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Expression–environment associations in transcriptomic heat stress responses for a global plant lineage

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Abstract

The increasing frequency and severity of heatwaves will intensify stress on plants. Given regional variation in heatwave exposure and expected differences in thermal tolerance between species it is unlikely that all plant species will be affected equally by climate change. However, little is currently known about variation in the responses of plants to heat stress, or how those responses differ among closely related species adapted to different environments. Here we quantify the response of 17 *Acacia* species (175 RNA-seq libraries), from across Australia's diverse biomes, to a multi-day experimental heatwave treatment to identify variation in transcriptomic and physiological responses to heat stress. Genes with known heat response functions showed consistent responses across *Acacia* species. Up to 10% of all genes and over 100 gene families showed significant clinal variation in the magnitude of their expression plasticity across species. Specifically, gene families linked to the temperature stress response were overrepresented among significant relationships with home range temperature conditions. Gene expression responses seen on the first day of the heatwave were more frequently associated with home range climates, while expression responses by day four were more commonly related to photosystem II acclimation. Comparative transcriptomics on non-model species has the potential to provide key information on stress response plasticity, especially when linked with our understanding of model species. Our study indicates that the pressing challenge to identifying potentially vulnerable species to climate change could be benefited by the further exploration of clinal variation in transcriptome plasticity.

KEYWORDS
Acacia, climate change, comparative transcriptomics, heatwave, local adaptation

1 | INTRODUCTION

Sessile plants rely on physiological responses to overcome high temperatures during a heatwave (Zhu, 2016). Heatwaves are defined as periods with abnormally high temperatures over multiple days and in plants this can lead to reduced photosynthetic efficiency and cell damage (Huang et al., 2019; Vico et al., 2019). Increased temperature stress on plants under warmer and more variable temperature regimes could have meaningful impacts on ecosystem primary productivity (Duffy et al., 2021; Xu et al., 2020), vegetation composition and the long-term persistence of ecosystems (Neumann et al., 2017; Trisos et al., 2020). The physiological responses of plants to ameliorate temperature stress are well known in model species (Chaudhary et al., 2020; Perrone & Martinelli, 2020; Zhu, 2016). However, with future climate scenarios projecting more frequent and intense heat extremes (Duffy et al., 2021), it is increasingly important to understand variation in the heat stress responses of non-model plants from diverse natural systems (Mokany et al., 2020).

The responses of plants to heat stress are wide ranging and include morphological, phenological, physiological and molecular responses that are known to be conserved across species (Wahid et al., 2007). To respond to heat stress, plants accumulate conserved stress proteins such as Heat Shock Proteins (HSPs), which are chaperone proteins involved with protein folding, aggregation, translocation and degradation (Chen, Feder, & Kang, 2018). These processes are essential for cellular stability when adverse temperatures negatively affect protein formation and stability (Chaudhary et al., 2020). Other examples of stress response gene ontologies include unfolded protein response, oxidative stress response, ubiquitination, signalling (e.g. phosphorylation) and transcription factors (Wahid et al., 2007; Zhang et al., 2022). Changes in mRNA expression are commonly used to evaluate the molecular response of organisms to stress (DeBiasse & Kelly, 2016; Rivera et al., 2021). Many genes known to be involved in heat stress responses are conserved (Zhu, 2016) and their responsiveness to stressful temperatures should have become adapted to local climates to improve the performance of plants (Calvo et al., 2020; Donelson et al., 2023).

To study local adaptation across environments, genomic SNP (Single-Nucleotide Polymorphism) markers are widely used to identify loci under selection through analyses of Genotype–Environment Associations (GEA, Ahrens et al., 2018; de Villemereuil et al., 2014; Frichot et al., 2013). However, differential gene expression has a high and under-evaluated potential to be an important molecular signature of thermal tolerance adaptation that could prove complementary to GEA methods (Price et al., 2022). Gene expression can be regulated by complex pathways making analyses of individual SNP markers potentially ineffective, due to epistatic interactions (Csilléry et al., 2018). Directly comparing gene expression plasticity across many related plant species, from a natural environmental cline in heatwave exposure, has not been explored with comparative transcriptomics to our knowledge. With this proposed method of Expression–Environment Associations (EEA), gene expression plasticity would be associated with climate metrics in the same way allele frequencies are associated with climate for GEA studies. Using comparative transcriptomics to quantify responses to standardised temperature exposures could help identify clinal variation in the magnitude of heat stress responses (DeBiasse & Kelly, 2016) and the aspects of transcriptomic stress responses that are most relevant for estimating adaptive capacity (Donelson et al., 2023).

Currently, physiological measurements of plant thermal tolerance often focus on Photosystem II (PSII) efficiency since photosynthetic activity is tightly linked with heat exposure (Mathur et al., 2014). As a result, PSII Heat Tolerance (PHT) is used to identify threshold temperatures at which PSII is significantly inhibited, often by measuring temperature-dependent change in basal chlorophyll fluorescence (expressed by the function T–F_0, Arnold et al., 2021). Interspecific variation in PHT and the capacity of species to acclimate PHT to changing conditions could be linked to several molecular pathways but this is still poorly understood (Mathur et al., 2014). No studies to our knowledge have explicitly associated interspecific variability in PHT acclimation with the magnitude of transcriptomic heat stress responses. This is a similar principle to the EEA method but in this case PHT plasticity is being associated with expression plasticity.

Our study plant lineage, *Acacia*, is known to have high PHT acclimation (Andrew, Arnold, et al., 2022). This genus is ubiquitous across Australia's varied bioclimatic regions and highly invasive in diverse climates around the world (Gallagher et al., 2011). There are over 1000 species of *Acacia* native to Australia and the diversification of the genus has been linked to its expansion into hotter and drier environments as the Australian continent became more arid since the Eocene (Renner et al., 2020). Frequent colonisation of new bioclimatic regions by *Acacia* in the past could be partially explained by high physiological plasticity and adaptive variation in genomic responses to climate extremes.

Using comparative transcriptomics we intend to identify clinal variation in gene expression plasticity across 17 *Acacia* species, in response to a controlled heatwave treatment. Although the direction of responses to heat stress are expected to be conserved across species in most cases (Chaudhary et al., 2020) we expect detectable variation in the magnitude of transcriptomic stress responses. Variation in transcriptomic responses could be a result of selection on genetic variation and/or adaptive plasticity regulated by epigenetic factors (Donelson et al., 2023). We expect clinal variation in responses, that is a potential signature of local adaptation, to be concentrated in gene families linked to transcriptomic heat stress responses (Nievola et al., 2017). Species from locations with more frequent heatwaves could be adapted to invest rapidly in their stress responses to manage negative consequences (e.g. protein misfolding or degradation). Conversely, we predict species from climates with relatively high mean annual temperature (MAT) to exhibit weaker molecular and PHT responses to the controlled heatwave due to these species having higher preferred temperature ranges.

2 | MATERIALS AND METHODS

2.1 | Summary

To improve our understanding of heatwave responses across related species from diverse climates, we cultivated 21 *Acacia* species using seed sourced from a single wild population per species that occupy a wide range of Australian climates (Figure 1a, Table S1) as well as being a phylogenetically diverse selection of species. After 4 months of growth in control temperature conditions (ca. 24°C day and 18°C night in 13–11 h cycles) plants were exposed to a 4-day heatwave (38°C day and 26°C night). Heatwave responses were quantified with coupled measurements of the transcriptome (gene expression) and PHT acclimation. Samples for transcriptomics were taken across 3 days: 1 day prior to heatwave (hereafter 'day prior'), at the onset of heatwave conditions (hereafter 'day one') and at the end of the heatwave (hereafter 'day four'). Accompanying measurements of PHT were taken the day prior and at day four using basal chlorophyll fluorescence (ca. 4–6 samples per species at each time point for transcriptomic and PHT data). A total of 175 samples from these species were sequenced from three time points across the heatwave, day prior $n=61$, day one $n=63$ and day four $n=51$. This controlled environment experiment quantifies the effect of the heatwave treatment on gene expression. To quantify heat stress frequency at species collection sites, we use the number of days greater than 14°C above the MAT at the source location. This climate metric varies greatly across Australia and the source sites for our study species (Figure 1a), with 14°C representing the difference in the daytime temperature between the control and heatwave in our experiment.

2.2 | Growth conditions and sampling

The seed for the study species were sourced from the seed banks of the Australian Tree Seed Centre and the Australian National Botanical Gardens (Table S1), which were all original wild-provenanced

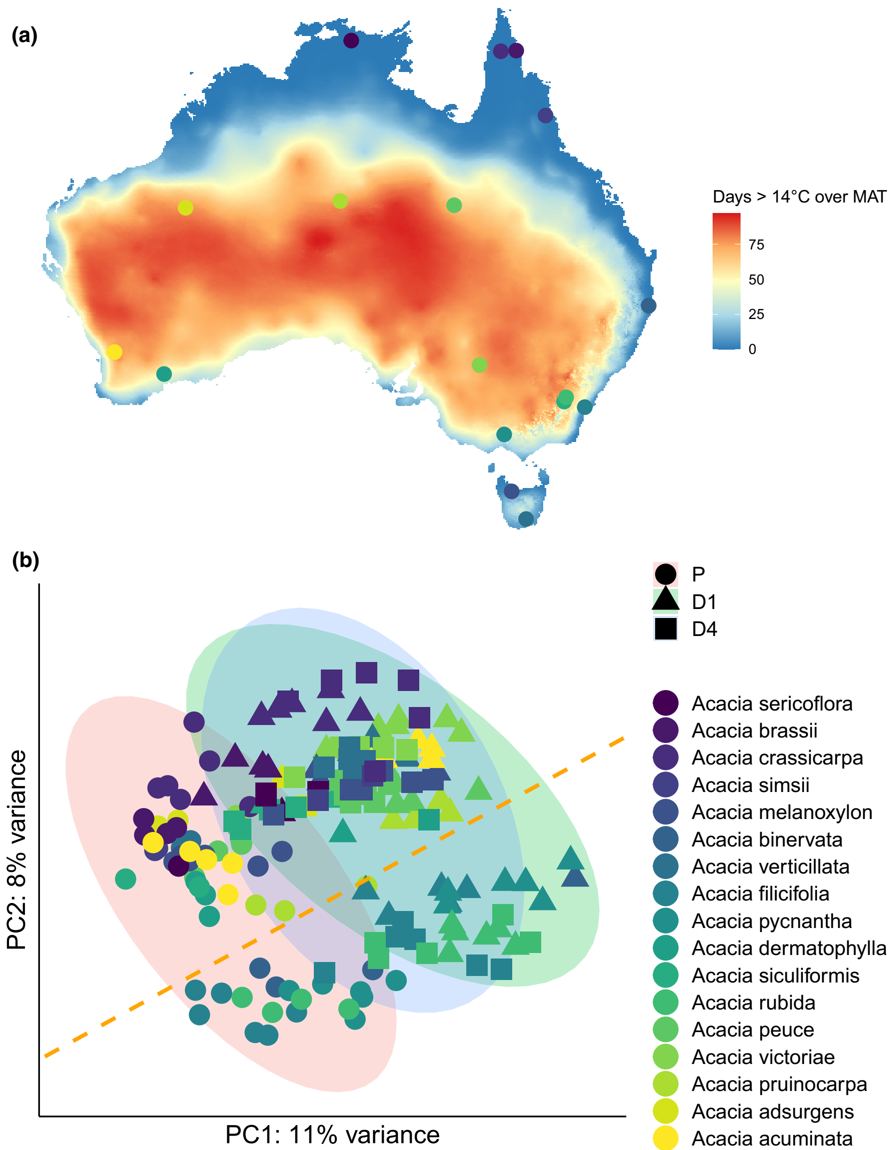

FIGURE 1 Seed collection sites and principal components analysis (PCA) of individual transcriptomes. (a) Climate layer for the number of days >14°C above local MAT overlaid with exclusive seed collection sites for each species. Shading of collection site points matches legend for (b) that plots the first two PCA axes for gene expression ordination. This PCA uses the set of 7274 genes that were expressed in all samples. There is no strong differentiation between individual species, but two broad clusters of species are evident (top left vs. bottom right, separated by dashed line) with the *Acacia* subclade *Botrycephalae* clustering separately in the lower cluster (including: *A. binervata*, *A. filicifolia*, *A. pycnantha* and *A. rubida*). Samples collected during the heatwave (triangles and squares clustering towards the upper right, D1 = day one and D4 = day four) show some differentiation from the pre-heatwave samples (circles clustering towards bottom left, P = day prior). The shaded ovals capture the distribution of these time point groupings.

collections. Seeds were sown over 2 days in August and grown in a temperature-controlled glass house in Canberra, Australia, for about 14 weeks. After germination seedlings were grown in individual pots (15 cm diameter ca. 2 L of soil) that were laid out in a randomised block design with 15 blocks of 40 pots (600 pots total, ca. 30 pots per species were planted) with two pots per species in each block. Full details for glasshouse growing conditions are provided in Andrew, Arnold, et al. (2022).

After the growing period, pots were moved to the Cropatron glasshouse (Australian Plant Phenomics Facility, https://www.plantphenomics.org.au/technologies/#greenhouses) which had a high level of temperature control. For 12 days after the move plants were left to acclimatise with temperature set to the same levels as growing conditions (24°C day and 18°C night). On day 13 after the move, 4 months after planting, a 4-day heatwave treatment started, 38°C day (13 h) and 26°C night (11 h). The duration and magnitude of this heatwave follows the Australian Bureau of Meteorology definition of at least 3 days of significantly above average maximum and minimum temperatures (http://www.bom.gov.au/australia/heatwave/knowledge-centre/). The heatwave was applied for 4 days so that samples could be taken after three full days of elevated maximum and minimum temperatures, above initial growing conditions. The maximum temperature of 38°C selected here represents the upper range of mean maximum temperature experienced during the warmest month of the year at the species' source locations (range 19.1–40.1°C, Table S1) and is experienced across regions in the Austral summer. Plants were watered each morning during the heatwave so there was no effect of droughting. Temperatures ramped up at 8 am and sampling was done between 10:30 am and 12 pm. Leaf samples for RNA extraction were taken the morning prior to the heatwave and on the first and fourth morning of the heatwave. Leaf samples were snap frozen with liquid nitrogen before being transported on dry ice to a −80°C freezer immediately after sampling.

Leaf samples were also collected the day prior and on day four of the heatwave to measure photosystem II heat tolerance (PHT) acclimation across the heatwave. The temperature-dependent chlorophyll fluorescence response (T–F_0 curve) of leaf samples was measured to calculate the T_{crit} and T_{max} PHT metrics (Arnold et al., 2021). For the chlorophyll fluorescence response curves, the inflection point referred to as the critical temperature, T_{crit}, represents the onset of damage, and the temperature at maximum F_0, T_{max}, corresponds to a temperature at which there could be sustained damage to the photosynthetic apparatus. Complete details of the system used are presented in Arnold et al. (2021) and the PHT data have been described in Andrew, Arnold, et al. (2022). Here, we test if changes in gene expression are related to acclimation in PHT, which we define as the increase in PHT (°C) from the day prior to day four of the heatwave.

2.3 | RNA extraction and RNA-seq library prep

For extracting total RNA from *Acacia* leaf and phyllode tissue, the best method for tissue homogenisation proved to be grinding leaf samples with liquid nitrogen in a mortar and pestle. After grinding, samples were returned to dry ice until 16 samples were ready to start the RNA extraction. The kit used for RNA extraction was the NucleoSpin RNA Plant and Fungi Kit (Macherey-Nagel, Germany) using the standard protocol except for an adjustment to the lysis buffer as suggested by Ishihara et al. (2016). The lysis buffer aliquot per sample included 400 μL of PFL and 50 μL PFR buffers from the NucleoSpin Kit, 100 μL Fruit-mate for RNA Purification (Takara, Japan) and 5 μL of ß-mercaptoethanol. Of the 21 species grown, only 17 species were sequenced at multiple time points across the heatwave treatment (Table S1) due to sequencing limitations.

After mRNA isolation with Oligo d(T)$_{25}$ Magnetic Beads (New England BioLabs, Australia), strand specific RNA-seq libraries were prepared using an in-house template switching protocol. The protocol for library preps is fully described in Paten et al. (2022). Two plates of 96 libraries were prepared using custom barcodes. Samples were sequenced on a single NovaSeq S4 flowcell (300 cycles, 2 × 150 bp), using a XP 4-lane splitter kit to split the two sample pools into two lanes each (i.e. set A on lanes 1 and 2 and set B on lanes 3 and 4). Sequencing was done at the Biomolecular Resource Facility at John Curtin School of Medical Research at The Australian National University.

2.4 | Sequence data

Quality assessment and filtering of reads was done with *fastp* program using default settings (Chen, Zhou, et al., 2018). The number of paired-end reads returned per library pre-filtering varied from 41.86 to 110.85 million (mean = 65.99 million) and on average 98% of reads were retained after filtering. Eight low-quality libraries, four technical replicates and five libraries for species sequenced at only one time point were excluded from further analysis. The retained 175 RNA-seq libraries from 17 species, were mapped to the *Acacia pycnantha* reference genome (McLay et al., 2022) using the *STAR* alignment program (Dobin et al., 2013). The STAR alignment used default settings aside from --*chimSegmentMin* set to 10 and --*alignEndsProtrude* set to 10. The number of reads successfully mapped ranged between 28.38 and 83.41 million (mean ± SD = 46.69 ± 10.22 million). The percentage of transcript sequences that mapped to a single locus ranged between 58.9% and 85.64%, mean ± SD = 71.73 ± 4.96%, with *A. pycnantha* individuals having the highest percentage of uniquely mapping sequence reads mean ± SD = 82.8 ± 2.07%. The percentage of reads that mapped to multiple loci ranged between 10.16% and 16.23%, mean ± SD = 13.33 ± 0.98%. The percentage of reads that did not map successfully to the reference genome ('too short' category) ranged between 2.68% and 30.5%, mean ± SD = 14.63 ± 5.04%, with *A. pycnantha* individuals having the lowest percentage of unmapped reads mean = 4.54%, SD = 2.07%.

These read alignment metrics show that a majority of reads mapped to genes with conserved sequences across the genus using the *A. pycnantha* reference genome. For genes that had transcript sequences consistently mapping to them across species, differences in expression between species could be due to a number of factors including recent within-species gene duplications or sequence divergence leading to the uneven mapping of reads. However, these effects should be consistent within species across time points allowing for relative changes in expression across time points to still be comparable across species. As a result, analyses of clinal variation across species focus on changes in the relative abundance of transcripts.

Strict filtering of genes was applied to remove genes that did not have transcripts consistently aligning to them and may not have a well-conserved sequence across our *Acacia* species. Only genes that were present in all libraries at a minimum frequency of 0.25 counts per million (minimum reads mapped ca 7) were kept for further analysis. This filtering to retain only genes being detected in all individuals reduced the number of potential genes from 56,398 annotated genes for the reference genome to 7274 retained genes. This filtering retained a high proportion of the reads that mapped to the reference genome with low variation between species (mean ± SD = 0.88 ± 0.02). This proportion did not vary much between species, showing that almost 90% of reads mapped to this set of core genes (approximately 13% of all genes) that had conserved sequences and/or consistent expression.

2.5 | Transcriptomic analyses

All analyses and visualisations of transcript count data were carried out using R version 4.0.5 (R Core Team, 2016). To test for Differentially Expressed Genes (DEGs) between the three time points the *limma + voom R* package was used (Law et al., 2014). For these three combinations of time points two tests were run. The first test used the standard method and an adjusted *p*-value threshold of .05 and the second test used a $\log_2$ FC threshold of 1 and adjusted *p*-value threshold of .05. The eBayes() and decideTests() functions were used to calculate *t*-statistics and adjusted *p*-values using default settings.

The normalised gene expression data from the DEG analyses were used in further analyses including a PCA using the *prcomp()* function. To test for overrepresented GO terms (Carbon et al., 2019) from the list of DEGs the *topGO R* package (Alexa & Rahnenfuhrer, 2019) was used. The topGO analysis used only genes included in the analysis and GO terms included for the *Acacia pycnantha* reference genome. Fisher exact tests were also run in *R* using the *fisher.test()* function to test for overrepresented PANTHER protein families (Thomas et al., 2022) in the list of significant DEGs. A Bonferroni correction of *p*-values from Fisher exact tests were applied using the *p.adjust()* function. The same methods were used to test for the overrepresentation of annotations among significant clinal relationships.

To test if differentiation in gene expression between species was associated with environmental distance Bray–Curtis dissimilarity was calculated for pairs of RNA-seq libraries using normalised expression values for the core set of 7274 genes. Bray–Curtis dissimilarity was calculated using the *vegan* package (Oksanen et al., 2018). The dissimilarity for library pairs that were from the same pair of species and the same time point, were averaged to get an average dissimilarity for each inter-species pair from each time point. Inter-species expression dissimilarity was plotted against the absolute difference between the environmental values for the two species source sites. Linear models were fitted for the three time points using the environmental differences in MAT and Days >14°C over MAT.

Modelling changes in HSP expression over the heatwave was done with Hierarchical Generalised Additive Mixed Models (HGAMMs) with the gam() function from the *mgcv* package (Wood, 2017) The HGAMMs had random factor levels for each gene with individual smoothers that had independent shapes but fixed wiggliness penalising terms across all levels (Pedersen et al., 2019).

2.6 | Association analyses

To test for clinal variation in fold change (FC) values across species, the $\log_2$ transformed FC values were calculated for each species when comparing day one against the day prior to the heatwave or day four against the day prior to the heatwave, so increases in expression during the heatwave were represented as positive values and decreases in expression as negative values. These FC values were used as response variables and the climate at the species' source locations and mean species PHT acclimation were used as predictors. The climate variables of MAT and average number of days per year getting to at least 14°C above MAT (hence, days >14°C above MAT) was calculated using AWAP daily maximum and minimum temperature data (Jones et al., 2009). The daily climate data for a 30-year period (1 January 1981 to the 31 December 2010) were used as a representative period from which seed was collected. The daily maximum and minimum temperatures were averaged per day for 10 × 10 km grid cells across Australia before averaging at each grid cell across the year to get the yearly average temperature. The mean of yearly averages for the 30 years was used for MAT for each grid cell (this layer was very highly correlated with WorldClim MAT layer (Fick & Hijmans, 2017), $R^2 = .99$). The number of days per year that reached a maximum temperature that was 14°C above the yearly average temperature (i.e. the average temperature for the specific year) was then calculated and averaged for the 30 years to determine the number of days >14°C above MAT climate metric (Figure 1a). The days 14°C above metric was calculated to capture temperature variability and the frequency at which temperatures reach levels above the local MAT that is comparable to the magnitude of our heatwave treatment (change from 24 to 38°C daytime temperatures). The days >14°C above MAT layer was not correlated with MAT but was correlated with WorldClim annual precipitation ($R^2 = .63$), with sites that have higher temperature variability being drier (this was tested based on our observations of Figure 1a). The latitude and longitude of seed collection sites were used to extract values for MAT and days

>14°C above MAT for each species. For our study species, the four predictor variables were not strongly correlated, with only T_{max} acclimation and days >14°C above MAT having a significant negative relationship ($p=.01$, $R^2=.40$).

For linear models, response and predictor variables were scaled to give semi-partial correlations (here after, 'semi-partial *r*'), the scaling set the mean is 0 and the standard deviation is 1 (Schielzeth, 2010). This scaling results in estimates that are similar to a Pearson's correlation coefficient and can be related to estimates of other response variables within models and between similarly structured models with the same predictors, with higher slopes indicating stronger relationships. However, all *p*-values and *t*-values remain unchanged due to scaling (Schielzeth, 2010). Linear models were also weighted by the total sample size for the two time points used to calculate FC values, such that species with smaller sample sizes are less influential. Where the linear relationships between changes in expression and predictors are significant, we might expect false positives before Bonferroni correction as well as false negatives due to our limited sample of species (16 species for day prior vs. day one and 12 species for day prior vs. day four models). We attempt to deal with this problem by testing which functional groups of genes (PANTHER protein families and biological process GO terms) have a higher-than-expected number of genes with significant relationships using Fisher exact tests. The mean FC values across PANTHER protein families and some GO terms were also averaged to look at clinal trends in responses at a more general functional level.

All plotting was done in R with most figures using *ggplot2* and related packages. All R code processed gene expression data and meta data for our main analyses are available with online data (Andrew & Mokany, 2023a). The original sequence data are also made available on the CSIRO's Data Access Portal (see Andrew & Mokany, 2023b for part 1 and Andrew & Mokany, 2023c for part 2).

3 | RESULTS

3.1 | General transcriptome response to heatwave conditions

The RNA-seq data for all samples were aligned to the reference genome of *Acacia pycnantha* to use a reference with complete gene assemblies and the best available gene annotations. As a result, analyses focus on genes with conserved sequences across the lineage. Approximately 88% (SD only 2% across all species) of all mapped reads aligned to this core set of 7274 conserved genes that represented about 13% of all annotated genes from the reference genome. There was however more variation in the percentage of all sequences that mapped to a single locus (range 58.9%–85.64%, mean ± SD = 71.73 ± 4.96%), with *A. pycnantha* individuals having the highest percentage of uniquely mapping sequence reads mean ± SD = 82.8 ± 2.07%. The general transcriptome response to the heatwave treatment was visualised using a PCA, that included the core set of 7274 genes detected in all individuals from the 17 *Acacia* species. The first two principal components accounted for 19% of the variance in the expression profiles; the first two axes mostly captured differentiation between the day prior and during the heatwave (Figure 1b), showing that the general response to the heatwave treatment was consistent at both day one and day four. We see no independent clustering of individual species using the first two principal components, suggesting that responses to heat at the level of individual genes could also be consistent in direction across species.

For our core set of genes we compared expression similarity among species for each time point to environmental distance between source sites. Overall expression dissimilarity was not correlated with environmental distance ($R^2 < .01$ for all three timepoints against the two environmental variables, Figure S1). This result shows that dissimilarity in normalised expression levels was not higher for pairs of species from more dissimilar environments.

3.2 | Genes responding across the heatwave treatment

A large proportion of genes showed significant changes in expression levels across the heatwave, as determined by combined analyses of DEGs, that included all species and were run for all three comparisons of time points (proportion of significant DEGs out of 7274; day prior vs. day one = 49.0% ($n=3563$), day prior vs. day four = 38.9% ($n=2833$) and day one vs. day four = 29.6% ($n=2153$), Table S2). When a threshold for significance of a minimum absolute log FC of 1 was applied (i.e. a minimum of a doubling or halving in expression levels), less than 2.5% of genes were found to be significant (165, 89 and 2 genes for the three comparisons respectively, Table S2, Figure S2).

To assess the functions being overrepresented in the DEG sets, we used PANTHER protein families (here after 'protein families', Table S3a,b) that group related genes with a shared function, and Gene Ontology terms (GO terms, Table S4a,b) that categorise genes based on the biological processes with which they are associated. Analyses identified many overrepresented annotations linked to stress responses. As an example of consistent trends over the heatwave, the response of most HSPs showed the abundance of transcripts increasing across species by the morning of day one, only hours after exposure to heatwave temperatures, followed by a partial reduction in abundance by the morning of day four without fully returning to pre-heatwave levels (Figure 2). In addition, we also see changes in HSP transcription factor abundance (Figure 2d), and other stress related transcription factors (Table S3a,b, e.g. MYB, NAC, WRKY and Two-component response regulator).

3.3 | Variation in stress responses associated with source climate

We expect the conserved transcriptomic heat stress responses seen in this experiment to show clinal variation across species. At the level

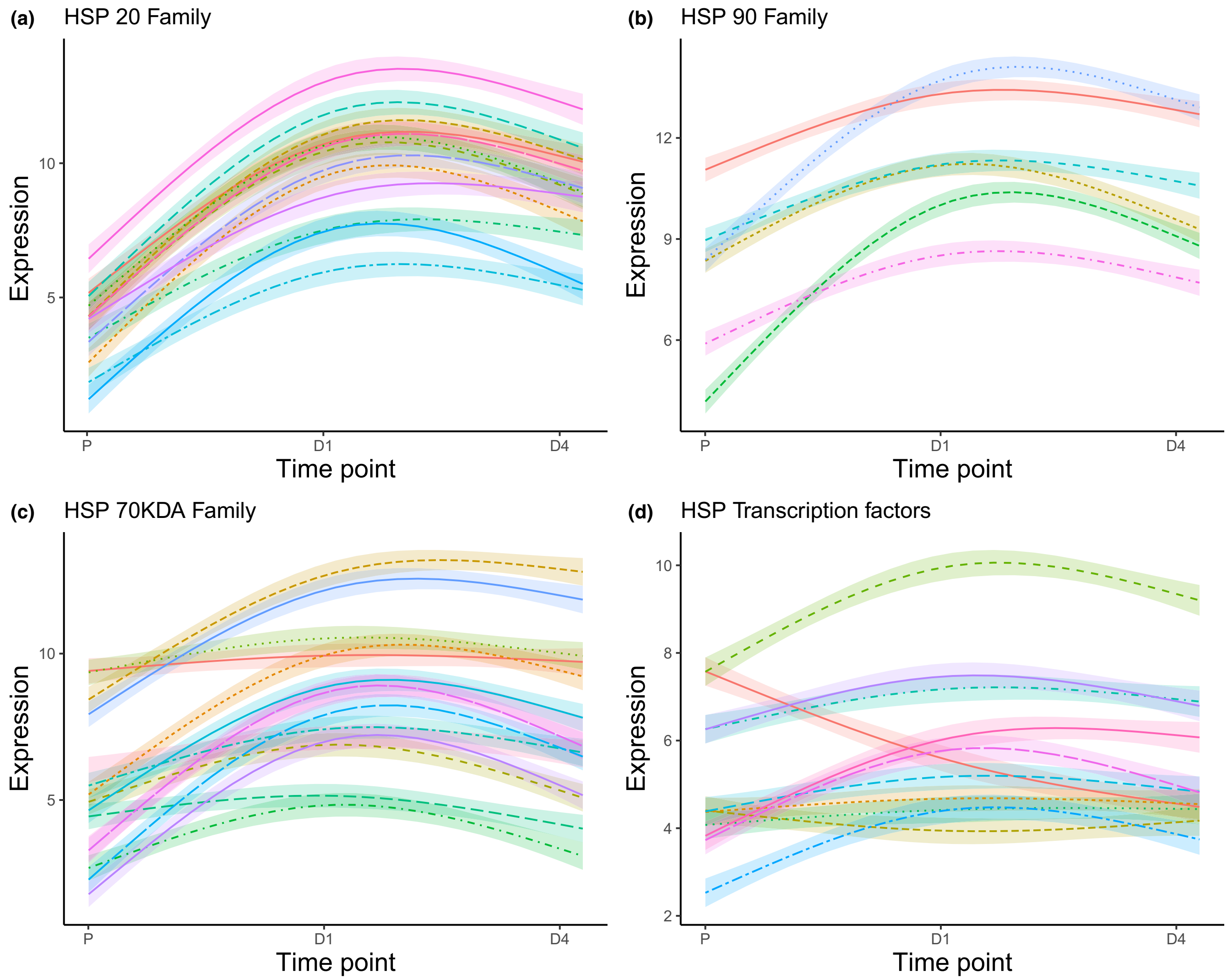

FIGURE 2 Changes in heat shock protein (HSP) expression over the heatwave. Each panel shows one of the four overrepresented HSP families, with generalised additive modelling (GAM) smoothers for each of the genes within families (smoothers show mean expression for all species and shaded area shows s.e.). Hierarchical GAMs were fitted with independent smoothers for each gene (coloured lines). Time point 'P' is for the day prior sampling, 'D1' for day one of the heatwave and 'D4' for day four of the heatwave. Many genes show a strong increase in expression going from the day prior to day one and some recovery by day four. However, some genes show minimal change in expression across time points (panels c and d), suggesting limited regulation, while others such as the HSP 20 family (a) change in parallel, suggesting a common pathway of regulation across genes. The expression values are the transformed values after normalisation for variation in library size.

of gene families we see stronger responses in species from locations with more frequent temperature fluctuations >14°C above MAT and weaker responses in species from more stable tropical climates with high MATs (Figure 3a,b). The average upregulation of HSPs had a marginal negative relationship with MAT ($p = .067$, $R^2 = .22$) and a significant positive relationship with the number of days >14°C above MAT at the seed source location ($p = .015$, $R^2 = .35$). The average FC for upregulated protein folding genes followed similar trends to HSPs with a significant negative relationship with MAT ($p = .005$, $R^2 = .44$, Figure 3). Response to heat genes that were upregulated also had a significant negative trend with MAT ($p = .003$, $R^2 = .47$) and all upregulated response to stress genes had a negative relationship nearing significance ($p = .077$, $R^2 = .21$).

Next we test if trends in the average response of gene families are replicated at the level of individual genes for the *Acacia* species studied. A small proportion of genes had significant clinal relationships with our selected climate metrics (range 4.45%–10.52% of genes were significant, Figure S3). We found that several protein families linked to heat stress responses were overrepresented among individual gene clinal relationships (Table 1 and Table S5). As expected, the mean FC of all genes within stress protein families also showed consistent linear relationships in many cases, to the trends of individual genes within those families (Table 1, see additional example in Table S6). Genes from the HSP families were most strongly associated with the number of days >14°C above MAT at source location. With the three major HSP families and

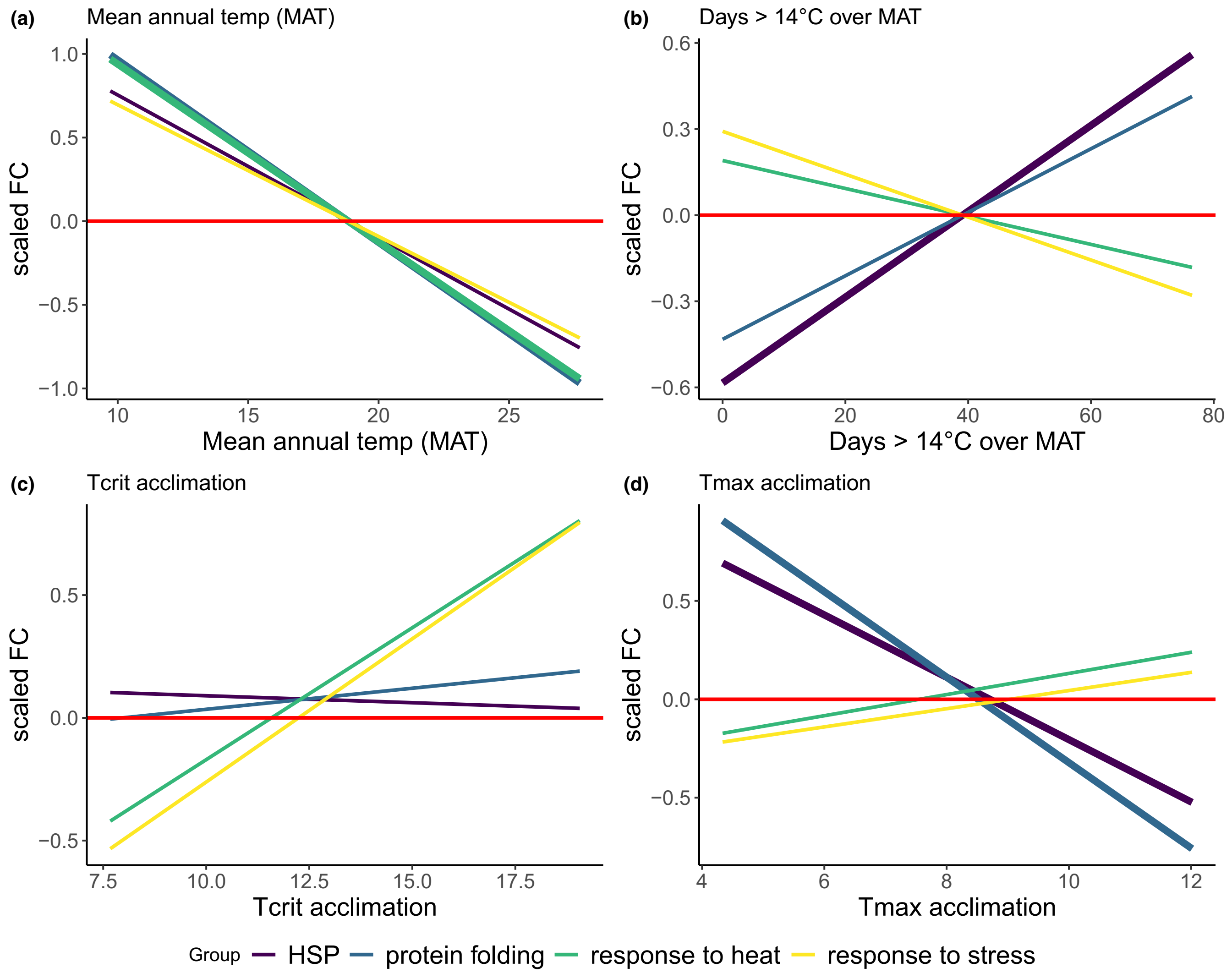

FIGURE 3 Clinal variation in average stress responses across species. The day prior versus day one $\log_2$ fold change (FC) values were averaged for upregulated stress response genes and plotted against climate and photosynthetic heat tolerance acclimation. (a) mean annual temperature at the source location (MAT), (b) average number of days annually >14°C above MAT at the source location, (c) leaf T_{crit} acclimation and (d) leaf T_{max} acclimation measured during the experiment. The mean FC for upregulated genes of each family were scaled so slopes are more comparable between functional groups. The red line shows the expected relationship for no variation between species. Significant relationships have bold lines. Genomic stress responses appear to be weaker for species from locations with high MATs and species with high T_{max} acclimation, while species from more variable climates (high number of days >14°C above MAT) had strong responses.

HSP transcription factors having a higher-than-expected number of significant positive relationships (fisher's exact test $p < .05$), indicating these genes were more strongly upregulated in species from more variable climates (Table 1, Tables S5 and S7). The mean expression levels of these four HSP families before the heatwave were only marginally correlated with days >14°C above MAT for the HSP 70 family ($t_{59} = -2.04$, $p = .045$, $R^2 = .07$). This indicates that clinal variation in transcriptome responses was much higher than clinal variation in expression levels prior to stress exposure for these HSP families. In contrast, individual HSP were less strongly upregulated in species from localities with high MAT (Table 1 and Table S6). Individual protein folding genes were also less strongly upregulated in species from locations with high MAT (Table S8, results for GO terms).

Of the genes with significant linear relationships with seed source climate, most tended to have standard deviations in FC values of less than 1 across species (Figure S3). In fact, variation in $\log_2$ transformed FC values across species had SD values less than 1 for over 91% of genes (day prior vs. day one = 91.1% and day prior vs. day four = 92%). The high frequency of low SD values and semi-partial r values near zero indicates that the response of *Acacia* species to the heatwave was relatively minimal or consistent for this large proportion of genes. However, it is also interesting to consider which protein families had the most varied responses across species. Of the 645 genes that had a FC SD > 1 (day prior vs. day one comparison) 588 of these genes had changes in expression that varied between positive and negative across species. For these genes with the most varied responses across species 37

TABLE 1 Responses associated with source climate and photosystem II heat tolerance (PHT) acclimation.

Protein family		Individual genes				Mean FC model	
PANTHER ID	Functional group	Sign/anno	Comp	% Pos	% Up	Semi-partial *r*	R^2
Number of days > 14°C over MAT models							
PTHR43888	Chaperone DNAJ	3/5	P vs. D1	100	100	**.7**	**.51**
PTHR10015	HSP TF	4/11	P vs. D1	100	100	**.67**	**.45**
PTHR19375	HSP 70	5/17	P vs. D1	100	100	**.72**	**.51**
PTHR11527	HSP 20	4/13	P vs. D1	100	100	.39	.17
PTHR11528	HSP 90	3/8	P vs. D1	100	100	**.73**	**.61**
PTHR48049	Glycosyltransferase	4/12	P vs. D1	100	0	**.44**	**.27**
PTHR10666	Ubiquitination	4/15	P vs. D1	75	100	.29	.08
PTHR24006	Deubiquitination	3/9	P vs. D1	66.7	0	.32	.2
PTHR12549	Demethylation	3/5	P vs. D1	66.7	100	.33	.13
PTHR31669	TF FAR1	3/10	P vs. D1	0	66.7	**−.63**	**.32**
Mean annual temperature (MAT) models							
PTHR11214	Glycosyltransferase	4/8	P vs. D1	100	50	**.57**	**.43**
PTHR43096	Chaperone DNAJ	4/8	P vs. D1	25	75	**−.52**	**.32**
PTHR45633	HSP 60 mitochondrial	3/5	P vs. D1	0	100	**−.59**	**.41**
PTHR11527	HSP 20	4/13	P vs. D1	0	100	−.42	.21
PTHR10972	Membrane	3/6	P vs. D1	100	0	**.66**	**.54**
PTHR11566	Membrane	3/6	P vs. D1	100	0	**.52**	**.36**
PTHR10257	Phosphorylation	3/6	P vs. D1	66.7	0	**.52**	**.33**
T_{crit} *PHT acclimation models*							
PTHR31669	TF FAR1	4/10	P vs. D1	75	50	**.66**	**.37**
PTHR23172	Membrane	3/6	P vs. D1	33.3	0	**−.47**	**.38**
PTHR10766	Membrane	5/7	P vs. D4	0	0	**−.62**	**.52**
PTHR13301	TF X-box	4/10	P vs. D4	0	25	−.29	.16
PTHR13832	Phosphorylation	6/24	P vs. D4	50	50	.21	.06
T_{max} *PHT acclimation models*							
PTHR43888	Chaperone DNAJ	4/5	P vs. D1	0	100	**−.68**	**.79**
PTHR19375	HSP 70	4/17	P vs. D1	0	100	**−.6**	**.49**
PTHR13902	Phosphorylation	2/6	P vs. D1	100	50	**.65**	**.55**
PTHR10666	Ubiquitination	3/15	P vs. D1	0	100	−.25	.06
PTHR10766	Membrane	3/7	P vs. D4	0	0	**−.53**	**.36**
PTHR12321	CpG binding	2/5	P vs. D4	100	100	.52	.31
PTHR11566	Membrane	2/6	P vs. D4	0	0	**−.49**	**.34**
PTHR11932	Ubiquitination	2/6	P vs. D4	100	50	.33	.11

Note: Models for individual genes used the $\log_2$ transformed fold change (FC) in expression as the response variable for two comparisons: day prior vs. day one (P vs. D1) and day prior vs. day four (P vs. D4). Footnote: The 'Sign/Anno' column shows the number of genes with significant relationships over the total number of annotated genes from that family that were tested. There were 922 protein family and model combinations tested of which 68 were significantly overrepresented for clinal relationships. Of the 68 significant protein families, 30 of particular interest are shown here (full results Table S6). The 'comp' column has the time point comparison being used for the response variable. The '% pos' is the percentage of significant genes with positive relationships, '% up' percentage of significant genes that were upregulated, 'semi-partial *r*' values are for the accompanying models on mean FC values across the protein family, and 'R^2' values are also for these mean FC models. Bold text indicates significant mean FC models.

protein families were overrepresented (Table S9). Notably these families included WRKY, Auxin and X-box transcriptions factors and also an Oxygenase superfamily, Thioredoxin and Glutathione *S*-transferases families that are related to the oxidative stress response (Table S9).

3.4 | Gene expression responses associated with PHT acclimation

All *Acacia* species showed rapid PHT acclimation, and all species maintained PHT thresholds well above the experimental heatwave temperature of 38°C (day four T_{crit} 53.1–59.0°C, and T_{max} 61.1–67.1°C, where the T_{crit} and T_{max} metrics represent the temperatures when damage to PSII begins and when irreversible damage occurs respectively). The average increases for both PHT metrics, between the day prior to day four was also high, with average T_{crit} acclimation at 12.1°C (range 7.7–19.1°C) and average T_{max} acclimation at 8.2°C (range = 4.4–12.1°C, Table S1). The average upregulation of HSPs had a significant negative relationship with T_{max} acclimation ($p = .02$, $R^2 = .36$, Figure 3d), which was similar to the negative relationship for the broader group of upregulated protein folding genes ($p = .03$, $R^2 = .33$, Figure 3d). Individual HSPs and protein folding genes were also less strongly upregulated in species that acclimated their T_{max} temperature to the greatest extent (Table 1, Tables S5 and S8). The small proportion of individual genes that had significant clinal relationships with PHT acclimation (range 5.66%–11.29% of genes significant, Figure S3) also included other overrepresented protein families linked to membrane structure and transporters that were generally being downregulated at day four and had negative relationships with PHT acclimation (see examples in Table 1 and Table S5).

4 | DISCUSSION

Relationships between source environment and the magnitude of *Acacia* transcriptomic stress responses could be signatures of local adaptation in the form of adaptive plasticity (Donelson et al., 2023). These clinal trends could help describe local adaptation and the strategies that help plant be successful in varied climates (Savolainen et al., 2013). We observed rapid responses in gene expression within hours of the heatwave treatment starting and these changes were still largely present at the end of the heatwave (Figure 1b and S1). Interestingly, most of the associations between changes in gene expression and PHT acclimation were for the responses seen between day four and the day prior (Table 1). This could be expected as these timepoints coincide with when PHT was measured. Conversely, the main associations between changes in expression and climate were more commonly linked to initial responses between day one and the day prior (Table 1). These results highlight the importance of timing for sample collection in gene expression studies, as changes in gene expression are highly plastic and can show different patterns across the stress exposure (Buchberger et al., 2019).

In the case of this study the response of each gene to the heatwave can be considered a trait and the response of some genes could have been correlated with each other or possibly with overall phylogenetic relatedness. Westoby et al. (2023) makes the argument that phylogenetic effects and local adaptation are complementary explanations and not mutually exclusive alternatives. For this study, genes that have a strong relationship with climate are not going to have this variation jointly explained by overall transcriptome dissimilarity due to the lack of collinearity between environment and transcriptome expression differentiation (Figure S1). For our questions it seems more relevant to evaluate what proportion of the total variance in gene expression responses can be explained by home range climate to assess the support for adaptive plasticity. Our experimental treatment only establishes the effect of the temperature treatment on gene expression while our comparative analyses is essentially an observational analysis that used a controlled environment to measure responses to a consistent temperature stimulus. The study also focuses on seed sourced from the wild to avoid adaptation to captive conditions or transgenerational epigenetic effects in the captive environment that could erode adaptive variation. This also means the seed maturation environment could also be influencing transcriptomic variation due to epigenetic effects. To accurately predict the adaptive capacity of species, variation in functional traits that is a result of selection and epigenetics should be considered together if they cannot be partitioned (Donelson et al., 2023).

Temperature stress can broadly be defined as biological responses to abnormal temperatures (Rosenfeld et al., 2022) and the transcriptome can capture part of the functional variation in these stress responses. One example of a consistent response to the heatwave treatment that showed clinal variation across species was the upregulation of HSPs (Figure 3b). This response is generally a reaction to the increasing frequency of misfolded proteins with higher temperatures (Wahid et al., 2007). Under normal conditions, HSPs will block HSP transcription factors. This suppression mechanism allows HSP transcription factors to be more active as misfolded proteins accumulate and engage HSPs resulting in the released transcription factors upregulating HSP expression (Zhang et al., 2022). In addition, the abundance of many HSP transcription factor transcripts also increased with the heatwave treatment (Figure 2d, Table S3a,b). The higher expression of HSP genes in species from climates with more variable daily temperatures is consistent with findings from *Eucalyptus grandis* using comparative proteomics (Maher et al., 2018). Finding a consistent trend across two taxonomic groups with both proteome and transcriptome data suggests variation in HSP upregulation could prove to be a powerful functional trait for assessing thermal tolerance adaptation in plants (Andrew, Gallagher, et al., 2022).

We also see some contrasting relationships between MAT at species source and number of days >14°C above MAT (Figure 3). The negative relationships between HSP upregulation and MAT are illustrative of weaker upregulation in tropical species from locations with high MAT and typically low temperature variability (Table 1, Table S5 and Figure 3, Figure S3). Seed source locations with high daily variability around the MAT are generally in the drier parts of central Australia (Figure 1a). Species from tropical regions with higher MAT and higher annual precipitation may also routinely rely on transpiration to keep leaf temperatures at levels suitable for maintaining photosynthesis and protein folding (Drake et al., 2018) and therefore also reduce the extent to which they invest resources in HSP production when temperatures increase. Additionally, tropical species adapted to high MAT are likely to have a higher optimal temperature for cellular processes (Huang et al., 2019), so the experimental change in temperature we implemented may not stimulate as large of a response in these species.

In addition to the EEA analyses, relationships between variation in transcriptomic stress responses and PHT acclimation could also help explain variation in thermal tolerance. High T_{max} acclimation could indicate that plants are acclimating well to the temperature stress and hence could have weaker responses for other indicators of stress. The weaker upregulation of HSP and chaperone genes in species with high T_{max} acclimation could mean these species also experienced less stress in the form of misfolded proteins. Alternatively, there may be a trade-off between the upregulation of these genes and T_{max} acclimation capacity. Gene expression responses associated with PHT acclimation also included other overrepresented protein families with relevant functions. Increases in temperature generally affect membrane permeability and fluidity (Zhang et al., 2022), therefore, gene families linked to cell membrane structure and function are expected to be associated with PHT acclimation. Negative relationships between the activity of membrane function genes and PHT acclimation (Table 1 and Table S6) suggest that species that had high PHT acclimation also more strongly downregulated these gene families, possibly to better maintain membrane fluidity and transportation rates. Additionally, several transcription factors, signalling, phosphorylation, and ubiquitination protein families had average changes in expression that were associated with PHT acclimation (Table S6). These families could provide insights into the mechanisms for regulating PHT acclimation that are currently not well explained (Mathur et al., 2014) or prove to be useful surrogate measures of PHT acclimation and overall response levels. Some examples of these groups are MYB transcription factors (PTHR31314) and Auxin response factor (PTHR31384) that are linked to abiotic stress responses (Ghanashyam & Jain, 2009; Liu et al., 2015).

4.1 | Interpretation of expression–environment associations

Genotype–environment association studies attempt to identify signatures of local adaptation through variation in population allele frequencies using markers such as genome-wide SNPs (Rellstab et al., 2015). We cannot directly compare our method to GEA studies of potentially adaptive alleles but we do find similar clinal trends in the potentially adaptive plasticity of gene expression responses (Price et al., 2022). For GEA analyses of SNPs even subtle trends in allele frequencies can prove biologically meaningful signatures of adaptation (Frichot et al., 2013) and this may also be the case for the subtle EEA results. The number of significant genes that were linked to relevant gene families for this comparative transcriptome analysis is relatively high and with significant overrepresentation (Table 1, Table S6). These results demonstrate the potential for detecting signatures of adaptive variation in the stress responses of non-model organisms with limited reference genome resources. Variation in transcriptomic responses could prove highly complementary to studies of genetic markers of adaptation that are more common (Ahrens et al., 2018). For example, we find that for all models of mean FC per protein family, 108 out of 154 (70.1%) of all significant relationships were plausibly linked to annotations relevant to stress responses (Table S6). In contrast, two studies of convergent evolution in plants found limited overlap of significant SNP markers, when a larger proportion of genes would be expected to be under selection across strong climate gradients (Steane et al., 2017; Yeaman et al., 2016). Studies of genome-wide SNP markers have several other technical differences due to other neutral influences on allele frequencies, that lead to genetic drift in allele frequencies that are used to assess both population structure and selection directly (Ahrens et al., 2018; de Villemereuil et al., 2014). However, the two different methods in combination could support each other's results and help remove the risk of false positives which is a constant challenge for these genomic methods. Our approach also differs from GEA in focusing on the relative changes or plasticity in expression rather than examining expression levels directly. The abundance of transcripts could vary between species for many reasons, but changes in expression (i.e. changes in the relative abundance of transcripts) in response to a stimuli could provide a consistent insight into physiological plasticity that is linked to species adaptive capacity (Bush et al., 2016). Looking at transcriptomic responses directly could also by-pass complex genetic architectures and epistatic effects by looking at the resulting expression patterns that may better match adaptation.

We explore how transcriptomic heat stress responses are associated with both the source climate of species and the PHT acclimation capacity of species. All four predictor variables were largely independent, though T_{max} acclimation and the number of days >14°C above MAT were significantly negatively related ($p = .01$, $R^2 = .40$). Therefore, we would expect the contrasting direction of relationships detected for these two variables (Figure 3b,d). We also identified complex interactions between how species acclimate PHT and other aspects of transcriptomic heat stress responses. For example, species from climates with more variable temperatures had high HSP upregulation but low T_{max} acclimation, possibly due to these species maintaining T_{max} nearer to maximum prior to the heatwave, hence having greater readiness for frequent heat stress (Andrew, Arnold, et al., 2022; Maher et al., 2018). Clear relationships between source climate and stress responses are not ubiquitous, with a previous review reporting that three out of eight studies that compared paired populations found greater expression responses in the thermally tolerant population compared to the thermally sensitive population (DeBiasse & Kelly, 2016). Our analyses of species from across a broad continuum of climates suggests these discrepancies in the differences between tolerant and sensitive populations could be partly explained by the climate variables used to define population sensitivity. Average climate metrics provide a good description of baseline environmental conditions but evolution is often driven by environmental extremes which shape demographic processes and impose selection pressure (Denny, 1993). The fact that we found contrasting relationships for our two environment metrics – average temperature and temperature variability (Figure 3a,b) – demonstrates the difficulties in identifying appropriate climate variables to consider when exploring clinal variation in gene expression responses and other functional traits.

For some protein families the direction of clinal trends and the average expression response to the heatwave treatment was highly consistent across genes from the same family (Table 1). The DEG analysis of all species also found that 49% of genes had a relatively consistent response to the first day of the heatwave treatment despite the potential for interspecies variation, this consistent response is also visualised in the PCA from Figure 1b. However, some genes did show highly varied responses to the heatwave treatment (Figure S3). These genes that have varied responses across species could also prove informative. Of the genes that had SD in FC values greater than 1 and a mix of upregulation and downregulation across species, in response to day 1 of the heatwave treatment, 37 protein families were overrepresented. Of these families those linked to oxidative stress, phosphorylation, and regulating gene expression stand out (Table S9). The accumulation of reactive oxygen species (ROS) is a universal symptom of stress and could also be a good indicator of temperature tolerance in plants. Interestingly, two protein families (PTHR47990 and PTHR24286) linked to the ROS stress response had negative relationships with PHT acclimation (Table S6) suggesting species with high acclimation had low ROS stress.

4.2 | Advancing expression–environment association methods

The transcriptomic responses of *Acacia* species have shown associations consistent with local adaptation theory, however, this variation has not yet been linked to fitness with experimental validation. To our knowledge, there are currently no similarly structured comparisons of genomic responses to heat stress across a large plant genera (Chaudhary et al., 2020). The analysis demonstrates new insight into how comparative transcriptomics can be used to describe clinal variation in stress responses (DeBiasse & Kelly, 2016). When trait plasticity is an active response to environment, molecular processes ultimately regulate the response. The abundance and activity of gene products can be regulated at several levels, including the epigenetic regulation of gene expression (Sonawane et al., 2017), mRNA regulation and disruption (Crisp et al., 2017; Wu et al., 2020), the regulation of mRNA translation to proteins, and the post-translation regulation of protein activity (Smythers & Hicks, 2021). Transcriptome sequencing provides a powerful tool for capturing detailed changes in mRNA expression that can, in part, explain changes in protein production which contribute to phenotypic plasticity (Todd et al., 2016). The power of comparative transcriptomics is that it is in the middle of many omics layers.

The explanatory power of the clinal trends in expression plasticity varied across Panther protein families and GO terms for our sample of *Acacia* species. The most relevant protein families with mean responses showing strong clinal trends had R^2 values that ranged between .27 and .79 (Table 1). For the more general trends of GO terms and all HSPs plotted in Figure 3 significant trends had R^2 values ranging between .33 and .47 (Table S7). For these broader trends we also report the slopes for the unscaled data that tell us more about the magnitude of the variation in expression plasticity. For example, HSP upregulation increased by 0.015 $\log_2$ FC units per day that temperatures are 14 over MAT per year. This relationship means for ever extra 10 days >14°C above MAT we would expect about an 11% stronger HSP response to heat. These clinal trends and general rules for defining the extent to which species should adapt to changing conditions, still need to be tested across more species and environments to further assess the explanatory power of these data prior to their potential use in decision making.

Our study is also unique in capturing responses in native species at multiple time points across a multiday heatwave, though sampling more time points and species will improve interpretation and statistical power (Table S1). For our study design we choose to maximise the number of included species to show clinal trends across species. Previously, most comparative transcriptomic studies on stress responses have focused on paired species comparisons (DeBiasse & Kelly, 2016). The extent to which we identify consistent changes in expression to the controlled heatwave and also clinal variation in these transcriptomic stress responses all supports the value of our study design and results. Combinations of functional traits and phenotypic plasticity are critical to how plants adapt to temperature extremes (Calvo et al., 2020; Des Marais et al., 2013). Standardised protocols for quantifying transcriptome responsiveness to stressors will need to be applied more broadly to grow our understanding of adaptive plasticity.

5 | CONCLUSIONS

Our set of diverse *Acacia* species responded dynamically and relatively consistently to our controlled heatwave based on transcriptome responses and PHT acclimation. For this study we mapped reads to the reference genome of *Acacia pycnantha* and were able to capture repeatable responses in a subset of 7274 genes that accounted for around 88% of all transcript sequences that successfully mapped to the reference genome. Taken together our results highlight how the sequence of genes and their response to stress can be highly conserved across species, making comparative studies across diverse taxa more tractable (Emms & Kelly, 2019). Clinal variation in responses could be seen within hours of starting the heatwave treatment. This rapid response demonstrates the importance of sensing changes in the environment for plants. The relationships between transcriptomic responses and PHT acclimation also suggests that the regulation of the two responses varies between species adapted to different biomes. Clinal variation in stress responses, found across species from strongly differentiated environments, could prove to be a powerful tool for deepening our understanding of local adaptation to temperature extremes. The results of this exploratory study suggest the possibility that climate change winners and losers (Hoffmann & Sgrò, 2011) could be identified by how well species match EEA that are documented for diverse plant species across climate gradients.

AUTHOR CONTRIBUTIONS

SCA, RVG and KM conceived the study. SCA, AKS and KM ran the experiment. PAA and VFB collected photosystem II heat tolerance data. SCA and CWC performed the lab work for RNA extraction and library preparation. TGBM and CJJ assisted with the incorporation of the *A. pycnantha* reference genome into the study. SCA generated the results with the assistance of all authors. All authors contributed to drafting the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests to disclose.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://doi.org/10.25919/mpk5-xr92.

DATA AVAILABILITY STATEMENT

As agreed with the funders of this research, raw sequence data are made publicly available on the CSIRO's Data Access Portal (part 1 = https://doi.org/10.25919/wa3k-0x90, Andrew & Mokany, 2023b and part 2 = https://doi.org/10.25919/ryte-pk64, Andrew & Mokany, 2023c). The RNA-seq library metadata, processed gene expression data, gene annotations, and additional data for analyses are also made available with all Rmarkdown scripts on CSIRO's Data Access Portal (https://doi.org/10.25919/mpk5-xr92, Andrew & Mokany, 2023a).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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