American Journal of Botany 88(3): 411–418. 2001.

CORRELATED EVOLUTION OF CHLOROPLAST HEAT SHOCK PROTEIN EXPRESSION IN CLOSELY RELATED PLANT SPECIES[1]

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Interspecific variation in chloroplast low molecular weight (cLMW) HSP (heat shock protein) expression was examined with respect to phylogeny, species specific leaf area, chlorophyll fluorescence, and mean environmental conditions within species ranges. Eight species of *Ceanothus* (Rhamnaceae) were heat shocked for 4 h at several different temperatures. Leaf samples were collected immediately after the heat shock, and cLMW HSP expression was quantified using Western blots. At 45°C species from the subgenus *Cerastes* had significantly greater cLMW HSP expression than species from the subgenus *Ceanothus.* Specific leaf area was negatively correlated with cLMW HSP expression after the 45°C heat treatment. In addition, chlorophyll fluorescence (F_v/F_m) 1 h after the heat shocks was positively correlated with cLMW HSP expression. Contrary to our prediction, there was no correlation between July maximum temperature within species ranges and cLMW HSP expression. These results suggest that evolutionary differentiation in cLMW HSP expression is associated with leaf physiological parameters and related aspects of life history, yet associations between climatic conditions within species ranges and cLMW HSP expression require further study.

Key words: *Ceanothus*; chlorophyll fluorescence; chloroplast low molecular weight heat shock protein; Rhamnaceae; specific leaf area.

There have been hundreds of biochemical studies of the heat shock protein (HSP) response in plants (Vierling, 1991), but very few have dealt with evolutionary and ecological variation within this protein class (Coleman, Heckathorn, and Hallberg, 1995; but see, Waters, 1995; Heckathorn et al., 1996; Waters, Lee, and Vierling, 1996; Downs et al., 1998; Feder and Hofmann, 1999). The evolutionary conservation of the heat shock response, and the fact that HSP expression is correlated with high temperature stress, has led to the hypothesis that HSPs protect cells from high temperature stress, and that HSP accumulation leads to increased thermotolerance. Recent mechanistic studies involving isolated chloroplasts and transgenic plants have supported this hypothesis (Heckathorn et al., 1996, 1998; Miyao-Tokutomi et al., 1998). It has been speculated that evolutionary change in the HSP response may be correlated with the frequency of temperature stress experienced by a species, and that differential regulation of HSPs may be a part of the suite of physiological and morphological adaptations characteristic of thermotolerant species (Heckathorn et al., 1996; Downs et al., 1998). Here we use eight species from the woody perennial genus *Ceanothus* that differ in both morphological traits traditionally associated with thermotolerance and in their distribution with respect to climatological parameters, to study evolutionary diversification in the HSP response with respect to these ecological characteristics.

HSPs are usually divided into five unique classes: HSP100, HSP 90, HSP 70, HSP 60, and low molecular weight (LMW) HSPs (17–30 kD). The LMW HSP class is particularly interesting in plants for several reasons. First, LMW HSPs dominate protein synthesis profiles during heat stress and can rapidly accumulate to >1% of total leaf protein under certain heat stress conditions (DeRocher et al., 1991; Hsieh et al., 1992). Secondly, the evolutionary diversification of the LMW HSP class is unique to plants. Other eukaryotes typically have only one to four different LMW HSPs, whereas plants have ~30–60 genes that are separated into at least six different gene families, each targeted to specific cellular compartments. Thirdly, there is variation in the LMW HSP response between species and artificially selected crop genotypes, and various studies show that this variation is correlated with the intrinsic thermotolerances of the genotypes (Ougham and Stoddart, 1986; Krishman, Nguyen, and Burke, 1989; Colombo et al., 1992; Weng and Nguyen, 1992; Frova and Gorla, 1993; Park et al., 1996, 1997) or environmental conditions within the species ranges (Downs et al., 1998).

It is thought that HSPs function as molecular chaperones by binding to partially folded or denatured proteins, thereby preventing irreversible aggregation and promoting correct folding (Lee, Pokala, and Vierling, 1995; Hook and Harding, 1997; Lee et al., 1997). Recently, Heckathorn et al. (1998) demonstrated that the chloroplast low molecular weight (cLMW) HSP plays a direct role in stabilizing the photosystem II (PSII) oxygen-evolving complex (OEC) proteins during heat stress and thereby promotes the maintenance of PSII electron transport. By adding purified cLMW HSP to isolated chloroplasts both before and after heat treatments, Heckathorn, Downs, and Coleman (1999) have shown that the cLMW HSP functions to protect OEC proteins from the denaturing effects of heat stress, but that the cLMW HSP does not reactivate proteins already denatured by heat stress. In addition, Miyao-Tokutomi et al. (1998) demonstrated that constitutive expression of the cLMW HSP in transgenic tobacco plants leads to an increase in PSII thermotolerance. As a direct result of these studies, the mechanistic functioning of the cLMW HSP is better known than for any other plant HSP. The conserved nature of the N-

[1] Manuscript received 9 November 1999; revision accepted 16 June 2000.

The authors thank Scott Heckathorn, Jim Coleman, and Craig Downs for assistance with protein methods and for providing the Ab_{met} primary antibody, Olle Bjorkman for the use of his fluorometer and for assistance with fluorescence measurements, Gina Kang and Carina Uraiquat for assistance with sample processing and Max Tuab for useful comments on this manuscript and helpful discussion of the results. Supported in part from a Tri-Agency (DOE, NSF, USDA) Training Grant in Plant Biology and an NSF Dissertation Improvement Grant (IBN-9902295) for C.K.

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terminal ''methionine-rich'' domain of the cLMW HSP allowed Downs et al. (1998) to produce an antibody that detects cLMW HSPs in heat-stressed plant tissue from a diverse assemblage of species representing five divisions of the plant kingdom. This, combined with the fact that heat-induced expression of the cLMW HSP is relatively easy to detect because the proteins are not constitutively expressed, make the cLMW HSP an ideal candidate for ecological and evolutionary studies designed to test the significance of variation in the HSP response.

Previous studies on the evolution of the HSP response in plants have operated at two extremes of evolutionary history. For instance, Downs et al. (1998) documented variation in the intensity of cLMW HSP expression following heat treatments for representative species from five divisions of the plant kingdom and tentatively correlated these results with mean environmental conditions within the species ranges. While useful for understanding the conserved nature of the cLMW HSP response, the resolution inherent to studies at this level is too coarse to make robust inferences about adaptation to a specific environment. At the other extreme, variability in the HSP response has been studied using thermotolerant varieties of agriculturally important crop species or transgenic plants (Marimiroli et al., 1986; Ougham and Stoddart, 1986; Fender and O'Connell, 1989; Krishman, Nguyen, and Burke, 1989; Jorgensen, Rosenow, and Nguyen, 1992; Weng and Nguyen, 1992; Frova and Gorla, 1993; Park et al., 1996; Miyao-Tokutomi et al., 1998). Although, there is evidence that suggests that the variation in the HSP response is associated with whole-plant thermotolerance in transgenic and artificially selected crop species, it is not yet clear whether this relationship exists in nature. Comparative studies of the HSP response for closely related species have been conducted in marine invertebrates and insects but not in plants. Results from these studies indicate that thermotolerant species have higher induction temperatures (Bosch et al., 1988; Gerhing and Wehner, 1995; Hofmann and Somero, 1996; Tomanek and Somero, 1996), greater HSP induction intensities (Bosch et al., 1988; Sanders et al., 1991; Hofmann and Somero, 1996) and a shorter HSP response duration following heat stress (Tomanek and Somero, 1996).

Ceanothus is composed of 55 woody perennial species that are morphologically and ecologically diverse. Traditionally *Ceanothus* has been divided into two subgenera, *Ceanothus* and *Cerastes,* based on phyllotaxy, leaf morphology and habit, and fruit characteristics. These subgeneric groups have recently been supported by phylogenies derived from both nuclear ITS and chloroplast *matK* DNA sequences (Hardig, Soltis, and Soltis, 2000; Jeong, Liston, and Myrold, 1997), but relationships between species within subgenera have been difficult to resolve, in part due to hybridization (McMinn, 1930, 1942; Brandegee, 1895; Howell, 1940; Hardig, Soltis, and Soltis, 2000). Species from the subgenus *Cerastes* display a xeromorphic leaf morphology typified by stomatal crypts and small, thick leaves that are resistant to drought, while species from the subgenus *Ceanothus* are sometimes deciduous and typically have much thinner leaves (McMinn, 1942; Nobs, 1963; Hickman, 1993). These differences in leaf morphology are associated with strategies of regeneration following fire. Species of *Cerastes* are generally obligate seeders, which do not develop deep root systems and are more drought tolerant, while species of subgenus *Ceanothus* are resprouters, which develop deeper root systems over time and are more sensitive to water stress (Davis, 1989; Thomas and Davis, 1989).

Variation in leaf size and thickness such as that exhibited by species of *Ceanothus* is often associated with a suite of physiological and plant growth parameters. Species with smaller, thicker leaves generally occur in more stressful environments and exhibit lower specific leaf area (SLA), slower growth rates, lower leaf nitrogen content, lower light-saturated photosynthetic capacity and dark respiration rates, and longer leaf life spans (Dijkstra and Lambers, 1989; Chapin, Autumn, and Pugnaire, 1993; Reich, Walters, and Ellsworth, 1997, Reich et al., 1998). These interrelationships suggest that SLA is at the nexus of a suite of covarying traits that may represent both evolutionary constraints and correlated responses to environmental conditions.

Ceanothus species also span a broad geographical range in relation to climatic conditions. If HSP expression is related to overall thermotolerance, then we would predict that evolutionary divergences in HSP expression (compared under controlled conditions) would be correlated with differences in the temperature regime experienced by different species. Although climatalogical and species range distribution information are usually only known at coarse scales, geographic information systems analysis (GIS) can provide a powerful tool to quantify the climatic conditions within species' ranges (Austin, Nicholls, and Margules, 1990; Westman, 1991; Franklin, 1998; Peterson, Soberón, and Sánchez-Cordero, 1999). Here we use a relatively coarse-scale GIS analysis of July maximum temperatures in relation to species' ranges (Ackerly et al., unpublished data) as a predictor of variation in cLMW HSP expression.

In this study, we examined the expression of cLMW HSPs following 4-h heat treatments at temperatures ranging from 30° to 50°C using eight species of *Ceanothus,* four from each of the subgenera described above. Based on these responses we address the following questions: (1) Is there significant divergence among species for cLMW HSP expression following heat shocks and if so, is this divergence primarily within or between subgenera? (2) Is variation in HSP expression correlated with leaf traits, particularly SLA and the maintenance of chorophyll fluorescence following heat shock? (3) Is variation in HSP expression associated with overall climatic variation in the ranges of different species?

MATERIALS AND METHODS

Eight species from the genus *Ceanothus* were obtained from native plant nurseries with verification of the wild provenance of their seed stocks. Four species from each subgenus were chosen: *C. integerrimus, C. griseus, C. hearstiorum,* and *C. cordulatus* from subgenus *Ceanothus,* and *C. cuneatus, C. gloriosus, C. maritimus,* and *C. prostratus* from subgenus *Cerastes.* In addition, one naturally occurring horticulturally selected variety of *C. maritimus,* ''Dr. Leicher's Dark,'' was used to test for fine-scale variation in the HSP response between closely related genotypes. Plants were grown in a glasshouse under a 14-h photoperiod and 1400 photon flux density (PFD), where temperatures did not exceed 27°C. After 3 mo there was a significant amount of new growth on all species. Heat shocks were conducted in forced-air temperature controlled growth chambers (EGC Corp., Chagrin Falls, Ohio, USA) at eight temperatures: 30°, 32.5°, 35°, 37.5°, 40°, 42.5°, 47.5°, and 50°C. Each temperature treatment was replicated three times, with one plant per species per replicate, and each replicate was conducted in a different growth chamber. Thus, there were nine plants, one per species, in each chamber during each heat shock event. Light conditions inside the chambers were not significantly different from inside the glasshouse.

Heat shocks were imposed for a duration of 4 h. Immediately after removing the plants from the chambers, 15–30 leaves from each plant were harvested, frozen in liquid nitrogen, and stored at −80°C. Protein analysis was performed on a subsample of a powderized homogenate of the 15–30 leaves. In addition, ten leaves from each plant were collected for dark-adapted, F_v/F_m chlorophyll fluorescence measurements after the 40°, 42.5°, 45°, 47.5° and 50°C treatments. F_v/F_m chlorophyll fluorescence was also measured on leaves from non-heat-stressed plants at 20°C. Leaves were dark-adapted for 1 h before measuring F_v/F_m using an OtpiSciences OS-500 fluorometer (Tyngsboro, Massachusetts, USA). Two leaves were collected from six individuals of each species (12 leaves total per species) for determination of specific leaf area (cm^2 area/g dry mass). Leaf area was measured using a LI-COR Li-3100 leaf area meter (Lincoln, Nebraska, USA). Leaf samples were dried at 50°C for 1 wk before dry mass measurement.

Protein methods—Total soluble leaf protein was extracted using a ceramic mortar and pestle with 25% w/v extraction buffer containing the following ingredients: 10% SDS (Sodium Dodecyl Sulfate), 1.5 mol/L Tris, 1 mmol/L PMSF (phenylmethylsulfonyl fluoride), 0.1 mol/L EDTA (ethylenediaminetetraacetic acid), 1 mol/L benzamidine, PVP (polyvinylpyrrolidone), PVPP (polyvinylpolypyrrolidone), DTT (dithiotheirtol), ascorbate, and the protease inhibitors antipain and leupeptin (modified from Heckathorn et al., 1996). Samples were ground thoroughly for 3 min, collected into a microfuge tube, and boiled immediately for 3 min. After centrifuging for 10 min the supernatant was collected and stored at −20°C. Total extracted protein concentration was determined using a Coomasie dot blot on Whatman filter paper and quantified using a Hewlett Packard ScanJet II (Palo Alto, California, USA) laser scanner (after Vincent et al., 1997). A standard curve, calculated using dilutions of a BSA protein standard, was used to infer sample protein concentration. Fifty micrograms of soluble protein were loaded on a 12.5% SDS-PAGE gel (following Laemmli, 1970). Gel loading mirrored the chamber replicates so that all of the species heat shocked at a given temperature and replicate were also loaded on the same gel. Each replicate was run on a separate gel. There were 27 total gels, one for each of three replicates at all nine different temperatures. Several of these gels were also run in duplicate to check for equal protein loading by Coomasie staining. A positive control (homogenized protein samples from a *C. griseus* plant heat shocked at 45°C for 8 h) was run on each gel to account for blot to blot variation in staining intensity. By running the samples in this manner, among-replicate variation is accounted for by normalization to the positive control.

After separation, the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane by Western Blot. Membranes were blocked for 5 h following transfer in a Tris/powdered milk solution. Transfer times were optimized on trials using the *C. griseus* positive control. The primary antibody, AB_{met} (provided by S. A. Heckathorn and J. S. Coleman; see Heckathorn et al., 1998), was synthesized in a rabbit from a fusion protein designed from the consensus sequence of the methionine-rich domain of the cLMW HSP. The consensus sequence used to design this antibody was derived from a broad range of plant taxa including *Pisum sativum, Zea mays, Arabidopsis thaliana, Glycine max, Triticum aestivum, Petunia hybrida,* and *Solanum tuberosum* (Downs et al., 1998). Optimal antibody dilutions were found by serial dilution so that resulting band intensities were within the linear range of detection. A 1/5000 dilution of Ab_{met} was incubated with the membrane overnight in a cold room. The alkaline phosphatase Goat anti-rabbit IgG secondary antibody (Sigma, St. Louis, Missouri, USA; 1/10 000 dilution) was incubated with the membrane for 2.5 h before development using the alkaline phospatase substrate. Intensity of the chloroplast 22 kD LMW HSP band was quantified using a Hewlett Packard ScanJet II and NIH Image software (http://rsb.info.nih.gov/nih-image). Optical density units were log transformed to account for non-normality in their distribution and are expressed relative to the positive control on each gel (100% positive control = 1).

GIS analysis—The presence or absence of *Ceanothus* species in each of 35 different subregions of California together with the elevational distribution of each species (Hickman, 1993), were used to create coarse-scale species range maps using ArcView GIS software (ESRI, Redlands, California, USA).

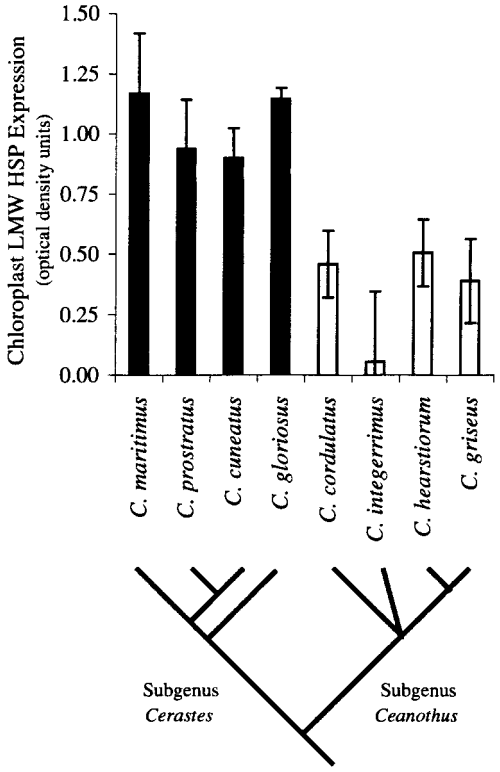

Fig. 1. Chloroplast LMW (low molecular weight) HSP (heat shock protein) expression as a percentage of a positive control for eight species of *Ceanothus.* The phylogeny of *Ceanothus* was drawn after Hardig, Soltis, and Soltis (2000) and Jeong, Liston, and Myrold (1997). Species from the subgenus *Cerastes* (dark bars) had greater cLMW HSP expression than species from the subgenus *Ceanothus* (open bars). Error bars represent 1 SE of the mean cLMW HSP optical density.

Climate maps of July maximum temperature and annual precipitation (obtained from the Oregon State University PRISM project; Daly, Neilson, and Phillips, 1994; Daly, Taylor, and Gibson, 1997) were intersected with the species range maps, resulting in a histogram of the percentage of each species range falling into several different temperature or precipitation classes. From this histogram, the mean July maximum temperature and mean annual precipitation inside each species distribution were calculated as an estimate of species differences in realized climatic niche distributions (see Austin, Nicholls, and Margules, 1990; Westman, 1991; Franklin, 1998).

Statistical analysis—Tests for significant differences between species and subgenera were performed using analysis of variance blocked by chamber/gel replicate. Correlations between cLMW HSP expression (mean of the three replicates) and mean July maximum temperature, annual precipitation, SLA, and F_v/F_m chlorophyll fluorescence were tested using nonparametric methods (the Spearman's rank correlation). In addition, we used independent contrasts (Felsenstein, 1985) to test for the correlated evolution of traits (correlation coefficients calculated using ACAP; Ackerly, 1997) and the dependence of these correlations on phylogenetic relationships between the species. The phylogeny for the eight species was based on recent molecular phylogenetic analyses (Hardig, Soltis, and Soltis, 2000; Jeong, Liston, and Myrold, 1997). One polytomy among species of subgenus *Ceanothus* was resolved in all three possible topologies and independent contrasts were calculated over the three resulting trees, using equal branch lengths; results did not differ over the three alternative trees, and results are reported for one arbitrarily chosen resolution (see Ackerly and Reich, 1999).

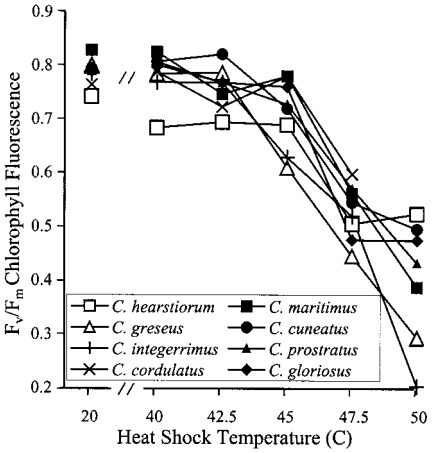

Fig. 2. F_v/F_m chlorophyll fluorescence decreased in all species with increasing temperature. Species from the subgenus *Cerastes* (closed symbols) typically maintained greater F_v/F_m ratios than did species from the subgenus *Ceanothus* (open symbols). Points represent the mean of 12 leaves from two different replicates at each temperature.

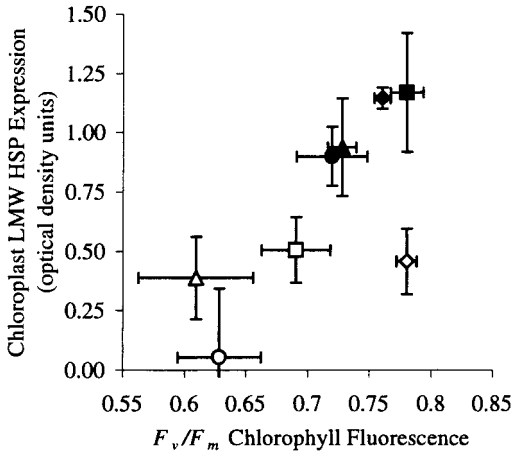

Fig. 3. Chloroplast LMW HSP expression measured immediately after 4-h, 45°C heat shocks was positively correlated with F_v/F_m chlorophyll fluorescence measured 1 h after the 45°C heat treatments. Error bars represent ±1 SE of the mean for both cLMW HSP expression and F_v/F_m chlorophyll fluorescence. Symbols follow Fig. 2 legend with the exception that the open circle represents *C. integerrimus* and the open diamond represents *C. cordulatus*.

RESULTS

Differences among species—All species expressed cLMW HSPs immediately after 4-h 45°C heat shocks. No cLMW HSP expression could be detected at lower temperatures (30°, 32.5°, 35°, 37.5°, 40°, and 42.5°C) or higher temperatures (47.5° or 50°C) for any species. At 45°C there was a significant difference in cLMW HSP expression between species (ANOVA, $F_{1,8} = 2.81$, $P = 0.032$). The horticulturally selected variety of *C. maritimus*, "Dr. Leicher's Dark," showed nearly identical cLMW HSP synthesis profiles as the wild *C. maritimus.* There was also a significant difference between the three replicates at 45°C (ANOVA, $F_{1,2} = 6.26$, $P = 0.01$), which could be due to random variation among chambers or gels. Duplicate Coomasie stained gels indicated equal protein loading for these replicates.

Phylogenetic association—Chloroplast LMW HSP expression at 45°C was associated with evolutionary lineage within the genus *Ceanothus.* Species from the subgenus *Cerastes* showed significantly greater cLMW HSP expression than species from the subgenus *Ceanothus* (ANOVA with species nested in clade, $F_{1,1} = 23.53$, $P < 0.001$, Fig. 1). Within subgenera there was not a significant difference between species (nested ANOVA, $F_{1,6} = 0.065$, $P = 0.69$).

Chlorophyll fluorescence vs. cLMW HSP—F_v/F_m chlorophyll fluorescence decreased with increasing heat shock temperature (Fig. 2). At each temperature there was a significant difference in F_v/F_m between species ($F_{1,8} = 6.23$, $P < 0.001$). In addition, species responded differently to increases in temperature ($F_{1,8} = 3.92$, $P < 0.001$). F_v/F_m following the 45°C heat treatments was correlated with cLMW HSP expression ($R = 0.722$, $P < 0.05$, Fig. 3). Species that expressed cLMW HSP to a greater extent during 4-h 45°C heat shocks maintained greater F_v/F_m chlorophyll fluorescence measured 1 h after the heat shock.

SLA vs. cLMW HSP—Species-specific SLA was negatively correlated with cLMW HSP expression ($R = -0.869$, $P < 0.01$; Fig. 4). Species with lower SLA (typically smaller, thicker leaves) had greater cLMW HSP expression than did species with high SLA.

Mean July maximum temperature—Mean July maximum temperature in the range of each species was not significantly correlated with cLMW HSP expression, though within each subgenus there was a negative trend (opposite to the predicted direction; Fig. 5). There was no correlation between species mean annual precipitation and cLMW HSP expression.

Independent contrasts—The correlation between cLMW HSP expression and SLA was weakened but still significant when analyzed with independent contrasts ($R = 0.767$, $P < 0.05$) perhaps because much of the variation was reflected in a single contrast between the two subgenera. The HSP-F_v/F_m relationship was not significant ($R = 0.662$, $P > 0.05$). The correlation be-

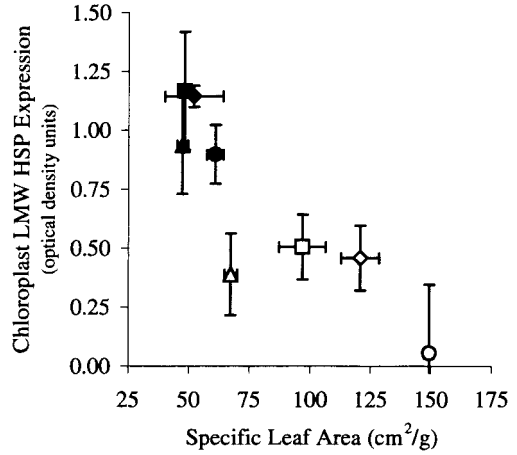

Fig. 4. Chloroplast LMW HSP expression vs. SLA (cm^2/g) of eight species of *Ceanothus.* Species with lower SLA had greater cLMW HSP expression. Error bars represent ±1 SE of the mean of both SLA and cLMW HSP optical density. Symbols follow Fig. 2 legend with the exception that the open circle represents *C. integerrimus* and the open diamond represents *C. cordulatus.*

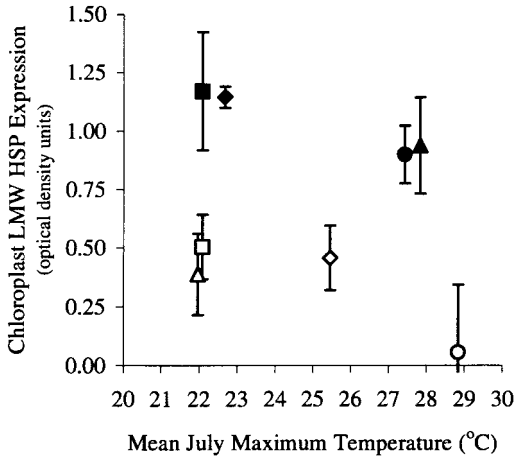

Fig. 5. Mean July maximum temperature calculated from the GIS analysis for each species vs. cLMW HSP expression following the 45°C heat treatment. Symbols follow Fig. 2 legend with the exception that the open circle represents *C. integerrimus* and the open diamond represents *C. cordulatus*. Error bars represent ±1 SE of the mean.

tween HSP expression and mean July maximum temperature was much stronger using independent contrasts, reflecting the negative trends within both subgenera ($R = -0.720$, $P < 0.05$).

DISCUSSION

We found significant differences in cLMW HSP expression among eight species of *Ceanothus.* This is the first study to document variation in expression of any HSP in closely related nonagricultural plant species and to examine this variation in the context of phylogenetic relationships and ecological variation among species. Expression levels of the cLMW HSP was significantly different between species of the two subgenera of *Ceanothus.* These two groups also differ in leaf traits and drought tolerance, and there was a negative correlation between HSP expression and specific leaf area (i.e., higher cLMW HSP per unit protein in thicker leafed species). There was a positive correlation between cLMW HSP expression and chlorophyll fluorescence, which supports the hypothesis that cLMW HSP expression functions to maintain PSII electron transport at high temperature. Interestingly, there was no correlation between cLMW HSP expression and measures of the mean July maximum temperature within the species range.

Species that were able to express cLMW HSPs during the 4-h 45°C heat treatments maintained greater PSII electron transport 1 h after the heat treatment. This result generalizes the functional relationship between cLMW HSP expression and the maintenance of PSII electron transport during heat stress that has been reported from studies involving isolated chloroplasts and transgenic plants (Heckathorn et al., 1998; Miyao-Tokutomi et al., 1998; Downs, Coleman, and Heckathorn, 1999) to the level of evolutionary divergence between closely related species. The cLMW HSP is translated in the cytosol and then transported to the chloroplast. Because our experiment used whole-leaf protein extracts we cannot be sure that the induced cLMW HSP expression we detected corresponded to entirely functional, fully localized proteins. The time course of F_v/F_m recovery may also be dependent on cLMW HSP expression (Heckathorn et al., 1996). Therefore it would also be interesting to study the time course of F_v/F_m recovery in relation to cLMW HSP expression at several sampling points following heat treatments rather than just the 1-h time point presented here.

Perhaps the most striking pattern revealed by this study was a negative correlation between cLMW HSP expression and SLA, which suggests that variation in the HSP response may be correlated with a suite of leaf-level and whole-plant physiological traits associated with carbon gain and stress. Species with small thick leaves (low SLA) generally occur in more stressful microclimates, have slower growth, lower leaf nitrogen content, light-saturated photosynthetic capacity and dark respiration rates, and longer leaf life spans (Dijkstra and Lambers, 1989; Chapin, Autumn, and Pugnaire, 1993; Reich, Walters, and Ellsworth, 1997; Reich et al., 1998). Despite the fact that cLMW HSP expression was not correlated with mean environmental temperatures taken from our GIS analysis, the fact that cLMW HSP expression was correlated with SLA suggests that the cLMW HSP response may be important for plants with stress-tolerating life histories. Because equal amounts of soluble protein were loaded from total leaf extracts for each species, it is unlikely that the correlation between cLMW HSP expression and SLA is simply an artifact resulting from variation in extraction efficiency, total protein content, leaf nitrogen levels, or other biochemical or experimental differences between species.

This experiment was conducted to test the hypothesis that HSP expression is greater in species that inhabit warmer geographic ranges, a pattern that has been observed in marine invertebrates (Sanders et al., 1991; Bosch et al., 1988; Hofmann and Somero, 1996), widely divergent plant species (Downs et al., 1998), and thermotolerant varieties of wheat (Weng and Nguyen, 1992). We used GIS analyses to estimate the mean climatic niche for each species. Surprisingly, we found no consistent relationship between HSP expression and an estimate of the temperature regime across the ranges of the different species. Other aspects of environmental variation, like the diurnal variation in air temperature, the frequency and duration of extreme temperature stress, average cloud cover or light intensity, and the timing and amount of precipitation may also be of biological importance with respect to the HSP response. In addition, the microclimate distribution with respect to local topography and vegetation cover may also be important for interpreting variation in the HSP response between species. Quantitative information about the geographic distribution of the species in this study was only available at coarse scales and did not include information on microclimatic affinities. In a related study, we found that *C. cuneatus* (subgenus *Cerastes*) occupies hotter microclimates than *C. oliganthus* (subgenus *Ceanothus*) where they co-occur at Jasper Ridge Biological Preserve (California, USA; Ackerly et al., unpublished data). This observation suggests that microclimate distributions of the two subgenera of *Ceanothus* may be quite important for predicting patterns of variation in HSP expression and that GIS analyses that do not incorporate local-scale distribution may be too coarse to detect significant relationships if they exist.

Results from a GIS analysis of *Ceanothus* species distributions show that although the two subgenera are markedly different in their leaf and life history characteristics, both of the subgenera reach their peak diversity north of San Francisco Bay (C. Knight, unpublished data). Species with low SLA, and high cLMW HSP expression (as assayed by this experiment) are commonly observed growing adjacent to other *Ceanothus*

species with much lower SLA (Davis, 1999). Therefore, the observed correlation between SLA and the cLMW HSP response may reflect different strategies for surviving in the same environment and explain why there was no correlation between mean July maximum temperatures within species ranges and cLMW HSP expression. Species of subgenus *Cerasetes* are typically more shallowly rooted than species of subgenus *Ceanothus,* which can lead to lower leaf and whole plant water potential during the summer drought (Barnes, 1974; Davis, 1989; Thomas and Davis, 1989) and may also cause higher realized leaf temperatures in the field due to decreased conductance. Species with low SLA, high cLMW HSP expression, and presumably other correlated traits as identified by Reich et al. (1998; Reich, Walters, and Ellsworth, 1997) may be seen as stress tolerators that can exploit marginal growing conditions by using conservative allocation and effective acute stress response systems to minimize costs associated with frequent and severe drought and thermal stress. Other species growing in the same locations may have a different set of traits that allow them to better exploit growing conditions during favorable parts of the season (e.g., high maximum photosynthetic rates and deciduousness) at the expense of carbon gain during stressful periods.

Three experimental factors should be considered that could influence patterns of HSP expression in this study: variation in leaf temperatures during heat shock, variation in antigenicity of the cLMW HSP from different species to the AB_{met} antibody, and the mechanisms underlying HSP expression immediately following heat shock. SLA could play an important role in determining leaf temperature under a variety of environmental conditions. For instance, thin leaves may heat up faster, yet may be able to modulate leaf temperature better than thick leaves because of higher stomatal conductance and lower specific leaf mass. With that in mind, we investigated the possibility that realized leaf temperatures varied between species during the temperature treatments. In a follow-up experiment, leaf temperatures were monitored for six leaves of each species during two 45°C heat treatments that were identical to the initial treatments. Thermocouples were attached to leaves with gas-permeable tape. Leaf temperatures during the two 4-h 45°C heat shocks were not significantly different between species or subgenera (Knight et al., unpublished data). Therefore, the correlation between SLA and cLMW HSP expression was not solely due to differences in realized leaf temperatures caused by biophysical interactions associated with variation in SLA between species.

The results for cLMW HSP expression presented here rely on antibody staining of Western Blots. Because equal micrograms of soluble protein were run for all species, optimized extraction protocols were identical between species, and no cLMW HSPs were detected at lower temperatures (30°–42.5°C), we feel that these intensities reflect intrinsic differences for inducible cLMW HSP expression between species. There remains the possibility that differences in antigenicity of the AB_{met} antibody towards the methionine-rich epitope of the cLMW HSP produced the resulting differences in our observations. Downs et al. (1998) found a qualitative association in diverse plant species between relative levels of cLMW HSP expression and estimates of habitat temperatures, despite the possibility of variation in antigenicity between species, and thus argue that their patterns reflect real differences in HSP accumulation. In addition, variation in antigenicity of the AB_{met} antibody may not be significant because of the conserved nature of the methionine-rich domain and the rest of the cLMW HSP (Waters, 1995; Waters, Lee, and Vierling, 1996).

Chloroplast LMW HSP expression was assayed immediately following 4-h heat treatments. This allows comparison between species for their ability to transcribe and translate cLMW HSPs at the treatment temperatures. We found that all species either could not, or did not, process a signal for cLMW HSP expression between 30° and 42.5°C and at 47.5° and 50°C. The variation seen at 45°C may have been due to mechanisms that are extrinsic to the HSP response itself (i.e., increased thermotolerance of the transcriptional and translational apparatus) or may reflect evolutionary changes in the signal transduction pathway for cLMW HSP synthesis (i.e., changes in promoter sensitivity to transcription factor binding, or other upstream regulatory changes). Because we observed cLMW HSP expression only after the 45°C heat treatment, our data do not support the hypothesis that HSP induction temperature evolution is correlated with the frequency or intensity of environmental stress within a species range. It may be that induction temperatures vary at a much finer scale than we could detect given that our treatments differed by 2.5°C. A finer resolution between temperature treatments would have been desirable, yet this is difficult in experiments involving whole plants and forced air growth chambers because leaf temperatures on a single plant can differ by several degrees. Before initiating the study we found that average variation in leaf temperature for leaves on a single plant in our growth chambers to be about 2.5°C. Therefore we chose the treatment temperatures to differ by 2.5°C to maximize the chances of detecting significant treatment differences and to reduce the probability of experimental error. Methods for decreasing the temperature difference between treatments typically involve detached leaves or leaf discs in small cuvettes or floating in water baths. Because detaching leaves or using leaf discs can be considered a stress in itself (certainly for leaf water potential, transpiration, and photosynthesis), we decided to use minimally invasive techniques involving whole plants.

Several questions concerning the evolution and functional significance of HSP expression merit further attention (see also Feder and Hofmann, 1999). For instance, does evolution in thermally stressful environments involve natural selection favoring more thermally stabile proteins, 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. Research on relative costs and benefits of HSP expression is also needed to understand the factors that favor increased or decreased HSP expression levels or induction temperatures (Coleman, Heckathorn, and Hallberg, 1995). Comparative studies involving species from contrasting environments are critical for understanding the adaptive and functional significance of HSP expression with regard to the evolution of thermotolerance. Studies of species with varying degrees of evolutionary relatedness will provide insight into different aspects of this question. Congeneric species from contrasting climates minimize the genetic differences between species and make it somewhat easier to isolate specific traits of interest. Studies of distantly related species will be valuable to examine evolutionary change in more conserved aspects of the HSP response.

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