

Variation in chloroplast small heat-shock protein function is a major determinant of variation in thermotolerance of photosynthetic electron transport among ecotypes of *Chenopodium album*

Deepak Barua^A, Craig A. Downs^B and Scott A. Heckathorn^{A,C}

^ADepartment of Biology, Syracuse University, 130 College Place, Syracuse, NY 13244, USA.

^BEnvirtue Biotechnologies, 1866-C East Market Street, Suite 164, Harrisonburg, VA 22801, USA.

^CCurrent address; EEES, University of Toledo, MS 604, Toledo, OH 43606, USA.

Corresponding author; email: scott.heckathorn@utoledo.edu

Abstract. Chloroplast small (low-molecular-weight) heat-shock proteins (csHsps) can protect photosynthetic electron transport (P_{et}), and quantitative variation in csHsps is correlated with thermotolerance of net photosynthesis and Photosystem II. However, the functional (i.e. protective) consequence of natural variation in csHsps is unknown. To investigate this, we used an *in vitro* assay to determine the contribution of csHsps to the tolerance of P_{et} to high temperatures in five ecotypes of *Chenopodium album* collected from habitats ranging from cool to warm, and we partitioned total P_{et} thermotolerance into basal and induced P_{et} components (without and with a pre-heat treatment, respectively, to induce csHsps). The ecotypes varied in total P_{et} thermotolerance and this was correlated with habitat temperature. Variation in total P_{et} thermotolerance was associated primarily with variation in induced P_{et} thermotolerance, and not with basal P_{et} thermotolerance. Variation in induced P_{et} was highly correlated with csHsp protection of P_{et} . Variation in csHsp function was associated with variation in csHsp content among ecotypes. These results are the first to demonstrate the direct functional consequences for natural variation in Hsps in plants, and show that functional variation is associated with evolutionary adaptation to specific habitats among ecotypes.

Keywords: natural variation, photosynthesis, stress proteins.

Introduction

Heat-shock proteins (Hsps) are important for protecting cells against high temperature and other stresses (e.g. Parsell and Lindquist 1994; Waters *et al.* 1996; Heckathorn *et al.* 1999). Most Hsps appear to protect proteins or membranes during heat stress or facilitate repair or degradation of damaged proteins following a stressful event. Hsps are highly evolutionarily conserved and occur in virtually every species in which they have been sought (Feder and Hofmann 1999), and one or more Hsps are produced in response to almost every environmental stress (Vierling 1991; Parsell and Lindquist 1994; Waters *et al.* 1996; Feder and Hofmann 1999; Heckathorn *et al.* 1999). These proteins can be grouped into families based on homology of sequence and function; e.g. Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp10, and the low-molecular-weight (*ca* 15–30 kDa), or small, Hsps (sHsps) (Parsell and Lindquist 1994). In plants, the sHsps are especially abundant and diverse and are

typically the most heat-responsive Hsps (Vierling 1991; Howarth and Ougham 1993). Plants are unique in that they can produce more than 20 sHsps (Vierling 1991; Howarth and Ougham 1993; Waters *et al.* 1996); most other taxa produce only one or a few sHsps (Arrigo and Landry 1994). At least five classes of sHsps are recognised in plants, two of which contain proteins that localise to the cytosol, and three that contain proteins that localise to the endoplasmic reticulum, mitochondria and plastids, respectively (Waters *et al.* 1996).

In spite of their highly conserved nature, qualitative and quantitative variation in Hsps has been demonstrated both among and within species (see references in Feder and Hofmann 1999). This variation has been shown to be at least partly heritable and, in most cases, associated with differences in stress tolerance. However, studies addressing variation among naturally occurring populations are few (Feder and Hofmann 1999). This is especially true in plants,

Abbreviations used: Ab, antibody; BSA, bovine serum albumin; csHsp, chloroplast small heat-shock protein; MV, methyl viologen; P_{et} , photosynthetic electron transport.

where most of the work has concentrated on commercially important species. Also, few, if any, studies have examined the functional consequences of either quantitative or qualitative natural variation in Hsps.

Recently, inter- and intraspecific variation in the content of the plastid-localised or chloroplast sHsp (csHsp) in plants has been documented and is positively correlated with photosynthetic and whole-plant thermotolerance (Heckathorn *et al.* 1996, 1999; Park *et al.* 1996, Joshi *et al.* 1997; Downs *et al.* 1998; Preczewski *et al.* 2000; Knight and Ackerly 2001). Further, using two different *in vitro* methods developed to assay protection of photosynthetic electron transport by csHsp, it was demonstrated that the csHsp can partially protect photosynthetic electron transport during heat, high-light, and oxidative stress (Heckathorn *et al.* 1998; Downs *et al.* 1999a, b). These *in vitro* results confirmed previous studies suggesting such a role for the csHsp (Schuster *et al.* 1988; Stapel *et al.* 1993; Heckathorn *et al.* 1996), have been confirmed *in vivo* (Heckathorn *et al.* 2002), and are supported by additional results from other recent studies with transgenic or mutant plants and cyanobacteria (Lee *et al.* 1998, 2000a, b; Miyao-Tokutomi *et al.* 1998; Nakamoto *et al.* 2000; Török *et al.* 2001). Because (1) natural variation in production of csHsp is widespread within and among species, (2) a specific function has been identified for the csHsp, and (3) this protective function can be quantified by an *in vitro* assay, the csHsp is an excellent model system for studying the functional consequences of quantitative or qualitative variation in Hsps.

Using the csHsp model system, we investigated the functional protective consequences of natural variation in csHsp among five ecotypes of *Chenopodium album* L. (lambs quarters) collected from a north–south gradient along the eastern coast of North America, from habitats that differed with respect to mean growing-season temperature. In this study, we determined the variation among these ecotypes in the thermotolerance of photosynthetic electron transport (P_{et}), including both basal and induced (or acclimated) components. We differentiate between basal and induced thermotolerance, because unlike the latter, the former is most likely due to mechanisms other than Hsps. Next, we determined the functional protective contribution of csHsp to induced photosynthetic thermotolerance. Finally, we determined whether variation in thermotolerance was associated with quantitative variation in csHsp among ecotypes.

The results demonstrate that there was ecotypic variation in photosynthetic thermotolerance and csHsp function that correlated with habitat temperature, that variation in csHsp function accounted for variation in induced thermotolerance, which explained most of the variation in total thermotolerance, and that variation in csHsp function was associated with differences in csHsp abundance among ecotypes. We believe that this is the first study to

demonstrate the direct functional consequences of natural variation in Hsps in plants, and to show that functional variation is associated with evolutionary adaptation to specific habitats among ecotypes.

Materials and methods

Plant material and growth conditions

Chenopodium album L. (lambs quarters) was grown from seed collected from multiple individuals from populations in Halifax (Nova Scotia), Syracuse (New York), Roanoke (Virginia), Columbia (South Carolina), and Athens (Georgia) (seeds within each population were then mixed before use). Plants of each ecotype were grown separately *en masse* (greater than 200 plants) in 5-cm-deep flats containing a top-soil/calcined-clay mix (1:1, v:v) in a greenhouse (natural photoperiod, up to 1300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, or photosynthetically active radiation, and temperatures ranging from 18–27°C, with occasional days up to 30°C). After plants were fully grown (8–10 weeks), but before flowering, they were moved into a temperature-controlled growth chamber and grown for 7 d at 22°C with a 14-h photoperiod at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, to ensure that chloroplast sHsp levels were negligible. Subsets of plants were then pre-heat-treated (at a moderately elevated temperature to allow for the accumulation of Hsps) in a growth chamber by increasing temperatures gradually from 22 to 40°C over 1.5 h and then holding plants at 40°C for 8 h, after which plants were harvested.

Protein extraction and electrophoresis

Proteins were extracted from recently expanded leaves by grinding tissue with a mortar and pestle in liquid nitrogen, and then grinding in extraction buffer containing 1% SDS, 100 mM Tris pH 8.0, 10 mM EDTA, 1 mM ϵ -amino caproic acid, 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 10 mM ascorbate, 10 mM dithiothreitol, 1 μM antipain and leupeptin, 10% glycerol (v:v), 10% sucrose (w:v), 2% polyvinylpyrrolidone and 2% polyvinylpyrrolidone (PVP) (w:v), and 0.05% bromophenol blue. Protein extractions were boiled for 2 min and then centrifuged at 21 000 *g* for 2 min to remove insoluble debris. Protein concentration of each sample was determined in triplicate by the Coomassie dye-binding method of Ghosh *et al.* (1988), using bovine serum albumin (BSA) as a standard.

Leaf proteins were fractionated by SDS-PAGE, with 16 \times 20 \times 0.15 cm 12% gels; equal total protein was loaded per lane (40 μg). Following SDS-PAGE, the proteins were transferred to polyvinylidenedifluoride (PVDF) membranes by electrophoresis, and then the membranes were probed with protein-specific antibodies and secondary antibodies conjugated to alkaline phosphatase. Antibodies to the chloroplast small Hsps were polyclonal rabbit antibodies raised against oligopeptides of conserved sequences (described by Downs *et al.* 1998). Secondary antibodies were detected with a colourimetric system consisting of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Chloroplast isolation and photosynthetic measurements

Chloroplasts were isolated by a combination of differential and density gradient centrifugation, as before (Downs *et al.* 1998; Heckathorn *et al.* 1998). First, chloroplasts were partially purified by differential centrifugation (5000 *g*) in a buffer containing 0.35 M sorbitol, 50 Hepes (pH 7.7), 5 mM EDTA, 5 mM ascorbate, 2 mM dithiothreitol, and 2% PVP (w:v). The resulting crude chloroplast pellet was resuspended in this same buffer and the chloroplasts were subjected to density gradient centrifugation (7000 *g*) using a step-gradient containing 80, 60, 40, and 20% Percoll in 50 mM Hepes (pH 7.7). Intact chloroplasts were collected from the gradients with a Pasteur pipette, pelleted by

centrifugation (5000 g), and resuspended in a small volume of 0.35 M sorbitol in 50 mM Hepes (pH 7.7). Chloroplast samples used in electron transport assays were diluted to 100 mM sorbitol and 25 µg chlorophyll mL⁻¹, to rupture the chloroplast envelope and yield thylakoid preparations, as described below.

Whole-chain photosynthetic electron transport was measured in isolated thylakoids, as described previously (Heckathorn *et al.*, 1998), by monitoring O₂ exchange from thylakoids in the presence of methyl viologen (MV), an artificial electron acceptor that receives electrons from PSI (thus intercepting flow from PSI to NADP⁺). O₂ exchange was monitored using a Clark-type O₂ electrode (Hansatech, King's Lynn, UK). Isolated chloroplasts were resuspended to 25 µg chlorophyll per mL in 50 mM Hepes (pH 7.75), 100 mM sorbitol, 4 mM MgCl₂, 4 mM NaCl, and 25 µM MV. Chlorophyll concentration of chloroplast suspensions was determined spectrophotometrically following extraction in DMSO (as in Heckathorn *et al.* 1998). Electron transport was monitored in thylakoid samples isolated from unstressed control and pretreated plants for at least 15 min at either 26 or 48°C, at 1000 µmol m⁻² s⁻¹ PAR, and with or without antiserum to chloroplast sHsp (Ab_{met}) to disrupt sHsp protection of electron transport; BSA was added as a negative control to ensure that observed protection was specific to sHsp (as in Heckathorn *et al.* 1998; Downs *et al.* 1999a, b). In preliminary experiments, inhibition of sHsp by Ab_{met} was titrated to determine the minimum amount of Ab_{met} sufficient to achieve maximum disruption (1:300); similar amounts of pre-immune serum had no effect on electron transport (not shown, but see Heckathorn *et al.* 1998; Downs *et al.* 1999a, b). Measurements were made only after allowing the O₂-electrode apparatus to thermoequilibrate for several hours at the treatment temperatures and equilibrating all reagents at these temperatures before their addition into the sample chamber. This prevented drift in the output signal at high temperature (as in Härndahl and Sundby 2001). Under conditions of equipment thermal equilibrium, observed changes in O₂ concentration with chloroplast samples were light-dependent.

Calculation of thermotolerance

Total (induced + basal) thermotolerance of P_{et} was calculated as P_{et} in pre-heat-treated plants measured at 48°C as a percentage of P_{et} in untreated plants at 26°C (i.e. pretreated at 48°C/control at 26°C). Induced thermotolerance of P_{et} was calculated as the relative increase in P_{et} at 48°C in pretreated v. control plants [i.e. (pretreated at 48°C – control at 48°C)/control at 26°C]. Basal thermotolerance of P_{et} was calculated as P_{et} of control plants at 48°C relative to control plants at 26°C (i.e. control at 48°C/control at 26°C).

Statistical analysis

Replicate values of P_{et} were derived from three independent chloroplast extractions, each using leaf material from more than 100 plants. Unless

indicated otherwise, results were first analysed by ANOVA. Following significant ANOVA results ($P < 0.05$), differences among treatments were analysed with Tukey's comparison of means test. Analysis (ANOVA) of the thermotolerance data (data expressed as % of control) was performed on both untransformed and transformed (arcsin of square root) data, as well as with non-parametric ANOVA, in case of non-normal distributions. In all cases, the three different analyses yielded similar results regarding treatment effects, so results from untransformed parametric ANOVA are reported here. Correlation analysis (Pearson's correlation coefficient) was conducted for analysis of cHsp contribution to P_{et} acclimation. Model-I least-squares regression was used to examine the relationship of thermotolerance to habitat.

Results

The five ecotypes of *C. album* used in this study came from habitats that differed in temperature and precipitation characteristics (Table 1). As expected, temperatures during the growing season were lower for the northern-latitude sites, compared with the southern-latitude sites. Drought stress and water availability are important abiotic factors that are also likely to influence Hsp accumulation (Ristic *et al.* 1998; Downs *et al.* 1999b). However, differences in water availability among habitats is unlikely to be a contributing factor in this case, as the warmer southern sites had higher total and average growing-season precipitation.

In vitro rates of whole-chain photosynthetic electron transport (P_{et}) decreased in chloroplast thylakoids when measured at 48°C v. 26°C ($P < 0.0001$, ANOVA) (Table 2). At 26°C, P_{et} was lower in thylakoids isolated from pre-treated plants compared with controls, indicating that the pre-treatment damaged P_{et} ($P < 0.0001$). However, at 48°C, P_{et} was higher in the pre-treated plants than in controls ($P < 0.0001$), indicating that acclimation of P_{et} to high temperatures occurred in the pre-treated plants. Pre-incubation of thylakoids with antibody to the chloroplast sHsp had no effect on P_{et} in controls at either 26 or 48°C, indicating that negligible amounts of sHsp were present in these samples (confirmed by immunoblotting, below). In contrast, addition of sHsp antiserum to pre-treated plants at 48°C, decreased P_{et} to rates comparable to those in controls measured at 48°C. This indicates that the sHsp was produced in all ecotypes (again, confirmed by immunoblotting) and

Table 1. Collection sites and habitat climate characteristics for the five ecotypes of *Chenopodium album* used in the study
Weather stations were chosen based on proximity to collection site and availability of data (30-year running averages from 1961–1990). The temperature data for the growing season were calculated from monthly climatological normals. The precipitation data are presented as growing season totals and monthly averages over the growing season

| Collection site | Weather station | Growing season | Mean | Temperature (°C) | | Precipitation (cm) | |
|-----------------|-----------------|----------------|-------|------------------|-------|--------------------|-------|
| | | | | Max. | Min. | Total | Mean |
| Halifax, Can | Portland, ME | June–Sept | 15.48 | 21.12 | 9.82 | 50.83 | 8.47 |
| Syracuse, NY | Syracuse, NY | June–Sept | 16.80 | 22.74 | 10.83 | 54.41 | 9.07 |
| Roanoke, VA | Roanoke, VA | May–Sept | 19.17 | 25.41 | 12.91 | 65.61 | 9.37 |
| Columbia, SC | Columbia, SC | Apr–Oct | 20.44 | 27.15 | 13.69 | 95.96 | 10.66 |
| Athens, GA | Athens, GA | May–Oct | 19.72 | 25.70 | 13.70 | 93.04 | 10.34 |

Table 2. Effect of high temperatures on *in vitro* photosynthetic electron transport in five ecotypes of *Chenopodium album*

Electron transport ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ chlorophyll s}^{-1}$) was measured at 26 and 48°C in thylakoids from unstressed control and pre-heat-treated plants. Pre-heat-treated plants were exposed to 40°C for 8 h to induce accumulation of chloroplast sHsp; sHsp was not detectable in controls. Antibodies to the chloroplast sHsp (Ab) were added to disrupt protective function of the sHsp; BSA, added to other samples as a control, had no effect (not shown). Results are means + s.e.; $n = 3$; significant differences between additions (none *v.* Ab) within each temperature \times pre-treatment combination are indicated by different superscript letters

| Ecotype | Addition | Control | | Pre-heat-treated | |
|-------------|----------|--------------------------|--------------------------|--------------------------|--------------------------|
| | | 26°C | 48°C | 26°C | 48°C |
| Nova Scotia | none | 60.6 (1.47) ^a | 9.4 (1.02) ^a | 47.5 (0.98) ^a | 16.4 (0.83) ^a |
| | Ab | 60.0 (0.96) ^a | 7.6 (0.37) ^a | 45.1 (0.88) ^a | 8.7 (1.03) ^b |
| New York | none | 43.7 (0.81) ^a | 9.4 (0.33) ^a | 34.5 (0.37) ^a | 18.6 (0.89) ^a |
| | Ab | 45.0 (1.39) ^a | 10.1 (1.02) ^a | 34.4 (0.93) ^a | 7.5 (1.00) ^b |
| Virginia | none | 51.6 (0.46) ^a | 13.4 (0.97) ^a | 41.3 (1.22) ^a | 20.3 (0.58) ^a |
| | Ab | 49.0 (2.18) ^a | 14.3 (0.94) ^a | 43.7 (0.46) ^a | 11.6 (0.37) ^b |
| S. Carolina | none | 59.1 (1.77) ^a | 12.9 (0.65) ^a | 50.5 (0.37) ^a | 34.5 (1.38) ^a |
| | Ab | 60.4 (0.98) ^a | 15.0 (0.83) ^a | 49.5 (1.28) ^a | 12.2 (0.83) ^b |
| Georgia | none | 72.5 (1.47) ^a | 16.4 (0.74) ^a | 56.1 (0.67) ^a | 38.6 (0.89) ^a |
| | Ab | 78.1 (3.89) ^a | 16.2 (0.73) ^a | 54.4 (0.74) ^a | 17.0 (0.73) ^b |

that antibody additions disrupt sHsp function (as in Heckathorn *et al.* 1998). Importantly, sHsp antiserum had no effect on P_{et} in pre-treated plants at 26°C, indicating that the chloroplast sHsp only affects P_{et} at high temperatures (as in Heckathorn *et al.* 1998). The addition of bovine serum albumin (BSA) had no effect on P_{et} (not shown), as shown in previous studies, in which neither BSA, purified IgG, or pre-immune serum affected P_{et} in this species or tomato (Heckathorn *et al.* 1998; Downs *et al.* 1999a, b).

To examine the contribution of csHsp to the induced increase in P_{et} with a pre-heat-treatment (induced thermotolerance), we compared the extent of antibody disruption of csHsp protective function at 48°C in pre-treated plants to basal P_{et} thermotolerance. If csHsps are responsible for induced P_{et} thermotolerance, then disruption of csHsp function by the antibody should decrease P_{et} in pre-treated plants at 48°C to levels comparable to the untreated control plants at 48°C. A strong correlation was observed between P_{et} in pre-stressed thylakoids treated with the csHsp antibody and P_{et} in untreated controls across the range of P_{et} examined (Fig. 1; $r=0.86$), indicating that most of the acclimation of P_{et} to high temperatures was attributable to production of csHsp in this study.

Not surprisingly, differences in P_{et} among populations were evident in this study (Table 2). For example, at 26°C, P_{et} varied among populations; however, this was not related to latitude. To illustrate, there was no statistically significant relationship (either by correlation or linear regression analysis) between either maximum or minimum growing-season temperatures and (non-relativised) rates of electron transport in control plants at 26°C (for maximum temperature, $r^2=0.086$ and $P=0.63$; for minimum temperature,

$r^2=0.123$ and $P=0.56$). The differences in P_{et} in control plants at 26°C preclude meaningful comparisons among populations of the effects of the various treatments using absolute rates of P_{et} , and instead require the use of relativised rates of P_{et} for such comparisons, which we utilise hereafter.

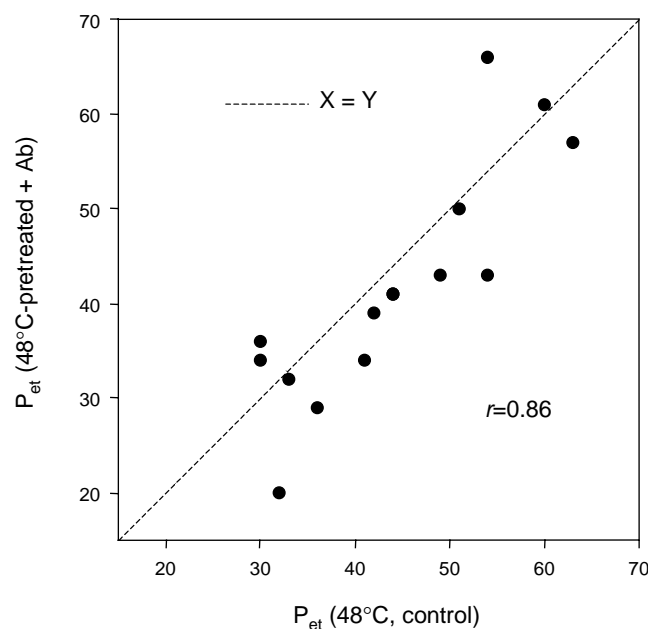


Fig. 1. The relationship between photosynthetic electron transport (P_{et}) in pre-treated plants at 48°C in the presence of antibodies to the chloroplast sHsp, used to disrupt sHsp function, (P_{et} , 48°C-pretreated + Ab) and P_{et} in untreated control plants at 48°C without antibody (P_{et} , 48°C-control). Results from correlation analysis (Pearson correlation coefficient) are shown.

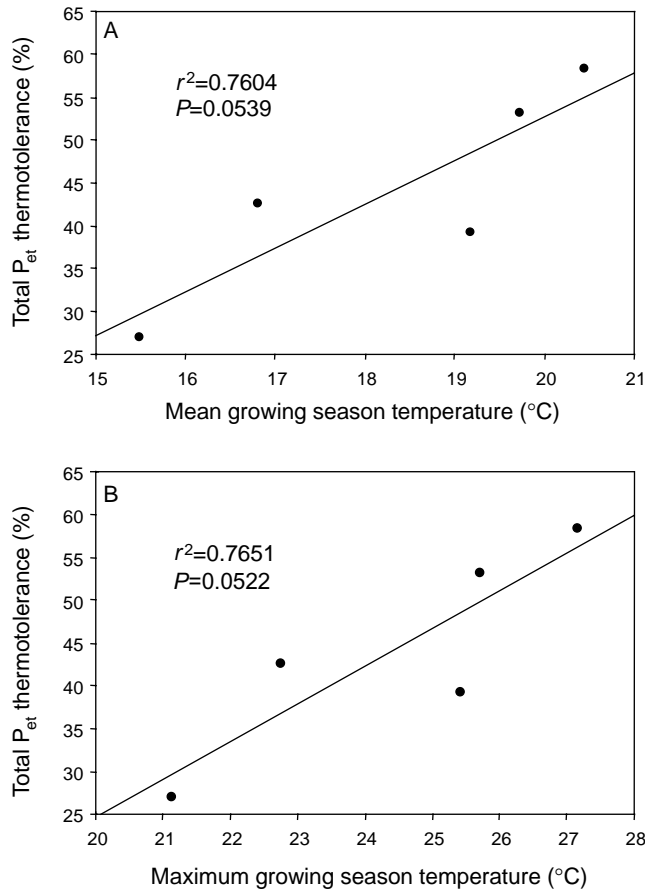


Fig. 2. The relationship between (A) mean or (B) maximum native growing-season temperature and the total thermotolerance (basal + induced) of photosynthetic electron transport (P_{et}) for the five populations of *Chenopodium album*.

To determine whether thermotolerance was associated with the habitat from which the populations originated, we examined the relationship between relative thermotolerance of P_{et} (calculated as described in the methods) and mean and maximum habitat temperatures. There were positive relationships among the five ecotypes, between mean and maximum growing-season temperatures of the sites, and thermotolerance of P_{et} (Figs 2A, B). Similar relationships were also obtained between the annual mean and maximum temperatures of the sites and the thermotolerance of P_{et} ($r^2=0.7728$, $P=0.0496$ for annual mean temperature v. thermotolerance of P_{et} ; $r^2=0.7665$, $P=0.0518$ for annual maximum temperature v. thermotolerance of P_{et}).

For normalised P_{et} , there were significant differences in thermotolerance of P_{et} among the populations (Fig. 3; $P<0.0001$). Since the plants used in this study were grown under identical conditions, the observed differences in P_{et} thermotolerance indicate that these five populations of *C. album* are distinct ecotypes. We then partitioned total P_{et} thermotolerance into basal and induced thermotolerance, and we observed differences among populations in both basal and induced P_{et} thermotolerance (Fig. 3; $P<0.0099$ and $P<0.0001$ respectively for basal and induced). However, there was substantially more variation in induced v. basal P_{et} thermotolerance among the populations, indicating that the variation in induced thermotolerance drives the observed variation in total P_{et} thermotolerance among the populations (note: similarities in basal thermotolerance also indicate that the plants were similarly acclimated to growth conditions). In addition, there were significant differences in the relative importance of induced v. basal thermotolerance among the populations (Fig. 4; $P<0.0015$), with substantially greater

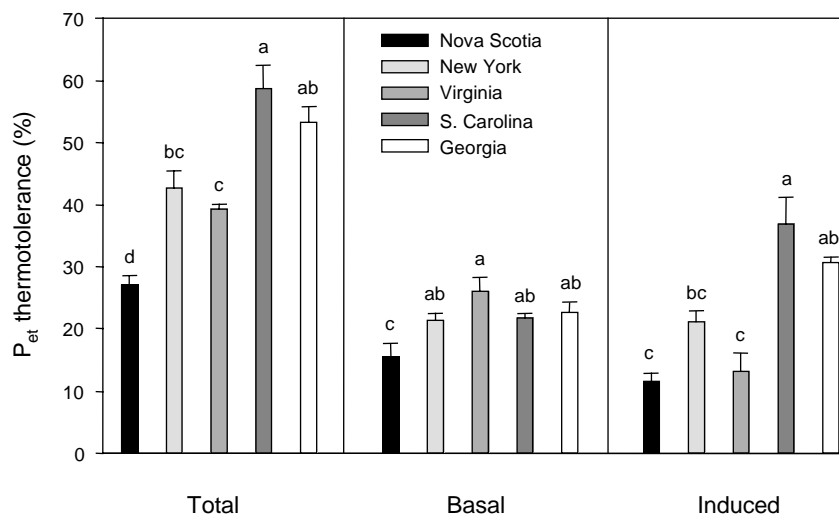


Fig. 3. Total (basal + induced), basal, and induced thermotolerance of photosynthetic electron transport (P_{et}) among five ecotypes of *Chenopodium album*. Differences among genotypes are indicated by superscript letters, following ANOVA ($P<0.0001$, $P=0.0099$, and $P=0.0001$, for total, basal, and induced P_{et} , respectively). Error bars = s.e.; $n = 3$.

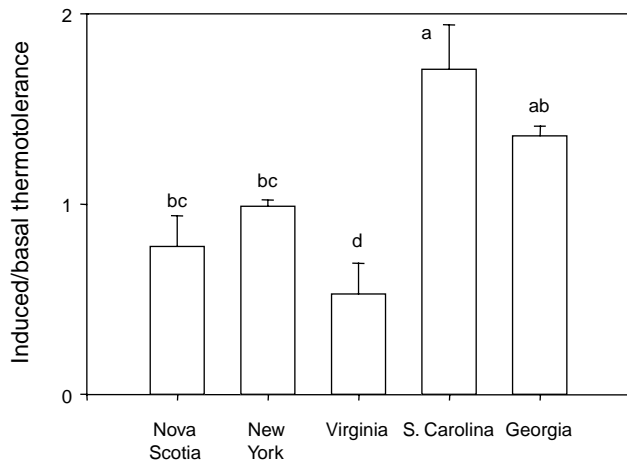


Fig. 4. The ratio of induced:basal thermotolerance of photosynthetic electron transport (P_{et}) among five ecotypes of *Chenopodium album*. Differences among genotypes are indicated by different superscript letters, following ANOVA ($P=0.0015$). Error bars = s.e.; $n = 3$.

importance of induced thermotolerance for the two southern populations native to warmer habitats.

To determine if variation in the contribution of chloroplast sHsp to P_{et} thermotolerance among ecotypes was associated with quantitative differences in the csHsp, we examined the accumulation of chloroplast sHsps of all ecotypes by gel electrophoresis and immunoblotting. The five ecotypes differed substantially in the quantity of chloroplast sHsp, per unit total leaf protein, produced in response to the Hsp-inducing pre-treatment (Fig. 5). The two southern ecotypes, from Georgia and South Carolina, exhibited much higher levels of chloroplast sHsp than the other ecotypes. The two northern ecotypes from New York and Nova Scotia made relatively low levels of sHsp, while levels of sHsp were intermediate in the Virginia ecotype. We note that faint sHsp bands in the southern ecotypes in Fig. 5 result from limited colour-development time of the immunoblot, to prevent over-exposure of bands in the northern ecotypes, rather than failure to produce sHsp. Previous

studies showed that the New York ecotype produces significant chloroplast sHsp when grown and heat-stressed under similar conditions, but that these plants make low levels of the sHsp relative to most other species (Downs *et al.* 1998, 1999a, b).

Discussion

Among the five ecotypes of *C. album* examined in this study, there was natural variation in the extent to which the chloroplast sHsps protected whole-chain P_{et} during high-temperature stress, and this variation in protective function was associated with differences in csHsp content among ecotypes. Protection of P_{et} by csHsp accounted for most of the observed acclimation of P_{et} after a pretreatment, which induced production of the csHsp. Total P_{et} thermotolerance among ecotypes was positively correlated with growing-season temperatures of the habitats from which the ecotypes were collected. Finally, there was greater variation in induced than in basal thermotolerance of P_{et} , indicating that these differences in induced thermotolerance drive variation in total thermotolerance.

To our knowledge, this is the first study to demonstrate functional consequences of natural variation in Hsps in plants, and shows that this functional variation is related to characteristics of the habitats to which different ecotypes are genetically adapted. While many studies have observed patterns in Hsp production among stress-tolerant and -sensitive genotypes, and which are often related to habitat characteristics, few studies have actually shown that natural quantitative or qualitative variation in Hsps contributes to variation in thermotolerance (Feder and Hofmann 1999). The comparative approach that we have used in this study is fairly common among investigators studying the ecological and evolutionary importance of physiological and other traits, but this approach typically provides only correlative evidence of links among physiological or organismal traits and habitat characteristics. In contrast, genetically engineered organisms permit one to vary a single trait against a constant genetic background and allow generation of variation beyond the range

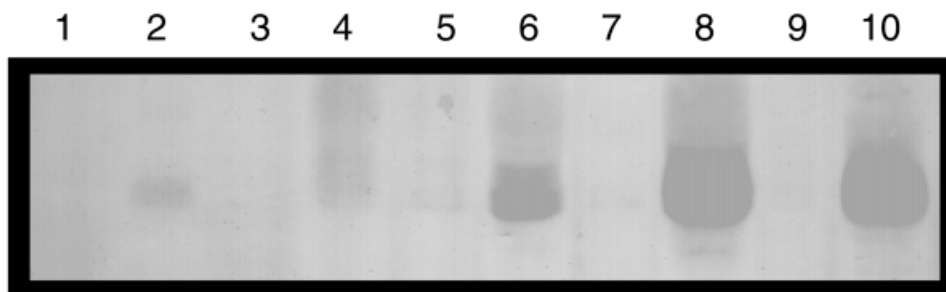


Fig. 5. The content of chloroplast sHsp in leaves of five ecotypes of *Chenopodium album*. All plants were grown at 22°C (controls = C) and then subsets of plants were heat stressed for 8 h at 40°C (heat-stressed = HS). Lanes 1 and 2 are *C. album* ecotype Nova Scotia (C and HS respectively), lanes 3 and 4 = New York (C and HS), lanes 5 and 6 = Virginia (C and HS), lanes 7 and 8 = South Carolina (C and HS), and lanes 9 and 10 = Georgia (C and HS). One of the three replicate gels is shown.

observed in natural populations (Feder and Krebs 1997; Ackerly *et al.* 2000), which permits rigorous investigation of the underlying links among genes, traits, organismal performance, and fitness in ecological and evolutionary studies (Feder *et al.* 1996). However, a transgenic approach fails to address the relevance of natural variation in traits (Feder and Krebs 1997). In the present study, the *in vitro* assay used allowed us to explicitly determine the adaptive significance of the natural variation in csHsp among ecotypes, by directly assessing the contribution of csHsp variation to variation in thermotolerance of a physiological trait, which was related to temperatures of the habitats from which the populations originated. The physiological parameter measured, photosynthetic electron transport, is known to be particularly heat-sensitive and is often a rate-limiting step for net photosynthesis at high temperatures (Weis and Berry 1988).

Although chloroplast sHsp protection accounted for most of the observed acclimation in P_{et} thermotolerance with a pre-treatment in this study, we emphasise strongly that these results should not be interpreted to mean that sHsps are the only important adaptation for protecting photosynthesis from high temperatures. It is likely that the relative importance of sHsps in protecting photosynthesis during heat stress varies during the time-course of heat stress, with temperature, among species, and for different aspects of photosynthetic metabolism. For example, we examined only P_{et} in this study, but in some situations, labile steps in the CO_2 fixation reactions may limit the overall rate of photosynthesis (e.g. Crafts-Brandner and Salvucci 2002). Also, most Hsps are known to be produced only over limited ranges of high temperatures and for limited time periods during heat stress (e.g. Howarth and Ougham 1993; Feder and Hofmann 1999). The temperature and length of time that plants were heat stressed in this study were chosen to optimise production of chloroplast sHsp. Other adaptations that protect photosynthesis from heat stress are likely to be more important earlier or later during the time course of heat stress or over different ranges of high temperatures; for example certain carotenoids and the xanthophyll cycle (Havaux 1998), protective solutes (Al-Khatib and Wiest 1990; Williams and Gounaris 1992; Lee *et al.* 1997; Georgieva *et al.* 2003), phosphorylation of thylakoid proteins (Rokka *et al.* 2000), and changes in chloroplast lipid composition (Murakami *et al.* 2000). In addition, it is virtually assured that the relative importance sHsps, and other adaptations, will vary among species. For example, we predict that sHsps would play a smaller role in species wherein basal thermotolerance of photosynthesis is the major determinant of overall photosynthetic thermotolerance, such as desert succulents, which have high levels of basal thermotolerance.

The mechanisms by which the chloroplast sHsps protect photosynthesis during stress are not fully understood. To date, chloroplast sHsps have only been demonstrated to

protect P_{et} , in particular PSII, or thylakoid membranes during stress (Heckathorn *et al.* 1998, 1999; Lee *et al.* 1998, 2000a, b; Miyao-Tokutomi *et al.* 1998; Downs *et al.* 1999a, b; Nakamoto *et al.* 2000; Török *et al.* 2001). Protection of PSII by sHsps occurs during oxidative and photoinhibitory high-light stress (Schuster *et al.* 1988; Stapel *et al.* 1993; Downs *et al.* 1999b; Lee *et al.* 2000b), as well as during heat stress, and the apparent specificity of sHsp protection for PSII with P_{et} may simply reflect the relative lability of PSII to these stresses (e.g. Weis and Berry 1988; Havaux 1998; Heckathorn, *et al.* 1998; Downs *et al.* 1999b).

Chloroplast sHsps do not appear to function in the repair of stress-related damage, but rather they function to prevent damage (Downs *et al.* 1999a). Consistent with this, csHsps only increased P_{et} at high temperatures in this study, as in a previous study using purified csHsp added to csHsp-deficient thylakoids (Heckathorn *et al.* 1998), but csHsp do not increase P_{et} in heat-stressed thylakoids when measured at normal temperatures (the protocol used by Härndahl and Sundby 2001). These observations are consistent with the temperature-dependent association of sHsps with thylakoids (Glaczinski and Kloppstech 1988; Eisenberg-Domovich *et al.* 1994) and their apparent mechanisms of protection. Accumulating evidence suggests that there are probably multiple mechanisms by which chloroplast sHsps function in protection: (1) as molecular chaperones to prevent, but not reverse, protein denaturation or aggregation (Török *et al.* 2001), (2) through membrane stabilisation in response to changes in fluidity at high temperatures (Török *et al.* 2001), and (3) possibly as a site-specific antioxidant (Hamilton and Heckathorn 2001). Lastly, given that chloroplast sHsps are found in both the stromal and membrane fractions of chloroplasts (Heckathorn *et al.* 2002, and references therein), and that they appear to protect via multiple mechanisms, it is likely that chloroplast sHsps fulfill other unidentified roles besides protection of P_{et} .

Acknowledgments

The authors thank EW Hamilton III, J Kathleen Mueller, WT Starmer, and the anonymous reviewers for helpful comments on the manuscript, Christine Mayer for assistance with statistical analyses, and Aaron Downs for technical assistance. We are grateful to Rowan Sage and Doug Jensen for providing seeds of specific ecotypes. This work was supported in part by grants from the USA National Science Foundation to SAH.

References

- Ackerly DD, Dudley SA, Sultan SE, Schmitt J, Coleman JS, Linder CR, Sandquist DR, Geber MA, Evans AS, Dawson TE, Lachowicz MJ (2000) The evolution of plant ecophysiological traits: recent advances and future directions. *Bioscience* 50, 979–995.

- Al-Khatib K, Wiest SC (1990) Solution effects on the thermostability of bean chloroplast thylakoids. *Crop Science* **30**, 90–96.
- Arrigo A, Landry J (1994) Expression and function of the low-molecular-weight heat-shock proteins. In 'The biology of heat shock proteins and molecular chaperones'. (Eds R Morimoto, A Tissieres and C Georgopoulos) pp. 335–373. (Cold Spring Harbor Laboratory Press: Plainview, NY)
- Crafts-Brandner SJ, Salvucci ME (2002) Sensitivity of photosynthesis in a C₄ plant, maize, to heat stress. *Plant Physiology* **129**, 1773–1780 doi:10.1104/PP.002170.
- Downs C, Heckathorn S, Bryan J, Coleman J (1998) The methionine-rich low-molecular-weight chloroplast heat-shock protein: evolutionary conservation and accumulation in relation to thermotolerance. *American Journal of Botany* **85**, 175–183.
- Downs CA, Coleman JS, Heckathorn SA (1999a) The chloroplast 22-Ku heat-shock protein: a luminal protein that associates with the oxygen-evolving complex and protects Photosystem II during heat stress. *Journal of Plant Physiology* **155**, 477–487.
- Downs CA, Ryan SL, Heckathorn SA (1999b) The chloroplast small heat-shock protein: evidence for a general role in protecting Photosystem II against oxidative stress and photoinhibition. *Journal of Plant Physiology* **155**, 488–496.
- Eisenberg-Domovich Y, Kloppstech K, Ohad I (1994) Reversible membrane association of heat-shock protein 22 in *Chlamydomonas reinhardtii* during heat shock and recovery. *European Journal of Biochemistry* **222**, 1041–1046.
- Feder M, Cartano N, Milos L, Krebs R, Lindquist S (1996) Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. *The Journal of Experimental Biology* **199**, 1837–1844.
- Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* **61**, 243–282 doi:10.1146/ANNUREV.PHYSIOL.61.1.243.
- Feder ME, Krebs RA (1997) Ecological and evolutionary physiology of heat-shock proteins and the stress response in *Drosophila*: complementary insights from genetic engineering and natural variation. In 'Stress, adaptation and evolution'. (Eds R Bijlsma and V Loeschcke) pp. 155–173. (Birkhauser-Verlag: Basel)
- Georgieva K, Fedina I, Maslenkova L, Peeva V (2003) Response of *chlorina* barley mutants to heat stress under low and high light. *Functional Plant Biology* **30**, 515–524 doi:10.1071/FP03024.
- Ghosh S, Gepstein S, Heikkila JJ, Dumbroff EB (1988) Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissue. *Analytical Biochemistry* **169**, 227–233.
- Glaczinski H, Kloppstech K (1988) Temperature-dependent binding to the thylakoid membranes of nuclear-encoded chloroplast heat-shock proteins. *European Journal of Biochemistry* **173**, 579–583.
- Hamilton EW, Heckathorn SA (2001) Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat-shock proteins, whereas Complex II is protected by proline and betaine. *Plant Physiology* **126**, 1266–1274.
- Härndahl U, Sundby C (2001) Does the chloroplast small heat-shock protein protect photosystem II during heat stress *in vitro*? *Physiologia Plantarum* **111**, 273–275.
- Havaux M (1998) Carotenoids as membrane stabilizers in chloroplasts. *Trends in Plant Science* **3**, 147–151 doi:10.1016/S1360-1385(98)01200-X.
- Heckathorn SA, Downs CA, Coleman JS (1999) Small heat-shock proteins protect electron transport in chloroplasts and mitochondria during stress. *American Zoologist* **39**, 865–876.
- Heckathorn SA, Downs CA, Sharkey TD, Coleman JS (1998) The small, methionine-rich chloroplast heat-shock protein protects Photosystem II electron transport during heat stress. *Plant Physiology* **116**, 439–444 doi:10.1104/PP.116.1.439.
- Heckathorn SA, Poeller GJ, Coleman JS, Hallberg RL (1996) Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. *Oecologia* **105**, 413–418.
- Heckathorn SA, Ryan SL, Baylis JA, Wang D, Hamilton III EW, Cundiff L, Luthe DS (2002) *In vivo* evidence from an *Agrostis stolonifera* selection genotype that chloroplast small heat-shock proteins can protect photosystem II during heat stress. *Functional Plant Biology* **29**, 933–944 doi:10.1071/PP01191.
- Howarth CJ, Ougham HJ (1993) Gene expression under temperature stress. *New Phytologist* **125**, 1–26.
- Joshi CP, Klueva NY, Morrow KJ, Nguyen HT (1997) Expression of a unique plastid-localized heat-shock protein is genetically linked to acquired thermotolerance in wheat. *Theoretical and Applied Genetics* **95**, 834–841 doi:10.1007/S001220050633.
- Knight CA, Ackerly DD (2001) Correlated evolution of chloroplast heat shock protein expression in closely related plant species. *American Journal of Botany* **88**, 411–418.
- Lee CB, Hayashi H, Moon BY (1997) Stabilization by glycinebetaine of photosynthetic oxygen evolution by thylakoid membranes from *Synechococcus* PCC7002. *Molecules and Cells* **7**, 296–299.
- Lee S, Prochaska DJ, Fang F, Barnum SR (1998) A 16.6-kilodalton protein in the cyanobacterium *Synechocystis* sp. PCC6803 plays a role in the heat shock response. *Current Microbiology* **37**, 403–407 doi:10.1007/S002849900400.
- Lee BH, Won SH, Lee HS, Miyao M, Chung WI, Kim IJ, Jo J (2000a) Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice. *Gene* **245**, 283–290 doi:10.1016/S0378-1119(00)00043-3.
- Lee S, Owen HA, Prochaska DJ, Barnum SR (2000b) HSP16.6 is involved in the development of thermotolerance and thylakoid stability in the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803. *Current Microbiology* **40**, 283–287 doi:10.1007/S002849910056.
- Miyao-Tokutomi M, Lee BH, Mizusawa N, Yamamoto N (1998) Active oxygen and photoinhibition of Photosystem II. In 'Photosynthesis mechanisms and effects III'. (Ed. G Garab) pp. 2097–2102. (Kluwer Academic: Dordrecht).
- Murakami Y, Tsuyama M, Kobayashi Y, Kodama H, Iba K (2000) Trienoic fatty acids and plant tolerance of high temperature. *Science* **287**, 476–479 doi:10.1126/SCIENCE.287.5452.476.
- Nakamoto H, Suzuki N, Roy SK (2000) Constitutive expression of a small heat-shock protein confers cellular thermotolerance and thermal protection to the photosynthetic apparatus in cyanobacteria. *FEBS Letters* **483**, 169–174 doi:10.1016/S0014-5793(00)02097-4.
- Park S, Shivaji R, Krans JV, Luthe DS (1996) Heat-shock response in heat-tolerant and non-tolerant variants of *Agrostis palustris* Huds. *Plant Physiology* **111**, 515–524.
- Parsell DA, Lindquist S (1994) Heat shock proteins and stress tolerance. In 'The biology of heat shock proteins and molecular chaperones'. (Eds R Morimoto, A Tissieres and C Georgopoulos) pp. 457–494. (Cold Spring Harbor Press: Cold Spring Harbor, NY)
- Preczewski PJ, Heckathorn SA, Coleman JS (2000) Photosynthetic thermotolerance is quantitatively and positively correlated with production of specific heat-shock proteins among nine genotypes of *Lycopersicon* (Tomato). *Photosynthetica* **38**, 127–134.
- Ristic Z, Yang G, Martin B, Fullerton S (1998) Evidence of association between specific heat shock proteins and drought and heat tolerant phenotype in maize. *Journal of Plant Physiology* **153**, 497–505.

- Rokka A, Ro EM, Hermann RG, Anderson B, Vener AV (2000) Dephosphorylation of Photosystem II reaction center proteins in plant photosynthetic membranes as an immediate response to abrupt elevation of temperature. *Plant Physiology* **123**, 1525–1535 doi:10.1104/PP.123.4.1525.
- Schuster GD, Even D, Kloppstech K, Ohad I (1988) Evidence for protection by heat-shock proteins against photoinhibition during heat-shock. *EMBO Journal* **7**, 1–6.
- Williams WP, Gounaris K (1992) Stabilisation of PSII-mediated electron transport in oxygen-evolving PSII core preparations by the addition of compatible co-solutes. *Biochimica et Biophysica Acta* **1100**, 92–97.
- Stapel D, Kruse E, Kloppstech K (1993) The protective effect of heat shock proteins against photoinhibition under heat shock in barley. *Journal of Photochemistry and Photobiology. B, Biology* **21**, 211–218 doi:10.1016/1011-1344(93)80185-C.
- Török Z, Goloubinoff P, Horváth I, Tsvetkova NM, Glatz A, *et al.* (2001) *Synechocystis* HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. *Proceedings of the National Academy of Sciences USA* **98**, 3098–3103 doi:10.1073/PNAS.051619498.
- Vierling E (1991) The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 579–620 doi:10.1146/ANNUREV.PP.42.060191.003051.
- Waters E, Lee G, Vierling E (1996) Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany* **47**, 325–338.
- Weis E, Berry JA (1988) Plants and high temperature stress. In 'Plants and temperature'. (Eds SP Long and FI Woodward) pp. 329–346. (The Company of Biologists Limited: Cambridge, UK)

Manuscript received 9 June 2003, accepted 25 August 2003