Brachypodium and the Abiotic Environment

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Abstract *Brachypodium distachyon* and its congeners are found in diverse environments throughout the temperate regions of the world. *Brachypodium* species also show considerable variation in life history strategy, with species representing short-season annual habits and other species persisting for multiple years. This variation in ecological setting and life history suggests the existence of considerable genetic diversity in adaptation to the abiotic environment, both in constitutive tolerance to local conditions and in the capacity of single genotypes to acclimate to changing or unpredictable conditions. We review the limited but growing empirical literature on the physiology, development, and molecular biology of the interaction of *Brachypodium* with the abiotic environment. We highlight how these findings inform studies of ecologically and agriculturally-important plant species, and identify areas of future research that will extend the utility of *Brachypodium* as a model genetic system for understanding plant-environment interactions.

Keywords *Brachypodium distachyon* • Drought • Abiotic stress • Cold • Adaptation • Acclimation • Plasticity • GxE

Introduction

Plants are confronted by a range of challenging environmental conditions that may vary on the scale of hours, days, months, years or even generations. Plant species have evolved diverse strategies to cope with this variable abiotic environment. These strategies include the evolution of both constitutive as well as inducible—plastic—traits that provide adaptive abiotic stress tolerance. *Brachypodium*

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distachyon is found in diverse environments across its native range surrounding the Mediterranean and Middle East. Natural populations of *B. distachyon* (*sensu* Schippmann 1991) occur from the mid-Atlantic islands east to Afghanistan, from north Africa into central France, spanning habitats near sea level to over 1800 m. *B. distachyon* has also successfully established invasive populations throughout temperate regions of the world as a result of recent human-mediated dispersal (Khan 1984). Other species of *Brachypodium* are found in equally diverse environments, albeit largely restricted to temperate climates. This habitat diversity suggests that populations of *B. distachyon*—and its congeners—may display varying degrees of local adaptation and/or a range of phenotypic plasticity that facilitate population persistence under many abiotic and biotic conditions. Here, we review the small but growing literature documenting molecular, physiological, and developmental aspects of *Brachypodium* interactions with the abiotic environment. We also discuss which of these features of *Brachypodium* vary within and between natural populations, and which are expressed facultatively (i.e. representing phenotypic plasticity or acclimation responses). Throughout, we will highlight the utility of *B. distachyon* and its congeners as reference genetic systems for temperate C3 cereal and biofuel crops, as well as discuss prospects for *Brachypodium* as a model for elucidating the evolution and ecology of plant-abiotic interactions. We focus on *Brachypodium* responses to soil drying and to temperature extremes, due to the limited number of studies assessing other aspects of plant-environment interactions.

As discussed in other chapters of the current volume, the genus *Brachypodium* is phylogenetically sister to a large clade of C3 grasses, the Pooideae. The Pooideae includes many cereal and forage crops and is broadly distributed in temperate climates around the world. Divergence time estimates from molecular analyses indicate that *B. distachyon* diverged from other Pooideae grasses ca. 32–39 mya, and from rice ca. 40–54 mya (The International Brachypodium Initiative 2010). Due to its phylogenetic position, its C3 photosynthetic system, and its adaptation to temperate climates, *B. distachyon* has been proposed as a genetic model system for Pooid cereal and forage species. For example, the growth form, physiology, and abiotic stress responses of *Brachypodium* should much better reflect characteristics of wheat (*Triticum*), barley (*Hordeum*) and rye (*Secale*) in comparison to the C4 grass *Zea mays* or sub-tropically-adapted *Oryza sativa*. Plants which exhibit C3 photosynthesis are generally distributed at higher latitudes, where the increased efficiency under high temperature afforded by C4 photosynthesis is less favorable. Higher-latitude grasses are also subject to a greater annual range of temperatures, including temperatures at or well-below 0 °C.

The *Brachypodium* genus has several interesting attributes for the analysis of plant-environment interactions. First, the genus contains both annual (e.g. *B. distachyon*; Brkljacic et al. 2011) and perennial species (e.g. *B. sylvaticum*; Steinwand et al. 2013) that may be contrasted to assess differential strategies of biomass acquisition and allocation between life history types. Moreover, perennial species vary in the persistence of their stems (Khan 1984) suggesting genetic diversity in biomass allocation strategies. Finally, species

(and, in some cases, populations within species) representing a range of ploidies allow for evaluation of classic questions in evolutionary ecology dealing with niche occupancy of diploid vs. higher-ploidy genetic systems.

Life History Variation Within and Among *Brachypodium* Species

Distressingly little has been published regarding the ecological setting and life history strategies of *Brachypodium* species. This gap in our understanding of basic ecology is unfortunate because variation in life history strategies can have a strong influence on plant adaptation to abiotic stress. In Fig. 1, we present annual patterns of precipitation and temperature for the site-of-origin for four commonly-studied *B. distachyon* accessions; the cool, wet winters and warm, dry summers typical of the Mediterranean climate are apparent in all four sites. These climate diagrams
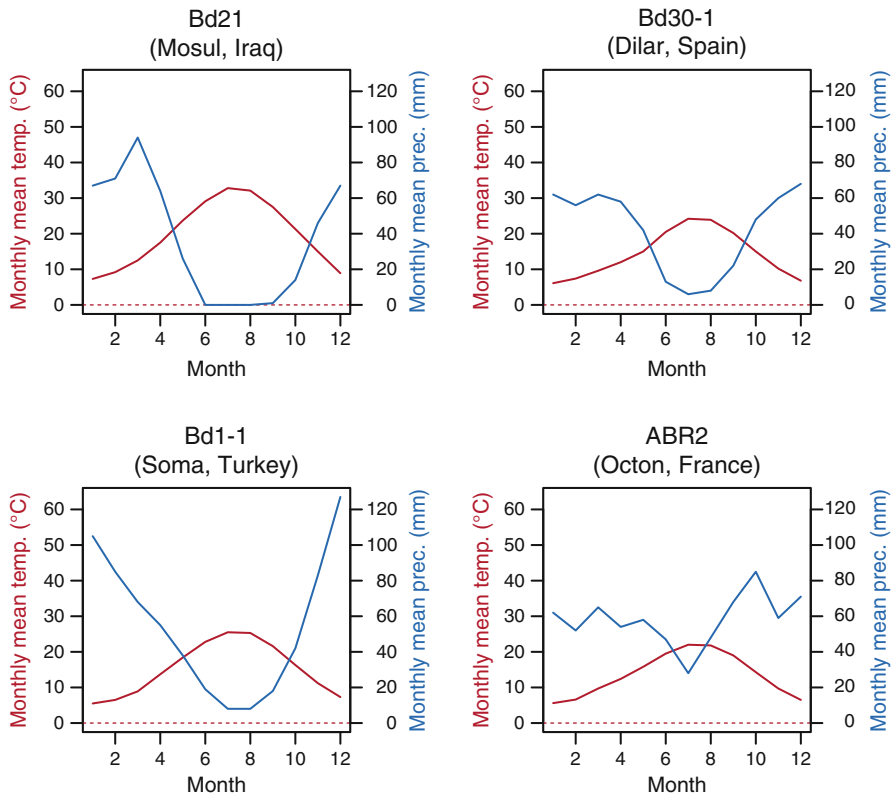

Fig. 1 Climate diagrams for sites of origin of four commonly-studied natural accession of *Brachypodium distachyon*. Climate data are from Hijmans et al. (2005)

also highlight the differences in water availability among populations during the portion of the year in which temperatures are likely conducive to growth. In southern Europe, *B. distachyon* is observed to germinate in the spring, setting seed in the early summer and senescing in the late summer and autumn (P. Catalan and A. Manzaneda pers. comm.). This represents an annual life history strategy, specifically a "spring-annual" strategy. The reference accession of *B. distachyon*, Bd21 from northern Iraq, and several other accessions descended from wild-collected plants are spring-annuals. These accessions flower readily under cultivation without extended cold treatment, or vernalization. Here, the timing of early spring establishment, rapid growth, and characteristics to escape hot and dry summer conditions—or to avoid their effects on cellular function—are paramount to ecological success and persistence.

In contrast to these rapid-flowering accessions, lab and greenhouse studies with *B. distachyon* reveal that many other accessions are relatively late flowering— "winter annuals"—and require cold stratification of seed or vernalization of adult plants to flower. Moreover, vernalization responses are often associated with flowering time responsiveness to photoperiod conditions (Schwartz et al. 2010; Ream et al. 2014). For example, the Bd18-1 accession originating from central Turkey requires >4 weeks of cold to transition from vegetative growth to flowering in lab studies (Schwartz et al. 2010). Seeds of many winter annual species germinate in the cool wet fall followed by establishment, slow and continuous growth through much of winter, and rapid growth and flowering in spring and early summer. In locations with exceptionally cold winter climates, plants may overwinter as dormant crowns or plants. It is likely that winter annual plants will be well established and larger in spring, with more stored resources, as compared to spring annual plants because of the head start this life history strategy affords them. The larger size may allow them to complete reproduction earlier in the spring before summer droughts, or to better avoid dehydration associated with drying environments because of expanded root systems and access to a deeper profile of soil moisture. Despite compelling evidence from lab studies for "overwintering" behavior in *B. distachyon,* we are not aware of any published reports of overwintering populations from field observations.

Life history strategies often show strong correlations with other developmental traits and physiological strategies for coping with abiotic stress. In some species spring-annual plants have higher growth rates, lower water use efficiency, and lower cold tolerance than do winter-annual plants. For example, accessions of *Arabidopsis thaliana* exhibit strong genetic tradeoffs between water-use efficiency and flowering time (McKay et al. 2003). This may reflect an adaptive axis associated with the evolution of drought-escaping spring annuals and drought tolerant winter annuals (Lovell et al. 2013; Kenny et al. 2014). Pleiotropic effects at a number of flowering time candidates (e.g., FRI and FLC) have been implicated as driving these tradeoffs (Mendez-Vigo et al. 2011; Lovell et al. 2013). These physiological correlates of life history have not been studied directly in *B. distachyon* but are not uncommon in related C3 grain crops. For example, quantitative trait loci (QTL) for carbon isotope discrimination (an integrated

measure of water-use efficiency in C3 plants) were associated with wheat heading date and plant height suggesting a possible pleiotropic relationship (Rebetzke et al. 2008). A better understanding of the genetic basis of these physiological tradeoffs, if they exist in *Brachypodium*, may give insights into constraints to drought adaptation in natural populations and facilitate efforts to improve crop tolerance to abiotic stresses (Juenger 2013).

While the annual *B. distachyon* has received the majority of attention from the research community, most *Brachypodium* species exhibit a perennial life history strategy. Perenniality is thought to evolve primarily as a compromise between allocation tradeoffs, survivorship risks, and subsequent fitness benefits (Bradshaw and Putwain 1972; Partridge and Harvey 1988). For example, plants can allocate limited resources to storage, maintenance, defense, growth and reproduction. Plants that allocate more resources to maintenance or storage, for example in belowground structures or meristems, are more likely to persist through extended periods of cold and drought and exhibit perennial-like life histories. These plants forgo current reproduction for increased survivorship but may accrue fitness benefits from reproduction in future growing seasons. In contrast, annual plants partition resources to maximize current reproduction at the expense of lower survivorship and future reproduction. As such, annual and perennial plants are also expected to have very different strategies for coping with the environment and abiotic stresses. The most obvious of these relates to annual variation in temperature: perennial species must cope with winter cold, whereas annuals likely only experience cold as seed if they germinate prior to the final winter frost, or if they persist until the first frost. Moreover, perennial plants are much more likely to shed leaf material and persist in a dormant state through extended periods of drought or cold. Unlike many short-lived annual species, perennial grasses utilize reserve meristems as perennating structures that likely facilitate regrowth following damage from abiotic stress. Differences among the perennial species of *Brachypodium* may be interesting in this regard (Schippmann 1991; Catalan and Olmstead 2000). *B. arbuscula* and *B. retusum* produce branched stems that may persist for many years. In contrast, the other perennial species (e.g. *B. sylvaticum*, *B. pinnatum*, and others) produce unbranched tillers that last for only 1–2 years. The perennials also differ in their investment in rhizomatous tissues. Most perennial species are capable of producing long rhizomes, whereas *B. mexicanum* produces much shorter rhizomes. Finally, perennial plants may exhibit considerable legacy effects across growing seasons associated with abiotic stress. This phenomenon is commonly observed in grassland communities, where poor precipitation in an earlier season results in meristem limitation in subsequent seasons and resulting lags in productivity. Comparative studies of annual and perennial *Brachypodium* may begin to provide insight into the mechanisms allowing plant persistence especially under stressful environmental conditions, and provide possible avenues for the development of hardy abiotic stress tolerance perennial grain and biofuel crops (Glover et al. 2010).

Our extended discussion of the interplay of life history and ecophysiology highlights the fact our poor understanding of the ecology of naturally occurring *Brachypodium* populations represents a significant impediment to interpreting plant

function in the context of the field environment. This information is critical as it provides context for the abiotic stress and selective regimes experienced by plants in nature and because it may help to interpret the adaptive significance of trait variation observed in realistic stress tolerance experiments. This gap can hopefully be addressed with future field demographic studies, the collection of new plant material across the natural geographical range of *B. distachyon,* and through careful curation of existing herbarium specimens.

Plant-Water Relations

Herbaceous plants have evolved a number of strategies or syndromes for surviving in the often harsh and unpredictable abiotic environment (Ludlow 1989). Under short-term non-lethal stress, many plants essentially cease photosynthesis and therefore growth. More moderate abiotic stress, including stresses that arise gradually, may allow for plants to acclimate to some degree. Considering first stress induced by soil drying, eco-physiologists recognize three very general non-exclusive syndromes of response. The first syndrome, dehydration avoidance, encompasses a suite of traits which plants deploy to prevent drying environments from resulting in dehydration of tissues and cells. Some features of dehydration avoidance observed in herbaceous plants include increased investment and growth in roots, increased water use efficiency, changes in cellular osmotic potential, and changes in cell wall characteristics. A second general syndrome is dehydration tolerance. These strategies are often deployed when reduced cellular water content becomes unavoidable, and include physiological responses such as the synthesis of proteins which stabilize cellular components to prevent damage or facilitate recovering from stress following water deficit. There are many parallels between dehydration tolerance and acclimation to severe cold, discussed below. Finally, herbaceous plants often rely on drought escape, which may include either genetically programmed early flowering (e.g. before an annually predictable end-of-season drying or terminal drought) or the induction of flowering in response to soil drying. Recent work points to a genetic basis of this latter aspect of drought escape (Han et al. 2013; Schmalenbach et al. 2014), though evidence for its frequency in natural populations is largely anecdotal. We will consider general aspects of plant response to other stressors in the following sections.

Ecological Context

Most of our limited understanding of *Brachypodium* performance in the field arises from work done in *B. distachyon.* Complicating matters, a recent comprehensive taxonomic reassessment of *B. distachyon* demonstrated that diverse natural collections previously described as ploidy variants of *B. distachyon* actually represent

three separate taxonomic entities: *B. distachyon*, *B. stacei*, and their allotetraploid hybrid, *B. hybridum* (Catalan et al. 2012). (See the chapter by Catalan in this volume for more on these considerations.) For our purposes, this taxonomic realignment means that those studies on *B. distachyon* published prior to 2012 which did not explicitly study known genotypes developed by the community cannot provide a clear picture of the specific ecology of *B. distachyon*. With that caveat, several studies point to the generally hardy nature of *B. distachyon sensu lato* under challenging environmental conditions. A study in a California grassland, representing the non-native range of *Brachypodium*, found that *B. hybridum* outcompeted resident native annual grasses during several years of drought (Armstrong and Huenneke 1993; note that these authors refer to the plants as "*B. distachyon*," though the plants were most likely *B. hybridum* as *B. distachyon sensu stricto* is unknown from California). This advantage increased over successive years of the drought, which the authors attributed to the larger seed size of *B. hybridum* facilitating rapid root growth early in the season. Rapid root establishment represents a drought avoidance strategy, allowing plants to outcompete neighbors for limited and dwindling soil moisture.

Natural populations of *B. distachyon* may show variation in drought escape strategy, as well. Aronson and colleagues performed a manipulative experiment contrasting the constitutive flowering times and drought-induced flowering response of desert (short season) vs Mediterranean-climate (longer season) Israeli populations of *B. distachyon* (Aronson et al. 1992). These authors found that, in a common garden, plants from the desert population flowered more rapidly than did Mediterranean plants regardless of the imposed watering regime. This result suggests that desert plants were genetically programmed to progress through their life cycle quickly, perhaps as an adaptation to flower before a predictable terminal drought. Strikingly, the authors also found differences in flowering response to soil water content. Mediterranean populations bolted and produced their first anthers more rapidly when soil moisture was limited, as compared to well-watered conditions. In contrast, desert plants show little drought response in the timing of bolting but significantly accelerated their progression through subsequent reproductive transitions (anthesis, seed set, and senescence). *B. distachyon sensu stricto* is not described from Israel, and so it is very likely that these populations represent *B. hybridum* and/or *B. stacei*. Future work may reveal that these genetic differences in flowering strategies may reflect divergence between the two species, or the hybrid nature of *B. hybridum*.

The role of hybridization in *B. distachyon* water relations was more directly assessed by Manzaneda and colleagues investigating Spanish populations of *B. distachyon* grown in the greenhouse (Manzaneda et al. 2012). By assessing integrated lifetime water use efficiency (WUE) of diverse natural genotypes, the authors found that allotetraploid *B. distachyon* (which we now know represents *B. hybridum*; see above) exhibited higher WUE and was generally found in more arid locations in Spain as compared to diploid *B. distachyon s.s.* Recent work assessing predicted ecological niches (using Ecological Niche Modeling) of the three species has suggested some ecological differentiation among the species

(López-Alvarez et al. 2015). Reinforcing the findings of Manzaneda and colleagues, ENM predicts that *B. distachyon* is found at higher elevations, experiencing cooler temperatures with more moisture than the comparatively lower-elevation, warmer, drier-adapted *B. stacei*. Allotetraploid *B. hybridum* is found to be largely intermediate in its climate preference compared to its two parental species. These patterns are particularly apparent on the Iberia peninsula, where all three species are found.

Additional ecological context for *B. distachyon* has been provided by two studies linking variation in DNA markers with composite indices of climate variation. These climate Genome Wide Association Studies (GWAS) offer a powerful way to infer putative functional relationships between natural variation and complex environmental parameters. Working with populations of Spanish *B. distachyon*, *B. hybridum*, and *B. stacei*, Hammami et al. identified fairly strong genetic clustering by species, and some correspondence between species and major axes explaining climate variation (Hammami et al. 2014). Dell'Acqua and colleagues extended this line of analysis by using next-generation sequencing on a smaller number of *B. distachyon* populations from Turkey, and performing both GWAS and genome scans for evidence of recent directional selection (Dell'Acqua et al. 2014). The higher genomic resolution afforded by this approach allowed the researchers to identify specific genes that were closely linked to climate-associated SNPs. The small sample size and deliberately conservative statistical analysis used in this analysis restricted power to some extent but the authors were able to identify a handful of genes putatively involved in local adaptation to climate. These included a MYB transcription factor, several other signaling proteins, and an enzyme hypothesized to play a role in wax synthesis (an important component of cuticles, and thus water retention in dry environments).

Developmental and Physiological Patterns of Response to Soil Drying

We currently lack a comprehensive understanding of how *B. distachyon*, and *Brachypodium* more generally, responds to soil drying. The extensive drought studies with wheat may illuminate key traits likely involved in drought adaptation in related C3 grasses like *Brachypodium*. Lack of progress on this key issue arises from an absence of manipulative experiments in the field environment, and from the lack of a consensus on how best to apply drying treatments in growth chambers or greenhouses. Moving forward, it is essential that investigators consider the confounding effects of light level, day length, relative humidity, growing media (including nutrient availability), and temperature on their experimental manipulation of water availability. Withholding water for 10 days from plants grown in a growth chamber set to conditions favorable for *Arabidopsis thaliana* will give dramatically different results than withholding water from plants grown in full

sunlight and spring temperatures representative of, for example, Mosul, Iraq (the collection locale for the reference Bd21 accession). The severity of stress, in terms of the rate of onset and the target soil water potential, will also have a considerable influence on experimental results, as will the life stage at which a stress is applied.

Natural accessions of *B. distachyon* show a range of responses to soil drying. Luo and colleagues withheld water from 57 soil-grown accessions until the wilting point, and assessed a range of developmental and physiological characters (Luo et al. 2011). Most accessions reached the wilting point in 6–7 days under the temperature and light conditions used. Generally, the dry-treatment plants had reduced leaf water content and reduced chlorophyll fluorescence (indicating reduced components of photosynthetic efficiency) and produced fewer leaves and tillers as compared to control plants. These observed effects of soil drying are typical of many small herbaceous plants (Chaves 2002). The authors characterized the accessions on a tolerance scale of their devising, finding that the BdTR2b, Bd3-1, and Bd18-1 were the most susceptible to soil drying and that B1-1, Tek-9 and Tek-10 were the least susceptible. Accessions along this continuum were most obviously differentiated by the extent to which leaf water content and chlorophyll fluorescence were affected by the soil drying. Interpreting results in relation to dehydration tolerance from this type of uncontrolled terminal drying experiment is difficult owing to interrelated functional effects of transpiration, photosynthetic rate, turgor pressure, and root:shoot biomass ratios. For example, the plants that wilted more quickly may simply have been larger plants that more rapidly exhausted soil moisture in a small pot; a supervised controlled dry-down experiment can control for these issues to some extent. We will return to the important issues of experimental design and ecophysiological interpretation at the end of this chapter.

The complex ways in which soil drying can affect plant tissues was carefully dissected by Verelst et al. (2013). Working with the reference accession of *B. distachyon*, Bd21, and using detailed understanding of leaf development from Arabidopsis and cereal crops, the authors distinguished among regions of the leaf in which cells are proliferating (e.g. undergoing mitosis), expanding, or mature. Leaves from plants grown under either moderate or severe soil drying were significantly shorter than control plants. Strikingly, the number of cells in stressed leaves was statistically indistinguishable from control leaves. Careful analysis of leaf development revealed that the reduction in leaf length without change in cell number was due to unabated cell division in the proliferation zone but greatly reduced cell expansion in the region of the leaf immediately distal to the meristematic tissue. The authors note that this result contrasts with data from diverse plant species—including *A. thaliana*, wheat, barley, maize and rice—showing both reduced cell division and reduced expansion under soil drying conditions. We will return to this study below when we consider molecular aspects of *Brachypodium* responses to soil drying.

As described earlier, roots play a key role in plant adaptation and acclimation to soil water availability. Chochois et al. recently described soil-drying response of 16 natural accessions of *B. distachyon*, and found substantial between-accession

differences in both root morphology and biomass (Chochois et al. 2015). These authors found that most measured root traits have very high heritability, and emphasize the important implications of a strong genetic basis for improving agriculturally-relevant traits. Under soil drying, most accessions increased investment in the primary seminal root (the root which arises from the seed during germination and forms the deep-penetrating tissue in this species), at the expense of roots arising from the coleoptile and leaf nodes. The extent to which this occurred, and the relative biomass allocation to different root types, varied considerably among accessions.

Molecular Responses of *Brachypodium* to Soil Drying

Researchers have utilized three inter-related avenues of research to explore the molecular aspects of *Brachypodium* response to soil drying. The first body of work builds on decades of work in reference plant species such as Arabidopsis, rice, and maize, and has identified apparent homologs of proteins demonstrated in these divergent species to play an important role in signaling responses to imposed stress. Perhaps unsurprisingly, the reference *B. distachyon* contains dozens of members of gene families known from other species to play an important role in environmental signaling. These families include the AREB/ABF, DREB, and WRKY families of environmentally-responsive transcription factors, as well as gene families more directly tied to regulation of growth (Mochida et al. 2011; Tripathi et al. 2012). Such *in silico* analyses are an excellent starting point to test hypotheses about conservation of gene function and the role of gene family member turnover in the evolution of environmental response, and these analyses also provide provisionary annotations essential for global analyses of gene expression.

A second avenue of research into the molecular responses of *Brachypodium* is to estimate directly global transcriptional responses to imposed soil drying (or a proxy). Building on the widely-cited and influential AtGenExpress study in *A. thaliana* (Kilian et al. 2007), which used a common Affymetrix microarray platform to assess transcriptional responses to a series of standardized environmental stressors in the lab, Priest et al. designed a microarray based on the Bd21 version 1.0 genome assembly (Priest et al. 2014). The Kilian dataset for *A. thaliana* has been hugely influential in subsequent studies of environmentally-responsive gene expression and was used extensively to annotate gene functions in the *A. thaliana* genome. (By extension, these annotations have been propagated across many additional plant genomes). Priest and colleagues used their microarray (BradiAR1b520742) to measure transcriptional response of Bd21 plants to a battery of stressors across a time series, including benchtop desiccation as a proxy for vegetative dehydration response. The resulting dataset is massive and several interesting patterns emerged. Desiccation stress results in the upregulation of ~1000 genes, with fairly little change in the magnitude of response over the sampled period of 1–24 h. By contrast, ~1000 genes are downregulated early during

desiccation and the number of downregulated genes grows substantially over time until the transcription of nearly 5000 genes are affected by 24 h. This latter result may be a simple effect of the plants shutting down most cellular processes as the leaves lose turgor from this very severe imposed stress. Indeed, function enrichment analyses of these downregulated genes indicated that many genes involved in cell cycle, DNA replication, and cell growth were turned off in their drying treatment. This transcriptional signature of cellular processes contrasts strongly with the results of Verelst et al. (2013), discussed above, finding unimpeded cell proliferation under moderate soil drying.

Priest et al. further looked for broad patterns in transcriptional network structure by clustering genes with similar transcriptional responses (e.g. upregulated over time during desiccation, but downregulated under elevated temperature or salinity) and then performing enrichment analysis on gene ontology and promoter sequence motifs. Such networks of co-regulated genes are excellent hypothesis generators that can narrow subsequent searches for key regulatory components such as transcription factors and sequence motifs associated with the regulation of target genes. The ABRE sequence motif, variants of which are frequently found to be associated with drought stress-responsive genes in many plant species (Shinozaki and Yamaguchi-Shinozaki 2007), was identified in this type of analysis. Interestingly, two groups of genes were downregulated under desiccation yet showed no overall enrichment for known cellular functions, despite the observation that most members of each group were associated with at least one annotated function. However, collectively, these groups were strongly enriched for several short sequence motifs in their promoter regions, suggesting a mechanism for their co-regulation even though the cellular functions of this coordinated regulation are not yet known.

Measuring the transcriptional response of diverse genotypes to environmental perturbation is another powerful tool for identifying the molecular genetic mechanisms of response and of cross-environment genetic correlations (Des Marais et al. 2013). A recent study of transcriptional responses to gradual soil drying across six natural accessions of *B. distachyon* revealed substantial among-accession diversity in response, or Genotype by Environment interaction (GxE) (Gordon et al. 2014). This analysis, which used mRNASeq to measure transcript abundance of ~15,000 genes, found 870 genes showed consistent treatment response across the six accessions while 300 genes responded to the treatment in a accession-specific manner (e.g. upregulated in one accession but downregulated in others). Multifactor experiments such as this are powerful because they are able to identify both genes that respond in a similar manner across diverse natural accessions, which possibly represent the conserved adaptive strategy of a species, and because they identify genes that respond differently in accessions. These latter genes, showing expression GxE, are of interest because they may reflect genes which are transcriptionally non-responsive under stressful conditions in some natural populations (e.g. because expressing the gene incurs a selective cost) but which are responsive to that stress in other populations (e.g. because NOT expressing them incurs a greater cost). As such, natural genetic diversity in transcriptional response can serve as the source material for the evolution of inducible or plastic response phenotypes and may

underlie local adaptation to climate (Lasky et al. 2014; Marchand et al. 2014). Studying expression GxE can also be a powerful means to make functional inferences about the molecular control of ecophysiological and developmental traits (Des Marais et al. 2012; Rengel et al. 2012).

The preceding studies focused exclusively on transcription levels of protein-coding genes, but increasing evidence suggests that small RNA species respond to soil drying as well. As a companion to the study of leaf developmental responses to drying, discussed earlier (Verelst et al. 2013), Bertolini and colleagues used next-generation sequencing technologies to characterize the complement of miRNA species in *B. distachyon* (Bertolini et al. 2013). These authors isolated both miRNA species previously described in other plant species as well as several miRNAs which appear to be unique to *B. distachyon*. Thirty-two miRNAs had distinct expression profiles when contrasting proliferating and expanding cells, and four miRNAs were significantly differentially expressed by soil drying in proliferating cells. Many of the miRNAs identified by Bertolini et al. using short-read sequencing were also identified by Budak and Akpinar (2011) using a microarray constructed of phylogenetically-conserved miRNA species.

Finally, conventional forward and reverse genetic approaches can be used to explore *Brachypodium* molecular responses to soil drying. We are aware of only a single study to date that has identified and validated a single gene effect for response to soil drying. Sun et al. assessed the functional effects of a WRKY transcription factor, BdWRKY36, and concluded that it was a positive regulator of drought response (Sun et al. 2014). BdWRKY36 appears to be constitutively expressed in all tissues, and is upregulated when leaves are exposed to benchtop desiccation, as well as at low temperature (4 °C) and high temperature (40 °C). The authors overexpressed BdWRKY36 in tobacco and found that, under soil drying as compared to control condition, this construct conferred increased leaf relative water content and chlorophyll abundance, decreased ion leakage and accumulation of reactive oxygen species (ROS), and increased expression of several well-known genes encoding ROS-scavenging proteins. The development of *B. distachyon* mutant populations through T-DNA insertion (Bragg et al. 2012) and TILLING (Dalmais et al. 2013) and will facilitate future efforts to discover genes involved in drought responses using forward genetic screens.

Brachypodium Response to Cold

Many populations of annual plants like *B. distachyon* experience severe cold, such as during season-ending frost, or during unusually cold temperatures in the early Spring. *Brachypodium* perennial species certainly experience severe cold, though we are unaware of any research addressing cold tolerance or acclimation response in these species. Annual plants which germinate in the Autumn use extended periods of cold as one of several vernalization cues to transition from vegetative to reproductive growth. The body of research dealing with *B. distachyon* flowering

is reviewed elsewhere in this volume. We focus here on research into the acclimation of *B. distachyon* to low temperatures.

Plants challenged with low temperatures often show two inter-related responses. First, they must survive the initial period of cold. Second, this initial cold can also stimulate an acclimation response that allows the plant to survive subsequent, often more severe, cold. Colton-Gagnon and colleagues assessed both of these aspects of cold response in seven natural accessions of *B. distachyon* (Colton-Gagnon et al. 2014). Four of these accessions were previously known to exhibit a "spring-annual" life history: these plants were competent to transition from vegetative to reproductive growth without a low temperature cue. The remaining three accessions exhibit typical "winter-annual" strategy: they require an extended cold cue to stimulate the transition to flowering. (Recall that the life history of these accessions has not been evaluated empirically in their native habitats.) These authors found that the seven accessions had largely similar non-acclimated cold tolerance, with 50 % mortality (LT50) at around $-8\ °C$. Acclimation via 28 days at $4\ °C$ reduced LT50 to around $-12\ °C$, again with no statistical difference in survival between "spring-annual" accessions (which might be inferred to experience cold temperatures infrequently) and "winter-annual" accessions (which presumably would experience cold temperatures). Cold acclimation in all accessions involved synthesis of fructans, free cellular Proline, and water soluble sugars. Accumulation of these molecules is frequently observed in response to low temperature and is generally believed to play a role in protecting cellular components from freezing damage. Fructan synthesis under cold and drought conditions is a key feature of Pooid grasses and may serve to provide a ready source of cellular energy or to stabilize cell membranes during osmotic stress (Hincha et al. 2000). Colton-Gagnon et al. caution that free cellular Proline concentration alone is a poor predictor of *Brachypodium* cold tolerance as the accessions studied showed wide variation in Proline abundance but little variation in cold tolerance. These authors conclude by arguing that the constitutive cold tolerance of *B. distachyon* "spring-annual" accessions does not match the general definition of temperate cereal species spring accessions, and argue instead that these accessions should be classified as having a "facultative" life history strategy.

Li et al. paired bioinformatic analysis of the Bd21 genome with comparative physiology and provide a nice evolutionary perspective on the cold tolerance of *B. distachyon* in relation to other members of the Pooideae (Li et al. 2012). They focus on genes encoding three groups of proteins believed to be core determinants of cold tolerance in Pooid grasses. The Ice Recrystallization Inhibition Proteins (IRIP) minimize cell damage by limiting ice crystal growth in the apoplast (Sidebottom et al. 2000). Based on their gene tree analysis, Li et al. conclude that IRIP proteins evolved before the divergence of *Brachypodium* from other Pooid grasses, but that independent radiations of these gene families occurred in the two lineages. C-repeat Binding Factor (CBF) transcriptional regulators are widely distributed in plants and regulate plant-wide response to low temperatures (Fowler et al. 2007). Interestingly, Li et al. find that an entire sub-family of CBFs—CBF4—is missing from *B. distachyon*, suggesting that this group of transcription factors

evolved subsequent to the divergence of *Brachypodium*. A third group of proteins believed to play a central role in Pooid cold tolerance are the Fructan Synthases (FST). FST genes are involved in the conversion of sucrose into fructose, which is polymerized to form fructans (see above). Li et al. show that *B. distachyon* has FST-like proteins, but that these proteins lack key domains associated with fructan synthase activity in core Pooids. Li et al. show that *B. distachyon* does accumulate fructans during cold acclimation, reinforcing the findings of Colton-Gagnon and colleagues, but that they accumulate fructans to a lesser extent than what is observed in core Pooid species.

The microarray study by Priest et al. (2014), described above in the context of transcriptional responses to soil drying, also assayed *B. distachyon* response to moderate cold treatment. Compared to the strong transcriptomic response observed under drying (~5000 genes downregulated after 24 h of desiccation), the response to 24 h of 4 °C treatment was quite moderate and dominated by the transcriptional induction of just a few hundred genes. This moderate response likely reflects the constitutive tolerance of *B. distachyon* to mild cold observed by Colton-Gagnon et al. (2014), described above. Priest and colleagues identified 63 genes which were upregulated by cold treatment over time but showed very little transcriptional response to their other treatments (desiccation, high temperature, and salt). These 63 genes were enriched for functions associated with transcriptional regulation, RNA metabolic processes, and regulation more generally; these genes might be interesting candidates to target for artificial selection as they may have limited pleiotropic effects in other stressful environments.

Brachypodium Responses to Elevated Temperatures

Relatively little is known about the molecular responses of *B. distachyon* or its congeners to stress due to elevated temperature. The proximate cause of elevated heat stress is the reduced efficiency—and biophysical instability at extreme temperatures—of cellular structures and processes. At the molecular level, this phenomenon has been studied in the greatest detail in plants as it relates to the photosynthetic apparatus (Allakhverdiev et al. 2008). Plants cope with elevated temperature stress on short time scales via biosynthesis of protective molecules (e.g. heat shock proteins) and via increased transpiration as a means of evaporative cooling. The efficacy of this latter response may be compromised if the temperature stress is accompanied by soil drying, which is often observed in the field environment.

Boden and colleagues assessed the response of Bd21 to slightly elevated temperature (27 °C), focusing particularly on the differential effects of this treatment when applied at the vegetative or reproductive stage (Boden et al. 2013). *A. thaliana* plants grown at 27 °C transition to flowering without the normally requisite long day cue but this pattern is not seen in *B. distachyon*. Moreover, *B. distachyon* plants grown at 27 °C do not transition to flowering earlier than plants

grown at 22 °C. These two observations suggest that 27 °C is not perceived as a stressful condition by the Bd21 accession, though the authors also find that the seeds of plants grown at 27 °C fill for a shorter duration and therefore weigh less at maturity than as compared to seeds of plants grown at 22 °C. Prior research in *A. thaliana* demonstrated a temperature-dependent effect of the occupancy of H2A. Z histones near the Transcriptional Start Sites (TSS) of genes, which inhibits transcription of those genes (Kumar and Wigge 2010). Increased temperature reduces H2A.Z occupancy, and thereby facilitates genome-wide transcriptional changes. The authors identified an apparent Bd21 ortholog of gene encoding an *A. thaliana* H2A.Z histone, *BdHTA9*, finding that its occupancy at the TSS of several candidate genes is not affected by elevated temperature in seedlings (i.e. vegetative tissue) but is affected in developing grains. This temperature effect in grains is not due to differences in transcription of *BdHTA9* itself. The authors surmise that H2A.Z histones may therefore account for some of the difference in transcriptional activity, and thereby development, when contrasting vegetative and reproductive tissues under elevated temperature. Their work also reiterates the important point that environmental stress is perceived differently by different parts of the plant.

The microarray study by Priest et al. (2014), also assayed *B. distachyon* response to high temperature treatment (24 h at 42 °C, a temperature which Bd21 plants may well have occasionally experienced in their native environment in northern Iraq; see Fig. 1). Here, the observed response was primarily transcript downregulation during the course of treatment. Just a few hundred genes were upregulated—dominated by heat shock proteins, genes associated with protein folding, and the dubiously helpful GO term "response to heat"—while over 1000 genes were downregulated after 12 h at 42 °C. Many of the downregulated genes reflected a slowing of plant growth at this high temperature: genes associated with the cell cycle, DNA replication and cell-wall biogenesis. In this latter respect, the high stress treatment imposed by Priest et al. had functionally similar effects to their desiccation treatment and may indicate that plants were conserving resources with the aim to resume growth following cessation of stress.

Interactions Between Environmental Stressors

Two outstanding questions in plant breeding and evolutionary genetics are how plants optimize fitness in multiple environments and how plants respond to combinations of stressful conditions such as heat and low soil water content, or abiotic and biotic stressors. A straightforward way to conceptualize this first issue is to ask whether a genetic locus affecting performance in one environment (e.g. drought) has pleiotropic effects in a second environment (e.g. high temperature). We recently reviewed the empirical literature on pleiotropy across environments, focusing on QTL studies in wild and crop species (Des Marais et al. 2013). We found that the preponderance of pleiotropic loci had much larger additive effects in

one environment than the other, or had a significant additive effect in only one environment. This pattern of trait expression is of interest because it suggests that fitness trade-offs between environments may be minimal for these naturally-segregating alleles, and is in contrast to the yield trade-offs often observed in studies of loss-of-function mutations or transgenic efforts to improve crop performance (Hall and Richards 2013).

At the molecular level, we can investigate mechanisms of cross-environment correlations by assessing whether different stressors invoke similar cellular responses. This has been explored in *B. distachyon* by the Priest et al. microarray study, discussed earlier (Priest et al. 2014). These authors found considerable overlap in the genes whose expression was induced under desiccation and under salt stress. Although these genes did not show any significant functional enrichments the authors did identify a number of enriched sequence motifs in their promoters, suggesting a common regulatory control for response to these two classic osmotic stressors.

A final, understudied, aspect of plant-abiotic environment interactions is the molecular, developmental, and physiological responses of plants to multiple stressors encountered at the same time (Rizhsky et al. 2004; Makumburage and Stapleton 2011; Prasch and Sonnewald 2013). While the field environment very often presents plants with complex challenges, such as high temperature in conjunction with low soil water content, or abiotic stress in conjunction with biotic stress, most molecular and physiological studies focus on just one imposed stress treatment at a time. *B. distachyon* is an excellent model system in which to address the mechanisms of combined stress. Its small stature facilitates the large, highly replicated, multi-factor experimental manipulations necessary for identifying the genetic basis of response to stressors encountered independently and in combination. We are not aware of any published studies looking at combined stressors in *Brachypodium*.

Moving Forward: *Brachypodium* as a Reference Genus for Plant-Abiotic Interactions

There is a growing need to improve crop productivity and to better understand plant adaptation in natural populations in the face of abiotic stress. In particular, changes in precipitation and temperature regimes driven by climate change are a potential threat to future crop productivity and natural ecosystems worldwide (Wheeler and von Braun 2013; Pacifici et al. 2015). *Brachypodium* is an emerging model system with extensive genetic, genomic, and natural variation resources that can be brought to bear on these issues (see other chapters in this volume). In particular, it is likely that natural populations of *Brachypodium* have evolved novel mechanisms of stress avoidance and tolerance that could be leveraged for improvement of several grain crops. A better understanding of natural variation in abiotic stress in *Brachypodium*,

especially at the molecular level, provides a unique opportunity for gaining insights into local adaptation and as well as novel avenues for improving crops. Here we outline some important summary points and future opportunities in studies of abiotic stress with *Brachypodium*:

1. Researchers should strive to impose realistic abiotic stresses to facilitate the discovery of adaptive responses and the interpretation of physiological and molecular mechanisms. Experimental design should carefully consider the timing and duration of stress treatment, the stage of plant development when imposed, and the possible interactions and indirect effects of manipulations (e.g., nutrient limitation or increased leaf temperature associated with soil drying). Matching stress assays with existing and well-established protocols for grain crops may facilitate the transfer of insights to important agricultural systems (Fischer 2011).
2. Plants experience the environment as a complex milieu of spatially and temporally varying conditions. Plants are remarkable in their ability to acclimate and physiologically respond to this complex, changing, and heterogeneous environment. Nevertheless, the vast majority of abiotic stress studies center on the manipulation of single environment factors across few levels of variation. We anticipate that combinations of stresses may induce signaling and physiological responses that are unique, that provide cross-protection, and that are especially relevant to crop improvement (Mittler and Blumwald 2010). We believe studies targeting multiple abiotic stresses in combination will be especially insightful for developing models of physiological functions and elucidating key molecular pathways.
3. We know surprisingly little about *Brachypodium* from natural populations and in a field setting. Additional information from field natural history observations, field experiments, and the continued collection of new germplasm will be key to the success of *Brachypodium* as an emerging model grass. In particular, life history and phenological data are critical needs as they determine the matching of environmental conditions with developmental transitions, the timing of stressful environments, and anticipated strategies for ecological adaptation.
4. Plant responses to abiotic stress often involve tradeoffs—traits that increase fitness or yield under stress come at a cost to performance in benign conditions (Des Marais et al. 2013). These pleiotropic relationships are a major limitation to traditional breeding programs and for adaptation in the wild. A central challenge in plant biology is to identify genetic constraints and through an understanding of their mechanism manipulate them for crop improvement. The rapid development of quantitative genetic and genomic approaches provide immense opportunity for teasing apart these mechanisms and for manipulating the costs and benefits of key abiotic stress tolerance traits.

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