1   Contrasting avoidance - tolerance in heat stress response from

2   thermally contrasting climates in *Arabidopsis thaliana*

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15   Running title: Avoidance – tolerance in heat stress

16   **Abstract**

17   Plants ameliorate heat stress by avoiding heat loading, reducing tissue

18   temperature through evaporative cooling, and/or through tolerance, i.e.

19   maintaining function at high temperature. Here *Arabidopsis thaliana* natural

20   populations from two ends of an elevation gradient in NE Spain were used to

21   ask: do plants from contrasting climates 1) show genetically based differences in

22   heat stress damage and 2) adopt different avoidance-tolerance patterns? Four

23   low- and four high-elevation populations were repeatedly exposed to high

24   temperature ($45^{o}C$) in a growth chamber at bolting stage. High temperature

25   induced 23% more inflorescence branches, 25% longer total reproductive branch

26   length, and 12% less root dry mass, compared with control. However summed

27   fruit length, hence fitness, decreased by 15%, populations did not differ

28   significantly in fitness reduction. High elevation populations showed more

29   avoidance, i.e. lower rosette temperature at $45^{o}C$. Low elevation populations

30   showed more tolerance, maintaining relatively higher photosynthetic rate at $45^{o}C$.

31   Avoidance was associated with high transpiration rate and flat rosette leaf angle.

32   Tolerance was negatively associated with heat shock protein 101 (Hsp101) and

33   salicylic acid (SA) accumulation. The divergent avoidance–tolerance patterns for

34   populations from thermally contrasting climates may indicate both constraints on

35   the evolution and contrasting adaptive divergence regulated by local climates.

36   **Keywords:** Heat stress; avoidance; tolerance; fitness; *Arabidopsis thaliana*;

37   geographic pattern.

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39 **Introduction**

40   Abiotic stresses, such as temperature and drought, are main range

41 limitation determinants. Heat stress imposed by daily temperature fluctuation can

42 cause severe damage to plants, including reduction of plant growth and

43 alterations in photosynthesis and phenology. Such disruptions are ultimately

44 likely to cause reduction in resources available for reproduction(Hasanuzzaman

45 et al. 2013). It is therefore quite likely that frequent heat stress will reorganize

46 allocation and physiology through selection for the highest fitness response to

47 high temperature events. Careful observation of the relationship between a

48 plant's thermal environment and the specific mechanisms of adaptation to heat

49 stress in wild populations is very limited, despite its likely relationship to

50 extinction at the warmer end of species' ranges.

51   In general, we define a heat stress as a diurnal temperature pattern in

52 which plants display reduced fitness compared to some other temperature

53 pattern (SØrensen 2001). Usually, the maximum stress temperature is about 10

54 to 15°C higher than the optimum for broadly distributed species and as little as

55 5°C higher than the optimum in species with narrow geographic ranges

56 (Lindquist 1986).

57   Plants have developed both long-term and short-term adaptations to high

58 temperature (Hong et al. 2003). Evolutionary adjustments of the timing of life

59 history events (Montesinos-Navarro et al. 2011) and further adjustments in the

60 timing of allocating of resources to rosette and inflorescence (Wolfe and Tonsor

61 2014), are mechanisms that allow annual plants to escape from the most

62   stressfully high temperature period by adjusting life cycle timing. To further

63   reduce or prevent stress from high temperature during the active growing

64   season, adaptive responses can be described as part of two main strategies,

65   avoidance and tolerance (Sakai and Larcher 1987).

66   Stress avoidance is a strategy through which plants adjust their internal

67   states in ways that reduce exposure to a potentially damaging environment

68   (Touchette et al. 2009, Puijalon et al. 2011). For most plants, leaves are the most

69   important structure for obtaining energy and carbon (but see (Earley et al. 2009)).

70   On average, avoidance can lower leaf surface temperature across growing

71   season by $4^{o}C$ compared to ambient temperature in cotton (Wiegand and

72   Namken 1966). Generally, the higher the air temperature, the larger the

73   differential between air and leaf can be (Linacre 1967, Wilson et al. 1987). Leaf

74   temperature thus becomes an ideal indicator to keep track of plants' heat

75   avoidance.

76   Avoidance can also be achieved by leaf orientation adjustment (Jones and

77   Corlett 1992, Zlatev et al. 2006). Many plants adjust leaf angle, thus reducing the

78   leaf area that is exposed to heat from sunlight (Bradshaw 1972, Huey 2002).

79   Populations originating from high temperature sites have higher leaf angle in heat

80   stress in *Arabidopsis thaliana* (Vile *et al.* 2012) and in *Aractostaphylos* species

81   (Shaver 1978, Ehleringer 1987, Fu 1989).

82   Avoidance can also be achieved through transpiration which is

83   immediately elevated at high temperature, thus cooling the leaf surface (Shah et

84   al. 2011). The threshold temperature that controls the relative rate of

85   transpiration is species-specific (Mahan 1990). The transpiration process is

86   closely connected with stomatal opening (Burke and Upchurch 1989). One

87   potential constraint on transpiration-driven heat stress avoidance is that high

88   temperature often co-varies with a dry environment in nature. Plants from drier

89   and warmer sites show higher water use efficiency, compensating for large water

90   losses due to transpirational cooling in *Boechera holboellii* populations (Knight et

91   al. 2006). However, phylogenetic analysis of 28 dominant species in a Mexican

92   evergreen shrubland also showed a correlation between steeper leaf angle and

93   low transpiration rate as an adaptation to dry climate (Falster and Westoby 2003,

94   Valiente-Banuet et al. 2010). These contrasting selection pressures on

95   transpiration in combined heat and drought stress further complicate the

96   evolution of avoidance in nature (Vile et al. 2012).

97   When internal temperatures rise sufficiently despite any avoidance

98   mechanisms possessed by the plant, heat stress can damage cells in a variety of

99   ways. High cellular temperature affects both cellular structural integrity and

100   protein function, causing membrane disruption as well as disruption of metabolic

101   function through production of reactive oxygen species (ROS) (Schöffl et al.

102   1998) and enzyme denaturation (Blum and Ebercon 1981, Reynolds et al. 1994,

103   Ismail and Hall 1999). Photosynthesis is especially heat-sensitive (Berry and

104   Bjorkman 1980, Reynolds et al. 1994, Sharkey et al. 2008). In plants

105   measurement of extent to which photosynthetic rate is depressed can be an

106   effective measure of functional disruption. Eventually, as temperatures rise

107   damage is sufficient to affect a plant's survivorship and fecundity (Senthil-Kumar

108   et al. 2007).

109   Heat tolerance is the ability of plants to minimize or repair damage while

110   experiencing a high internal temperature (Touchette et al. 2009, Puijalon et al.

111   2011). Heat tolerance mechanisms include protection and repair of damaged cell

112   structures, structural proteins, and enzymes (Shah et al. 2011). While heat

113   tolerance is complex (Kotak et al. 2007) and incompletely understood, it is known

114   to involve up-regulation of two classes of molecules: heat shock proteins

115   (thereafter, Hsps) (Queitsch 2000b, Hong and Vierling 2001, Wang et al. 2004)

116   and plant hormones (Larkindale 2004, He et al. 2005, Larkindale and Huang

117   2005). Hsps are a group of molecular chaperones involved in dissolving and

118   refolding aggregated cellular proteins, under both normal and stressful conditions

119   (Hartl 1996). Members of the Hsp100/ClpB family have shown a significant role

120   in heat tolerance in *Saccharomyces cerevisiae* (Hsp104) (Sanchez and Lindquist

121   1990) and *Arabidopsis thaliana* (Hsp101) (Queitsch 2000a, Hong and Vierling

122   2001). Plant hormones, especially salicylic acid (SA), are a common stress

123   response. SA reduces reactive oxygen species (ROS) accumulation and affects

124   a great many other processes in the plant. SA expression modulation and its

125   effects are not fully understood, but SA's importance for a variety of stress

126   responses is well documented (Delaney et al. 1994, Klessig and Malamy 1994,

127   Clarke et al. 2004, Yuan and Lin 2008, Vlot et al. 2009).

128   Plants in environments with frequent heat stress tend to evolve greater

129   tolerance (Huey 2002), but how avoidance varies in adaptation to heat stress

130   remains unclear. Avoidance and tolerance can have different costs and benefits

131   depending on complex aspects of the growth environment. Thus different

132   environments may favor tolerance or avoidance in response to the balance of

133   selection acting on the mechanisms involved in each strategy (see for example

134   for drought tolerance / herbivory avoidance (Siemens and Haugen 2013), for

135   irradiance and water availability (Sánchez-Gómez et al. 2006)). It is however a

136   mystery how avoidance and tolerance work together to contribute at the whole

137   plant fitness level. The question of the potential relationship between avoidance

138   and tolerance, different strategies responding to the same stimuli, is of primary

139   ecological interest, as it may reveal constraints that limit the evolution of the traits

140   involved in the response and variation in a broad range of architectural traits of

141   canopies, leaves and stems, all involved in these strategies.

142   In this study we use a set of wild-collected *Arabidopsis thaliana*

143   populations that exhibit clines in many traits in association with a climate gradient

144   (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Wolfe and

145   Tonsor 2014). Previous work indicates that the climate gradient includes

146   gradients in both temperature and precipitation. Thus we are particularly

147   interested in heat stress in the context of water use.

148   We ask two questions. Do populations from thermally contrasting climates:

149   1) Display the same reductions in fitness with heat stress?

150   2) Exhibit contrasting avoidance and tolerance strategies?

151   Four low- and four high- elevation populations, each with four genotypes,

152   were collected from northeastern Spain. In this area, climate is highly correlated

153   with elevation (Montesinos-Navarro et al. 2009, Wolfe and Tonsor 2014). Low

154   elevation populations experience hotter and dryer conditions, while high elevation

155   populations experience colder and wetter conditions. In this study, we first looked

156   at the fitness effect of repeated heat stress episodes for all the plants. We then

157   compared avoidance and tolerance strategies in heat stress response in plants

158   from the contrasting climates. We further explored possible causally-connected

159   traits for both avoidance and tolerance. For avoidance, we looked at rosette

160   angle and transpiration rate. For tolerance, we looked at the accumulation of

161   Hsp101 and SA.

Materials and Methods

Materials and heat treatment

164   Plant lineages were collected from NE Spain as seeds (Montesinos-

165   Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al.

166   2012, Wolfe and Tonsor 2014) and grown for at least three generations in

167   common controlled environmental conditions to remove any maternal

168   environmental variance that might otherwise have carried over from the field. The

169   geographic locations of these populations can be seen in Fig.S1 (adapted from

170   Fig.1 in Montesinos-Navarro et al. 2001). Seeds were germinated and

171   maintained at 22°C for 3 weeks (16 hrs light/8 hrs dark, 200 μM m^{-2}s^{-1}) after 5-

172   day stratification at 5°C in the dark. Since these populations are more likely to

173   experience heat stress at the bolting stage in nature, heat treatments were

174 performed at bolting stage (stage 6 - 6.10 based on Table 1 in Boyes et al.

175 2001). Seedlings therefore experienced a 4-week vernalization at 5°C (10 hrs

176 light / 14 hrs dark, 150 μM m^{-2}s^{-1}), to synchronize flowering time. After return to

177 control growth conditions (16 hrs light/8 hrs dark, 200 μM m^{-2}s^{-1}) plants were

178 checked every day and those at the bolting stage were transferred to a separate

179 chamber for the heat treatment. Growth, control, and heat treatment were all

180 conducted in our Conviron PGW36 controlled environment growth chambers

181 (http://www.conviron.com) at the University of Pittsburgh. Plants from each

182 population were blindly partitioned into two groups and randomly ordered across

183 populations in each group. One group was the control group, in which plants

184 were maintained at 22°C all the time, the other was the heat treatment group, in

185 which chamber air temperature was increased steadily over 15 minutes to reach

186 45°C, maintained at 45°C for 3 hrs and then brought back to 22°C. This

187 treatment was repeated twice a week from each plant's first heat treatment till

188 harvest (following method of Larkindale & Vierling 2008). We harvested all the

189 plants 60 days after their first heat treatment. Avoidance and tolerance measures

190 were performed during the first heat treatment, while fitness was estimated at

191 harvest.

192 **Fitness quantification**

193 We were able to measure total plant fitness 60 days after the first heat

194 treatment, since plants were at that time nearly completely senesced. As

195 explained in the results, there were two distinct types of fruits, aborted and

196 mature. The distinction between the aborted and mature was visually obvious.

197   Sampling a substantial number of aborted fruits showed that they contained no

198   viable seeds. Only mature fruits were included in the fitness quantification. The

199   length of the fruit in *Arabidopsis* is highly correlated with the number of seeds

200   within the fruit (Alonso-Blanco et al. 1999). Thus we used summed fruit length as

201   a measure of fitness. We measured the length of five randomly chosen normal

202   and mature fruits, two from the main (apical meristem) stem and three from

203   secondary (lateral meristem) stems, to estimate the average fruit length. We then

204   counted the total fruit number for each plant. Fitness, here summed fruit length,

205   is equal to the fruit number times the average fruit length, similar to Wolfe &

206   Tonsor (2014).

207   **Resource allocation quantification**

208   We anticipated that heat stress would change resource allocation to the

209   reproductive system and re-shape the reproductive structures, reflected in

210   changes in reproductive branch lengths, number of branches and dry mass. To

211   assess the potential change in resource allocation to reproduction, we partitioned

212   the plants into rosettes, inflorescences and roots, dried them at 65°C for at least

213   three days, and recorded the dry mass of each component. Prior to drying we

214   also measured the length of all the reproductive portions of inflorescence

215   branches (length from insertion of the lowest fruit to the apex) with a Map Wheel

216   (scalex.com) and counted the number of basal branches in each plant.

217   **Avoidance characterization**

218   A direct metric to indicate the level of avoidance is the difference between

219   rosette temperature and air temperature. For rosette temperature, a

220   thermocouple was placed in the center of the rosette, not touching the rosette

221   surface, 15mins after the heat stress initiation. For air temperature, a

222   thermocouple was suspended at height of the apical meristem of the tallest

223   inflorescence in free air. For each plant the difference between rosette

224   temperature and air temperature was calculated as the difference between the

225   temperatures of these two thermocouples. To better understand the physiological

226   basis for variation in rosette temperature among populations, rosette angle and

227   transpiration rate were also quantified. To measure leaf angle, the whole rosette

228   was photographed from four vantage points 90 degrees apart. The rosette angle

229   is the angle of a plant's most recently fully developed leaf to the horizontal line

230   and was measured in ImageJ in each image (Schneider et al. 2012). We then

231   averaged the four angles as a measure of rosette angle for each plant. We

232   simultaneously measured transpiration and photosynthetic rate using a LiCor

233   6400XT gas exchange analyzer. A custom-made *Arabidopsis* single-leaf cuvette

234   was used and one most recently fully expanded leaf was held in the cuvette until

235   gas exchange became steady (see Fig.S2). Five measurements of carbon

236   assimilation and transpiration were recorded at 12-second intervals and the

237   measures were then averaged. Immediately following gas exchange

238   measurement, each leaf was imaged and leaf area was calculated in ImageJ

239   (Schneider et al. 2012). The photosynthetic and transpiration rates were

240   calculated as rate per area.

241   **Tolerance characterization**

242   We use relative photosynthetic rate compared with control to quantify heat

243   tolerance. Photosynthetic rate was measured as described above with the LiCor

244   6400XT gas exchange analyzer. To determine the relationship of Hsp101 and SA

245   accumulation to tolerance, we collected leaf samples and quantified Hsp101 and

246   SA immediately following heat treatment. Two newly fully developed leaves, one

247   for Hsp101 and the other for SA quantification, were collected right after heat

248   treatment, freeze dried and weighed. Hsp101 quantification was measured via

249   western blot as described in Tonsor *et al.* 2008. SA was quantified with HPLC as

250   described in Zhang et al. 2015.

251   **Statistical Analyses**

252   For each measure, we performed a separate ANOVA analysis to look at

253   whether the measure showed significant difference at the level of elevation group,

254   population nested within elevation group, heat treatment and the elevation group

255   * heat treatment interaction, using Proc GLM in SAS (SAS Institute 2005).

256   Elevation and population were treated as fixed effects.

257   To assess the relationship between these potential covariates and our

258   avoidance and tolerance measures, a multivariate analysis of variance

259   (MANOVA) was used both for avoidance and tolerance in Proc GLM (SAS

260   Institute 2005). For avoidance, the difference between air temperature and

261   rosette temperature, hereafter DeltaT, was treated as the dependent variable,

262   with rosette angle, transpiration rate, heat treatment, elevation group and the

263   elevation group * heat treatment interaction as independent variables. For

264   tolerance, photosynthetic rate was treated as the dependent variable, while

265 Hsp101 accumulation, SA accumulation, heat treatment, elevation group and the

266 elevation group * heat treatment interaction were treated as independent

267 variables. The nested effects, population nested within elevation group, genotype

268 nested within population, were also included in the MANOVA for both avoidance

269 and tolerance analyses.

270 To further explore the direct relationship between transpiration rate and

271 avoidance we performed univariate regression of DeltaT on transpiration rate.

272 Likewise we tested for relationships between Hsp101/SA and tolerance by

273 regressing photosynthetic rate on Hsp101 or SA the independent variables, using

274 Proc REG (SAS Institute 2005).

275 **Results**

276 **Heat stress caused reproductive disruption and fitness reduction**

277 Two types of heat stress disruptions of reproduction were observed. First,

278 in some cases heat stress damaged the apical meristem of flowering stems

279 (Fig.S3). After the death of the apical meristem, additional secondary stems were

280 initiated. Second, even if the apical meristem survived, heat nevertheless often

281 led to failure of fertilization or early fruit abortion for flowers with active gametes

282 at the time of the heat stress (Fig.S4). After heat stress, the apical meristem

283 recovered growth but the fruits from the damaged portion of apical meristem did

284 not successfully mature. Dissection of fruits from damaged apical meristem

285 showed no viable seeds. We did not count such fruit in our measure of total fruit

286 lengths.

287   Plants from low elevation produced greater total fruit length than plants

288   from high elevation (p<0.0001) across both heat stress and control treatments.

289   Across the experiment as a whole, plants from low elevation produced more

290   basal branches (p<0.0001), greater reproductive length (p<0.0001), and lower

291   root dry mass (p<0.0001) than plants from high elevation. Plants from both low

292   and high elevations showed 15% reduction in total fruit length when exposed

293   repeatedly to 45°C (p=0.0007, Fig.1a). Under heat stress, all populations

294   showed about 25% longer reproductive length (p=0.01, Fig.1c), while only high

295   elevation populations showed 23% more basal branches (p=0.005, Fig.1b) and

296   12% less root mass (p=0.0001, Fig.1d). We did not see a significant difference in

297   the above responses to heat stress between low vs. high elevation populations

298   (Table S1).

299   **High elevation populations showed greater avoidance**

300   All plants maintained rosette temperatures that were statistically

301   significantly different from ambient air temperature under all conditions (Fig.2).

302   The direction of the difference in rosette temperature depended on the ambient

303   temperature. All plants, regardless of elevation of origin, increased their rosette

304   temperature relative to ambient temperature under the 22°C control condition.

305   High elevation populations increased about 1.2°C more than low elevation

306   populations (high vs. low: 24.4°C vs. 23.2°C, p<0.0001).

307   When exposed to heat stress, however, both low and high elevation

308   populations maintained rosette temperature significantly and substantially lower

309   than ambient air temperature, on average by 7.7°C across all populations. High

310   elevation populations reduced rosette temperature 1.8°C more than low elevation

311   populations (low vs. high: 38.2°C vs. 36.4°C, p = 0.004). We saw greater heat

312   stress avoidance in high elevation populations (Fig.2).

313   Considering both the control and heat stress treatments together, high

314   elevation populations exhibit greater rosette temperature homeostasis than low

315   elevation populations (Fig.2).

316   **Low elevation populations showed greater tolerance**

317   Heat tolerance is the ability of a plant to perform normal plant functions

318   when exposed to high temperature. Here we measured photosynthesis, one of

319   the key plant functions, as a measure of tolerance (Fig. 3). Greater

320   photosynthetic rate indicates relatively higher tolerance. We saw a significantly

321   lower photosynthetic rate in low compared to high elevation populations under

322   the control temperature (p<0.0001).

323   However, with a 45°C heat stress, low elevation populations showed no

324   significant change in photosynthetic rate, while high elevation populations

325   significantly reduced their photosynthetic rate (Elevation group*Heat treatment

326   interaction: p=0.003, Table S1). Low elevation populations were significantly

327   more heat tolerant than high elevation populations (p<0.0001).

328   **Avoidance was positively associated with high transpiration rate and flat**

329   **rosette angle**

330   We measured rosette angle and transpiration rate as potential traits

331   associated with avoidance (Fig. 4, Fig.5, Table S1).

332   Rosette angle differed between low and high elevation populations

333   regardless of treatment, with low elevation populations exhibiting sharper rosette

334   angle (low vs. high mean rosette angle: p=0.0005). However, under our

335   measurement protocol the rosette angle was not significantly affected by heat

336   treatment (Fig. 4a).

337   High elevation populations showed significantly higher transpiration rate

338   than low elevation populations under the control temperature (p<0.0001, Fig.4b).

339   Transpiration rate was significantly increased with heat treatment (p<0.0001),

340   with high elevation populations increasing significantly more than low elevation

341   populations (p=0.0002).

342   Our MANOVA analysis explained 94% of the variation in avoidance

343   (p<0.0001, Table 1). Significant interaction effects were observed between

344   elevation groups and heat treatment (p<0.0001), indicating that low and high

345   elevation populations have evolved different responses to heat. We also saw a

346   significant effect of rosette angle (p=0.005). However, we did not see a significant

347   effect of transpiration rate. We observed highly significant nested effects for both

348   population and genotype (p<0.0001 for both).

349   To further explore the direct relationship between transpiration rate and

350   avoidance, separate regression analyses in the two heat treatments of DeltaT on

351   transpiration rate showed a significant positive relationship between transpiration

352   rate and DeltaT at 45 $^{\circ}$C (p=0.009, Fig.5), e.g., higher transpiration rate was

353   associated with higher DeltaT. We did not see a significant relationship between

354   transpiration rate and DeltaT in the control (Fig.5). Even though we did not detect

355   a significant effect of transpiration rate on DeltaT in the MANOVA analysis, this

356   separate analysis confirmed its direct effect on DeltaT at 45 ^{o}C. We consider

357   these apparently contradictory results for transpiration rate's effects in the

358   discussion.

359   **Tolerance was negatively associated with Hsp101 and SA accumulation**

360   With heat stress, Hsp101 accumulation was significantly increased 22 fold

361   and 8 fold for low and high elevations populations, respectively (Fig. 6a,

362   $p < 0.0001$, Table S1). However, because of the large variation within each

363   elevation group, we did not detect a significant difference between low vs. high

364   elevation populations in Hsp101 accumulation in the heat treatment (Fig.6a).

365   Both free and total SA were higher in low elevation populations than in

366   high elevation populations across the experiment as a whole (Fig. 6b, free

367   salicylic acid: $p = 0.0001$; total salicylic acid data not shown). However, with heat

368   treatment, high elevation populations significantly increased free and total SA,

369   while low elevation populations showed no significant difference compared to

370   control (Fig. 6b) in the 45 ^{o}C heat stress.

371   Our MANOVA explained 93% of the variation in tolerance ($p < 0.0001$,

372   Table 2). Significant effects of Hsp101 accumulation ($p = 0.005$), total SA

373   ($p < 0.0001$), free SA ($p = 0.005$) were observed. The interaction between elevation

374   group and heat treatment ($p = 0.01$) was also significant (Table 2), indicating

375   evolved differences in elevation groups in their response to high temperature.

376   Univariate regression analysis of photosynthetic rate on Hsp101

377   accumulation showed a significant negative relationship, e.g., higher Hsp101

378   accumulation was associated with lower photosynthetic rate (p=0.03, data not

379   shown). A negative relationship was also found between photosynthetic rate and

380   free SA accumulation (p=0.005). These two univariate analyses are concordant

381   with the results of the MANOVA.

Discussion

383   Here we have shown that a 45°C repeated heat stress imposed

384   periodically starting at the bolting stage is a significant heat stress for genetic

385   lines collected from natural populations of *Arabidopsis thaliana* in NE Spain,

386   since it caused significant decrease in fruit production compared to a benign

387   control temperature (Fig.1). We then showed that, although both avoidance and

388   tolerance were observable in all populations in response to heat stress, high

389   elevation populations manifested more avoidance (Fig.2) and low elevation

390   populations showed more tolerance (Fig.3). Our mechanistic analyses further

391   showed that avoidance was positively associated with high transpiration rate and

392   flat rosette angle (Fig.4, Fig.5, Table 1), while tolerance was negatively

393   associated with Hsp101 and SA accumulation (Fig.6, Table 2). The 8 populations

394   used in this study are part of 17 populations along a climate gradient described in

395   previous studies. In those prior studies we observed strong clines in many traits

396   (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-

397   Navarro et al. 2012, Wolfe and Tonsor 2014). We previously showed that

398   population genetic analyses strongly support the hypothesis that this cline results

399   from local adaptation along a climate gradient associated with altitude

400   (Montesinos et al. 2009, Montesinos-Navarro et al. 2011). Likewise the contrast

401   in strategy in low vs. high elevation populations observed in this study indicates

402   differential evolutionary responses to heat stress associated with adaptation to

403   Mediterranean low elevation vs. interior high elevation climates.

404   Despite the importance of heat stress, there is very little work that

405   examines genetically based adaptive differentiation among lineages in heat

406   avoidance and heat tolerance. However, much work has been done on other

407   abiotic stresses. Abiotic stresses, drought stress and salt stress in particular,

408   show a similar pattern of avoidance vs. tolerance in various plant species.

409   Farrant et al. (1999) reported a negative relationship between drought avoidance

410   and tolerance in three desiccation-tolerant angiosperm species (M.Farrant et al.

411   1999). Five herbaceous wetland plant species showed varying combinations of

412   avoidance and tolerance in response to short-term drought stress (Touchette et

413   al. 2007). Four of the species showed an avoidance strategy while all five

414   species also showed a tolerance strategy. Rahman et al. (2011) compared the

415   relative contribution of avoidance and tolerance to drought stress in two kiwifruit

416   species, finding *Actinidia deliciosa* had lower avoidance and higher tolerance

417   than *Actinidia chinensis* (Rahman et al. 2011). Similarly, Touchette et al. (2009)

418   showed a contrasting response to salt stress in marsh halophytes *Juncus*

419   *roemerianus* and *Spartina alterniflora*, in which *Juncus roemerianus,*

420   experiencing transient salt stress exposure, showed salt avoidance and *Spartina*

421   *alterniflora,* with frequent long-term salt exposure, showed salt tolerance

422   (Touchette et al. 2009). A diverse array of abiotic stresses share some common

423   pathways at both physiological and molecular levels (Pastori and Foyer 2002),

424   suggesting that we might expect a similar pattern as we learn more from heat

425   stress. Further exploration of the constraints on the evolution of the two

426   strategies at morphological, physiological, genomic and gene expression level

427   can provide insights in understanding the distribution pattern of plants and how

428   adaptive responses evolve over time.

429   The contrast in evolved relative importance of avoidance and tolerance

430   between our two climatic study regions indicates disruptive selection on heat

431   stress response between the high and low elevation regions of our source

432   populations. This disruptive selection, selecting for greater avoidance at high

433   elevation but greater tolerance at low elevation, is likely the result of different

434   costs for each strategy depending on the local physical environment.

435   In *Impatiens capensis* early season drought stress selects for avoidance

436   but later drought stress favors tolerance (Heschel & Riginos 2005). *Nicotiana*

437   *tabacum* shows a sequential response in drought stress, first avoidance then

438   tolerance, indicating avoidance is favored in short-term stress but tolerance is

439   favored in long-term stress (Riga and Vartanian 1999). This is in accordance with

440   our study in *Arabidopsis thaliana* as well. When the observed avoidance and

441   tolerance patterns were put in the context of climate, we saw interesting

442   associations of response to heat with climate of population origin. For example,

443   we know that annual precipitation in high elevation is 550mm greater than

444   precipitation in low elevation (Wolfe and Tonsor 2014) and continues longer into

445   the summer season (Montesinos-Navarro et al. 2009). This may allow greater

446   transpiration in high elevation populations, contributing to the greater ability to

447   avoid high temperature we observed among high elevation populations. Studies

448   on drought and salt stress also showed that plants that have greater access to

449   water adopt avoidance rather than tolerance (Touchette et al. 2007, Touchette et

450   al. 2009). Similarly, the average annual temperature is up to 11°C higher in our

451   low elevation sites compared to high elevation sites (Wolfe and Tonsor 2014);

452   thus populations from low elevation are constantly exposed to higher

453   temperatures compared to high elevation populations. This, combined with the

454   lower availability of water for transpirative cooling, may explain why low elevation

455   populations are more tolerant and less resistant than high elevation populations.

456   We found an increase in rosette temperature in the 22°C control but a

457   decrease in the 45°C heat treatment compared to the ambient temperature for all

458   populations. The difference between rosette temperature and air temperature is

459   positive in cool but negative in hot air. The air temperature at which one observes

460   zero air-leaf temperature differential has been called the "equality temperature"

461   (Linacre 1964). The equality temperature is often around 30°C in well-watered,

462   thin-leaved plants (Linacre 1967) but is species-specific (Savvides et al. 2013).

463   For example, cotton has an equality temperature of 27°C (Upchurch and Mahan

464   1988). Based on our data and assuming a linear response, we can draw

465   approximate equality temperatures at 25.4^{o}C and 27°C for low elevation and high

466   elevation populations, respectively. This 1.6^{o}C difference in equality temperature

467   reflects an intraspecific differentiation in homeostatic control in natural

468   *Arabidopsis* populations. In a range of 22 – 45°C, high elevation populations

469   were more homeostatic than low elevation populations (Fig.2). Our study also

470   supported Mahan and Upchurch's (1988) proposal that plants are capable of at

471   least limited homeothermy.

472   Transpirational cooling is one of the most important transient avoidance

473   mechanisms in plants (Burke and Upchurch 1989). The importance of

474   transpiration and homeostatic control in meristem temperature has been shown

475   for cucumber and tomato plants (Savvides et al. 2013), as well as cotton (Burke

476   and Upchurch 1989). Our study also revealed a direct positive relationship

477   between the transpiration rate and avoidance (Fig.5), even though we could not

478   detect a significant effect in the combined MANOVA analysis (Table 1).

479   Measures of transpiration rate are noisy, especially at high temperature.

480   *Arabidopsis* populations exhibit a high level of genetic homogeneity. Most of the

481   measured trait variance in studies of natural populations of *Arabidopsis* is

482   between populations and regions (e.g. Montesinos et al. 2009). The certainty of

483   assignment to population and elevation, the high trait variance among

484   populations and regions, and the high error variance in transpiration measures

485   mean that most of the causal variance is absorbed at the population and region

486   level in the MANOVA, leaving little variance directly attributed to transpiration

487   rate.

488   It is also important to emphasize that our transpiration rate measures were

489   conducted on a single leaf. Transpirational cooling of the rosette involves

490   interactions of the complex stacked leaf structure of the rosette and its interaction

491   with the micro-climate in which plants reside. The rosette temperature depends in

492   a complex way on the aggregate functioning of all the individual leaves in the

493   rosette. Whole rosette transpiration is influenced not only by the leaf properties

494   under uniform conditions, but also on the much more complex influence of

495   rosette structure. The structure leads to variation in the rates of energy loading

496   to individual leaves. It also determines convective and conductive transfer of heat

497   to the surrounding air. These factors further influence the steepness of the water

498   vapor diffusion gradient, and temperature gradient around individual leaves. A full

499   understanding of control over air and tissue temperature within rosettes will

500   require study of the rosette as a functional unit.

501   Leaf angle did not show the significant hyponastic response we expected

502   in this study. This is likely because we measured this trait too soon after heat

503   stress. A constitutively steep leaf angle is a long-term adaptive trait to deal with

504   water deficit, high radiation load, or high temperature (Fu 1989, Falster and

505   Westoby 2003, Valiente-Banuet et al. 2010). Plants from low latitude showed

506   much steeper leaf angle compared with high latitude plants in 21 European

507   *Arabidopsis thaliana* ecotypes, and all plants displayed steeper leaf angle in

508   response to extended vernalization period (10, 20, or 30 days) (Hopkins et al.

509   2008). Physiologically-driven changes in leaf angle can be elicited by a variety of

510   environmental conditions, including heat. Leaf angle movement generally

511   requires observation over a number of hours (Ehleringer 1987). However, in our

512   study, we measured leaf angle 15 min after heat treatment started. We

513   hypothesize the leaf angle might change if given repeated and prolonged heat

514   stress period and measured later in the treatment.

515   Hsp101 and SA accumulation both had very high variation (Fig.6) in this

516   study. In prior published studies plants were assayed for Hsp101 and SA

517   accumulation in seedling stage when plants have not yet developed any

518   functional avoidance mechanisms. Seedlings therefore experience a heat stress

519   temperature that is equal to the ambient heat treatment temperature. Thus in

520   these prior studies, Hsp101 and SA accumulation is less variable and is

521   distinguishable among populations (Tonsor et al. 2008, Zhang et al. 2014, Zhang

522   et al. 2015a). However, adult plants can adopt both avoidance and tolerance in

523   heat stress response. As shown in Fig.2, the actual rosette temperature for a

524   45°C heat stress is 36.4^{o}C for high elevation populations vs. 38.2^{o}C for low

525   elevation populations, on average. Hsp101 accumulation increases rapidly in the

526   range of 34-40°C among *Arabidopsis* plants collected from natural populations

527   (Tonsor et al. 2008). The observed variation in this study in the actual rosette

528   temperature might explain why we did not detect a significant difference in

529   Hsp101 accumulation between low vs. high elevation populations at the 45°C

530   heat stress (Fig.6a); based on our past studies the various genetic lines used

531   here are very likely to be variable in expression in uniform temperature, and differ

532   in their DeltaT. As a result they are highly variable in their responses in an

533   experiment like the one reported here. To the best of our knowledge this is the

534   first time that Hsp101 has been quantified in adult plants that experienced

535   variable plant tissue temperature despite uniform ambient temperature. In

536   addition our Hsp101 measurement method and Hsp101 expression itself are

537   both highly variable at the level of both biological and technical replicates (data

538   not shown). In this study, we estimated the necessary sample sizes based on

539   prior seedling experiments, under conditions in which variation in heat avoidance

540   was not possible. However, in retrospect based on the results of this study, an

541   estimated sample size of 96 (48 samples for each elevation group) would be

542   needed to distinguish the difference in low and high elevation populations in their

543   response to the 45°C heat.

544   In a previous experiment, we detected a cline in SA in genetic lines

545   collected along our study system's elevation gradient, when measured in a 22°C

546   environment (Zhang et al. 2015b). In the present study, even with the large

547   variation in rosette temperature observed, we still detected a significant

548   difference in free SA accumulation comparing low and high elevation populations

549   at both control and 45°C heat stress temperatures (Fig.6b). The total SA

550   accumulation for the low elevation populations was indistinguishable in the

551   control vs. the 45°C heat treatment, while the total SA value was significantly

552   increased by about 180% in the 45°C heat treatment for the high elevation

553   populations, compared with their control (data not shown

554   Hsp101 and SA expression are both rapidly up-regulated with heat stress.

555   We observed a negative association between both Hsp101 and SA and

556   photosynthetic rate. This suggests that the accumulation of Hsp101 and SA are

557   upregulated when cellular or subcellular damage is sensed by the plant, i.e. the

558   same conditions in which photosynthesis declines.

559   The high avoidance ability under high temperature in adult plants, as we

560   saw in Fig.2, indicates the necessity of connecting lab studies with more accurate

561   reflections of field conditions. Previous heat stress response studies focus on

562   seedlings under controlled lab conditions, yet it is at the reproductive stage that

563   Arabidopsis and other spring annuals and biennials most often encounter high

564   temperatures.

565   Because our study and others (Helliker and Richter 2008, Broitman et al.

566   2009, Helmuth et al. 2010) demonstrate that adult plants can maintain leaf and

567   rosette temperature that differs substantially from ambient temperature due to the

568   avoidance mechanisms, studies of heat tolerance and stress responses will be

569   most fruitful if done in reference to plant tissue temperature instead of ambient

570   temperature. However, even with these refined and comprehensive measures,

571   mysteries still exist regarding how whole plants respond to heat stress in nature.

572   Transcriptome sequencing data from these low vs. high elevation populations

573   under heat stress could provide more detailed information about the gene

574   networks for universal and regional-specific stress response.

575

579   **Contributions by authors**

580   NZ and SJT designed the experiment. NZ, PC, JN and JH performed the

581   experimental work. NZ and SJT performed analyses. All co-authors discussed

582   and interpreted results prior to writing. Writing was done by NZ and SJT.

583   **Acknowledgement**

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791

Figure Legends

793   **Figure 1.** Heat stress disruption as measured by comparing trait values between

794   45°C heat stress and control for plants originating both low elevation and high

795   elevations. Black = control; Red = 45°C heat stress. Figure shows the means of

796   each elevation group under each treatment, and the bars are standard errors.

797   See supplementary Table 1 for results from statistical analyses.

798

799   **Figure 2.** Rosette temperature, a measure of heat avoidance, comparing plants

800   from low vs. high elevation populations, under 45°C heat stress and control.

801   Black = control; Red = 45°C heat stress. Figure shows the means of each

802   elevation group under each treatment, and the bars are standard errors. Dash

803   lines show the ambient temperature for control (22°C) and heat treatment (45°C).

804   Notice the standard error for the low vs. high elevation populations under the

805   control is very small, so it is invisible in the figure.
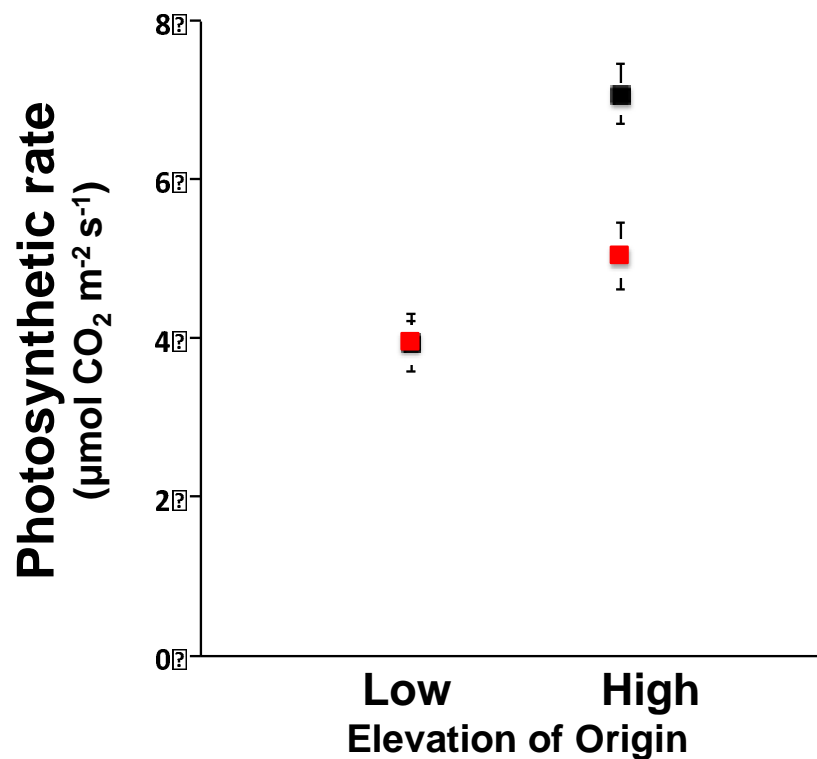
806

807   **Figure 3.** Photosynthetic rate, a measure of heat tolerance, for the low vs. high

808   elevation populations under the 45°C heat stress and control. Black = control;

809   Red = 45°C heat stress. Figure shows the means of each elevation group under

810   each treatment, and the bars are standard errors. Note the photosynthetic rates

811   for low elevation control and 45°C heat stress treatments overlap each other.

812

813   **Figure 4.** Two potential avoidance mechanisms compared for the low vs. high

814   elevation populations under 45°C heat stress and control. Black = control; Red =

815   45°C heat stress. Figure shows the means of each elevation group under each

816   treatment, and the bars are standard errors.

817

818   **Figure 5.** The relationship between transpiration rate and DeltaT (the difference

819   between air and rosette temperature), comparing 45°C heat stress and control.

820   Data points displayed are genotype means at both control and heat treatment

821   groups. Statistical analysis was done with individual plant trait values (total

822   samples = 84) using Proc REG in SAS. The regression line for the 45°C heat

823   stress is: DeltaT = **0.23***Transpiration rate **+ 4.11**. The slope is statistically

824   significant (p=0.009).

825

826   **Figure 6.** Two tolerance mechanisms, Hsp101 ratio and free salicylic acid, and

827   their relative value in low vs. high elevation populations under 45°C heat stress

828   and control. Black = control; Red = 45°C heat stress. Figure shows the means of

829   each elevation group under each treatment, and the bars are standard error.

830   Notice in b) the free salicylic acid concentration for the control overlapped with its

831   concentration at the 45°C heat stress. The Hsp101 ratio is its concentration

832   relative to our biological standard, in which we heat treated a combination of leaf

833   samples at 45°C and used it as a quality control on gel-to-gel variation in western

834   blot, see details on materials and methods in Tonsor et al. 2008. The unit for b)

835   free salicylic acid in the figure is ug/g per leaf dry mass.

836

837   **Supplementary Materials:**

838   **Figure S1.** Geographic location of the 8 populations (adapted from Fig.1 in

839   Wolfe and Tonsor 2014, New Phytologist). Map showed all 16 Arabidopsis

840   thaliana populations in northeastern Spain. The eight populations used in this

841   study were highlighted with black dots.

842

843   **Figure S2.** Using single leaf cuvette to quantify photosynthetic rate and

844   transpiration rate. Plants shown were at the pre-bolting stage. Here we only use

845   this photo to show the equipment used for quantification. We do not have a figure

846   to show the measurement at the bolting stage.

847

848   **Figure S3.** Heat stress caused more inflorescences to be induced. Red arrows

849   showed the regions where the stems were damaged, and green arrows showed

850   new branches initiated after heat stress. Figure shows both the whole

851   reproductive part and the detailed heat stress disruption of shoot apices.

852

853   **Figure S4.** Heat stress reduced fruit quality. Damage occurred in the middle of

854   the main stem at a time when that stem segment was near the stem apex. After

855   heat stress, the main stem growth recovered, but the fruits did not succeed to

856   mature, possibly because of pollen inviability. Dissection on fruits of this kind

857   showed that they did not produce viable seeds. Figure shows both the damage

858   on the whole reproductive part and a enlarged view of section of the stem

859   containing heat-damaged fruit. In the enlargment, the short red lines mark

860   damaged fruits and the short green lines mark normally developed fruit. The

861   damaged fruits are much smaller compared to a normal fruit. The smaller dashed

862   rectangles highlight additional stems with similar damage.

863

864   **Table S1.** ANOVA table for each variable measured in this study.

865   **Table 1.** ANOVA table for avoidance using Rosette temperature as a dependent

866   variable (here DeltaT was used, which is the difference between ambient

867   temperature and rosette temperature for normality), and rosette angle,

868   transpiration rate, as well as heat treatment and climate of origin and their

869   interaction as potential causal factors. This model explains 94% variation we saw
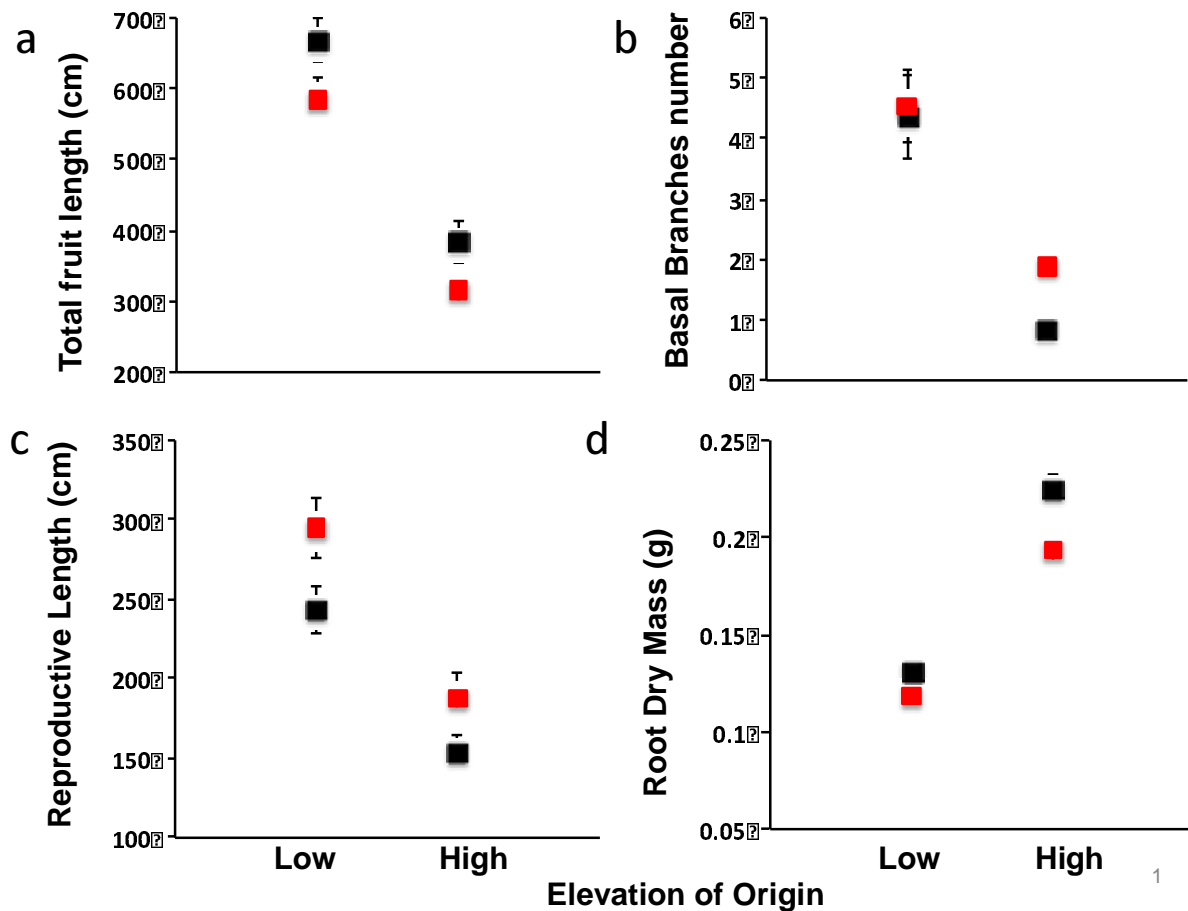
870   in avoidance.

871

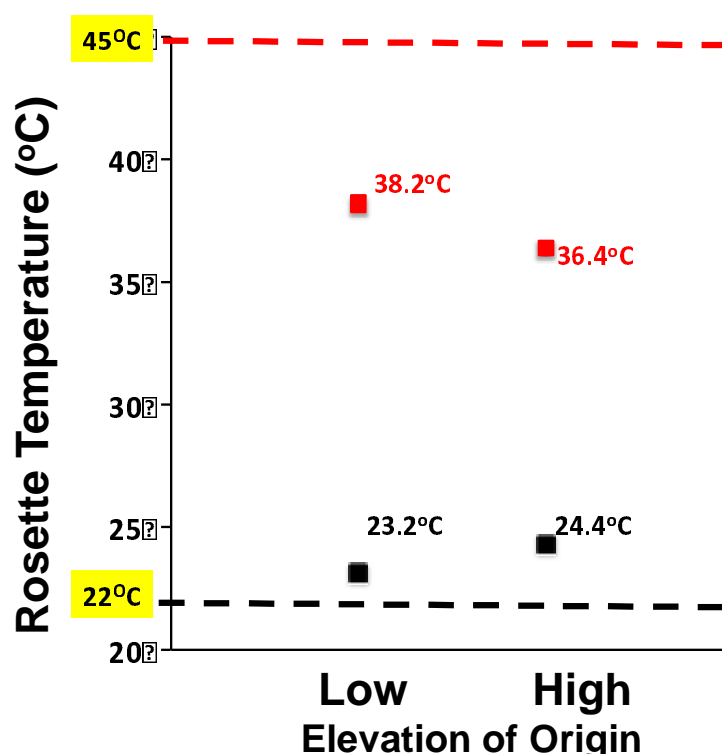| Source	DF	Type III SS	Mean Square	F Value	Pr>F
Full Model (R^2=0.94)	32	3713.83*	116.06	108.77	<.0001
Heat treatment	1	1984.17	1984.17	1859.58	<.0001
Rosette Angle	1	8.52	8.52	7.98	0.005
Transpiration Rate	1	0.12	0.12	0.11	0.74
Elevation group	1	0.31	0.31	0.29	0.59
Population (Elevation group)	6	236.60	39.43	36.96	<0.0001
Genotype(Population * Elevation group)	21	242.67	11.56	10.83	<0.0001
Elevation group * Heat treatment	1	48.69	48.69	45.63	<0.0001
Error	240	256.08*	1.07		
Corrected Total	272	1075.98*			

872
873
874   * Sum of Squares.

875 **Table 2.** ANOVA table for tolerance using photosynthesis rate as a dependent,

876 and Hsp101, free and total salicylic acid as well as heat treatment and climate of

877 origin and their interaction as potential causal factors. This model explains 93%

878 variation we saw in tolerance.

879

Source	DF	Type III SS	Mean Square	F Value	Pr>F
Full Model (R^2=0.93)	32	1384.38*	43.26	47.36	<0.0001
Heat treatment	1	1.95	1.95	2.13	0.14
Elevation group	1	64.53	64.53	70.63	<0.0001
Population (Elevation group)	6	243.19	40.53	44.37	<0.0001
Genotype(Population * Elevation group)	20	617.81	30.89	33.81	<0.0001
Hsp101 accumulation (log)	1	7.41	7.41	8.11	0.005
Total salicylic acid accumulation (log)	1	73.11	73.11	80.02	<0.0001
Free salicylic acid accumulation (log)	1	7.51	7.51	8.22	0.005
Elevation group * Heat treatment	1	5.90	5.90	6.46	0.01
Error	112	102.32*	0.91		
Corrected Total	144	1486.70*			

880
881
882 * Sum of Squares.
883

884
885 **Figure 1.** Heat stress disruption as measured by comparing trait values between

886 45°C heat stress and control for plants originating both low elevation and high

887 elevations. Black = control; Red = 45°C heat stress. Figure shows the means of

888 each elevation group under each treatment, and the bars are standard errors.

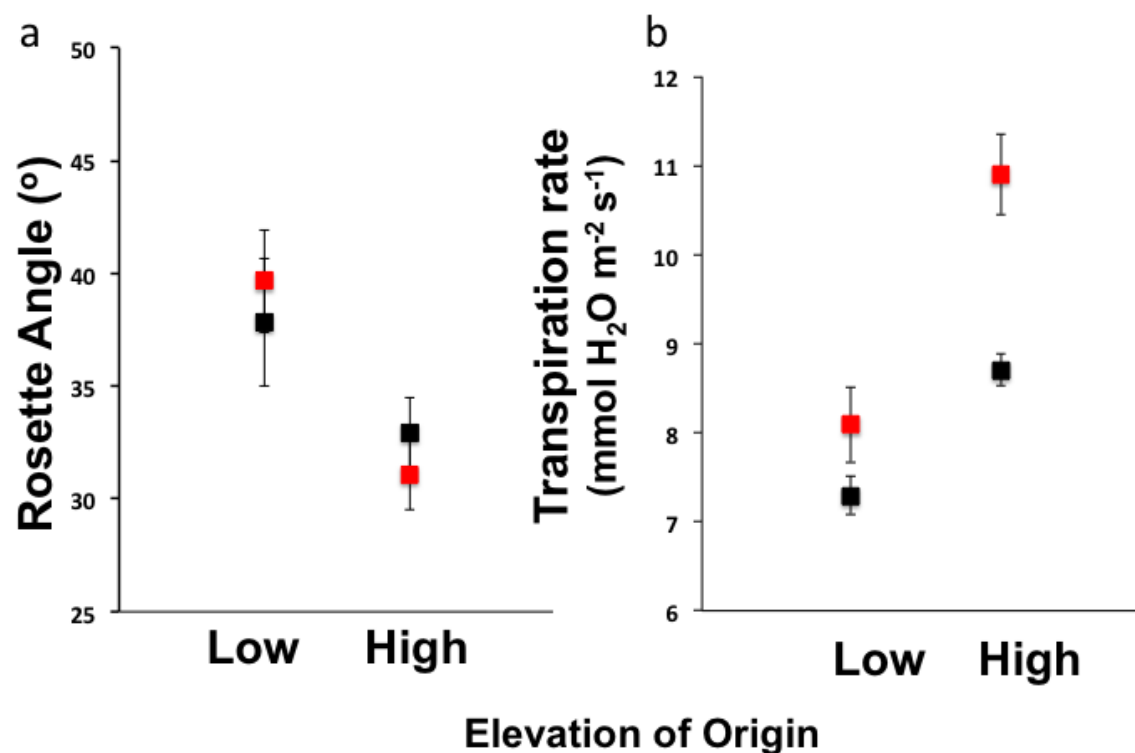889 See supplementary Table 1 for results from statistical analyses.

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891

Figure 2. Rosette temperature, a measure of heat avoidance, comparing plants from low vs. high elevation populations, under 45°C heat stress and control. Black = control; Red = 45°C heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors. Dash lines show the ambient temperature for control (22°C) and heat treatment (45°C). Notice the standard error for the low vs. high elevation populations under the control is very small, so it is invisible in the figure.
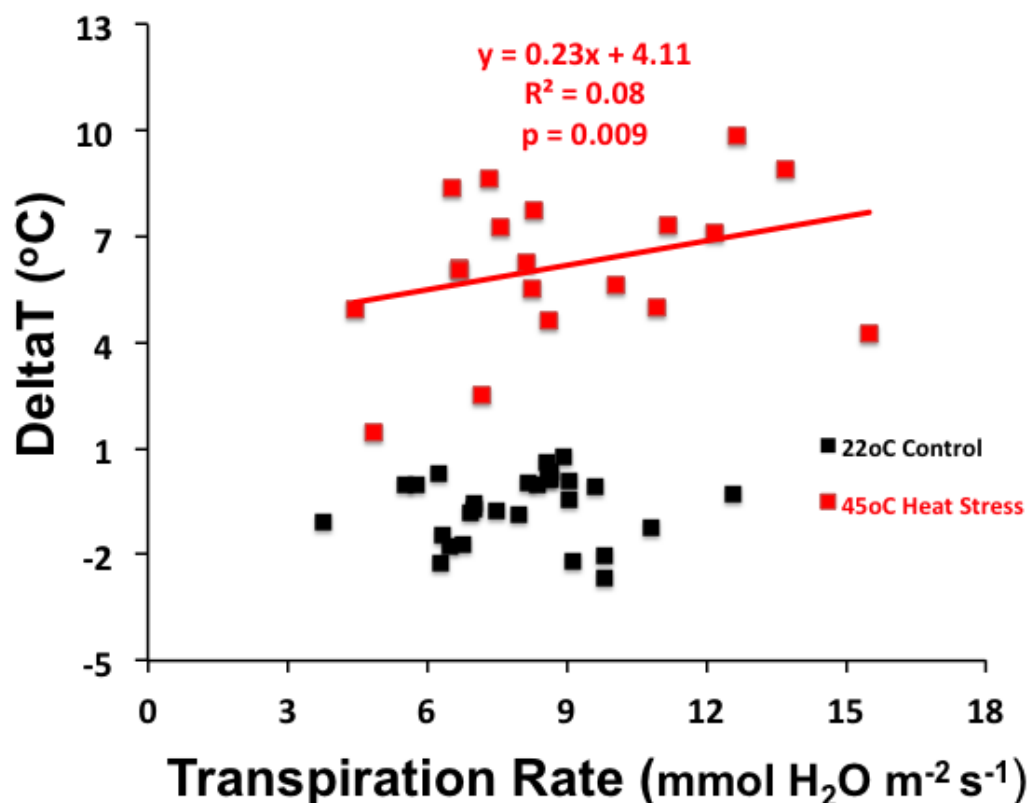
899

900

Figure 3. Photosynthetic rate, a measure of heat tolerance, for the low vs. high
elevation populations under the 45°C heat stress and control. Black = control;
Red = 45°C heat stress. Figure shows the means of each elevation group under
each treatment, and the bars are standard errors. Note the photosynthetic rates
for low elevation control and 45°C heat stress treatments overlap each other.
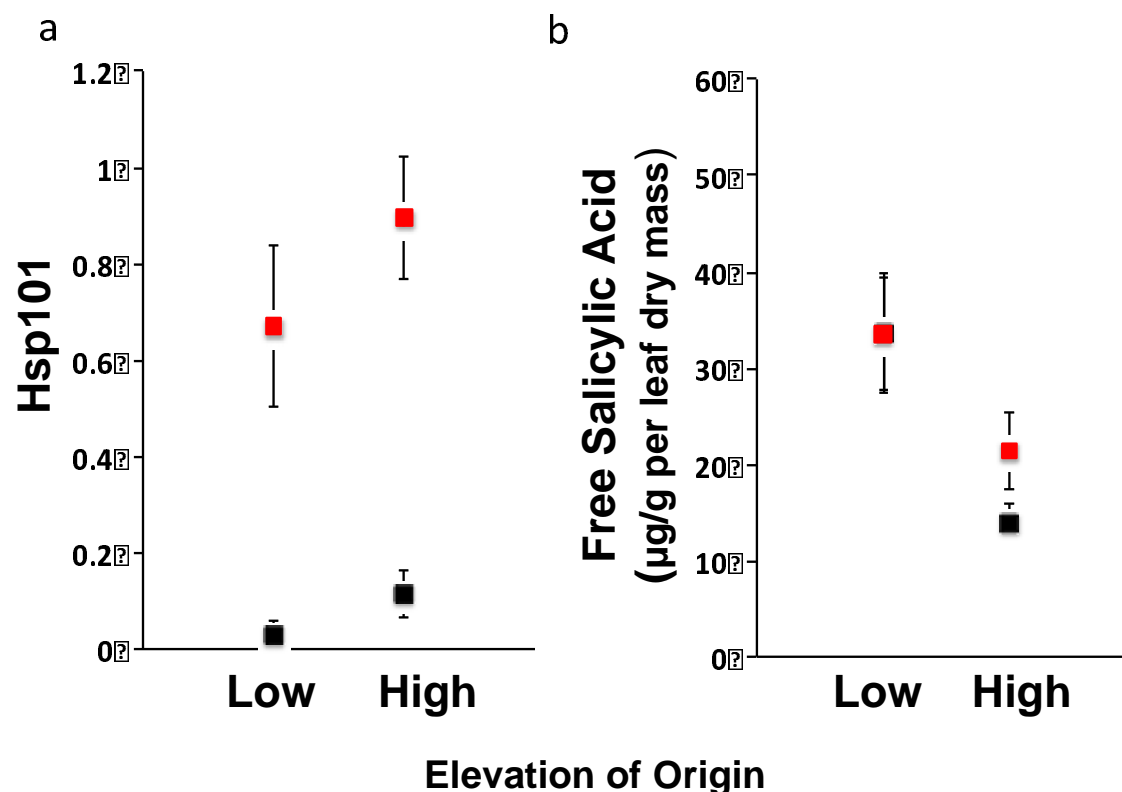
906

Figure 4. Two potential avoidance mechanisms compared for the low vs. high elevation populations under 45°C heat stress and control. Black = control; Red = 45°C heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors.

914

Figure 5. The relationship between transpiration rate and DeltaT (the difference between air and rosette temperature), comparing 45°C heat stress and control. Data points displayed are genotype means at both control and heat treatment groups. Statistical analysis was done with individual plant trait values (total samples = 84) using Proc REG in SAS. The regression line for the 45°C heat stress is: DeltaT = **0.23***Transpiration rate **+ 4.11**. The slope is statistically significant ($p=0.009$).

922

923

Figure 6. Two tolerance mechanisms, Hsp101 ratio and free salicylic acid, and their relative value in low vs. high elevation populations under 45°C heat stress and control. Black = control; Red = 45°C heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard error. Notice in b) the free salicylic acid concentration for the control overlapped with its concentration at the 45°C heat stress. The Hsp101 ratio is its concentration relative to our biological standard, in which we heat treated a combination of leaf samples at 45°C and used it as a quality control on gel-to-gel variation in western blot, see details on materials and methods in Tonsor et al. 2008. The unit for b) free salicylic acid in the figure is ug/g per leaf dry mass.