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# The roles of divergent and parallel molecular evolution contributing to thermal adaptive strategies in trees

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## Abstract

Local adaptation is a driver of biological diversity, and species may develop analogous (parallel evolution) or alternative (divergent evolution) solutions to similar ecological challenges. We expect these adaptive solutions would culminate in both phenotypic and genotypic signals. Using two *Eucalyptus* species (*Eucalyptus grandis* and *Eucalyptus tereticornis*) with overlapping distributions grown under contrasting 'local' temperature conditions to investigate the independent contribution of adaptation and plasticity at molecular, physiological and morphological levels. The link between gene expression and traits markedly differed between species. Divergent evolution was the dominant pattern driving adaptation (91% of all significant genes); but overlapping gene (homologous) responses were dependent on the determining factor (plastic, adaptive or genotype by environment interaction). Ninety-eight percent of the plastic homologs were similarly regulated, while 50% of the adaptive homologs and 100% of the interaction homologs were antagonistical. Parallel evolution for the adaptive effect in homologous genes was greater than expected but not in favour of divergent evolution. Heat shock proteins for *E. grandis* were almost entirely driven by adaptation, and plasticity in *E. tereticornis*. These results suggest divergent molecular evolutionary solutions dominated the adaptive mechanisms among species, even in similar ecological circumstances. Suggesting that tree species with overlapping distributions are unlikely to equally persist in the future.

KEYWORDS

eucalyptus, local adaptation, plasticity, reciprocal transplant, RNA-seq, transcriptomics

## 1 | INTRODUCTION

Temperature is known to be extremely important in plant adaptations to their local environment (Moles et al., 2014), as it strongly affects photosynthesis and leaf characteristics (Ahrens, Byrne, et al., 2019; Aspinwall et al., 2017, 2019), while significantly predicting the distribution of heritable traits (Ahrens et al., 2020). Plants adapt to different temperature conditions through the selection of beneficial mutations. Mutations can have various effects on phenotype and most molecular studies focus on mutations within coding regions that

result in changes to the amino acid sequence (Booker et al., 2017). While changes in coding regions do change the adaptive potential of a population or species, it has become increasingly clear that mutations in *cis*- and *trans*-regulatory regions are consistently important for evolutionary change in many types of organisms (Ahrens et al., 2021; Todesco et al., 2022) and can directly influence gene expression (Albert & Kruglyak, 2015). Changes in gene expression can result in plastic (depending on environment) or genotypic (depending on genotype) responses. Indeed, the molecular mechanisms determining the ability to respond to temperature variation is an important facet of evolutionary biology where the biological process of local adaptation can happen either in parallel or divergently across species and populations (Stern, 2013).

Local adaptation occurs when a population shows increased fitness in its local environment compared to nonlocal environments (Kawecki & Ebert, 2004). In plants, reciprocal transplant studies have been used to identify populations that are locally adapted by measuring physiological differences across contrasting environments (Ahrens, Mazanec, et al., 2019; Sork et al., 1993). Our ability to measure local adaptation has improved substantially with the proliferation of next-generation sequencing techniques (Sork, 2017), and by using transcriptomics, we can now answer fundamental ecological questions about wild populations by quantifying and comparing gene expression patterns (Alvarez et al., 2015). As such, the coupling of reciprocal transplant and genomic studies can now elucidate the role of single or multiple genes underpinning adaptive divergence, increasing our understanding of local adaptation. Yet, the use of reciprocal transplant experiments to understand differential gene expression for non-model plant systems is somewhat rare (Alvarez et al., 2015; Boshier et al., 2015; Voelckel et al., 2017), and unknown in long-lived tree species. However, some studies have used transcriptomics or RNA sequencing (RNA-seq) to understand how trees respond to stressful environments (Cokus et al., 2015; Mead et al., 2019; Spokevicius et al., 2017). In particular, RNA-seq has been used to elucidate how different *Eucalyptus* species (Myrtaceae) respond to drought stress and revealed that transcript expression significantly differs between species with overlapping gene ontology (GO) patterns (Spokevicius et al., 2017). Indeed, it is common for studies to identify local adaptation at the population level within species, but are these patterns of local adaptation repeatable? Transcriptomics data can be used to uncover similar or different mechanisms of adaptation between species.

If a common gene is found to be used in local adaptation patterns among two sister species, they can have either evolved divergently or in parallel. Divergent evolution, in a gene sense, occurs when an ancestral gene (homolog) evolves different solutions to the same environmental challenge (Schluter, 2001). In an RNA-seq framework, this could look like the same gene expressing in different directions (upregulated for one species and downregulated for another). Alternatively, molecular parallel evolution occurs when an ancestral gene, from two distinct species, evolves in the same way when subjected to the same conditions and is reproducible (Arendt & Reznick, 2008; MacLean & Bell, 2003). These adaptive responses can be applied to changing signals of gene expression as well and are apparent in studies exploring differential gene expression patterns where plants are grown under different climate conditions (Gould et al., 2018). Perhaps the best evidence of parallel evolution has been established in controlled environments on single-celled organisms such as yeast and bacteria (Butlin et al., 2014) and has also been identified in two species of *Drosophila* from two environmentally different populations, resulting from spatially varying selection (Zhao et al., 2015). One comparative study showed parallel evolution among social insects (Hymenoptera) and found that while different genes may change among species there is substantial overlap among pathways and biological functions (Berens et al., 2015). More comparative studies are required to determine if similar patterns can be found across kingdoms, including trees with long, overlapping generation times, long-distance gene flow, and high recombination rates. Do trees have similar evolutionary patterns evident among congeneric tree species exposed to shared selection pressures?

Our ability to measure local adaptation has improved substantially with the proliferation of next-generation sequencing techniques (Sork, 2017), and by using transcriptomics, we can now answer fundamental ecological questions about wild populations by quantifying and comparing gene expression patterns (Alvarez et al., 2015). As such, the coupling of reciprocal transplant and genomic studies can now elucidate the role of single or multiple genes underpinning adaptive divergence, increasing our understanding of local adaptation. Yet, the use of reciprocal transplant experiments to understand differential gene expression for non-model plant systems is somewhat rare (Alvarez et al., 2015; Boshier et al., 2015; Voelckel et al., 2017), and unknown in long-lived tree species. However, some studies have used transcriptomics or RNA-seq to understand how trees respond to stressful environments (Cokus et al., 2015; Mead et al., 2019; Spokevicius et al., 2017). In particular, RNA-seq has been used to elucidate how different *Eucalyptus* species (Myrtaceae) respond to drought stress and revealed that transcript expression significantly differs between species with overlapping GO patterns (Spokevicius et al., 2017).

In similar ways, gene expression patterns can reveal the underlying genomic differences between species and populations in reciprocal transplant studies revealing patterns of local adaptation, even when measured differences between traits are small or nonexistent. Indeed, these data can be used in conjunction with phenotypic traits (e.g., photosynthetic capacity and growth) to identify the underlying genetic pathways in which selective pressures are acting upon that control those traits. Therefore, by using multidisciplinary approaches to understand the connection between phenotypic, genetic and environmental changes, we can better understand the strength and prevalence of local adaptation, within and among species.

*Eucalyptus* species are dominant in eastern Australian ecosystems and many of them occupy large climate gradients. The overlapping distributions of some *Eucalyptus* species elicit questions about the evolutionary development of their adaptive mechanisms; for example, have they developed similar mechanisms to deal with

similar temperature challenges? Here, we study the differential trait and transcriptome responses within two *Eucalyptus* species (*Eucalyptus grandis* W. Hill and *Eucalyptus tereticornis* Sm.) that occupy similar geographic and climate space to address three hypotheses: (1) species' differential responses to temperature are driven by genotypic, plastic and local adaptation processes, (2) species with common ancestry and overlapping distributions have developed similar gene and trait expression patterns (parallel evolution) of thermal adaptation and (3) associations between gene expression and important functional traits is similar across species. To address these hypotheses, we grew distinct *E. grandis* and *E. tereticornis* populations in glasshouse conditions under two contrasting growing temperatures in a reciprocal transplant design. We found that adaptation was driven mostly by divergent evolution but coevolving genes among species had a 50% chance of evolving in parallel.

## 2 | METHODS AND MATERIALS

### 2.1 | Species and collections

Two widespread *Eucalyptus* species (*E. grandis* and *E. tereticornis*) were selected for this study because they both occur within tropical and temperate regions along a shared temperature gradient in eastern Australia (Figure 1a). The species are in the subgenus Symphyomyrtus (which includes about 470 species); however, they are in different sections within the subgenus (*E. grandis* is in section Transversaria and *E. tereticornis* is in section Exsertaria; Steane et al., 2011).

To explore the adaptation to temperature within temperate and tropical regions, two replicate natural populations were selected from each region for both species (eight populations total; Table 1). All populations had similar coastal (<100 km from the Pacific Ocean) and elevation (<500 m altitude) environments to minimize the effect of climatic factors other than temperature. Seeds of the selected populations were obtained from the Australian Tree Seed Centre (CSIRO).

### 2.2 | Growing conditions

The seeds were germinated in a nursery at Western Sydney University (Richmond, NSW, Australia), and grown as tube stock for 2 months in HIKO V-93 seedling trays before they were established into the experimental growth conditions. Seedlings were transplanted in late spring into polyvinyl chloride (PVC) pots (15 cm diameter × 40 cm height) containing 10 kg of dry loamy-sand soil (containing

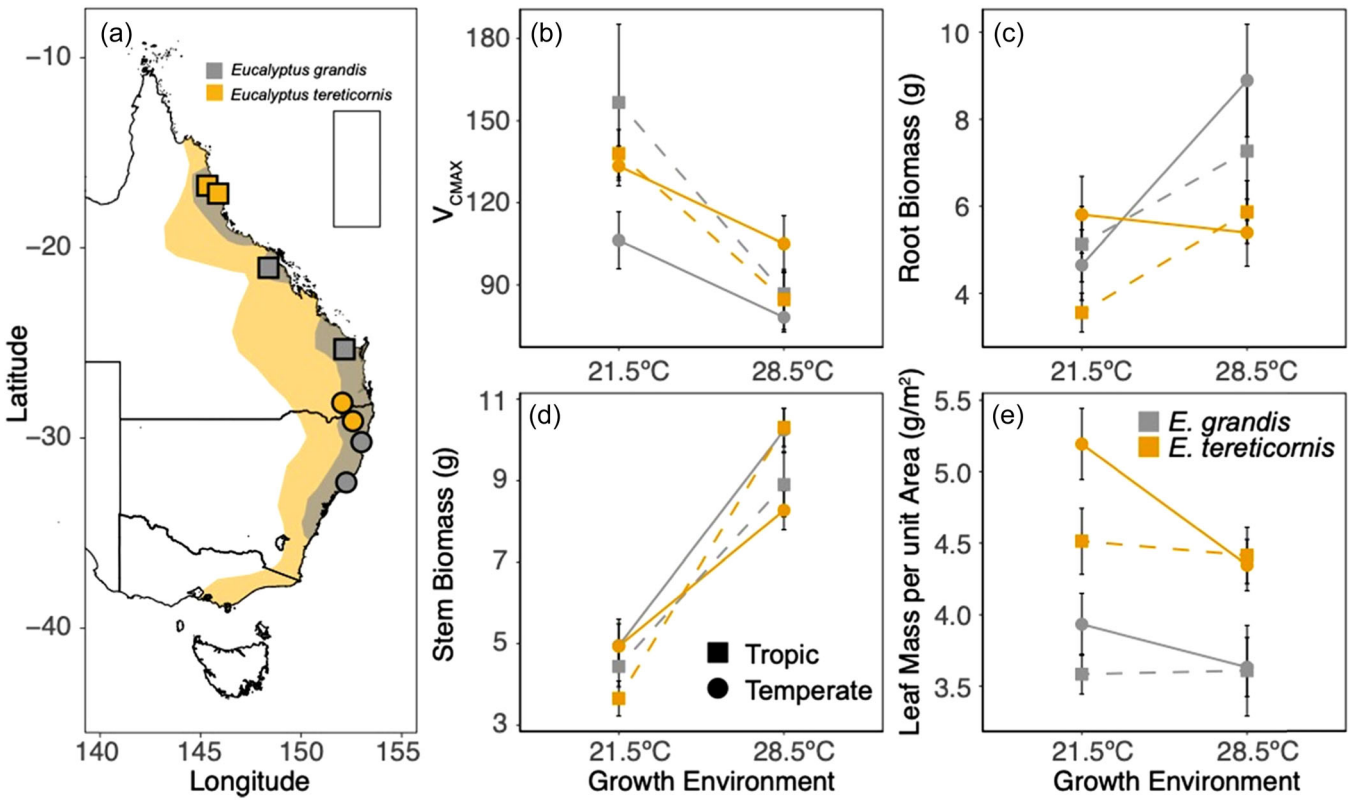


**FIGURE 1** Map of the study species distribution (a) and the populations used within the study. Differences in four trait-means among two *Eucalyptus* species and two growing conditions. Significant differences are provided in Table 2.

**TABLE 1** Population location and climatic details for each of the eight populations

| Seedlot | Sp. | Lat | Long | Location | State | Region | BIO1 | BIO10 |
|---|---|---|---|---|---|---|---|---|
| 19313 | *Eucalyptus grandis* | −32.333 | 152.25 | Bulahdelah SF | NSW | Temp | 17.3 | 21.8 |
| 20678 | *E. grandis* | −30.203 | 153.003 | Orara West SF | NSW | Temp | 18.4 | 22.8 |
| 16893 | *E. grandis* | −25.330 | 152.160 | Brooweena SF | QLD | Trop | 20.8 | 25.5 |
| 19307 | *E. grandis* | −21.043 | 148.376 | Finch Hatton Gorge | QLD | Trop | 20.2 | 24.3 |
| 18732 | *Eucalyptus tereticornis* | −29.100 | 152.580 | Selection Flat | NSW | Temp | 18.9 | 23.9 |
| 17762 | *E. tereticornis* | −28.150 | 152.050 | Warwick | QLD | Temp | 16.8 | 22.3 |
| 20469 | *E. tereticornis* | −16.733 | 145.333 | Copperlode | QLD | Trop | 23.2 | 25.8 |
| 20355 | *E. tereticornis* | −17.150 | 145.867 | Little Mulgrave Deeral | QLD | Trop | 24.1 | 26.8 |

*Note*: BIO1, mean annual temperature (°C); BIO10, mean temperature of the warmest quarter (°C); Lat, latitude; Long, longitude; Temp, temperature/southern; Trop, tropic/northern.

86.5% sand and 9.5% clay with moderate fertility; Menangle sandy loam; Drake et al., 2015). For the duration of the experiment, all seedlings were irrigated to field capacity daily and fertilized (500 ml Aquasol at 1.6 g $l^{-1}$; Yates Australia) every 3 weeks to minimize resource limitation.

We employed a fully factorial experimental design where temperate and tropic-origin populations of both Eucalyptus species (eight populations) were grown under two different temperature regimes, simulating temperate and tropic conditions. Seedlings were paired based on stem length and basal diameter, to minimize initial seedling size differences, and then randomly assigned into one of two temperature conditions. Glasshouse bays were used to simulate the temperature conditions in temperate and tropical (mean diel temperatures of 21.5°C and 28.5°C, respectively) regions (Drake et al., 2015). Two replicate bays were established with five plants of each population to avoid artefacts and minimize confounding effects from individual bays. In each glasshouse bay, the air temperature was controlled at four set-points over the 24-h period to simulate a natural diel temperature cycle. The average temperature range for the diel temperature cycle was 9°C, with a mid-day maximum temperature (between 10:00 and 16:00 h; 21.5°C and 28.5°C), a night-time minimum temperature (between 20:00 and 06:00 h; 12.5°C and 19.5°C), and a moderate morning and evening temperature (between 06:00–10:00 and 16:00–20:00 h; 17°C and 24°C).

## 2.3 | Physiological traits

Plant responses were estimated across a range of scales and processes from leaf to root. Four physiological traits were measured within a week of RNA sampling: maximum photosynthetic carboxylation rate ($V_{cmax}$), leaf mass per unit area (LMA), stem biomass (SBM) and root biomass (RBM). All plants were sampled after growing in their temperature treatments for 140 days following establishment.

The $V_{cmax}$ measurement represents the maximum rate parameter of enzyme kinetic processes driving photosynthesis (Farquhar et al., 1980). In brief, $V_{cmax}$ was estimated from A/Ci curves taken using a LICOR LI-6400XT gas analyzer, following the methods in Aspinwall et al. (2017). The LICOR conditions were maintained with constant conditions for leaf temperature 30°C, PAR light 1800 µmol $m^{-2}$ $s^{-1}$, flow 500 mol $s^{-1}$ and relative humidity 40%–60% during the $CO_2$ response curves ($CO_2$ reference set points 50, 100, 150, 230, 330, 400, 650, 1200, 1500, 1800 µmol $CO_2$ $mol^{-1}$). All curves were examined throughout the measurement period and repeat measures taken to ensure adequate replication ($n = 7–9$ plants). Each A/Ci curve was parameterized using the Farquhar model of C3 photosynthesis (Farquhar et al., 1980). The model estimates $V_{cmax}$ as the maximum rate of Rubisco carboxylation (µmol $m^{-2}$ $s^{-1}$). The model was fitted using nonlinear regression in R using the function fitaci in the R Package plantecophys (Duursma, 2015).

LMA is an important trait in the leaf economic spectrum (Osnas et al., 2013), providing information on the trade-off between carbon allocation to leaf structural integrity against leaf light capture. Five fully expanded leaves were collected from the upper canopy of five replicate plants for each population and growth temperature treatment combination. Leaves were scanned on an A4 flatbed scanner at 150 dpi resolution, and images were analyzed using WinFolia software (https://www.plant-image-analysis.org/software/winfolia) to estimate the total leaf area. The fresh leaf material was immediately dried at 70°C for 48 h before being weighed on a 4-decimal place analytical balance. The leaf dry mass was divided by the total leaf area to obtain the LMA (g $cm^{-1}$).

A destructive harvest was conducted to estimate the biomass components of plant growth as they can be related to a plant's ecological niche (Graham et al., 2000). Above-ground growth (SBM) and below-ground growth (RBM) were measured as adaptive traits to their local temperature conditions. All leaf material was removed from the plant, the main stem was then cut at soil level, and root material was washed of soil debris before all organs were dried in an oven at 70°C for 48 h and weighed with an analytical balance to obtain SBM and RBM.

Differences in physiological traits were estimated using a linear model approach with the `lm` function in R. All models were setup as:

trait.value ~ genotype*environment for each species separately. The results were plotted using the ggplot2 package in R.

## 2.4 | RNA-seq

Sampling of leaf material forRNA-seq was linked to the gas exchange measurements via sampling paired leaves at the same developmental stage and canopy position (selecting the third and fourth fully expanded leaves from the growth tip in the upper canopy fully exposed to light) sampled at the same time of day (1000–1200). Leaf discs (three 6.3 mm diameter Sigma-Aldrich cork-borer) were sampled from an intact leaf, and immediately immersed into liquid nitrogen before storage at −80°C. Total RNA was extracted using a modified cetyltrimethylammonium bromide method specified for plant species (White et al., 2008).

Library preparation and sequencing were performed by the Next-generation sequencing facility at Western Sydney University. In brief, total RNA samples were first treated with DNase I following the manufacturer's protocol (Qiagen), before poly(A) mRNA selection library preparation was carried out using oligo d(T) magnetic beads (Dynabead mRNA purification kit; Invitrogen). The mRNA was then fragmented at 94°C using divalent cations, and each fragment of mRNA was synthesized to cDNA. Sequencing adapters were then ligated to each fragment, amplified by PCR, and purification of amplified cDNA molecules. Sequencing took place on an Illumina HiSeq. 2000 (Illumina Inc.), producing 100-bp paired-end reads.

## 2.5 | Filtering and abundance estimation

Adapters and low-quality reads (below a threshold quality of three) were removed from the raw sequences using Trimmomatic V 0.38 (Bolger et al., 2014). A sliding window of four bases was then used to remove any segments where the average quality per base drops below 15, and all sequences under 40 base pairs were removed using Trimmomatic V 0.38. Next, the quasi-align function within Salmon v 0.7.0 (Patro et al., 2017) was used to estimate transcript abundance. Before alignment, an index was created using the *E. grandis* v 2.0 transcriptome downloaded from the EUCANEXT database (Nascimento et al., 2017) using the index function. The quasi-align function was then used to align the raw reads of each sample to the generated index to give a normalized estimate of transcript abundance (transcripts per million [TPM]) for each sample.

## 2.6 | Gene expression

To investigate general patterns within species, we ran multi-dimensional scaling (MDS) analysis using the plotMDS function in R to explore how each sample compared to one another and to investigate which treatment, if any, provided an overarching explanation for the patterns observed. To investigate general patterns between species, we created coexpression networks for each species in the WGCNA (Langfelder & Horvath, 2008) and igraph libraries (Csardi & Nepusz, 2006), and then performed a conditional uniform graph test (cugtest function in the SNA library) between the two networks. We used the gcor function to estimate the correlation of the two networks (cmode = 'order'; indicating draws are uniform over both networks) using Monte Carlo simulations to estimate the null model and providing pseudo *p* values to test if the two networks are more different than expected.

## 2.7 | Gene–trait association

To reveal the relationship between gene expression and physiological traits, we used the R package mixOmics to explore associations to understand how plant traits may be coordinated with gene expression and also qualitatively assess parallel evolution between species. We focused on the same four traits (LMA, SBM, RBM and $V_{cmax}$) discussed above. We first ran sparse partial least squares (spls command) analysis to reduce the dimensionality and explain the relationship between the two datasets (gene expression and trait). We then created networks between physiological traits and gene expression patterns using the network command with a correlation cutoff of 0.85 to identify highly correlated patterns among traits and species. Lower cutoff rates were explored (e.g., 0.75) revealing thousands of associations. The network was saved as a graphml file and imported into cytoscape v 3.7.1 for visualisation (Shannon et al. 2003). Similar patterns of pleiotropy between traits (both the number and type of genes) would support our expectation of parallelism as the dominant mode of evolution to thermal pressures.

## 2.8 | Differential expression analyses

To assess transcripts which might be significantly differentially expressed (DE) between treatments and species, comparisons were performed using the package edgeR v3.26 (Robinson et al., 2010) in R (R core development team 2018). As the design of the experiment was a fully factorial design where two populations were grown in two separate growing climates, we used the makeContrasts argument to obtain significant differences between treatments, as described in the edgeR manual. We made within-species contrasts: (1) between temperature treatments (environmental contrasts [ENV]), (2) between populations (genotype contrasts [GEN]) and (3) among temperature treatments and populations (genotype × environment interaction contrasts [GEI]). Importantly, this GEI contrast represents an antagonistic pleiotropy version of local adaptation where all samples are combined. Our data cannot support multiple contrasts to explore other versions of local adaptation. Contrasts were run with the glmLRT function and *p* values were adjusted by applying a Benjamini–Hochberg false discovery rate (FDR). Significance patterns of differential expression were identified with an adjusted *p* value (FDR value) of 0.05.

The transcripts for both species were mapped on *E. grandis* during the alignment phase because of the shared ancestry between the species. Therefore, we refer to shared genes as homologs, and genes can be identified as parallel or divergent evolution for all three contrasts depending on their expression profiles. But first, we calculated the expected number of overlapping genes to evolve in parallel or divergently for each contrast (ENV, GEN and GEI) by using a hypergeometric distribution ($C_{hyper}$) technique described in Yeaman et al. (2018). This analysis provided the proportion of genes expected to be overlapped, the standard deviation and the metric $C_{hyper}$ provides a quantitative representation of how much more overlap is observed compared to expected. This was performed twice, genes with similar expression profiles (parallel) and different expression profiles (divergent) for each contrast and a *p* value was estimated using the approach in the R library dgconstraint to ask if $C_{hyper}$ is greater than zero.

We compared the direction of differential expression between homologs in all three contrasts to identify consistent (up-up or down-down; parallel) or contrasting (up-down and down-up; divergent) regulation patterns. We tested if the gene profile groups (parallel and divergent) were significantly different than 0.5 for each contrast, such that both profiles had an equal chance of occurring, for example, is parallel evolution more likely to occur in the ENV contrast compared to divergent evolution? To do this, we used a binomial test in R (bionom.test) to test if the observed proportions are significantly different than random change (~0.5 proportion).

We also ran coexpression network analysis on the significant genes for each combination of contrast and species. Using the WGCNA library we developed the networks and plotted them using *igraph*. We used the R libraries *statnet* and *SNA* to calculate gene expression network topographical metrics. These included degree (how many ties do the nodes in these networks have on average), transitivity (the degree to which these networks can break down into subgroups), centralization (is the network dominated by one or a few nodes?) and betweenness (the number of times a node lies on the shortest path between other nodes). While these gene expression topology networks and metrics can be used to inform on the similarity of adaptive variation between contrasts and species, genes with greater betweenness are known to be more strongly correlated with the evolutionary rate (Hahn & Kern, 2005) and these nodes are known to bridge gaps between clusters (Ravasz et al., 2002).

To compare species in a known gene function important for temperature response, we isolated heat shock protein (HSP) and terpene synthase protein (TSP) expression profiles by identifying the *E. grandis* homologs of previously characterized genes.

## 2.9 | Gene ontology

While different profiles of significantly DE genes may be present, genes may still contribute to the same pathway, signifying a degree of parallel evolution. Therefore, to detect similarities between pathways, GO enrichment analysis was performed on all groups of genes identified as significantly DE for all three contrasts (i.e., ENV, GEN and GEI). To explicitly test differences between species, we used a $\chi^2$ test to see if species' GO pathways were significantly different from one another for the two main effects and interaction effect by using the chisq. test function in R. Likewise, GO networks were explored among the group of genes that were found to be associated with each of the four traits. For GO enrichment analyses, we imported the *E. grandis* gene names into the Plant Transcriptional Regulatory Map (Tian et al., 2020). The outputs were recorded with GO IDs, GO terms and significance value.

**TABLE 2** Significant differences between traits using generalized linear models

| Species | Trait | Genotype | | Environment | | GEI | |
|---|---|---|---|---|---|---|---|
| | | *T* value | *p* | *T* value | *p* | *T* value | *p* |
| *Eucalyptus grandis* | $V_{cmax}$ | **2.78** | **0.01** | −1.88 | 0.07* | −1.80 | 0.09* |
| | RBM | 0.28 | 0.78 | **2.46** | **0.02** | −0.87 | 0.39 |
| | SBM | −0.57 | 0.57 | **5.88** | **<0.001** | −0.66 | 0.51 |
| | LMA | −1.06 | 0.30 | −0.91 | 0.37 | 0.71 | 0.49 |
| *Eucalyptus tereticornis* | $V_{cmax}$ | 0.33 | 0.74 | **−2.08** | **0.05** | −1.31 | 0.20 |
| | RBM | **−2.21** | **0.03** | −0.41 | 0.69 | 1.89 | 0.07* |
| | SBM | −1.25 | 0.22 | **5.22** | **<0.001** | **2.89** | **0.007** |
| | LMA | **−2.24** | **0.03** | **−2.78** | **0.01** | 1.74 | 0.09* |

*Note*: *p* values < 0.05 are given in bold.

Abbreviations: GEI, Genotype × environment interaction; LMA, leaf mass per unit area; RBM, root biomass; SBM, stem biomass; $V_{cmax}$, a maximum rate parameter of enzyme kinetic processes driving photosynthesis.

*Marginally significant ($p < 0.1$).

## 3 | RESULTS

### 3.1 | Physiological traits

Physiological traits showed significant variability within species among regions and temperatures (Table 2; Figure 2); however, this variability was not consistent among species. For instance, GEN effects were identified for $V_{cmax}$ in *E. grandis* and RBM and LMA for *E. tereticornis*. ENV signatures were identified RBM and SBM for *E. grandis* and for all traits but RBM for *E. tereticornis* (Table 2). The only GEI and pattern of local adaptation among species was identified for *E. tereticornis* for SBM where the tropical population responded with decrease SBM to lower temperature and the temperate population responded with an increase in SBM to higher temperature. However, $V_{cmax}$ was marginally significant in *E. grandis*, and RBM and LMA were marginally significant in *E. tereticornis*. The marginally significant GEI pattern for $V_{cmax}$ in *E. grandis* shows that the temperate populations decrease with increasing temperature and the tropical populations increase with decreasing temperature. The marginally significant GEI pattern for RBM in *E. tereticornis* suggests that tropical populations respond with a decrease in RBM to lower temperature compared to the temperate populations which do not change with an increased temperature. The marginally significant GEI pattern for LMA in *E. tereticornis* suggests that the temperature populations decrease with an increase in temperature compared to the tropical populations that do not change with a lower temperature. The only trait that showed consistent effects among species was SBM, which showed an ENV effect for both species.

### 3.2 | Gene expression

Mapping of the RNA-seq output to the *E. grandis* transcriptome yielded expression data for 16 576 genes, with a 70%–80% mapping rate. The MDS of the transcript expression profiles showed strong environmental signals, driven by growth temperatures (Figure 2). Species exhibited different genome-wide patterns; for example, *E. grandis* growing in 21.5°C clustered closer together while the opposite occurred for *E. tereticornis*. However, in both cases, there was no obvious genotypic (GEN; grouping of colours) or interaction (GEI; grouping of one colour is more distant than the other) effects evident. We found some similarities between species using the correlation analysis among coexpression networks ($r^2 = 0.12$, $p < 0.05$), which indicates that the topologies have some overlapping patterns but a large proportion of the variation is unexplained (88%) in the correlation analysis.

### 3.3 | Gene–trait associations

An array of associations was identified between gene expression and the trait measurements, which highlight differences among species. In *E. grandis*, nine genes were found to be significantly associated with SBM, RBM and $V_{cmax}$ (Figure 3a). GO analysis of these nine genes were found to be within the cellular component pathway (Supporting Information: Table S1), and the putative gene functions included oxidoreductase, glycoprotein and abscisic acid occurring across six chromosomes (Supporting Information: Table S1). Furthermore, 16 genes are associated with both SBM and RBM, 13 genes link SBM and $V_{cmax}$, and 20 genes link RBM and $V_{cmax}$. SBM, RBM and $V_{cmax}$ also showed the most associations in *E. grandis* had more similar number of genes per individual trait compared to *E. tereticornis*. However, LMA had fewer associations with gene expression and none of these were linked to other traits. *E. tereticornis* showed no overlapping expression patterns between any of the four trait measurements. There were many more associations with RBM compared to the other three traits (Figure 3b).

### 3.4 | Differential gene expression

Generalized linear models identified significant DE (FDR≤ 0.05) genes for the three contrasts within each species (Figure 4). The contrasts between growing conditions (ENV) identified the highest number of DE genes with 283 and 203 genes for *E. grandis* and *E. tereticornis*, respectively (Figure 4a). The contrasts between temperate and tropic regions revealed numerous DE genes (Figure 4b), where 226 and 197 genes were found to be significant for *E. grandis* and *E. tereticornis*

**TABLE 3** Topology measures for the gene coexpression network analysis in Figure 5

| | | Degree (SD) | Betweenness (SD) | Transitivity | Centralization |
|---|---|---|---|---|---|
| ENV | *Eucalyptus grandis* | 315.4 (94.1) | 66.4 (71.8) | 0.8384788 | 0.2348676 |
| | *Eucalyptus tereticornis* | 169.7 (67.8) | 65.2 (101.8) | 0.7939989 | 0.3290446 |
| GEN | *E. grandis* | 64.2 (22.8) | 135.2 (309.0) | 0.7844478 | 0.6368213 |
| | *E. tereticornis* | 77.9 (33.8) | 93.3 (175.9) | 0.6965927 | 0.5127201 |
| GEI | *E. grandis* | 102.6 (44.9) | 78.1 (154.3) | 0.8826315 | 0.4226348 |
| | *E. tereticornis* | 17.2 (10.1) | 29.8 (69.6) | 0.8867292 | 0.5958736 |

*Note*: SDs are given in parentheses for the metrics that were measured per node.

Abbreviations: ENV, environmental contrasts; GEI, genotype × environment interaction contrasts; GEN, genotype contrasts; SD, standard deviation.

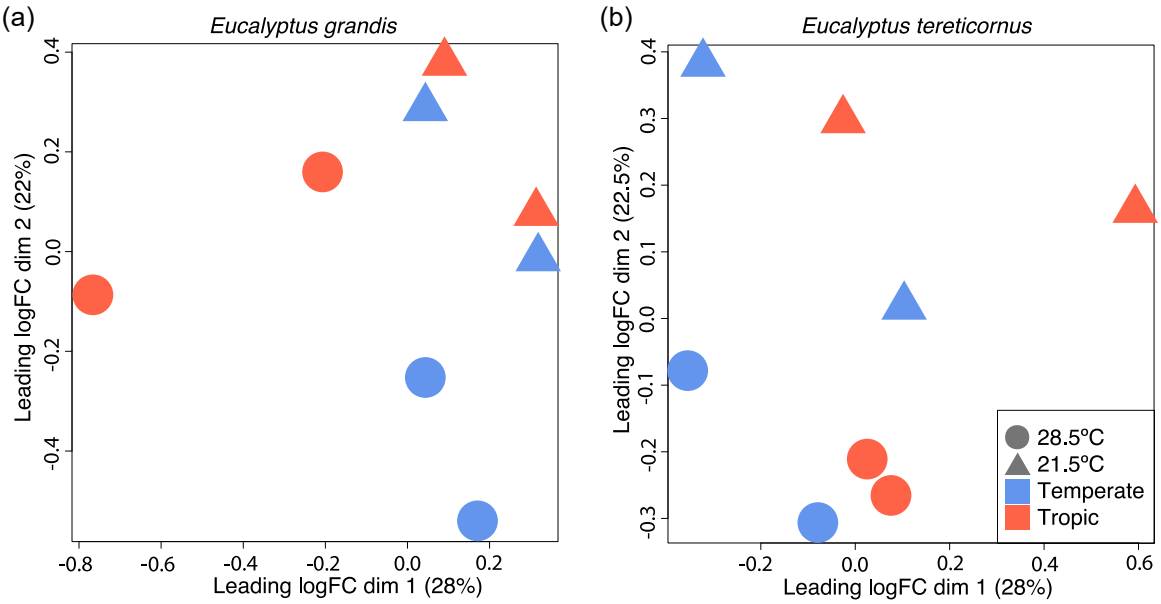


**FIGURE 2** Multidimensional scaling for all 16 576 genes based on the log(fold change) of the transcripts per million for each gene. Species are *Eucalyptus grandis* (a) and *Eucalyptus tereticornis* (b). Colour of the points represents genotype and shape indicates growth temperature. [Color figure can be viewed at wileyonlinelibrary.com]

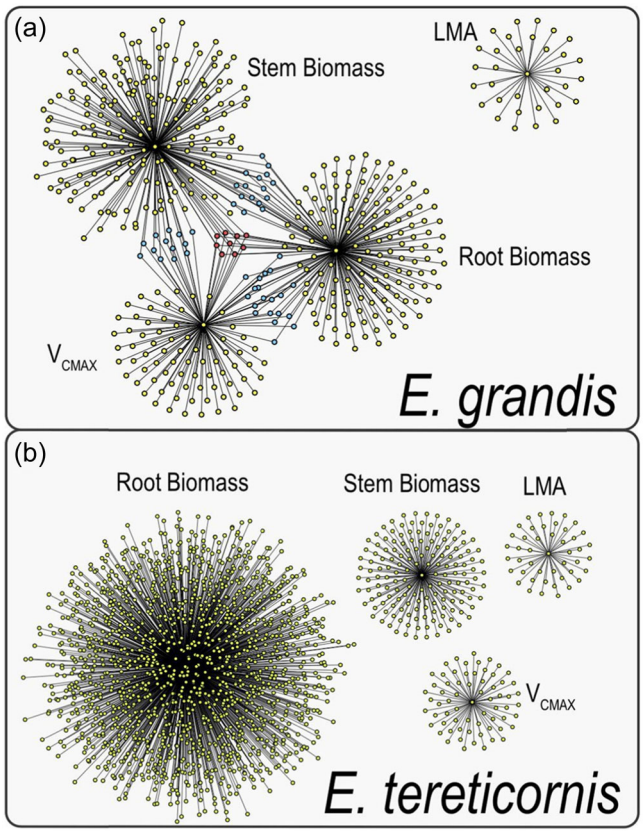


**FIGURE 3** Significant gene expression correlations with physiological traits for (a) *Eucalyptus grandis* and (b) *Eucalyptus tereticornis*. Each point represents one gene and lines indicate correlation between gene expression and trait measurements. Some genes are associated with multiple traits, signifying pleiotropy. [Color figure can be viewed at wileyonlinelibrary.com]

respectively. Finally, the contrasts between reciprocal and home environments (GEI) revealed the fewest DE genes within each species (Figure 4c), particularly for *E. tereticornis* (74 genes), which had less than half found in *E. grandis*. The number of common DE genes found in both species varied markedly within each contrast (Figure 4); 61 genes were found to be common for environmental effects, 20 for genotypic effects and 5 for GEI effects.

Genes that are both DE and show gene-trait associations provide further evidence for important functional relationships. In our analyses, we found genes of interest in *E. grandis* (48 genes) and *E. tereticornis* (79 genes) (Supporting Information: Table S2). For *E. grandis*, the only genes that were in common between trait association and DE were plastic (Supporting Information: Table S2). These GO functions were within photosynthetic processes and were associated with SBM and $V_{cmax}$. Whereas genes exhibiting both DE and gene-trait associations for *E. tereticornis* were found for all three effects (ENV, GEN and GEI) in RBM (Supporting Information: Table S2), but SBM was only found significant for the ENV effect.

We also conducted the gene-trait analysis on the gene groups that were significant for each combination of contrast and species (Figure S1). Pleiotropy was evident for both species in the ENV contrast among different traits with *E. grandis* having correlations with RBM and SBM, and *E. tereticornis* also including $V_{cmax}$, but there were more genes correlated with SBM in *E. grandis* and more genes correlated with RBM in *E. tereticornis*. There were large differences between species for the GEN contrast, where *E. grandis* had 21 genes correlated with $V_{cmax}$ while *E. tereticornis* only had one gene correlated with LMA. There were no genes correlated with the GEI contrast in *E. grandis* and two genes, one each correlated with SBM and LMA in *E. tereticornis*. Similarly, both genome-wide and

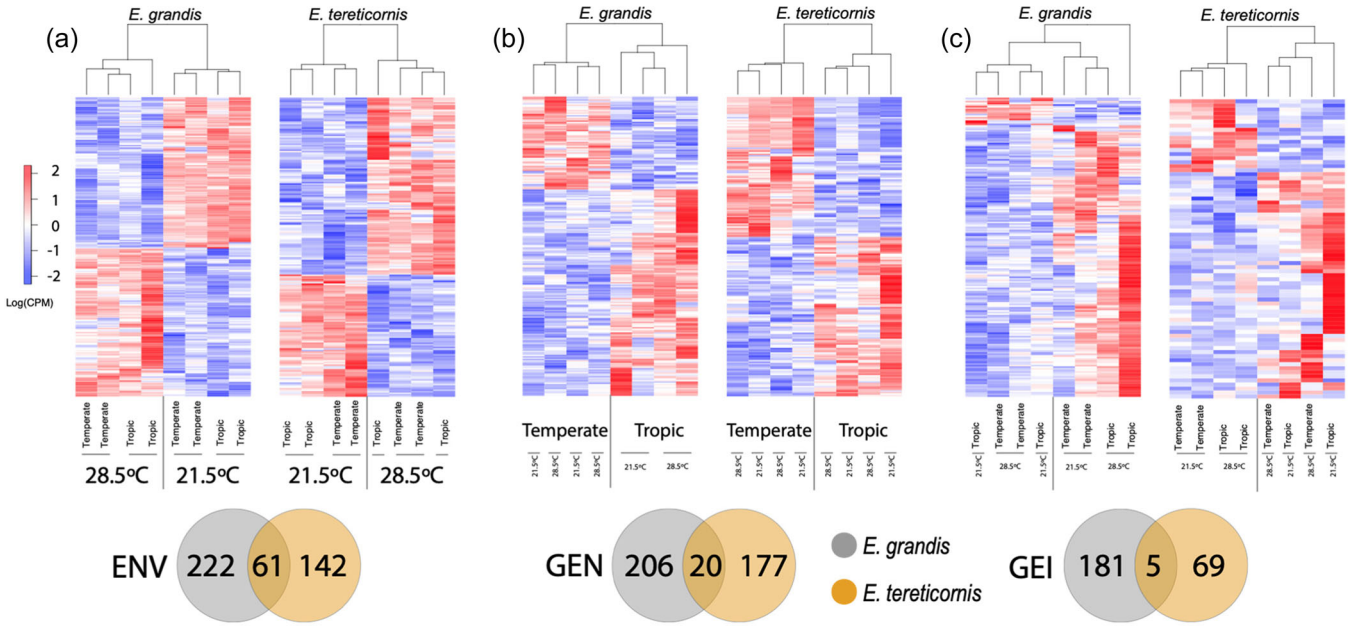


**FIGURE 4** Genes found to be differentially expressed (FDR ≤ 0.05) within species and contrasts. Differential gene expressions are displayed as log(counts per million). Environmental contrasts (ENV; a), genotype contrasts (GEN; b) and genotype× environment interaction contrasts (GEI; d). The Venn diagrams display the number of genes found to be significantly differentially expressed in both species, and the number of genes that are uniquely differentially expressed in either species. [Color figure can be viewed at wileyonlinelibrary.com]

significant genes revealed qualitatively different patterns between species.

The differences between species were mostly characterized by different pathways as shown by GO enrichment analysis. The $\chi^2$ test revealed that the GO pathways with differential expression between the two species were significantly different from one another for all three effects ($p < 0.001$; Supporting Information: Figure S2 and Table S3). For GEN, ENV and GEI effects there were 1, 6 and 15 GO terms that corresponded between species, respectively. The remaining GO pathways were independent from one another. That is 50 (GEN), 151 (ENV) and 160 (GEI) GO enriched pathways identified for *E. grandis* were not highlighted in *E. tereticornis*; likewise, 171 (GEN), 45 (ENV) and 33 (GEI) GO enriched pathways identified for *E. tereticornis* were not highlighted in *E. grandis*. This highlights the unique regulatory process associated with adaptation to temperature.

The coexpression network analyses between contrasts revealed very different patterns among species, indicating adaptive divergence (Figure 5). Two modules (node colours) were estimated for all contrasts except for GEI in *E. tereticornis* with only one module. The topology metrics generally agree with this interpretation (Table 3), but the standard deviations for the centrality metrics of degree and betweenness have large standard deviations indicative of a large amount of variation. There are similarities with betweenness in the ENV contrast and with transitivity in the GEI contrast. Generally, the degree and centralisation metrics provide the largest differences in the coexpression for adaptive contrasts. The genes with the greatest betweenness in the *E. grandis* GEN combination were

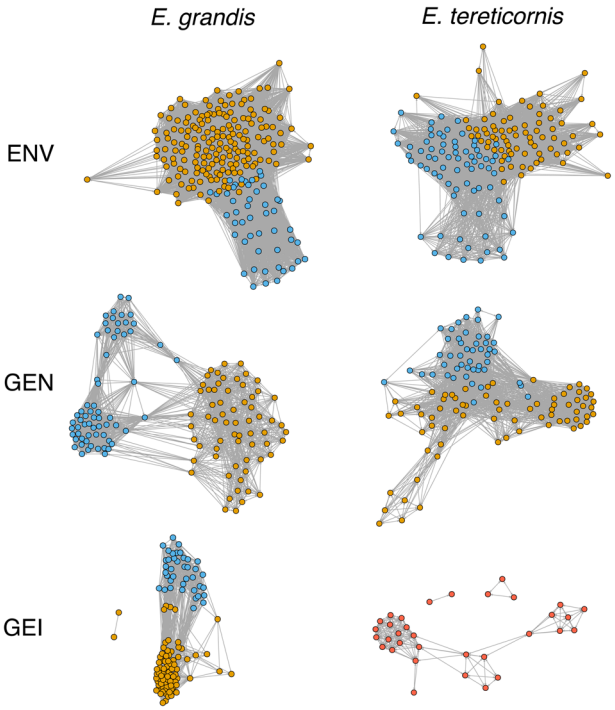


**FIGURE 5** Coexpression network analysis for the significant genes for each of the six contrast/species combinations. Node colours indicate assignment to a module, all networks have two modules except for *Eucalyptus tereticornis*:ENV combination which has one. Environmental contrasts (ENV), genotype contrasts (GEN), and genotype x environment interaction contrasts (GEI). [Color figure can be viewed at wileyonlinelibrary.com]

Eucgr.B00139 (2056 with a proportion of 9.66 compared to the mean) and Eucgr.D01461 (1990 with a proportion of 9.35). Eucgr.B00139 is an AMP-dependent synthetase and ligase family protein and Eucgr.D01461 is a heavy metal transport/detoxification superfamily protein. The genes with the greatest betweenness in the *E. tereticornis* GEN combination were Eucgr.E04153 (1276 with a proportion of 13.7) and Eucgr.F02623 (661 with a proportion of 7.08). Eucgr.E04153 is a cysteine-rich RLK (RECEPTOR-like protein kinase) and Eucgr.F02623 is an O-methyltransferase 1 protein.

The homologs among species that were significantly differentiated due to ENV showed similar responses (Figure 5a), in that all but one gene pair acclimated in the same gene expression fold change direction (60 genes). For GEN effects, homologous pairs showed 10 genes which were expressed in the same direction (parallel), while the other 10 genes were expressed in opposite directions (divergent) (Figure 5b). The five homologs that were in common within the GEI contrasts were all expressed in opposite directions (divergent; Figure 5c). These five homologs occurred across four chromosomes and had unrelated functions (Supporting Information: Table S4), highlighting the divergent processes of local adaptation.

We tested these patterns of adaptation against expectations to see if these patterns could be due to chance. The ENV contrast showed significant overrepresentation of parallel evolution ($C_{hyper}$ = 30.8; $p < 0.001$; Figure 5d) and no significance for divergent evolution ($C_{hyper}$ = 1.3; $p = 0.97$). Parallel evolution was significantly greater than the 0.5 expectations ($p < 0.001$). The GEN contrast showed significant overrepresentation of parallel and divergent evolution ($C_{hyper}$ = 10.7; $p < 0.001$ for both), and a gene was just as likely to be parallel or divergent ($p = 0.59$). The GEI contrast showed significant overrepresentation of divergent evolution ($C_{hyper}$ = 4.6; $p = 0.001$) but not for parallel evolution ($C_{hyper}$ = 0.9; $p = 1$). Overlapping genes between species in the GEI contrast are more likely to be divergent than parallel ($p = 0.03$) (Figure 6).

We found variations in HSPs and TSPs. In total 31 HSP and seven TSP genes were significantly DE in at least one contrast (Table 4). Most of the HSPs found to be DE for *E. grandis* showed a GEN effect (17 genes), while the HSPs found in *E. tereticornis* showed an ENV effect (19 genes). *E. grandis* also had six genes with an ENV effect (two of which are shared with *E. tereticornis*) along with three genes with a GEI effect. *E. tereticornis* had no GEN or GEI HSP genes. There were fewer TSPs identified overall, but most of these were GEIs (*E. tereticornis* three genes; *E. grandis* one gene), with two genes having a GEN effect (*E. grandis*) and only one gene with an ENV effect (*E. tereticornis*).

## 4 | DISCUSSION

Reciprocal transplant experiments provide a powerful approach to explore patterns of local adaptation (Sork, 2017), however, an understanding of molecular mechanisms of local adaptation are often lacking (Kenkel et al., 2013; Lohman et al., 2017; Voelckel et al., 2017). In this study, we combined growth and physiological traits with transcriptomics to provide unique insights into the adaptive and plastic processes enabling species to respond to different temperature conditions. Results here indicate that closely related *Eucalyptus* species, occupying very similar distributions, largely show different evolutionary responses to temperature. Within species, we found that differential expression was substantial across environments, genotypes and their interactions. While among species, we found evidence that many genes have evolved fundamentally different expression patterns, with only a small fraction of genes showing parallel responses, and associations between gene expression and traits indicate species have evolved fundamentally different connections between gene expression, phenotype and environment.

### 4.1 | Patterns of plasticity and adaptation within species

The overarching patterns of differential expression for both species are driven largely by differences in temperature (ENV). However, despite these strong ENV patterns, there were underlying patterns attributable to GEN and GEI. Therefore, we accept our first hypothesis that both species show expression and trait patterns driven by adaptation (GEN), plasticity (ENV) and local adaptation (GEI) in response to growth temperature differences. By growing these genotypes in glasshouses, we were able to control environmental factors (e.g., soil, water availability, daily sunlight fluctuations and relative humidity) so that the differences measured within this study were due to temperature effects alone. Therefore, we can surmise that the two temperatures applied in this study singularly drove all three effects.

Patterns of adaptation (GEN) in gene expression are indicative of genetic differentiation among regions, and may directly affect gene expression through changes in *cis*-regulatory regions. It is known that the presence of standing genetic variation within regulatory regions can enhance a species' ability to persist under harsh climate conditions (Todesco et al., 2022), and adaptive variation within *cis*-regulatory regions have been identified in other eucalypts (Ahrens et al., 2021). Further, the link between gene expression and trait response is evident in both species, directly suggesting that the adaptive variation among genes can affect adaptive traits.

In *E. grandis*'s case, the genes exhibiting significant associations between the three traits are indicative of a plantwide connection through pleiotropic processes. While annotation of these genes was generally associated with photosynthetic processes, the relationship between growth and photosynthetic traits are not unusual. For example, the link between maximum carboxylation and growth has been found in other species, where increased $V_{cmax}$ increases photosynthetic capacity, therefore increasing biomass (Way & Oren, 2010). The intrinsic mechanisms identified in the cellular component relating to RBM, SBM and $V_{cmax}$ control many traits. In *E. grandis*, the number of genes associated with RBM, SBM and $V_{cmax}$ were similar, while the genes that were found to be associated with *E. tereticornis* traits had no overlap. In *E. tereticornis*, the number of genes

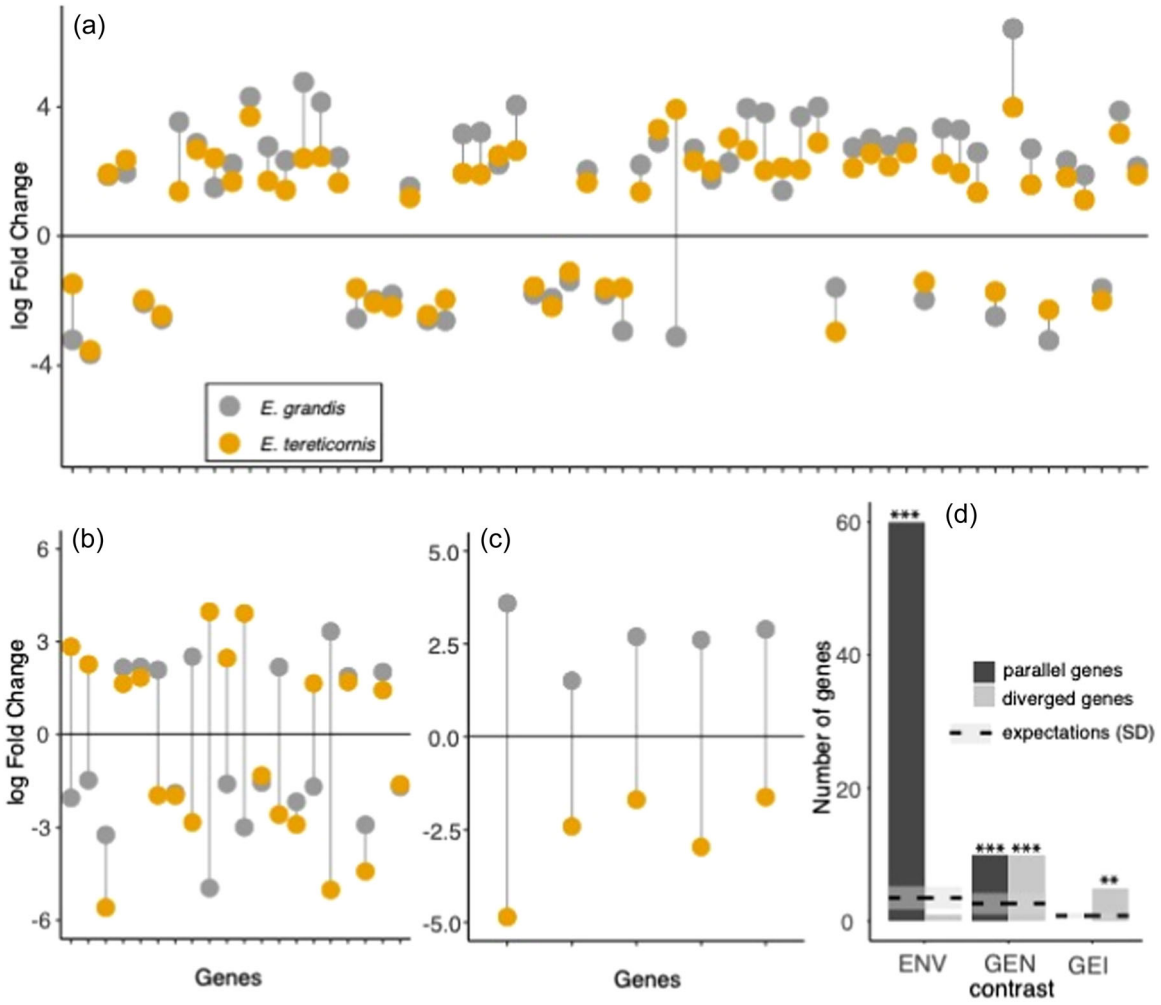


**FIGURE 6** The differentially expressed (FDR ≤ 0.05) homologs in common between the two species within each of the three contrasts (a) environment (ENV; plastic), (b) genotype (GEN; adapted) and (c) genotype-environment interaction (GEI; locally adapted). The grey lines connect homologous gene-pairs between species. The *x*-axes are the genes (unlabelled) and the *y*-axis is the log fold-change between the two groups (i.e., (a) environment = 21.5°C vs. 28°C; (b) genotype = temperate genotypes versus tropic genotypes; and (c) GEI = home versus reciprocal) within each species (denoted by colour). (d) The number of homologs evolving in parallel (dark) or divergently (light) compared to expectations (dashed line) for each contrast (ENV, GEN, GEI). Observed homologs were tested for an alternative hypothesis of being greater than expectations using a one-sided binomial test. ***$p < 0.001$; **$p < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

associated with RBM far exceeded those associated with the other three traits. These results highlight a stark contrast in GEN signals between species, which is characteristic of divergent evolution.

Likewise, we found functional traits that showed response patterns indicative of plasticity (ENV), thereby providing evidence that *Eucalyptus* species rely on short-term responses to temperature. Indeed, plastic responses to temperature were found in the photosynthetic capacity of *E. tereticornis* and *E. grandis* when grown at five different growth temperatures ranging from 18°C to 32°C (Crous et al., 2018). Similarly, we found the same response in *E. tereticornis* and a similar trend in *E. grandis* (marginally significant for $V_{cmax}$ for the ENV effect). Plasticity is an important component when considering the impacts of changing climate on long-lived trees because it may provide the time needed for important functional and molecular traits to evolve or for migration to occur (Fox et al., 2019; Lande, 2009).

The GEI patterns of differential expression were found in both species, but only one species exhibited a GEI pattern in a single trait (although there were several traits that were marginally significant at an $\alpha < 0.1$ for both species). These patterns suggest the presence of physiological local adaptation. However, the signals of local adaptation are much more pronounced when looking at gene expression data. Even so, molecular and physiological patterns of local adaptation together indicate that populations may have lower fitness in new environments as climate change continues to apply pressure on natural forest systems (Reed et al., 2011). While we were unable to find a similar study on plant species for a good comparison, patterns of local adaptation and plasticity were found within the yellow monkeyflower (*Mimulus guttatus*) between coastal and inland sites in California grown in situ (Gould et al., 2018). However, it is difficult to elucidate which climate/environmental factors were

**TABLE 4** A list of differentially expressed (FDR ≤ 0.05) heat shock proteins genes and terpene synthases

| Gene ID | description | *Arabidopsis* | GEN | ENV | GEI |
|---|---|---|---|---|---|
| Eucgr.I00929 | Heat shock factor 4\|nucleus | AT4G36990 | | | |
| Eucgr.J01972 | Heat-shock protein 17; HSP20-like chaperones superfamily protein | AT1G53540 | | | |
| Eucgr.B02899 | Heat shock protein binding | AT1G71000 | | | |
| Eucgr.H04692 | Heat shock protein 21\|plastid | AT4G27670 | | | |
| Eucgr.H02866 | Heat shock protein 60\|mitochondrion | AT3G23990 | | | |
| Eucgr.F03980 | Heat shock protein 70B\|cytosol | AT1G16030 | | | |
| Eucgr.L03563 | Heat shock protein 70\|cytosol | AT3G12580 | | | |
| Eucgr.G00235 | Chloroplast heat shock protein 70-2\|plastid | AT5G49910 | | | |
| Eucgr.K00295 | Heat shock protein 90.1\|cytosol | AT5G52640 | | | |
| Eucgr.A02734 | Heat shock protein 90.1\|cytosol | AT5G52640 | | | |
| Eucgr.K02521 | Heat shock protein 101\|cytosol | AT1G74310 | | | |
| Eucgr.D02070 | Mitochondrion-localized small heat shock protein 23.6\|mitochondrion | AT4G25200 | | | |
| Eucgr.D02073 | Mitochondrion-localized small heat shock protein 23.6\|mitochondrion | AT4G25200 | | | |
| Eucgr.L02860 | 17.6 kDa class II heat shock protein\|cytosol | AT5G12020 | | | |
| Eucgr.C00664 | Heat shock transcription factor A2\|multiple | AT2G26150 | | | |
| Eucgr.C03056 | Heat shock transcription factor A2\|multiple | AT2G26150 | | | |
| Eucgr.K03325 | Heat shock transcription factor A6B\|nucleus | AT3G22830 | | | |
| Eucgr.K00238 | Heat shock transcription factor B2A\|nucleus | AT5G62020 | | | |
| Eucgr.D00597 | DNAJ heat shock family protein\|cytosol | AT2G20560 | | | |
| Eucgr.J02231 | DNAJ heat shock N-terminal domain-containing protein\|plastid | AT5G23240 | | | |
| Eucgr.G02581 | DNAJ heat shock N-terminal domain-containing protein\|plastid | AT5G23240 | | | |
| Eucgr.L02648 | DNAJ heat shock N-terminal domain-containing protein | AT1G56300 | | | |
| Eucgr.A01949 | No description | AT1G06460 | | | |
| Eucgr.J01959 | Heat shock protein 18.2 | AT5G59720 | | | |
| Eucgr.J01957 | Heat shock protein 18.2 | AT5G59720 | | | |
| Eucgr.J00981 | Heat shock protein 18.2 | AT5G59720 | | | |
| Eucgr.I02136 | Class III heat shock protein (HSP17.4-CIII) | AT1G54050 | | | |
| Eucgr.G01045 | Heat shock 70 kDa protein, mitochondrial | AT5G09590 | | | |
| Eucgr.D00856 | HSA32 (heat-stress-associated 32); catalytic | AT4G21320 | | | |
| Eucgr.D02334 | BAG6 (BCL-2-associated athanogene 6); calmodulin binding / protein binding | AT2G46240 | | | |
| Eucgr.F02898 | Class I-related small heat shock protein-like (HSP15.7-CI) | AT5G37670 | | | |
| Eucgr.E03610 | Terpene synthase 14\|plastid | AT1G61680 | | | |
| Eucgr.E03562 | Terpene synthase 14\|plastid | AT1G61680 | | | |
| Eucgr.E03563 | Terpene synthase 03\|cytosol | AT4G16740 | | | |
| Eucgr.E03115 | Terpene synthase 21\|cytosol | AT5G23960 | | | |
| Eucgr.K00828 | Terpene synthase-like sequence-1,8-cineole\|plastid | AT3G25830 | | | |
| Eucgr.K00879 | Terpene synthase-like sequence-1,8-cineole\|plastid | AT3G25830 | | | |
| Eucgr.K00881 | Terpene synthase 03\|cytosol | AT4G16740 | | | |

*Note*: Genes were found to show significantly different expression for genotype effects (GEN), environmental effects (ENV), and genotype–environment interactions (GEI). Grey, *Eucalyptus grandis* and yellow, *Eucalyptus tereticornis*. The Eucalyptus homolog (Gene ID), its gene description, and Arabidopsis orthologous ID are provided for each gene.

driving these patterns in the monkeyflower. Another comparable study found intraspecific gene expression variation, similar to patterns of local adaptation, within valley oak (*Quercus lobata*) when exposed to drought (Mead et al., 2019), even though the species' ecophysiological traits were static across populations.

Physiological differentiation within the *Eucalyptus* group is known to exhibit strong patterns of adaptation and plasticity (Aspinwall et al., 2017; Pfautsch et al., 2016). However, the genetic mechanisms controlling those phenotypes remain unknown. A recent study revealed different responses in HSPs and TSPs among four *Eucalyptus* species when subjected to a heatwave simulated 17°C increase in temperature for 4 days (Aspinwall et al., 2019), and found that all species used HSPs to respond to thermal stress. This heatwave study elucidated the response to extreme events, and differs from ours in that our study explored the relative difference between *Eucalyptus* species under long-term growth conditions. There were still some similarities found between our study and Aspinwall et al. (2019), in that there were differences of HSP and TSP expression between species, which can be an important indication for physiological inhibition for species that overexpress these proteins (Aspinwall et al., 2019). Indeed, these HSP responses differed markedly between species, with *E. tereticornis* having a mostly plastic (ENV) response and *E. grandis* having a mostly genotypic (GEN) response to temperature. This type of general response pattern has been found in other systems where significant gene regulation was found in reference samples exposed to stress but not in tolerant individuals (Roelofs et al., 2009). If this pattern holds true for the two eucalypt species, *E. grandis* may be more tolerant to thermal environments. Our findings are notable because we found that HSP responses, which are generally associated with extreme events, differed under moderate changes in growth temperatures.

## 4.2 | Patterns of evolution among species

The differences between species are far greater than the similarities for traits and expression, indicating evolutionary divergence. The species-specific genes found to be significant for ENV suggest different plastic responses among species. These genes were found to be from different GO pathways, indicating each species has adapted different strategies to overcome challenges associated with changes in temperature. Although we expected higher correlation between GO pathways (suggestive of parallel evolution), this was not the case; therefore, we refute our hypothesis of strong signals of parallel evolution between similar species occupying similar habitats. Overall, the differences between species indicate that evolution in eucalypts is largely not predictable, suggesting there are myriad ways for plants to adapt to similar environmental challenges. One caveat to this argument is that we are specifically looking at differences in gene pathways related to contrasting growth temperatures, it is likely that many gene or GO pathways that could result in similar physiological characteristics. This means that while there were low amounts of parallelism at the gene level, there could be higher amounts of parallelism at the trait level.

The GEN similarity between species illustrates some evidence for parallel evolution. However, these genes are only a small proportion of the genes that show significant patterns of differential expression. This is not surprising because the rate of parallel evolution among these species is within the estimated rate of parallel evolution within genes, ranging from 0% to 20% (reviewed in Kassen, 2014). These results suggest that congenerics, occupying very similar environments, evolve different mechanisms to adapt to temperature. Indeed, we uncovered very different mechanisms underlying the broad trait differences. In this study, we found that changes in gene expression occur at different rates among genes. Random mutations could be causing the different patterns of adaptation across the same environments culminating in different gene and trait responses, particularly genes that are associated with RBM in *E. tereticornis*, a pattern not observed in *E. grandis*. Indeed, mutations are known to change gene expression but genes are not equally sensitive to mutations (Landry et al., 2007). Hence, we hypothesize this type of divergent evolution is likely indicative of other *Eucalyptus* species and not the exception; however, more studies on additional species would need to be performed to confirm these results.

The interpretations between homologs among species significant for the ENV effect are nuanced. For example, all but one homolog identified under the ENV effect show the same expression pattern, indicating that they function in similar ways, they have been conserved from the most recent common ancestor or both. On the other hand, the homolog showing a different expression pattern is likely due to selection. Indeed, this type of selection on gene expression plasticity is becoming an important factor in short- and long-term climatic adaptation (Armarego-Marriott, 2021).

The five genes in common among species in the GEI effect represent an excellent example of divergent evolution, in that while the genes showed patterns of local adaptation for both species, their responses were the opposite of one another. This finding suggests that the homologs have different functions within their adaptive pathways. In trees, different physiological responses to the same drought conditions have been shown in ponderosa pine (limiting transpiration) and trembling aspen (carbon dense tissues), even though they have similar distributions (Anderegg & Hille RisLambers, 2015); however, these species have very different evolutionary histories and are part of different Families. Our finding of five common genes with different responses, in addition to 10 genes showing different regulation patterns in the GEN effect, is surprising because we expected that the same genes would undergo the same selection process, particularly for species within the same genus. Interestingly, these homologs may be linked to other genes in epistatic or pleiotropic interactions (Lind et al., 2018). Effects stemming from pleiotropy can have consequences on fitness (Chevin et al., 2010), as was found in *Escherichia coli* grown under different glucose treatments (Ostrowski et al., 2008). Likewise, epistatic interactions have been found to yield significant changes to fitness in bacteriaphage (Sackman & Rokyta, 2018), although phages are

known to have higher rates of parallelism than other organisms (Wichman & Brown, 2010).

With these studies in mind, we can assume that in more complex genomes, such as those found in trees, that epistasis and pleiotropy play an important role in shaping adaptation to new environments and are likely important for using the same pathways and genes in different ways to optimize their relationship with their environment. Therefore, we reiterate Lind et al. (2018) suggestion that research into gene interactions would be worthwhile and complement recent studies that found epistatic interactions to be an important component of growth in hybrid *Eucalyptus* species (Tan et al., 2018). While the effects of gene interactions on fitness are inherently difficult to quantify (Bailey et al., 2015), particularly in long-lived species such as trees, it will continue to be an important area of inquiry to better understand the mechanistic basis for species adaptation to their environment. Further, it would be beneficial to compare and contrast gene expression from this environmentally controlled experiment to natural populations, potentially estimating the variance associated with naturally occurring gene expression profiles of local adaptation to thermal pressures.

## 5 | CONCLUSIONS

Understanding the molecular mechanisms contributing to local adaptation to different temperature conditions is fundamental to evolutionary biology and provides an essential step in developing predictions for the capacity to respond to global warming. In response to temperature, the evolutionary signal among the two *Eucalyptus* species consists of mostly divergent signatures of selection, with limited evidence supporting evolution occurring in parallel. This reinforces three important findings that (1) evolution is generally nondeterministic for long-lived, highly outcrossing species, (2) species' adaptive potentials are not equal, even when occupying the same environmental space and (3) gene–trait correlation networks differ among species. These novel findings have implications for the diversification and maintenance of biodiversity, in that broadly distributed trees appear unconstrained in their capacity to adapt to temperature, and selection can act upon diverse gene functions in unpredictable ways when responding to temperature. While we found that there may be a common temperature response across species to help with species management, their ways of dealing with the common temperature response are largely divergent. Consequently, rising temperatures associated with climate change will likely differentially impact species with similar distributions, requiring adaptive management strategies to independently consider the diverse adaptive and plastic mechanisms among species.

## ACKNOWLEDGEMENTS

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in DRYAD at https://doi.org/10.5061/dryad.mkkwh713n. Raw and filtered RNA seq data will be available via DRYAD upon acceptance along with the R code, physiological, experimental, and environmental data.

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