Research Article

Small Heat Shock Protein Responses Differ between Chaparral Shrubs from Contrasting Microclimates

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Small heat shock protein (sHsp) responses were studied for two evergreen perennial shrubs in the northern California chaparral; one common on warm, south-facing slopes (*Ceanothus cuneatus*), and the other on cooler, north-facing slopes (*Prunus ilicifolia*). Small Hsp expression was induced experimentally for field collected leaves. Leaf collections were made where the species co-occur. Small Hsp expression was quantified using two antibodies, one specific to a chloroplast 22 kD sHsp and another that detects a broad range of sHsps. Differences between chloroplast sHsp accumulation, which protects thermally labile proteins in PSII, and the general sHsp response were examined. The species from the cooler microclimate, *Prunus*, had a lower induction temperature and accumulated greater levels of sHsps at low temperatures. Both *Prunus* and *Ceanothus* reached peak sHsp expression at 42°C. The species from the warmer microclimate, *Ceanothus*, had greater sHsp expression at higher temperatures. Chloroplast sHsp expression generally tracked sHsp expression in *Ceanothus*, but in *Prunus* general Hsps were elevated before chloroplast sHsps. Variation between species for sHsp expression (induction temperatures, accumulation levels, and the duration of expression) coupled with the costs of Hsp synthesis, may contribute to differences in the abundance and distribution of plants across environmental gradients.

1. Introduction

There have been hundreds of biochemical studies of the heat shock protein (Hsp) response in plants (see [1, 2] for recent reviews), but previous studies have almost exclusively involved experimentally grown plants in controlled environments. Therefore, while our knowledge of the functional roles of Hsps has been rapidly expanding, there is still relatively little information concerning Hsp expression for plants growing in their natural environments. Of the few studies that have examined Hsp expression in the field, three made no report of Hsp expression in leaves [3–5], and the others examined Hsp expression agricultural fields [6, 7]. In addition, a lack of comparative studies has precluded a general synthesis of how genetic differentiation for the Hsp response might contribute to adaptive evolution.

Leaf temperatures in the field can easily exceed those that lead to increased Hsp expression in artificial environments. Short duration temperature extremes (~15 minutes) can lead to increased Hsp expression [8]. Correlated abiotic stresses such as decreased water availability, high light, or low nutrients can also affect Hsp expression [9]. Plastic changes in protein synthesis, such as the Hsp response, may constitute a significant resource cost for plants, and significantly affect the distribution and abundance of species.

In plants, the functional roles of the small Hsps (sHsps) have received considerable attention. Small Hsps dominate protein synthesis during and after high temperature stress and can rapidly accumulate to greater than 1% of total leaf protein under some conditions [1, 10–14]. While most eukaryotes have just a few sHsps, in plants the protein class has duplicated and diversified to include 20–50 nuclear encoded genes [15, 16]. The evolutionary conservation of sHsps, combined with their diversification in plants, has led to the hypothesis that sHsps play an important role for whole plant thermal tolerance [17, 18].

This study was designed to address two questions. (1) How do induction temperatures and expression levels of
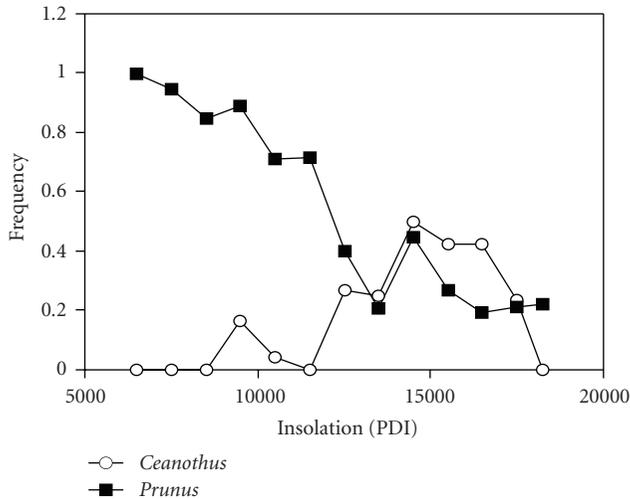
Figure 1: Microclimate distribution of Ceanothus cuneatus (open circles) and Prunus ilicifolia (closed circles). Potential diurnal insolation (PDI) was calculated using topographic position from an digital elevation model and a annual solar radiation model (figure reproduced from [19]).

sHsps differ between species with contrasting microclimate distributions? We addressed this questions using two evergreen perennial shrubs in the northern California chaparral; *Ceanothus cuneatus* codominant on warm, south-facing slopes, and *Prunus ilicifolia* a dominant species on cooler, north-facing slopes (Figure 1). Field leaf collections were made at sites where *Ceanothus* and *Prunus* cooccurred to control for other factors that could lead to variation in sHsp accumulation. The experiment was conducted using field collected leaf material to gain some realism for the natural context of sHsp expression at the expense of controlled environment assurances. (2) How does chloroplast sHsp expression track general sHsp expression? It is known that the chloroplast sHsp helps protect thermally labile proteins in PSII. Are chloroplast sHsps more or less temperature responsive compared to the general sHsp response?

2. Materials and Methods

Field work was conducted in the northern California chaparral at Jasper Ridge Biological Preserve (JRBP) on the campus of Stanford University. Ackerly et al. [19] quantified the microclimate distribution of shrub species at JRBP using line transects and vegetation plots combined with a solar insolation model. *Ceanothus cuneatus* is a codominant with *Adenostoma fasciculatum* on the high solar exposure south-facing slopes while *Prunus ilicifolia* is a codominant on the cooler north-facing slopes (Figure 1). Leaf temperatures for *Prunus* and *Ceanothus* were measured in the field using a Campbell Scientific data logger with two AM25T thermocouple multiplexers (Campbell Scientific, Logan, UT, USA). Twenty-five leaves of each species were measured at the microclimate extremes, and where the species cooccurred, by adhering 36 gauge type E thermocouples to the underside of

leaves (Figure 4). One minute average leaf temperatures were recorded throughout the spring and summer.

2.1. Experimentally Induced sHsp Expression. The temperature dependence of sHsp expression was examined experimentally for field-collected leaves of *Ceanothus* and *Prunus*. Leaves with no visible sign of physiological stress were collected in March 2001 before they experienced significant naturally occurring heat stress. Leaf collections were made at sites where the species cooccurred to control for other factors that might influence sHsp expression. Three replicate groups of 15 leaves per species were subjected to a series of temperature treatments (32, 34, 36, 38, 40, 42, 45°C) in air circulating chambers submerged in temperature-controlled water baths (Oven Industries, Mechanicsburg, PA, USA). We used detached leaves because it was the only method available to achieve precise and constant leaf temperatures for field collected leaves. Leaves were placed on top of a piece of moist filter paper inside the chamber to prevent drying. Leaf temperatures did not vary by more than 0.1°C (both between leaves and between set temperature and leaf temperature). Temperature treatments lasted 4 h. Following the heat treatment, leaves were allowed to recover for 4 h under low light. They were then frozen in liquid N and stored at minus 80°C until protein extraction.

The photochemical efficiency of PSII was measured for a subset of leaves following the heat stresses using the ratio of variable to maximum fluorescence (F_v/F_m). F_v/F_m was measured after a 0.7 second saturating actinic light pulse of approximately 12,000 μmol $* $ m$^{-2} * $ s^{-1} (Hansatech FMS2 fluorometer, King's Lynn, UK). Leaves were dark adapted for 30 min before F_v/F_m measurements.

2.2. Protein Methods. The conserved nature of the N-terminal "methionine-rich" domain of the chloroplast sHsp allowed Downs et al. [18] to produce an antibody that detected chloroplast sHsps in heat-stressed plant tissue from a diverse assemblage of species representing five divisions of the plant kingdom. A similar strategy was used to design a polyclonal antibody that cross-reacts with many small heat shock proteins [20]. Heckathorn and Downs graciously provided these antibodies for this study.

Total soluble leaf protein was extracted using a ceramic mortar and pestle with an extraction buffer made from the following ingredients: 3% w/v SDS (sodium dodecyl sulfate), 10% v/v 1.5 M Tris, 1% v/v 1 mM PMSF (phenylmethyl-sulfonyl fluoride), 2% v/v 0.1 M EDTA (ethylenediaminete-traacetic acid), 0.5% v/v 1 M ε-amino-n-caproic acid, 1% v/v 1 M benzamidine, 2% w/v PVP (polyvinylpyrrolidone), 4% w/v PVPP (polyvinylpolypyrrolidone), 0.1% w/v DTT (Dithiothreitol), 0.2% w/v Ascorbate, and 0.1% v/v of the protease inhibitors antipain and leupeptin (modified from [9]). We found that the soluble protein concentration of the extract sample varies considerably with the ratio of extraction buffer to fresh weight of leaf tissue and with the duration of incubation, mixing and grinding with the extraction buffer. Therefore, for all samples we added 2 mL of the extraction buffer to 1 gram of fresh leaf material and continued grinding the leaf tissue in the mortar and pestle

for 10 minutes after pulverizing the leaf tissue on liquid N. Samples were boiled for 4 min, centrifuged for 15 min at 15,000 revolutions per min, and the supernatant was collected and stored at −80°C.

The soluble protein concentration of the extract was determined using a Coomasie dot blot on Whatman filter paper (#4) and quantified using a Hewlett Packard ScanJet II laser scanner (Palo Alto, California, USA, after [21, 22]). Sample concentrations were inferred from a standard curve of BSA serial dilutions of known concentration. 40 ug of soluble protein was loaded on a precast 5%–20% TRIS-glycine SDS-PAGE gels (BIO-RAD, Hercules, California, USA). A positive control was run on each gel to account for blot-to-blot variation. Following separation, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane by Western Blot. Membranes were blocked for 2 h following transfer in a Tris/powdered milk solution. The optimal antibody concentrations were found by serial dilution so that resulting band intensities were within the linear range of detection. The PVDF membranes were incubated overnight at room temperature with a 1/3000 dilution of the polyclonal sHsp antibody (obtained from a generous gift from Heckathorn), or a 1/5000 dilution of the chloroplast sHsp antibody, both of which were followed by a 1.5 h incubation with a alkaline phosphatase conjugated secondary antibody (Sigma, St. Louis, Missouri, USA; 1/10000 dilution). Accumulation levels of sHsps were quantified using a Hewlett Packard ScanJet II and Scion Image for Windows image analysis software (available free at http://www.scioncorp.com/), following development with the alkaline phosphatase substrate. sHsp accumulation levels are expressed as a percentage of the positive control.

3. Results

3.1. Differences between Species for sHsp Expression. There was a significant difference between *Prunus* and *Ceanothus* for the temperature dependent accumulation of sHsps (Figure 2). There was a significant difference for general sHsp expression, (ANOVA, $F = 6.21$, $P = .01$, Figure 2(a)), and chloroplast sHsp expression (ANOVA, $F = 5.32$, $P = .01$, Figure 2(b)). At low temperatures *Prunus*, which is common on the cooler north-facing slopes, had greater levels of general sHsp expression as well as for the chloroplast sHsp (Figures 2(a) and 2(b)). Both species exhibited peak general sHsp expression after the 42°C treatment. Chloroplasts sHsp expression peaked at 40°C for *Prunus* and at 42°C in *Ceanothus*. After the 32°C treatment low levels of sHsps were expressed for *Prunus*, while only trace amounts were present for *Ceanothus*. Neither species expressed chloroplast sHsps following the 32°C treatment. Both *Prunus* and *Ceanothus* expressed general sHsps after the 45°C temperature treatment. Chloroplast sHsp expression was significantly reduced following the 45°C treatment in *Prunus* while only somewhat reduced in *Ceanothus*.

Photosynthetic thermal tolerance, measured by the fluorescence parameter F_v/F_m, was significantly different between species (ANOVA $F = 4.68$, $P = .05$, Figure 2(c)). *Ceanothus* had greater at F_v/F_m higher temperatures
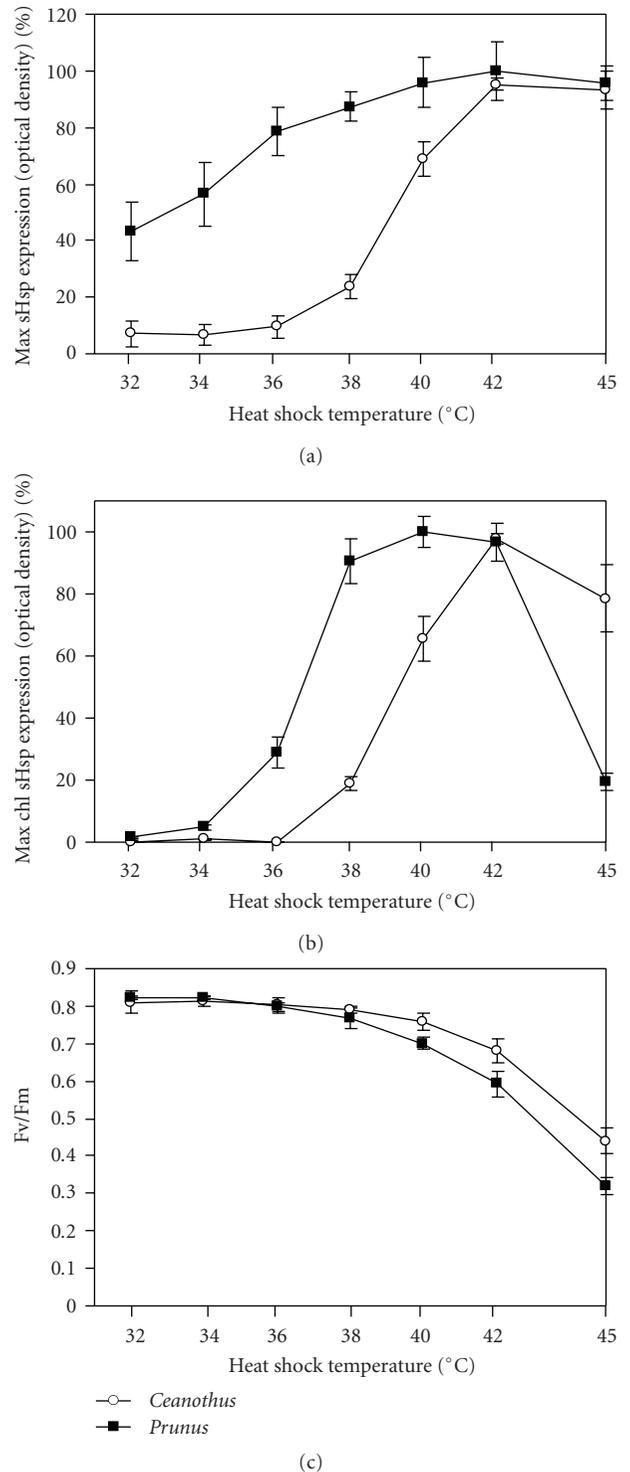

(a)

(b)

(c)

FIGURE 2: (a) The temperature-dependent decline for F_v/F_m following temperature treatments in Ceanothus and Prunus. General sHsp responses (b) and chloroplast sHsp expression following the same temperature treatments as (a) In (a), (b), and (c) Prunus is represented with a solid square and Ceanothus an open circle.

(36°C+). At low temperatures there was a positive correlation between F_v/F_m and sHsp expression, but at high temperatures the relationship became negative.
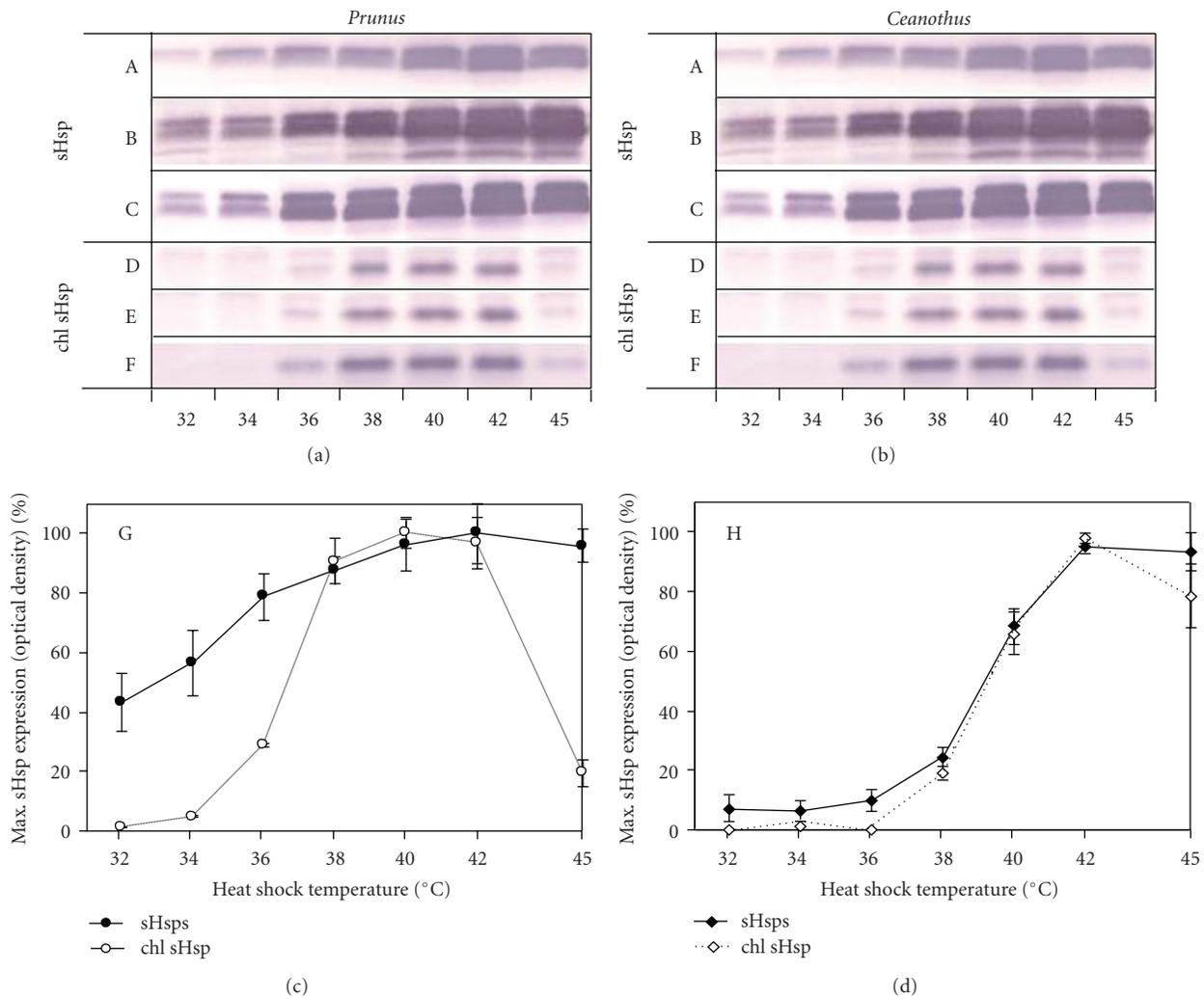
Figure 3: Experimentally induced sHsp expression for *Ceanothus* and *Prunus* after a series of temperature treatments (32 to 42°C on 2°C intervals and 45°C). Panels A, B, and C are western blots of the three replicates using the general sHsp antibody. Panels D, E, and F are western blots of the three replicates using the chloroplast sHsp (chl. sHsp) antibody. Panels G, and H are the optical quantification of the three replicates. Error bars are SE for the three replicates.

3.2. Differences between the Chloroplast and General sHsp Response. Chloroplast sHsp expression generally tracked general sHsp accumulation in *Ceanothus*, except following the 45°C treatment, when chloroplast sHsp expression was slightly reduced compared to general sHsp expression (Figure 3H). In *Prunus,* general sHsps were expressed at lower temperatures compared to chloroplast sHsps (Figure 3G), and the chloroplast sHsp was significantly down regulated following the 45°C temperature treatment compared to the general sHsps response.

3.3. Leaf Temperature Variation. Where the species cooccurred (where leaf collections were made for this study) leaf temperatures were not significantly different between *Ceanothus* and *Prunus* (ANOVA, $F = 6.13$, $P = .01$). However, leaf temperatures varied considerably where the species are most common in the field (i.e., at opposite extremes of the microclimate gradient at our field site,

Figure 4). Leaf temperatures for *Ceanothus* on the warmer south facing slope exceeded 32°C on most days, while temperatures were rarely that hot for *Prunus* on the cooler north facing slope. On most days leaf temperatures differed by more than 10°C between *Prunus* and *Ceanothus* at these microclimate extremes. Solar radiation is the primary factor that establishes these microclimate differences. Figure 4 shows six days of leaf temperature measurements. When solar radiation was variable, microclimate leaf temperature differences were not as great (see the second day in Figure 2).

4. Discussion

We observed significant differences for sHsp accumulation between chaparral shrubs with contrasting microclimate affinities. *Prunus illicitifolia*, which is common on cooler north-facing slopes (Figures 1 and 4) had greater sHsp expression at lower temperatures compared to *Ceanothus*
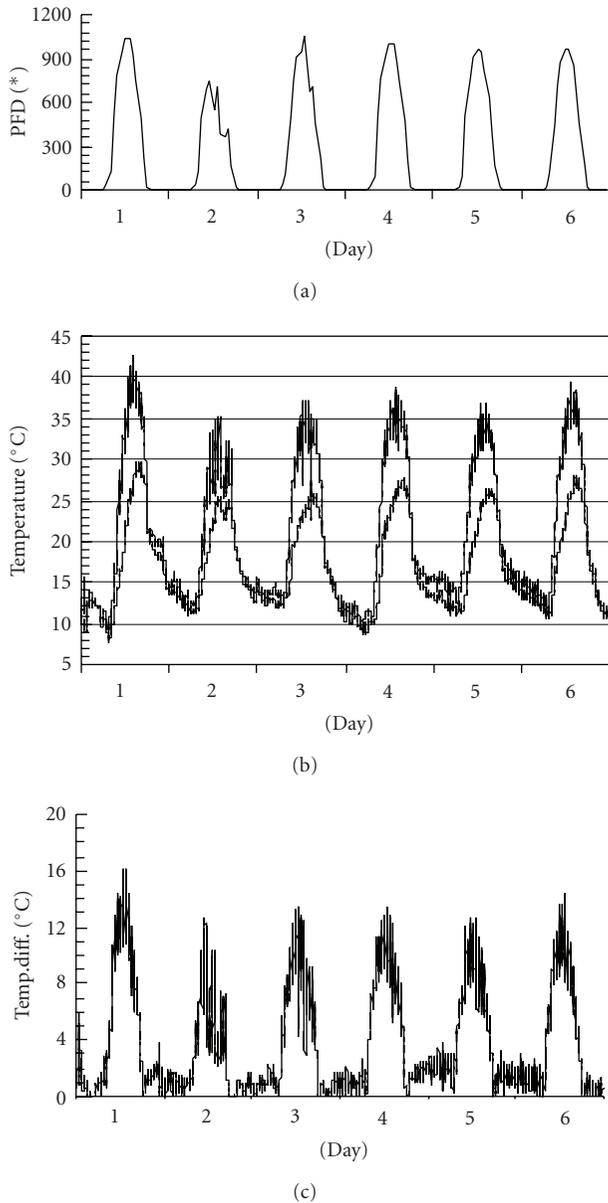
(a)

(b)

(c)

FIGURE 4: (a) Daily time course of photon flux density (PFD), (b) leaf temperature on north and south facing slopes (leaf temperatures were warmer on the south facing slope), and (c) the leaf temperature difference between the two microclimates. The leaf temperature measurements were the average of 25 sun leaves for each microclimate.

cuneatus, an abundant species on warmer south-facing slopes. *Prunus* also had lower photosynthetic thermal tolerance (Figure 2(c)) and a reduced capacity to synthesize sHsps at high temperatures. Knight and Ackerly [8] documented a similar pattern in *Encelia* for comparisons between *Encelia farinosa*, a species abundant in the Mojave desert, and *Encelia californica*, a species common on the cooler coastal bluffs of southern California.

In a review article of photosynthetic thermal tolerance based on gas exchange and fluorescence studies, Berry and

Bjorkman [23] suggested that 42°C is a critical thermal breakpoint for photosynthetic performance. Both *Ceanothus* and *Prunus* reached peak sHsp expression after 4 hours 42°C temperature treatments. The *Encelia* species studied by Knight and Ackerly [8] also reached peak sHsp expression at 42°C after similar temperature treatments. These observations reinforce Berry and Bjorkman's suggestion that 42°C is a critical thermal threshold for plants.

Increased sHsp expression may constitute a significant resource cost for plants and contribute to differences in the distribution and abundance of species across microclimate gradients. Leaf temperature measurements where *Prunus* is abundant indicated that it rarely experiences leaf temperatures in the field that lead to significant increases in sHsp expression (based on our experimental measurements of sHsp expression). However, *Ceanothus*, growing on the hot, south-facing slopes, frequently exceeded leaf temperatures that induced sHsp expression in our experiment. Conservative allocation strategies involving long leaf lifespan, high leaf nitrogen content, and reduced maximal photosynthetic performance [24] may help sustain sHsp expression for plants growing in thermally stressful habitats.

Variation in the sHsp response between species and artificially selected crop genotypes has previously been correlated with whole plant thermotolerance [25–31]. However, most of these studies measured Hsp expression after a single temperature. For example, Downs et al. [18] sampled a broad taxonomic group and found that most species synthesized the chloroplast sHsp following a 40°C heat stress, and species from warmer climates accumulated greater concentrations of chloroplast sHsp than species from cooler climates. However, Knight and Ackerly [32] found no correlation between expression levels of the chloroplast Hsp following 45°C temperature treatments and mean environmental conditions within the geographic ranges of eight species of *Ceanothus*. Comparisons of sHsp induction temperatures gleaned from full temperature-dependent induction profiles may provide more reliable comparisons between species than single temperature comparisons.

Accumulating evidence suggests that both the chloroplast and mitochondrial sHsps are important for the maintenance of photosynthetic and respiratory electron transport during and after high temperature stress. Heckathorn et al. [31] added purified chloroplast sHsp to isolated chloroplasts both before and after heat treatments and demonstrated that the chloroplast sHsp protects PSII oxygen evolution. In another study, Downs and Heckathorn [20] demonstrated functional inactivation of the mitochondrial sHsp, by protein-specific antibodies, leads to decreased levels of oxidative phosphorylation in isolated mitochondria, and that addition of purified mitochondrial sHsps to preheat stressed mitochondria leads to increased levels of oxidative phosphorylation. Both the chloroplast and mitochondrial sHsp are nuclear encoded and therefore the isolated organelles in the previous studies could not synthesize the sHsps *denovo*. In addition, Miyao-Tokutomi et al. [33] demonstrated that constitutive expression of the chloroplast sHsp in transgenic tobacco plants leads to increased PSII thermotolerance.

In our study, chloroplast sHsp expression tracked general sHsp expression in *Ceanothus*, with the exception that following the 45°C temperature treatment the chloroplast sHsp expression was somewhat reduced compared to general sHsp expression. However, in *Prunus*, chloroplast sHsp expression was not induced until higher temperatures, and was also significantly reduced following the 45°C treatment. Given that previous investigators have shown that the 22 kD chloroplast sHsp protects thermally labile proteins of PSII it is surprising that the chloroplast sHsp is not upregulated until higher temperatures compared to the general sHsp response. The temperature-dependent induction of chloroplast sHsps was quite similar between the two species. Perhaps this similarity is due to the conserved nature of the proteins involved in the C3 photosynthetic apparatus. Differences between species for the general sHsp response may be more informative if these proteins protect more derived proteins perhaps uniquely adapted in thermally sensitive species.

The symptomatic and protective roles of sHsps lead to conflicting hypotheses concerning the correlation between sHsp expression and whole plant or photosynthetic thermotolerance. The evolution of thermotolerance may partly involve the evolution of greater individual protein thermostability [34, 35]. Such possibilities may partly explain why some studies have demonstrated a positive relationship between HSP expression and plant thermotolerance [18, 25, 26, 30, 36, 37], while several other studies have observed no relationship [13, 29, 32, 38].

The results for sHsp expression presented here rely on immunological assays using antibodies constructed against consensus regions of the methionine-rich chloroplast sHsp and for the "small heat shock domain" common to all sHsps [15, 16]. The method is limited by the fact that differences in antigenicity of our antibodies between species may have contributed to contrasting results between species. However, Downs et al. [18] was able to detect the chloroplast sHsp expression in a diverse assemblage of plant species using the same antibody used here. While differences between species for sHsp expression at any particular temperature may be difficult to infer due to antigenicity effects, general trends for induction temperatures, peak accumulation temperatures, or thermal maxima for Hsp expression may still be reliable comparisons. Variation in antigenicity may be less of a problem for the general sHsp antibody which may detect many of the 30–60 different sHsps.

Several questions concerning the evolution and functional significance of Hsp expression merit further attention. For instance, does evolution in thermally stressful environments involve natural selection favoring more thermally stable proteins [35], or better mechanisms for maintaining proteins in their folding competent states (e.g., Hsps)? Understanding the importance of these alternative strategies will aid in interpreting variation in induction temperature or expression levels of Hsps between species. Comparative studies involving species from contrasting environments are critical for understanding the adaptive and functional significance of Hsp expression. Research on relative costs and benefits of Hsp expression is also needed to better understand selection pressures influencing the Hsp response.

It is also possible that the altered Hsp expression profiles that we observed are the result of differential evolutionary histories. For example, members of the genus *Prunus* are more typically found in cooler environments, and therefore, the *Prunus ilicifolia* that we studied here may have had different Hsp induction profiles as a result of this evolutionary history.

Abbreviations

sHsp: Small heat shock protein
F_v/F_m: Variable to maximal fluorescence
PSII: Photosystem II.

Acknowledgments

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