Molecular signatures of heat tolerance and sensitivity in the alpine plant *Wahlenbergia ceracea* during moderate warming

Running title: Molecular basis of tolerance during warming

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Abstract

Understanding the molecular basis of plant heat tolerance helps to predict the consequences of climate warming on plant performance, particularly in vulnerable environments. Our current understanding comes primarily from studies in *Arabidopsis thaliana* (Arabidopsis) and select crops exposed to short and intense periods of heat stress. In this study we sought to characterise the transcriptomic responses of *Wahlenbergia ceracea* (*W. ceracea*), an Australian alpine herb, to growth under sustained moderate warming. We compared responses of pre-defined tolerant and sensitive lines, based on measures of photosynthetic thermostability, to growth under cool (24/15 ºC day/night) or warm (30/20 ºC day/night) temperatures to identify the pathways involved in heat tolerance and acclimation. Under warmer growth temperatures, *W. ceracea* up-regulated genes involved in RNA metabolism, while down-regulating those involved in photosynthesis and pigment metabolism. In tolerant lines, genes related to photosystem II and light-dependent photosynthetic reactions were more strongly down-regulated. This suggests that the regulation of electron transport and its components may be involved in thermal acclimation. Our results also highlight the importance of hormonal gene networks, particularly those responsive to ethylene, during longer-term moderate warming. In conclusion, our results point to post-transcriptional processes and the stabilisation of the electron transport chain as candidate mechanisms for thermal acclimation in heat-tolerant *W. ceracea*. The study also revealed many temperature-responsive genes with no detectable ortholog in Arabidopsis, whose characterization may enhance our understanding of physiological acclimation and have relevance for the biotechnological improvement of threatened species and crops.

Keywords: heat tolerance, photosynthesis, acclimation, mRNA sequencing, transcription factors, climate warming.

Introduction

Alpine environments, which are characterised by high spatio-temporal variability in climatic conditions including steep temperature and precipitation gradients across complex topography, are predicted to be severely impacted by global warming (Gobiet *et al.* 2014). Australia's alpine and subalpine regions harbour many plant species that are vulnerable to climate change (Sritharan *et al.* 2021). Plants that occupy distinct elevations exhibit differing levels of phenotypic plasticity, which suggests that there are distinct molecular processes facilitating growth and reproduction (Nicotra *et al.* 2015). However, genomic studies on native plant species that occupy these environments remain under-represented (Geange *et al.* 2021). Such studies, including transcriptomics, have the potential to reveal the genomic architecture of traits under selection, their variation, and plasticity in response to climate change factors (Oomen & Hutchings 2017, 2022), while identifying the molecular basis of physiological attributes and acclimatory responses that may underlie resilience to harsh climates (Rashid *et al.* 2020). Transcriptomic profiles also have the potential to inform conservation planning (Hansen 2010; Oomen & Hutchings 2022). Therefore, expanding the application of genomics methodology across natural populations of native species has the potential to produce both fundamental and practical insights.

Understanding plant responses to heat stress is intensely pursued due to projected increases in mean surface temperatures (+3 °C by 2100) as a result of global warming (IPCC 2023). Many studies focus on short, intense heat events, referred to as heat-shock, which can be used to infer the pathways that contribute to thermal acclimation (Wang *et al.* 2020). Heat-shock causes several changes to the physical and chemical properties inside cells, which in turn affect cellular function (Lippmann *et al.* 2019). Many enzymes lose their native folding thus impairing their catalytic function, with disrupted activity at temperatures even slightly above their optimum (Daniel *et al.* 2008; Hobbs *et al.* 2013). These denatured or unfolded proteins can also disrupt protein homeostasis and form toxic insoluble aggregates (Richter *et al.* 2010). Heat can disrupt photosynthetic electron flow, which can lead to an increased concentration of reactive oxygen species (ROS), which cause oxidative damage to proteins, DNA, and lipids (Suzuki and Mittler, 2006). High temperatures also increase membrane fluidity, altering their permeability to water, ions, and solutes, and affecting transmembrane proteins (Niu & Xiang 2018; Cano-Ramirez *et al.* 2021). These changes in membrane stability can directly affect the function of photosystems and photosynthetic reaction centres (Hugly *et al.* 1989). Therefore, heat stress can severely impact plant growth and fitness in the absence of appropriate acclimatory responses.

Plant molecular responses to heat stress have been extensively explored in models and crops, such as Arabidopsis and rice. Such studies reveal that heat responses involve the regulation of genes encoding heat-shock proteins (HSPs), which are predominantly chaperones and transcription factors (TFs) (Richter *et al.* 2010). The molecular chaperones, such as HSP70, HSP90, HSP100, and small HSPs, bind unfolded proteins preventing their aggregation and, in some cases, induce their correct re-folding or degradation (Richter *et al.* 2010). The heat-shock transcription factors (HSFs) regulate the expression of HSPs in response to many abiotic stresses, including heat (Andrási *et al.* 2021; Haider *et al.* 2022). In addition, many phytohormones, such as abscisic acid (ABA) and ethylene, influence the response to increased temperature by regulating processes, such as sucrose metabolism and ROS detoxification, which can influence growth and stress tolerance (Li *et al.* 2020a). The combination of such molecular responses helps maintain cellular function under elevated temperature, thereby facilitating heat tolerance (Yamori *et al.* 2014; de Pinto *et al.* 2015). Experimental heat-shocks, which often involve a temperature change of >10 ºC (Mittler *et al.* 2012), have been useful to identify the first responders to heat stress. However, plants also make metabolic and physiological adjustments to optimize their photosynthetic performance under more moderate (change of <10 ºC) and prolonged warming (Larkindale & Huang 2004; Way & Yamori 2014; Yamori *et al.* 2014). Responses to longer-term warming may involve distinct pathways compared to acute responses (Wang *et al.* 2018, 2020). For example, acclimation to moderate or gradual warming in plants can be achieved by increasing the thermal optimum for photosynthesis, the electron transport rate, and/or stabilising RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE (RUBISCO) ACTIVASE (Way & Yamori 2014; Yamori *et al.* 2014). Thus, it is vital that we extend our understanding of acclimation responses to moderate and continuous warming. This is likely more reflective of the gradual and sustained increase in mean temperature that plants must cope with as a result of climate warming.

Here, we study the molecular response of *Wahlenbergia ceracea*, an herbaceous perennial that grows across Australia's alpine regions, to sustained moderate warming. For this, we generate an annotated genome assembly and infer functional attributes based on identifying Arabidopsis orthologs for thousands of genes, thus providing preliminary functional annotation. We then assess gene regulation in response to moderate warming, including the construction of gene regulatory networks. We have previously demonstrated that *W. ceracea* has the capacity to acclimate to warmer growth temperatures, as evidenced through sustained changes in photosynthetic thermostability (Notarnicola *et al.* 2021; Arnold *et al.* 2022). We treated previously established lines of *W. ceracea*, categorized as heat-sensitive and tolerant based on the thermostability of their photosystems (PS) (Arnold *et al.* 2024), to a prolonged (2 weeks) moderate warming to determine whether heat-tolerant lines invoked different patterns of gene regulation

commensurate with effective acclimation compared to sensitive lines. We observed distinct regulation of genes associated with post-transcriptional processes, phytohormone signaling (particularly ethylene and abscisic acid), and the stabilisation of the electron transport chain during thermal acclimation. These findings highlight multiple candidate pathways that may contribute to heat tolerance in an alpine plant and underline the importance of expanding the breadth of species investigated in plant genomics.

Materials and Methods

Plant germplasm

Seeds of *W. ceracea* were collected in the field from mature capsules (F_0 generation), across the subalpine-alpine elevation range of the species (1590 – 2095 m a.s.l., Kosciuszko National Park, NSW, Australia) and subjected to controlled crosses to generate F_3 lines (**Figure S1**, **Table S1**). Tolerant and sensitive lines were established previously, where individuals from F_3 lines were grown in the glasshouse under optimal (24/18 ºC day/night) or warm (30/25 ºC day/night) growth temperatures (Arnold et al., 2024). Heat tolerance was assessed by measuring temperature-dependent change in basal chlorophyll fluorescence (T-F_0), where T_{max}, the temperature at maximum fluorescence in a T-F_0 curve, represents the point of irreversible membrane damage. We ranked lines using their average T_{max} and selected the six F_3 lines with the highest and lowest heat tolerance respectively for the following experiment (tolerant lines average T_{max} = 56.07 °C; sensitive lines average T_{max} = 50.83 °C; ΔT_{max} = 5.24 °C; **Table 1**).

Plant growth and treatments

Seeds from F_3 plants were germinated in seed raising mix in 14 × 8.5 × 5 cm pots in the glasshouse under 28/20 ºC to maximise germination and establishment (Arnold *et al.* 2022; Notarnicola *et al.* 2023). Subsequently, plants were grown under controlled conditions (28/20 ºC, 60 % relative humidity, 14-hour photoperiod, 1200 µmol m^{-2} s^{-1}) using a Growth Capsule (Photon Systems Instruments, Brno, Czech Republic). Individual plants were transplanted into individual pots (6.5 × 6.5 × 16 cm) and supplemented with slow-release fertilizer (Osmocote Plus, Scotts Australia). Two weeks after transplantation, plants were subject to either cool [24/15 ºC day/night, representative of sub-alpine summer temperatures (Arnold *et al.* 2024)] or warm temperatures (30/20 ºC day/night). Plants grown at 30 ºC have reduced fitness (seed number and viability) but are not sterile nor do they exhibit severe photoinhibition [Maximum PSII efficiency, F_v/F_m > 0.70; (Notarnicola *et al.* 2021)]. Plants were grown for a further two weeks by which time they had produced new leaves that were acclimated to the respective temperature conditions. Our study consisted of 12 independent lines that were grown in two

conditions in biological triplicate (**Figure S1**, **Table 1**). We randomly arranged three replicate plants for each line in three blocks (one replicate per block) that were equivalent for the two treatments. Plants were bottom watered as needed and supplemented with liquid fertiliser every two weeks (Yates Thrive, Yates Australia). We harvested approximately 100 mg leaf tissue samples per plant from the youngest, fully-expanded, healthy leaves. Leaves were harvested in a randomized order between 11:00 and 16:30 hours, and immediately flash-frozen in liquid nitrogen and stored at -80 ºC. Time of harvesting and developmental stage were recorded for each sample (non-flowering n = 56; early bud n = 16).

Genome sequencing and assembly

Genomic DNA was extracted from an F_2 individual (F_2.209, **Table S1**) that represents a mixed pedigree from F_0 plants that occupied relatively low (F_0.1613 x F_0.1606, ~1750 m) and high (F_0.1525 x F_0.1524, ~1900 m) elevations, which was grown in the glasshouse under cool conditions (24/15 ºC). High-molecular weight DNA was extracted using an SDS-based protocol that is described in detail on protocols.io: https://dx.doi.org/10.17504/protocols.io.bss7nehn (Jones *et al.* 2021). Size selection was performed to obtain fragments ≥10 kb with a Short Read Eliminator (Circulomics) followed by isolating 12 kb fragments using a SageELF (Sage Science). A PacBio Sequel II long-read native DNA sequencing library was prepared for Single Molecule, Real-Time sequencing (Eid *et al.* 2009) according to the manufacturer's SMRTbell Express Template Prep Kit 2.0 (PacBio). Sequencing was performed on one 8M SMRT cell, using the circular consensus sequencing mode to generate high-accuracy HiFi reads. Raw reads were inspected with NanoPlot (De Coster *et al.* 2018). Reads mapping to non-plant contigs were identified using BlobTools and excluded from analysis (Laetsch & Blaxter 2017). The remaining reads were used for genome assembly with Canu using PacBio HiFi settings and a genome size estimate of 0.9 Gb (Koren *et al.* 2017; Nurk *et al.* 2020). The assembly was deduplicated using purge_dups (Guan *et al.* 2020). Repeats were annotated and masked using RepeatModeler2 and RepeatMasker (Flynn *et al.* 2020). RepeatMasker results were summarised with "One code to find them all" on fuzzy settings (Bailly-Bechet *et al.* 2014). Genome annotation was performed using BRAKER2 in epmode (Brůna *et al.* 2021). Gene predictions were guided by all eudicot orthogroups available in OrthoDB v.10 (Kriventseva *et al.* 2019) and the mRNA sequencing dataset generated here. For the latter, trimmed reads across all samples were merged and aligned to the genome using Subjunc (Liao *et al.* 2013). The amino acid and coding sequences were drawn from the braker.gtf output using "getAnnoFastaFromJoingenes.py" from Augustus (Stanke *et al.* 2008). We tested for completeness of the

genome assembly and gene annotation using Benchmarking Universal Single-Copy Orthologs (BUSCO) (Simão *et al.* 2015). We refer to the predicted gene annotations as the transcriptome.

mRNA sequencing and analyses

Total RNA was extracted from snap-frozen and ground leaf tissue using the Spectrum™ Plant Total RNA Kit (Merck, New Jersey, USA) with on-column DNase I digestion. Purified RNA was quality checked for DNA contamination and integrity using semi-denaturing agarose-gel electrophoresis and the LabChip GX Touch (PerkinElmer). RNA was quantified with ND-1000 Spectrophotometer (NanoDrop Technologies) and Qubit 4 Fluorometer (RNA HS kit, Thermo Fisher Scientific). Library preparation and sequencing were conducted by Deakin Genomics Centre. The cDNA libraries were constructed with 250 ng RNA using the Illumina stranded mRNA prep ligation protocol, as per manufacturer's instructions except that reaction volumes were scaled by one-half and 12 PCR cycles were used for library amplification. Paired-end sequencing (2 × 150 bp) was conducted on the NovaSeq6000 (Illumina, California, USA).

Raw reads were trimmed for adapters and low-quality base calls (PHRED <20) using Trim Galore (Babraham Bioinformatics) with default settings. Trimmed reads were inspected using MultiQC (Ewels *et al.* 2016), prior to transcript quantification with Kallisto (Bray *et al.* 2016). Quantification was performed based on our assembled transcriptome. To do this, the BRAKER2 GTF output file was used to extract target gene sequences from the genome assembly in FASTA format using GffRead (Pertea & Pertea 2020). This was used to generate an index for Kallisto. Transcript abundances were summarised to gene-level counts with Tximport, using the 'length-scaled transcripts per million' equation (Soneson *et al.* 2015). For this purpose, we used all sequences in the transcriptome with predicted exons, start, and stop codon annotations. Only genes with >10 counts in 18 samples were retained for analysis using FilterByExpr from edgeR with default parameters (Robinson *et al.* 2010). We calculated scaling factors to normalise gene counts based on effective library size using the calcNormFactors function in edgeR using the Trimmed Mean of M-values (TMM) method. Observational-level and sample-specific quality weights were assigned with the voomWithQualityWeights function in the limma package to reduce heteroscedasticity of the data from different samples and transform the mRNA sequencing read count for linear modelling (Smyth 2004; Liu *et al.* 2015). Lines were treated as random effects: within line correlation was estimated with duplicateCorrelation and quality weights were re-assigned as above (Smyth *et al.* 2005). We used the lmFit function in limma to fit linear models to expression. Initial models utilized a pseudo-factor that combined tolerance and temperature ($\text{Tolerant}_{\text{cool}}$, $\text{Sensitive}_{\text{cool}}$, $\text{Tolerant}_{\text{warm}}$, and $\text{Sensitive}_{\text{warm}}$; n per group = 18), as well as the developmental stage ('non-flowering' and 'early bud'), and collection time. However, preliminary analysis did not reveal clustering of samples by

developmental stage nor harvest time. Therefore, these factors were not included in the final models. The full model for identifying differentially expressed genes (DEGs) was: gene expression ~ 0 + (tolerance_temperature), random effect = line. The exclusion of the intercept from the model and use of a combined factor allowed us to calculate differences in gene expression between pre-determined contrasts (**Table 2**).

To assign functional information to predicted genes, we detected orthologs between *W. ceracea* and Arabidopsis based on amino acid sequences using OrthoFinder with default settings (Emms & Kelly 2019). To improve the phylogenetic inference of orthologs and gene trees, OrthoFinder was run with the following proteomes, in addition to those of *W. ceracea* and Arabidopsis: *Eucalyptus grandis*, *Glycine max*, *Manihot esculenta*, *Oryza sativa*, *Solanum lycopersicum*, *Vitis vinifera*, *Zea mays*, *Chlamidomonas reinardtii*, *Marchantia polymorpha*, *Physcomitrium patens*, and *Selaginella moellendorfii*. Enrichment tests for functional terms were performed using Metascape. Multi-gene analyses were performed separately for up- and down-regulated genes, using the deduplicated Arabidopsis ortholog of predicted *W. ceracea* genes (**Supplementary Data 1**), to compare enriched functional terms between tolerant and sensitive lines. Genes were annotated using the default catalogue, except 'Drug' (DrugBank) and 'Disease & Drugs' (ChatGPT) were replaced with KEGG Pathway terms. Enrichment analyses were based on GO Biological Process, KEGG Pathway, and WikiPathway catalogues with default settings (minimum overlap = 3; P-value cut-off = 0.01; minimum enrichment factor = 1.5). TFs were associated with Arabidopsis orthologs of up- and down-regulated genes, separately, for tolerant and sensitive lines using TF2Network (Kulkarni *et al.* 2018).

Results

Genome assembly and annotation

To investigate the transcriptomic response of *W. ceracea* to moderate warming, we first produced an annotated reference genome assembly. To do this, we performed PacBio long-read sequencing on genomic DNA extracted from a single F_2 individual (F_2.209, **Table S1**). The total nuclear DNA content for *W. ceracea* was estimated at 1.8 Gb by flow cytometry. The genome assembly using Canu was 802.965 Mb, which accounts for approximately 87% of its haploid genome size (0.9 Gb). Testing for gene space completeness reported 91.5% of Embryophyte BUSCOs as complete. However, the assembly recorded low contiguity as the number of contigs was 1,876, the contig N_{50} was 1.125 Mb, and the contig NG_{50} was 0.985. This fragmentation may be due to the presence of transposons that were identified at the end of existing contigs. Indeed, 72.19% of the assembly were masked as transposons and almost all contigs (99.5%) contained at least one. Next, we annotated our assembly to make functional insights. We

utilized our mRNA sequencing dataset across 72 individuals (*see below*), which were combined into a single file and aligned against our assembly. Providing the aligned reads to BRAKER2 for gene prediction identified 223,827 exons for 87,730 proteins. BUSCO testing for completeness of our protein annotation reported 95.7% of Embryophyte BUSCOs as complete. To establish how many genes could be identified across our experiment, based on our annotation, we quantified the predicted transcripts in individual mRNA sequencing samples using Kallisto. On average, mapping efficiency across samples was approximately 69.7%, corresponding to 13.3 – 63.1 million mapped reads per sample (**Supplementary Data 2**). These were mapped to 38,257 transcripts, which could be summarised to 37,307 genes with Tximport (Soneson *et al.* 2015). To gain functional information on the predicted genes, we identified orthologous genes across a range of plant species, including Arabidopsis, using OrthoFinder (Emms and Kelly, 2019). This revealed that *W. ceracea* shared the highest number of orthogroups with *Manihot esculenta* (9,357), *Glycine max* (9,275), and *Vitis vinifera* (9,239), among the species analysed (**Table S2**). We found 8,854 orthogroups between *W. ceracea* and Arabidopsis, and a total of 11,205 orthologs between the two species (**Table S2**, **Supplementary Data 3**). Overall, approximately 81.4% of DEGs (*see below*) could have an ortholog identified in Arabidopsis. Taken together, this genome and gene annotations formed the basis of our investigation of heat-responsive gene regulation.

Heat-tolerant lines of *W. ceracea* regulate genes related to photosynthesis, RNA processing, and translation in warm conditions

To investigate differential gene regulation under moderate warming between heat-tolerant and sensitive *W. ceracea*, we performed mRNA sequencing across 12 lines of *W. ceracea* grown in either cool or warm conditions (**Table 1**). These lines were previously established and categorized as either heat-tolerant or heat-sensitive based on the thermostability of their photosystems (Arnold *et al.* 2024). Of the 37,307 predicted genes, 22,585 were considered as expressed in our dataset (>10 counts in 18 samples). Principal Component Analysis (PCA) revealed separation along PC2 (8.83%), which reflected the differing growth conditions (**Figure 1A**). In contrast, PC1 explained 14.10% of the variation but did not associate with any measured variable and may reflect genetic differences. To ensure we could observe a robust temperature response, we first verified that all *W. ceracea* lines responded to the warmer conditions (contrast I). Indeed, we could detect 6,140 DEGs in response to temperature (**Table 2, Figure 1B**). Of these, 1,646 were detected when considering all lines (contrast I) but were not significant when tested within tolerant or sensitive lines (contrasts II and III), presumably due to reduced statistical power at smaller sample sizes. Nonetheless, both tolerant and sensitive lines showed pronounced warm-induced changes in gene expression, which were comparable between lines (**Figure S2**). Furthermore, as might be expected, many of the DEGs and their functions were similar between the overall response (contrast I)

and line-specific responses (contrasts II and III) (**Figure S3**, **Supplementary Data 4**). However, we focus on differences in gene expression changes in tolerant and sensitive lines under warm conditions (contrasts II and III) in order to explore possible molecular mechanisms contributing to heat tolerance in *W. ceracea*. When comparing transcriptomic responses to warming between lines, we identified 2,946 (up: 1,461; down: 1,485) and 3,721 (up: 2,056; down: 1,665) differentially expressed genes in tolerant and sensitive lines under warm conditions, respectively (**Table 2**, **Supplementary Data 5-6**). While 1,634 DEGs were common between tolerance groups, 1,312 and 2,087 were temperature-responsive only in tolerant or sensitive lines, respectively (**Figure 1B**). In tolerant lines, we could not detect an orthologous gene in Arabidopsis for the three most significantly up-regulated genes. Therefore, these may represent novel heat-responsive genes or possibly non-coding RNAs for which homology can be difficult to trace.

To understand the broader functions of heat-responsive genes in *W. ceracea*, we identified over-represented GO terms in 1,760 up-regulated (tolerant: 747, sensitive: 1,013) and 1,830 down-regulated (tolerant: 861, sensitive: 969) genes, for which we could detect an Arabidopsis ortholog, using Metascape (**Table 2**, **Supplementary Data 1**). Amongst the up-regulated genes, there were multiple enriched terms related to RNA processing and translation (**Figure 2A**). On the other hand, down-regulated genes were enriched for terms related to the chloroplast and photosynthesis (**Figure 2B**). Strikingly, the sensitive lines demonstrated a more significant enrichment of terms related to post-transcriptional regulation of gene expression among the up-regulated genes, such as RNA modification and processing. The opposite was observed for down-regulated genes, where tolerant lines exhibited a stronger enrichment for various terms including photosynthesis, plastid organization, and thylakoid membrane organization. This suggests that sensitive lines may have perturbed RNA regulation whilst tolerant lines may have attempted to suppress photosynthetic processes.

We further investigated the expression patterns of individual genes contributing to the enriched functional terms that may potentially explain differences in T_{max} between lines. We first inspected photosynthesis-associated DEGs, since T_{max} relates to photosynthetic performance and photosystem II thermostability. The GO and KEGG term 'photosynthesis' comprised 114 *W. ceracea* DEGs that were orthologs to 74 Arabidopsis genes (**Figure 3**). These genes encoded for components of the antennae and complexes involved in electron transport, such as *PROTEIN PHOSPHATASE 1* (*PPH1*), *LIGHT HARVESTING COMPLEX* (*LHC*) *A2* (*LHCA2*), *LHCA2*1*, *LHCB5*, *LHCB7*, *OXYGEN EVOLVING COMPLEX 33* (*OEC33*), and *ATP SYNTHASE C1* (*ATPC1*). All these genes were significantly down-regulated in both tolerant and sensitive lines (**Supplementary Data 5-6**) except for *PPH1*, *LHCA2* (tolerant lines only, contrast II), and *OEC33* (overall response, contrast I). Strikingly however, expression

of these genes tended to be much lower in tolerant lines, compared to sensitive lines, under warm conditions.

Since many heat-induced genes encode for HSPs, many of which function as chaperones and proteases, we expected to see a strong enrichment of genes related to 'protein folding'. Interestingly, while this term was enriched among up-regulated genes in tolerant lines (contrast II), it was not in sensitive lines (contrast III). We investigated further to see whether this was occurring specifically at HSP encoding genes. Among the DEGs related to protein folding, five were HSPs that were all up-regulated in warm conditions, especially in sensitive lines (**Figure S4**). Taken together, we observed strong repression of photosynthesis-related genes in response to warming, especially in tolerant lines, which also appear to up-regulate many non-HSP genes involved in maintaining protein assembly and structure.

Heat-sensitive *W. ceracea* repress gene networks related to plant growth and development under warm conditions

We hypothesized that altered activity of gene regulatory networks could contribute to the differences in heat tolerance between our *W. ceracea* lines. Since we observed contrasting heat-responsive gene regulation between heat tolerant and sensitive lines, we interrogated whether these differences could be explained by altered TF activity. We constructed gene regulatory networks, using TF2Network (Kulkarni *et al.* 2018), for up- and down-regulated genes in tolerant and sensitive lines, separately (**Figure 4**, **Supplementary Data 7**). We identified 22 and eight TFs from up-regulated DEGs in sensitive and tolerant lines, respectively; of which seven were detected in both (**Figure 4A**). Interestingly, only one TF (NGA4) was associated with up-regulated genes from tolerant lines. Many more TFs were associated with down-regulated genes, with 381 and 98 detected in sensitive and tolerant lines, respectively, of which 83 were in common (**Figure 4B**). These results suggest that sensitive lines tend to have stronger transcriptional regulation in response to heat stress, possibly a reflection of their physiological sensitivity to the effects of warming. Furthermore, a bias towards TFs identified amongst down-regulated genes suggests a tendency towards TF inactivation for transcriptional repression in plants grown under warm conditions.

We next explored the functions of the identified TFs based on characterized orthologs in Arabidopsis. There were too few tolerant-specific TFs for GO enrichment testing. Nonetheless, when looking at individual TFs, there were numerous related to hormone signaling amongst those targeting down-regulated genes. On the other hand, many TFs were predicted from sensitive-specific DEGs or those detected in both tolerant and sensitive lines. When exploring functional terms amongst the Arabidopsis orthologs of sensitive-specific TFs targeting down-regulated genes, we observed enrichments

for terms relating to biosynthetic processes, development, and hormone signaling (**Figure 4C**), pointing towards sensitive lines halting growth and development. Among those TFs identified in both sensitive and tolerant lines, we observed enrichments related to ABA signaling and light stress. Additionally, multiple TFs involved in heat response, such as HSF4, were identified amongst these common TFs. Since many TFs were identified targeting down-regulated genes, we tested whether this regulation may reflect altered expression of the TF-encoding gene under warming conditions. Indeed, 47 TFs also displayed differential expression in at least one contrast (**Figure 5**). Among those, *HSF4*, *BASIC HELIX-LOOP-HELIX* (*bHLH*) *104*, *BASIC LEUCINE ZIPPER DOMAIN* (*bZIP*) *28*, and *LHY/CCA1-LIKE 1* (*LCL1*) are involved in heat responses. Many TFs related to ABA or ethylene signaling, and developmental processes were also observed. Therefore, tolerant and sensitive lines of *W. ceracea* appear to directly regulate the expression of a subset of genes encoding TFs under warm conditions in distinct patterns, which possibly relate to underlying differences in signaling pathways and/or susceptibility to heat stress.

Discussion

Elucidating the molecular responses of native plants to warmer temperatures could improve our ability to prepare for the impacts of climate change, especially in vulnerable environments such as the Australian alps. Here, we present a genome assembly, with associated transcriptome, of the Australian alpine herb *W. ceracea*. We utilize these resources to investigate gene regulation under warmer temperature in pre-defined heat-tolerant and sensitive lines (Arnold *et al.* 2024). We identified thousands of heat-responsive genes for which we infer functional information based on Arabidopsis orthologs. Notably, we identified hundreds of genes with altered regulation between sensitive and tolerant lines. Overall, under warm conditions, we found that tolerant lines regulate genes associated with translation, chloroplast organization, and photosynthesis, whereas sensitive lines prioritized RNA processing and carbohydrate metabolism. We could not identify an ortholog in Arabidopsis of 23.9% of the DEGs in our study; these may represent novel genes or long non-coding RNAs that are transcriptionally regulated by heat. This suggests that studying new genes and/or gene variants in more native species could improve our ability to predict responses to climate change, engineer stress-resilient crop varieties, and inform conservation strategies. Below we discuss the importance of these results for heat tolerance in *W. ceracea* and pinpoint knowledge gaps for further investigation.

Down-regulation of photosynthesis-associated genes is linked to heat tolerance in *W. ceracea*

The temperature corresponding to the maximum basal chlorophyll fluorescence (T_{max}) reflects the thermal threshold for photosynthesis beyond which thylakoid membranes are disrupted, PSII centres are

inactivated, and photosynthetic activity declines (Schreiber & Berry 1977; Ilík *et al.* 2003; Ducruet *et al.* 2007). Therefore, higher T_{max} should reflect an ability to sustain photosynthesis at higher temperatures. However, the genes that influence T_{max} are yet to be identified. Here, we observed that heat tolerant *W. ceracea* lines (higher T_{max}) down-regulated genes associated with thylakoid membrane organization and light harvesting reactions in warm conditions. These patterns are highlighted by expression changes in photosynthesis-related genes, including those encoding PSII components (*OEC33*, *ATPC1*) or antenna complexes (*LHCAs* and *LHCBs*, **Figure 3**). Similar observations have been made in *Nothofagus pumilio* where photosynthesis-associated genes, including those involved in light harvesting and chlorophyll biosynthesis, were down-regulated (Estravis-Barcala *et al.* 2021). This suggests *W. ceracea* is attempting to inhibit photosynthetic electron transport from PSII as a photoprotective mechanism to avoid PSI photoinhibition, which can otherwise lead to redox imbalance and impaired photochemistry (Zivcak *et al.* 2015; Jiang *et al.* 2021). We had also hypothesized that differential regulation of HSPs, chaperone proteins involved in the re-folding or degradation of misfolded proteins that accumulate during heat stress (Richter et al., 2010), may explain differences in heat tolerance. Contrary to expectations, HSPs were significantly up-regulated only in sensitive lines, suggesting a more severe reaction in these lines because of their sensitivity to heat. However, tolerant lines showed an enrichment for warming-induced genes related to protein folding while sensitive lines did not (**Figure 2A**). Inspection of these genes revealed the presence of many encoding chaperones, including *BINDING PROTEIN 1* (or *HSP70-11*), *CALNEXIN 1*, and *CALRETICULIN 1A* (**Supplementary Data 5**), which are involved in maintaining the native state of nascent proteins (Sun *et al.* 2021). These observations may reflect an attempt to maintain protein homeostasis (proteostasis), especially of photosynthesis-associated proteins. Indeed, after short-term light stress, a transcriptional up-regulation of *de novo* protein synthesis appears to counteract destabilization of photosynthesis-related proteins to maintain proteostasis (Li *et al.* 2022). Therefore, it would be insightful to investigate protein stabilities under long-term warming in *W. ceracea* and relate these to the changes in gene expression observed here, especially with regards to photosynthesis and protein folding related genes. Nonetheless, these results suggest that tolerant lines repress photosynthesis and prioritize fidelity of actively synthesised proteins in response to sustained warming.

RNA modifications and post-transcriptional regulation in native plants

Post-transcriptional regulation refers to a variety of processes occuring after transcription that can influence mRNA stability and translation (Sieburth and Vincent, 2022). Such processes are dynamic and coordinated with transcription during stress to manage protein production (Smith *et al.* 2024). This regulation is also impacted by the presence of RNA modifications, which can be stress-dependent (Prall *et al.* 2023). Whilst the study of plant RNA biology is rapidly expanding in models and some crops

(Manavella *et al.* 2023), their study in native plant species remains an unexplored frontier. We present evidence that genes involved in RNA-related processes, including RNA modification, RNA processing and transport, and translation were up-regulated under warmer temperatures (**Figure 2A**). This potentiates the involvement of post-transcriptional regulation in heat acclimation in *W. ceracea*. Such observations align with reports of abiotic stressors, such as heat and salt, impacting RNA stability through changes to RNA secondary structure or modifications (Anderson *et al.* 2018; Kramer *et al.* 2020). In Arabidopsis, heat triggers LA RELATED PROTEIN 1A (LARP1A) to associate with EXORIBONUCLEASE 4 (XRN4) to degrade over 4,500 transcripts, including some in a co-translational manner, which was required for survival at high temperatures (Merret *et al.* 2013). We also observed up-regulation of genes related to RNA splicing. While this occurs co-transcriptionally, it can have post-transcriptional impacts by, for example, instigating nonsense-mediated decay, a form of RNA surveillance that degrades translationally inert mRNAs (Drechsel *et al.* 2013). Similar to our observations, heat triggered the induction of genes related to the RNA splicing and translation in *Nothofagus pumilio*, Arabidopsis, and *Populus tomentosa* (Estravis-Barcala *et al.* 2021). Heat-induced alternative splicing can also produce protein variants with altered biochemical properties, such as thermostability, which can influence thermal tolerance (Reddy *et al.* 2013). Examples of this can be observed for RUBISCO ACTIVASE in wheat (Scafaro *et al.* 2019; Degen *et al.* 2020) and HSFA2 in Arabidopsis (Liu *et al.* 2013). These results highlight the potential of directly studying these mechanisms in native plants to better understand their ecological and evolutionary relevance, which is now possible with the proliferation of methods to assay these processes on a genome-wide scale (Prall et al., 2023).

Phytohormone-regulated gene networks may contribute to differences in heat sensitivity in *W. ceracea*

By building gene regulatory networks we associated hundreds of TFs with down-regulated genes in sensitive lines (**Figure 4B**). These sensitive-specific TFs may reflect the underpinning cause of their heat sensitivity, although a mechanistic link to physiological changes needs to be established. These TFs were enriched for functions related to biosynthetic processes, leaf and flower development, and hormone signaling, especially ethylene (**Figure 4C**). With regards to biosynthetic processes and development, this may reflect a consequence of having greater sensitivity to heat rather than the cause. However, the observation of orthologs of ETHYLENE-INSENSITIVE-LIKE 3 (EIL3) and multiple ETHYLENE RESPONSE FACTORs (ERFs) suggest that regulation by ethylene signaling could contribute to differences in heat tolerance (**Supplementary Data 7**). Indeed, ethylene signaling, through EIL3 and multiple ERFs, can stimulate heat responses by inducing HSFs (Huang *et al.* 2021; Poór *et al.* 2022). For example, rice seedlings treated with the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, had

increased chlorophyll *a* content, increased expression of HSFs, including HSFA1 and HSFA2A/C/D/E/F, and increased activity of detoxification enzymes that were associated with greater heat tolerance compared to mock-treated seedlings (Wu & Yang 2019). Interestingly, EIL3 over-expression was correlated with reduced hydrogen peroxide, increased proline, and increased survival under drought and salt stress in Arabidopsis and mulberry (Liu *et al.* 2019). Therefore, it is plausible that the down-regulation of gene networks regulated by EIL3 contributes to reduced heat tolerance in sensitive lines of *W. ceracea*. Leaf senescence is also induced by ethylene, through EIL3, as well as by increased temperature (Li *et al.* 2013; Kim *et al.* 2020). When relatively short-lived plants, such as *W. ceracea*, experience stress, ethylene-induced genes may be promoting cell turnover for nutrient remobilization to reproduction while activating defenses that ameliorate the impacts of heat stress (Schippers *et al.* 2015; Sade *et al.* 2018). This might be consistent with the repression under warm conditions of photosynthetic components in tolerant lines, which did not show the repression of genes associated with ethylene-responsive TFs (**Figure 2B**). Furthermore, prior work showed that *W. ceracea* plants with higher T_{max} produced more flowers (Notarnicola *et al.* 2021). Together, these results link ethylene signaling and nutrient management to heat tolerance in *W. ceracea*, thus warranting further investigation into whether there is a mechanistic linkage.

We also observed an enrichment of TFs, associated with down-regulated genes in sensitive lines, which respond to cytokinins (CKs) whose levels decline during heat stress (Dobrá *et al.* 2015). CKs can influence a range of processes including cell division, sugar accumulation, and stomatal opening, via reduced hydrogen peroxide generation, which may, collectively, contribute to heat tolerance (Song *et al.* 2006; Cortleven *et al.* 2019). Indeed, active CKs, especially zeatin, was associated with membrane stability and ameliorating oxidative damage during repeated heat stress in *Pinus radiata* (Escandón *et al.* 2016). Furthermore, Arabidopsis mutants deficient in cytokinin levels or signaling also showed impaired heat responses (Cerný *et al.* 2014). These reports align with our observations that tolerant lines down-regulated genes encoding key detoxification enzymes such as ASCORBATE PEROXIDASE (APX) 1, APX3, and STROMAL APX (sAPX) under warm conditions (**Supplementary Data 5**). The hypothesis is that tolerant lines maintain cytokinin signaling whereas sensitive lines do not, hence the latter showing an enrichment of cytokinin-associated TFs with down-regulated genes. This could be the result of maintaining, or accumulating, cytokinin levels during stress thus preserving their protective effects against oxidative damage. To test this would require cytokinin quantification and correlation with enzyme activity related to ROS detoxification (e.g. APX activity) in future studies.

Contrary to our expectations, we could only associate two HSFs with DEGs, namely HSFC1 (down-regulated genes in sensitive lines) and HSF4 (down-regulated genes in both tolerant and sensitive

lines). However, we linked numerous (14) TFs from the bZIP protein family to down-regulated genes (**Supplementary Data 7**). One possibility is that bZIPs are more relevant for longer term responses to warming than HSFs. Observations in Arabidopsis support this assertion, where HSFs were differentially regulated after short-term heat-shock but bZIPs became up-regulated with prolonged warming (Wang *et al.* 2020). There is also functional evidence that bZIPs contribute to heat tolerance. For example, bZIP60, which was associated with down-regulated genes in tolerant lines, promotes the heat-shock response in maize via induction of *HSFTF13* and other genes associated with chlorophyll catabolism and chloroplast protein turnover (Li *et al.* 2020b). We also identified bZIP28 herein, which has been reported to act in concert with HSFA2 to activate heat stress response pathways in Arabidopsis (Kataoka *et al.* 2017). These observations suggest that the activity of bZIPs may be important in *W. ceracea* and potentially other native plants for long-term heat tolerance. Despite identifying over 400 TFs from our gene regulatory networks, we only observed 45 of their encoding genes to be differentially expressed (**Figure 5**). However, the activity of many TFs, including HSFs, are influenced by post-translational modifications, especially phosphorylation (Richter *et al.* 2010). Therefore, to extend beyond our observations of differential regulation of TFs will be to investigate the phosphorylation status of those that were associated to down-regulated genes but not in and of themselves, differentially expressed. In conclusion, this study has assembled foundational genomic resources for *W. ceracea* and utilized these to identify candidate mechanisms contributing to heat tolerance and heat sensitivity in this Australian native plant.

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Data availability

Genome assembly and predicted annotations, including nucleotide and protein sequences, are available at figshare (10.6084/m9.figshare.27890484). The mRNA sequencing data is available at NCBI under GEO accession GSE274951.

Author Contributions

RFN, ABN, PAA, and DRG designed experiments. RFN conducted the experiment, RNA extractions, bioinformatic and statistical analysis, and wrote the manuscript. ABN and PAA assisted with sample harvesting. JH, AWJ, and BS performed DNA extraction, sequencing, and genome assembly and annotation. SL performed Illumina-based sequencing. DRG and ZF contributed to bioinformatic and statistical analysis. DRG contributed to RNA extractions. BS and SL contributed ideas to the manuscript. ABN, PAA, and DRG critically revised the manuscript.

Tables

Table 1: Thermal tolerance metrics and category across F_3 lines established in Arnold et al. (2024).

F3 line ID	Tolerance	T_{max} (± SD)
F3.485	Tolerant	55.48 (± 1.80)
F3.491	Tolerant	54.86 (± 1.32)
F3.495	Tolerant	56.14 (± 4.04)
F3.479	Tolerant	55.76 (± 3.02)
F3.467	Tolerant	57.10 (± 0.86)
F3.483	Tolerant	57.06 (± 0.23)
F3.457	Sensitive	49.32 (± 0.33)
F3.439	Sensitive	50.35 (± 0.42)
F3.475	Sensitive	50.51 (± 0.46)
F3.487	Sensitive	50.16 (± 0.96)
F3.477	Sensitive	52.52 (± 0.91)
F3.437	Sensitive	52.11 (± 3.20)

Table 2: Numbers of differentially expressed genes in *W. ceracea* for each contrast performed and their biological interpretation.

Contrast	Comparison	DEGs	Down-regulated (orthologs)	Up-regulated (orthologs)
I - Overall response	($Tolerant_{warm}$ + $Sensitive_{warm}$) - ($Tolerant_{cool}$ + $Sensitive_{cool}$)	6,140	2,932 (1,653)	3,208 (1,540)
II - Response in tolerant lines	$Tolerant_{warm}$ - $Tolerant_{cool}$	2,946	1,485 (861)	1,461 (747)
III - Response in sensitive lines	$Sensitive_{warm}$ - $Sensitive_{cool}$	3,721	1,665 (969)	2,056 (1,013)

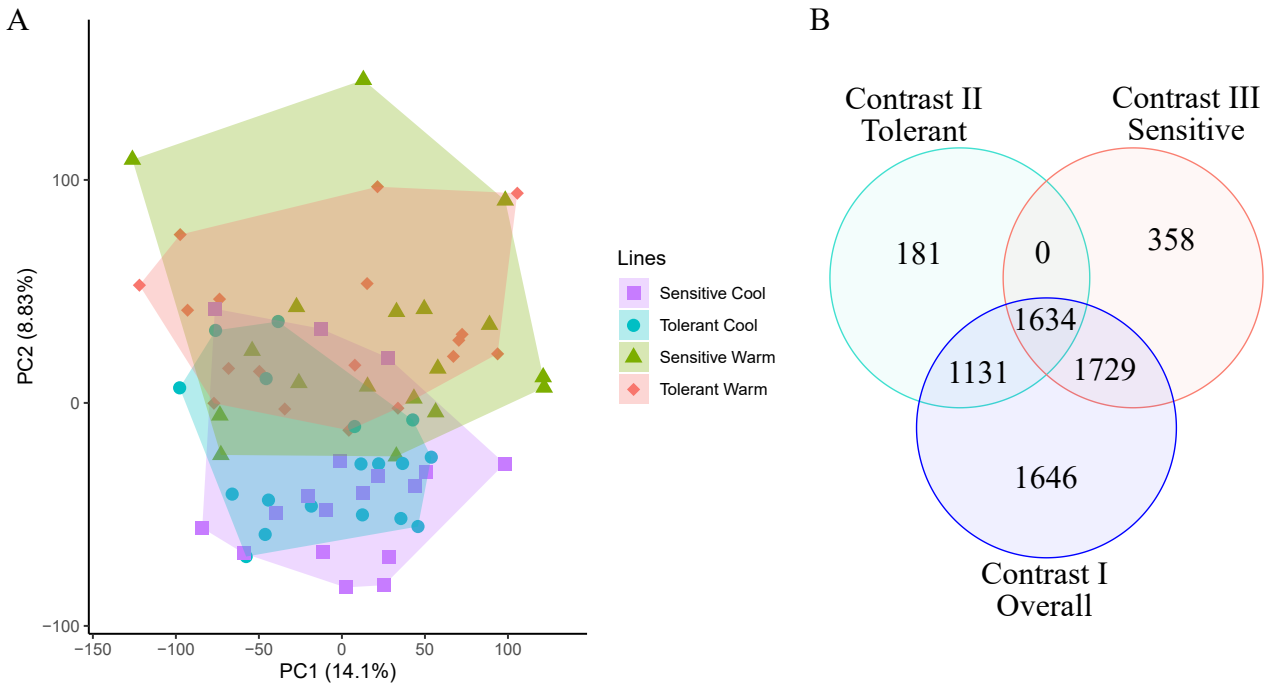

Figure 1 Differential expression analysis reveals warming-responsive changes in gene expression between tolerant and sensitive lines of *W. ceracea*
(A) PCA plot on gene level expression across all samples
(B) Venn diagram of heat-responsive DEGs detected in each contrast defined in Table 2.

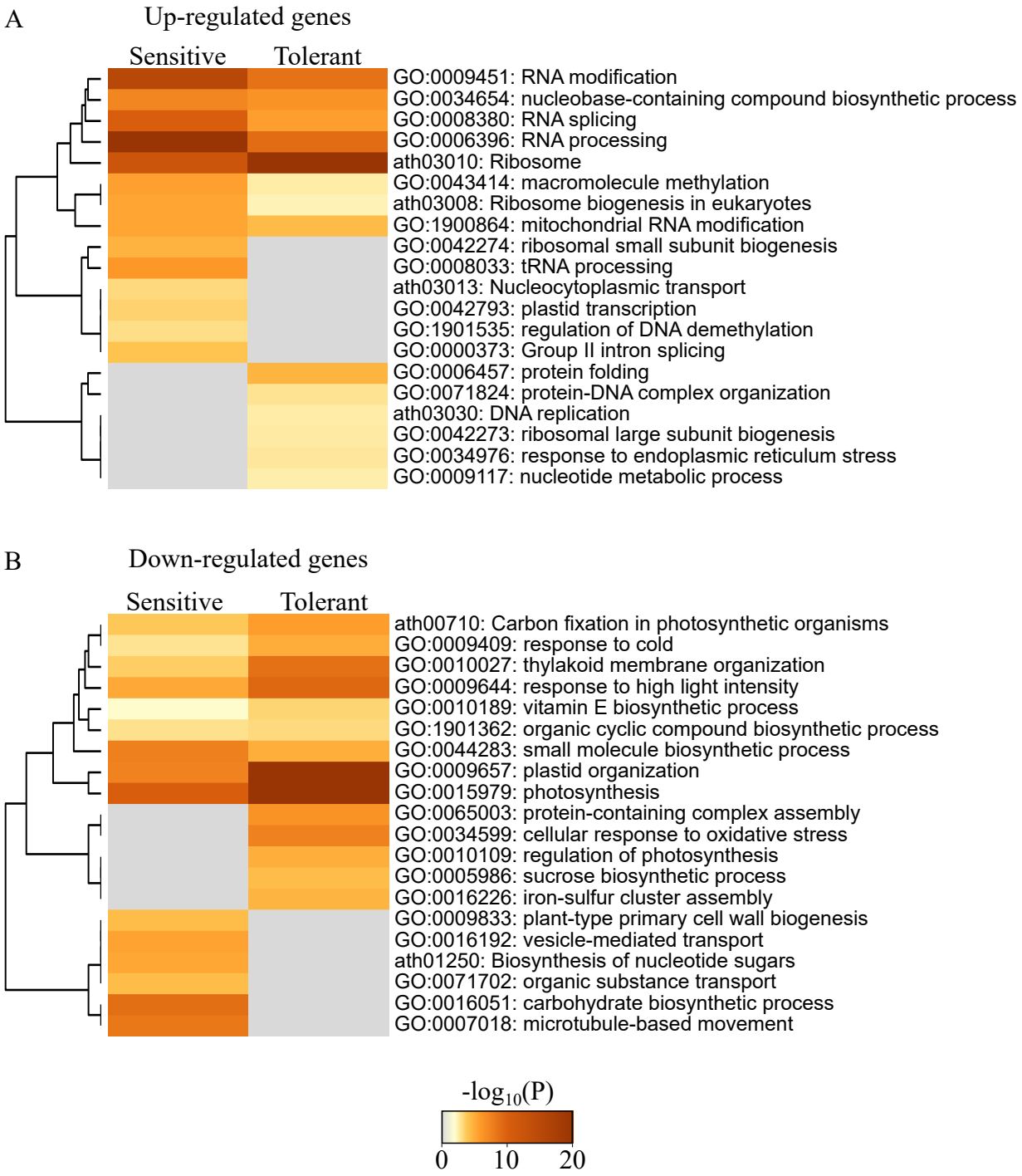

Figure 2 Functions of heat-responsive pathways in tolerant and sensitive lines of *W. ceracea*
(A-B) Heatmaps with one-dimensional hierarchical clustering of significantly enriched GO Processes and KEGG Pathway terms in (A) up-regulated and (B) down-regulated genes identified in tolerant and sensitive lines. Significant terms were clustered by Metascape; the terms with the highest -log (P-values) in each cluster were chosen as representatives. Cell colour denotes statistical significance (-$\log_{10}$ P-value; grey presents non-significant terms).

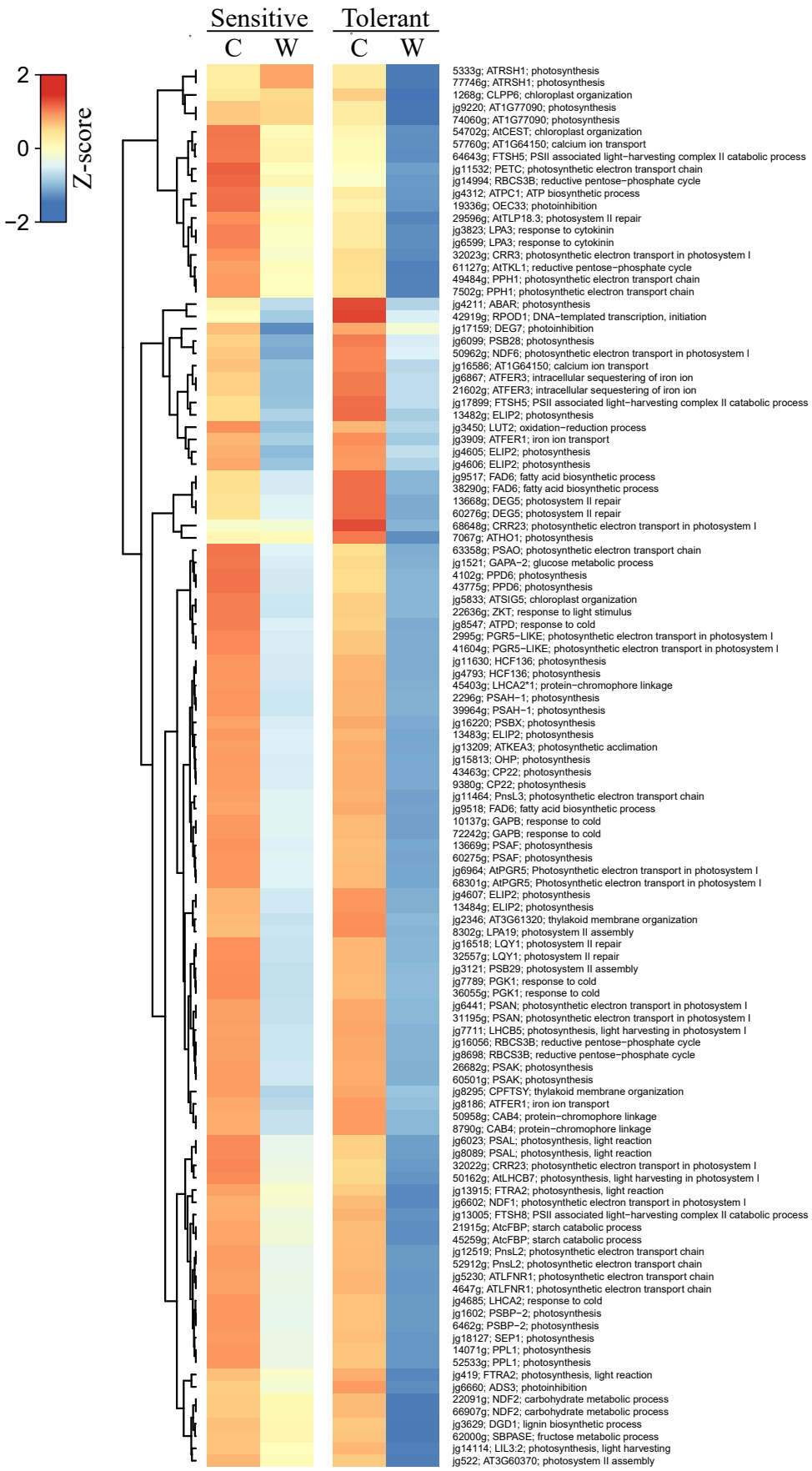

Figure 3 Expression of photosynthesis-associated genes in heat tolerant and sensitive lines of *W. ceracea*
Heatmap with one-dimensional hierarchical clustering of mean expression levels, normalized as Z-scores, of photosynthesis-associated DEGs. Labels denote abbreviated gene IDs, Arabidopsis gene symbols, and function. C = cool conditions; W = warm conditions.

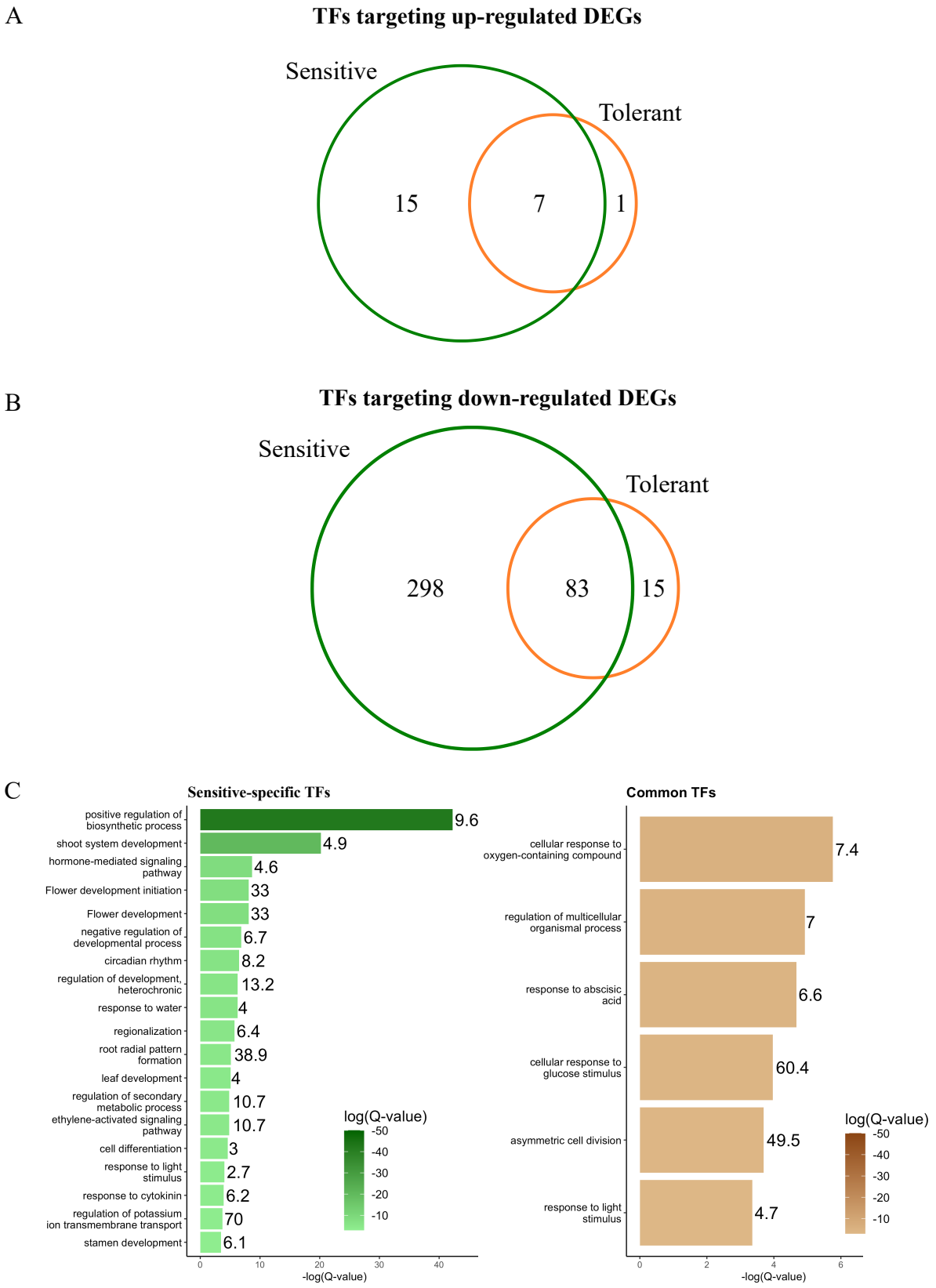

Figure 4 Transcription factors predicted to target heat-responsive genes
(A-B) Venn diagrams of the number of TFs identified by constructing gene regulatory networks with *TF2Network* using (A) up-regulated and (B) down-regulated genes detected in heat sensitive and tolerant lines of *W. ceracea*.
(C) Bar charts representing enriched functional terms for TFs, based on detected Arabidopsis orthologs, identified as targeting down-regulated genes in sensitive lines or both (common). Numbers denote the fold enrichment of genes in each category.

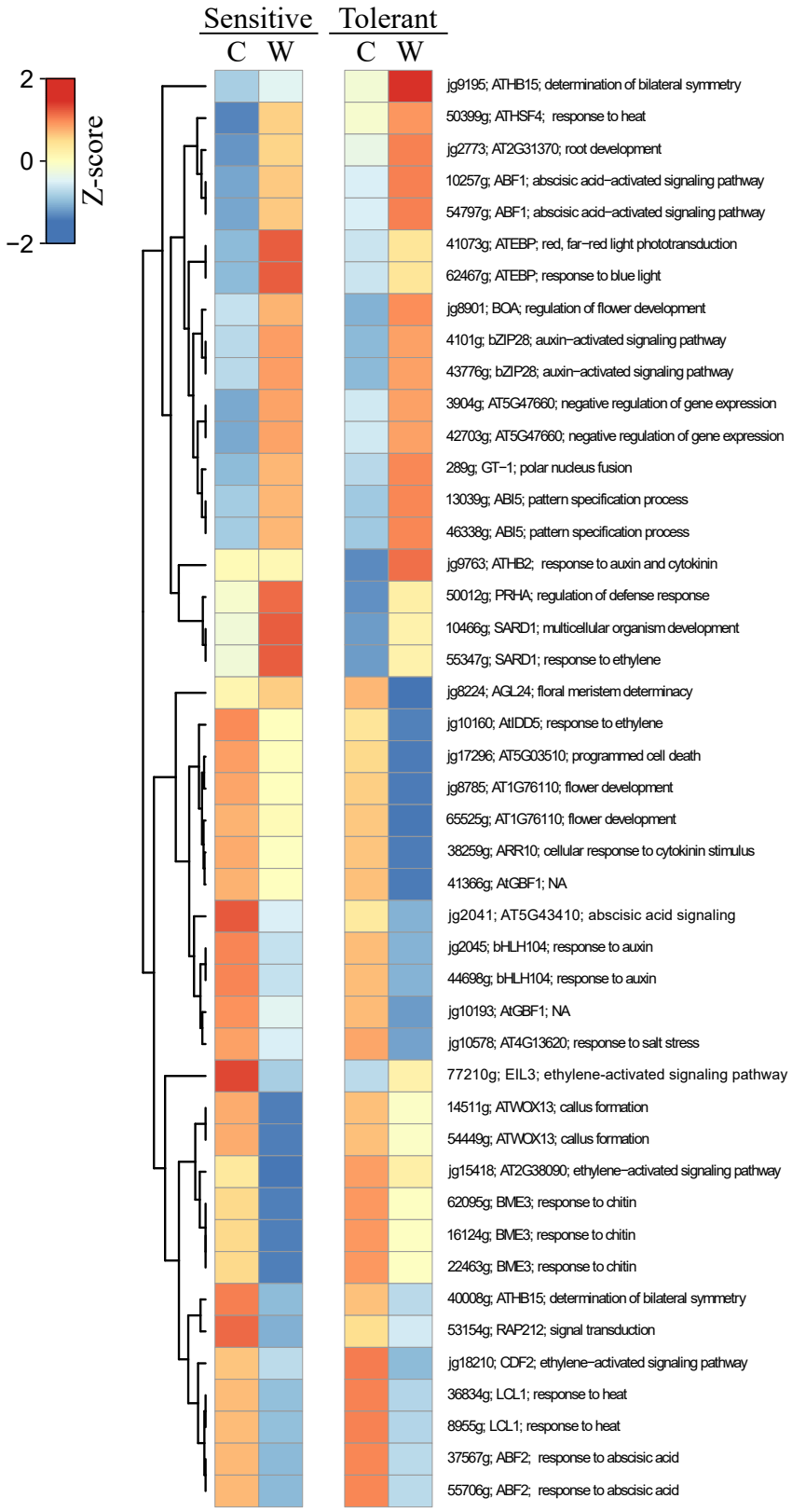

Figure 5 Mean expression of differentially expressed genes encoding transcription factors
Heat map with one-dimensional hierarchical clustering representing mean expression (Z-scores) of transcription factors identified by TF2Network that were also differentially expressed under warm conditions. Expression across *W. ceracea* paralogs was averaged. Labels denote *W. ceracea* gene ID, Arabidopsis gene symbol, and the most significant GO process. C = cool conditions; W = warm conditions.