leading to a reduction in costs for the wine industry thanks to better plant establishment.

4. Project aims

The main objective of this research was to determine whether long-term epigenetic memory of stress could be used for propagation and breeding purposes in grapevine. To achieve this, we aimed to:

- Elucidate the temporal scale of long-term maintenance of stress-induced epigenetic modifications in grapevine.
- Determine whether epigenetic memory of stress confers grapevines with a measurable advantage against future episodes of the same other stresses.
- Compare how different vegetative propagation methods affect the maintenance of epigenetic memory of stress.
- Investigate the genomic contexts where stress-induced epigenetic differences persist and where they do not.

Originally, we planned to work with plants exposed to stress in 2013 by Dr Everard Edwards (CSIRO) during a prior Wine Australia-funded project and analysed from an epigenetic point of view during the incubator project UA 1402. As those plants no longer existed, we sourced new plants with known mother vines and tested their resilience to stress in comparison with control (unstressed) plants in experiments over two years.

As planned, we studied the effect of different propagation methods on gene expression. For this, plants exposed to stress and control environments were used as donor material (ortets) to generate new plants (ramets) using two propagation systems (propagation from dormant buds and layering). The effect of vegetative propagation on the epigenetic memory of stress was to be studied by comparing the resilience to stress of ramets plants generated from control and stress ortets propagated through each of the methods mentioned before.

5. Method

Experiment 1 (Year 1)

Callused, dormant cuttings of Cabernet Sauvignon vines were obtained from a commercial nursery (Yalumba Nursery, Nuriootpa, SA) and the parent vine recorded. These were trimmed back to two buds and rooted in tubes of UC mix soil in a propagator with bottom heat. The rooted cuttings were transplanted into 24 cm diameter pots, trimmed to a single shoot and grown in a greenhouse until several mature leaves were present. In all, 120 cuttings from six parent vines were produced.

The vines were given unique numbers and randomly assigned to one of four treatments, with the caveat that vines from a single parent were spread between the treatments to ensure that treatment response was not potentially confounded by parentage. The treatments were:

- T0: control conditions (fully irrigated, approx. 27°C air temperature);
- T1: water stress (reduced irrigation, approx. 27°C air temperature);
- T2: high-temperature stress (fully irrigated, 40°C+ air temperature);
- T3: dual stress (reduced irrigation, 40°C+ air temperature).

Prior to the application of the treatments the plants were assigned to five blocks, each containing six replicates of the four treatments, and grown under greenhouse conditions, with daytime air temperature limited to 27°C, night-time air temperature maintained at a minimum of 20°C, ambient light and irrigated to field capacity daily.

The two water stress treatments (T1 & T3) were applied on 15 February 2017, by removing the drippers and monitoring the stomatal conductance (g_s) on a near-daily basis using a Delta-T AP4 Porometer (MEA, Magill, SA). Measurements were made on a single leaf per treatment per block between 10 and 11 a.m. Small amounts of irrigation were provided as required to maintain g_s at 50-75 mmol m^{-2} s^{-1}. After ten days of water stress, the high-temperature stress was applied by changing the greenhouse cooling to limit air temperature to 45°C during the day and heat the greenhouse to a minimum of 30°C during the night. Daytime heating was as a result of passive natural warming due to high insulation, whereas night-time temperatures were maintained with an active heating system. The control temperature vines (T0 & T1) were removed and placed in a matched greenhouse at 27/20°C. The high-temperature event was maintained for two days (26-27 February 2017), following which the previous temperature regime was restored and the control temperature treatments replaced in the original greenhouse. Irrigation was then restored to all plants.

In addition to the monitoring of g_s, stem water potential (Ψ_s) and leaf temperature were measured on seven occasions, in conjunction with leaf sampling for molecular biology. At each of these sampling dates, the youngest fully expanded leaf was used for stomatal conductance measurement and the second youngest fully expanded leaf used for Ψ_s. For the latter measurement the leaf was bagged with a silvered plastic zip-lock bag (PMS Instruments, Albany, OR, USA) for a minimum of 20 minutes, then the petiole cut, the leaf placed in a Scholander-type pressure chamber (Soil Moisture Equipment Corp model 3000 Plant Water Status Console, ICT Instruments, Armidale, NSW) and the

balance pressure determined. At the same time, the temperature of the youngest fully expanded leaf and the next youngest leaf was recorded with a Fluke 63 infra-red thermometer (RS Instruments, Smithfield, NSW), following which the two oldest still expanding leaves (i.e. the two leaves above the youngest fully expanded leaf) were harvested, immediately snap frozen in liquid nitrogen and then stored at -80°C until further use.

This measurement and sampling strategy was undertaken:

- immediately prior to any treatment imposition (9 Feb 2017);
- during water stress, but immediately prior to the heat stress (26 Feb 2017);
- at 4 p.m. on the first day of heat stress (26 Feb 2017);
- pre-dawn on the second day of heat stress (27 Feb 2017);
- at 4 p.m. on the second day of heat stress (27 Feb 2017);
- on the day following the heat stress, but prior to re-watering (28 Feb 2017); and
- after two weeks of recovery (15 Mar 2017).

Table 1. Sampling points (and abbreviations used) in relation to treatments in experiment 1.

Sampling times	Abbr.	Description
1	ST1	1st day plant acclimation to glasshouse
2	ST2	1st day of drought treatment
3	ST3	11th day of drought treatment and 1st day of heat treatment
4	ST4	12th day of drought treatment and 2nd day of heat treatment
5	ST5	Day of stress removal
6	ST6	Plant physiological recovery, 16 days after removal of stress treatments

Experiment 2 (Year 2)

At the end of the 2016/17 season, post-leaf fall, the vines used in experiment 1 were split into two groups and propagated via dormant cuttings or layering. For the dormant cuttings, the single cane was cut four buds from the origin on the main stem and trimmed to sections of approximately five buds, callused and then propagated as for the vines in experiment 1. For the layering, empty pots were prepared as for experiment 1, then the cane of the mother vine, bent over and pegged into the empty pot, with at least two buds below the soil surface (cover page photograph). After the propagated vines had viable shoots, the cane was cut between the two pots and both the mother vine and layered vine trimmed to a single shoot. All vines (mother and propagated), were maintained under controlled greenhouse conditions as for experiment 1 until use.

Pairs of vines (mother and propagated) were assigned to two treatments (control or dual-stress) and one of four blocks, such that there were four daughter vines for each propagation method (dormant cutting and layering) from each of the four experiment 1 treatments (T0-T3) and the matching mother vines, giving a total of 64 vines per

treatment. These were then assigned to one of four blocks, with one mother and daughter pair per propagation type and experiment 1 treatment per block.

As with experiment 1, water stress was imposed by removing drippers and monitoring stress using measurements of g_s on approximately 12 vines per treatment, with small additional water provided as required to maintain g_s in the 50-75 mmol m^{-2} s^{-1} range. The water stress treatment was started on 23 January 2018, with heat stress generated for two days using natural insolation, as previously, on 4-5 February 2018.

On the first day of dual stress, g_s was measured on every vine (128 in total) at approximately 4 p.m., then a single set of measurements and sampling (Ψs, leaf temperature, g_s, snap-frozen leaves for molecular biology) was undertaken as for experiment 1 during the afternoon for the second of dual stress, with the exception that it was not possible to take g_s measurements on every leaf (2-3 replicates per 2017/18 treatment combination per propagation type).

No further sampling or measurements were undertaken for experiment 2.

Gene expression analyses

Gene expression was quantified from 95 leaf samples in year 1 (five samples per timepoint/treatment). More specifically, sampling time 1 contained five control (unstressed) samples only. Sampling time 2 contained five control samples and five drought-treated samples. Other sampling times contained five samples for each group (control, drought, heat and combined treatment). In year 2, gene expression was quantified from 128 samples in 32 groups with four replicates per group. Comparisons were made between propagation methods where mother vines had been propagated by layering or from dormant buds between years 1 and 2. To test for memory of the previous year's stress, comparisons were made between each year 1 group and its propagule exposed to drought and heat in year 2 or unstressed.

RNA extraction

Total RNA was extracted from 100 mg of frozen and ground powder of the second and third youngest leaves from each plant using the SpectrumTM Plant Total RNA Kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's Protocol. RNA quality and quantity were determined by spectrophotometric analysis (NanoDropTM 1000, Thermo Fisher Scientific, Wilmington, DE, USA) and ExperionTM RNA StdSens Chips (BIO-RAD, USA). Only samples showing nanodrop 260/280 and 260/230 absorbance ratios between 1.8-2.2 and RNA quality indicator (RQI) above 7 were kept for analysis.

Library preparation and Illumina sequencing

Four µg of total RNA from each sample was used for ribosomal depletion using Dynabeads® mRNA Purification Kit following the manufacturer's instruction (Thermo Fisher Scientific, USA). Five µL ribosomal depleted RNAs were used to prepare RNA-seq libraries using the NEBNext® UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, USA) following manufacturer's instructions except that first strand cDNA synthesis samples were incubated at 42°C for 50 minutes. AMPure XP Beads (Beckman Coulter, USA) were used for the purification of the double-stranded cDNA, the

ligation reaction and the PCR reaction. cDNA library size and quality were estimated using 1K ExperionTM DNA High Sensitivity Chips (BIO-RAD, USA) and Agilent Bioanalyzer DNA 1000 and High Sensitivity kits (Agilent, Santa Clara, CA). Finally, individually indexed libraries were diluted to 10nM and pooled prior sequencing. 75bp single-end sequence reads were generated using the Illumina NextSeq 500 High Output platform (Australian Genome Research Facility).

Bioinformatic analysis RNAseq

Sequence quality control was performed with a set of quality checks in FastQC format (https://biof-edu.colorado.edu/videos/dowell-short- read-class/day-4/fastqc-manual) following adaptor removal as in Lindgreen (2012). Subsequently, the alignment tool (HISAT2) (Kim *et al.*, 2015; Khalil-Ur-Rehman *et al.*, 2017) was used for mapping the sequencing reads to the 12X grapevine genome reference (http://plants.ensembl.org/Vitis_vinifera/Info/Index).

Gene expression levels were estimated in RStudio using the *edgeR* and Limma packages (Robinson *et al.*, 2010; Ritchie *et al*., 2015). The raw mapped data of each sample were standardised by counts per million (CPM) and genes lower than 1 CPM were removed. The filtered data were normalised for differential expression analysis as described previously (Lun *et al.*, 2016). Between control and each treatment, a false discovery rate adjusted P-value<0.01 was adopted to determine differential expression using Benjamini and Hochberg's algorithm. The heat maps of gene expression pattern in drought, heat and drought/heat treatments were generated using the '*pheatmap'* package on RStudio (Wang *et al.*, 2010).

Multidimensional scaling (MDS) analysis

Multidimensional scaling (MDS) was used to visualise the global difference and similarity in gene expression patterns.

DNA methylation analysis

For total genomic DNA extractions, 60 mg of leaf tissue from each sample was cryogenically ground using a Geno/Grinder© 2010 Automated cell lyser, (SPEX sample prep, Metuchen, NJ, USA). DNA extractions were undertaken utilising the LGC genomics oKtopure™ liquid handling robot and sbeadex™ chemistry (LGC limited, Berlin, Germany). The manufacturer's protocol was followed using an LGC 'maxi-prep' DNA extraction kit with the following modifications; addition of 2% w/v PVP-40 (Sigma-Aldrich, Steinheim, Germany) to the proprietary lysis buffer prior to incubation, plus the addition of 7 units of RNase per mL of lysis buffer prior to extraction (Sigma-Aldrich, Steinheim, Germany). Following extraction, DNA concentration was measured using PicoGreen© quantification reagents (Thermo Fisher Scientific, Wilmington, DE, USA). DNA purity determination was made using Thermo Scientific NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All DNA samples were diluted to a working concentration of 20 ng/µL.

EpiGBS library preparation and sequencing

DNA methylation at cytosine bases was assessed using the method and analysis pipeline of van Gurp et al. (2016) for reduced representation bisulfite sequencing

(EpiGBS). Four hundred ng DNA for each sample was digested with the restriction enzymes NsiI-HF and Csp6I. Adaptors containing methylated cytosines were ligated onto digested DNA in a multiplex design and DNAs pooled. Fragments in the size range 200-600 bp (peak at 300 bp) were selected using 0.8X Agencourt AMPure magnetic beads (Beckman Coulter, USA) and nicks repaired with DNA polymerase and a 5-methylcytosine dNTP mix (New England Biolabs, USA). Genotyping by sequencing libraries were prepared from the nick-repaired libraries by PCR of 14 cycles of 98°C for 10s, 65°C for 15s, 72°C for 15s in the presence of 5 µL Kappa HiFi HotStart + Uracil (2x) and 3 pmol each forward and reverse Illumina sequencing primer. Libraries' fragment sizes and quality were checked using the Agilent Bioanalyzer High Sensitivity DNA kit (as before) and 110 Gb 150 bp paired-end sequencing reads were generated using the Illumina HiSeq 2500 platform (NovoGene, China).

Bioinformatic analysis epiGBS

Raw sequencing data were processed using the custom-built analysis pipeline of https://github.com/thomasvangurp/epiGBS. The 19 million loci output from the pipeline were analysed for differential methylation with the DSS in R tool available at Bioconductor (https://bioconductor.org/packages/release/bioc/html/DSS.html) based on the beta-binomial distribution.

6. Results and discussion

Plant physiology

The aim of the experimental treatments was to generate a combination of water and heat stress. As it is impractical to generate a water stress on the same timescale as heat stress, the water stress treatments were imposed prior to the heat stress treatments, with the intention that the vines would be in a relatively steady stress condition, but that the duration of this prior to the heat stress would not be excessive. Glasshouse conditions were intended to replicate conditions that grapevines are exposed to in the field during the middle of the growing season and be similar for experiment 1 (Figure 1) and experiment 2 (Figure 2). In general this was achieved, with daytime maximum air temperature ranging between 25 and 35°C in both experiments and night-time temperatures between 20 and 25°C (Figures 1 & 2, bottom). There was a difference in vapour pressure deficit (VPD), however, with maximum VPD in experiment 1 approximately 2 kPa, whereas in experiment 2, VPD was approximately 3 kPa (Figures 1 & 2, top). This difference would result in a greater evaporative demand on the vines in experiment 2, which may explain the slower rate of drying under the water deficit in experiment 1 (Figure 3) compared with experiment 2 (Figure 4).

The two-day heat stress events generated in the two experiments were also intended to represent heatwave conditions in the field and were also similar across the two experiments, reaching the same average daytime maximum temperature, albeit with a greater day-to-day variation in experiment 1 than experiment 2 (Figures 1 & 2, bottom). As with the control conditions, the VPD was higher during the heat stress event in experiment 2 than experiment 1 (Figures 1 & 2, top).

Experiment 1 allowed the interaction between heat stress and water stress to be examined, something that was not possible with experiment 2 due to resource constraints.

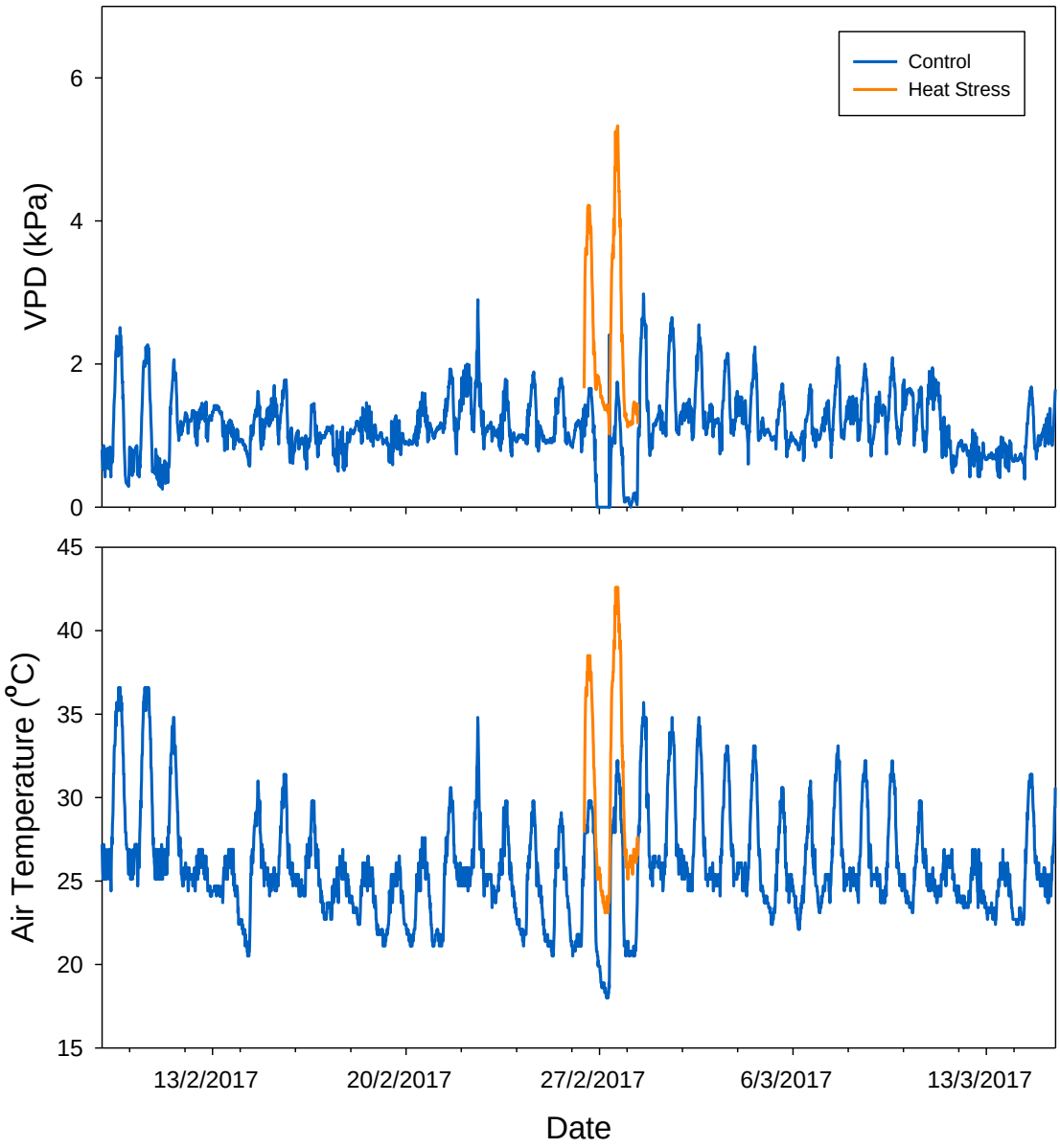


Figure 1. Air temperature (bottom) and vapour pressure deficit (VPD, top) during experiment 1.

As would be expected from the literature, reduced soil water availability in the water deficit treatments led to a drastic reduction in g_s, reduced Ψ_s and pre-dawn water potential (Ψ_{pd}) and a higher leaf temperature than the control (Figure 5). During the heat stress period, the well-watered vines exhibited similar stress indicators, with lower Ψ_s and Ψ_{pd} than the control. However, g_s was not reduced, but was actually higher than control on the first day of heat stress (Figure 5). The impacts on water potential were likely a result of the very high evaporative demand under the high temperature conditions as a result of the three-fold increase in VPD (Figure 1). The combination of stresses resulted in an additive effect on all three physiological parameters measured (Figure 5).

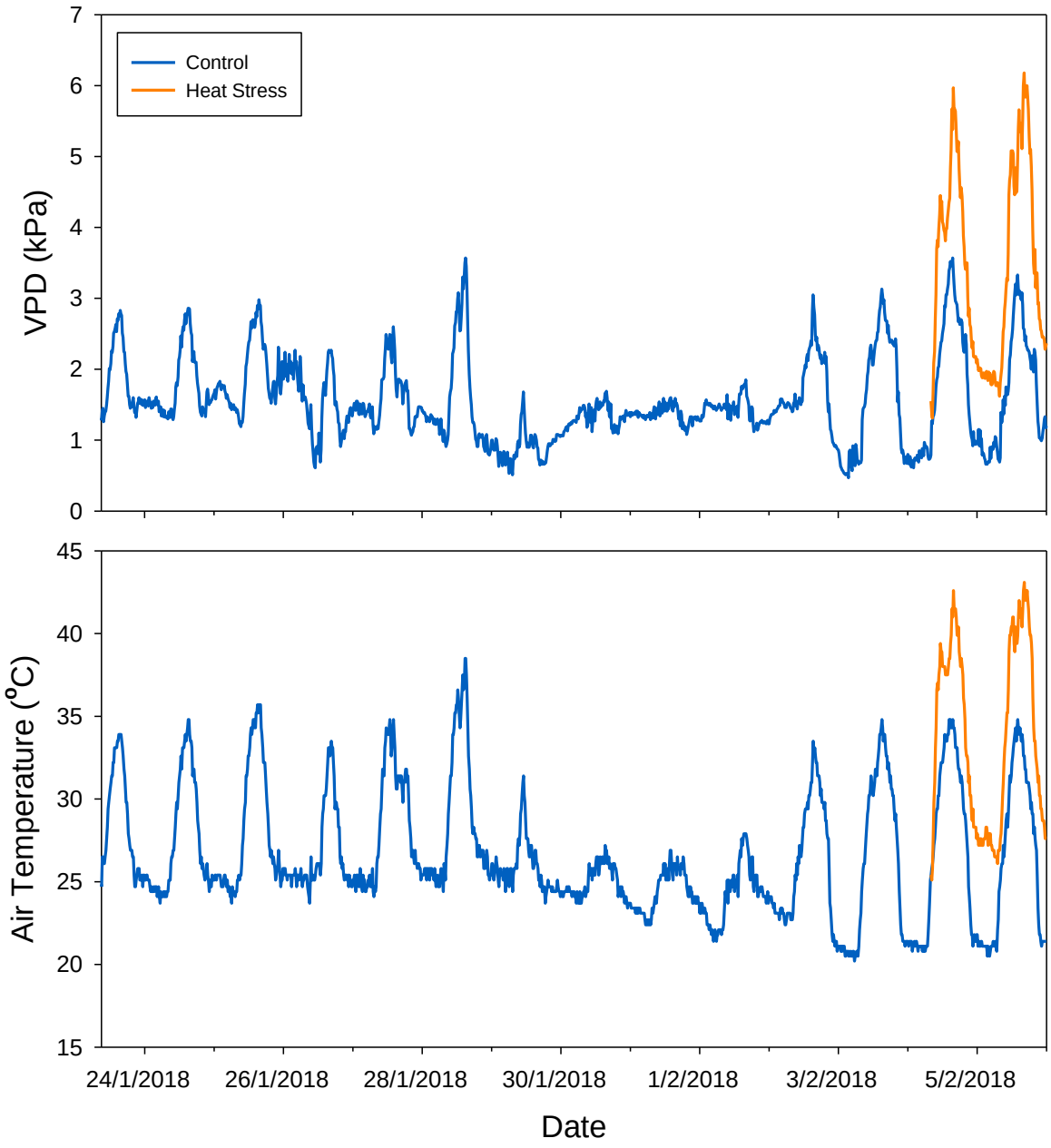


Figure 2. Air temperature (bottom) and vapour pressure deficit (VPD, top) during experiment 2.

Leaf temperature was higher than under heat stress alone, despite being in the same atmosphere, and stomatal conductance and Ψ_s were both lower than under water stress alone. However, Ψ_{pd} was not affected by heat stress, indicating that the water status of the vines under the combined stress had recovered as far as the available soil water permitted over the night.

Recovery from the heat event was rapid, with no measured parameter being worse than the equivalent vines (control or drought only) at the first post-heat stress measurement time following, only a few hours after return to control temperature (Figure 3 & 5).

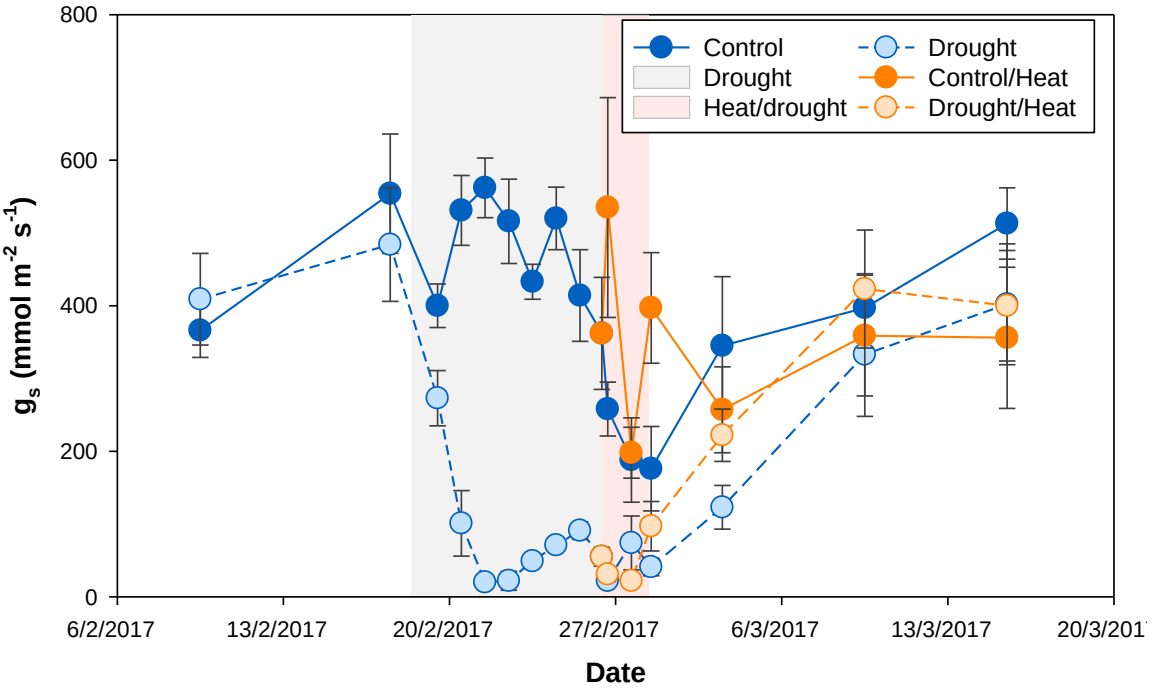


Figure 3. Stomatal conductance (g_s) of vines under full irrigation or water deficit, before, during and after a heat-stress event in experiment 1.

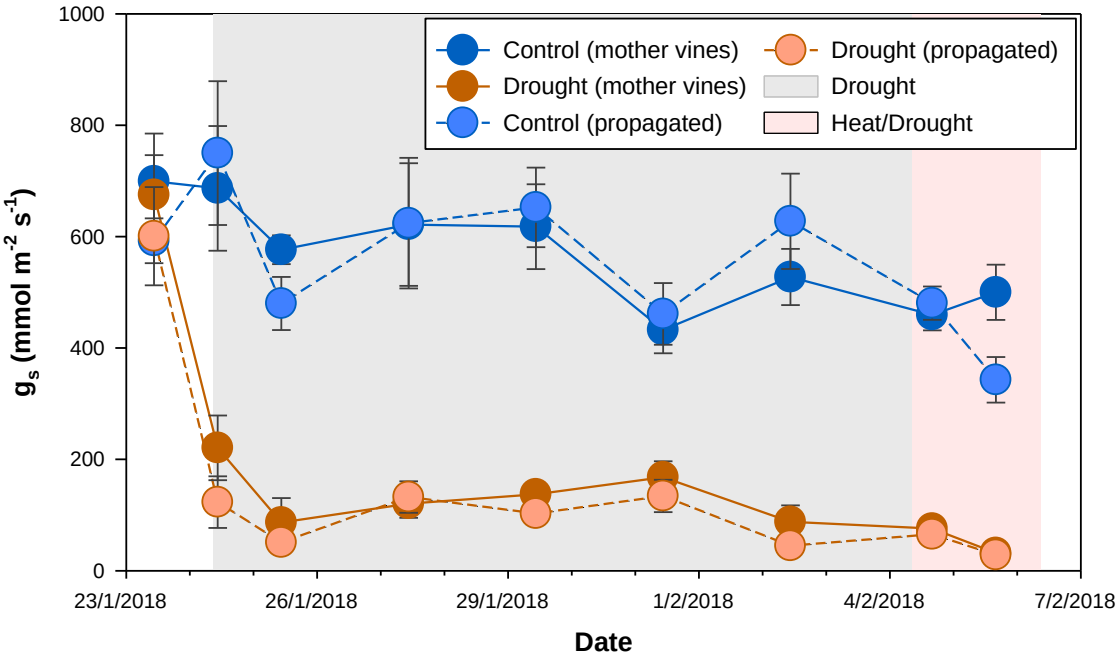


Figure 4. Stomatal conductance (g_s) of mother and daughter vines under control or dual-stress (water deficit followed by water deficit and heat stress combined) in experiment 2.

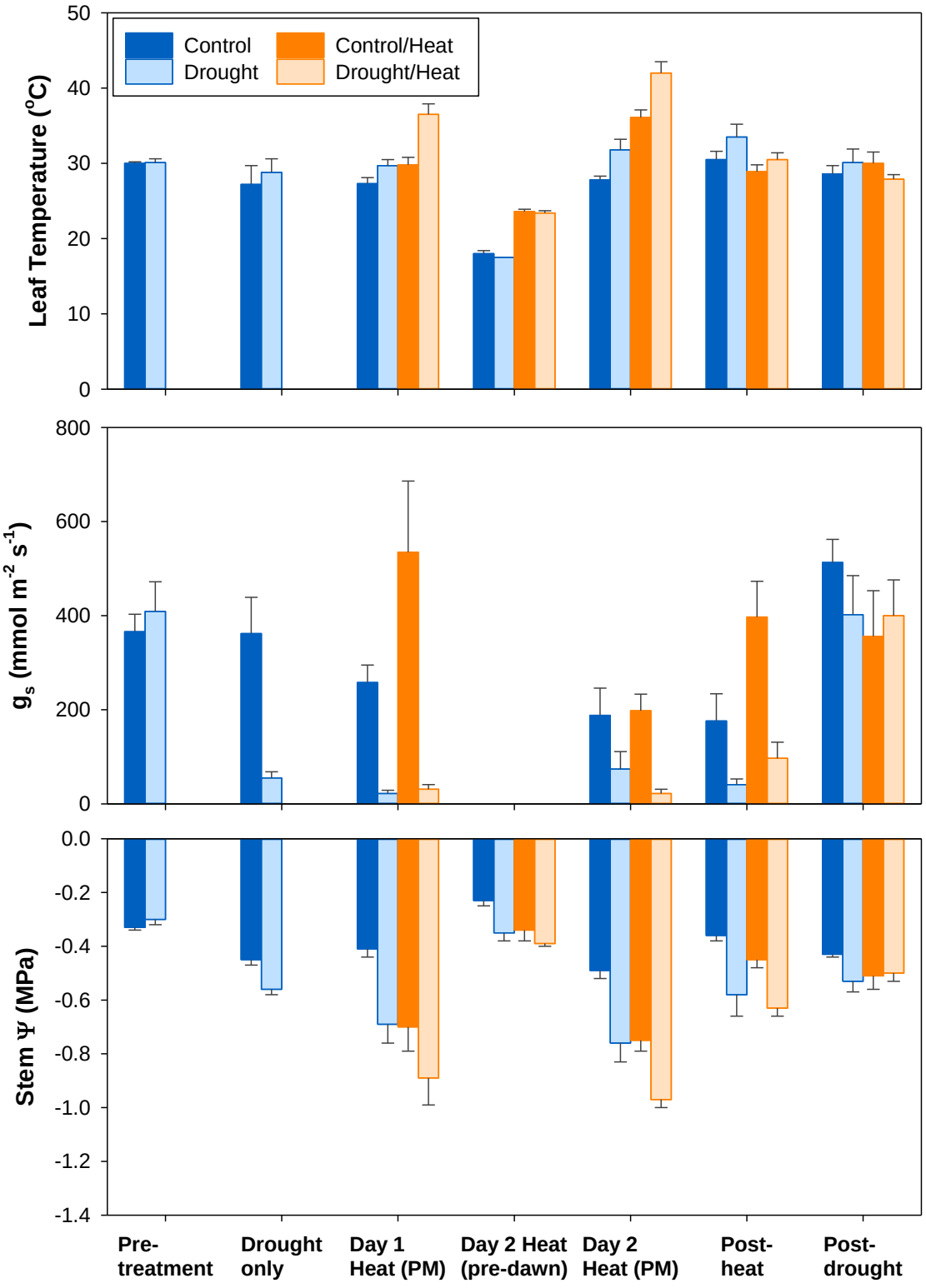


Figure 5. Leaf temperature (top), stomatal conductance (g_s, middle) and stem water potential (Ψ_s, bottom) at leaf biomass sampling times during experiment 1.

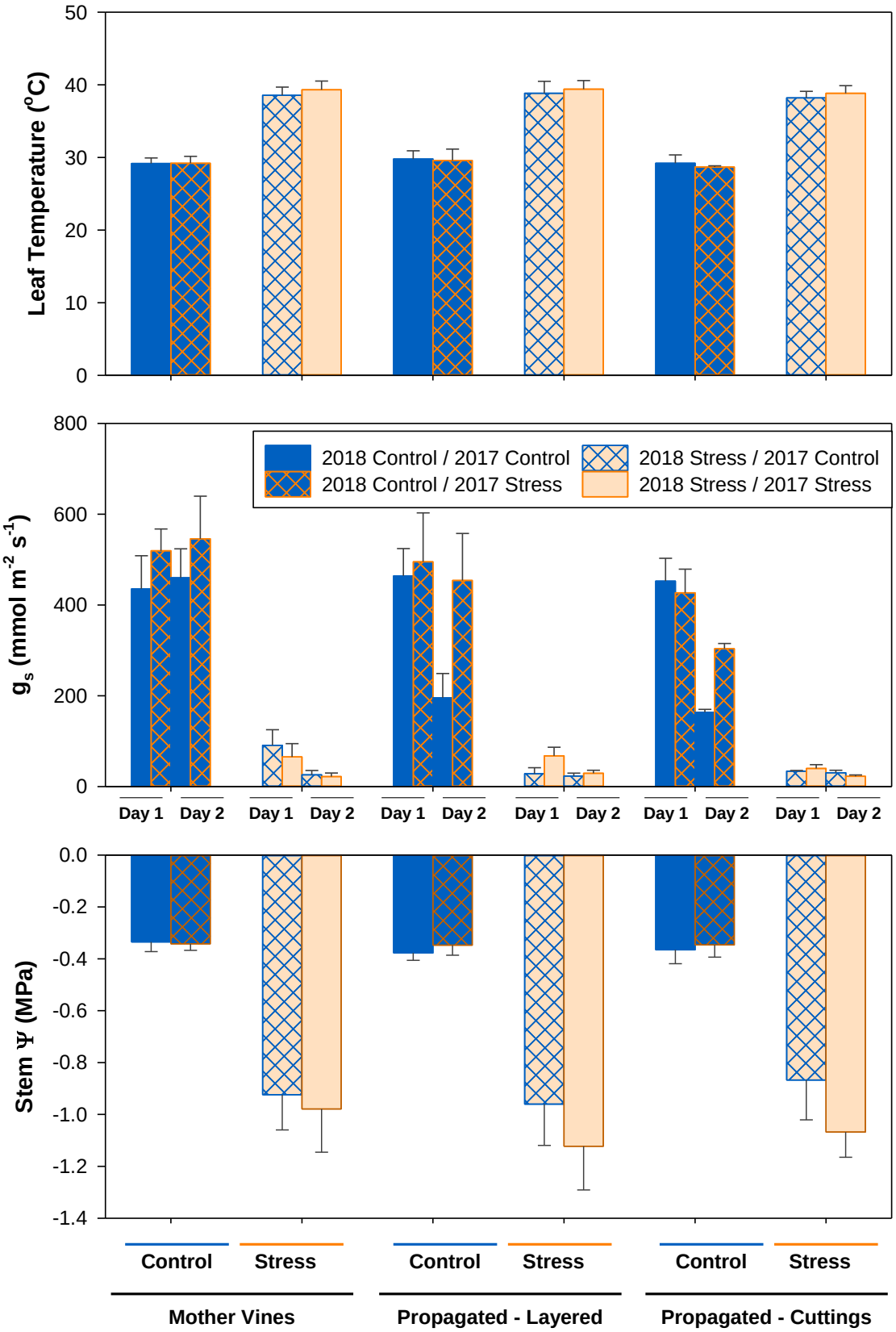


Figure 6. Leaf temperature (top), stomatal conductance (g_s, middle) and stem water potential (Ψ_s, bottom) on day one (g_s only) and day two of dual stress in experiment 2.

The basic response of the vines exposed to the dual stress in experiment 2 was identical to experiment 1, with greatly reduced g_s and Ψ_s relative to the control (Figure 6). Moreover, there was no statistically significant effect of vine age, either the propagation type or the past history (experiment 1 treatment) on those responses. Oddly, the daughter vines under control conditions had a significantly lower g_s than the mother vines under control conditions ($p=0.018$, $n=20$) on the second day of measurement. Given this was not the case on any of the other days (Figure 4), it seems likely it was an artefact, although the cause is not obvious.

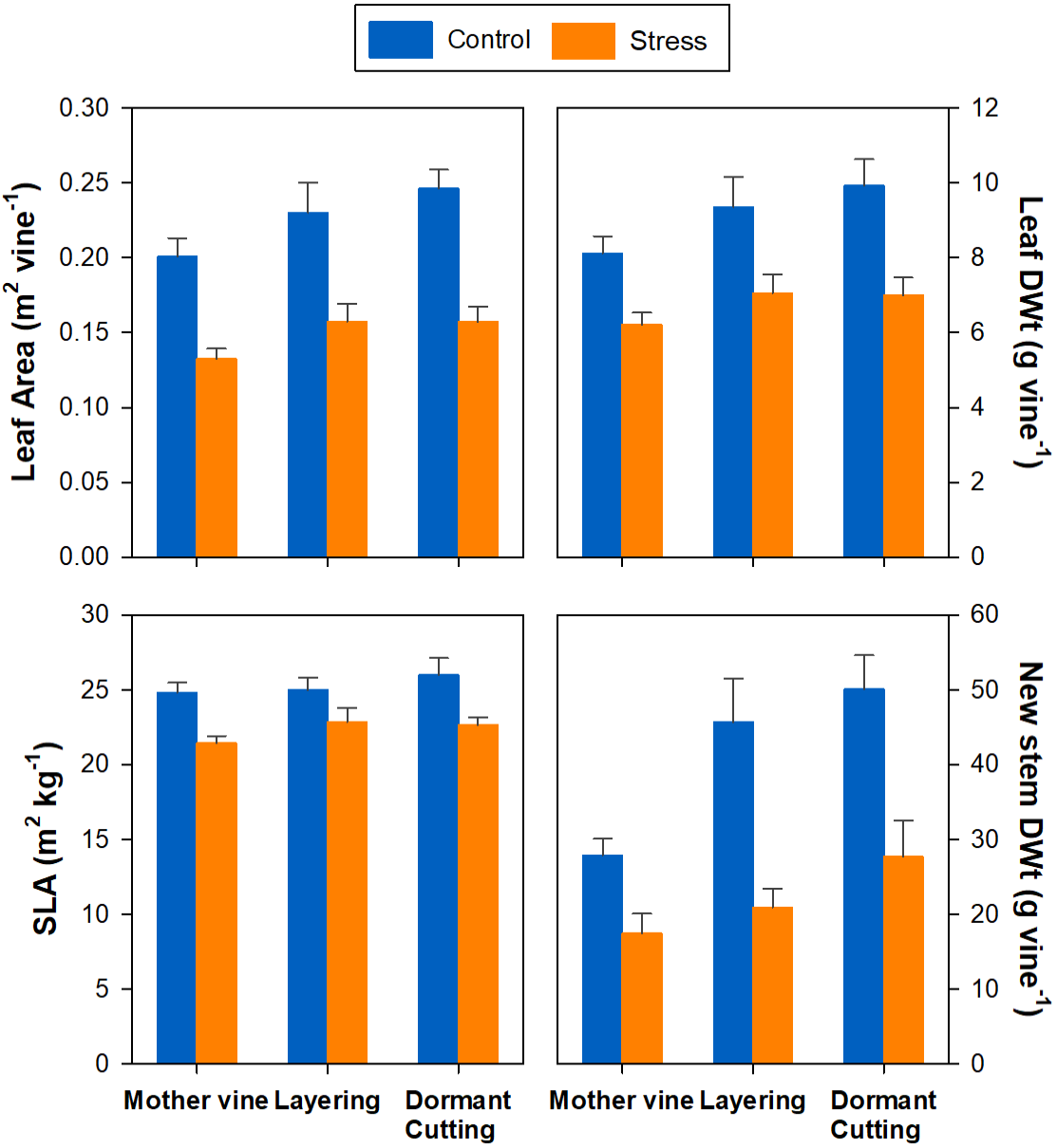


Figure 7. Plant biomass measurements at harvest on day two of the dual stress in experiment 2.

Although there was no impact of past history of stress (experiment 1 treatment) on the leaf physiology, it was possible that the longer-term stress response may have been impacted. To test for this, the effect of water stress on vine growth was examined through a destructive harvest of the new growth (Figure 7). Water stress had a significant negative effect on stem elongation, leaf biomass and leaf area, but there was no effect of vine history (experiment 1 treatment) on that response (data not shown).

Gene expression

Each sample was sequenced to a depth exceeding 20 million sequence reads per sample and, following adaptor trimming, average read alignment rate to the *Vitis vinifera* reference genome assembly in all samples was 77%.

Gene expression grouped with treatment and with sampling time for year 1 (Figure 8). Control plants, during treatment (brown, green, aquamarine and blue circles) and after treatment (ST6, magenta circle and all symbols) occupied the top quadrants of the multidimensional scaling (MDS) plot. Samples collected from stressed plants at ST3 and ST4 occupied the furthest distance from samples collected at ST1 and ST6. Samples collected from stressed plants under the first day of each stress (ST2 of drought stress and ST3 of heat stress) and the first day of the stress condition removal (ST5) occupied an intermediate space between samples at ST1/ST6 and ST3/ST4 (Figure 8). Non-stressed samples and stressed samples were separated globally from each other.

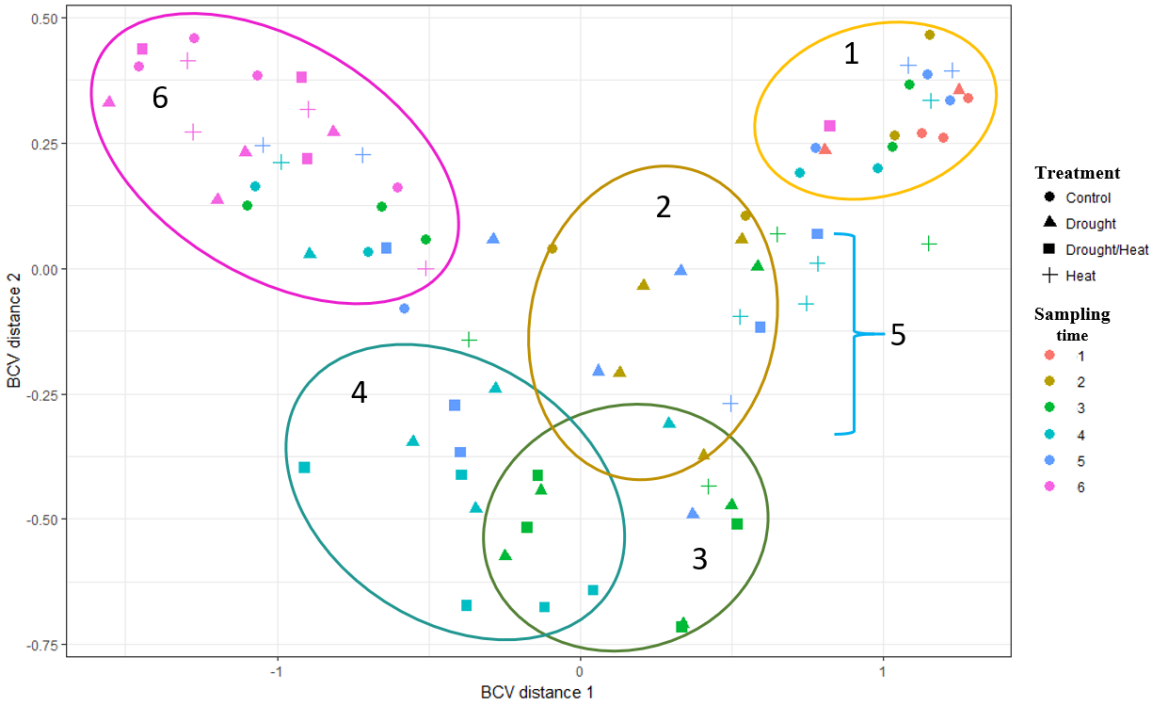


Figure 8. Effect of drought, heat and drought/heat stress on grapevine transcriptome. The normalised sequencing data were used for multidimensional scaling (MDS) analysis. Four symbols indicate the four treatments, respectively, circle (control), triangle (drought), cross (heat) and square (drought/heat). Six colours indicate the six sampling times. **1**, 1st day plant acclimation to glasshouse; **2**, 1st day of drought treatment; **3**, 11th day of drought treatment and 1st day of heat treatment; **4**, 12th day of drought

stress and 2nd day of heat treatment; **5**, Day of stress removal; **6**, 16 days after removal of stress treatments. The separation distance is based on log2 fold change. The more similar expression pattern shows the closer distance.

To interpret and analyse the RNA-seq data, the differential expression of transcripts was examined between treatments and control (drought vs. control, heat vs. control, drought and heat vs. control). The differentially up-regulated and down-regulated genes were identified for the drought, heat and combined drought and heat treatments, respectively. Differential expression analysis revealed that more up-regulated and down-regulated genes were identified in the combined drought and heat treatment than either individual stress. More genes were over- than under-expressed in the combined treatment and with drought, whereas the numbers of genes over or under-expressed with heat treatment alone did not differ significantly (Table 2).

Table 2. Number of up-regulated (top row) and down-regulated (bottom row) differentially expressed genes induced by drought, heat and drought and heat stress at different sampling times (p-value= 0.01).

	ST3	ST4	ST5	ST6
Drought	4	0	0	0
	1	0	0	0
Heat	0	183	37	0
	0	156	38	0
Drought + Heat	506	2355	1507	0
	344	1403	243	0

Genes differentially expressed early (ST3) under drought stress were also differentially expressed following subsequent heat stress (combined drought and heat (DH) ST3) (Figure 9).

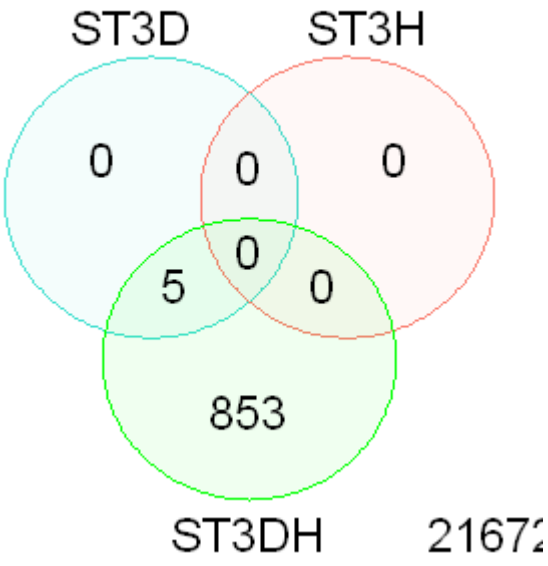


Figure 9. Number of differentially expressed genes at ST3 in the drought (D), heat (H) and dual stress (DH) treatments and their overlap.

Following heat stress treatment (ST4) there was considerable overlap (284 genes) between genes differentially expressed with heat stress alone (H) and in response the combined treatment (DH) (Figure 10).

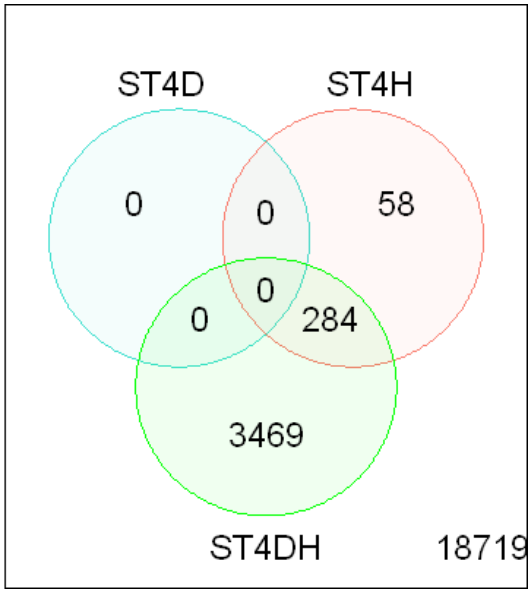


Figure 10. Number of differentially expressed genes at ST4 in the drought (D), heat (H) and dual stress (DH) treatments and their overlap.

ST4 was also the most responsive time point with the largest number of differentially expressed genes. Only 48 genes were differentially expressed at each sampling time point during drought and heat treatment, i.e. immediately upon treatment and remaining differentially expressed through treatment and after stress removal (Figure 11).

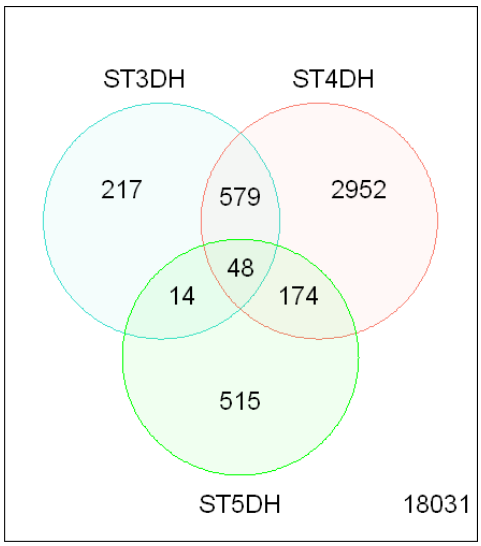


Figure 11. Number of differentially expressed genes at ST3, 4, and 5 in the drought (D), heat (H) and dual stress (DH) treatments and their overlap.

To classify differentially expressed genes under drought and heat stresses, gene ontology (GO) enrichment analysis was performed to investigate over-represented putative biological processes, molecular function and cellular components functions (Young *et al*., 2010). Table 3 shows the biological functions enriched in expression after removal of drought and heat stress. Biological functions for response to stress were significantly over-represented.

Table 3. List of biological functions of genes enriched in expression following dual drought and heat stress.

GO ID	GO Name	Expected Sample Enrichment	Actual Sample Enrichment	p-value
9644	response to high light intensity	1.5969	14	5.565437E-11
9408	response to heat	1.4638	12	2.211185E-9
34976	response to endoplasmic reticulum stress	1.2547	10	4.409343E-8
42542	response to hydrogen peroxide	1.4068	10	1.496375E-7
46777	protein autophosphorylation	1.749	11	1.793768E-7
6457	protein folding	5.3801	19	5.8697E-7
44255	cellular lipid metabolic process	0.0951	2	6.639447E-5
9684	indoleacetic acid biosynthetic process	0.4753	4	9.446524E-5
19761	glucosinolate biosynthetic process	1.1216	6	0.0001250817
72593	reactive oxygen species metabolic process	0.1141	2	0.0001309099
10155	regulation of proton transport	0.8365	5	0.0001753634
48574	long-day photoperiodism, flowering	0.1331	2	0.0002258536
6517	protein deglycosylation	0.1331	2	0.0002258536
7050	cell cycle arrest	0.1331	2	0.0002258536
19288	isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway	3.0037	10	0.0002303866
6655	phosphatidylglycerol biosynthetic process	0.8935	5	0.0002542225
70838	divalent metal ion transport	0.6654	4	0.0004936103
10103	stomatal complex morphogenesis	1.4068	6	0.0005167221
6984	ER-nucleus signalling pathway	0.1711	2	0.0005268521
10206	photosystem II repair	0.1711	2	0.0005268521

35066	positive regulation of histone acetylation	0.1711	2	0.0005268521
9610	response to symbiotic fungus	0.1711	2	0.0005268521
6013	mannose metabolic process	0.1711	2	0.0005268521
10286	heat acclimation	0.3992	3	0.0005975739
18279	protein N-linked glycosylation via asparagine	0.1901	2	0.0007420356
6597	spermine biosynthetic process	0.1901	2	0.0007420356
9903	chloroplast avoidance movement	0.2091	2	0.001005929
9658	chloroplast organisation	1.6159	6	0.001185482
35556	intracellular signal transduction	2.6425	8	0.001400225
30003	cellular cation homeostasis	0.8555	4	0.00158988
45036	protein targeting to chloroplast	0.5133	3	0.001601271
6399	tRNA metabolic process	0.5133	3	0.001601271
9904	chloroplast accumulation movement	0.2471	2	0.001694913
16573	histone acetylation	0.2852	2	0.002621287
45087	innate immune response	0.5893	3	0.002704017
6098	pentose-phosphate shunt	2.3764	7	0.002733563
10304	PSII associated light-harvesting complex II catabolic process	0.3042	2	0.003180982
6526	arginine biosynthetic process	0.3042	2	0.003180982
9073	aromatic amino acid family biosynthetic process	1.0076	4	0.003298858
6338	chromatin remodelling	0.6274	3	0.003413036
9773	photosynthetic electron transport in photosystem I	0.6274	3	0.003413036
6499	N-terminal protein myristoylation	1.0266	4	0.003579691
6468	protein phosphorylation	4.8668	11	0.003854261
52696	flavonoid glucuronidation	4.3155	10	0.004333332
6569	tryptophan catabolic process	0.3422	2	0.004506312
8295	spermidine biosynthetic process	0.3422	2	0.004506312
19252	starch biosynthetic process	2.1292	6	0.005628081
6541	glutamine metabolic process	0.3802	2	0.006120943
46856	phosphatidylinositol dephosphorylation	0.3802	2	0.006120943
6629	lipid metabolic process	2.7186	7	0.006156704
10114	response to red light	0.7414	3	0.006273713
6816	calcium ion transport	0.7604	3	0.006867959

6006	glucose metabolic process	0.3992	2	0.007041504
9800	cinnamic acid biosynthetic process	0.3992	2	0.007041504
10218	response to far red light	0.7794	3	0.007498251
16556	mRNA modification	1.2357	4	0.007891264
9813	flavonoid biosynthetic process	4.6957	10	0.008014653
6559	L-phenylalanine catabolic process	0.4372	2	0.00911699
17148	negative regulation of translation	0.4372	2	0.00911699
6012	galactose metabolic process	0.4372	2	0.00911699
9627	systemic acquired resistance	0.8365	3	0.009613913
9414	response to water deprivation	1.3117	4	0.01009741
5985	sucrose metabolic process	0.4563	2	0.01027454
9744	response to sucrose	1.3308	4	0.01070868
45454	cell redox homeostasis	3.0037	7	0.01092728
10468	regulation of gene expression	0.4753	2	0.01151343
42343	indole glucosinolate metabolic process	0.4753	2	0.01151343
6357	regulation of transcription from RNA polymerase II promoter	0.8935	3	0.01208332
9723	response to ethylene	0.8935	3	0.01208332
10073	meristem maintenance	0.5133	2	0.01423873
48439	flower morphogenesis	0.5133	2	0.01423873
50832	defence response to fungus	1.4448	4	0.01491242
9630	gravitropism	0.9505	3	0.01492563
9637	response to blue light	0.9505	3	0.01492563
2237	response to molecule of bacterial origin	0.5703	2	0.01895491
16117	carotenoid biosynthetic process	1.5399	4	0.01916228
9867	jasmonic acid mediated signalling pathway	1.5589	4	0.02009816
9409	response to cold	1.0456	3	0.02053497
272	polysaccharide catabolic process	0.5893	2	0.02069607
98542	defence response to other organism	0.5893	2	0.02069607
7033	vacuole organisation	0.6083	2	0.0225221
6342	chromatin silencing	1.1216	3	0.02584119
23	maltose metabolic process	1.673	4	0.02634155

48364	root development	0.6464	2	0.0264287
19375	galactolipid biosynthetic process	0.6464	2	0.0264287
9887	organ morphogenesis	0.6654	2	0.028509
19344	cysteine biosynthetic process	1.711	4	0.02866854
48573	photoperiodism, flowering	1.2167	3	0.03353206
10264	myo-inositol hexakisphosphate biosynthetic process	0.7224	2	0.03525428
48443	stamen development	0.7224	2	0.03525428
42742	defence response to bacterium	1.825	4	0.03641249
16310	phosphorylation	0.7414	2	0.03766933
6636	unsaturated fatty acid biosynthetic process	0.7414	2	0.03766933
10200	response to chitin	1.2737	3	0.03872029
42538	hyperosmotic salinity response	0.7604	2	0.04016674
45893	positive regulation of transcription, DNA-templated	4.5816	8	0.04181385
48193	Golgi vesicle transport	1.3308	3	0.04434153
31408	oxylipin biosynthetic process	0.7985	2	0.04540586
6406	mRNA export from nucleus	0.8175	2	0.04814602
43085	positive regulation of catalytic activity	1.3688	3	0.04832946

The number of significantly differentially expressed genes between T00 and T33 was higher than between T00 and T03 mother plants both when plants were used for layering or dormant cutting propagation (Table 4). In both cases the number of differentially expressed genes was higher in mother plants used to generate layered propagules (Table 4). Plants exposed to combined drought and heat treatment for two consecutive years (primed plants) (i.e. T33) presented a higher number of differentially expressed genes compared to plants exposed to control conditions for two consecutive years (naïve plants) (i.e. T00) than those exposed to control conditions in year one and to combined drought and heat treatment during year 2 (naïve plants) (i.e. T03). In both cases the number of differentially expressed genes was higher in mother plants used to generate layered propagules (Table 4). This increased response to stress suggests a potential priming at a molecular level by the previous year's environmental treatment.

Table 4: Number of differentially expressed genes (adjusted P-value<0.01) between plants grown under control conditions for two years (T00) and naïve plants exposed to drought/heat stress during year 2 (T03) and between T00 plants and primed plants exposed to drought/heat stress during year two (T33).

Propagation method	T00 vs T03	T00 vs T33	Common genes (Year 2)	Common genes (both years and propagation methods)
Dormant cuttings	284	1686	200	67
Layered	4242	5376		

In year 2, following combined drought and heat treatment, 4242 genes were differentially expressed in plants propagated by layering compared with only 284 differentially expressed genes in plants propagated from dormant buds. Two hundred of these differentially expressed genes were common between the two propagation methods. In control plants there was no difference between the two propagation methods and thus there was an interaction between treatment and propagation method with gene expression in layered plants much more sensitive to treatment than in bud-propagated plants. The intersection between year 1 (ST4) and year 2 differentially expressed genes following drought and heat stress showed that the majority of genes responding to stress treatment were in common between the two years. 67 genes were consistently differentially expressed in both years and propagation methods with drought and heat treatment. Of those that have been functionally annotated to date, Small Heat Shock Protein (HSP20-like) genes were significantly over-represented – five of the top 20 genes - and are likely to be important in regulation of the grapevine genome in response to drought and heat. Other differentially expressed genes included several involved in sugars metabolism including hexosyltransferases and Galactinol-sucrose galactosyltransferase, an oxioreductase involved in auxin catabolism and transcription elongation factor spt6 involved in histone binding and chromatin silencing.

DNA methylation

We generated 103.6 Gb sequence data for methylation profiling. After preliminary analysis of these data with the custom bioinformatic pipeline, we did not detect any differentially methylated regions with any of the treatments. However, the overall number of methylated bases called was extremely low and biologically unlikely in any plant genome. We therefore consider this method has not worked and this does not allow us to make any conclusions about DNA methylation. We will look for the opportunity to re-analyse the samples in future using a different method.

7. Outcomes and conclusions

The main objective of this research was to determine whether long-term epigenetic memory of stress could be used for propagation and breeding purposes in grapevine.

1) We were able to elucidate the temporal scale of long-term maintenance of stress-induced modifications in grapevine. Drought, heat and combined drought and heat negatively affected plant growth and influenced water relations. In both drought only and combined drought and heat experiments, stomatal conductance and stem water potential were reduced by the plant, whereas in the heat only treatment stomatal conductance increased. Post-stress, stomatal conductance broadly returned to control levels, However, stomatal conductance of vines that were subject to the combined stress was higher than the drought only vines for most of the recovery period. The experimental results clearly demonstrated that combined stresses had an impact on the vines similar to that seen in previous work and that the heat and drought stresses were additive in nature. Gene expression differed with sampling time during the course of stress treatments and recovery so that more genes were differentially expressed as the drought and heat treatment progressed and these genes were no longer differentially expressed 16 days after removal of stress in any treatment.
2) Although there was no measurable impact on the plant's phenotype from the treatment to which a vine was exposed in experiment 1 and its response to stress in experiment 2 (almost 12 months later), at the gene expression level, primed plants showed a higher response to subsequent stress than naïve plants.
3) Different vegetative propagation methods did not significantly influence plant physiology in response to stress, but naïve mother plants used to generate layered plants showed a higher response to combined drought/heat stress than those used to generate dormant cuttings. Further analysis of the generated data is needed to assess the effect of priming on the gene expression of the different types of propagules included in this study. Nonetheless, the successful implementation of the experimental work offers a platform to assess any epigenetic changes observed, as well as any interactions between the stresses on the transcriptome.
4) Memory of stress did not confer grapevines with a significant measurable advantage against future episodes of drought and heat stress. Plant physiology was not significantly influenced, either positively or negatively, by the previous year's stress exposure.
5) A core collection of differentially expressed genes identified here, common to both propagation methods, is likely to be important for response to the stress.
6) The genomic context of stress-induced changes could not be satisfactorily explored at the level of DNA methylation during the course of this project. We chose the most up-to-date method of DNA methylation analysis that would generate the most data but, unfortunately, in our hands this method did not provide a reliable result. An improvement or change of methodology could change this outcome.

We conclude that there are significant differences between Cabernet Sauvignon molecular responses to drought, heatwaves or combined drought and heat stress. Genetic variation for the core collection of responsive genes and for those that differ

between treatments could be sought for use in plant breeding. Although primed plants showed a higher shift in gene expression to combined drought/heat stress than naïve plants, the failure to generate satisfactory results at a DNA methylation level does not allow us to determine whether this was due to epigenetic priming induced by the environmental treatments imposed in these experiments. Further analysis of the gene expression and DNA methylation datasets generated here, in combination with more detailed phenotypic characterisation of naïve and primed plants would be needed to determine whether long-lived plants could be primed to deal with these abiotic stresses more effectively than naïve ones.

8. Recommendations

We identified a core set of important genes for response to drought and heat stress. We recommend further research into genetic and varietal variation for these genes of interest for breeding.

We identified a significant interaction between propagation method and molecular response to abiotic stress. We also recommend that this interaction be explored in future research that should investigate whether yield and berry characteristics are influenced by the marked change in gene expression in layering versus cutting propagation methods.

With the knowledge gained in this project, we recommend implementing further phenotypic analysis throughout the reproductive phase to determine whether the increase in gene expression changes observed in primed plants is conferring any significant tolerance to stress. We also recommend targeting DNA methylation analyses and analysing histone modification to determine whether changes in gene expression are regulated epigenetically and with the different propagation methods as this will inform breeding for the future to resist climate change. Our results do not preclude an impact of epigenetic priming response to stress in the same season and further research in this field will be required to establish any role of epigenetic modifications in altered gene expression with response to abiotic stress.

9. Appendix 1: Communication

Communication to industry and the scientific community has been an integral part of this project. Results have been presented at a range of industry and scientific meetings throughout the life of the project. The outcomes of this project have been communicated (to date) as follows:

Presentations:

- It is not all about location, location, location (when you talk about wine). EpiCSA & Adelaide RNA Special Interest Group Monthly Seminar Series, 28 April 2017 (http://www.thewaite.org/event/epicsa-seminar-april2017/).
- Use of DNA methylation markers for horticultural research. Department of Horticulture, School of Agriculture, food and Environmental, University of Kentucky, USA, 10 July 2017.
- Use of DNA methylation markers for agricultural, medical and environmental epigenetics research. Centre for Integrative Ecology (Deakin University), 25 August 2017 (https://cie-deakin.com/seminars-2017/).
- Effect of vegetative propagation on epigenetic memory on perennials plant species. Environmental Epigenomics stream. ComBio 2017, Adelaide, 5 October 2017. (http://www.combio.org.au/combio2017/).
- The relevance of epigenetic response to abiotic stress in complex crop genomes. 40th New Phytologist Symposium: Plant epigenetics - from molecular mechanisms to ecological relevance, Vienna, Austria, September 2017.

10. Appendix 2: Intellectual Property

The information generated by this project will enter the public domain as scientific manuscripts and will then be accessible for future reference.

11. Appendix 3: References

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12. Appendix 4: Staff

List of key staff engaged on the Project

Name	Role	Organisation
Dr Penny Tricker	Principal Investigator (from Jan 2018)	ACPFG / University of Adelaide
Dr Carlos Rodriguez Lopez	Principal Investigator (to Dec 2017)	University of Adelaide / University of Kentucky
Dr Everard Edwards	Investigator	CSIRO
Dr Ute Baumann	Investigator	ACPFG / University of Adelaide
Dr Roberta De Bei	Advisor	University of Adelaide
Kiflu Tesfamichael	Technical Officer	University of Adelaide
Annette Boettcher	Technical Officer	CSIRO
Mario Fruzangohar	Bioinformatician	ACPFG / University of Adelaide
Yikang Hu	MSc candidate / Technical Assistant	University of Adelaide

13. Appendix 5: Budget reconciliation

A budget reconciliation will be provided separately.