Int. J. Plant Sci. 175(4):442–449. 2014.
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1058-5893/2014/17504-0005$15.00 DOI: 10.1086/675573

DROUGHT RESPONSE DIVERSIFICATION IN AFRICAN *PROTEA* SPECIES

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Editor: Christina Caruso

Premise of research. Evolutionary radiations can be driven by physiological tolerances. *Protea* is a species-rich genus in the Cape Floristic Region of South Africa and provides the opportunity to examine whether drought response traits may have contributed to differentiation in this stress-tolerant genus. Commonly utilized drought responses might be indicative of traits important to species diversification in stressful environments.

Methodology. We studied how greenhouse-grown plants of six white *Protea* species physiologically responded to drought stress. We measured leaf-level physiological traits such as stomatal conductance, temperature, pubescence, chlorophyll, and abscisic acid (ABA) content.

Pivotal results. Most traits showed similar drought responses across all six species and 29 populations we examined. Only for foliar ABA content, leaf hair density, and foliar chlorophyll content did species respond to drought in different ways, indicating that some differences in plasticity might be important to this evolutionary radiation.

Conclusions. Our data support stomatal conductance as a trait important to stress tolerance across a range of environmental conditions. Moreover, population variation in the plasticity of physiological traits might be important to evolutionary trajectories in this system.

Keywords: Protea, drought stress, stomatal conductance, ABA content, leaf temperature, plasticity.

Introduction

Physiological tolerances can set a fundamental limit on range size (Hacker and Bertness 1995; Heschel et al. 2004; Lee et al. 2009); nonetheless, the precise physiological traits contributing to species' ranges are not well established (Lovell et al. 2009; Bibee et al. 2011). The role of physiology in species distribution patterns might be better understood by analyzing evolutionary radiations. In the Cape Floristic Region (CFR) of southwestern South Africa, strong rainfall gradients suggest that differentiation in drought response traits might play a key role in allowing lineages to persist across the entire CFR (Yates et al. 2009; Carlson et al. 2011). Thus, understanding drought physiology in a species-rich lineage of the CFR might elucidate the role of drought physiology diversification in species radiations.

The particular characters involved in evolutionary responses to drought are debated (Farnsworth 2004; Nicotra and Davidson 2010). Drought response traits are notoriously interconnected, and trade-offs with growth can limit their adaptability (Maherali et al. 2008); e.g., trade-offs between water use and carbon gain constrain the extent to which water loss can be reduced to relieve drought stress (Heschel et al. 2002). Decreased stomatal conductance (g_{ST}) in response to drought concomitantly reduces water efflux and carbon dioxide influx, limiting carbon assimilation. In high light conditions, a decrease in stomatal conductance that reduces the leaf boundary layer can also increase leaf temperature (Bazzaz 1979; Jones 1992; Heschel and Hausmann 2001), further reducing carbon assimilation by promoting photorespiration reactions (Kobza and Edwards 1987). Although cuticular wax and leaf pubescence can ameliorate such temperature increases (Bibee et al. 2011), membrane instability and a loss of chlorophyll can still result from thermal stress and negatively impact photosynthesis (Schrader et al. 2004; Barker et al. 2008). Both membrane instability and decreased chlorophyll content can contribute to lowered photosystem efficiency or the efficiency of photon capture for light reactions (i.e., chlorophyll fluorescence). Therefore, adapting to drought is difficult because of trade-offs between water use, carbon assimilation, and growth potential (Caruso et al. 2006).

One approach to better understanding the adaptive nature of drought response traits is to examine them at basal levels of organization, such as at the hormonal level (Sultan 2000; Farnsworth 2004). The phytohormone abscisic acid (ABA) is an example of such a trait. Due to the wide-ranging effects of changes in ABA sensitivity/endogenous content (Zhang et al. 2006), this phytohormone could be a central mechanism behind the evolution of drought tolerance (Heschel and Hausmann 2001). Intercellular levels of ABA increase across species

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Manuscript received July 2013; revised manuscript received October 2013; electronically published April 4, 2014.

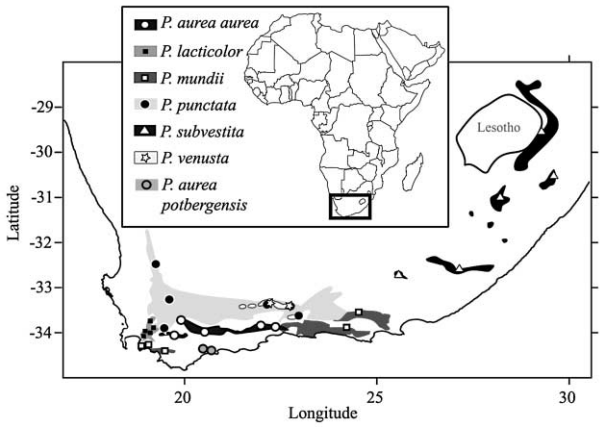

Fig. 1 Sampling locations for the seven *Protea* taxa (six species, one of which had two subspecies) in this study.

in response to drought stress (Farnsworth 2004). An increase in intercellular ABA causes stomatal closure and, as a result, increased water-use efficiency (WUE = carbon assimilation rate/stomatal conductance), thereby providing a mechanism for minimizing water consumption during plant growth (Davies et al. 1990; Heschel and Riginos 2005). ABA endogenous content also directly influences life history via interactions with flowering time genes (e.g., *FCA* and *FLC*; Chiang et al. 2009; Taiz and Zeiger 2010), so ABA would seem to be a phenological and physiological target of natural selection. Thus, it is plausible to suggest that plant populations could evolutionarily respond to drought selection via changes in ABA leaf content and/or ABA leaf sensitivity (Boggs et al. 2010).

Here we examine physiological traits associated with drought stress across plants derived from wild populations of white *Protea* species. Previous work with white *Protea* has demonstrated that species differ in the extent to which photosynthetic rates and specific leaf area change between wet and dry seasons, indicating the potential for drought response differentiation (Carlson and Holsinger 2012). Carlson and Holsinger (2012) also detected consistent decreases in carbon assimilation with dry conditions, but the degree of plasticity in these traits was variable between species. We measured functional traits related to drought stress tolerance on members of *Protea* section *Exsertae* in a controlled greenhouse environment. We considered the following experimental questions: How do white *Protea* species respond physiologically to drought stress in a greenhouse environment, and do species differ in their responses? Decreased photosystem efficiency/chlorophyll content, increased ABA content, and decreased stomatal conductance are likely physiological responses to drought stress.

Material and Methods

Population Background

In early 2008, seeds from seven *Protea* taxa (six species, one of which had two subspecies) were collected from across their respective distributions (fig. 1): *Protea aurea* ssp. *aurea* (*aur*), *P. aurea* ssp. *potbergensis* (*aurpot*), *P. lacticolor* (*lact*), *P. mundii* (*mund*), *P. punctata* (*punc*), *P. venusta* (*venu*), and *P. subvestita* (*sub*). Annual rainfall across the sampled populations ranged from 255 to 2069 mm, and median monthly rainfall was <50 cm for *aur*, *punc*, and *venu* populations and >50 cm for *lact*, *mund*, and *sub* populations (see Carlson et al. 2011 for rainfall details).

Experimental Setup

Seeds were used to establish lines in the University of Connecticut (Storrs) greenhouse in 2009 following the protocol in Prunier et al. (2012). These seeds were derived from the same maternal lines as those used to establish two common gardens in South Africa (Carlson et al. 2011; Carlson and Holsinger 2012) and for the greenhouse study of Prunier et al. (2012). Twenty-nine populations from the seven taxa were represented in the greenhouse (total 300 plants, 3–14 plants per population per treatment). The soil consisted of a well-drained matrix of sand, peat moss, perlite, and charcoal (the substrate was primarily sand and peat). All species were regularly watered and fertilized prior to the start of this experiment. Drought stress was imposed on the white *Proteas* in this greenhouse in July 2010 by withholding water from approximately half of the plants for ~2 wk, with one watering midway through the drought treatment (note: this watering was necessary to prevent mortality, indicating that all species were experiencing significant drought stress). Nondrought treatment plants were watered approximately twice a week throughout that same period. Drought stress intensity in the greenhouse was characterized using time-domain reflectometry (TDR; HydroSense probe, Campbell Scientific). The volumetric water content (VWC) of each pot was measured with the TDR probe. Light and relative humidity (RH) conditions were measured with a photosynthetically active radiance quantum-light sensor (Field Scout, Spectrum Technologies) and an RH probe (Traceable Humidity Pen, Fisher Scientific), respectively. We measured the following traits on all plants: endogenous foliar ABA content, water use (stomatal conductance), chlorophyll fluorescence, leaf chlorophyll content, leaf temperature, and leaf hair density (i.e., pubescence). Measurements were taken while the drought treatment was imposed in July 2010 (~5 d after midpoint watering during the drought treatment); plants were randomly measured across both treatments to avoid an association between species and time. ABA content was determined later from leaves sampled during this drought stress episode.

Leaf Physiology Methods

Stomatal conductance measurements. Stomatal conductance measurements (g_{ST} = mmol H_2O m^{-2} s^{-1}) were taken with a steady-state leaf porometer (SC1, Decagon Devices) on every plant. The porometer recorded the temperature, date, and time for each measurement. Stomatal conductance measurements were recorded only when ambient light levels were ≥700 μmol photons m^{-2} s^{-1}. In order to keep light levels consistent between measurements, each leaf was exposed to 1000 μmol photons m^{-2} s^{-1} of supplemental diode light. Stomatal conductance was measured on the abaxial side of recently fully expanded *Protea* leaves; also, care was taken to position leaves in the cuvette precisely the same way for every measurement

Table 1

Nested ANOVA for Species, Population (Nested within Species), and Drought Treatment on Soil Moisture Availability during Weeks 1 and 2 of the Drought Treatment

Effect	Soil moisture week 1	Soil moisture week 2
Treatment	1, 1,238.47*	1, 1,256.22*
Population (species)	22, .96	22, .81
Species	6, 1.76	6, 1.09
Treatment × population	22, .92	22, 1.17
Treatment × species	6, 2.02	6, .83

Note. Data are df, *F* values.
* $P < 0.001$.

(note: white *Protea* are bifacial, but abaxial stomata were examined here for consistency between measurements). Recently expanded leaves that were three nodes from the primary shoot apex were chosen for measurement. Because these data were collected per unit area per unit time, stomatal density should not be the primary determinant of species differences in conductance. Conductance values may be set in part by the number and size of stomata, but they will also be greatly influenced by cuticular wax and ABA (Bibee et al. 2011). In fact, previous work on these same species demonstrated that stomatal density and stomatal size were not significantly correlated with stomatal conductance (Carlson and Holsinger 2012).

Chlorophyll fluorescence, chlorophyll content, leaf temperature, and leaf hairs. Fluorescence, chlorophyll content, temperature, and pubescence measurements were taken on the same recently fully expanded leaves as stomatal conductance. Fluorescence (F_v/F_m) was measured with an OS-30p chlorophyll fluorometer (Opti-Sciences), chlorophyll content (mg) was determined with an Opti-Sciences chlorophyll meter (CCM-200), leaf temperature was assessed with an infrared temperature probe (Extech Instruments), and leaf hair density was estimated with digital photographs. All four measurements were taken in the same part of the leaf across plants. Leaf temperature was measured on the adaxial side of leaves at the midrib and taken when light levels were ≥700 μmol photons m^{-2} s^{-1}; leaf temperature was determined ~1 min before each stomatal conductance measurement. Chlorophyll content was estimated on all plants after temperature and conductance were measured (note: only 3 d of drought stress are necessary to observe a decline in foliar chlorophyll content; Alberte and Thornber 1977). F_v/F_m or photon capture efficiency was assessed on dark-adapted leaves ~1 h after sunset. Fluorescence was measured on a subset of the total sample after conductance and temperature readings were taken on a given leaf. Leaves were clamped with dark-adapting clips for ~5 min prior to each measurement. F_v/F_m was based on 20 s of fluorescence readings from 0 to 1000 μmol photons m^{-2} s^{-1} of red light and calculated as $(F_o - F_m)/F_m$. Leaf hair density was estimated on a subset of the plants across both moisture treatments. A digital photo was taken of the same leaf used to assess leaf temperature, and ImageJ (National Institutes of Health, Bethesda, MD) was used to capture the images and calculate leaf hair area on adaxial surfaces. A 1.5-cm^2 area was sampled for each leaf hair density estimate.

ABA Quantification Methods

Leaf harvest. One leaf sample per plant was harvested from every greenhouse plant (~0.5 g leaf tissue per sample); the harvested leaf was the same leaf on which the aforementioned traits were measured. Leaves were placed on ice and transferred to a −80°C freezer immediately after harvest and shipped to Colorado College in Colorado Springs, Colorado, on dry ice. Leaves were stored in the dark at −20°C until starting the extraction procedure.

ABA extraction. Leaves were weighed and then lyophilized for 24 h. Dried tissue was ground with a Polytron tissue grinder (Brinkmann Instruments) in an ABA extraction buffer containing methanol, butylated hydroxytoluene, and citric acid monohydrate (Walker-Simmons 1987; Bibee et al. 2011). The lyophilized material was incubated in the extraction buffer for 12 h at 4°C and then spun at 2000 *g* for 10 min; dried supernatant was resuspended in Tris-buffered saline (with $MgCl_2$; Boggs et al. 2010). Enzyme-linked immunosorbent assay (ELISA) was used to quantify ABA in leaf extracts. Serial dilutions of ABA (+/− mixed isomers, Sigma-Aldrich) were used to generate standard curves with an Optima plate reader (BMG Labtech) set to absorbance at 405 nm. ELISA reactions were performed in low light (to prevent ABA degradation) in microtiter trays. ABA tracer and substrate antigen/antibody reactions (Agdia) were quantified with an Optima plate reader to generate molar concentrations per milligram of leaf extract (Boggs et al. 2010). All absorbance readings were considered relative to a blank sample in each set of ELISA reactions.

Statistical Methods

All statistical analyses were performed in JMP, version 7.0.2 (SAS Institute, Cary, NC). For all ANOVA models, residual variation was normally distributed. Nested ANOVAs (standard least squares) were used to examine the effects of species, population (species), drought treatment, and their interactions on soil water content, stomatal conductance, leaf temperature, leaf hair density, leaf chlorophyll content, and leaf ABA content. Population was nested within species and treated as a random effect in analyses; drought treatment and species were

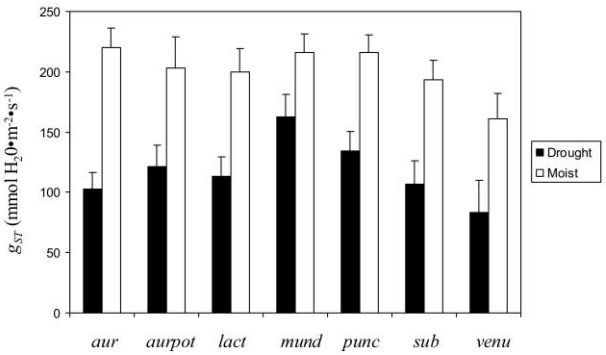

Fig. 2 Mean stomatal conductance (g_{ST}) for white *Protea* species in drought and moist greenhouse conditions. Abbreviations are as follows: *aur* = *P. aurea* ssp. *aurea*, *aurpot* = *P. aurea* ssp. *potbergensis*, *lact* = *P. lacticolor*, *mund* = *P. mundii*, *punc* = *P. punctata*, *sub* = *P. subvestita*, and *venu* = *P. venusta*.

considered fixed factors. For the fluorescence model, sample size issues precluded the use of every population (two populations were excluded). Also, in our analyses of leaf hair area, the population × treatment interaction term was excluded due to sample size constraints.

Tukey's honest significant difference (HSD) contrasts on least square means were conducted when significant or marginally significant effects were detected in our ANOVA models. Where appropriate, contrasts were conducted to examine whether species from dry environments differed in drought response physiology. In order to examine relationships between some traits, Pearson product-moment correlations were calculated across species for each drought treatment. Correlations between ABA content and stomatal conductance as well as between conductance and leaf temperature were selected to examine a priori expectations about the associations between transpiration, hormonal control, and temperature.

Stomatal conductance and leaf temperature data were adjusted for effects of measurement time and date to account for conductance changes throughout a given day (Heschel et al. 2002). Conductance and temperature data were regressed against measurement time and day; residuals from these linear regressions were added to the conductance and temperature grand means. Ambient temperature was not included in the time and date conductance models because environmental conditions were taken into account with time and day effects. These time-adjusted conductance and leaf temperature data were used in all analyses.

Results

Greenhouse Conditions

Greenhouse conditions were warm and moist (temperature: 25°–30°C, reaching 35°C in the afternoon on sunny days; average RH: 54%). For moist treatment plants, soil moisture averaged ~14%–15% VWC. For drought treatment plants, soil moisture averaged ~3%–4% VWC. Significant differences in VWC were detected between drought treatments regardless of species or population identity (table 1). Light conditions in the greenhouse ranged from ~500 to 800 μmol photons m^{-2} s^{-1}.

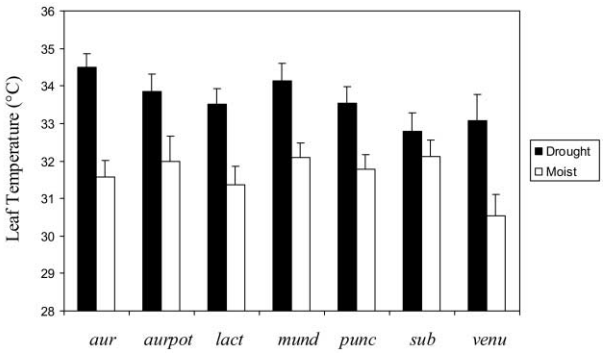

Fig. 3 Mean leaf temperatures for white *Protea* species in drought and moist greenhouse conditions. Abbreviations are as in fig. 2.

Stomatal Conductance

Across all the study species, plants responded to drought stress by significantly decreasing stomatal conductance values (fig. 2; table 2, significant treatment effect). These data indicated that all species were experiencing drought stress. Also, there were marginally significant overall species differences in conductance responses regardless of drought treatment (table 2, marginally significant species effect).

Leaf Temperature and Leaf Hairs

Across all the study species, leaf temperatures significantly increased under drought conditions (fig. 3; table 2, significant treatment effect). Neither a significant effect of species nor of population was detected on leaf temperature (fig. 3; table 2). Nonetheless, a trend in species differences was observed: *sub* plants had the lowest mean leaf temperatures under drought conditions (fig. 3). Interestingly, *sub* plants also had significantly greater leaf hair density than the other species (table 2, significant species effect; fig. 4). Across all species, leaf temperature and stomatal conductance were more strongly correlated in drought conditions ($r = -0.51$, $P < 0.0001$) than in moist conditions ($r = -0.28$, $P < 0.01$).

Table 2

Nested ANOVA for Species, Population (Nested within Species), and Drought Treatment on Physiological Traits

Effect	g_{ST}	Leaf temperature	Leaf hair area	Chlorophyll	Fluorescence	ABA
Drought treatment	1, 69.98***	1, 58.47***	1, .053	1, .15	1, .29	1, 6.82**
Population (species)	22, 1.16	22, 1.45	11, 2.38	22, .74	20, 1.15	22, 1.75*
Species	6, 1.94****	6, 1.02	6, 2.94*	6, 2.49*	6, .75	6, 1.53
Treatment × population	22, .84	22, 1.10	NA	22, 1.60*	20, .69	22, 2.01**
Treatment × species	6, .68	6, 1.23	6, 1.82	6, 0.54	6, .28	6, 2.42*

Note. Data are df, *F* values. Leaf temperature and stomatal conductance values (g_{ST}) were adjusted for measurement time. ABA = foliar abscisic acid concentration per milligram fresh mass, NA = nonapplicable effect due to sampling issues. Replication was as follows (abbreviations are as in fig. 2): drought treatment (D), *aur* = 32; D, *aurpot* = 19; D, *lact* = 23; D, *mund* = 17; D, *punc* = 22; D, *sub* = 20; D, *venu* = 12; moist treatment (M), *aur* = 21; M, *aurpot* = 12; M, *lact* = 22; M, *mund* = 31; M, *punc* = 31; M, *sub* = 25; M, *venu* = 17.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

**** $P < 0.10$.

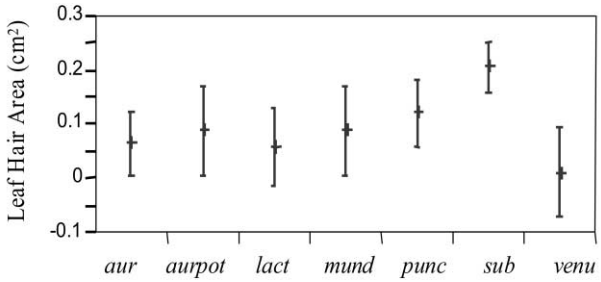

Fig. 4 Mean leaf hair density ± 1 SE in area units for white *Protea* species across moisture conditions in the greenhouse. Abbreviations are as in fig. 2.

Leaf Chlorophyll Content and Fluorescence

Significant species differences in foliar chlorophyll content were detected regardless of drought treatment (fig. 5; table 2, significant species effect). *Lact* and *punc* plants had significantly higher overall amounts of leaf chlorophyll than the other taxa regardless of treatment (fig. 5; both species were significantly different from all other species; $P = 0.05$, Tukey's HSD). Interestingly, within species, population responses to drought were significantly variable with regard to leaf chlorophyll content (table 2, significant population [species] × treatment effect). Neither a significant effect of drought treatment nor of species was detected on chlorophyll fluorescence (table 2). The mean fluorescence of >0.70 (F_v/F_m) across all species and treatments indicated that the experimental plants had relatively efficient photosystems and that drought had not damaged photon capture potential (fig. 6).

Leaf ABA Content

ABA production in response to drought depended on species (table 2, significant species × treatment interaction term); e.g., *sub* and *venu* plants produced more ABA in response to drought than the other species (fig. 7; both species were significantly different from all other species in drought conditions; $P = 0.05$, Tukey's HSD). In contrast, *aur* and *punc* plants did not significantly increase foliar ABA in response to drought stress (fig. 7; $P = 0.05$, nonsignificant Tukey's HSD for both species contrasting drought vs. moist conditions). Significant population differences in foliar ABA content were detected in response to drought treatment (table 2, significant population [species] × treatment interaction term). Also, increased ABA content was correlated with decreased stomatal conductance values for *sub* and *venu* ($r = -0.59$, $P = 0.09$) in drought conditions but not significantly correlated in moist conditions ($r = 0.13$, $P = 0.61$). ABA and conductance were not significantly correlated across the other species for either drought treatment. This correlation for *sub* and *venu* was probably not the result of differences in the severity of drought stress for these taxa because no species differences were observed in either stomatal conductance (table 2) or soil VWC (table 1).

Discussion

Protea *Species and Foliar ABA Differences*

The *Protea* species examined were drought tolerant via mechanisms to avoid desiccation; however, the specific suite of traits involved with this tolerance varied by species. Of the white *Protea* species studied here, *venu*, *aur*, and *punc* inhabit the driest conditions in the wild, with *sub* occupying dry, savannah-like conditions. Thus, we might more closely consider the physiological responses of these taxa to determine whether any drought response traits might be particularly important to water relations. All the species sampled demonstrated significant plasticity in stomatal conductance in response to drought stress, but some trait differences were observed between species; e.g., *sub* and *venu* were the only species to employ plasticity in ABA. Differences in the degree of plasticity among *Protea* species has been observed previously for other traits. In a common garden study using many of the same species and source populations, Carlson and Holsinger (2012) found that *punc* had relatively low plasticity in light-saturated photosynthetic rates between the wet and dry seasons in South Africa, whereas *aur* had relatively high plasticity (*sub* and *venu* were not included in the study). Therefore, among these closely related taxa (Valente et al. 2010), there is a lack of stasis in the evolution of different types of plasticity; i.e., none of these lineages seem to be constrained to evolve along a particular physiological axis.

All the species examined here responded to drought stress by decreasing stomatal conductance to some degree; however, while *sub* and *venu* produced significantly more ABA in drought, foliar ABA concentration did not increase with drought stress for *aur* and *punc*. Because the degree of conductance plasticity was indistinguishable among species, *aur* and *punc* were potentially more sensitive to small changes in ABA in order to effectively close stomata (Heschel and Hausmann 2001). This is supported by the fact that ABA content and stomatal conductance were negatively correlated only for *sub* and *venu*. Therefore, the precise mechanism of stomatal closure probably varied by taxon; ABA concentration and sensitivity seem to be evolutionarily labile traits. Interestingly, ABA concentration seemed to be the most variable trait ex-

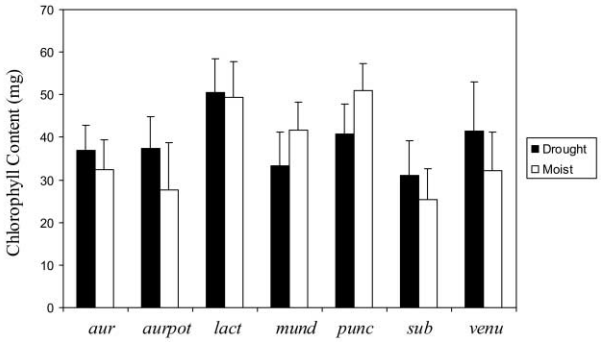

Fig. 5 Mean leaf chlorophyll content for white *Protea* species in drought and moist greenhouse conditions. Abbreviations are as in fig. 2.

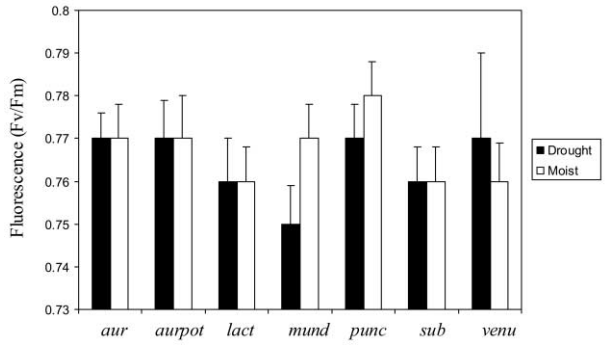

Fig. 6 Mean fluorescence for white *Protea* species in drought and moist greenhouse conditions. Abbreviations are as in fig. 2.

amined; i.e., a significant population × treatment interaction term was detected for ABA content (table 2). ABA may be under strong selection in dry conditions, but selection does not seem to cull variation from lineages. Variation in ABA response might provide fitness advantages across seasons and between years for perennial species such as members of the genus *Protea*. Benign seasons/years might select for decreased foliar ABA and increased gas exchange, while stressful times might select for the opposite. Moreover, if drought and high light covary in a particular environment, then decreased ABA might be a target of selection (Heschel and Hausmann 2001). Lower levels of ABA would promote increased gas exchange rates and boundary layer maintenance, thereby ameliorating temperature stress. An alternate explanation is that each species spans a broad range of environmental conditions, and populations within species have become locally adapted (Prunier and Holsinger 2010). Previous common garden work on these same *Protea* species supports this explanation. Carlson et al. (2011) found significant differences in leaf area, length-width ratio, growth rate, and specific leaf area among conspecific populations grown in a common garden, and these trait differences were related to variation in drought stress and cold stress in their home environment. A subsequent study by Prunier et al. (2012) further showed that the pattern of trait variation along environmental clines within species was often idiosyncratic among species; i.e., populations of *mund* and *punc* in warmer areas had relatively heavy roots, whereas *aur* populations in warmer areas had relatively light roots.

Species Divergence and Leaf Characteristics

Leaf pubescence might promote variability in ABA responses. All the white *Protea* species examined have some degree of leaf pubescence on adaxial surfaces; these leaf hairs may help to maintain a leaf boundary layer and prevent overheating. However, *sub* is a unique species in that it utilizes both increased leaf hair density and increased ABA content to ameliorate stress. Interestingly, the *sub* populations in this study do not occupy the driest sites sampled (based on mean annual precipitation), but *sub* populations are typically found in bright savannah habitats (Rebelo 2006), making leaf temperature as well as water use potential targets of selection (Bazzaz 1979; Bibee et al. 2011). Moreover, *sub* populations are unique in that they occur in grasslands characterized by summer rainfall and episodic drought (Rebelo 2006).

Leaf hairs might be critical in maintaining decreased leaf temperature in drought conditions. *Sub* plants had relatively low leaf temperatures under drought stress (fig. 3), yet they also produced significantly more ABA than the other species (with the exception of *venu*; fig. 7). Increased ABA levels should have led to higher leaf temperatures due to decreases in stomatal conductance/boundary layer, but pubescence may have provided a mechanism for circumventing the trade-off between water loss and temperature. Our correlation analyses support this assertion because stomatal conductance and leaf temperature were negatively correlated regardless of treatment. Therefore, increased leaf pubescence might provide a mechanism whereby ABA responsiveness can evolve despite tradeoffs between decreased stomatal conductance, loss of leaf boundary layer, increased leaf temperature, and photorespiration.

All white *Protea* species in this study maintained functional photosystems in drought conditions. Despite some leaf chlorophyll content differences between species, chlorophyll fluorescence was unaffected by either stress or lineage. It is unknown whether leaf pigments, succulence, or thylakoid membrane characteristics might help to maintain photosystem integrity; however, given population differentiation for specific leaf area, a measure of sclerophylly (Carlson et al. 2011), it seems likely that sclerophylly is an important trait in photosynthetic homeostasis. Moreover, these data suggest that white *Protea* are able to effectively photosynthesize during stress.

Conclusions

Given the ubiquity of conductance responses to drought, there might be strong selection on stomatal conductance in dry conditions for these *Protea* species. The lack of differences in species' conductance responses to drought (table 2, nonsignificant species × treatment interaction term for g_{ST}) suggests that selection for decreased stomatal conductance in drought may have increased species similarity in plasticity; i.e., conductance plasticity is evolutionarily conserved across these white *Protea* taxa. However, within species, we detected population variability in ABA levels, a primary mechanism influ-

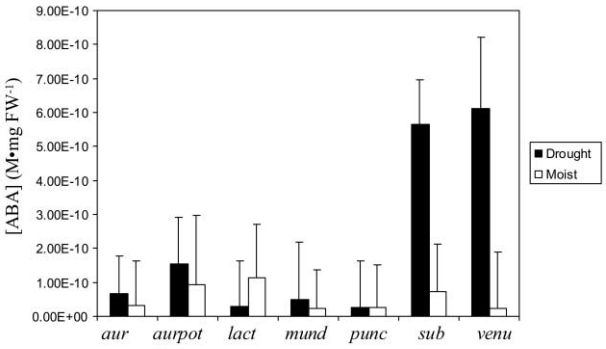

Fig. 7 Mean molar abscisic acid (ABA) foliar concentration per milligram fresh mass (FW) for white *Protea* species in drought and moist greenhouse conditions. Abbreviations are as in fig. 2.

encing stomatal closure. This discrepancy in species similarity might be a consequence of selection for other traits that covary with ABA levels such as leaf temperature and flowering time. Species dissimilarity in ABA content might also be a consequence of different trait-environment relationships among species (Prunier et al. 2012) or differences in the timescale of drought response among species; e.g., when Carlson and Holsinger (2012) compared plasticity between wet and dry seasons, they also found that all white *Protea* species had similar degrees of plasticity in conductance and other stomatal traits. However, interannual variation in stomatal pore index was detected for one species.

Drought response traits are certainly important to evolutionary radiations in the white *Protea* species studied here. Observed species-level differences in ABA and leaf characteristics support this assertion. In particular, stomatal conductance seems to be a trait important to drought tolerance across all species, but the degree of ABA plasticity is variable between species. This variability might be important in the ability of certain species to maximize carbon assimilation under moist conditions and to ameliorate temperature stress. The fact that different suites of traits have evolved for different taxa also indicates that functional homeostasis in drought might be maintained with mechanisms tailored to particular microclimates. Moreover, for ABA and chlorophyll content, significant variation existed among populations of some species. Therefore, given the lack of consistent trait differentiation among the most drought tolerant taxa, it seems possible that the maintenance of genetic variation among populations for drought response traits is as important to *Protea* speciation as the precise identity of the traits.

Acknowledgments

We thank R. Prunier for her work to establish the *Protea* populations in the University of Connecticut greenhouse and C. Morse and K. Theiss for their help with experimental setup and greenhouse collection maintenance. We also thank K. Tebo at the University of Connecticut and C. Noble, D. Sison, and D. Winkelblech at Colorado College for logistical support. The manuscript benefited from comments by R. T. Heschel and two anonymous reviewers. Our research was supported by a faculty-student collaborative research grant provided by Colorado College as well as a National Science Foundation Research Opportunity Award to K. E. Holsinger and M. S. Heschel. Additionally, we thank T. Rebelo at the South African National Biodiversity Institute as well as staff and reserve managers of the following South African organizations for their logistical support and permission in seed collections: Cape Nature (permit AAA-0005-00093-0028), Esemvelo KZN (1789/2008), and the Eastern Cape Parks Board and Department of Water Affairs and Forestry (CRO.23/08CR).

Literature Cited

Alberte RS, JP Thornber 1977 Water stress effects on the content and organization of chlorophyll in mesophyll and bundle sheath chloroplasts of maize. Plant Physiol 59:351–353.

Barker DH, GGR Seaton, SA Robinson 2008 Internal and external photoprotection in developing leaves of the CAM plant *Cotyledon orbiculata*. Plant Cell Environ 20:617–624.

Bazzaz FA 1979 The physiological ecology of plant succession. Annu Rev Ecol Syst 10:351–371.

Bibee K, K Shishido, RP Hathaway, MS Heschel 2011 Population differentiation of *Impatiens capensis* (Balsaminaceae) at the range limit. Int J Plant Sci 172:211–219.

Boggs J, K Loewy, K Bibee, MS Heschel 2010 Phytochromes influence stomatal conductance plasticity in *Arabidopsis thaliana*. Plant Growth Regul 60:77–81.

Carlson JE, KE Holsinger 2012 Developmental plasticity in *Protea* as an evolutionary response to environmental clines in the Cape Floristic Region. PLoS ONE 7:e52035. doi:52010.51371/journal.pone.0052035.

Carlson JE, KE Holsinger, R Prunier 2011 Plant responses to climate in the Cape Floristic Region of South Africa: evidence for adaptive differentiation in the Proteaceae. Evolution 65:108–124.

Caruso CM, H Maherali, M Sherrard 2006 Plasticity of physiology in *Lobelia*: testing for adaptation and constraint. Evolution 60:980–990.

Chiang GCK, D Barua, EM Kramer, RM Amasino, K Donohue 2009 Major flowering time gene, *FLOWERING LOCUS C*, regulates seed germination in *Arabidopsis thaliana*. Proc Natl Acad Sci 106:11661–11666.

Davies WJ, TA Mansfield, AM Hetherington 1990 Sensing soil water status and the regulation of plant growth and development. Plant Cell Environ 13:709–719.

Farnsworth E 2004 Hormones and shifting ecology throughout plant development. Ecology 85:5–15.

Hacker SD, MD Bertness 1995 Morphological and physiological consequences of a positive plant interaction. Ecology 76:2165–2175.

Heschel MS, K Donohue, NJ Hausmann, J Schmitt 2002 Population differentiation and natural selection for water-use efficiency in *Impatiens capensis* (Balsaminaceae). Int J Plant Sci 163:907–912.

Heschel MS, NJ Hausmann 2001 Population differentiation for abscisic acid responsiveness in *Impatiens capensis* (Balsaminaceae). Int J Plant Sci 162:1253–1260.

Heschel MS, C Riginos 2005 Mechanisms of selection for drought stress tolerance and avoidance in *Impatiens capensis* (Balsaminaceae). Am J Bot 92:37–44.

Heschel MS, SE Sultan, S Glover, D Sloan 2004 Population differentiation and plastic responses to drought stress in the generalist annual, *Polygonum persicaria*. Int J Plant Sci 165:817–824.

Jones HG 1992 Plants and the microclimate: a quantitative approach to environmental plant physiology. Cambridge University Press, New York.

Kobza J, GE Edwards 1987 Influences of leaf temperature on photosynthetic carbon metabolism in wheat. Plant Physiol 83:69–74.

Lee JE, C Janion, E Marais, BJ van Vuuren, SL Chown 2009 Physiological tolerances account for range limits and abundance structure in an invasive slug. Proc R Soc B 276:1459–1468.

Lovell J, J Gibson, MS Heschel 2009 Disturbance regime mediates riparian forest dynamics and physiological performance, Arkansas River, CO. Am Midl Nat 162:289–304.

Maherali H, ME Sherrard, MH Clifford, RG Latta 2008 Leaf hydraulic conductivity and photosynthesis are genetically correlated in an annual grass. New Phytol 180:240–247.

Nicotra AB, A Davidson 2010 Adaptive phenotypic plasticity and plant water use. Funct Plant Biol 37:117–127.

Prunier R, KE Holsinger 2010 Was it an explosion? using population genetics to explore the dynamics of a recent radiation within *Protea* (Proteaceae L.). Mol Ecol 19:3968–3980.

Prunier R, KE Holsinger, JE Carlson 2012 The effect of historical legacy on adaptation: do closely related species respond to the environment in the same way? J Evol Biol 25:1636–1649.

Rebelo AG 2006 Protea atlas project. http://www.proteaatlas.org.za/.

Schrader SM, RR Wise, WF Wacholtz, DR Ort, TD Sharkey 2004 Thylakoid membrane responses to moderately high leaf temperature in Pima cotton. Plant Cell Environ 27:725–735.

Sultan SE 2000 Phenotypic plasticity for plant development, function and life history. Trends Plant Sci 5:537–542.

Taiz L, E Zeiger 2010 Plant physiology. 5th ed. Sinauer, Sunderland, MA.

Valente LM, G Reeves, J Schnitzler, IP Mason, MF Fay, TG Rebelo, MW Chase, TG Barraclough 2010 Diversification of the African genus *Protea* (Proteaceae) in the Cape biodiversity hotspot and beyond: equal rates in different biomes. Evolution 64:745–760.

Walker-Simmons M 1987 ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol 84:61–66.

Yates MJ, GA Verboom, AG Rebelo, MD Cramer 2009 Ecophysiological significance of leaf size variation in Proteaceae from the Cape Floristic Region. Funct Ecol 24:485–492.

Zhang J, W Jia, J Yang, AM Ismail 2006 Role of ABA in integrating plant responses to drought and salt stresses. Field Crop Res 97:111–119.