Climate gradients explain population-level divergence in drought-induced plasticity of functional traits and gene expression in a South African *Protea*

Melis Akman[1,2], Jane E. Carlson[3,4], Andrew M. Latimer[1]

[1] Department of Plant Sciences, UC Davis

[2] Department of Plant and Microbial Biology, UC Berkeley

[3] Department of Biology, Nicholls State

[4] Gulf Coast Network Inventory and Monitoring Program, National Park Services

Abstract

Long term environmental variation often drives local adaptation and leads to trait differentiation across populations. Additionally, when traits can change in an environment-dependent way through phenotypic plasticity, the underlying genetic variation will also be under selection, but only in the inducing environment. Both of these processes will create a landscape of differentiation across populations in trait means as well as their plasticity. However, studies uncovering environmental drivers of this variation are scarce. With this work, we studied drought responses in seedlings of a shrub species from the Cape Floristic Region, the common sugarbush (*Protea repens*). We measured morphological and physiological traits as well as whole transcriptomes in 8 populations that represent both the climatic and the geographic distribution of this species. We found that there is substantial variation in how populations respond to drought, but we also observe common patterns such as reduced leaf size and thickness and upregulation of stress- and down-regulation of growth-related gene groups. Both environmental heterogeneity and milder source site climates were associated with higher plasticity in various traits and co-expression gene networks. By uncovering associations between traits, trait plasticity, co-expression gene networks with source site climate, we showed that temperature plays a bigger role in shaping these patterns when compared to precipitation, in line with recent changes in the region due to climate change. We also found that traits respond to climatic variation in a context dependent manner: some associations between traits and climate were apparent only under certain growing conditions.

Keywords: phenotypic plasticity, climatic variation, trait variation, transcriptome, *Protea repens*, drought, climate change

INTRODUCTION

Populations can persist in changing environmental conditions through three mechanisms: by tracking their climatic niche in space through migration or dispersal, by shifting their niche through adaptation, and/or by matching traits to the new environment through functional phenotypic plasticity (Chevin *et al.* 2010). For plants, plasticity can be the fastest, since it involves rapid adjustments of morphology, physiology and gene expression within a single generation. Like mean trait values, the form and magnitude of trait plasticity can be under selection and thus plasticity can also vary among populations along environmental gradients (Geber & Griffen 2003). Plasticity in key growth-related plant traits, including stomatal density and conductance, growth rate, and leaf morphology has been observed to vary among populations in long-lived woody plants (e.g. Aubin *et al.* 2016) as well as herbaceous annuals (Jacobs & Latimer 2012). Recent studies have demonstrated that population differences in plasticity might have climatic drivers (Pratt & Mooney 2013; Lázaro-Nogal *et al.* 2015). However, examples of underlying environmental processes that maintain population differentiation in plasticity are still scarce.

Functional plasticity can provide at least short-term benefits to individual plants upon changing environmental conditions (Wennersten & Forsman 2012). The fitness consequences of plasticity will often be context dependent, however, as is demonstrated for many functional traits (e.g. Etterson 2004). For example, plants generally adjust many features of leaf morphology to optimize photosynthesis at different light levels, and tune stomatal behavior to adjust water use efficiency depending on soil moisture levels. While levels and patterns of plasticity can have strong, context-dependent fitness consequences, it remains less clear how often differential plasticity confers environment-specific fitness benefits (Forsman 2015). In a few cases, differential plasticity has been directly shown to be adaptive: for example, Dudley & Schmitt (1996) showed that plastic stem elongation was beneficial at high but not low plant densities. Other studies have characterized patterns of plasticity that suggest local adaptation: for example, Sultan (2003) showed that plasticity in allocation to leaf light capture was more pronounced in a generalist species than a high-light specialist. Nicotra *et al.* (2015) showed that plasticity in seedlings of an alpine plant was higher and also favored in low elevation but not in high elevation populations. Resurrection studies using historical and modern *Daphnia magna* populations showed evolution of plasticity for heat tolerance and fish predation (Stoks *et al.* 2016; Cuenca

Cambronero *et al.* 2018). In a field study using *Boechera stricta*, Wadgymar *et al.* (2018) showed differential germination plasticity along elevational gradients. Apart from these studies, our understanding of the role of adaptive evolution in differential plasticity remains largely theoretical (West-Eberhard 2003; Ghalambor *et al.* 2015).

In light of these studies and theoretical predictions (Bradshaw & Hardwick 1989; Grime 1994; Schlichting & Pigliucci 1995; Alpert & Simms 2002), we expect that local adaptation should promote differential plasticity under two main circumstances. First, plants from environments with strong and relatively predictable forms of spatial or temporal heterogeneity should be more plastic than those from homogeneous environments (Ackerly *et al.* 2000; Van Kleunen & Fischer 2005; Baythavong & Stanton 2010; Scheiner 2013; but see Palacio-López *et al.* 2015). Second, plants from harsh climates are expected to be less plastic than those from milder climates (Vitasse *et al.* 2013; Gugger *et al.* 2015), because the fitness costs of environmental mismatch should be greater in resource-poor conditions (Alpert & Simms 2002). Elevation contrasts provide one example of this latter pattern, as plants from colder, higher-elevation environments tend to have both lower and less plastic growth rates than plants from warmer sites (Nicotra *et al.* 2015).

The form and magnitude of functional plasticity which organisms express can be expected to influence the vulnerability of populations to environmental change. Thus, understanding the factors that drive differentiation among populations in functional plasticity, and the consequences of these plasticity differences for population vulnerability, therefore, is an important challenge for global change research. The importance of plasticity for population persistence is well-grounded in theory (Nicotra *et al.* 2010; Chevin *et al.* 2010) and borne out in some experimental work (e.g. Anderson & Gezon 2015; Lázaro-Nogal *et al.* 2015; Sgrò *et al.* 2016). Studies that have incorporated plasticity variation in predictive models of species distribution have yielded a range of results; in some cases, plasticity is projected to cause slow loss at the warmer range edge (Valladares *et al.* 2014) or open new habitats for colonization (Muñoz *et al.* 2014), while in others plasticity is projected to accelerate range loss (Duputié *et al.* 2015). The gaps in empirical knowledge about both the functional basis and environmental landscape of plasticity variation creates a challenge for these models to accurately predict how and under what scenarios plasticity will buffer populations in the face of climate change.

Our study species originates from the Cape Floristic Region (CFR), a biodiversity hotspot with a climatically stable past (Cowling 2009), that is predicted to be strongly affected by climate change. Climate models project warming up to 3 °C by 2070 (Wilson *et al.* 2015), and precipitation projections being more variable, most models project drying in the super-diverse western mountains (Hewitson & Crane 2006; Wilson *et al.* 2015). Our study species, *Protea repens* (common sugar bush), is part of a lineage that has radiated over the past 5 to 18 million years to produce 112 species, ~85% of which are endemic (Rebelo 2001; Sauquet *et al.* 2009). Along the environmental gradients *Protea species* inhabit, we have evidence for local adaptation and context-dependent benefits in several traits (Latimer *et al.* 2009; Carlson *et al.* 2011, 2016; Prunier *et al.* 2012; Akman *et al.* 2016; Mitchell *et al.* 2018). There is also evidence for differential developmental plasticity and drought plasticity in leaf traits of several other *Protea* species (*Protea sect. Exsertae*: Carlson & Holsinger 2012; Heschel *et al.* 2014), however, the extent to which this reflects local adaptation remains unknown. Upon rapidly shifting environmental conditions, plasticity will play an important role for survival in this species considering its long generation time. Thus, uncovering patterns of plasticity can inform vulnerability predictions for this species under climate change.

A particularly important form of environmental variation in the CFR is drought. Some Mediterranean-climate regions including the CFR, have recently experienced unprecedented drought. Such droughts are projected to be more likely in the near future. Recent droughts have caused large mortality events in trees (Allen *et al.* 2010; Young *et al.* 2017). Understanding the patterns and effects of functional plasticity to drought is thus a vital issue for plant vulnerability in many semi-arid regions. Measurements of plasticity in gene expression, in which tens of thousands of "phenotypes" can be measured simultaneously, are particularly valuable for investigating the causes and consequences of plasticity. Gene expression patterns can change within seconds of a stimulus, long before larger biochemical shifts and morphological changes become apparent. Gene expression patterns can also be linked to morphological and physiological adjustments, providing insights into the genetic basis of adaptation and/or plasticity (Whitehead 2012; Akman *et al.* 2016). Previous studies have revealed patterns of adaptive plasticity in gene expression in fish (Morris *et al.* 2014; Schneider *et al.* 2014; Dayan *et al.* 2015), plants (Lasky *et al.* 2014; Colicchio *et al.* 2015) and corals (Granados-Cifuentes *et al.* 2013; Barshis *et al.* 2013). These studies demonstrate that gene expression profiling can usefully complement standard functional trait measurements to help understand the genetic basis and physiological

135 significance of plasticity and how it varies across populations. With this study, we aim to
136 answer two questions: 1) How do populations of *P. repens* respond to drought and how
137 do these responses vary across populations? and 2) Do the climatic conditions of the
138 source sites covary with traits, gene expression and their plasticity? To do so, we first
139 uncover the consequences of a severe, experimentally imposed drought by measuring
140 traits and gene expression patterns in seedlings in a dry-down experiment. Then we test
141 for population differentiation in trait means and drought-response plasticity in functional
142 traits, performance, and gene expression patterns, and evaluate associations between
143 patterns of population differentiation and their potential climatic drivers.

144 MATERIALS AND METHODS

145 Species description

146 *Protea repens* L. is a common evergreen shrub endemic to South Africa, with
147 particularly high abundance in semi-arid fynbos of the CFR (Rebelo 2001). This
148 species has narrow, sclerophyllous leaves and reaches 2-4 m in height. Growth is
149 sympodial, with new shoots and leaves generally emerging in the months before
150 flowering. Plants flower between Feb-Aug for 1-3 months, with eastern populations
151 blooming later than western populations. Inflorescences are dense heads of 70-150
152 protandrous florets, subtended by pink or white bracts. Each floret can produce a
153 single-seeded fruit, although seed set per head averages under 30%. All viable and
154 nonviable fruits remain within a woody, serotinous infructescence until the adult plant is
155 killed by fire, which occurs at ~10-13 year intervals in the fynbos (with variation among
156 geographic regions; (Van Wilgen *et al.* 2010). Fruits disperse by tumbling, and seeds
157 germinate during the next rainy season (May-Aug for much of the CFR). Due to the
158 Mediterranean rainfall pattern in the western CFR, some western population sites
159 receive far more wintertime precipitation than do eastern population sites.

160 Field sampling and climate data

161 We collected seeds from 8 wild populations of *P. repens* spanning much of its
162 geographic distribution in the CFR (**Figure 1A**). Between Feb. and May 2011, we visited
163 these 8 sites as part of a larger sampling effort in *P. repens* (see Carlson *et al.*
164 2016). At each site, we collected infructescences from 20-40 plants along a transect
165 through each population's center, and we air-dried infructescences until they opened
166 and single-seeded fruits could be removed. We stored seeds in envelopes at room

167 temperature until the greenhouse study. At each seed collection site, we also recorded
168 GPS coordinates near the population center, and we used these points to extract
169 elevation and six climate metrics for rainfall and temperature from GIS layers. A
170 principal component analysis performed on these data confirmed that the sample sites
171 cover most of the range of variation in major environmental factors (**Figure 1B**). The 6
172 climate data layers were from the South African Atlas of Agrohydrology and Climatology
173 (Schulze 1997 with higher resolution for 4 and 5 below) (Schulze 2007 for the rest) and
174 were based on 30 year climate averages, extremes, or variances. The simple or
175 composite climate layers used were (1) mean annual rainfall, (2) average daily
176 maximum temperature for January (summer), (3) average daily minimum temperature
177 for July (winter), (4) rainfall seasonality, measured as the percent of annual precipitation
178 to fall within a single month (5) inter-annual rainfall variability, measured as the
179 coefficient of variation in mean annual rainfall across years and (6) extreme temperature
180 range within a year, measured as the difference between January maximum and July
181 minimum temperatures (2 minus 3), which is strongly correlated with the difference
182 between the maximum minus the minimum monthly temperature using 50 year
183 extremes (R=0.95). Our seventh variable was altitude, which was extracted separately
184 from ASTER GDEM (Daac 2011). Most of these layers have been used in our earlier
185 work on *P. repens*, except for (5) and (6), which were added to join rainfall seasonality
186 (4) as measures of environmental heterogeneity. Locations of each population along
187 these axes can be estimated from **Figure 1B** or viewed in **Table S1**. Only two pairs of
188 climate variables were strongly correlated with each other: July minimum temperature
189 with altitude (Pearson's R= -0.91) and January maximum temperature with annual
190 temperature range (annual max. minus min. temp., R=0.70; see **Table S2** for all
191 correlation coefficients). Rainfall seasonality in our dataset is positively associated with
192 wintertime rainfall (total for Dec-Feb, Pearson's R=0.80) and negatively associated with
193 summertime rainfall (total for June-August, Pearson's R=-0.90).

194 **Experimental setup and dry-down treatments**

195 In late July 2014, we germinated 938 seeds from 114 maternal lines (per population
196 means: 117 seeds from 14 lines) for a dry-down experiment. Seeds were sown on low
197 nutrient mix (50% bark, 50% sand) in trays of 100 individual plugs 2x2 cm in size. Low-
198 nutrient mix is standard for growing *Protea* species because they naturally occur on low-
199 nutrient soils. Trays were placed in a long-day growth chamber set for 8 hours of 8℃
200 (nighttime) and 16 hours of 20℃ (daytime). After 1.5 months, 419 seeds had

germinated, and these were subsequently transplanted into small, deep pots (5 cm diameter x 17 cm deep) in trays of 50 each, in the same low nutrient mix. Transplants were given an additional two weeks indoors under growth lights before being transferred to a single greenhouse bench at Nicholls State University Farm, Thibodaux, LA.

Two weeks post-greenhouse transfer and two weeks prior to the dry-down treatment, 316 seedlings from 8 populations were reorganized into thirteen trays of 25 in a completely randomized design. The drought experiment began in late October 2014, when greenhouse conditions were warm but relatively dry, and thus most similar to summer conditions in their native range. The 2.5 months-old seedlings were assigned to one of two treatments: regular watering (~every 3 days) or 12 days of water deprivation. Efforts were made to include a replicate from each maternal line within each treatment, which we succeed at for 80% of lines. Total sample size ranges from 19 to 20 plants per population per treatment.

Trait measurements and transcriptome sampling

At 6 days and 12 days into the drought experiment, plants were measured for functional traits and leaves were sampled for transcriptomics. Our sampling design is depicted in **Figure S1**. Functional traits that were measured at both time-points included plant height (cm), stomatal conductance, leaf area, leaf length:width ratio, specific leaf area (fresh leaf area/dry leaf mass), stomatal density (number per mm^2), stomatal pore length (mm) and stem pigmentation. Stem pigmentation, measured as stem hue, was derived using Endler's segment classification (Maia *et al.* 2013), based on the average of two reflectance spectra readings (400-700 nm) taken at mid-stem (JAZ, Ocean Optics). On the segment classification scale for hue, higher values represent redder stems that contain higher levels of anthocyanin. Stomatal conductance was measured using a steady-state leaf porometer (SC-1, Decagon) on one leaf per plant, with an additional adjacent leaf measured simultaneously if the chamber was not filled by a single leaf due to small leaf size. All leaf measurements were taken from the most recently fully expanded leaf from the stem apex. Immediately following conductance measurement, the leaf was removed to measure leaf area, width, length and stomatal traits. Fresh leaves were digitally scanned shortly after harvest at 400 dpi on a flatbed scanner. After scanning, acrylic peels were taken from the adaxial leaf surface and placed on cellophane, which were later viewed under a light microscope at 40x for stomatal measurements (see Carlson *et al.* 2016). Although height and pigmentation were measured on all plants on both day 6 and day 12 of the treatments (mean sample size

235 per population=39), all other traits were measured on only a subset of plants on each of
236 these days, typically in association with the transcriptomic data (10-20 individuals per
237 population for each day). Two additional variables were calculated by subtracting day 6
238 from day 12 values on the same plant: pigment accumulation (day 12 hue - day 6 hue)
239 and growth (day 12 height - day 6 height). A principle component analysis was
240 performed on the traits measured separately for day 6 and day 12, and shown in **Figure**
241 **2C-D**.

242 A smaller subset of plants had one leaf per plant harvested for whole
243 transcriptome sequencing and gene expression analysis (n=120 plants total, or 4 per
244 population per treatment per day, except no ALC on day 12 due to insufficient number
245 of individuals because of lower germination in this population). Plants with leaves
246 removed on day 6 were not sampled for transcriptomics on day 12, to avoid possible
247 shifts in gene expression caused by leaf removal. As a consequence, gene expression
248 and most traits were measured in one set of plants on day 6 and in a separate set on
249 day 12. Transcriptome sampling took place prior to any other measurements on a given
250 day and consisted of collecting one leaf for RNA extraction per plant (the 2nd or 3rd
251 youngest leaf from the tip) into a 1 mL tube and snap-freezing immediately in liquid
252 nitrogen. These samples were stored at -80℃ until library preparation. All sampling for
253 transcriptome sequencing was done between 10 AM and 11 AM, and functional trait
254 measurements were done between 10AM and 2PM each day. Both transcriptomics and
255 other trait measurements were done in a random order. On day 12, we harvested a
256 subset of plants for root length and carbohydrate storage measurements (n=9 per
257 population including the 4 transcriptome plants and 5 additional plants).

258 After completing all trait and transcriptome sampling, we cut plants at the root-
259 shoot junction and placed aboveground tissues in a 10 mL tube and snap-froze the
260 samples in liquid nitrogen for future carbohydrate analyses. Belowground tissues were
261 gently removed from the soil, rinsed clean and stored in a separate tube and also snap-
262 frozen. For these 9 plants per population, we also measured the length of the longest
263 root. Tubes containing plant tissues were stored in a -80℃ freezer for ~1 month, then
264 freeze-dried for biomass and carbohydrate analyses. Dried tissues for above- and
265 belowground material was measured and ground. Ten to hundred and fifty mg of ground
266 material was used for carbohydrate analyses as described in (Akman *et al.* 2012).
267 Briefly, carbohydrates and starch were extracted with 70% methanol solution. The
268 extract was used for soluble carbohydrate content measurements and the pellet

269 including starch was treated enzymatically with α-amylase and amyloglucosidase
270 resulting in hexose sugars. Soluble carbohydrates and hexose sugar concentration was
271 measured with a modified version of anthrone method using fixed glucose standards.
272 We also measured biomass but biomass and carbohydrate content were highly
273 correlated, and thus only carbohydrate content results are shown (dry mass and soluble
274 sugars Pearson's R = 0.90 and 0.94; starch Pearson's R = 0.64 and 0.81 for
275 belowground and aboveground, respectively).

276 **Gene expression analyses and bioinformatics**

277 Please see Supplementary Materials and Methods for transcriptome library preparation
278 and sequencing details. Raw reads were trimmed and demultiplexed with trimmomatic
279 (Bolger *et al.* 2014) and fastx toolkit (Hannon, 2010). By using bowtie (Langmead &
280 Salzberg 2012), trimmed reads were mapped to a previously published de-novo
281 transcriptome of *P. repens* (Akman *et al.* 2016) assembled by using sequences of three
282 individuals from 19 populations that also include all of our focal populations. Before
283 reads were mapped to the transcriptome, a further clustering of the contigs were done
284 using cd-hit (Fu *et al.* 2012). The final number of contigs in this final assembly was
285 168515. Read counts were extracted using eXpress (Pachter 2011), and ranged
286 between 2.46 to 21.80 million reads per sample with an average of 6.02 million reads.
287 Differential gene expression analyses were performed using edgeR. Contigs with <1
288 counts per million (cpm) were excluded from the analyses. For each time point,
289 differentially expressed genes per population were revealed using pairwise comparisons
290 using negative binomial models. The common set of differentially expressed genes was
291 uncovered by extracting the overlap of differentially expressed genes for each
292 population at day 12 only. We then used linear models in edgeR, by including
293 sequencing lane as a fixed factor, to detect contigs that showed variation between
294 treatments and among populations (population x treatment). Multi-dimensional scaling
295 (MDS) analysis was performed for each time point to visualize general patterns of gene
296 expression for all the samples using the top 500 genes that explain the variation the
297 most (**Figure 2A-B and Figure S6**).

298 Co-expression gene networks were computed using WGCNA (Langfelder &
299 Horvath 2008) with consensus clustering option for the 2 time-points. Normalized and
300 cpm-filtered read counts were transformed with "voom" in limma package for using in
301 WGCNA. A soft-threshold of 6 was used for clustering and 14 co-expressed gene
302 networks were constructed. The number of genes within each gene network varied

303 between 49 (GN13) to 7682 (GN1). The gene network "grey" (called GN0 in this study)
304 as constructed by WGCNA for genes that do not fit any gene network has 3124 genes.
305 A representative "eigen gene" value, which is essentially the first principal component
306 axis for all genes within the network, was used for further analyses of climate and trait
307 associations. By using gene orthology of our genes with Arabidopsis (Akman *et al.*
308 2016), we also tested for Gene Ontology (GO) enrichment for the 13 networks. This
309 analysis was performed in Gorilla (Eden *et al.* 2009), using the network genes as target
310 set and the whole gene set as the background. Contigs within each module, GO
311 enrichments and the genes with the highest module membership for a single network
312 are provided in **Table S3**.

313 **Statistical analyses**

314 *How do populations respond to drought and how do these responses vary?*

315 We tested whether drought significantly affected functional and performance traits in
316 *Protea repens* using linear mixed models. We used a total of 23 plant metrics in our
317 analyses, including 8 traits on day 6, and 14 traits or performance indices on day 12.
318 We analyzed each trait on each day separately, because preliminary analyses on both
319 days combined revealed significant time-point effects for almost every response
320 variable. Each model included the fixed effects of treatment, population, and the
321 population by treatment interaction (G x E) and random effects of maternal line. Models
322 for stomatal conductance included the added random effect of air temperature during
323 measurement. In these models, the treatment-by-population interaction term indicates
324 whether there was differential plasticity among populations, or in other words, that
325 plants from different populations responded differently to the treatment (G x E effect).
326 These analyses were performed using the lme4 package (Bates *et al.* 2012) in R 3.1
327 (Team 2014). To obtain p-values for each fixed effect, we successively dropped one
328 predictor from the full model and used a chi-square test to compare AIC scores between
329 models. Given the large number of statistical tests that this entailed, we also adjusted p-
330 values according to estimated false discovery rates (R package "fdrtools").

331 Gene expression analyses followed the same general approach as for traits. As
332 response variables, these analyses focused on levels of expression in the 14 co-
333 expressed gene networks (GN) that were produced through the gene clustering analysis
334 (see *Gene expression analyses and bioinformatics* section above). Models for gene
335 networks included fixed effects of treatment, population, and their interaction, random

effects for maternal line, and an additional random effect for sequencing lane (1 or 2). We also tested for genes that are differentially expressed among populations for the two treatments and time-points separately. A Venn diagram (Oliveros 2016) showing the genes in these four groups are given in **Figure 3B** . For example, the group of genes that are differentially expressed among populations under drought at day 6 is shown in purple color on the diagram. Gene ontology analysis was performed for detecting enrichments in these four groups using GOrilla as explained above.

Do the climatic conditions of the source sites covary with traits, gene expression and their plasticity?

To test whether characteristics of the home climate predicted cross-population variation in traits and gene expression, we used mixed effect models. In these models, traits and gene networks (GN) were response variables, the climate variables were fixed effect terms and maternal line nested in population was used as random effect variables. For testing the significance of the fixed factors, we calculated p-values based on chi-square tests as explained above. FDR corrections were applied to p-values in three groups: functional traits, performance traits and gene networks. Day 6 and Day 12 FDR corrections were also applied separately. For variables with significant climate and treatment interaction, each treatment was also analyzed separately to estimate slopes and test their significance within treatments. These are depicted as divided squares in **Figure 4**.

To characterize plasticity for individual traits and to quantify the direction and magnitude of this plasticity, we used maternal line plasticity indices. For each maternal family, we calculated plasticity as the family mean trait value in the wet treatment minus the family mean trait value in the drought treatment, divided by the overall family mean trait value. We used mixed effect models to determine whether characteristics of the home climate predicted cross-population variation in plasticity of traits and gene expression. In our analyses, plasticity in traits and gene networks (GN) were response variables, the 7 climate variables were fixed effect variables and population was included as a random effect. Each climate variable was tested against each response variable in a separate model, with day 6 and day 12 measurements analyzed separately (lmer, R program). FDR corrections were applied to p-values in three groups: functional traits, performance traits and gene networks. Day 6 and Day 12 FDR corrections were also applied separately.

369 # RESULTS

370 ## How do populations respond to drought and how do these responses vary?

371 *Drought responses are stronger in gene expression patterns compared to traits*

372 Six days into the experiment, only three of the 8 traits differed significantly between
373 treatments: unwatered plants had shorter stems, lower stomatal conductance and more
374 variable SLA. At the later stage, treatment effects became pronounced for more traits
375 measured (12 of 15): unwatered plants had lower stomatal conductance, decreased
376 height, slower growth (from day 6 to day 12), larger SLA, smaller leaf area, denser
377 stomata, smaller stomatal pore size, increasing pigment accumulation, less
378 carbohydrates aboveground (but not belowground). In the earlier stages of the stress
379 imposition, morphological and physiological traits did not show responses as
380 pronounced as those observed in gene expression patterns (**Figure 2**). In contrast to
381 most of the traits, the treatment effect was significant for over half of the gene networks
382 already at day 6 into the treatment. This trend of more pronounced gene expression
383 response compared to morpho-/physiological traits is visible both in co-expression gene
384 networks (**Table 2**, **Figure S2**) and Multi-dimensional Scaling (MDS) analyses (**Figure**
385 **2A-B**), which indicates a lag between the adjustments in gene expression and trait
386 onset. However, we should also note that the MDS for gene expression analyses is
387 based on the top 500 genes that explain the most variation, and we might not have
388 measured morpho-physiological traits that might also respond as quickly. For both gene
389 expression patterns as seen in MDS and morpho-physiological traits in PC analyses,
390 axis 1 and 2 explain the variation between treatments and among populations,
391 respectively. Notably, population V (Baviaanskloof, a high-elevation eastern site) does
392 not show a clear treatment effect in traits at the later stage, although the effect is quite
393 pronounced in the gene expression data (**Figure 2B&D** and **3A**).

394 At day 6 into the treatment, unwatered plants had higher values for gene
395 networks 2, 4, 5, 6, and 7 and lower values for gene networks 1, 9 and 11, relative to
396 watered plants (**Table 2**, **Figure S4**). These modules show gene enrichment in GO
397 categories for ATPase activity (GN2), oxidoreductase activity (GN4), cytoskeleton
398 organization (GN5), photorespiration (GN6), response to high light intensity and
399 temperature (GN7; note that p-value<0.0005, fdr-corrected p-value is not significant),
400 cell wall organization (GN1) and pyridine-containing compound metabolic process
401 (GN9). As the stress advanced after 12 days into the drought treatment, watered and

402 unwatered plants differed significantly for most traits and gene networks. Significant
403 treatment responses were detected for 10 of 14 gene networks, 8 of 10 functional traits,
404 and 4 of 5 performance measures (**Tables 1-3**, **Figures S3-5**). In addition to the gene
405 networks with treatments effects at day 6, GN3 (tropism and carbohydrate metabolism
406 related, and shows associations with leaf area, height and aboveground soluble
407 carbohydrates) and GN13 (homeostasis related) also had evident treatments effects at
408 day 12, with GN3 having lower values and GN13 higher values under drought.

409 *Drought up-regulates stress related genes and down-regulates growth related genes*

410 The number of genes differentially regulated between earlier and later stages of the
411 stress and among populations differed significantly (**Figure 3B**). For example,
412 population G (Garcia's Pass) does not have any genes differentially expressed at day 6,
413 indicating either a lower level of stress or lack of accurate perception and timely signal
414 transduction of the environmental cues to gene expression phenotypes. In contrast,
415 population B (Bredasdorp) had several hundred differentially expressed genes by day 6.
416 All populations had at least several hundred differentially expressed genes by day 12.
417 Two populations (C-Cederberg and S-Swartberg) showed few differentially expressed
418 genes initially, but over 1500 differentially expressed genes by day 12 (**Figure 3A**).
419 Although the number of genes regulated upon drought stress was highly variable, we
420 found 110 and 63 genes to be consistently up- or down- regulated, respectively, among
421 populations (**Table S4**). Homology of these genes with Arabidopsis show that up-
422 regulated genes include heat shock proteins, auxin signalling and ABA catabolism
423 genes, genes related to drought stress and photosynthesis, and a leaf amylase;
424 whereas down-regulated genes included ARF genes (involved in cell elongation and
425 division), and salt stress-, fatty acid and amino acid biosynthesis-related genes.

426 *Population differentiation in stress response becomes more evident as the stress*
427 *advances*

428 A population effect is evident in most of the traits, 5 out of 8 functional traits and 6 out of
429 7 performance traits were significantly different between the populations at day 6, and
430 all traits except for root length showed a population effect at day 12 (**Table 1 and 3**).
431 We also observed G x E effects (variation in population responses to drought) to a
432 limited extent on day 6, and to a greater extent on day 12 (**Table 1**). At day 6, only SLA
433 and gene network 13 (enriched for homeostasis GO term) showed variation in
434 responses to drought across populations (i.e., population x treatment interaction

435 significant; **Table 1**). Specific leaf area was lower under drought for four, mostly western
436 populations (R, G, C and F), but it was higher under drought in the remaining 4
437 populations (V, A, B, S;**Table 1**, **Figure S3**). At day 12, populations showed variation in
438 their response to drought for SLA, stomatal size, pigment accumulation, stomatal
439 conductance, stem growth rate, starch biomass aboveground, sugar biomass above
440 and belowground. In addition to GN13 at day 6, GN7 expression (linked to high light
441 intensity and high temperature GO term) also showed a G x E effect at day 12 (**Table**
442 **2**).

443 In addition to gene network responses, we also tested for gene expression
444 responses in single genes. Differential expression patterns of some genes varied across
445 populations (significant G x E interaction), including 13 contigs on day 6, rising to 23 on
446 day 12. These included genes associated with ATP synthase, cell redox homeostasis,
447 translation, response to cadmium ion, cellulose biosynthesis and heat shock response.
448 For example, contig 9970 showing homology with a heat shock gene in Arabidopsis
449 (AT5G12020) is up-regulated in all the populations 12 days after the treatment started,
450 but this increase is variable among populations (**Figure S7**).

451 For each treatment, we also tested for genes expressed differentially among
452 populations (testing for population effect) and time-points separately. This analysis
453 reveals which genes are differentially expressed among populations, indicating
454 population differentiation, within a treatment and a timepoint. With these analyses, we
455 find 851 and 668 genes to be differentially regulated among populations for the control
456 treatment, and 717 and 786 for drought treatment at day 6 and 12, respectively (FDR-
457 corrected p-value<0.01). We also observed some overlap between these groups
458 (**Figure 3B**); 45 of the genes being differentially expressed among populations in both
459 of the treatments for both days. Interestingly, the majority of genes differentially
460 expressed in a treatment did not overlap between the two time-points and this holds
461 true for both of the treatments (e.g. purple and pink in **Figure 3B**). Likewise, the genes
462 differentially expressed in the different treatments did not show overlap within a single
463 time-point (e.g. purple and yellow in **Figure 3B**). These results show that together with a
464 strong treatment effect, there is a pronounced time-point effect in our experiment for
465 gene expression (note that the plants were all harvested at the same time of the day but
466 6 days apart). This is also evident from our data for both traits and gene expression as
467 there is a significant time-point effect or interaction involving the time-point for most
468 traits and gene expression networks. We also found that the differentially expressed

genes among populations in watered conditions show enrichments for processes related to translation and peptide biosynthesis, whereas in dry conditions we find oxidative and heat stress related categories.

Do the climatic conditions of the source sites covary with traits, gene expression and their plasticity?

Gene networks and functional traits correlate with temperature-related source-site climate variables

In order to assess climatically driven trait and gene expression variation among populations, we tested for correlations between source-site climate variables and co-expression gene networks and functional traits. We found several gene networks that covary with climatic variables (**Figure 4**; boxed combinations). These include GN 3, 4, 5, 9 and 13 at day 6, and GN3, 4, 5, 6, 7, 10, 12 and 13 at day 12. Most of these gene networks showed strong correlations with elevation and the closely related variables July (winter) minimum temperature and January (summer) maximum temperature. We also observed correlations between mean annual precipitation and GN6, 10 and 13, and stomatal conductance. Of the performance traits, stem growth showed correlations with elevation, summer maximum and winter minimum temperature. Aboveground starch content co-varied with elevation, mean annual precipitation and intra-annual precipitation variation. Similarly, aboveground soluble carbohydrates showed associations with elevation.

We tested for climate by treatment interaction which revealed if associations between traits and the climate are significant for only one of the treatments. We found that gene expression patterns for GN7 at day 12 show a positive correlation with elevation (and minimum winter temperature) only for plants under drought conditions. Contrastingly, the correlations between GN12 and elevation, and GN13 and mean annual precipitation are significant only for watered plants. Similarly for stem growth and aboveground starch, the correlations with elevation are significant only for watered plants, and stomatal conductance shows a correlation with mean annual precipitation only under watered conditions.

Plasticity differentiation is associated with climate

We tested if plasticity in traits and gene expression reflect signatures of climatic effects. In order to do this, we used a subset of climate and trait pairs that already show

trait/gene expression variation among populations, as well as trait/ gene expression patterns that show a climate x treatment effect (associations between the climate holds true only at a single treatment). At day 6, plasticity in GN5 (related to cytoskeleton organization) is the only gene network that show a correlation with a climatic variable: low elevation populations showed higher plasticity in gene expression patterns of this gene network (**Table 4**). At day 12, we found that plasticity in GN10 (related to translation) and GN13 (linked to homeostasis) covaries with mean annual precipitation. For GN10, populations from both the driest and the wettest sites show higher plasticity (note that plasticity has both positive and negative values dependent on the directionality of the response), and populations from wetter sites show higher plasticity for GN13 (**Table 4**, **Figure 5D**). Additionally, plasticity in stem growth correlates with both elevation and summer maximum temperature. High plasticity in growth is observed in populations from low elevation and warmer sites (**Figure 5A**). Similar to GN13, plasticity in leaf area shows correlations with mean annual precipitation with higher plasticity in populations from wetter sites (**Figure 5B**). Leaf conductance and pigment accumulation shows correlations with temperature heterogeneity (temperature range within years). For both pigmentation and conductance, higher temperature heterogeneity, which is correlated with maximum summer temperature, leads to higher plasticity (**Figure 5C**). Finally, pigment accumulation plasticity also shows correlations with summer maximum temperature, with populations from coolest and warmest sites showing the highest plasticity. However, pigment-related associations are driven by a single population in Cederberg, the most northern population we studied. We should also note that this trend is driven by the direction of plasticity in this population for pigment content, which is opposite to what we observe in all the other populations:, plants from the Cederberg population have higher pigment under drought and lower pigments when watered.

DISCUSSION

Local adaptation can lead to divergence among populations along environmental gradients. This divergence in genetic variation is often reflected in the phenotypic variation in morphology, physiology as well as the underlying gene expression variation. Plasticity in these, as any other phenotypic character, can also be under selection, creating matching patterns between plasticity differentiation and the source site climatic variation among populations. In this study, we combined a greenhouse drought experiment with extensive measurements of physio/morphological traits and RNA-seq

535 data for 8 common sugarbush populations collected along an environmental gradient to
536 uncover patterns of climatic selection on these traits and their plasticity. We show that
537 functional traits and gene expression patterns are associated with the climate, and
538 these associations are highly dependent on the environmental conditions the traits are
539 measured in. More importantly, we show that plasticity in the functional traits and gene
540 expression patterns also are associated with climatic conditions of the source
541 population sites. Together, our results suggest that local adaptation affects evolution of
542 both trait means and their plasticity along a climate gradient in *Protea repens*.

543 *Drought imposes a complex stress regulating many gene expression networks*

544 Drought has been a strong driver of plant evolution in the Cape Floristic Region and
545 other Mediterranean climate zones (Allsopp *et al.* 2014). When water availability is
546 limited, as it typically is for several months during the summer in most of the region,
547 plants experience a suite of complex stresses such as accumulation of reactive oxygen
548 species, starvation, lower photosynthesis rates and turgor loss (Ramachandra Reddy *et*
549 *al.* 2004; West *et al.* 2012). Therefore, selection is expected to act on several genes
550 involved in these stresses, enabling plants to cope with the severe effects of drought.
551 Indeed, several studies have shown that various pathways including ABA-dependent
552 pathways, carbohydrate metabolism, ROS scavenging and photosynthesis are
553 regulated under drought (Miller *et al.* 2010; Heschel *et al.* 2014). In concordance with
554 drought being a complex stress acting on several pathways, we observe regulation of
555 10 out of 13 gene networks under drought conditions. For example, the genes with the
556 highest gene network membership of GN5 (related to cytoskeleton organization) are
557 downregulated under drought in all the populations, and this gene network also shows
558 correlations with both elevation and traits such as height, leaf area and aboveground
559 sugar content. Cytoskeleton organization related pathways were also shown to be
560 highly hampered during drought stress in Arabidopsis (Bhaskara *et al.* 2017), linking this
561 process to reduced growth. These results make this gene network a prime candidate for
562 controlling elevation-dependent growth patterns. A general pattern similar to that of GN5
563 genes is seen in other gene networks important for growth (such as GN1 related to cell
564 wall organization and other carbohydrate metabolism related gene networks, and shows
565 significant correlations with conductance). This “shut-off” of growth also explains the
566 shorter stems and smaller plant carbohydrate biomass aboveground under 12 days of
567 drought in all of the populations. Additionally, the genes that were consistently up-
568 regulated among all the populations were genes involved in stress response. Together

569 these results indicate a trade-off in which resources are channeled from growth to
570 stress acclimation. A similar trade-off has been observed across many plant species for
571 herbivory (Kempel *et al.* 2011; Züst *et al.* 2015), cold stress (Loehle 1998) and drought
572 (Haugen *et al.* 2008). We also detected reduced stomatal conductance in all
573 populations in the drought treatment, and thus a potential drop in photosynthesis might
574 also further contribute to the energy deficit, which might hamper growth and efficient
575 acclimation to the stress. The only gene network, GN6 (enriched for photorespiration
576 related genes) that shows a correlation with conductance at the later stage of the stress
577 is also correlated with leaf area, aboveground starch and above and below ground
578 soluble carbohydrates. Interestingly, we also find a correlation between this gene
579 network and mean annual precipitation suggesting local adaptation to water availability
580 via regulation of these genes.

581 *Drought induces smaller and thinner leaves*

582 Upon 12 days of drought stress, we see smaller leaves (except for population V) and
583 higher SLA (thinner leaves). This reduction in leaf size overall is potentially due to a
584 reduced investment in photosynthetic architecture. Previously, we showed that leaf area
585 measured in a common garden increases with source site precipitation, potentially
586 maximizing photosynthesis with water availability (Carlson *et al.* 2016). These patterns
587 are observed in other species within micro and macro scales. For example, Peppe et al.
588 (2011) showed that leaf size decreases with precipitation across many species which
589 also holds true here within the single species *Protea repens*. Additionally, we also see a
590 drop in stomatal pore size coupled with a higher stomatal density under drought
591 conditions. Previous studies show both positive and negative trends between aridity and
592 stomatal density or no relationship at all (Stenström *et al.* 2002; Pearce *et al.* 2006; Yu
593 *et al.* 2008; Franks & Beerling 2009; Skelton *et al.* 2012; Carins Murphy *et al.* 2014).
594 Our finding is consistent with a common garden study of 19 *P. repens* populations
595 sourced from a range of climates, in that plants from hotter sites with drier summers had
596 smaller and denser stomates (Carlson *et al.* 2016).

597 *Associations with the climate are context dependent*

598 When there are fitness consequences of a trait state that is induced conditionally in
599 response to environmental stressors, with no fitness costs/benefits when those
600 stressors are absent, genetic variation that controls this modified trait state (plastic
601 response) will be under selection only under the inducing stressful environment (Van

602 Dyken & Wade 2010; Ledón-Rettig *et al.* 2014). Strikingly, we observed climatic
603 correlations in gene expression and traits that only exist in either one of the treatments
604 (**Figure 4**). Gene network 7 (GN7), for example, shows a correlation with elevation and
605 winter minimum temperature only when the plants are under drought stress. Gene
606 ontology enrichments in this module point to regulation by high light and temperature
607 stress. Overall patterns show that best representative gene of this network (with the
608 highest similarity to eigen gene of PC1) show a higher expression in the high elevation
609 populations. A similar gene network in our previous study in a common garden was also
610 enriched for GO terms related to high light intensity and temperature, and this module
611 showed correlations with stem diameter and stomatal density, but surprisingly not with
612 any climate variables. Gene network 7 in this study also shows significant correlations
613 with height at both timepoints and SLA in the earlier timepoint (**Table S5**). Populations
614 from warmer sites show lower GN7 values (note that within a gene network there could
615 be genes that are regulated in opposite directions among populations, and thus it is not
616 possible to infer if a gene network is up- or down-regulated) which translates to taller
617 plants and thicker leaves. The top members of this gene network with Arabidopsis
618 homology are down-regulated in populations coming from higher elevation; a
619 nucleotide-sugar transporter gene (AT4G32390), a zinc-finger family protein
620 (AT2G38970), MTP8 (AT3G58060), 4CL3 involved in last step of phenylpropanoid
621 pathway - the upstream pathway anthocyanins are derived from (AT1G65060) and
622 PRA1.B1 (AT3G56110). We also observe a weak correlation between stem
623 pigmentation and temperature. Plants from warmer sites (higher maximum summer
624 temperature) have higher pigmentation accumulation (p-value =0.029, fdr-corrected
625 value = 0.106). Previous studies showed a negative effect of heat stress on plant growth
626 (Olszyk *et al.* 1998; Hasanuzzaman *et al.* 2013; Bita & Gerats 2013), and anthocyanin
627 accumulation as a protective mechanism against heat, cold, oxidative stress and UV
628 radiation (Hatier & Gould 2009; Steyn *et al.* 2009; Zeng *et al.* 2010; Wang *et al.* 2013).
629 The higher amount of anthocyanins accumulated in populations originating from warmer
630 sites in this study and their taller stature may accordingly be due to stronger selection in
631 warmer environments for production of anthocyanins to provide protection against
632 reactive oxygen species. In a parallel finding, under drought conditions, differentially
633 expressed genes among populations were enriched for only oxidative stress related
634 genes, suggesting there has also been selection on expression of these genes arising
635 from different levels of heat/drought stress along the climate gradient.

636 Contrastingly, GN13 shows a correlation with mean annual precipitation only in
637 watered conditions. This gene network also shows correlations with leaf area, SLA,
638 stomatal pore size and aboveground carbohydrate content. Gene network 13 is a
639 module related to homeostasis and the best representative of this module shows
640 homology to an Arabidopsis heat shock protein (AT5G59720). Heat shock proteins are
641 shown to be involved in responses to stresses such as heat, cold, UV radiation, salt and
642 drought (Chen *et al.* 2018). Under control conditions, plants from wetter source sites
643 express genes in this network less compared to populations from drier sites, and show
644 a bigger up-regulation, eventually reaching similar levels of expression for all
645 populations under drought. In drier environments, higher constitutive expression of
646 these genes might be crucial for a prompt response to drought via regulation of stomatal
647 pore size, leaf size and thickness. However, populations that do not experience drought
648 as often might not benefit from constitutive expression of these genes, creating a
649 mismatch between costs and benefits. These patterns indicate local adaptation in
650 plasticity of these genes through a relaxed selection under wet environments, and
651 directional selection under drought.

652 Similar to GN13, we also observe a correlation between GN12 and elevation
653 under wet conditions: populations from high elevation show higher expression of genes
654 in this network. Although this gene network is not enriched for gene ontologies, the
655 gene network is constituted of photosynthesis and chloroplast related genes, and shows
656 correlations with leaf area. Unlike GN13, we observe a variable population response
657 under drought: some populations have lower eigen gene values and some have higher
658 under drought conditions compared to controls. This implies that some genes are up
659 and some are down-regulated in a population specific manner. Under drought it is likely
660 that most populations reduced their photosynthetic rate to very low values and thus
661 patterns are not present, as evidenced from the generally low conductance values in the
662 drought treatment.

663 *Temperature exerts a stronger selection pressure than precipitation*

664 Previously, we showed that functional traits and gene expression patterns as measured
665 in a common garden with older plants (1.5 year old plants vs 2 months-old plants in this
666 study) correlated with source site climate. Although using fewer populations in this
667 study, we observed similar patterns in those correlations. For example, in both studies
668 we observe associations between gene networks and minimum winter temperature,
669 elevation and mean annual precipitation. We also found that the climate variable that

has the most correlations with the gene networks to be elevation/minimum winter temperature (2 variables highly correlated, Pearson's R = -0.91, **Table S2**), which was also true for the common garden study. Although Protea populations throughout the CFR should experience drought at least occasionally if not frequently, the drought metrics used in this study are not as strong predictors of trait variation as temperature/elevation is under the tested conditions (drought and control in a greenhouse and a common garden). Even if our selection of leaf traits was biased away from those likely to reflect drought adaptations (which we consider unlikely), this could not be the case for gene expression networks, which include all major groupings of coexpressed genes detected. Gene expression networks should therefore reflect an unbiased pattern in plant responses, and these networks also had a high number of correlations with home-site temperature or elevation.

Possible explanations for the dominance in temperature/elevation correlations are (1) we have not identified the most influential metrics of drought stress in the CFR, such that we fail to detect these associations; or (2) temperature-gradients across the CFR have shaped trait evolution in *Protea repens* more strongly than rainfall-gradients recently. This could have arisen because *P. repens* plants experience drought less frequently than expected due to rapidly growing roots that reach and remain in the water table despite water shortages at the surface. It could also be because drought is an inconstant selection pressure, relative to temperature. Populations in the CFR experience drought periodically, and although some of the populations we studied experienced a decrease in precipitation over the past 115 years, this effect is not as strong as the increase in temperature over the same period (**Figure 6**, Mason & Jury 1997; Kruger & Shongwe 2004). Compared to a periodic drought cycle that varies widely in strength, this temperature increase potentially imposes a more stable and continuous stress and thus a stronger selection force. Additionally, the fact that precipitation patterns are affected in a more variable way compared to more stable increases in temperature might make it harder to detect patterns of selection in response to precipitation changes across climate gradients even under drought conditions as in our experiments. For example, a recent meta-analysis of 21 plant traits from 25354 species concluded that temperature is a stronger driver of plant trait evolution compared to precipitation (Moles *et al.* 2014).

Carbohydrate reserves play a role in survival

703 Surprisingly, we found a positive correlation between survival under drought and
704 precipitation; plants from wetter sites survived longer under drought. Populations from
705 wetter sites also showed higher stomatal conductance and shoot starch indicating
706 higher photosynthetic rates. The fact that populations from wetter sites surviving better
707 under drought is counterintuitive. However, in a sudden drought onset, like the one
708 imposed in this experiment, carbohydrate reserves might be a decisive factor in
709 survival, especially in earlier stages of development, and thus if plants from wetter sites
710 have built up larger reserves, they may survive longer (O'Brien *et al.* 2014).

711 *Both climatic heterogeneity and favorable environmental conditions shape plasticity*
712 *evolution*

713 Here, we also tested if plasticity is favored and thus selected for in heterogeneous
714 and/or milder climatic conditions. A heterogeneous environment in theory should
715 promote plasticity evolution as this can allow for a better match between the phenotype
716 and the changing environment, potentially surpassing cost of plasticity. Similarly, in mild
717 environments, plasticity can arise through a relaxed selection in which a mismatch
718 between the phenotype and the climate does not impose strong fitness costs. We found
719 evidence for both of these patterns for the functional traits and the gene expression
720 patterns. These results support that plasticity can evolve under both milder and
721 heterogeneous conditions. However in this study, we have more evidence for growth
722 plasticity evolving under milder conditions than heterogeneous environments, i.e. higher
723 plasticity for growth and GN5 (cytoskeleton organization) in populations from lower
724 elevation/warmer sites (representing milder climates). Although heat stress can be
725 detrimental, in the CFR cold stress may impose a stronger selective pressure for growth
726 (Wilson *et al.* 2015). In fact, in a study of 56 Proteaceae species looking at climate and
727 plant trait correlations, Mitchell et al, showed the most strongly supported evolutionary
728 correlations to be between winter minimum temperature (next to mean annual
729 temperature and elevation) and plant height (Mitchell *et al.* 2018). They also note that
730 this negative effect of elevation on plant height might be coupled potentially with
731 shallower soils in the high elevation sites (Campbell & Werger 1988). These combined
732 effects might create harsher environments which increase the cost of mismatch
733 between phenotype and the environment, thus decreasing fitness of more plastic
734 individuals. We also find higher plasticity in wetter sites for GN13 and leaf area, and
735 again these sites represent milder climates in this drought-prone region.

Similar to trait means, we also find more associations between temperature related variables and trait plasticity, once again suggesting that temperature may create a stronger or more consistent selective force compared to precipitation in this system. Interestingly, the only correlations we observe for heterogeneity are also related to temperature. Both plasticity in conductance and pigment content increases with increasing heterogeneity. In environments where temperature change across seasons is high, higher plasticity in stomatal conductance (thus photosynthesis and transpiration rates) might decrease the phenotype-environment mismatch and increase plant fitness, thus be selected for in these environments. Likewise, adjusting levels of anthocyanins through plasticity in response to temperature changes might effectively protect plants against abiotic stressors, thus promoting selection on higher plasticity.

CONCLUSION

Here, we show that both phenotypic traits and gene expression, and their plasticity can be under selection along a climate gradient. However, detecting associations between these traits and the environmental variables can be a challenging task as these associations are context-dependent, and thus can be detected only when they are measured under certain conditions. We also found that trait variation can be explained better with temperature related-variables rather than precipitation related variables, potentially due to the fact that the recent temperature increases associated with climate change impose a stronger and more continuous selective pressure compared to the more variable changes in precipitation. This study also provides insights into the drivers of plasticity evolution: we found more evidence for selection for plasticity in mild environments (or selection against plasticity in harsher environments) compared to directional selection in heterogeneous environments. We also demonstrate that integration of gene expression variables into studies of climate trait associations proves valuable for detecting molecular mechanisms involved in selection and by representing a comprehensive palette of traits that are shaped by both neutral and selective evolutionary forces.

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REFERENCES

Ackerly DD, Dudley SA, Sultan SE *et al.* (2000) The evolution of plant ecophysiological traits: Recent advances and future directions: new research addresses natural selection, genetic constraints, and the adaptive evolution of plant ecophysiological traits. *Bioscience*, **50**, 979–995.

Akman M, Bhikharie AV, McLean EH *et al.* (2012) Wait or escape? Contrasting submergence tolerance strategies of Rorippa amphibia, Rorippa sylvestris and their hybrid. *Annals of botany*, **109**, 1263–1276.

Akman M, Carlson JE, Holsinger KE, Latimer AM (2016) Transcriptome sequencing reveals population differentiation in gene expression linked to functional traits and environmental gradients in the South African shrub Protea repens. *The New phytologist*, **210**, 295–309.

Allen CD, Macalady AK, Chenchouni H *et al.* (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest ecology and management*, **259**, 660–684.

Allsopp N, Colville JF, Verboom GA (2014) *Fynbos: Ecology, Evolution, and Conservation of a Megadiverse Region*. Oxford University Press.

Alpert P, Simms EL (2002) The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? *Evolutionary ecology*, **16**, 285–297.

Anderson JT, Gezon ZJ (2015) Plasticity in functional traits in the context of climate change: a case study of the subalpine forb Boechera stricta (Brassicaceae). *Global change biology*, **21**, 1689–1703.

Aubin I, Munson AD, Cardou F *et al.* (2016) Traits to stay, traits to move: a review of functional traits to assess sensitivity and adaptive capacity of temperate and boreal trees to climate change. *Environmental Review*, **24**, 164–186.

Barshis DJ, Ladner JT, Oliver TA *et al.* (2013) Genomic basis for coral resilience to climate change. *Proceedings of the National Academy of Sciences*, **110**, 1387–1392.

Bates D, Maechler M, Bolker B (2012) lme4: Linear mixed-effects models using S4 classes.

802 Baythavong BS, Stanton ML (2010) Characterizing selection on phenotypic plasticity in
803 response to natural environmental heterogeneity. *Evolution; international journal of*
804 *organic evolution*, **64**, 2904–2920.
805 Bhaskara GB, Wen T-N, Nguyen TT, Verslues PE (2017) Protein Phosphatase 2Cs and
806 Microtubule-Associated Stress Protein 1 Control Microtubule Stability, Plant Growth,
807 and Drought Response. *The Plant cell*, **29**, 169–191.
808 Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment:
809 scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in plant*
810 *science*, **4**, 273.
811 Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina
812 Sequence Data. *Bioinformatics* .
813 Bradshaw AD, Hardwick K (1989) Evolution and stress—genotypic and phenotypic
814 components. *Biological journal of the Linnean Society. Linnean Society of London*, **37**,
815 137–155.
816 Campbell BM, Werger MJA (1988) Plant Form in the Mountains of the Cape, South
817 Africa. *The Journal of ecology*, **76**, 637–653.
818 Carins Murphy MR, Jordan GJ, Brodribb TJ (2014) Acclimation to humidity modifies the
819 link between leaf size and the density of veins and stomata. *Plant, cell & environment*,
820 **37**, 124–131.
821 Carlson JE, Adams CA, Holsinger KE (2016) Intraspecific variation in stomatal traits, leaf
822 traits and physiology reflects adaptation along aridity gradients in a South African
823 shrub. *Annals of botany*, **117**, 195–207.
824 Carlson JE, Holsinger KE (2012) Developmental Plasticity in <italic>Protea</italic> as an
825 Evolutionary Response to Environmental Clines in the Cape Floristic Region. *PloS*
826 *one*, **7**, e52035.
827 Carlson JE, Holsinger KE, Prunier R (2011) Plant responses to climate in the Cape
828 Floristic region of South Africa: Evidence for adaptive differentiation in the Proteaceae.
829 *Evolution; international journal of organic evolution*, **65**, 108–124.
830 Chen B, Feder ME, Kang L (2018) Evolution of heat-shock protein expression underlying
831 adaptive responses to environmental stress. *Molecular ecology*.
832 Chevin L-M, Lande R, Mace GM (2010) Adaptation, plasticity, and extinction in a
833 changing environment: towards a predictive theory. *PLoS biology*, **8**, e1000357.
834 Colicchio JM, Monnahan PJ, Kelly JK, Hileman LC (2015) Gene expression plasticity
835 resulting from parental leaf damage in Mimulus guttatus. *The New phytologist*, **205**,
836 894–906.

837 Cowling RM (2009) From winter wet to winter dry: Fynbos diversity and evolution along
838 the west–east gradient. *South African journal of botany: official journal of the South*
839 *African Association of Botanists = Suid-Afrikaanse tydskrif vir plantkunde: amptelike*
840 *tydskrif van die Suid-Afrikaanse Genootskap van Plantkundiges*, **2**, 386–387.
841 Cuenca Cambronero M, Beasley J, Kissane S, Orsini L (2018) Evolution of thermal
842 tolerance in multifarious environments. *Molecular ecology*, **27**, 4529–4541.
843 Daac LP (2011) ASTER L1B. *Sioux Falls, South Dakota: USGS/Earth Resources*
844 *Observation and Science (EROS) Center*.
845 Dayan DI, Crawford DL, Oleksiak MF (2015) Phenotypic plasticity in gene expression
846 contributes to divergence of locally adapted populations of Fundulus heteroclitus.
847 *Molecular ecology*, **24**, 3345–3359.
848 Dudley SA, Schmitt J (1996) Testing the Adaptive Plasticity Hypothesis: Density-
849 Dependent Selection on Manipulated Stem Length in Impatiens capensis. *The*
850 *American naturalist*, **147**, 445–465.
851 Duputié A, Rutschmann A, Ronce O, Chuine I (2015) Phenological plasticity will not help
852 all species adapt to climate change. *Global change biology*, **21**, 3062–3073.
853 Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z (2009) GOrilla: a tool for discovery
854 and visualization of enriched GO terms in ranked gene lists. *BMC bioinformatics*, **10**,
855 48.
856 Etterson JR (2004) Evolutionary potential of Chamaecrista fasciculata in relation to
857 climate change. I. Clinal patterns of selection along an environmental gradient in the
858 great plains. *Evolution; international journal of organic evolution*, **58**, 1446–1458.
859 Forsman A (2015) Rethinking phenotypic plasticity and its consequences for individuals,
860 populations and species. *Heredity*, **115**, 276–284.
861 Franks PJ, Beerling DJ (2009) Maximum leaf conductance driven by CO2 effects on
862 stomatal size and density over geologic time. *Proceedings of the National Academy of*
863 *Sciences of the United States of America*, **106**, 10343–10347.
864 Fu L, Niu B, Zhu Z, Wu S, Li W (2012) CD-HIT: accelerated for clustering the next-
865 generation sequencing data. *Bioinformatics* , **28**, 3150–3152.
866 Geber MA, Griffen LR (2003) Inheritance and Natural Selection on Functional Traits.
867 *International journal of plant sciences*, **164**, S21–S42.
868 Ghalambor CK, Hoke KL, Ruell EW *et al.* (2015) Non-adaptive plasticity potentiates rapid
869 adaptive evolution of gene expression in nature. *Nature*, **525**, 372–375.
870 Granados-Cifuentes C, Bellantuono AJ, Ridgway T, Hoegh-Guldberg O, Rodriguez-
871 Lanetty M (2013) High natural gene expression variation in the reef-building coral

872 Acropora millepora: potential for acclimative and adaptive plasticity. *BMC genomics*,
873 **14**, 1–12.
874 Grime JP (1994) The role of plasticity in exploiting environmental heterogeneity.
875 *Exploitation of environmental heterogeneity by plants: ecophysiological processes*
876 *above-and belowground*, **1**, 19.
877 Gugger S, Kesselring H, Stöcklin J, Hamann E (2015) Lower plasticity exhibited by high-
878 versus mid-elevation species in their phenological responses to manipulated
879 temperature and drought. *Annals of botany*, **116**, 953–962.
880 Hannon GJ Fastx-toolkit. 2010. *GNU Affero General Public License*, **706**.
881 Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M (2013) Physiological,
882 biochemical, and molecular mechanisms of heat stress tolerance in plants.
883 *International journal of molecular sciences*, **14**, 9643–9684.
884 Hatier J-HB, Gould KS (2009) Anthocyanin Function in Vegetative Organs. In:
885 *Anthocyanins: Biosynthesis, Functions, and Applications* (eds Winefield C, Davies K,
886 Gould K), pp. 1–19. Springer New York, New York, NY.
887 Haugen R, Steffes L, Wolf J *et al.* (2008) Evolution of drought tolerance and defense:
888 dependence of tradeoffs on mechanism, environment and defense switching. *Oikos* ,
889 **117**, 231–244.
890 Heschel MS, Evankow A, Wolfson KB, Carlson JE, Holsinger KE (2014) Drought
891 Response Diversification in African Protea Species. *International journal of plant*
892 *sciences*, **175**, 442–449.
893 Hewitson BC, Crane RG (2006) Consensus between GCM climate change projections
894 with empirical downscaling: precipitation downscaling over South Africa. *International*
895 *Journal of Climatology*, **26**, 1315–1337.
896 Jacobs BS, Latimer AM (2012) Analyzing reaction norm variation in the field vs.
897 greenhouse: Comparing studies of plasticity and its adaptive value in two species of
898 Erodium. *Perspectives in plant ecology, evolution and systematics*, **14**, 325–334.
899 Kempel A, Schädler M, Chrobock T, Fischer M, van Kleunen M (2011) Tradeoffs
900 associated with constitutive and induced plant resistance against herbivory.
901 *Proceedings of the National Academy of Sciences of the United States of America*,
902 **108**, 5685–5689.
903 Kruger AC, Shongwe S (2004) Temperature trends in South Africa: 1960--2003.
904 *International Journal of Climatology*, **24**, 1929–1945.
905 Langfelder P, Horvath S (2008) WGCNA: an R package for weighted correlation network
906 analysis. *BMC bioinformatics*, **9**, 559.

907 Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature*
908 *methods*, **9**, 357–359.
909 Lasky JR, Des Marais DL, Lowry DB *et al.* (2014) Natural Variation in Abiotic Stress
910 Responsive Gene Expression and Local Adaptation to Climate in Arabidopsis thaliana.
911 *Molecular biology and evolution*, **31**, 2283–2296.
912 Latimer A, Silander JA Jr, Rebelo AG, Midgley GF (2009) Experimental biogeography:
913 the role of environmental gradients in high geographic diversity in Cape Proteaceae.
914 *Oecologia*, **160**, 151–162.
915 Lázaro-Nogal A, Matesanz S, Godoy A *et al.* (2015) Environmental heterogeneity leads
916 to higher plasticity in dry-edge populations of a semi-arid Chilean shrub: insights into
917 climate change responses. *The Journal of ecology*, **103**, 338–350.
918 Ledón-Rettig CC, Pfennig DW, Chunco AJ, Dworkin I (2014) Cryptic genetic variation in
919 natural populations: a predictive framework. *Integrative and comparative biology*, **54**,
920 783–793.
921 Loehle C (1998) Height growth rate tradeoffs determine northern and southern range
922 limits for trees. *Journal of biogeography*, **25**, 735–742.
923 Maia R, Eliason CM, Bitton P-P, Doucet SM, Shawkey MD (2013) pavo : an R package
924 for the analysis, visualization and organization of spectral data (A Tatem, Ed,).
925 *Methods in ecology and evolution / British Ecological Society*, **I**.
926 Mason SJ, Jury MR (1997) Climatic variability and change over southern Africa: a
927 reflection on underlying processes. *Progress in Physical Geography: Earth and*
928 *Environment*, **21**, 23–50.
929 Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species
930 homeostasis and signalling during drought and salinity stresses. *Plant, cell &*
931 *environment*, **33**, 453–467.
932 Mitchell N, Carlson JE, Holsinger KE (2018) Correlated evolution between climate and
933 suites of traits along a fast-slow continuum in the radiation of Protea. *Ecology and*
934 *evolution*, **8**, 1853–1866.
935 Moles AT, Perkins SE, Laffan SW *et al.* (2014) Which is a better predictor of plant traits:
936 temperature or precipitation? (A Helm, Ed,). *Journal of vegetation science: official*
937 *organ of the International Association for Vegetation Science*, **25**, 1167–1180.
938 Morris MRJ, Richard R, Leder EH *et al.* (2014) Gene expression plasticity evolves in
939 response to colonization of freshwater lakes in threespine stickleback. *Molecular*
940 *ecology*, **23**, 3226–3240.

941 Muñoz NJ, Farrell AP, Heath JW, Neff BD (2014) Adaptive potential of a Pacific salmon
942 challenged by climate change. *Nature climate change*, **5**, 163.
943 Nicotra AB, Atkin OK, Bonser SP *et al.* (2010) Plant phenotypic plasticity in a changing
944 climate. *Trends in plant science*, **15**, 684–692.
945 Nicotra AB, Segal DL, Hoyle GL *et al.* (2015) Adaptive plasticity and epigenetic variation
946 in response to warming in an Alpine plant. *Ecology and evolution*, **5**, 634–647.
947 O'Brien MJ, Leuzinger S, Philipson CD, Tay J, Hector A (2014) Drought survival of
948 tropical tree seedlings enhanced by non-structural carbohydrate levels. *Nature climate*
949 *change*, **4**, 710.
950 Oliveros JC (2016) Venny. An interactive tool for comparing lists with Venn's diagrams.
951 2007--2015.
952 Olszyk D, Wise C, VanEss E, Tingey D (1998) Elevated temperature but not elevated
953 CO2 affects long-term patterns of stem diameter and height of Douglas-fir seedlings.
954 *Canadian journal of forest research. Journal canadien de la recherche forestiere*, **28**,
955 1046–1054.
956 Pachter L (2011) Models for transcript quantification from RNA-Seq. *arXiv [q-bio.GN]*.
957 Palacio-López K, Beckage B, Scheiner S, Molofsky J (2015) The ubiquity of phenotypic
958 plasticity in plants: a synthesis. *Ecology and evolution*, **5**, 3389–3400.
959 Pearce DW, Millard S, Bray DF, Rood SB (2006) Stomatal characteristics of riparian
960 poplar species in a semi-arid environment. *Tree physiology*, **26**, 211–218.
961 Peppe DJ, Royer DL, Cariglino B *et al.* (2011) Sensitivity of leaf size and shape to
962 climate: global patterns and paleoclimatic applications. *The New phytologist*, **190**,
963 724–739.
964 Pratt JD, Mooney KA (2013) Clinal adaptation and adaptive plasticity in Artemisia
965 californica: implications for the response of a foundation species to predicted climate
966 change. *Global change biology*, **19**, 2454–2466.
967 Prunier R, Holsinger KE, Carlson JE (2012) The effect of historical legacy on adaptation:
968 do closely related species respond to the environment in the same way? *Journal of*
969 *evolutionary biology*, **25**, 1636–1649.
970 Ramachandra Reddy A, Chaitanya KV, Vivekanandan M (2004) Drought-induced
971 responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of*
972 *plant physiology*, **161**, 1189–1202.
973 Rebelo A (2001) Saso proteas: a field guide to the proteas of Southern Africa.

Sauquet H, Weston PH, Anderson CL *et al.* (2009) Contrasted patterns of hyperdiversification in Mediterranean hotspots. *Proceedings of the National Academy of Sciences*, **106**, 221–225.

Scheiner SM (2013) The genetics of phenotypic plasticity. XII. Temporal and spatial heterogeneity. *Ecology and evolution*, **3**, 4596–4609.

Schlichting CD, Pigliucci M (1995) Gene regulation, quantitative genetics and the evolution of reaction norms. *Evolutionary ecology*, **9**, 154–168.

Schneider RF, Li Y, Meyer A, Gunter HM (2014) Regulatory gene networks that shape the development of adaptive phenotypic plasticity in a cichlid fish. *Molecular ecology*, **23**, 4511–4526.

Schulze RE (1997) *South African atlas of agrohydrology and-climatology*. Water Research Commission, Pretoria, South Africa.

Schulze RE (2007) *South African atlas of climatology and agrohydrology: WRC Report 1489/1/06*. Water Resource Commission.

Sgrò CM, Terblanche JS, Hoffmann AA (2016) What Can Plasticity Contribute to Insect Responses to Climate Change? *Annual review of*.

Skelton RP, Midgley JJ, Nyaga JM, Johnson SD, Cramer MD (2012) Is leaf pubescence of Cape Proteaceae a xeromorphic or radiation-protective trait? *Australian journal of botany*, **60**, 104–113.

Stenström A, Jónsdóttir IS, Augner M (2002) Genetic and environmental effects on morphology in clonal sedges in the Eurasian Arctic. *American journal of botany*, **89**, 1410–1421.

Steyn WJ, Wand SJE, Jacobs G, Rosecrance RC, Roberts SC (2009) Evidence for a photoprotective function of low-temperature-induced anthocyanin accumulation in apple and pear peel. *Physiologia plantarum*, **136**, 461–472.

Stoks R, Govaert L, Pauwels K, Jansen B, De Meester L (2016) Resurrecting complexity: the interplay of plasticity and rapid evolution in the multiple trait response to strong changes in predation pressure in the water flea Daphnia magna. *Ecology letters*, **19**, 180–190.

Sultan SE (2003) Phenotypic plasticity in plants: a case study in ecological development. *Evolution & development*, **5**, 25–33.

Team RC (2014) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

1007 Valladares F, Matesanz S, Guilhaumon F *et al.* (2014) The effects of phenotypic
1008 plasticity and local adaptation on forecasts of species range shifts under climate
1009 change. *Ecology letters*, **17**, 1351–1364.
1010 Van Dyken JD, Wade MJ (2010) The genetic signature of conditional expression.
1011 *Genetics*, **184**, 557–570.
1012 Van Kleunen M, Fischer M (2005) Constraints on the evolution of adaptive phenotypic
1013 plasticity in plants. *The New phytologist*, **166**, 49–60.
1014 Van Wilgen BW, Forsyth GG, De Klerk H (2010) Fire management in Mediterranean-
1015 climate shrublands: a case study from the Cape fynbos, South Africa. *Journal of*
1016 *Applied*.
1017 Vitasse Y, Hoch G, Randin CF *et al.* (2013) Elevational adaptation and plasticity in
1018 seedling phenology of temperate deciduous tree species. *Oecologia*, **171**, 663–678.
1019 Wadgymar SM, Mactavish RM, Anderson JT (2018) Transgenerational and Within-
1020 Generation Plasticity in Response to Climate Change: Insights from a Manipulative
1021 Field Experiment across an Elevational Gradient. *The American naturalist*, **192**, 698–
1022 714.
1023 Wang H, Fan W, Li H *et al.* (2013) Functional characterization of dihydroflavonol-4-
1024 reductase in anthocyanin biosynthesis of purple sweet potato underlies the direct
1025 evidence of anthocyanins function against abiotic stresses. *PloS one*, **8**, e78484.
1026 Wennersten L, Forsman A (2012) Population-level consequences of polymorphism,
1027 plasticity and randomized phenotype switching: a review of predictions. *Biological*
1028 *reviews of the Cambridge Philosophical Society*, **87**, 756–767.
1029 West AG, Dawson TE, February EC (2012) Diverse functional responses to drought in a
1030 Mediterranean-type shrubland in South Africa. *New*.
1031 West-Eberhard MJ (2003) *Developmental Plasticity and Evolution*. Oxford University
1032 Press.
1033 Whitehead A (2012) Comparative genomics in ecological physiology: toward a more
1034 nuanced understanding of acclimation and adaptation. *The Journal of experimental*
1035 *biology*, **215**, 884–891.
1036 Wilson AM, Latimer AM, Silander JA (2015) Climatic controls on ecosystem resilience:
1037 Postfire regeneration in the Cape Floristic Region of South Africa. *Proceedings of the*
1038 *National Academy of Sciences*, **112**, 9058–9063.
1039 Young DJN, Stevens JT, Earles JM *et al.* (2017) Long-term climate and competition
1040 explain forest mortality patterns under extreme drought. *Ecology letters*, **20**, 78–86.

1041 Yu H, Chen X, Hong Y-Y *et al.* (2008) Activated expression of an Arabidopsis HD-
1042 START protein confers drought tolerance with improved root system and reduced
1043 stomatal density. *The Plant cell*, **20**, 1134–1151.
1044 Zeng X-Q, Chow WS, Su L-J, Peng X-X, Peng C-L (2010) Protective effect of
1045 supplemental anthocyanins on Arabidopsis leaves under high light. *Physiologia*
1046 *plantarum*, **138**, 215–225.
1047 Züst T, Rasmann S, Agrawal AA (2015) Growth--defense tradeoffs for two major anti-
1048 herbivore traits of the common milkweed Asclepias syriaca. *Oikos* , **124**, 1404–1415.

Table 1. Results of linear mixed models testing functional trait and performance differences of *Protea repens* seedlings at 6 and 12 days into the dry down experiment. The model compares values between watered and unwatered treatments as well as among 8 wild-sourced populations and the population by treatment interaction. Significant values are bolded.

Response variable	Fixed effects	*Fixed effects at 6 days*				*Fixed effects at 12 days*			
		df	Chi-sq	P-value	q-val	df	Chi-sq	P-value	
Leaf area (cm^2)	Population	7	49.91	**<0.0001**	**<0.0001**	6	69.16	**<0.0001**	**<0.0001**
	Treatment	1	0.17	0.678	0.881	1	23.39	**<0.0001**	**<0.0001**
	Pop. × Trt.	7	8.61	0.282	0.423	6	13.3	0.039	0.050
SLA (cm^2/g)	Population	7	5.15	0.642	0.881	**6**	**15.35**	**0.018**	**0.027**
	Treatment	**1**	**12.13**	**0.0005**	**0.002**	1	73.14	**<0.0001**	**<0.0001**
Log transform	Pop. × Trt.	**7**	**19.23**	**0.008**	**0.027**	**6**	**16.71**	**0.010**	**0.017**
LWR	Population	7	67.48	**<0.0001**	**<0.0001**	6	78.55	**<0.0001**	**<0.0001**
	Treatment	1	0.06	0.814	0.881	1	1.86	0.173	0.210
	Pop. × Trt.	7	3.47	0.839	0.881	6	1.52	0.958	0.958
Stomatal density	Population	**7**	**17.46**	**0.015**	**0.040**	**6**	**29.73**	**<0.0001**	**0.0001**
	Treatment	1	3.07	0.080	0.192	**1**	**12.95**	**0.0003**	**0.0007**
	Pop. × Trt.	7	9.56	0.215	0.369	6	3.59	0.732	0.765
Stomatal pore size (µm)	Population	7	3.87	0.795	0.881	**6**	**21.92**	**0.001**	**0.002**
	Treatment	1	0.04	0.844	0.881	**1**	**27.68**	**<0.0001**	**<0.0001**
	Pop. × Trt.	7	3.05	0.881	0.881	**6**	**14.27**	**0.027**	**0.038**
Stem pigmentation	Population	7	34.13	**<0.0001**	**<0.0001**	7	38.51	**<0.0001**	**<0.0001**
	Treatment	1	1.85	0.174	0.336	1	0.42	0.515	0.579
	Pop. × Trt.	7	10.12	0.182	0.336	7	5.71	0.574	0.630
Stem height (cm)	Population	**7**	**105.34**	**<0.0001**	**<0.0001**	7	121.24	**<0.0001**	**<0.0001**
	Treatment	**1**	**5.9**	**0.015**	**0.040**	1	49.4	**<0.0001**	**<0.0001**
	Pop. × Trt.	7	4.1	0.768	0.881	7	6.86	0.443	0.511
Stomatal conductance	Population	7	0	0.254	0.406	**6**	**17.52**	**0.008**	**0.014**
	Treatment	**1**	**0**	**<0.0001**	**<0.0001**	**1**	**104.7**	**<0.0001**	**<0.0001**
	Pop. × Trt.	7	0	0.148	0.323	**6**	**23.43**	**0.0007**	**0.001**

Table 2. Results of linear mixed models comparing eigengene values (E0-E13) at 6 and 12 days into the dry down experiment for watered and unwatered plants of *P. repens*. Significant values are bolded.

	Fixed effects	*Fixed effects at 6 days*				*Fixed effects at 12 days*			
		df	Chi-sq	p-value	q-val	ddf	Chi-sq	p-value	q-val
E0	Population	**7**	**24.89**	**0.0008**	**0.0042**	**6**	**80.38**	**<0.0001**	**<0.0001**
	Treatment	1	2.05	0.152	0.281	1	0.77	0.380	0.479
	Pop. × Tr.	7	5.72	0.573	0.655	6	8.51	0.203	0.294
E1	Population	**7**	**18.59**	**0.010**	**0.042**	**6**	**16.49**	**0.011**	**0.026**
	Treatment	**1**	**91.40**	**<0.0001**	**<0.0001**	**1**	**123.77**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	11.30	0.126	0.252	6	6.71	0.348	0.457
E2	Population	7	8.91	0.259	0.363	6	13.87	0.031	0.0566
	Treatment	**1**	**13.01**	**0.0003**	**0.0018**	**1**	**50.11**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	9.65	0.210	0.351	6	3.22	0.781	0.841
E3	Population	7	8.56	0.286	0.387	**6**	**18.70**	**0.005**	**0.013**
	Treatment	1	0.07	0.796	0.836	**1**	**9.88**	**0.00167**	**0.005**
	Pop. × Tr.	7	9.30	0.232	0.351	6	1.47	0.962	0.962
E4	Population	7	16.16	0.024	0.078	**6**	**36.29**	**<0.0001**	**<0.0001**
	Treatment	**1**	**7.52**	**0.006**	**0.028**	**1**	**75.50**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	6.04	0.536	0.643	6	3.80	0.704	0.799
E5	Population	7	12.91	0.074	0.177	**6**	**26.58**	**0.0002**	**0.0007**
	Treatment	**1**	**13.37**	**0.0003**	**0.0018**	**1**	**68.66**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	9.32	0.231	0.351	6	2.90	0.821	0.841
E6	Population	7	12.84	0.076	0.177	6	11.76	0.067	0.113
	Treatment	**1**	**56.14**	**<0.0001**	**<0.0001**	**1**	**107.13**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	9.00	0.253	0.363	6	7.72	0.260	0.352
E7	Population	7	15.34	0.032	0.096	**6**	**19.99**	**0.003**	**0.008**
	Treatment	**1**	**67.46**	**<0.0001**	**<0.0001**	**1**	**86.20**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	10.67	0.154	0.281	**6**	**14.79**	**0.022**	**0.049**
E8	Population	7	15.11	0.035	0.098	6	3.69	0.719	0.799
	Treatment	1	0.65	0.420	0.540	1	1.49	0.222	0.311
	Pop. × Tr.	7	9.96	0.191	0.334	6	4.10	0.663	0.796
E9	Population	7	12.56	0.084	0.186	**6**	**23.63**	**0.0006**	**0.0019**
	Treatment	**1**	**26.83**	**<0.0001**	**<0.0001**	**1**	**102.74**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	6.21	0.515	0.636	6	13.97	0.030	0.057
E10	Population	7	1.76	0.972	0.972	6	10.58	0.102	0.165
	Treatment	1	0.04	0.840	0.860	1	0.13	0.723	0.799
	Pop. × Tr.	7	5.46	0.604	0.655	6	14.12	0.028	0.056
E11	Population	7	13.34	0.064	0.168	6	12.34	0.055	0.096
	Treatment	**1**	**31.91**	**<0.0001**	**<0.0001**	**1**	**64.04**	**<0.0001**	**<0.0001**
	Pop. × Tr.	7	9.27	0.234	0.351	6	6.33	0.388	0.479
E12	Population	7	7.05	0.424	0.540	6	9.38	0.154	0.231
	Treatment	1	2.75	0.097	0.204	1	0.06	0.808	0.841
	Pop. × Tr.	7	5.53	0.596	0.655	6	9.41	0.152	0.231
E13	Population	7	16.84	0.018	0.063	6	14.14	0.028	0.056
	Treatment	1	0.26	0.608	0.655	**1**	**30.16**	**<0.0001**	**<0.0001**
	Pop. × Tr.	**7**	**17.85**	**0.013**	**0.0496**	**6**	**16.57**	**0.011**	**0.026**

Table 3. Results of linear mixed models testing for differences in growth and carbohydrate storage between watered and unwatered *Protea repens* seedlings and among 8 populations at the end of the dry-down experiment (day 12). . Significant values are bolded.

Response variable	Fixed effects	df	Chi-sq	p-val	q-val
Soluble sugars mass (g) belowground	Population	**7**	**55.57**	**<0.0001**	**<0.0001**
	Treatment	**1**	**6.33**	**0.012**	**0.019**
	Pop. × Trt.	**7**	**17.52**	**0.014**	**0.022**
Soluble sugars mass (g) aboveground	Population	**7**	**80.08**	**<0.0001**	**<0.0001**
	Treatment	**1**	**25.69**	**<0.0001**	**<0.0001**
	Pop. × Trt.	**7**	**24.27**	**0.001**	**0.002**
Starch mass (g) belowground	Population	**7**	**21.06**	**0.004**	**0.008**
	Treatment	1	0.10	0.748	0.765
	Pop. × Trt.	7	4.57	0.712	0.763
Starch mass (g) aboveground	Population	**7**	**57.40**	**<0.0001**	**<0.0001**
	Treatment	**1**	**60.10**	**<0.0001**	**<0.0001**
	Pop. × Trt.	**7**	**100.76**	**<0.0001**	**<0.0001**
Growth rate (height on Day 12-6)	Population	**7**	**34.85**	**<0.0001**	**<0.0001**
	Treatment	**1**	**194.34**	**<0.0001**	**<0.0001**
	Pop. × Trt.	**7**	**26.33**	**0.0004**	**0.0009**
Root length (mm)	Population	6	9.36	0.154	0.192
	Treatment	**1**	**6.66**	**0.010**	**0.017**
	Pop. × Trt.	6	6.3	0.391	0.463
Stem pigment accumulation	Population	**7**	**15.47**	**0.031**	**0.041**
	Treatment	**1**	**5.08**	**0.024**	**0.035**
	Pop. × Trt.	**7**	**15.58**	**0.029**	**0.040**

Table 4. Significant effects from mixed models testing associations between climate variables and plasticity measures, for traits and eigengenes on day 6 and day 12.

Day	Plasticity measure (RDPI)	Climate variable	P-val.	Slope	Plasticity high in pops. from:
Day 6	GN5	Elevation	0.015	0.0005	lower elevation
Day 12	GN10	Mean annual precip.	0.035	0.0014	wettest and driest sites
Day 12	GN13	Mean annual precip.	0.213	-0.0014	wetter sites
Day 12	Growth	Elevation	0.014	-0.0005	lower elevation
Day 12	Growth	Summer max. temp.	0.016	0.0709	warmer sites
Day 12	Leaf area	Mean annual precip.	0.019	0.0006	wetter sites
Day 12	Conductance	Temperature range	0.007	0.1235	higher heterogeneity
Day 12	Pigment content	Temperature range	0.039	0.0474	higher heterogeneity
Day 12	Pigment content	Summer max. temp.	0.041	0.0477	coolest and warmest sites

FIGURES

Fig 1. Map of CFR and locations of sampling sites (A); Principal component analysis for the climatic variables used in this study

Fig 2. Multi-dimensional scaling (MDS) plots for gene expression patterns at day 6 (A) and day 12 (B); principal component analysis of measured traits at day 6 (C) and day 12 (D) into the drought treatment.

Fig 3. Number of differentially expressed genes for the 8 populations for after 6 and 12 days of the drought treatment (A). Note that population A is not sampled and does not have any DE genes at day 12. Number of differentially expressed genes among populations under control and drought conditions for day 6 and 12 separately (B).

Fig 4. Heatmap of standardized regression coefficients for climate variables as predictors and traits and gene networks (GN) as response variables for measurements (A) on day 6 or (B) day 12. For variables with significant interaction effects, each treatment was analyzed separately to estimate slopes and test their significance within treatments. Solid boxes indicate a significant assosication and the box is divided in cases where there is a significant treatment and climate effect, indicating that the climate association was significant only in one of the treatments. The upper half of the box represents the watered treatment and the lower half represents the dry treatment. Stars indicate significant correlations for either of the two treatments.

Fig 5. Response curves for the studied 8 populations under well-watered and drought conditions and the associations between plasticity and climate variables for growth (A), leaf area (B), stomatal conductance (C) and gene network 13 (D).

Fig 6. Mean annual precipitation (A) and mean annual temperature (B) averaged across the 8 population sites between 1901-2016.

Supplementary info

Table S1. Population source site climatic information

Table S2. Correlations between climatic variables

Table S3. WGCNA GO categories and highest gene network membership genes

Table S4. Up and down-regulated genes for all the populations under drought

Table S5. Gene network and trait correlations

Figure S1. Sampling scheme

Figure S2. WGCNA sample clustering

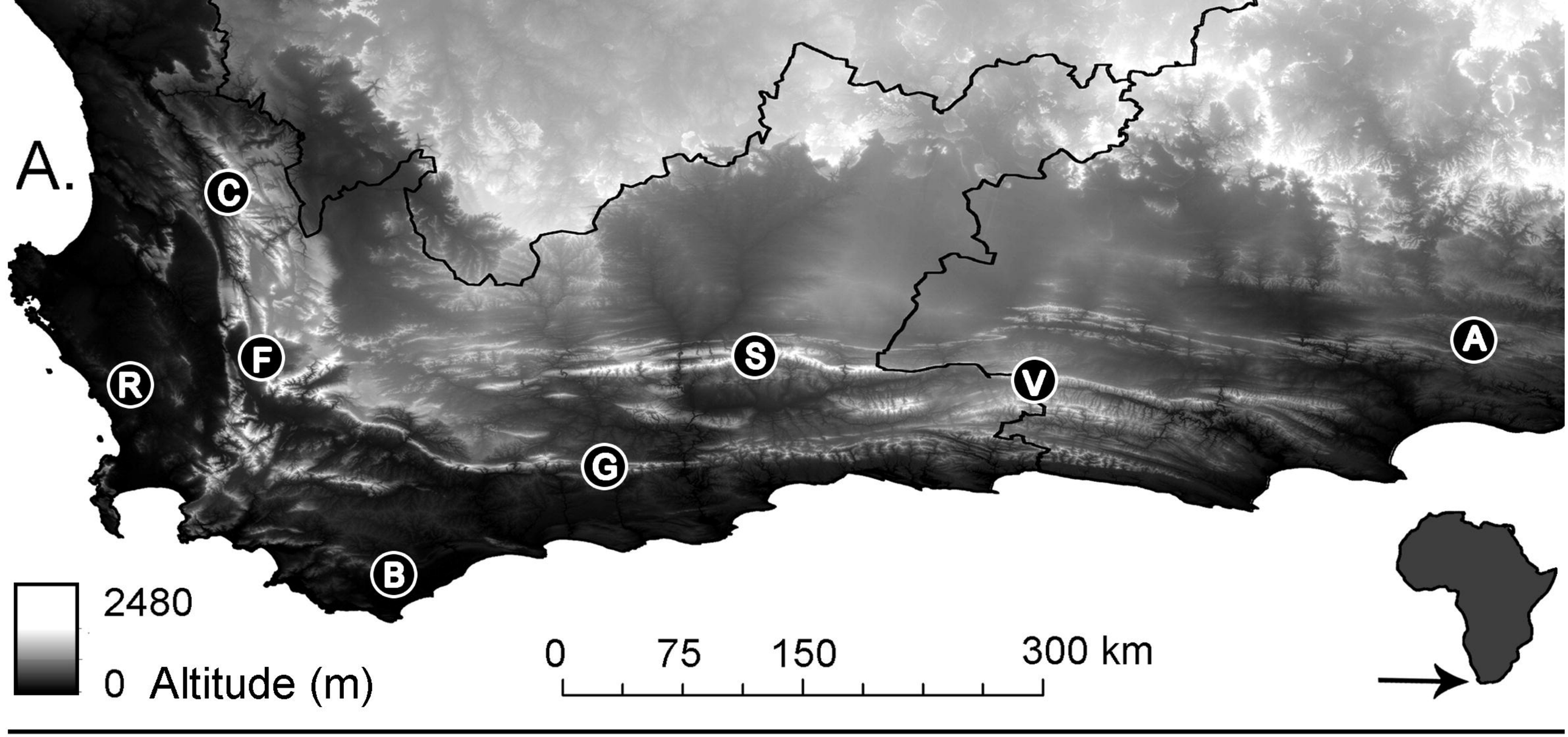
Figure S3. Functional traits response curves

Figure S4. Gene network response curves

Figure S5. Performance traits response curves

Figure S6. MDS plot with all samples for day 6 and day 12

Figure S7. Contigs that show a population x treatment effect, from figure 3


A.
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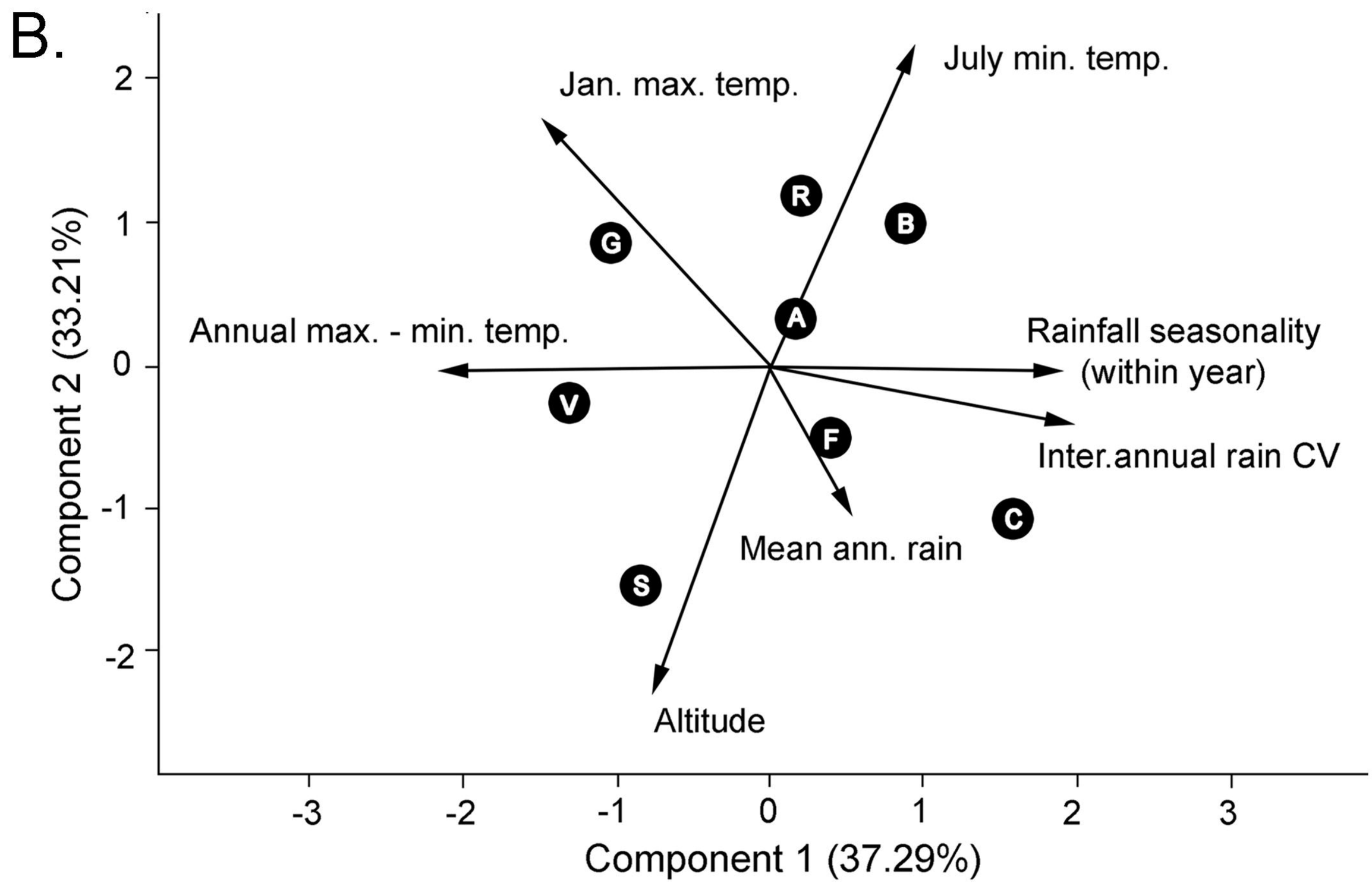
B.
Jan. max. temp.
July min. temp.
Annual max. - min. temp.
Rainfall seasonality (within year)
Inter.annual rain CV
Mean ann. rain
Altitude
R
B
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V
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S
Component 2 (33.21%)
Component 1 (37.29%)
2
1
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-1
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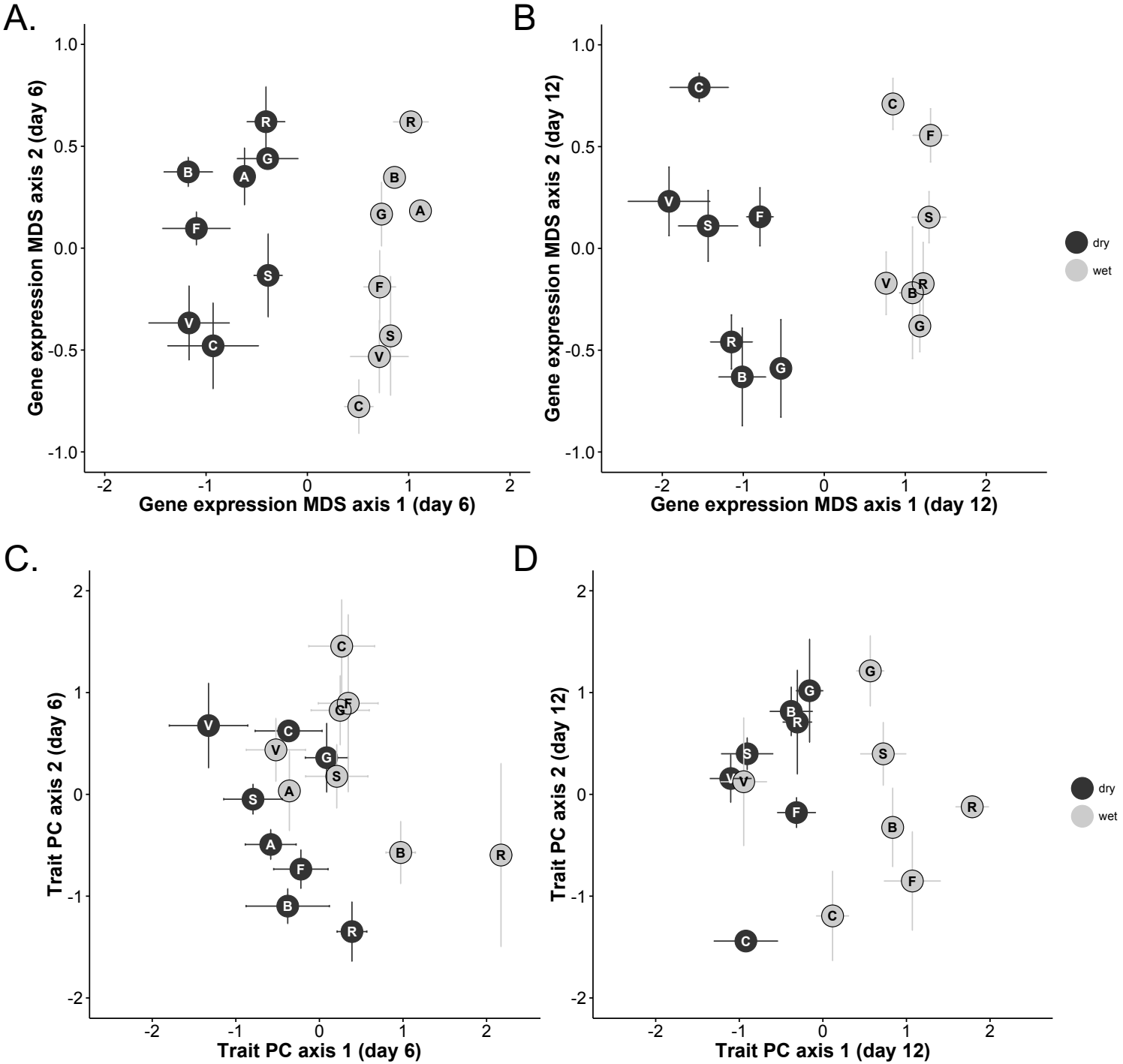
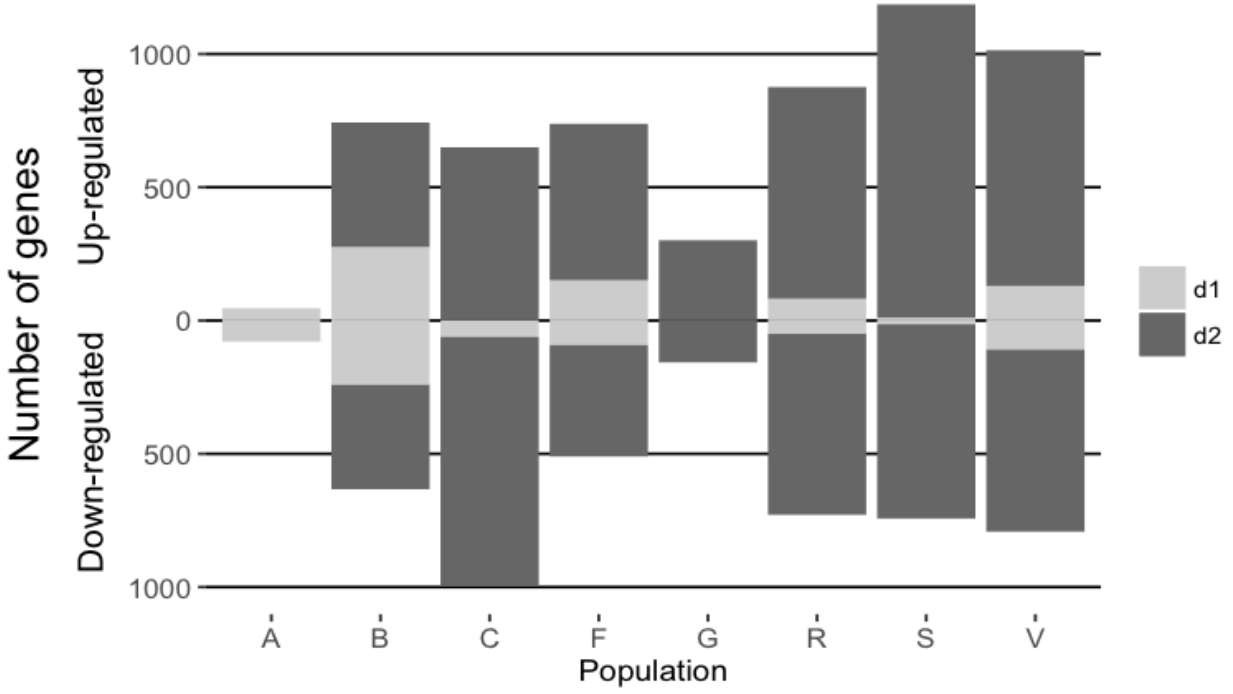
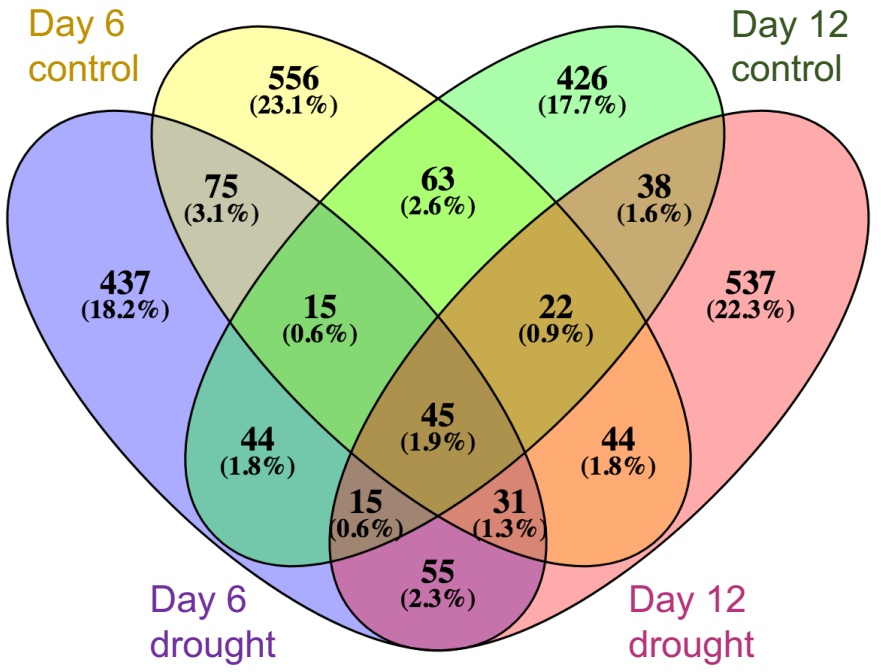
2
3

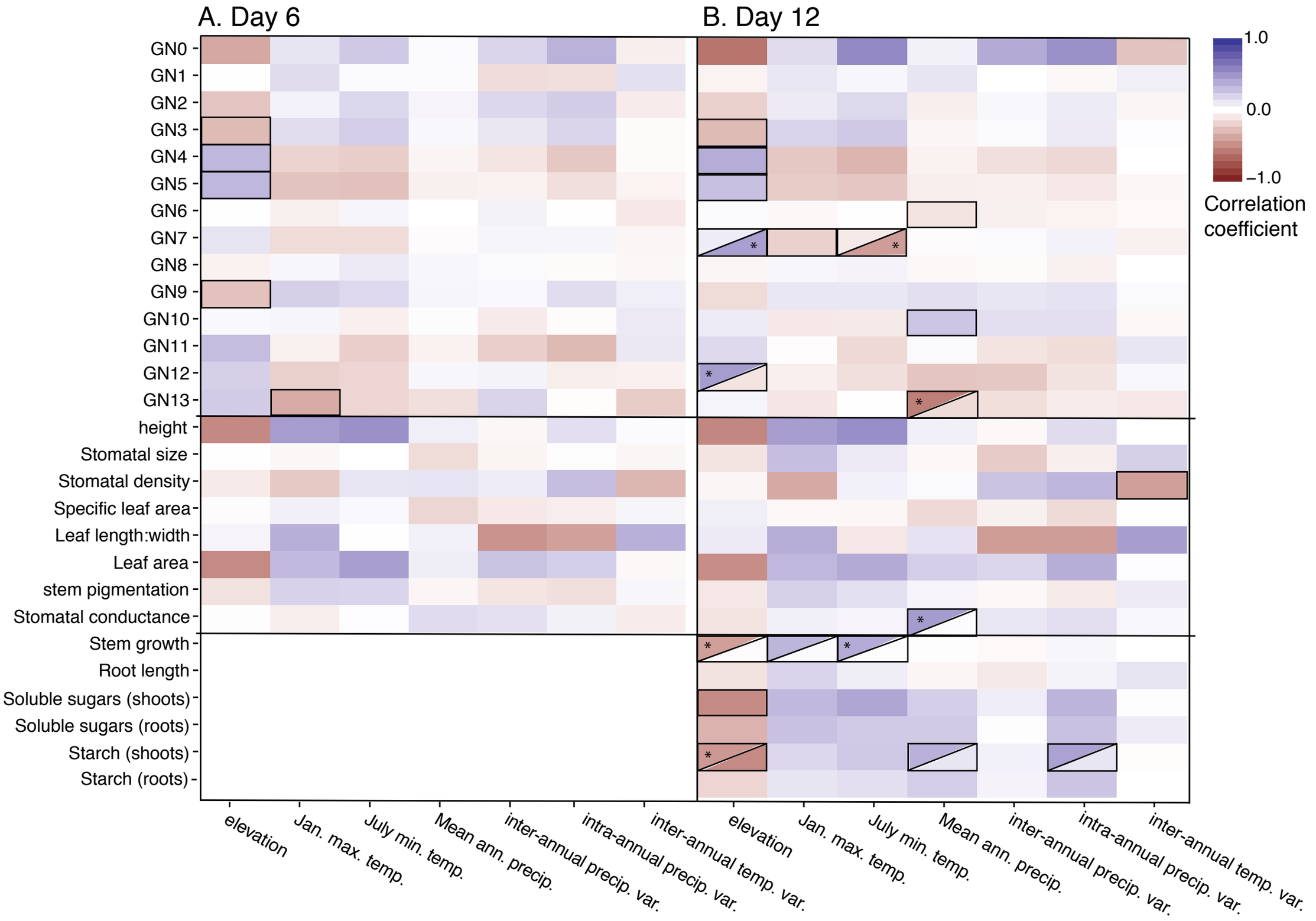

A.
B
C.
D
Gene expression MDS axis 2 (day 6)
Gene expression MDS axis 1 (day 6)
Gene expression MDS axis 2 (day 12)
Gene expression MDS axis 1 (day 12)
Trait PC axis 2 (day 6)
Trait PC axis 1 (day 6)
Trait PC axis 2 (day 12)
Trait PC axis 1 (day 12)
dry
wet

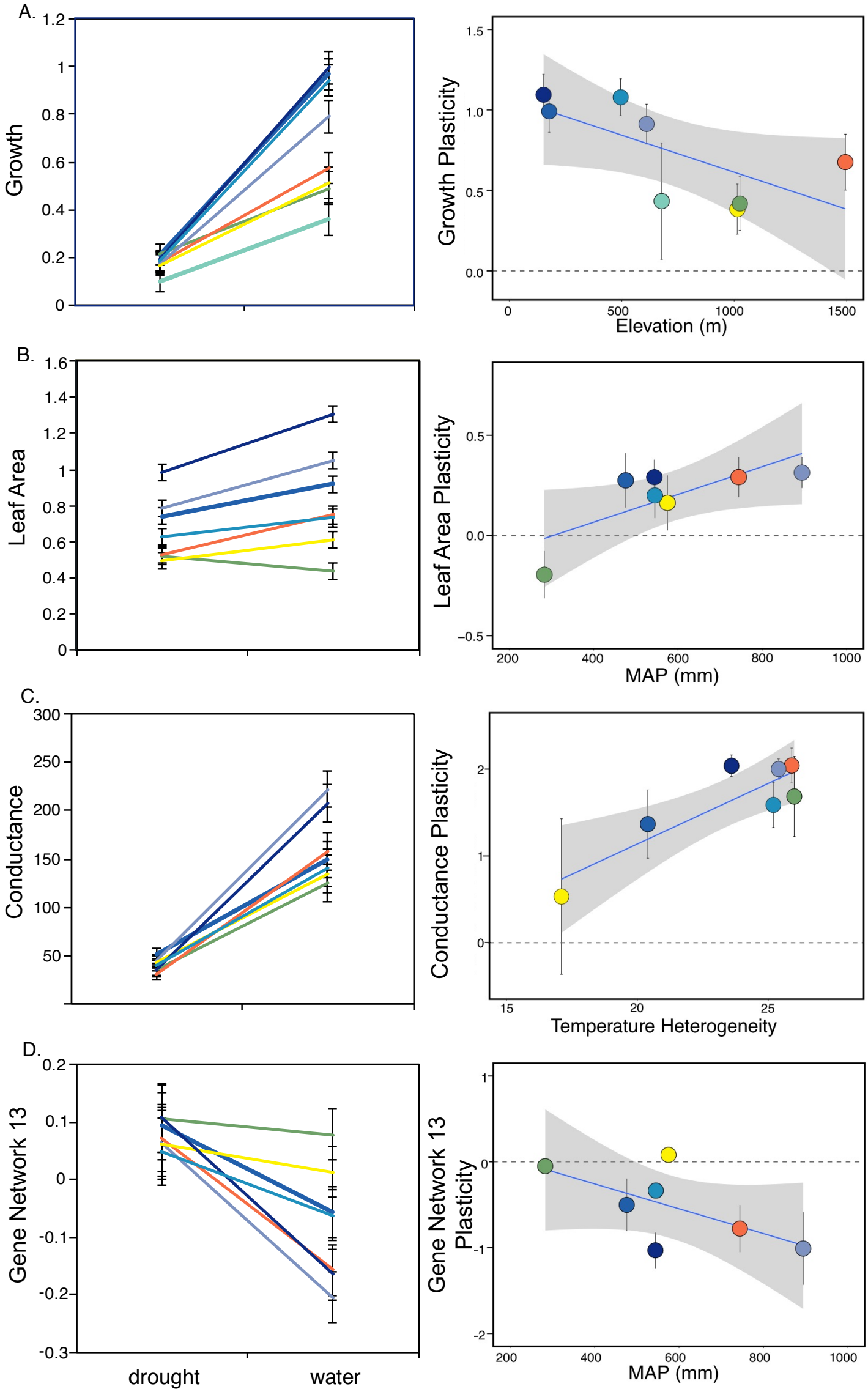
A.

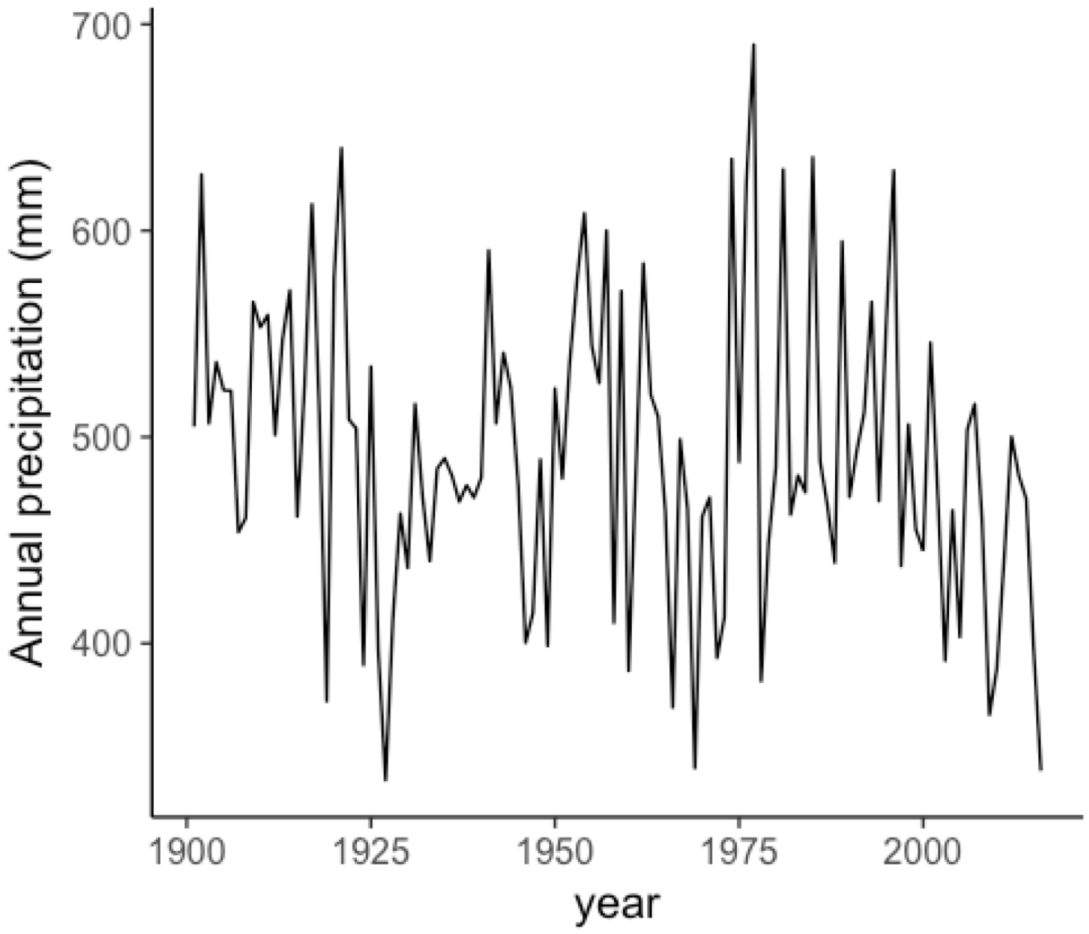

Number of genes
Up-regulated
Down-regulated
1000
500
0
500
1000
A
B
C
F
G
R
S
V
Population
d1
d2

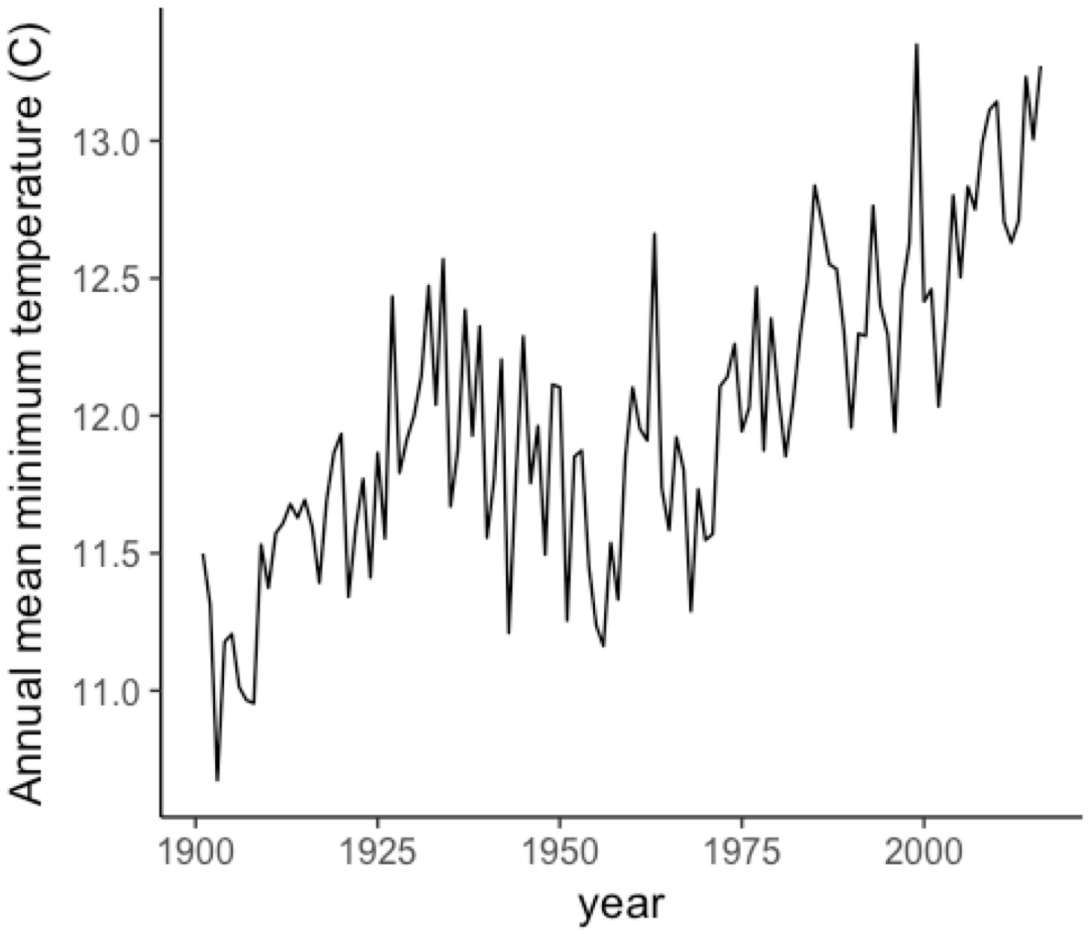
B.


Day 6 control
Day 12 control
Day 6 drought
Day 12 drought
556 (23.1%)
426 (17.7%)
437 (18.2%)
537 (22.3%)
75 (3.1%)
63 (2.6%)
38 (1.6%)
15 (0.6%)
22 (0.9%)
45 (1.9%)
44 (1.8%)
44 (1.8%)
15 (0.6%)
31 (1.3%)
55 (2.3%)


A. Day 6
B. Day 12
GN0
GN1
GN2
GN3
GN4
GN5
GN6
GN7
GN8
GN9
GN10
GN11
GN12
GN13
height
Stomatal size
Stomatal density
Specific leaf area
Leaf length:width
Leaf area
stem pigmentation
Stomatal conductance
Stem growth
Root length
Soluble sugars (shoots)
Soluble sugars (roots)
Starch (shoots)
Starch (roots)
elevation
Jan. max. temp.
July min. temp.
Mean ann. precip.
inter-annual precip. var.
intra-annual precip. var.
inter-annual temp. var.
1.0
0.0
−1.0
Correlation coefficient


A.
Growth
Growth Plasticity
Elevation (m)
B.
Leaf Area
Leaf Area Plasticity
MAP (mm)
C.
Conductance
Conductance Plasticity
Temperature Heterogeneity
D.
Gene Network 13
Gene Network 13 Plasticity
MAP (mm)
drought
water


Annual precipitation (mm)
700
600
500
400
1900
1925
1950
1975
2000
year


Annual mean minimum temperature (C)
13.0
12.5
12.0
11.5
11.0
1900
1925
1950
1975
2000
year