Withstanding heat waves: proteomic analysis of adaptive thermotolerance in *Eucalyptus grandis* seedlings

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Declaration

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- Mass spectrometry analysis of peptides was performed by Dr Mehdi Mirzaei.
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- Owen Watson assisted with the processing of leaf samples.
- Yunqi Wu, Samantha Emery and Azadeh Fallahbaghery assisted with the optimisation of the protein extraction protocol.
- Associate Professor Brian Atwell assisted in the experimental design.
- My supervisors Associate Professor Ian Wright, Dr Rachael Gallagher and Dr Mehdi Mirzaei assisted with the experiment design, acquisition of data and editing my drafts.

All other research described in this report is my own original work.

Timothy Maher

Note to examiners

This thesis is written in the form of a journal article from the *Journal of Ecology* adhering to most of the author guidelines (Appendix II) apart from instances where the guidelines go against the Macquarie University thesis formatting requirements.

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Data accessibility

The following supplementary files can be found in the Dropbox folder " Supplementary data files- *E. grandis* TMT analysis" via the following link:

https://www.dropbox.com/s/v8d7oinpobhhp9p/Supplementary%20data%20files-%20E.%20grandis%20TMT%20analysis.xlsx?dl=0

TMT 1-Provenance 1: E. grandis dataset (all 3284 proteins identified)

TMT 2- Provenance 4: E. grandis dataset (all 3171 proteins identified)

TMT 3- Provenance 5: E. grandis dataset (all 3051 proteins identified)

TMT 4- Provenance 6: E. grandis dataset (all 3180 proteins identified)

TMT 5- Provenance 3: E. grandis dataset (all 3208 proteins identified)

TMT 6- Provenance 2: E. grandis dataset (2933 all proteins with expression values, fold changes)

Significantly diff expressed: All 532 proteins found to be differentially expressed

HSPs differentially expressed: All heat shock proteins differentially expressed

Top 10 upregulated: The top ten most upregulated proteins for each provenance under 42°C

DE across all: Proteins significantly differentially expressed across all provenances under 42°C

Contents

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Abstract

Heat wave events have been implicated in an increasing number of large scale die-off events in eucalypt-dominated forest throughout Australia. Being sedentary, plants in part rely on the expression of a specific set of proteins for heat acclimation and survival. However, the specific patterns of protein expression that underlie variability in important ecological traits like thermotolerance are rarely investigated in native plant species.

This study tested the capacity of six provenances of *Eucalyptus grandis* (Flooded Gum) arrayed along a strong temperature gradient to respond to experimental manipulations of heat shock. Recently sequenced*, E. grandis* is a dominant species in forests along the east coast of Australia and a key species in forestry plantings. A comparative proteomic approach was used to characterise types and abundances of proteins involved in the heat shock response between seedlings grown from different provenances. We focused on seedlings as they are most vulnerable to intense heat conditions at the soil surface and to have limited access to soil moisture due to shallow root systems. *Synthesis*: Seedlings from southern provenances that experience more frequent heat stress, demonstrated higher levels of acquired thermotolerance and may have greater adaptive capacity to cope with increased heat waves under climate change.

Introduction

Heat waves: an increasing phenomenon under future climates

Extreme climatic events are increasing in frequency and severity as a result of anthropogenic climate change with the potential for significant negative impacts on biological systems (Della-Marta *et al.* 2007; De Boeck *et al.* 2010; Fishcher & Schär 2010). Of particular concern is the increase in heat wave conditions across many regions globally (Perkins, Alexander & Nairn 2012). While heat waves are differently defined around the world according to local climate characteristics, they are typically described as continuous periods of above average temperatures and are largely a summertime phenomenon (Teskey *et al.* 2015). The proportion of the global land surface experiencing anomalous temperatures – defined as > +3 standard deviations warmer than baseline period 1951-1980 – increased an order of magnitude by the period 2006-2011 (Hansen, Sato & Ruedy 2012).

Instances of record-breaking monthly temperatures are also rising (Coumou & Robinson 2013; Coumou, Robinson & Rahmstorf 2013). For example, since 2000, continental Europe has experienced the five largest heat wave events since record keeping began in 1871 (Barriopedro *et al.* 2011). Over coming decades, this trend in heat wave frequency and intensity is projected to continue to increase (Meehl & Tebaldi 2004; Teskey *et al.* 2015). However the potential impact of heat wave conditions on species and communities remains relatively poorly characterised as studies focus largely on gradual climatic trends, such as increases in average temperature and their potential effects on biological systems (Jentsch, Kreyling & Beierkuhnlein 2007).

Although heat waves are a regular summertime phenomenon in Australia, projected increases in their frequency and intensity may have negative consequences for its biota. In Australia, heat waves are commonly defined using an Excess Heat Factor index which compares high temperature conditions over a three-day period to conditions in both the preceding month and the 95th percentile of a climatological average (Nairn & Fawcett 2013). The frequency, duration and severity of heat waves varies across Australia, with southern parts of the continent, where daily summertime temperature fluctuations are largest, shown to be most vulnerable (Tryhorn & Risbey 2006). By contrast, northern parts of Australia experience fewer and less extreme heat waves due to the coincidence of wet season conditions during summer which limit daily temperature fluctuations (Nairn & Fawcett 2013).

Heat wave events also occur with large inter-annual variability across Australia. Individual years may experience multiple or much longer heat wave events (up to 5-days of above 95th percentile temperatures) (Nairn & Fawcett 2013; Perkins & Alexander 2013). Heat wave patterns have changed both temporally and spatially in Australia over recent decades (Tryhorn & Risbey 2006; Alexander *et al.* 2007; Perkins, Argüeso & White 2015) with rates of extreme climatic events increasing faster relative to average changes in climate conditions (Alexander *et al.* 2007). A number of projections show that these patterns, like global trends, are expected to increase in frequency, intensity and duration in the coming century (Alexander *et al.* 2007; Cowan *et al.* 2014).

Plant responses to heat-wave conditions

Plants, being sessile, lack an ability to behaviourally respond to external stimuli and are limited in their capacity for leaf-cooling by the availability of water. These factors make plants particularly vulnerable to fluctuations in environmental conditions (Feder & Hofmann 1999). At the cellular level, many biological processes undergo large changes in response to particular temperature thresholds (Easterling 2000). Beyond these thresholds plants undergo heat stress which can impair cell functioning and induce permanent damage to tissues and membranes (Kregel & Sieck 2002; Wahid *et al.* 2007). For example, proteins which are particularly heat sensitive may become misfolded, begin to aggregate and be transported to incorrect locations within the cell, disrupting normal physiological functioning (Vierling 1991).

Vital processes such as photosynthesis and photorespiration are also temperature dependent and their interruption may have serious flow-on effects for growth (Teskey *et al.* 2015). For instance, a manipulative experiment on tree seedling performance under heat wave conditions showed substantial reductions in gas exchange and biomass in exposed seedlings relative to controls, though the interactive effects of elevated CO_2 and high water availability tempered this response (Ameye *et al.* 2012).

Organisms have evolved molecular pathways that allow the detection of even subtle changes in ambient temperature and mounting of suitable heat stress response (Mittler, Finka & Goloubinoff 2012). However, it is unclear how adequate these adaptive mechanisms may be under the magnitude of expected increases in heat stress due to climate change (Liu & Huang 2000). During a heat stress response, plants may upregulate the expression of certain thermotolerance proteins to aid in establishing an equilibrium of metabolic processes that

maintain normal cell function at elevated temperatures (Mittler *et al.* 2012). Thermotolerance is categorised as being either basal or acquired. Basal thermotolerance describes the inherent ability of an organism to survive excess heat, for example protein stability under heat. In contrast, acquired thermotolerance describes the acclimation ability provided directly by the accumulation of thermotolerance proteins (Bokszczanin & Fragkostefanakis 2013).

At larger scales, the influence of temperature on function influences both the geographic distribution of plants (Bokhorst *et al.* 2007; Zimmermann & Yoccoz 2009) and their survivorship (Niu *et al.* 2014; Myburg *et al.* 2014). Recent increases in extreme heat events attributed to human influence on the climate system are having direct and indirect effects on vegetation globally. For example, the number of large-scale forest die-back events increased over the last century, with heat and drought stress among the key drivers (Allen *et al.* 2010). A review of worldwide events of large scale forest die-back by Allen *et al.* (2010) identified 88 instances of forest die-back linked to climatic changes since 1970. These different events ranged from more local increases in tree mortality to regional-scale forest mortality. Key examples include the widespread forest die-off over southern parts of Europe between 2003-2008 and an almost four-fold increase above background mortality in boreal forest of Western United States between 1955-2007 (van Mantgem *et al.* 2009).

Although increased drought stress is the most common driver for these forest mortality events, heat waves have also shown to be an important contributing factor (Allen *et al.* 2010). Drought and heat stress often co-occur and this interplay leads to an intensification of their respective effects, with excessive heat exacerbating moisture stress in plants (Anderegg, Kane & Anderegg 2013). However, there are also instances of mass tree die-off events in the absence of drought stress (Anderegg *et al.* 2013). The effect of climate induced forest die-off events, which change community dynamics and lead to shifts in tree species distribution, can cascade through ecosystems by affecting the wider composition of flora and fauna (Butt, Pollock & McAlpine 2013).

Increased incidence of heat waves have also been implicated in forest mass mortality events and reduction of primary production in Australia (Fensham & Holman 1999; Matusick *et al.* 2013; Ross & Brack 2015). For instance, Australia's Mediterranean-type forests – which have evolved under regular climate fluctuations and periods of heat disturbance – suffered a severe large-scale forest die-off event in 2011 centred on Northern Jarrah Forest in the southwest of Western Australia (Matusick *et al.* 2013). This mass die-off event was triggered by a combination

of multiple years of record dry and hot temperatures and a prolonged record breaking heat wave event (9 days above 35°C) during summer. Tree crowns of a number of species were affected resulting in collapse of the canopy across a number of ecosystems in this region (Matusick *et al.* 2013). In an initial survey of the 16,515 ha of affected forest, Matusick *et al.* (2013) reported that 74% of all previously living stems were found to be dead or dying, compared to 11% in paired control plots. On reassessment of the affected sites the following spring, these authors recorded a final stem mortality of 26% compared to 5% for control plots. This die-back occurred despite the adaptations of tree species in Mediterranean plant communities which increase survivability under extreme and frequent climatic fluctuations such as embolism repair mechanisms (Nardini *et al.* 2014) and volatile stress defence compounds in leaves (Loreto *et al.* 2014). Although, Mediterranean plant communities are considered resilient relative to other Australian forest types, these die-back events indicate that increases in heat waves may exceed their adaptive capacity (Matusick *et al.* 2013). As a result, the community composition and structure may be dramatically altered and may compromise the ecosystem services they provide (Mueller *et al.* 2005; Guardiola-Claramonte *et al.* 2011).

It is important to note that large-scale die-off events have also negatively affected forestry output and harvesting globally, significantly depleting short and mid-term timber stocks (Anderegg *et al.* 2013). Heat waves can reduce timber production by impacting the carbon fixation ability of trees among natural and cultivated stands (Anderegg *et al.* 2013). A study by Bastos *et al.* (2014) demonstrated that forest regions corresponding to the highest temperature anomalies during the 2003 and 2010 European heat waves experienced reductions of −6.7 TgC and −40.7 TgC of the average annual productivity of these respective forest regions (equivalent to losses of about 20% and 50%). Model simulations of productivity showed that the European heat wave of 2003 led to the largest Europe-wide reduction in forest productivity of the past century (20% below the 1960–1990 average) (Ciais *et al.* 2005). Given the potential economic impact of forestry production loss, investigating responses in those species and populations which may be at risk of large-scale tree die-off under temperature extremes is essential.

Vulnerability of trees to heat waves

Different plant growth forms may respond to heat wave conditions in diverse ways. For example, some of the characteristics of trees may make them particularly vulnerable to relatively rapid changes in climatic conditions, including a perennial life-cycle which is characterised by lengthy time to maturity and seed production relative to annual or biennial species. This may result in a

significant lag in tree species responding to changes in climate (Reyer *et al.* 2013) because longer generation times may decrease the rate at which heritable adaptations to novel conditions can be passed to offspring and the rate at which new beneficial mutations can accumulate (Reyer *et al.* 2013). For example, Rehfeldt, Wykoff & Ying (2001) approximate that coniferous tree species require 2-12 generations (200- 1200 years) to start accumulating adaptations to changes in climatic conditions. In addition, trees face the added challenge of maintaining an adequate supply of water to leaves which are held long-distances from the soil surface, high in the canopy. Preventing and repairing embolism in the transpiration stream is crucial for maintaining basic functions such as photosynthesis and respiration (Hubbard *et al.* 2001). Many tree species function with narrow hydraulic safety margins and under extreme dry conditions may be more vulnerable to drought induced embolism than other plant growth forms (Lens *et al.* 2013).

Heat waves may also differentially affect plants at different demographic stages, in particular during the seedling phase. A successful seedling phase is crucial for the establishment of plant populations making it essential to understand the impact of climate change on this key demographic phase. Seedlings have been shown to be particularly sensitive to environmental stresses, resulting in greater mortality relative to other life-history stages (Cook 1979; Antonio Gazol and Ricardo Ibáñez 2012). Compared to mature trees, they possess characteristics that may make them particularly vulnerable to damage from heat wave conditions. For example, seedlings typically possess shallow root systems, high transpirational surface area to volume ratios, and are close to the soil surface where air temperatures can be much higher than the ambient air (Donovan, Mausberg & Ehleringer 1993). Root systems increase proportionally with size and seedling roots are typically too shallow to access stable groundwater supplies (Niinemets 2010). Therefore, unlike mature trees, seedlings are highly reliant on surface soil water for growth, increasing the likelihood of water stress.

Limited access to water may also reduce seedling ability to use transpirational cooling to maintain optimal leaf temperatures for photosynthesis under heat stress (Cui & Smith 1991; Donovan *et al.* 1993). Field based evidence shows that under climate stress, younger stands - especially seedlings - often have much higher mortality rates than mature trees (Valladares *et al.* 2014). Therefore, a clear understanding of the resilience of forests in response to heat events requires further investigation of what factors influence seedling survival (Niinemets 2010).

Proteomics as a tool for understanding plant adaptive capacity to climate change

Identifying the specific cell pathways that underlie particular stress responses is an enduring theme in plant biology, but their inherent complexity has made this a challenging task (Ungerer, Johnson & Herman 2008). Prior to the genomic era, molecular-biological approaches provided limited insights by only identifying individual genes, at any one time, associated with abiotic stress (Hirayama & Shinozaki 2010). However, advances in molecular techniques and genome sequencing of species have made it possible to characterise genome-wide changes in expression. This has given rise to the pursuit of systems biology which combines the technologies and corresponding fields of transcriptomics, proteomics and metabolomics (Abril *et al.* 2011) and provide opportunities for much broader investigation of the abiotic stress response at different genomic levels (Hirayama & Shinozaki 2010). Unlike former methods, these techniques are not based on assumptions of which genes are important, but instead provide a survey of those pathways involved in responding to the variable of interest (Feder & Walser 2005).

Transcriptomic studies which characterise variation in gene expression through measuring mRNA levels are most commonly used to decode expression pathways important to stress tolerance (Diz & Calvete 2016). However, studies have shown transcript abundance correlates poorly with protein abundance (Papakostas *et al.* 2012; Diz & Calvete 2016) largely because post-transcription mechanisms play a big part in influencing protein abundance (Plomion *et al.* 2006). Because of this, variation in expressed proteins may offer a more accurate representation of an individual's phenotype, which is the basis of selection (Diz & Calvete 2016).

Proteomics studies can be used to identify protein markers which are linked to physiological performance under a particular stress and use their abundance to compare the level of stress tolerance between natural populations (Kosova & Renaut 2011). One well-studied and important class of proteins involved in the heat stress response pathway are heat shock proteins (HSPs). Acting as molecular chaperones, HSPs function by attaching to and refolding denatured proteins, transporting proteins to the correct location within the cell, preventing abnormal protein aggregation and disposing of degraded proteins (Wahid *et al.* 2007; Zhang *et al.* 2015). Although this group of proteins are most strongly expressed in response to heat stress, they are fundamental to the general stress response and are induced to some degree by many environmental stressors (Barua, Downs & Heckathorn 2003). HSPs have been strongly conserved throughout evolution and are found across all taxa from bacteria to mammals (Feder & Hofmann 1999).

A number of HSP sub-classes are recognised according to their molecular weights and sequence homology: Hsp100, Hsp90, Hsp70, Hsp60 (Chaperonins) and the small HSPs (sHSPs) with molecular weights between (15–30 kDa) (Georgopoulos & Tissières 1994). Within these sub-classes, HSPs may differ in their localisation, function and relative abundance in the cell (Feder & Hofmann 1999). Compared to other taxa, the sub-class of sHSPs is particularly diverse and abundant in plants and the most reactive to heat (Vierling 1991; Howarth & Ougham 1993). For instance, plants possess up to 20 different sHSPs compared to only a few types present in other taxa (Waters, Lee & Vierling 1996). A number of transgenic experiments in *Arabidopsis thaliana* L. as well as other plant species have confirmed the link between increased HSP levels and improved physiological functioning under heat stress (reviewed by Grover *et al.* 2013). For instance, Neta-sharir *et al*. (2005) demonstrated increased thermotolerance in tomato, by manipulating the over-expression of Hsp21. Another study showed that the inhibition of HSP expression in an *A. thaliana* mutant resulted in reduced thermotolerance under heat stress (Lee & Schöffl 1996). HSPs are therefore vital elements in the plant heat response and serve as informative molecular markers for thermotolerance.

In investigating the ecological importance and role of HSPs, their potential costs must also be considered. The benefits of these acquired thermotolerance mechanisms in plants suggest that through evolution their expression should be maximized under heat stress (Feder & Hofmann 1999). However, a number of studies in fruit flies have demonstrated that the frequency and degree of HSP induction under extreme temperatures can have negative fitness effects on growth, reproduction and survival (Feder *et al.* 1992; Krebs & Feder 1998; Silbermann & Tatar 2000). One study in particular showed that increases in levels of HSP 101 in *A. thaliana* under heat stress were accompanied by an increasing reduction in root allocation (Tonsor *et al.* 2008). This may be explained by the synthesis of heat tolerance proteins taking up a large proportion of the cell's energy and nutrient potential and thereby reducing the cells ability to produce the necessary biomolecules involved in other ongoing cell processes (Feder & Hofmann 1999). Alternatively, the increased expression of heat tolerance proteins may lead to concentrations that have toxic effects and that interfere with other cell processes (Feder & Hofmann 1999). This has been thought to be responsible for plants relying more on basal thermotolerance mechanisms instead of acquired thermotolerance mechanisms to withstand extreme heat in some circumstances (Barua, Heckathorn & Coleman 2008; Amano, Iida & Kosuge 2012)

A variety of other types of proteins have also shown to be upregulated and are thought to be involved in the heat stress response (Kosova & Renaut 2011). These include proteins and protein classes such as Pir proteins, ubiquitins, dehydrins, late embryogenesis abundant (LEA) proteins, thioredoxin h, glutathione S-transferase and dehydroascorbate reductase , cytosolic Cu/Zn-superoxide dismutase and Mn-peroxidase (Bokszczanin & Fragkostefanakis 2013). These are thought to serve a number of roles in the heat stress response such as countering the effects of oxidative stress and dehydration. However, these protein species seem to be less important than HSPs in acquired thermotolerance under acute heat stress (Grover *et al.* 2013).

Study aim: understanding heat stress at the molecular level in natural populations arrayed along a temperature gradient

In this study, we investigate the relative capacity of seedlings grown from natural populations of *Eucalyptus grandis* W. Hill ex Maiden*,* collected along a latitudinal gradient of increasing mean annual temperature (MAT), to withstand and recover from extreme heat events. Our questions are tested using a comparative proteomics approach which characterises differentially expressed proteins in seedlings exposed to simulated extreme heat events in growth chambers. We characterise and compare protein level responses to heat waves across six seedling provenances based on: the entire complement of proteins extracted (the 'proteome'), but focusing especially on HSPs – an important suite of proteins known to be crucial in heat response.

To date, most proteomics studies on heat stress in plants have been based upon classical model species such as *Arabidopsis* and agricultural plants such as rice, wheat and grapes (Neilson *et al.* 2010; Mirzaei *et al.* 2012; George *et al.* 2015). These species, which are often economically important crops , have been artificially selected for a range of traits such as stress resistance and increased growth rate (Leakey *et al.* 2009). Therefore, the types and abundances of HSP expression in these species may provide limited insight into heat responses which have evolved among natural plant populations (Leakey *et al.* 2009).

While former studies have investigated phenotypic differences between populations of *Eucalyptus* species over broad environmental gradients in Australia (Dillon *et al.* 2014; Drake *et al.* 2015), we are aware of no studies exploring evolved differences in HSP expression and heat tolerance under heat stress in this genus. This paucity of studies may be largely due to a lack of annotated sequence data for investigating *Eucalyptus* species. One recent key advance is the genome sequencing of two dominant *Eucalyptus* species, *E. grandis* and *E. camaldulensis* (Myburg

et al. 2014). In particular, *E. grandis* is a suitable candidate species for investigating heat tolerance mechanisms because it occurs across a broad temperature gradient on the east coast of Australia, increasing the likelihood of adaptive variation to different heat conditions. The findings of this research will aid in predicting *E. grandis* population resilience under increased extreme heat events and inform climate change planning for this species. In addition, this research will benefit forestry practitioners by identifying more heat resilient provenances of *E. grandis*.

Eucalypts offer an informative model through which plant heat response can be investigated in an Australian context. The genus *Eucalyptus* comprises over 900 species, subspecies and varieties which dominate the majority of forest ecosystems across Australia (Butt *et al.* 2013). Eucalypts are the dominant tree species in a wide variety of vegetation types throughout Australia and are often regarded as a foundation species in their respective communities (Grattapaglia *et al.* 2012). A number of eucalypt species are also important for industry and are widely cultivated across the globe as a source of hard wood timber and pulp (Grattapaglia *et al.* 2012).

Native eucalypt forests and woodlands have recently undergone a number of large scale die off events (Fensham & Holman 1999; Bennett *et al.* 2013; Ross & Brack 2015), which may indicate dominant species are rapidly approaching or exceeding their climatic tolerance limits (Butt *et al.* 2013). Although the dispersal potential of *Eucalyptus* seeds is typically high (Booth *et al.* 2015), the range distribution of many *Eucalyptus* species is constrained by other environmental factors, such as soil type and drainage, which may impede their tracking of suitable climate conditions (Hughes 2012). Due to these factors, understanding the adaptive capacity for eucalypts to respond to heat waves *in situ* is vital for long-term climate change adaptation planning and also maintaining yields in forestry.

Forest-tree species have long been known to show substantial adaptive variation between populations in response to environmental stress (Matyas 1996). This was initially established in forestry through common-garden experiments, where stands sourced from different provenances (population at a defined geographical location) are grown under the same conditions to test relative performance and survival (Matyas 1996). Over time, these studies have demonstrated that plant species display strong clinal variation in genes and functional traits over natural environmental gradients through local adaptation (González-Martínez, Krutovsky & Neale 2006). While these studies originally focused on tree traits relevant to industry, they have gone on to

demonstrate intraspecific variation in a range of morphological, phenological and growth characteristics over environmental gradients (González-Martínez *et al.* 2006). For instance, climate driven differences in the concentration of foliar nitrogen and photosynthetic performance have been demonstrated across latitudinal gradients of increasing mean annual temperature in widely distributed forest trees (Oleksyn *et al.* 1998; Tjoelker *et al.* 2008). It is also likely that differences in the temperature regime between populations arrayed along a latitudinal gradient should lead to intraspecific variation in the expression of proteins involved in thermotolerance.

Despite being highly conserved across taxa, the abundance and types of HSPs present under heat stress have been shown to vary both within and between species (Feder & Hofmann 1999; Barua *et al.* 2008). Recent studies have set out to investigate the level of natural population variation in HSPs and how this relates to the background conditions from which organisms are sourced (Amano *et al.* 2012). Such studies often set out to establish whether individuals from less stressful environments produce a different heat response under extreme conditions compared to those from a more stressful environment (Feder & Hofmann 1999). Studies have addressed these questions by characterizing the molecular mechanisms of natural populations along naturally occurring gradients of environmental stress (Barua *et al.* 2003, 2008; Zhang *et al.* 2015). These studies have shown trends between heat tolerance protein expression and heat tolerance along a heat gradient (Knight & Ackerly 2001). However, the majority of these studies have focused on animal species such as fruit flies (Hoffmann, Sørensen & Loeschcke 2003) or commercial cultivars of plants such as maize (Ristic *et al.* 1998) and there remains a paucity of studies investigating variation in HSP content between natural populations (Feder & Hofmann 1999; Zhang *et al.* 2015)

In this study, we set out to test the following questions about heat stress response in seedlings grown from populations arrayed along a gradient of increasing temperature which were exposed to heat wave conditions in growth chambers:

(1) Is there a relationship between the differential expression of proteins associated with heat shock and the temperature conditions associated with each seedling provenance? We predict differences in the proteome and specifically in HSP content which correlate positively with increasing local temperature of seedling provenances. That is, we expect that, relative to seedlings from the cooler southern edge of the range, seedlings from warmer northern locations (see Fig. 2.) are likely to express a greater abundance of HSPs and proteins associated with heat stress response due to long-term adaptation to hotter conditions.

(2) Does the magnitude of the heat stress affect the differential expression of proteins? Seedlings were exposed to one of three heat wave treatments (28 °C, 35 °C, 42 °C for four consecutive days). We predicted increases in the abundance of heat stress response proteins with increasing temperatures and expect response to correlate with the adaptive conditions experienced along the temperature gradient in the field.

Materials and Methods

Study species

E. grandis, is a subtropical hardwood tree species naturally distributed across a broad latitudinal gradient on the east coast of Australia from Newcastle in New South Wales to the Daintree region of northern Queensland (Fig. 1). It occurs as a series of disjunct populations that are largest at the southern extent of its range and become increasingly smaller and scattered towards the northern extent (Drake *et al.* 2015). A dominant member of plant communities in fertile alluvial soils, *E. grandis* also likely provides food resources and habitat for a wide variety of vertebrates and invertebrates (Pellow *et al.* 2009).

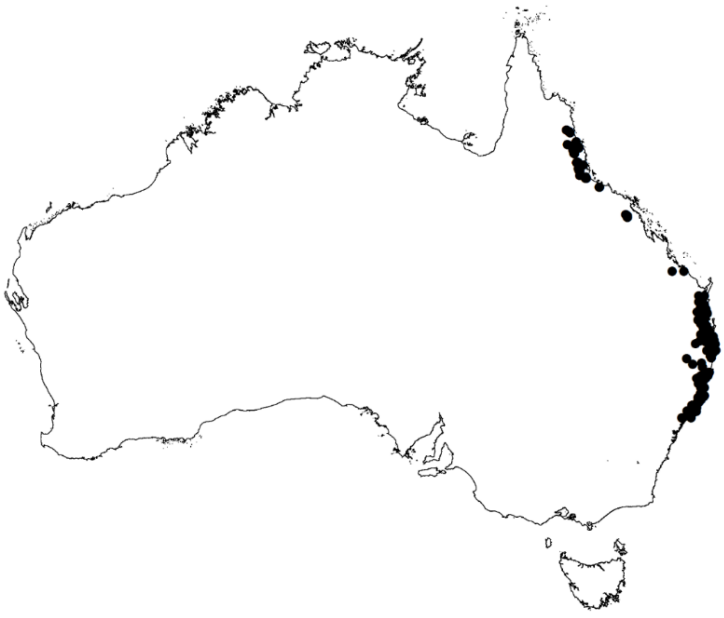

Fig. 1. Map of natural distribution *E. grandis* along the east coast of Australia.

E. grandis is one of the largest species in the genus, reaching between 45-55m at maturity and as a result is a key timber plantation species with major economic importance both in Australia and overseas, especially in Brazil and South Africa (McMahon *et al*. 2010). Its ease of cultivation, straight form, fast growth and utility in a variety of end products underpins its status as one of the most widely grown hardwood species globally, planted in over 100 countries on more that 20 million ha[2] (Myburg *et al.* 2014, McMahon *et al*. 2010).

Provenance selection

Seed provenances of *E. grandis* were sourced from the CSIRO Australian Tree Seed Centre (ATSC) (Canberra, ACT). Seed was previously collected from > 10 adult trees at each of six coastal, low altitude provenances spanning the natural distribution of *E. grandis* (Fig. 2). Here we use provenance to describe populations that are genetically and geographically distinct. Due to the sample distance between provenances, it is reasonable to believe that the chosen provenances are distinct with limited gene flow occurring between them. Provenance locations are listed in Table 1. Provenances were selected by matching all potential seed sampling locations for *E. grandis* seed-lots held within the ATSC to gridded climate data. Climate data (monthly mean, maximum, and minimum temperature averages between 1950-2000 at the respective sites) was sourced from ANUCLIM1.0 via e-MAST (http://www.emast.org.au/models/anuclimate-1-0/) and matched to locations in a geographic information system (ArcGIS 10.2; ESRI Corporation). We also measured potential heat stress exposure for each seedling provenance as the average number of days across a climatological reference period (1960-1990) which were 15°C above the mean (*sensu* Barua *et al.* 2008). Daily maximum temperature data collected by the Bureau of Meteorology was downloaded from the Australian Water Availability Project (www.csiro.au/awap/) and percentages calculated across all data in the reference period. A temperature increase of 15°C in addition of the mean was chosen as it is threshold known to induce heat stress in many plant species (Wahid *et al.* 2007).

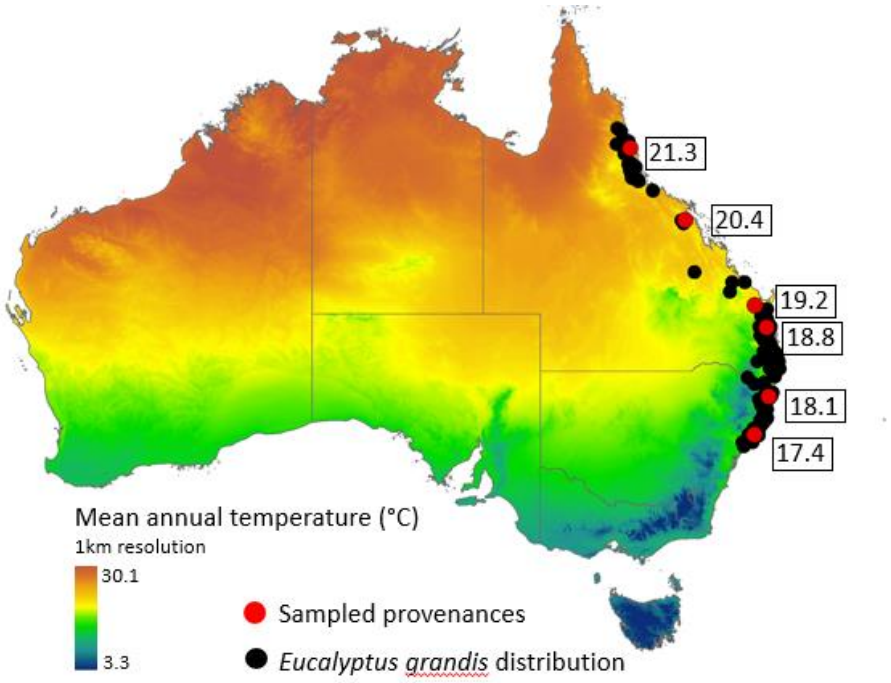

Fig. 2. Mean annual temperature map for Australia over the period of 1970-2000 showing the natural distribution of *E. grandis* and the geographic location of seed provenances included in the study. Boxes contain the average mean annual temperature conditions (°C) during the period 1950-2000 at the respective sites.

Table. 1. Location of *E. grandis* seed provenances included in experimental heat wave manipulations in growth chambers. Seedlot numbers correspond to those held within the CSIRO Australian Tree Seed Centre. Temperature data is extracted from gridded average climate conditions during the period 1970-2000 at the respective sites. Days >+15◦C represents number of days a year (1960-1970) in excess of 15◦C of the mean annual temperature at each of the respective provenances.

Provenance	Seedlot	Collection site	Co-ordinates	Mean annual temperature (◦C)	Minimum (◦C)	Maximum (◦C)	Range (◦C)	Days >+15◦C (%)
1	19307	Gadgarra National Park, QLD	17°18'00.0"S 145°43'58.8"E	21.3	11.9	29.2	17.3	0.3
2	16893	Finch Hatton Gorge, QLD	21°04'01.2"S 148°37'01.2"E	20.4	7.4	28.7	21.3	0.1
3	15875	Brooweena State Forest, QLD	25°33'00.0"S 152°16'01.2"E	19.2	8.2	30.3	22.1	6.4
4	20678	Baroon Pocket, Maleny, QLD	26°42'00.0"S 152°52'58.8"E	18.8	7.1	28.1	21	1.4
5	19313	Orara West State Forest, NSW	30°19'58.8"S 153°00'00.0"E	18.1	5.5	27.6	22.1	2.6
6	17563	Bulahdelah State Forest, NSW	32°19'58.8"S 152°15'00.0"E	17.4	6	27.2	21.2	3.78

Experimental design

E. grandis growth conditions

E. grandis seedlings were grown in a common garden design at the Macquarie University Plant Growth Facility under the following controlled glasshouse conditions: temperature 28/22 ± 2°C (day/night, 13/11h), supplementary lighting on a daily 12-hour cycle (6am to 7pm) (Philips GreenPower LED lights) at an illumination of 600 µmol $m^{-2}s^{-1}$ and watering every second day. Seeds from the same provenance were germinated together in tube stocks containing native all-purpose soil mix with a covering of vermiculite to maintain moisture. Glasshouse conditions were chosen in in order to minimise any plant stress effects.

At a height of 3-4cm, 18 seedlings from each provenance were size matched and transplanted individually into 800ml pots using the same native all-purpose soil mix and labelled with provenance information (Fig. 3). At this stage in a previous trial study conducted in these facilities, seedling leaves of *E. grandis* began to exhibit signs of UV deficiency under glasshouse conditions (Fig. S-1). This has been attributed to plastic glasshouse roofing preventing adequate UV transmission for normal development of *Eucalyptus* saplings (Haigh 2007, unpublished data). This was resolved in the final experiment by translocating plants outside and into direct sunlight for a period of 2 hours during the most intense UV levels of the day (11am-2pm). Plants had constant access to water and were only placed outside on days when temperatures fell within the range of glasshouse temperatures (22-28°C). Throughout the growth period, seedlings received constant access to water and Yates Nutricote slow-release plant fertilizer containing no phosphorus (N:P:K = 19.1:0:11.9; Yates Australia, Padstow, NSW, Australia). Variation in glasshouse growth conditions between provenances was circumvented by random weekly shuffling of seedlings to avoid blocking effects. Plants were grown for 137 days with the most uniform seedlings from each provenance retained for the experiment.

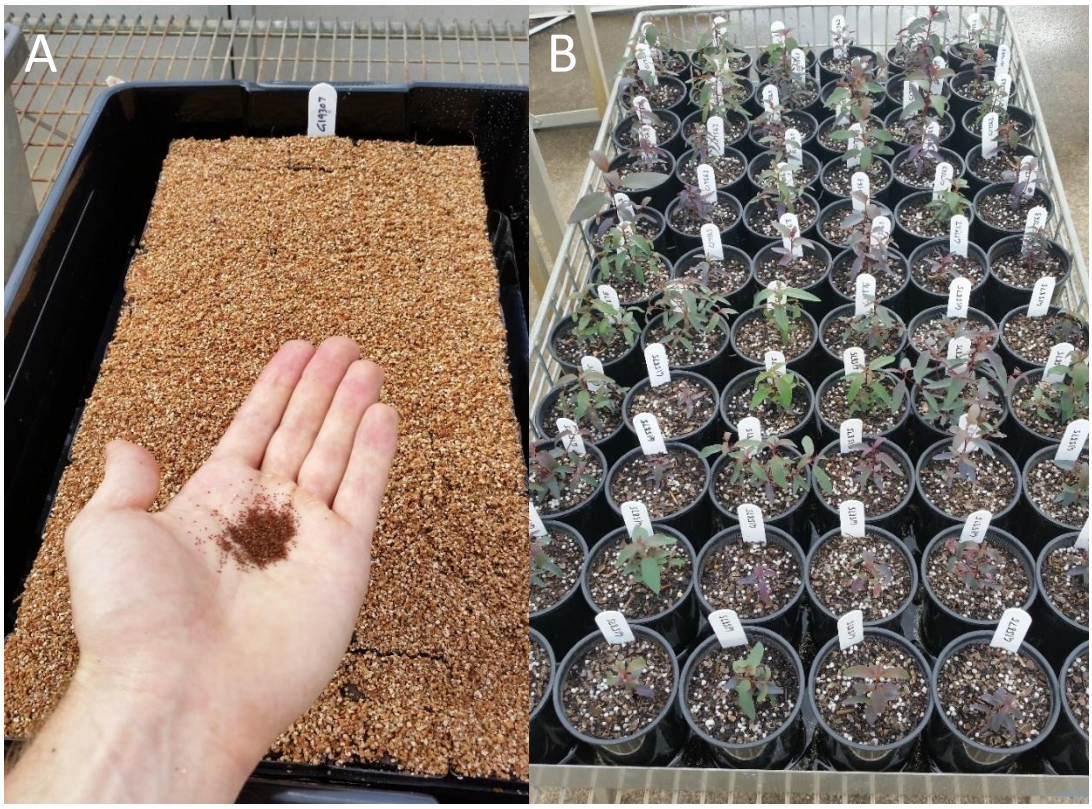

Fig. 3. (A) Germination conditions for individual provenances (B) Newly, individually potted 1-month old *E. grandis* saplings under glasshouse conditions.

Heat stress treatment

A manipulative heat experiment was conducted on seedlings from each provenance to mimic heat stress induced by summer heat wave conditions. This was achieved by randomly assigning three seedlings from each provenance to each of three heat treatments in growth cabinets (model PG.15.18.9 TD.5x100R; Thermoline Scientific, Wetherill Park, NSW, Australia) over four days: high intensity (42°C /26°C day/night), medium intensity (35°C /26°C day/night) and control (28°C /22°C day/night) (Fig. 4). For heat manipulations temperatures, 35°C was selected at the intermediate temperature treatment as it represents 10-15°C above the mean annual growing temperature of provenances, which is the threshold at which plants usually begin to encounter heat stress. In addition, 42°C represents the upper end of extreme summer temperatures experienced on the east coast of Australia.

Conditions were controlled across growth cabinet treatments with all plants receiving a 13h daily photoperiod at an illumination of 600 µmol $m^{-2}s^{-1}$ (+/- 50 µmol $m^{-2}s^{-1}$) and constant access to water and fertilizer. On day four of heat shock treatments, while conditions were maintained, 1 gram of leaf material was harvested from each plant and immediately stored in liquid nitrogen at -20°C for subsequent proteomic analysis. In summary, a total of 54 individually potted *E. grandis* seedlings were used in the experiment with three replicates of each provenance and heat treatment combination (six provenances x three temperature conditions x three replicates). This manipulative experimental approach is designed to isolate temperature stress response in *E. grandis* seedlings by avoiding confounding abiotic stressors such as moisture, lighting, soil and biotic interactions such as disease and predation encountered in field experiments.

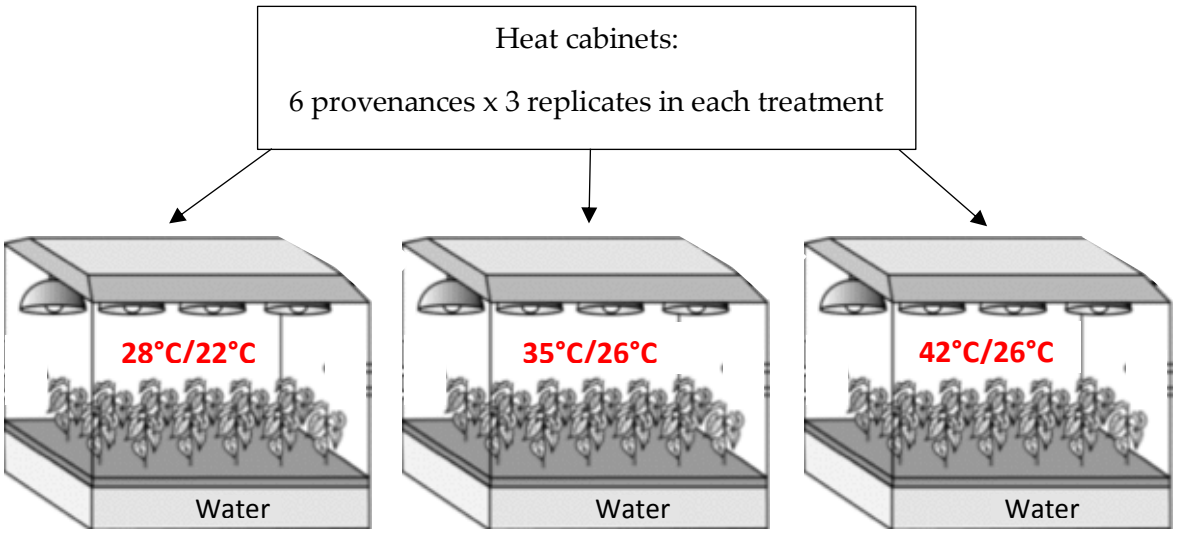

Fig. 4. Configuration of *E. grandis* seedlings in growth cabinets for the heat manipulation experiment used in this study. Three different temperature conditions were used in the study: high intensity (42°C /24°C day/night), medium intensity (35°C /24°C day/night) and control (28°C /24°C day/night.

Protein extraction and quantification

Eucalyptus leaves are generally known to contain a number of compounds that interfere with proteomic analysis (Abril *et al.* 2011). In a trial experiment, a TCA/acetone based extraction protocol and a phenol-ammonium acetate based extraction protocol were both optimised and compared for their effectiveness in extracting *E. grandis* proteins. However, the phenol-

ammonium acetate approach, which has previously been shown to be more effective in purifying protein samples in recalcitrant study species (Saravanan & Rose 2004), proved to provide the best extraction results in terms of yield and reproducibility and was used in the study.

Leaf samples were first ground into a fine powder using a mortar and pestle in liquid nitrogen. Proteins were then extracted from 200mg of the resulting powder with 0.8ml phenol (Tris buffered, pH 8.0) and 0.8ml of dense sodium dodecyl sulfate (SDS) (30% sucrose, 3% SDS, 0.1 Tris-HCl (pH 8.0), 5% β-mercaptoethanol) in a 2ml Eppendorf tube. Samples were vortexed and then centrifuged 10000 × g for 3 min (4 °C), and the resulting upper phenol phase was transferred to a new 5ml Eppendorf tube. Proteins were then precipitated from samples by the addition of five volumes of ice-cold methanol/0.1M ammonium acetate at -20°C, overnight. Protein samples were then pelleted by centrifugation at 10,000 × g for 5 min (4 °C). The supernatant was removed and pellets were washed twice with 1mL of 0.1M ammonium acetate in cold methanol and one more time with 1mL of cold 80% acetone. Samples were vortexed and centrifuged at 5000 × g for 5 min (4 °C), between wash steps. Following this, the supernatant was removed and the pellets were air-dried, before being re-suspended in 2% SDS in 50mM Tris-HCl (pH 8.8). Samples then underwent ultra-sonication at 30,000Hz for 3 seconds to completely dissolve the remaining pellet and then kept at -20°C overnight. Protein samples were then reduced at a concentration of 5mM of dithiothreitol (DTT) for 15min at room temperature and subsequently alkylated at a concentration of 10mM of iodoacetamide for 30 min in the dark at room temperature. Alkylation was then quenched in a final concentration of 10mM of DTT.

Protein samples were then precipitated using the methanol-chloroform protocol (Wessel & Flügge 1984). First, four parts of methanol were added to one part sample, followed by three parts chloroform and one part RO water, vortexing between steps. The mixture was then centrifuged at 1000 × g for 2 min (4 °C). The solvent layers were then slowly decanted to collect the protein pellet which was then washed with 2mL of ice-cold methanol. The supernatant was removed and the pellet left to dry before resuspension in 8M urea in Tris-HCl (pH. 8.8). Pellets were then completely dissolved by ultra-sonication at 30,000Hz with a 3 second burst and placed immediately on ice. The protein concentration of samples was then quantified in triplicate with a BCA assay, using bovine serum albumin (BSA) as the standard. Protein products were verified by visualisation on a gel. For gel electrophoresis, aliquots containing 40µg of protein, were taken from 8 protein samples at random and combined with 5 µl DTT and 2µl of loading buffer and

heated at 95 degrees for 2 min. The samples were then loaded to wells of a Bi-Rad 10% Tris–HCl precast gel and separated at -70mv for 30 min. The resulting gel was stained in colloidal Coomassie Blue solution for 2 hours and then de-stained in RO water overnight. Protein bands were visualised on the gel.

Protein purification and digestion

Based on the BCA results, aliquots containing 500µg of protein were transferred from each sample to new 2ml Eppendorf tubes. Samples were then diluted with four volumes of 50mM Tris to reduce urea concentration to less than 2 M, before digestion with trypsin (Promega, Madison, WI) at a 1:100 enzyme: protein ratio overnight at 37°C. Samples were then acidified to a final pH of 2-3 with 1% trifluoroacetic acid (TFA). Samples were desalted using C-18 SPE cartridges (Sep-Pak, Waters Corporation, Milford, MA) attached to a 3ml luer-lok syringe and plunger. One at a time, Sep-Pak cartridges were equilibrated by washes with 100% methanol, 1% formic acid/ 5% acetonitrile (ACN) solution, and 80% acetonitrile/ 0.5% acetic acid solution. Samples were then loaded into syringes with 2ml of 1% formic acid/ 5% acetonitrile solution and passed through C-18 SPE cartridges at a slow rate (approx. 0.5ml/min) by manually applying pressure. The flow through was collected in separate 2ml Eppendorf tubes and stored for further inspection in case peptides happened to pass through. Cartridges were subsequently washed with 2ml of 1% formic acid/ 5% acetonitrile solution. Lastly, peptide samples were eluted into new 2ml Eppendorf tubes with 1.8 ml of 80% acetonitrile, 0.5% ccetic acid in Mili-Q water and then dried under vacuum overnight. Once dried, peptides were resuspended in 200mM HEPES buffer (pH 8.8). Peptide samples were then quantified in triplicate in a MicroBCA assay (Thermo Scientific- Rockford, IL) using bovine serum albumin (BSA) as the standard. Using the MicroBCA results, aliquots of 70ug of peptides were taken from each sample and transferred to new 2ml Eppendorf tubes and made up to the same volume with additional HEPES buffer ready for tandem mass tag (TMT) labelling.

TMT labelling

A set of 10-plex TMT labelling reagents (Thermo Scientific, USA) were removed from the freezer and allowed to reach room temperature. Labels were individually solubilised in 41µl of anhydrous acetonitrile, and occasionally vortexed for 5 minutes. The 10 labels were then added in aliquots of 20µ separately to the 10 individual peptide samples in each of the six-10plex experiments and left at room temperature for 1 hour, vortexing occasionally. For each 10-plex experiment, the three

control replicates were labelled with tags 126, 127N, 127C, the three 35°C replicates with tags 128N, 128C, 129N and the three 42°C with tags 129C, 130N, 130C, respectively. A reference sample with the same peptide concentration was used as the tenth sample for each 10-plex experiment. Populations two to six shared a common reference made by pooling equal volumes of all samples from population 2. For population 1 a duplicate of a single control replicate was used as a reference sample instead. After 1 hour, 8 µl of 5% hydroxylamine was added to samples to quench the TMT reaction. Samples were then vortexed and at kept at room temperature for 15 minutes. All TMT labelled samples within a 10-plex experiment were then pooled in a 2ml Eppendorf tube and then dried under vacuum overnight. This sample mixture was resuspended in 1% TFA and desalted using C-18 SPE cartridges (Sep-Pak, Waters Corporation, Milford, MA) as previously described. Eluted peptides were then dried again under vacuum and resuspended in 1ml of 0.1% formic acid ready for sample fractionation.

Peptide fractionation

Pooled peptide samples were fractionated by high pH reverse phase high-performance liquid chromatography to reduce sample complexity. Fractionation was performed by Chi-Hung Lin from the Australian Proteome Analysis Facility to the following specifications. Samples were analysed using an Agilent 1260 quaternary pump with an Agilent extend C18 column (2.1mm x 150 mm, 3.5 µm) with UV detection at 210nm. The column was first primed with buffer A (5 mM ammonia, pH ~10.4) before samples were resuspended using the same buffer, then injected and adsorbed to the column. Peptides were then gradually eluted in fractions with an increasing gradient (3-90%) of buffer B (Buffer B: 90% ACN/ 5 mM ammonia) over 85 minutes, at a flow rate of 0.3 mL/min at room temperature. For each 10-plex experiment, a total of 64 fractions of varying volumes were collected in a 96-well plate and suspended in 1% formic acid. Fractions were then pooled into eight in 2ml Eppendorf tubes, based on UV absorbance before being dried under vacuum and resuspended in 100 µl of 1% TFA. Separate stage tips were then used to desalt fractions. Stage tips consisted of SDB-RPS resin pushed to the bottom of 200µl pipette tips. Samples were loaded into stage tips placed in 1.7ml collection tubes and centrifuged at 2000 × g for 10 minutes until the solution had completely passed through. The flow through was kept and stored. Stage tips were then washed with 200µl of 0.2% TFA by centrifugation at 2000 × g for 10 minutes. Finally, stage tips were transferred to 1.7ml collection tubes and centrifuged with the addition of 100µl of 5% ammonium hydroxide/ 80%ACN to elute peptides. Eluted fractions were then dried under vacuum

and stored at −60 °C until analysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Nanoflow LC–MS/MS analysis of peptides

Fractions were reconstituted in 30 µl of 0.1% formic acid, with a final peptide concentration of approximately 0.4 µg/µl, vortexed and transferred to vials ready for analysis using LC-MS/MS. Sample analysis was conducted by Dr Mehdi Mirzaei at the Australian Proteome Analysis Facility (APAF) (Macquarie University, NSW) using a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled to an EASY-nLC1000 (Thermo Scientific). Peptide fractions were loaded on to the trapping column (75 µm id. × 100 mm, C18 HALO column, 2.7 µm bead size, 160 Å pore size) in 5ul volumes, before being eluted through the separation column with a linear gradient of 1-30% solvent B (99.9% ACN/0.1% FA) over 170 minutes directly into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap MS and ion trap MS/MS acquisition.

For data collection, MS spectra were surveyed at the range of 350-1850 m/z with a precursor isolation width of 0.7 m/z, resolution of 70,000 at m/z 400 and an AGC (Automatic Gain Control) target value of 1×10^6 ions using Xcalibur software (Thermo Scientific). For identification of TMT-labelled peptides, the ten most abundant ions were selected for higher energy collisional dissociation (HCD) fragmentation. HCD normalised collision energy was set to 35% and fragmentation ions were detected in the Orbitrap at a resolution of 70,000. Target ions that previously selected for MS/MS were dynamically excluded for 90 sec. For accurate mass measurement, the lock mass option was enabled using the polydimethylcyclosiloxane ion (m/z 445.12003) as an internal calibrant. Note that, because of the order in which samples were analysed in the study, each TMT 10-plex corresponded to following provenance: TMT 1=Provenance 1, TMT-2= Provenance 4, TMT-3= Provenance 5, TMT-4= Provenance 6, TMT 5= Provenance 3 and TMT-6= Provenance 2.

Data Analysis

Protein identification and quantification

The raw mass data from LC–MS/MS analysis was imported to Proteome Discoverer V1.3 (Thermo Scientific) and using a local MASCOT server (Matrix Science, London, U.K.) protein identification and

relative abundance data were generated. For protein identification MS/MS spectra were searched against the downloaded Uniprot *E. grandis* proteome database (44150 proteins, accessed August 7th 2016). Peptide identifications were made using the following database search parameters in MASCOT: Search type: MS/MS Ion search; MS tolerance: ±10 ppm; Enzyme: trypsin with one missed cleavage; fragment mass error: 0.1 Da; Peptide Mass Tolerance: >15 ppm; Carbamidomethylation of cysteine and 10-plex TMT tags on lysine residues and peptide N-termini set as a static modification and oxidation of methionine and deamidation of asparagine and glutamine residues as a variable modification. In addition, peptide sequences were searched against a reversed-sequenced decoy database to determine false discovery rates (FDR). Search results were further filtered using FDR cut off at 1%, only peptides with a score >15 and below the Mascot significance threshold filter of p = 0.05 were included and single peptide identifications required a score equal to or above the Mascot identity threshold. Proteins with at least two unique peptides were regarded as confident identifications. Relative quantitation of proteins was achieved by pairwise comparison of TMT reporter ion intensities, using the ratio of the labels for each of treatment replicates (numerator) versus the labels of their corresponding control replicates (denominator).

Identification of differentially expressed proteins

To determine differential protein expression one-way ANOVA analyses, with temperature as a fixed effect, were conducted on the log transformed protein ratios of samples with respect to the selected reference, which was typically a pool of all samples. The overall distribution of log transformed reporter ion ratios to the common reference was generated in an overlapped kernel density plot to check the data met the normality assumption of ANOVA. A one-way ANOVA was performed for every protein in the set for each provenance separately, to identify those that displayed patterns increased or decreased abundance between temperature treatments. This analysis was conducted using the TMTPrepPro package in R. The analysis requires an added input of an Excel spreadsheet containing the experimental design and sample location for each run. Parameters were set so that a 1.5 change in fold count was needed for differential expression (with no limit to the number of counts per peptide). Proteins were considered significantly differentially expressed if the ANOVA p-value was <0.05 and the maximum observed fold change was at least 1.5. All differentially expressed proteins between the control and 42°C were grouped based on their presence or absence for each of the six populations. Of these, we focused on the proteins found to be uniquely up and up-regulated in each provenance under 42°C. Linear regressions were conducted in MS Excel 2016 to

test the relationship between HSP abundance under 35°C and 42°C with climate parameters across the six *E. grandis* provenances.

Bioinformatic analysis of E. grandis proteins

The recent genome sequencing of *E. grandis* has enabled the use of a range of bioinformatics tools to identify the functional role of specific proteins in response to heat stress and detect the broader biological pathways in which they may operate to generate thermotolerance in this species.

Protein annotation and functional analysis

To characterize the proteins identified functionally, all identified proteins were further categorized into biological functions using a gene ontology-term (GO) analysis. Initially, GO information for differentially expressed proteins was extracted for all proteins, where available, from the Uni-Prot database. Proteins were then classified based on their biological process using the R-based tool PloGO (Pascovici *et al.* 2012), using a selected subset of biological process annotations of interest for this project.

Pathway analysis of differentially expressed proteins

In addition to gene ontology information, biological pathway information was extracted for all proteins identified as differentially expressed under heat treatment in *E. grandis*. All differentially expressed proteins were introduced into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway-mapping tool (http:// www.genome.jp/kegg/mapper.html), using the protein Uniprot ID and the trend (increasing or decreasing with temperature stress) identified in the analysis. The top ten pathways identified in decreasing order of the number of proteins contained from the dataset were tabulated, and the pathways of biological interest were discussed.

Results

Seedling post-treatment survival

All potted *E. grandis* seedlings survived growth cabinet heat treatments, with no discernible physical differences in plant health across conditions (control, 35°C and 42°C) at the end of 4 days. Seedling survival was still 100% two months post-treatment.

Abundance of proteins identified and quantified in *E. grandis*

The MASCOT search identified and quantified the following number of proteins across temperature treatments for each of six *E. grandis* provenances respectively with a protein false discovery rate (FDR) at 1%: provenance 1-3284, provenance 2- 2933, provenance 3-3208, provenance 4-3171, provenance 5-3051, provenance 6-3180. Provenances are numbered in order of occurrence along the east coast of Australia, beginning with Provenance 1, the most northern *E. grandis* provenance, Gadgarra NP, QLD, and ending with Provenance 6, the most southern *E. grandis* provenance, Bulahdelah State Forest, NSW.

Data quality check

An initial quality check of the overall data was performed using '*TMTPrepPro'* software (an in house bioinformatics tool). The overall distribution of log transformed protein fold ratios compared to the common reference (pool of all samples) were visualised in a boxplot and a density plot. The boxplot showed similar distributions of protein expression ratios across the groups, with no significant outliers (See Fig. S-2 in Appendix 1). The density plot showed that log-transformed ratios were centred at zero, with no significant outliers, satisfying the normality assumption for subsequent ANOVA analyses (See Fig. S-1 in Appendix 1). The log-transformed ratios from provenances two to six, with instances of missing values (proteins not identified for a particular condition) removed from the dataset, were also visualised using a principal component analysis in TMTPrepPro (Fig. 5). PCA scores showed no significant outliers across runs. Samples grouped based on treatment showed distinct clustering patterns, in particular those from the 42°C treatment.

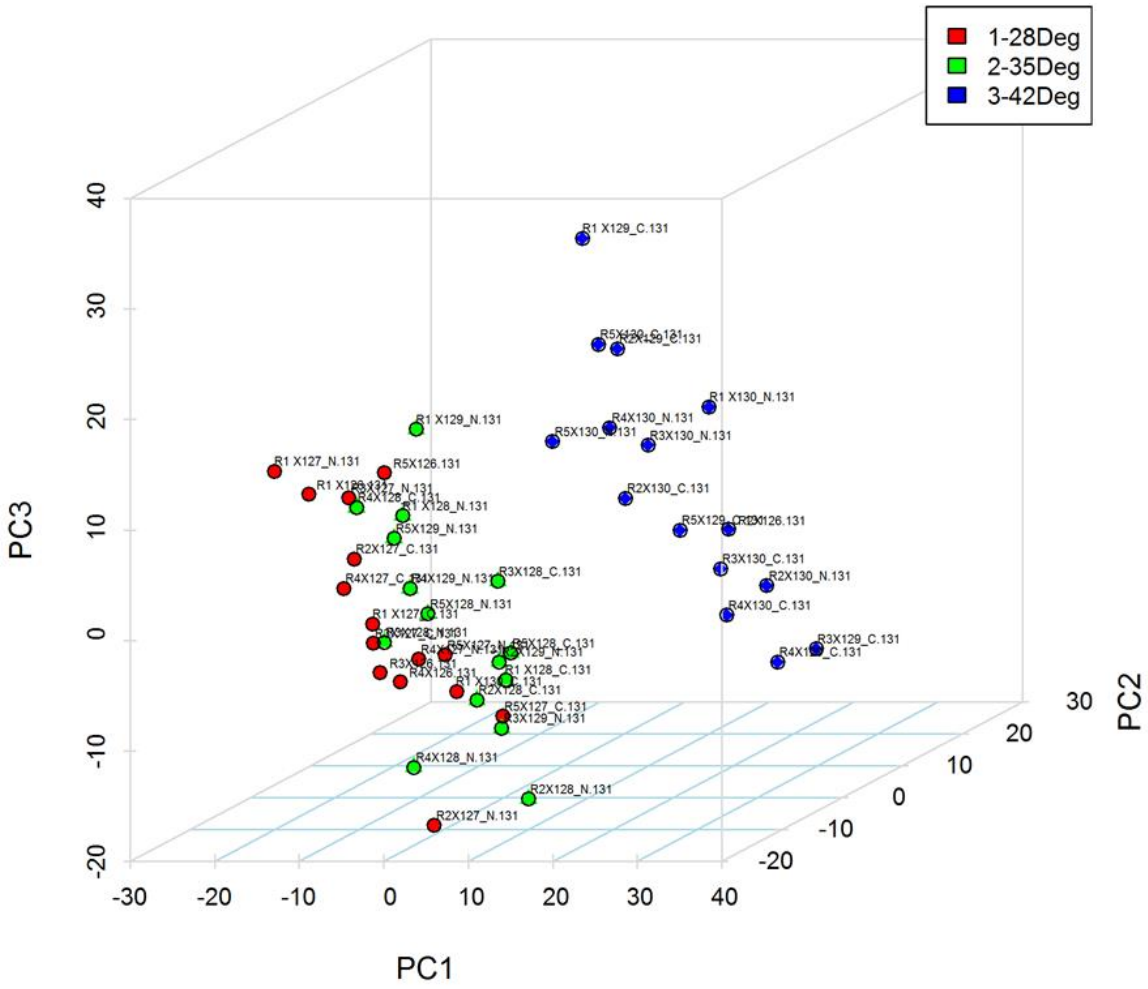

Fig. 5. Three-dimensional plot of the PCA scores from populations two to six for the first three components. Heat treatments showed distinct clustering patterns, in particular the 42°C treatment**.**

Quantification of heat responsive proteins

ANOVA analysis identified 532 proteins from the total dataset that were significantly differently expressed (p-value <0.05) in heat treated plants (either 35°C or 42°C) compared to control plants in one or more provenances. Of proteins differentially expressed, 299 were found to be down-regulated and 233 up-regulated in response to heat treatments. The breakdown of up and down-regulated proteins for each heat treatment across provenances is shown in Fig. 6. As expected many more proteins were differentially expressed in the 42°C treatment than the 35°C treatment compared to controls. The ten most upregulated proteins in response to 42°C for each provenance is provided in the Dropbox spreadsheet entitled “Top 10 upregulated”. Among these, HSPs consistently showed the largest increases in abundance across provenances. Fold increases among proteins ranged up to 62.13 compared to the control. Note that, as a quality check step, those

proteins common to all provenances and differentially expressed (P<0.05) in at least one were visualised in a heat map using a hierarchical clustering procedure in TMTPrepPro (Fig. 7). The heat map generated shows a good separation of the conditions regardless of the provenance, especially for the 42°C samples. Further hierarchical analyses were conducted on differentially expressed proteins for each individual run to test that replicates showed reproducible patterns of expression across treatments (see Fig. S-3 in Appendix).

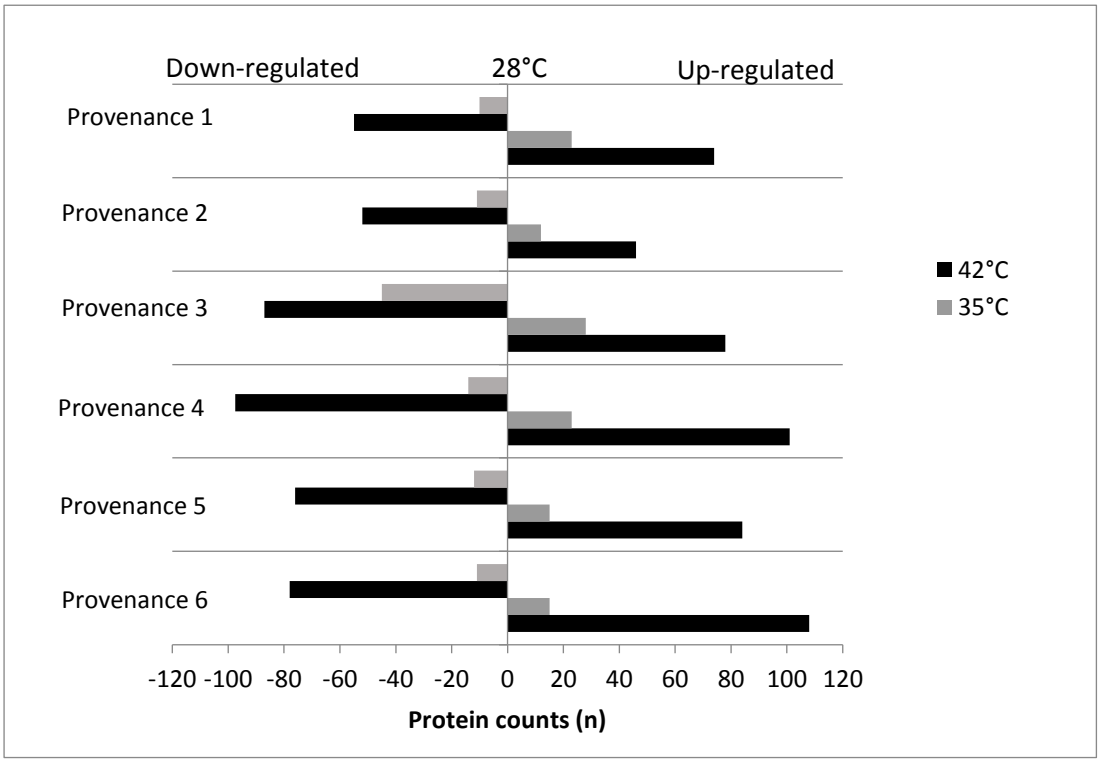

Fig. 6. Breakdown of the number of significantly up and down- regulated proteins for both the 35°C intermediate and 42°C extreme heat treatments across seedlings sourced from six *Eucalytpus grandis* provenances. Heat treatments were imposed on 137-day old seedlings in growth cabinets. Provenances on the Y-axis are arranged in order of occurrence moving north to south along the east coast of Australia. P1 = Gadgarra NP, QLD; P2 = Finch Hatton Gorge, QLD; P3= Brooweena State Forest, QLD; P4= Baroon Pocket, Maleny, QLD; P5= Orara West State Forest, NSW; P6= Bulahdelah State Forest, NSW.

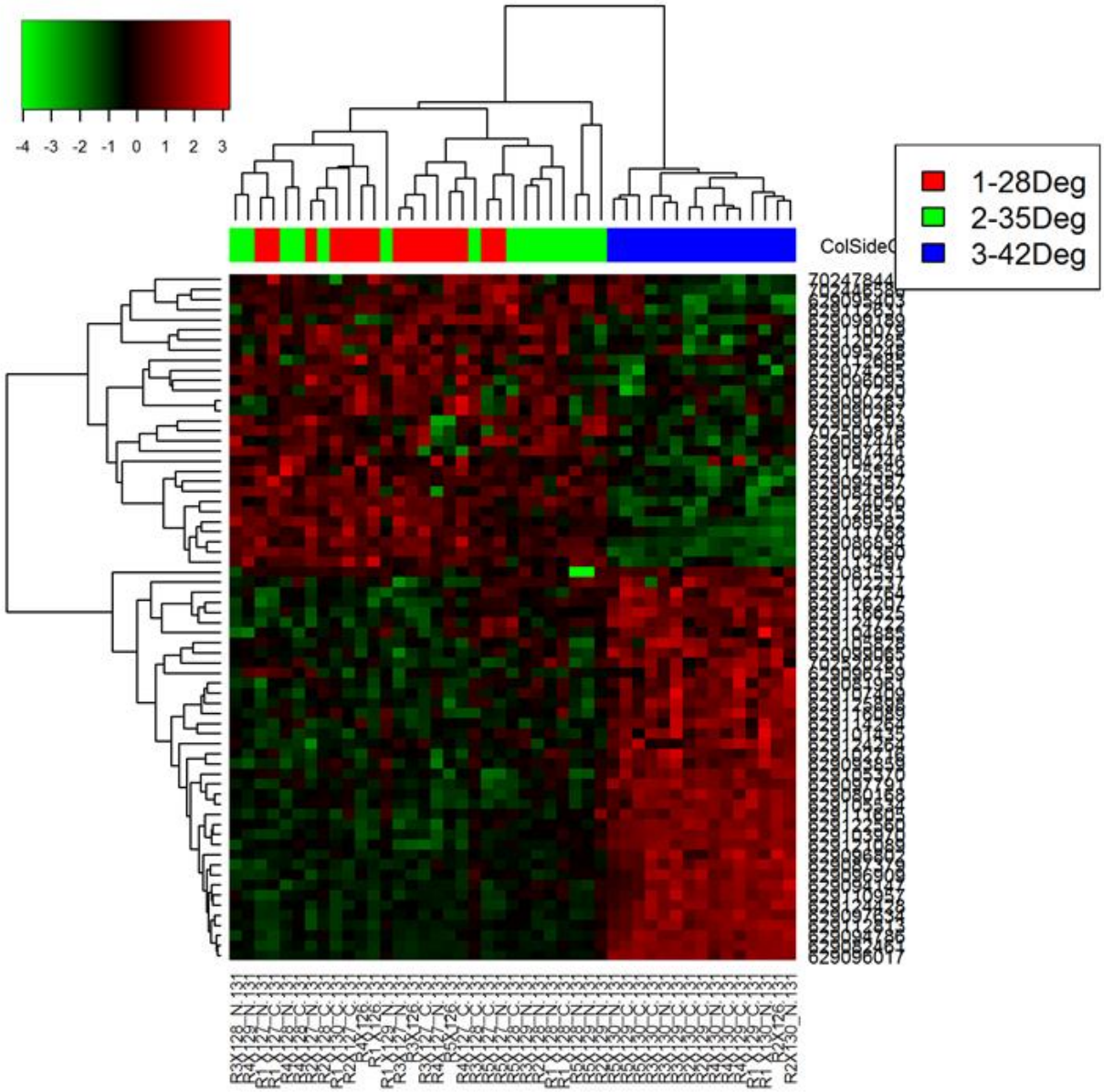

Fig. 7. Heat map (hierarchical clustering) of all labelled samples from provenances 2-6 showing expression profile for proteins common to all and differentially expressed (P<0.05) in at least one population. Column colours indicate treatment type. Redness indicates degree of increase in protein expression, greenness degree of decrease in expression and black no significant change in expression.

Uniquely expressed heat responsive proteins

Proteins differentially expressed at 42°C compared to the control were sorted into categories according to their absence and presence across the six provenances. We focus here on the number of proteins uniquely differentially expressed in each provenance under the 42°C

treatment as these showed the most marked response (Fig. 8). See Fig. S-4 for the complete the complete absence and presence table in Appendix.

The largest category belonged to Provenance 3 (Brooweena State Forest, QLD) with 98 uniquely differentially expressed proteins, while Provenance 2 (Finch Hatton Gorge, QLD) was found to have the lowest with 38. In terms of up-regulation at 42°C, Provenance 6 (Bulahdelah State Forest, NSW) had the highest number of uniquely up-regulated proteins with 32 and Provenance 2 (Finch Hatton Gorge, QLD) had the lowest number with 12. Provenance 3 (Brooweena State Forest, QLD) had the highest number of uniquely down-regulated proteins with 68 and Provenance 1 (Gadgarra NP, QLD) had the lowest with 23. Across all provenances, a total of 19 proteins were found differentially expressed- 18 proteins were consistently upregulated and 1 consistently downregulated in response to the 42°C treatment. HSPs made up the majority of these proteins. No proteins were consistently differentially expressed across provenances in response to the 35°C treatment.

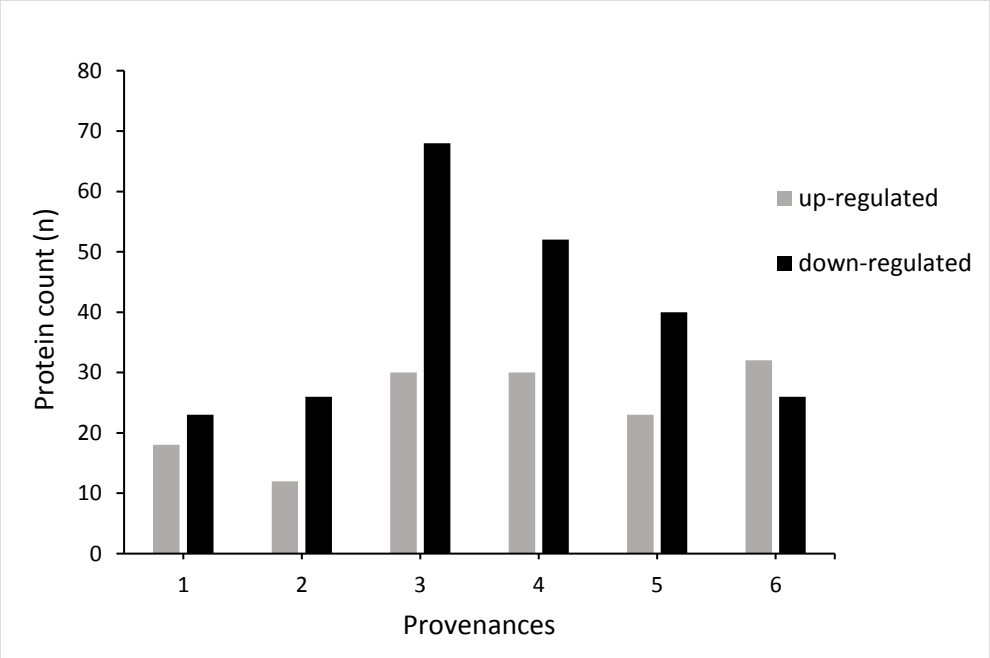

Fig. 8. The number of proteins uniquely up and down-regulated under the 42°C treatment for each of the six *E. grandis* provenances in the study: 1 – Gadgarra NP, QLD; 2 – Finch Hatton Gorge, QLD; 3 – Brooweena State Forest, QLD; 4– Baroon Pocket, Maleny, QLD; 5 – Orara West State Forest, NSW; 6 – Bulahdelah State Forest, NSW.

Provenance temperature characteristics and HSP expression

The temperature characteristics for each of the sampled provenances of *E. grandis* are provided in Table 1. As expected, the mean annual temperature (MAT) showed a decreasing trend between provenances moving from north to south. The diurnal temperature range was similar across all sites except for the northern most provenance, Gadgarra NP, QLD, which has a smaller range by at least 3°C.

The heat stress index which indicated the average annual frequency of days exceeding 15°C above the MAT (1960 to 1990) for each respective provenance showed large variability and did not vary predictably with latitude. The largest number of days exceeding this threshold belonged to Provenance 6 (Bulahdelah State Forest, NSW) with 13.8. Provenance 2 (Finch Hatton Gorge, QLD) was found to have the lowest number with 0.1 days.

As expected, plants grown under the 42°C showed a consistently significant increase in HSP content compared to the 35°C treatment (Table 2). A total of 29 HSPs from 3 different HSP classes (HSP20, HSP60, HSP70) were found to be significantly upregulated in response to the 42°C treatment in one or more provenances (see Drop box spreadsheet entitled “HSPs differentially expressed”). This was compared to 10 HSPs, belonging two different HSP classes (HSP20, HSP70) identified in response to the 35°C treatment. In terms of the number of HSPs upregulated between provenances, the most southern provenance (Bulahdelah State Forest, NSW) displayed the highest number with 24 out of a total 29 HSPs. Provenance 2 (Finch Hatton Gorge, QLD) showed the lowest number with 16 out of the 29 HSPs upregulated.

The relative abundance of the identified HSP classes across each provenance was calculated using the mean of pooled fold count ratios in each HSP class across each treatment and provenance (Fig. 9). Of the HSPs identified the greatest increases in abundance across provenances was observed in sHSPs. The other classes HSP60 and HSP70 also showed consistent increases in abundance across provenances. Provenance 6 (Bulahdelah State Forest, NSW) showed the highest HSP expression in HSP20 and HSP70, whereas HSP60 expression was highest in Provenance 5 (Orara West State Forest, NSW). In response to 42°C, sHSPs showed the greatest differences in abundance across provenances, whereas fold count increases in HSP60 and HSP70 were much more similar across provenances. For sHSPs and overall HSP content, Provenance 6 (Bulahdelah State Forest, NSW) showed the greatest abundance, while Provenance 2 (Finch Hatton Gorge, QLD) had the lowest.

Table 2. Two-sample *t*- test comparisons of mean abundance for HSP60, HSP70, HSP20 and total HSP content between the 35°C and 42°C treatments across provenances.

HSP class	Measure	Provenance 1	Provenance 2	Provenance 3	Provenance 4	Provenance 5	Provenance 6
HSP60	**Mean 35°C**	1.060165	0.904231	1.097406	1.104117	1.10233	1.166449
	Mean 42°C	1.421116	1.33075	1.639306	1.595513	1.64225	1.705537
	T-Value	-3.65	-6.14	-5.22	-13.1	-5.1	-8.74
	DF	2	2	3	2	3	3
	P-Value	0.0338	0.0128	0.0069	0.0029	0.0073	0.0016
HSP70	**Mean 35°C**	1.404952	1.069879	0.99858	1.18068	1.223553	1.140044
	Mean 42°C	2.386053	2.326621	2.333188	2.911439	3.24967	2.857419
	T-Value	-1.89	-3.04	-4.6	-2.88	-2.34	-3.08
	DF	8	7	8	8	7	9
	P-Value	0.0478	0.0095	0.0009	0.0103	0.0261	0.0066
HSP 20	**Mean 35°C**	1.686966	1.256064	1.63413	1.377199	1.267933	1.68918
	Mean 42°C	5.287689	4.788228	9.370353	7.375565	5.853056	13.16688
	T-Value	-2.91	-3.89	-2.33	-6.12	-5.26	-2.16
	DF	8	10	8	8	11	10
	P-Value	0.0098	0.0015	0.024	0.0001	0.0001	0.0279
HSP total	**Mean 35°C**	1.47656	1.140383	1.285077	1.253965	1.230896	1.395032
	Mean 42°C	3.491763	3.421624	5.24999	4.636646	4.398295	7.438603
	T-Value	-4.09	-4.09	-2.48	-4.7	-5.02	-2.3
	DF	21	21	20	20	22	23
	P-Value	0.0003	0.0003	0.011	<0.0001	<0.0001	0.0153

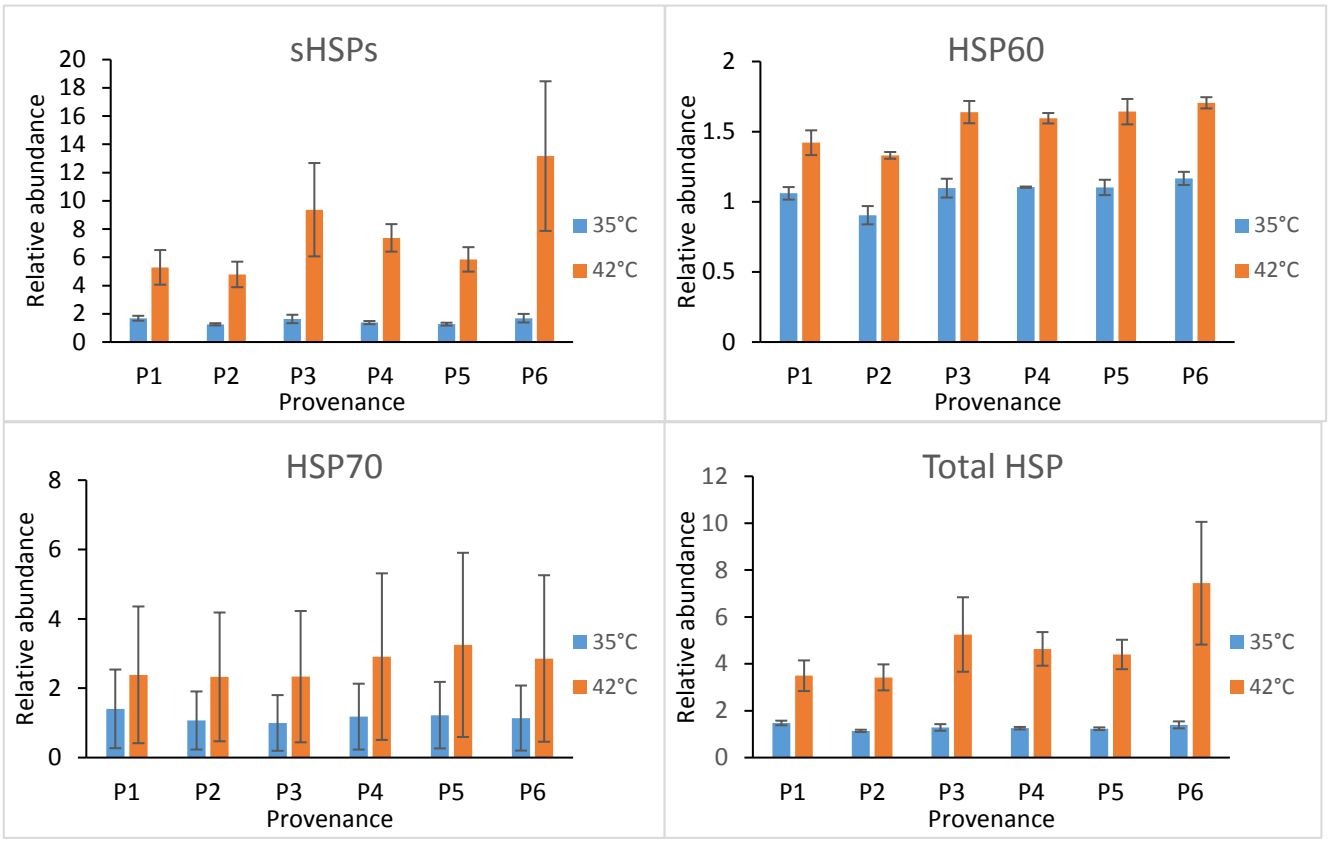

Fig. 9. Relative abundance of (a) small heat shock protein class (sHSPs), 9b) HSP60, (c) HSP70 and (d) total HSP content in response to both the intermediate 35°C (blue bars) and extreme 42°C (orange bars) treatment for each of the six *E. grandis* provenances in the study. Relative HSP abundance was calculated as the mean of pooled fold counts of proteins in each HSP class for each treatment and for each population. Error bars represent ± SE. P1, Gadgarra NP, QLD; P2, Finch Hatton Gorge, QLD; P3, Brooweena State Forest, QLD; P4, Baroon Pocket, Maleny, QLD; P5, Orara West State Forest, NSW; P6, Bulahdelah State Forest, NSW.

Relationship between HSP content and provenance climate

Regressions of the relative abundance of HSP content under 42°C against MAT of provenance were non-significant for sHSPs (β= – 0.707, $R^2_{adjusted}$=0.374, P = 0.116), HSP70 (β= – 0.747, $R^2_{adjusted}$= 0.448, P = 0.088) or total HSP content (β= – 0.807, $R^2_{adjusted}$= 0.565, P = 0.052), but showed a consistently negative correlation, contradicting out original prediction (Fig. 10). HSP60 showed a significant relationship negative relationship with MAT (β = – 0.881, $R^2_{adjusted}$= 0.721, P = 0.020). HSP content under 35°C showed no significant relationship with MAT of provenances across HSP classes, sHSP (β = 0.042, $R^2_{adjusted}$= -0.248, P = 0.936), HSP60 (β = – 0.686, $R^2_{adjusted}$= 0.338, P = 0.133), HSP70 (β = 0.359, $R^2_{adjusted}$= -0.089, P = 0.485), total HSP (β = 0.118, $R^2_{adjusted}$= -

0.233, P = 0.824). In contrast, the six provenances showed a strongly significant positive relationship under 42°C between HSP content and days exceeding >+15°C of MAT at provenances (1960 to 1990) for sHSP (β = 0.970, $R^2_{adjusted}$= 0.927, P = 0.001) and total HSP (β = 0.977, $R^2_{adjusted}$= 0.942, P = 0.001) (Fig. 10). However, only a weak positive correlation was observed in HSP60 (β = 0.729, $R^2_{adjusted}$= 0.415, P = 0.100) and HSP70 (β =0.193, $R^2_{adjusted}$= -0.203, P = 0.714). HSP content under 35°C also showed no significant relationship with days exceeding >+15°C of MAT at provenances sHSP (β = 0.563, $R^2_{adjusted}$= 0.146, P = 0.245), HSP60 (β = 0.661, $R^2_{adjusted}$= 0.296, P = 0.153), HSP70 (β = – 0.350, $R^2_{adjusted}$= -0.097, P = 0.497), total HSP (β = 0.338, $R^2_{adjusted}$= -0.107, P = 0.512).

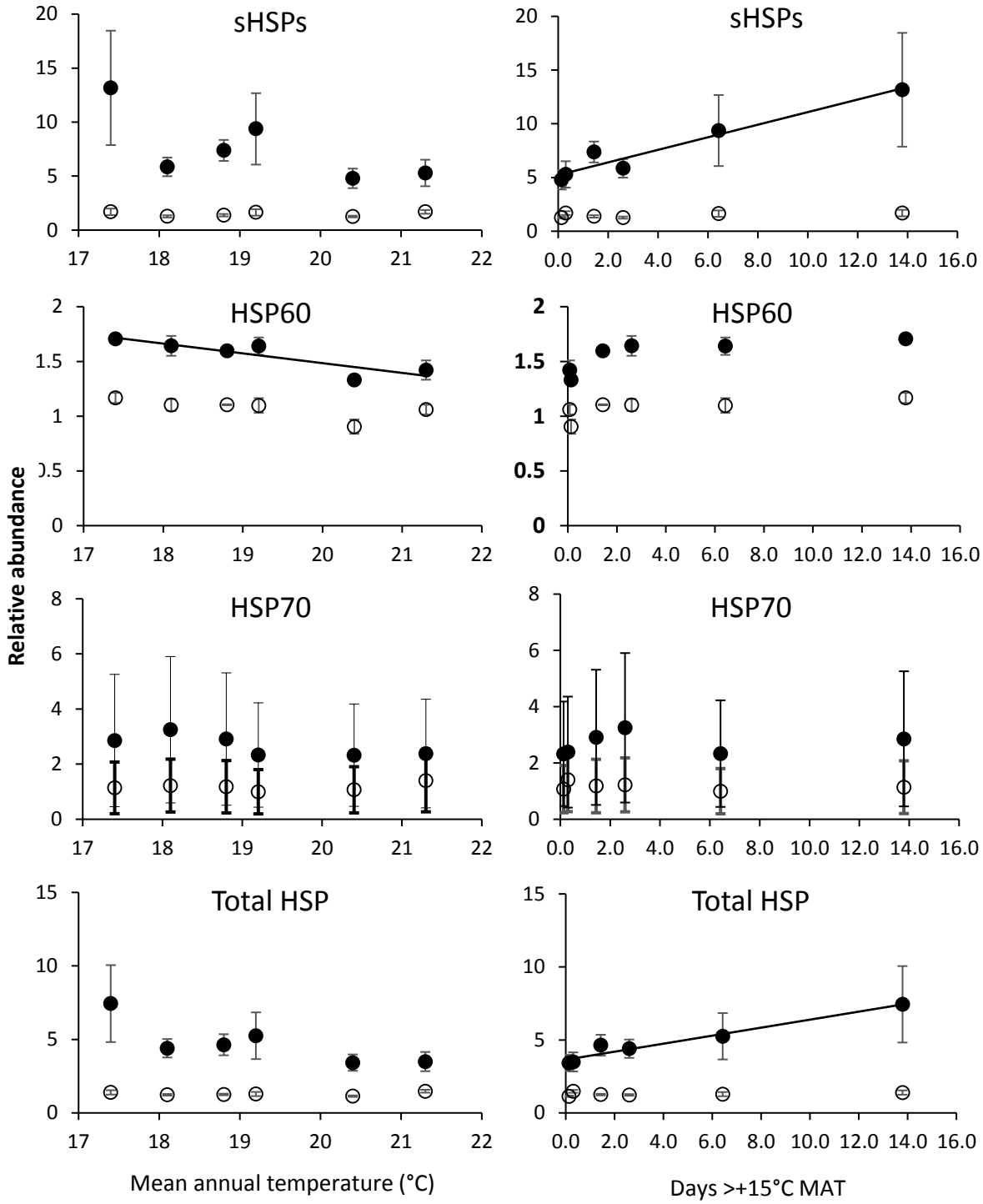

Fig.10. Regression analysis of the relationship between HSP content and both mean annual temperature and average annual days in excess of +15°C at each of the study provenances. This was assessed between MAT for the three different classes of HSPs and total HSP content for plants grown in 35°C and 42°C treatment. (42°C, closed circles: sHSPs (β = – 0.707, $R^2_{adjusted}$=0.374, P= 0.116), HSP60 (β = – 0.881, $R^2_{adjusted}$= 0.721, P=0.020), HSP70 (β = – 0.747, $R^2_{adjusted}$= 0.448, P= 0.088) or total HSP content (β = – 0.807, $R^2_{adjusted}$= 0.565, P=0.052). 35°C, open circles sHSP (β

=0.042, $R^2_{adjusted}$= -0.248, *P*=0.936), HSP60 (*β* = – 0.686, $R^2_{adjusted}$= 0.338, *P*=0.133), HSP70 (*β* =0.359, $R^2_{adjusted}$= -0.089, *P*= 0.485), total HSP (*β* =0.118, $R^2_{adjusted}$= -0.233, *P*=0.824).
This was assessed between average days >+15°C MAT for the three different classes of HSPs and total content for plants grown in 35°C and 42°C treatment (42°C, closed circles: sHSP (*β* = 0.970, $R^2_{adjusted}$= 0.927, *P*= 0.001) HSP60 (*β* =0.729, $R^2_{adjusted}$= 0.415, *P*= 0.100) and HSP70 (*β* =0.193, $R^2_{adjusted}$= -0.203, *P*=0.714) and total HSP (*β* = 0.977, $R^2_{adjusted}$= 0.942, *P*= 0.001). (35°C, open circles: sHSP (*β* = 0.563, $R^2_{adjusted}$= 0.146, *P*= 0.245), HSP60 (*β* = 0.661, $R^2_{adjusted}$= 0.296, *P*= 0.153), HSP70 (*β* = – 0.350, $R^2_{adjusted}$= -0.097, *P*= 0.497), total HSP (*β* = 0.338, $R^2_{adjusted}$= -0.10, *P*= 0.512). Error bars are ± SE. Lines only fitted for significant regressions.

Functional classification of heat responsive proteins

In order to interpret the broader biological roles that the identified heat responsive play in the response to heat in *E. grandis*, they were functionally categorised according to the gene ontology information. Because *E*. *grandis* has only recently been sequenced few protein annotations are currently available relative to other more established plant model species (Myburg *et al.* 2014). Approximately 72% of differentially expressed proteins had available GO information and were functionally classified. From GO analysis, the heat responsive proteins were found to be involved in 17 different biological processes (Fig. 11). The relative percentages of DEPs classified into each GO category are shown in Figure 5. In terms of up-regulation, the two most common categories belonged to the very general biological processes of metabolic process and protein metabolic process. More importantly, response to stress and response to stimulus made up the next largest categories. In terms of down-regulation, the most proteins were involved in metabolic process, biosynthetic process and carbohydrate metabolic process, respectively.

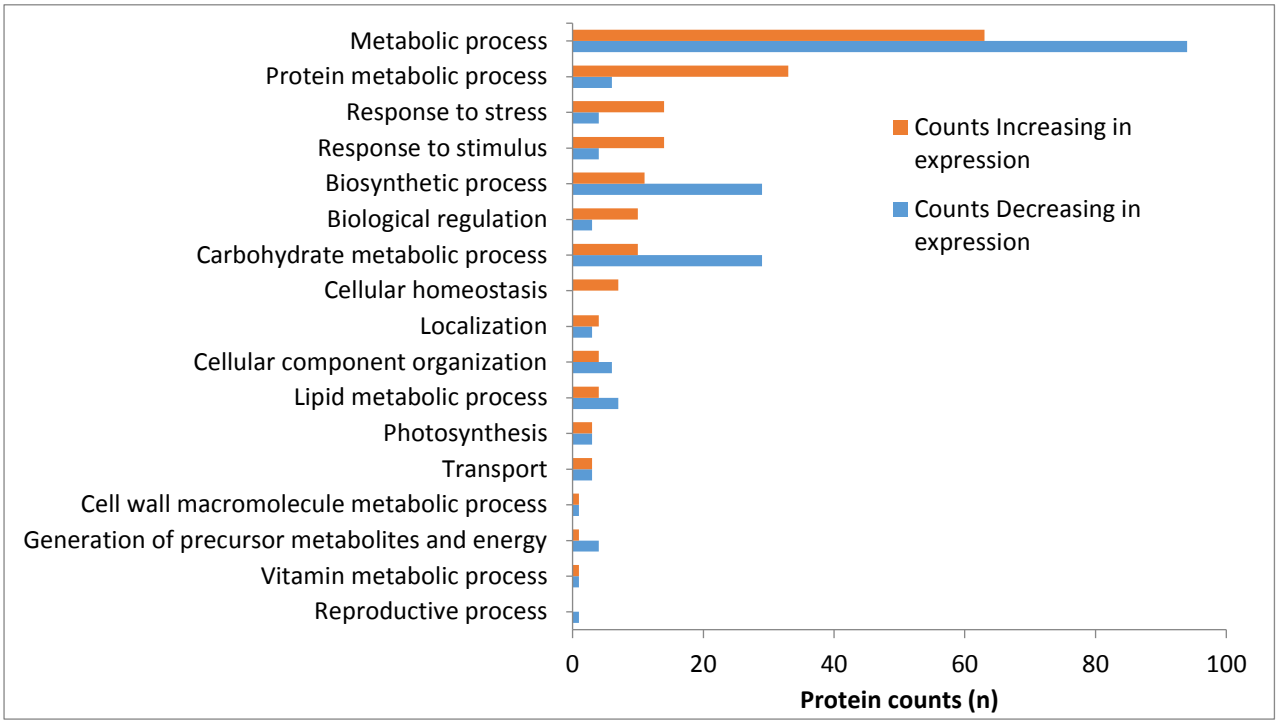

Fig. 11. Proteins differentially expressed under heat treatments (either 35°C or 42°C) classified into biological processes according to their gene ontology information. Proteins are further divided based on decreasing (blue bars) or increasing expression (orange bars) in response to heat treatment.

Protein pathway analysis

To look the broader biological processes important to heat adaptation in *E. grandis*, heat responsive proteins were mapped to their associated metabolic pathways using the Kyoto Encyclopaedia of Genes and Genomes database. Differentially expressed proteins were mapped to a total of 64 pathways. The top 10 pathways and the number of proteins involved in each are shown in Table 3. The largest number of mapped protein sequences belonged to the pathway category metabolic pathways (51 sequences) followed by Biosynthesis of secondary metabolites (39 sequences). However, we only selected 2 pathways of interest, which have previously been implicated in the plant stress response for further discussion. This included the Protein processing in endoplasmic reticulum pathway which was significantly up-regulated in response to heat treatments, and the Phenylpropanoid biosynthesis pathway which was significantly down-regulated. See Fig. S-5 and Fig. S-6. In Appendix 1 for illustrations of these metabolic pathways.

In addition, the proteins identified in each respective pathway and their abundances across conditions is provided in Table S-1 and Table S-2 of Appendix 1.

Table 3: The top 10 molecular pathways heat responsive proteins in *E. grandis* were assigned to using KEGG and the associated number of differentially expressed proteins that were mapped from the study.

Pathway ID	Pathway name	Number of proteins mapped
egr01100	Metabolic pathways	51
egr01110	Biosynthesis of secondary metabolites	39
egr04141	Protein processing in endoplasmic reticulum	21
egr01230	Biosynthesis of amino acids	8
egr01200	Carbon metabolism	7
egr00940	Phenylpropanoid biosynthesis	7
egr00010	Glycolysis / Gluconeogenesis	7
egr00500	Starch and sucrose metabolism	7
egr00941	Flavonoid biosynthesis	6
egr04626	Plant-pathogen interaction	5

Discussion

Summary of study

This study used a quantitative proteomics approach to investigate adaptive variation in protein mechanisms of heat tolerance between naturally occurring *E. grandis* provenances. Specifically, we tested the adaptive differences in the leaf HSP content in response to different heat intensities and with respect to different temperature regimes between provenances of *E. grandis* seedlings. We further explored the larger proteome response to heat treatments and identified biological processes which may be important in heat acclimation in *E. grandis* for further investigation. This was achieved in a manipulative heat experiment, exposing four-month old *E. grandis* seedlings, from six provenances arrayed across a gradient of increasing annual mean temperature, to simulated heat-wave conditions over four days. Leaves were then subjected to proteomic analyses to compare differences in the protein expression profile across provenances and temperature conditions. The main goal of the study was to use adaptive variation in HSP expression between provenances to predict the relative thermotolerance of *E. grandis* seedlings to heatwaves under future climate change.

Hypothesis 1: HSP expression will increase predictably with temperature conditions at collection sites of *E. grandis* provenances

The findings of the present study confirmed distinct changes in the leaf proteome between the six study provenances of *E. grandis* in response to heat treatments. As expected, HSPs were consistently the most up-regulated group of proteins in response to the extreme heat treatment and showed varied expression across provenances. HSP levels at 42°C showed a consistently negative trend with annual mean temperature, contradicting our initial prediction. Instead, we found that differences in the expression of small HSPs and overall HSP content were strongly positively correlated with the heat stress index of each provenance, which was measured as the average number of days (1960 to 1990) exceeding +15°C of the MAT for a particular provenance. This meant that the cooler southern edge population actually displayed the largest sHSP and overall HSP abundance and warmer edge populations displayed the lowest abundance under the 42°C treatment. This finding suggests that the frequency of extreme heat events may be more important than temperature averages in influencing the evolved sHSP response between *E. grandis* provenances. This result supports previous findings on the role of temperature extremes

in predicting geographic variation in HSP content between plant populations (Barua, Heckathorn & Coleman 2008). Similarly, Amano *et al*. (2012) showed the importance of fluctuations in habitat temperature in underpinning evolved species differences in HSP content between aquatic plants. Since HSPs are most important for survival during periods of acute heat stress, the frequency of heat stress in a habitat is likely to impose greater selection pressures on the evolved HSP content of populations compared to mean annual temperature.

Individual HSP classes also showed different expression trends between provenances. The HSP60 and HSP70 class showed very slight trends of increase in expression across provenances compared to sHSPs. These trends were non-significant for both MAT and heat stress frequency across provenances, apart from HSP60 which showed a significant negative relationship with MAT. The abundance of sHSPs in the 42° treatment compared to the other HSP classes is unsurprising since they are widely considered the most heat responsive class of HSPs in plants (Waters *et al.* 1996; Heckathorn, Downs & Coleman 1999; Nakamoto & Vígh 2007). However, the strong correlation with heat stress frequency suggest that expression of sHSPs may be more strongly influenced by exposure to extreme temperature fluctuations and as a result may be a stronger marker of thermotolerance.

sHSPs have been the subject of many plant studies of heat shock, likely due to their wide diversity (Waters *et al.* 1996). For instance, several ecological and evolutionary studies into temperature response have shown how the abundance of various sHSPs is strongly correlated with plant survival and fitness under heat stress (Downs et al. 1998; Knight & Ackerly 2001; Barua *et al.* 2003). For instance, Downs *et al.* (1998) showed how the abundance of a chloroplast sHSP was consistently positively correlated with thermotolerance in each of six evolutionarily divergent angiosperm species. Similarly, sHSP transcripts were most highly expressed in eight common bean varieties under heat shock known to have the highest thermotolerance and to have originated from regions with more frequent heat stress (Luiz Simões-Araújo, Gouvêa Rumjanek & Margis-Pinheiro 2003). Together these studies indicate that sHSP mechanisms are a very conserved and an important component of acquired thermotolerance in plants – a finding supported by our data in *E. grandis*.

Our findings have important implications for the potential adaptive response of *E. grandis* populations under increasing heat stress projected under future climates (Nairn & Fawcett 2013). In particular, the clear relationship between intraspecific differences in sHSP expression and heat

stress exposure detected in this study implies that there may be a gradient in adaptive capacity in *E. grandis.* Specifically, southern populations (e.g. P6, Bulahdelah State Forest, NSW; P3, Brooweena State Forest, QLD; P4, Baroon Pocket, Maleny, QLD; P5, Orara West State Forest) may have superior molecular-level adaptive potential than more northern populations (e.g. P1, Gadgarra NP, QLD; P2, Finch Hatton Gorge) increasing their ability to cope with heat wave conditions. In addition to HSP content, the 42°C treatment resulted in large differences in the number of proteins significantly differently upregulated across provenances. Interestingly, the patterns of increase showed some similarities to the trend of sHSP content across provenances. Notably, P1 and P2 showed the lowest overall proteome response to the 42°C treatment and P6 showed the largest number of up-regulated proteins. This finding was also reflected by the number of proteins uniquely up-regulated across provenances with P1 and P2 showing the lowest number of unique mechanisms and P6 showing the largest number. This finding may suggest a less adaptive heat shock response among provenances from more northern climates.

Hypothesis 2: Increased heat intensity will maximise HSP expression across *E. grandis* provenances.

Heat intensity had a significant effect on the HSP expression levels between provenances in the study. As predicted the extreme heat treatment (42°C) elicited a significantly greater HSP response, for all HSP classes, compared to the intermediate heat treatment (35°C) across provenances. This suggests that the increased expression of HSP levels may be an adaptive response to extreme temperatures. However, previous studies on different taxa have also demonstrated that HSP abundance declines with exposure to increasing heat stress (Roberts, Hofmann & Somero 1997; Sørensen, Dahlgaard & Loeschcke 2001; Zatsepina *et al.* 2001; Barua *et al.* 2008). For instance, a study comparing HSP response in populations of the perennial shrub *Chenopodium album* L. showed that HSP abundance decreased in most populations in response to increasing experimental temperatures (Barua *et al.* 2008). Furthermore, the study showed that HSP expression decreased more among plant populations from more fluctuating climates in response to heat treatment than those from more stable climates. These authors hypothesised that decreasing HSP expression in fluctuating climates may result from an increased reliance on basal mechanisms of thermotolerance in *C. album* populations. Basal thermotolerance describes the inherent ability of plants to survive excess temperatures which is the product of adaptations such as increased protein stability which allows proteins to function normally at elevated temperatures. This is compared to acquired thermotolerance which describes the acclimation

ability provided by the accumulation of induced molecular mechanisms such as HSPs in response to stress.

Although explicitly testing of basal versus acquired thermotolerance in *E. grandis* as outside the scope of this study future work may explore the balance between these thermotolerance mechanisms in acclimating to heat stress along environmental gradients. Previous studies have suggested that habitats of chronic heat stress may favour the evolution of basal thermotolerance mechanisms over the fitness costs of regular HSP accumulation (Sørensen *et al.* 2001; Zatsepina *et al.* 2001; Tonsor *et al.* 2008). However, it is possible that in the habitat conditions that *E. grandis* experiences in the field, heat stress events occur at a frequency at which the benefits of acquired thermotolerance in HSP expression are not yet outweighed by its costs (e.g. reduced survival (Feder *et al.* 1992) and growth (Tonsor *et al.* 2008)) and are favourable compared to basal mechanisms.

Compared to the 42°C treatment, the intermediate 35°C treatment led to a similar level of expression of all HSP classes across provenances. This was surprising given that long term adaptation of provenances to different climate characteristics would be expected to result in different temperature thresholds of HSP induction and therefore detectable differences in HSP content. This may also suggest that 35°C was too high a temperature to observe these base differences in the HSP induction thresholds between provenances. Future experiments may instead use temperatures below the heat stress threshold of 10 - 15°C above ambient temperature used in this study to explore the differences in HSP induction threshold between provenances. In addition to HSP expression it is interesting to note that, compared to 35°C, the 42°C treatment also resulted in much greater number of up-regulated proteins and therefore overall proteome response to heat stress.

Functional classes of differentially expressed proteins and their pathways

Proteins differentially expressed under the 42°C treatment were predominantly involved in the following top functional categories: metabolic processes, protein metabolism, stress response and biosynthesis. These are the key biological processes we would expect to see up-regulated under heat stress and this result parallels previous studies on protein response to heat stress in rice (Gammulla *et al.* 2010) and grapes (George *et al.* 2015). Functional classification of differentially expressed proteins was not feasible at the provenance level in the present study, but the overall

data provides a complementary perspective to the information on heat response at the species level.

Of the multiple metabolic pathways significantly upregulated in *E. grandis* under heat stress we will focus our attention on the protein processing in the endoplasmic reticulum (ER). This pathway is responsible for the production of correctly folded secretory and membrane proteins and also the disposal of misfolded proteins (Liu & Howell 2010). In addition to this, it acts in detecting and responding to environmental stresses (Liu & Howell 2010) . Under stress conditions, sensitive folding machinery in the ER begins to breakdown and misfolded proteins begin to accumulate (Liu & Howell 2010). This triggers an unfolded protein response in the ER which results in increased expression of genes underlying protein folding machinery – such as HSPs and other molecular chaperones – in order to restore normal protein and cell function (Vitale & Boston 2008). However, the unfolded protein response may also trigger programmed cell death in events of extreme or chronic stress. This pathway has previously been shown to respond to a range of environmental stresses in plants including heat stress (Gao *et al.* 2008; Liu & Howell 2010). The overexpression of certain proteins of this pathway have also shown to confer stress tolerance in plants (Alvim *et al.* 2001; Valente *et al.* 2009). The prominence of this pathway in this study highlights it as a key cellular process of adaptive significance in the heat stress response of *E. grandis* at the species level, though the basis of expression patterns across the environmental gradient remain for future research.

By contrast, the phenylpropanoid biosynthesis pathway was significantly down-regulated in response to heat stress at the species-level in *E. grandis*. This pathway is responsible for the production of large range of phenolic compounds such as anthocyanin, stilbene and flavonoids which are involved in many different plant functions (Vogt 2010). Importantly, phenolic compounds of this type are known to be vital for protecting vegetative tissues and especially leaf cells against a number of biotic and abiotic stresses (Dixon & Paiva 1995). Heat stress has also been show to result in increases in some phenylpropanoids (Wahid *et al.* 2007) and has been associated with increased thermotolerance in cropping plants, such as watermelon (Rivero *et al.* 2001). Interestingly, the expression of some phenylpropanoids has also been shown to vary inversely with HSP expression under heat stress (Walter 1989) . For instance a study found that the normal induction of phenol compounds in parsley cells under light and fungal stress, were over-ridden by the addition of heat shock and was associated with an increase in HSP content (Walter 1989). This finding supports the results in the current study *E. grandis* which show a

consistent pattern of down-regulation in phenylpropanoid biosynthesis at the same HSPs are upregulated. Therefore, this pattern of down-regulation of the phenylpropanoid biosynthesis pathway in the heat stress response in *E. grandis* may warrant further investigation.

Conclusions on the protein-level heat stress response of *E. grandis*

Adaptive differences in acquired thermotolerance may affect seedling survival and wider demographic processes in *E. grandis* under projected increases in extreme heat events. This study provided initial insights into molecular mechanisms important to heat adaptation at the protein level in *E. grandis* seedlings. Our comparative analysis across *E. grandis* provenances indicated that the magnitude of temperature fluctuation over a baseline period (% days +15°C above the mean) was a stronger predictor of HSP expression levels than mean annual temperature. This suggests that seedlings from provenances that experience more frequent heat stress, which are located at the southern edge of range, may have greater adaptive capacity to cope with increased heat waves under climate change compared to those from more stable climates at the northern edge of the range. This finding is important for decision makers in informing climate adaptation strategies for *E. grandis* and, perhaps, for a wider suite of phylogenetically-related *Eucalyptus* species.

Although the study inferred thermotolerance based on HSP markers known to be important in heat adaptation, we were unable to link observed HSP content with relative physiological tolerance of *E. grandis* provenances under heat stress. Therefore, future studies may measure physiological traits sensitive to heat – such as photosynthetic performance or respiration rate – to test the relative physiological performance of seedling provenances. In addition, heat responsive proteins in the study were linked to their associated biological functions and metabolic pathways to better understand the broader cellular processes important in acclimation to extreme heat in *E. grandis*. This is an interesting direction for further investigation and may lead to the identification of novel biomarkers of thermotolerance, which may assist forestry practitioners in selecting stands of *E. grandis* that maximize yield under increased heat stress.

A limitation of the experimental approach used here was the sampling of the leaf proteome only at the end of the heat shock treatment. This did not allow for insight into the initial accumulation of HSP content in the first hours of heat shock or other potentially important temporal changes in individual HSPs and HSP class content between provenances throughout the treatment period. In addition, the lab conditions used in the experiment, which exposed plants to extreme stress for extended periods may not reflect the heat shock response of *E. grandis*

provenances in natural habitats. This may therefore limit the inferences we can make about the HSP levels that are important for acquired heat tolerance *in situ* in this species. Therefore, further studies may use more ecologically realistic lab conditions or conduct field studies to more accurately investigate adaptive variation in HSP content under heat stress. Despite these limitations, this study makes a contribution to the growing field of quantitative proteomics and provides potentially interesting insights into the ecology and evolution of plant traits in non-model and non-cropping species at the molecular level.

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Appendices:

Appendix I:

Fig. S-1. Comparison of *E. grandis* saplings of the same age (approximately four months) and provenance between the trial and final experiment. (A) Trial experiment sapling with leaves showing signs of UV deficiency (B) Final experiment sapling with UV-supplementation showing normal leaf development.

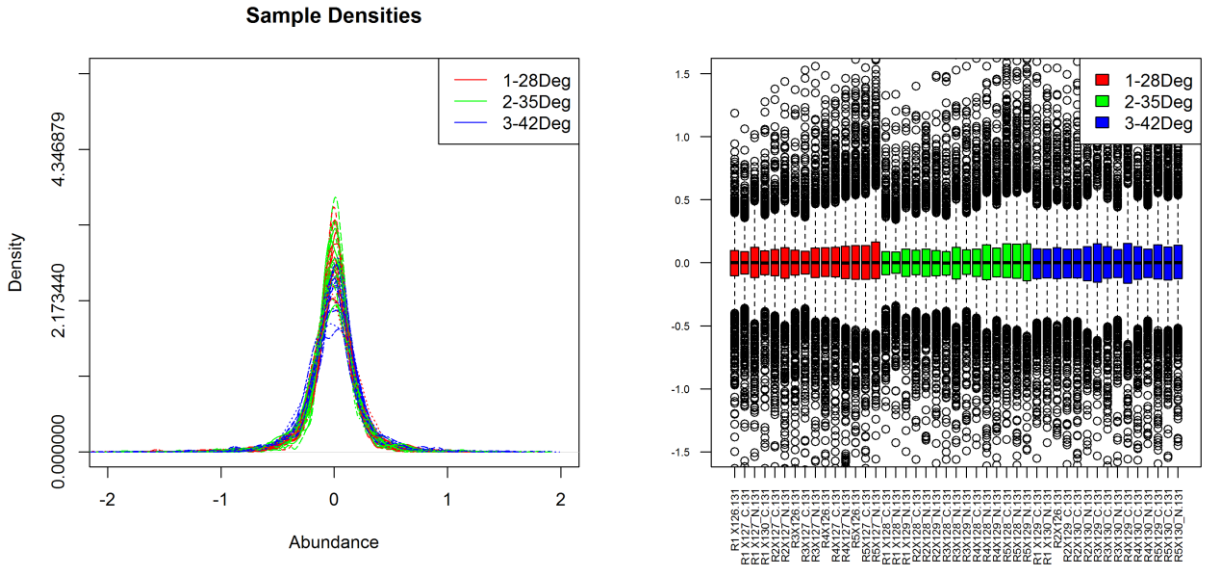
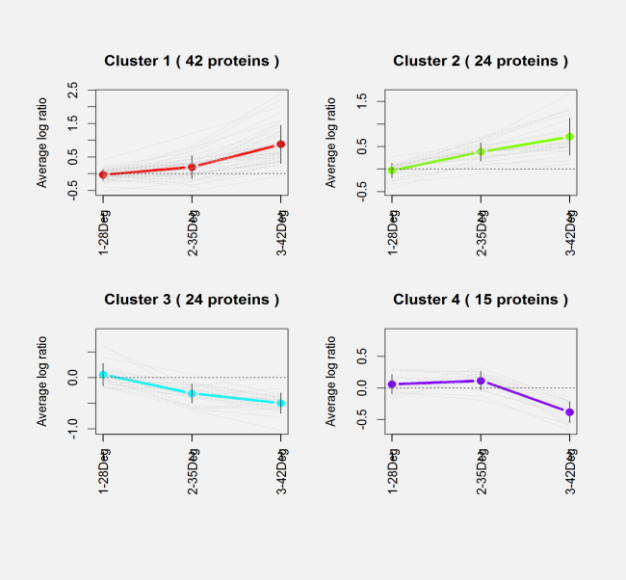


Fig. S-2. A) Density plot of all sample log ratios compared to a common reference approximating a normal curve. (B) Boxplot of all sample log ratios compared to the common reference grouped by treatment.



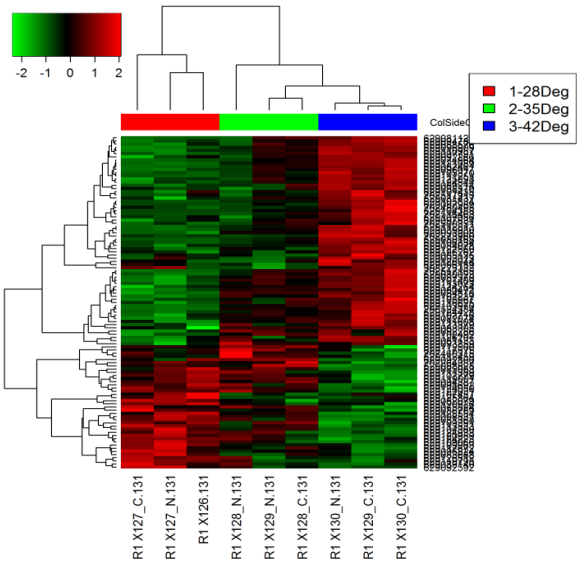


Fig. S-3. Hierarchical clustering of the differentially expressed proteins under heat stress for run 1. (A) Cluster diagram grouping differentially expressed proteins in Run 1 with similar expression trends. The coloured trend bar indicates the mean trend in expression of proteins in each cluster across conditions. (B) Example for Run 1 of individual heat maps generated for each provenance based differentially expressed proteins. Redness indicates degree of increase in protein expression, greenness degree of decrease in expression and black no significant change in expression.

Population						No. of proteins
P1	P2	P3	P4	P5	P6	
0	0	0	0	0	1	58
0	0	0	0	1	0	63
0	0	0	0	1	1	7
0	0	0	1	0	0	82
0	0	0	1	0	1	6
0	0	0	1	1	0	3
0	0	0	1	1	1	2
0	0	1	0	0	0	98
0	0	1	0	0	1	15
0	0	1	0	1	0	4
0	0	1	0	1	1	2
0	0	1	1	0	0	7
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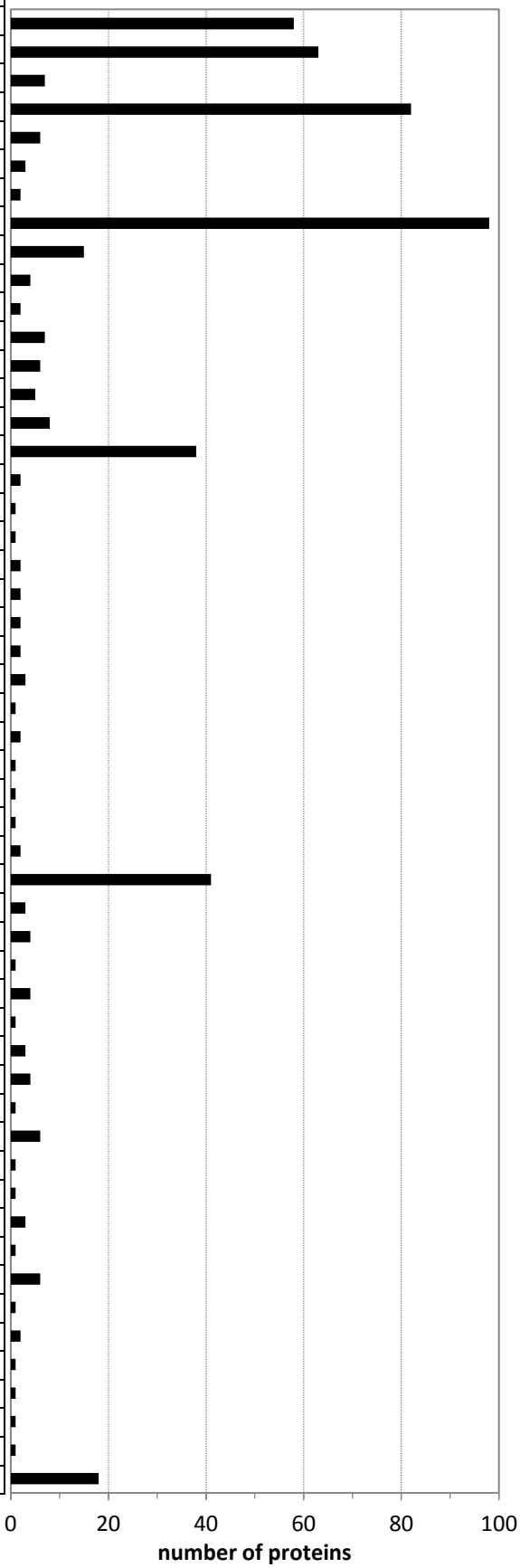

Fig. S-4. Complete presence/absence table with all categories of uniquely differentially expressed proteins across populations under the 42°C treatment.

Table S-1. Heat responsive enzymes identified in the first pathway of interest in the study: protein processing in endoplasmic reticulum.

Pathway	Enzyme description	KEGG enzyme ID
Protein processing in endoplasmic reticulum	small heat shock protein, chloroplastic-like	egr:104415724
	dnaJ protein homolog	egr:104421364
	18.1 kDa class I heat shock protein-like	egr:104422425
	18.1 kDa class I heat shock protein-like	egr:104422440
	18.1 kDa class I heat shock protein	egr:104422443
	18.1 kDa class I heat shock protein-like	egr:104422450
	heat shock 70 kDa protein-like	egr:104423239
	heat shock protein 83	egr:104424446
	small heat shock protein, chloroplastic-like	egr:104424548
	protein disulfide isomerase-like 2-3	egr:104424708
	17.4 kDa class I heat shock protein-like	egr:104432744
	heat shock protein 90-1	egr:104433348
	B-cell receptor-associated protein 31	egr:104435278
	hsp70 nucleotide exchange factor fes1-like	egr:104436723
	23.6 kDa heat shock protein, mitochondrial-like	egr:104443693
	heat shock protein 90-1-like	egr:104443842
	heat shock protein 83-like	egr:104448346
	15.7 kDa heat shock protein, peroxisomal	egr:104449754
	22.0 kDa class IV heat shock protein	egr:104450758
	18.1 kDa class I heat shock protein-like	egr:104452453
	endoplasmin homolog	egr:104456658

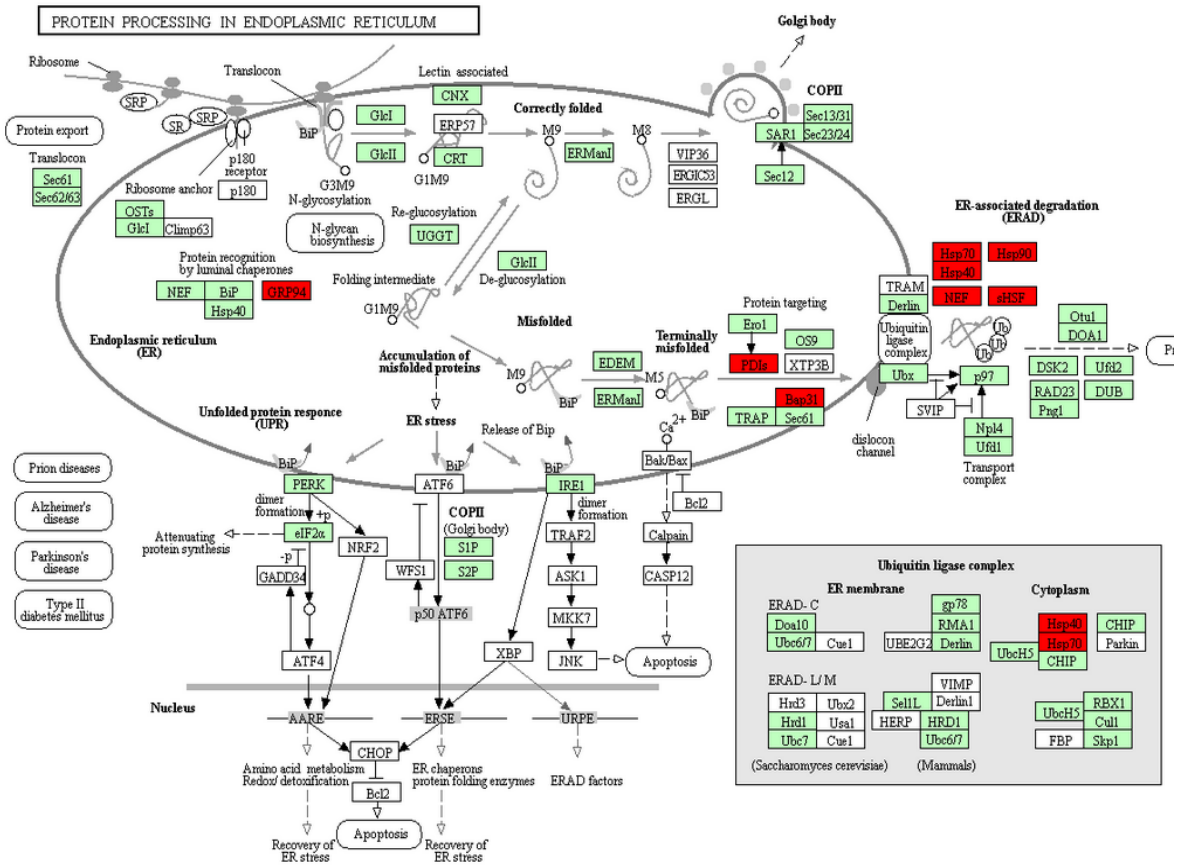

Fig. S-5. Protein network diagram of the protein processing in endoplasmic reticulum pathway up-regulated in the study. Red highlighted cells represent proteins identified as up-regulated in response to heat treatments.

Table S-2. Heat responsive enzymes identified in the second pathway of interest in the study: Phenylpropanoid

biosynthesis.

Pathway	Enzyme	Enzyme ID
Phenylpropanoid biosynthesis	probable mannitol dehydrogenase	egr:104418344
	trans-cinnamate 4-monooxygenase	egr:104422338
	probable cinnamyl alcohol dehydrogenase 1	egr:104444153
	caffeic acid 3-O-methyltransferase-like	egr:104445601
	isoliquiritigenin 2'-O-methyltransferase-like	egr:104449527
	shikimate O-hydroxycinnamoyltransferase-like	egr:104450636
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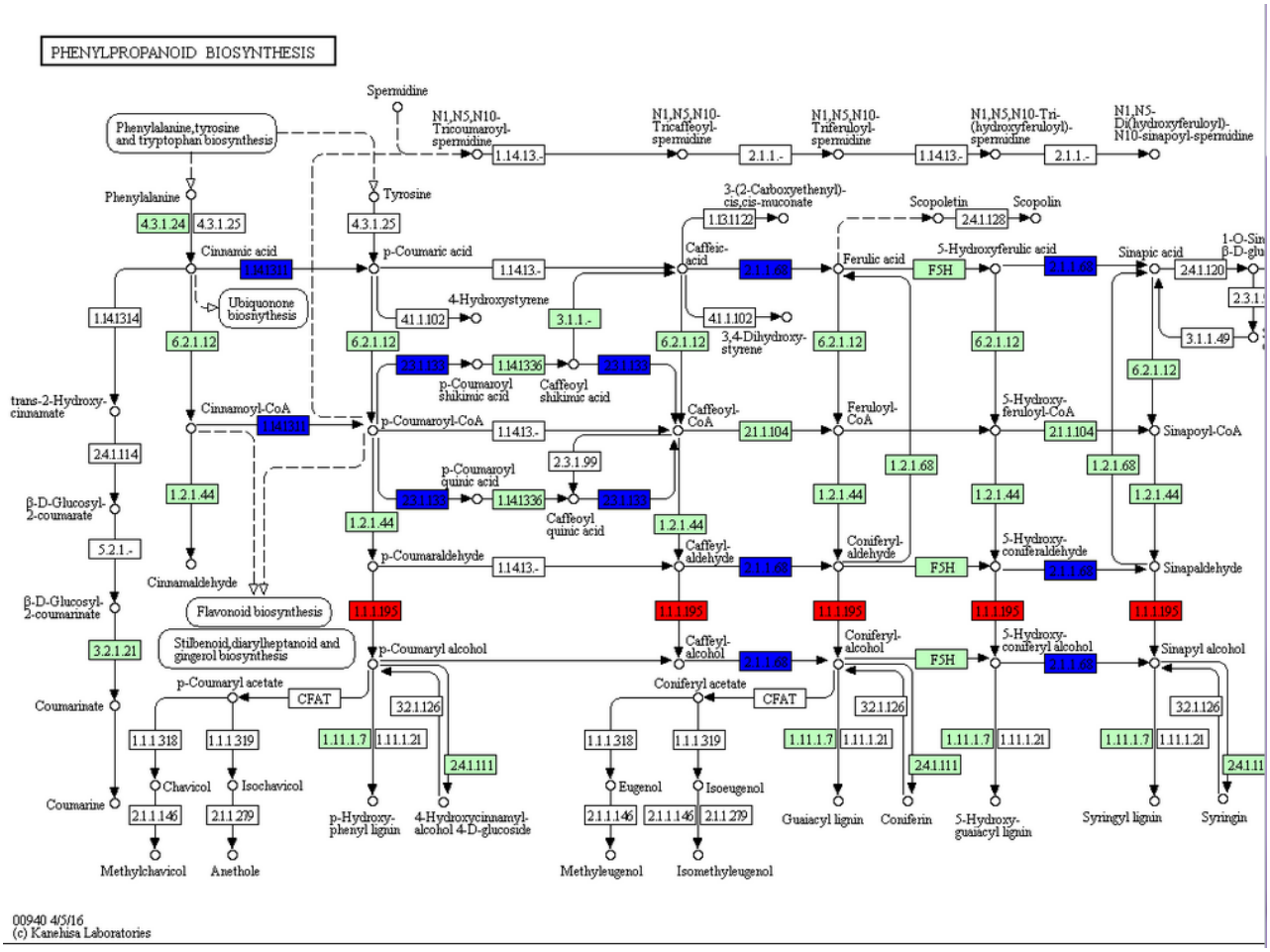

Fig. S-6. Protein network diagram of the phenylpropanoid biosynthesis pathway found to be down-regulated in the study. Blue highlighted cells represent proteins identified as down-regulated in response to heat treatments and red cells represent proteins identified as up-regulated.

Appendix II: Journal of Ecology author guidelines

Manuscript Structure

Submitted manuscripts must be double spaced with sequential line numbers throughout the entire document. Pages should be numbered consecutively, including those containing acknowledgements, references, tables and figures.

Standard Papers. Standard papers should not normally be longer than 12 printed pages (a page of printed text, without figures or tables carries c. 800 words). The typescript should be arranged as follows, with each section starting on a separate page.

Title page. This should contain:

- a concise and informative title (as short as possible). Do not include the authorities for taxonomic names in the title.
- a list of authors' names with names and full addresses of institutions. Author first names should be provided in full.
- the name of the correspondence author, indicated using an asterisk, to whom decisions and, if appropriate, proofs will be sent. Email address, fax number and current address, if different from above, should be provided.
- a running headline of not more than 45 characters.

Summary. This is called the Abstract on the web submission site. The Summary must not exceed 350 words and should list the main results and conclusions, using simple, factual, numbered statements. The final point of your Summary must be headed '*Synthesis*', and must emphasize the key findings of the work and its general significance, indicating clearly how this study has advanced ecological understanding. This policy is intended to maximize the impact of your paper, by making it of as wide interest as possible. This final point should therefore explain the importance of your paper in a way that is accessible to non-specialists. We emphasize that the Journal is more likely to accept manuscripts that address important and topical questions and hypotheses, and deliver generic rather than specific messages.

Key-words. A list in alphabetical order not exceeding ten words or short phrases, excluding words used in the title. One of the keywords must be the subject category that you select for your manuscript as part of the submission process (e.g. "Habitat fragmentation", "Dispersal", etc.).

Introduction. Explain the reasons for carrying out the work, outline the essential background and clearly state the nature of the hypothesis or hypotheses under consideration.

Materials and methods. Provide sufficient details of the techniques employed to enable the work to be repeated. Do not describe or refer to commonplace statistical tests in this section but allude to them briefly in Results.

Results. State the results and draw attention in the text to important details shown in tables and figures.

Discussion. Point out the significance of the results in relation to the reasons for doing the work, and place them in the context of other work.

Author's Contributions. All submissions with more than one author must include an Author's Contributions statement. All persons listed as authors on a paper are expected to meet ALL of the following criteria for authorship:
- substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, or drafting the article or revising it critically for important intellectual content;

- final approval of the version to be published;
- agreement to be accountable for the aspects of the work that they conducted and ensuring that questions related to the accuracy or integrity of any part of their work are appropriately investigated and resolved.
Acquisition of funding, provision of facilities, or supervising the research group of authors without additional contribution are not usually sufficient justifications for authorship.
The statement should include an explanation of the contribution of each author. We suggest the following format for the Author Contributions statement:
AB and CD conceived the ideas and designed methodology; CD and EF collected the data; EF and GH analysed the data; AB and CD led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Acknowledgements. If authors refer to themselves as recipients of assistance or funding, they should do so by their initials separated by points (e.g. J.B.T.). Do not acknowledge Editors by name.

Data Accessibility. To enable readers to locate archived data from papers, we require that authors list the database and the respective accession numbers or DOIs for all data from the manuscript that have been made publicly available. An example of what this section should look like can be found in the **Data Archiving Q&A**.

References (see **Specifications**).

Tables (see **Specifications**). Each table should be on a separate page, numbered and accompanied by a legend at the top. They should be referred to in the text as Table 1, etc. Do not present the same data in both figure and table form or repeat large numbers of values from Tables in the text.

Figures (see **Specifications**). Figures and their legends should be grouped together at the end of the paper, before the appendices (if present). If figures have been supplied as a list at the end of the text file (as recommended), they should appear above their respective legend. The word figures should be abbreviated in the text (e.g. Fig. 1; Figs. 2 and 3), except when starting a sentence. Photographic illustrations should also be referred to as Figures.

Appendices / Supporting information. Please note that *Journal of Ecology* no longer publishes Appendices in the printed version, but supporting information may be published in electronic form. Instructions for the preparation of supporting information are available **here** and general instructions on supporting information can be found **here**.

Please combine multiple files of the same type into one file (e.g. if you have several supplementary tables in Excel please combine these into one Excel file).

In order to promote the advancement of science through the process of documenting and making available the research information and supporting data behind published studies, the editors of this journal strongly encourage authors to make arrangements for archiving their underlying data.

Units, symbols and abbreviations. Authors are requested to use the International System of Units (SI, Système International d'Unités) where possible for all measurements (see Quantities, Units and Symbols, 2nd edn (1975) The Royal Society, London). Note that mathematical expressions should contain symbols not abbreviations. If the paper contains many symbols, it is recommended that they should be defined as early in the text as possible, or within a subsection of the Materials and methods section. Journal style for time units are: s, min, h, days, weeks, months, years.
Scientific names. Give the Latin names of each species in full, together with the authority for its name, at first mention in the main text. Subsequently, the genus name may be abbreviated, except at the beginning of a sentence. If there are many species, cite a Flora or checklist which may be consulted for authorities instead of listing them in the text. Do not give authorities for species cited from published references. Give

priority to scientific names in the text (with colloquial names in parentheses, if desired).

Manufacturers' names. When a special piece of equipment has been used it should be described so that the reader can trace its specifications by contacting the manufacturer; thus: 'Data were collected using a solid-state data logger (CR21X, Campbell Scientific, Utah, USA)'.

Mathematical material. Where ever possible, mathematical equations and symbols should be typed in-line by keyboard entry (using Symbol font for Greek characters, and superscript and subscript options where applicable). Do not embed equations or symbols using Equation Editor or Math Type, or equivalents, when simple in-line, keyboard entry is possible. Equation software should be used only for displayed, multi-line equations and equations and symbols that can not be typed. Suffixes and operators such as d, log, ln and exp will be set in Roman type; matrices and vectors in bold type; other algebraic symbols in italics; and Greek symbols in upright type. Make sure that there is no confusion between similar characters like l ('ell') and 1 ('one'). If there are several equations they should be identified by an equation number (e.g. 'eqn 1' after the equation, and cited in the text as 'equation 1').

Number conventions. *Text*: Numbers from one to nine should be spelled out except when used with units; e.g. two eyes but 10 stomata; 3 years and 5 kg. *Tables*: Do not use an excessive number of digits when writing a decimal number to represent the mean of a set of measurements (the number of digits should reflect the precision of the measurement).

Figures (including photographs). Please note that your paper will be published more quickly if instructions on figure content and figure format are followed carefully. Vector graphic figures will give the best possible online publication quality.

All illustrations are classified as figures and should be numbered consecutively (Fig. 1, Fig. 2, etc.) and placed in a list at the end of the document. Figures should be cited in the text as, e.g., Fig. 1, Fig. 1a,b, Figs 1 and 2 (no period after Figs), or, if starting a sentence, Figure 1. Each figure must have a legend, presented separately from the figure (i.e. as text rather than as part of the image). The legend should give enough detail so that the figure can be understood without reference to the text. Information (e.g. keys) that appears on the figure itself should not be duplicated in the legend. The figure legend label should be abbreviated, in bold, and end in a period (e.g. Fig. 1.). The figure legend text should end in a full stop.

Please ensure that artwork is prepared to fit across one or two columns or two-thirds width (71 mm, 149 mm or 109 mm, respectively). Wherever possible, figures should be sized to fit into a single column width. To make best use of space, you may need to rearrange parts of figures (e.g. so that they appear side by side). If figures are prepared that will require reduction, please ensure that symbols, labels, axes text, etc., are large enough to allow reduction to a final size of about 8 point, i.e. capital letters will be about 2 mm tall. Lettering should use a sans serif font (e.g. Helvetica or Arial) with capitals used for the initial letter of the first word only. Bold lettering should not be used within the figure. Units of axes should appear in parentheses after the axis name, as required. All lettering and symbols must be proportioned, clear and easy to read, i.e. no labels should be too large or too small. Figures should not be boxed (superfluous bounding axes) and tick marks must be on the inside of the axes if possible. Label multi-panel figures (a), (b), (c), etc., preferably in the upper left corner. Use greyscales (e.g. 0, 20, 40, 60, 80, 100%) in preference to pattern fills where possible. If colour figures are submitted for colour online publication only, ensure that after conversion to greyscale they remain entirely intelligible for the black-and-white print publication of your paper.

Colour figures (including photographs) must be accompanied by the **Colour Work Agreement Form**. The cost for colour printing must be met by the author (currently £150 for the first figure, £50 thereafter,

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File formats. At the time of submission, or after acceptance of the manuscript for publication, figure files should be supplied as follows. Photographic figures should be saved in tif format at 300 d.p.i. (or failing that in jpg format with low compression). Line figures should be saved as vector graphics (i.e. composed of lines, curves, points and fonts; not pixels) in pdf, eps, ai, svg or wmf format, or embedded as such in Word, as this enhances their display when published online. Combination figures (those composed of vector and pixel/raster elements) should also be saved in pdf, eps, ai, svg or wmf format where possible (or embedded as such in Word). If line figures and combination figures cannot be saved in vector graphics format, they should be saved in tif format at high resolution (i.e. 600 d.p.i.) (do not save them in jpg format as this will cause blurring). If you are unsure about the quality of your figures, please inspect a small portion by zooming in to check that fonts, curves and diagonal lines are smooth-edged and do not appear unduly blocky or burred when viewed at high magnification. Note that line and combination figures supplied in tif format are downsampled for online publication, authors should therefore preferentially opt for vector graphic formats for these figure types (note, however, that for print publication full resolution files will be used). For full instructions on preparing your figures please refer to our **Electronic Artwork Information for Authors** page.

Tables and table captions. Tables should be provided in editable format (rather than as embedded images or separate PDF files) with the columns clearly defined (for example, separated tabs or using the table function in Word or similar word processor software).

Data Accessibility. A list of databases with relevant accession numbers or DOIs for all data from the manuscript that have been made publicly available should be included in this section. For example:

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- Phylogenetic data: TreeBASE Study accession no. Sxxxx
- R scripts: uploaded as online supporting information
- Sample locations, IMa2 input files and microsatellite data: DRYAD entry doi: xx.xxxx/dryad.xxxx

Citations and references. Citation to work by four or more authors in the text should be abbreviated with the use of *et al.* (e.g. Able *et al.* 1997). Work with the same first author and date should be coded by letters, e.g. Thompson *et al.* 1991a,b. Citations should be listed in chronological order in the text and separated by a semi-colon, e.g. Zimmerman*et al.* 1986; Able *et al.* 1997.

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- Boutin, C. & Harper, J.L. (1991) A comparative study of the population dynamics of five species of Veronica in natural habitats. *Journal of Ecology*, **79**, 199-221.
- Clarke, N.A. (1983) The ecology of dunlin (*Calidris alpina* L.) wintering on the Severn estuary. PhD thesis, University of Edinburgh, Edinburgh.
- Pimm, S.L. (1982) *Food Webs*. Chapman and Hall, London.
- Sibly, R.M. (1981) Strategies of digestion and defecation. *Physiological Ecology* (eds C. R. Townsend & P. Calow), pp. 109-139. Blackwell Scientific Publications, Oxford.

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