Title: Promises and challenges of eco-physiological genomics in the field: tests of drought responses in switchgrass.

Running Title: Physiological genomics of drought in switchgrass.

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One-sentence summary: Physiological and gene expression analyses across field and greenhouse experiments highlight that diverse gene expression patterns can produce physiologically similar responses to soil water deficits.

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ABSTRACT

Identifying the physiological and genetic basis of stress tolerance in plants has proven to be critical to understanding adaptation in both agricultural and natural systems. However, many discoveries were initially made in the controlled conditions of greenhouses or laboratories, not in the field. To test the comparability of drought responses across field and greenhouse environments, we undertook three independent experiments using the switchgrass reference genotype Alamo AP13. We analyzed physiological and gene-expression variation across four locations, two sampling times and three years. Relatively similar physiological responses and expression coefficients of variation across experiments masked highly dissimilar gene expression responses to drought. Critically, a drought experiment utilizing small pots in the greenhouse elicited nearly identical physiological changes as an experiment conducted in the field, but an order of magnitude more differentially expressed genes. However, we were able to define a suite of several hundred genes that were differentially expressed across all experiments. This list was strongly enriched in photosynthesis, water status and reactive oxygen species responsive genes. The strong across-experiment correlations between physiological plasticity—but not differential gene expression—highlight the complex and diverse genetic mechanisms that can produce phenotypically similar responses to various soil water deficits.

INTRODUCTION

Crop productivity and wild plant distributions are governed by the availability of soil moisture (Axelrod, 1972; Boyer, 1982; Ciais et al., 2005). The impact of drought and soil water deficit in agriculture is estimated to be the largest abiotic determinant of yield (Boyer, 1982; Araus, 2002), while drought is also considered a primary cause of speciation and adaptation in nature (Stebbins, 1952). Dehydration avoidance and other drought adaptive strategies permit plants to survive or maintain growth during periodic droughts (Blum, 1996; Chaves et al., 2003; Chaves, 2004). Specifically, phenotypic plasticity of stomatal conductance, water foraging and growth traits (among many others) may effectively maintain homeostasis of leaf water potential despite soil water deficits.

Leaf water potential is a bellwether of the physiological impact of water deficit (Jones, 2007). Under drought, decreasing water availability results in reduced leaf water potentials and a sequence of physiological responses including reduced photosynthesis, growth rate and ultimately, fitness (Taiz and Zeiger, 2014). Plants therefore seek to maintain homeostasis of leaf water potential, with the highest (least negative) values supporting the most efficient functioning of photosynthesis and other metabolic processes in most species (Lawlor and Fock, 1978; Turner and Begg, 1981; Kramer and Boyer, 1995; Cornic and Massacci, 1996; Jones, 2007). Plants that exhibit dehydration avoidance strategies compensate for soil water deficit through phenotypic plasticity of gene expression (Verslues et al., 2006; DesMarais and Juenger, 2010; DesMarais et al., 2013; Lovell et al., 2015) and downstream physiological phenotypes (Levitt, 1980), among others.

To understand plant stress responses, it is critical to determine the physiological and genetic underpinnings of drought adaptation in both field and laboratory conditions (Travers et al., 2007; Gaudin et al., 2012). A common finding among such studies is that physiological and gene expression responses to drought vary considerably depending on the severity and temporal dynamics of drying soil (Chaves et al., 2003; Barker et al., 2005; Malmberg et al., 2005; Mittler, 2006; Mishra et al., 2012). Natural soil moisture variation, which has shaped adaptive responses to drought in wild populations, is not necessarily recapitulated by controlled (often, “shock”) laboratory experiments. For example, single abiotic stresses rarely occur in isolation in the field (Mittler, 2006). Instead, wild and crop plants respond to the combination of diverse stressors such

as drought, heat and salinity simultaneously and at both molecular (e.g. Rizhsky et al., 2002; Rizhsky et al., 2004; Suzuki et al., 2005) and physiological (e.g. Heyne and Brunson, 1940; Craufurd and Peacock, 1993; Machado and Paulsen, 2001) levels. Therefore, inquiries into evolved plant stress responses are perhaps best served by experimental conditions that emulate selective agents in the field. Given that the extent and severity of stress causes qualitatively different physiological responses, it is not necessarily surprising that several studies have found relatively weak genetic correlations between laboratory phenotypes and those collected in the field (e.g. Weinig et al., 2002; Malmberg et al., 2005; Anderson et al., 2011; Mishra et al., 2012).

Soil properties and biota can also affect plant growth and physiology (Meisner et al., 2013; Schweitzer et al., 2014), which may be exacerbated by contrasts between growth in potting mix or in native soil (Rowe et al., 2007; Heinze et al., 2016). The observation that field-grown plants have different root systems and greater total water storage than those in greenhouse pots is of particular importance to water relations (Poorter et al., 2012a). Short-term drought stress in the field may be buffered by access to larger volumes of soil and more complex root-soil-water dynamics, conditions poorly represented in most controlled settings.

The field of experimental design has been fundamentally shaped by a central problem of biology: that it is notoriously difficult to control environmental factors in the field (Jones, 2013). A classic solution is to increase biological replication, but this is generally not feasible with costly and time-sensitive physiological and genetic assays (Poorter et al., 2012b; Marchand et al., 2013). Despite these difficulties, understanding the effects of drought in field conditions is necessary because it is in these settings that yield is impacted and selection is acting to shape adaptive responses to stress. Here, we determine how the interplay between drought severity, planting condition (e.g., field, potted, greenhouse) and sampling timing impacts physiological and genomic responses to drought in the C_4 perennial grass, *Panicum virgatum* (switchgrass). To accomplish this, we used observations collected from clonally replicated individuals of the "AP13" switchgrass genotype (derived from the Alamo cultivar), which is the genome reference for this important biofuel crop and dominant member of mesic tall grass prairie ecosystems. The Alamo cultivar is a southern lowland accession that has high vigor and performance across a variety of climatic conditions. Replicates were grown in three separate soil moisture manipulation experiments with distinct rooting environments: medium sized pots in a greenhouse, large containers in a field setting, and in native soil under rainout shelters. In all three of these experiments, we collected leaf-level physiological and whole-genome gene expression data from droughted and control plants.

Combined, the three experiments represent contrasts in drought experimental manipulations (i.e., the extent, timing and duration of drought), plant characteristics (i.e., age, maturity and size), and broadly fit with the concepts of best practice for physiological analysis of drought responses (Poorter et al., 2012b). Contrasting these experimental design considerations allows us to address how edaphic and climactic conditions impact links between gene expression and physiological phenotypic plasticity. Specifically, we assessed three fundamental questions pertaining to physiological genomics in the field: 1) How consistent is phenotypic plasticity to drought across experiments? 2) Which soil moisture deficit responses vary across sites, years and timing of sampling? 3) How does plasticity of physiological and gene expression phenotypes co-vary within and across experiments? To assess these questions, we tested how leaf physiology and whole-genome gene expression responded to the effects of drought treatments, leaf water potential and sampling time (midday and predawn). Such analyses permitted inference of the number, relative effect size and identity of differentially expressed (plastic) genes. Overall, our results suggested that differences in leaf water potential and diurnal patterns were the major drivers of gene expression variation. Furthermore, we observed consistent physiological plasticity

across greenhouse dry-down and field precipitation manipulation experiments, but extreme variability in the number of differentially expressed genes.

RESULTS

Physiological and gene expression variation across experiments: The AP13 *P. virgatum* accession was clonally replicated and grown in three experiments from 2011-2014: (1) a greenhouse dry-down in 3.74 L pots ("greenhouse", expression data from this experiment was published previously, Meyer et al. (2014)), (2) 1400 L PVC cylinders in field conditions ("cylinder"), and (3) spaced plants grown directly in the field under 18 m x 73 m rainout shelters ("shelter"; Fig. 1A; Aspinwall et al. (2013)). The shelter experiment was further subdivided into (3a) a 2012 experiment, where six distinct watering treatments were employed at a single field site in Temple, TX, and (3b) experiments in 2013-2014 where two drought treatments were contrasted at sites in Austin and Temple, TX (Fig. 1A).

Predawn leaf water potential (Ψ_{leaf}) varied considerably between watering treatments in each experiment (Table 2), reflecting the physiological impacts of drought. However, plant physiology also varied with the time of sampling (cylinder and shelter experiment), location and year (shelter experiment, Table 2). Within the 2013-2014 shelter experiment, most plants grown in the Austin site exhibited more negative midday Ψ_{leaf} values than those in Temple (Fig. 1B). Consistent with reductions in midday Ψ_{leaf}, we also observed plasticity of both photosynthetic rate (A) and stomatal conductance (g_s); both physiological parameters significantly declined in the drought treatments of the 2012 shelter, cylinder and greenhouse experiments (Table 2).

Both the physical effects of water deficit and genetic control of gene expression may drive physiological plasticity. To assess the extent of genetic responses to drought, we quantified gene expression in mature leaves in each experiment using the previously described high-throughput "TAG-seq" protocol (Meyer et al., 2011). We evaluated how distinct each treatment was with respect to the expression data using principal component analyses (PCA) (Fig. 1C) and visualized PCAs of the transposed expression matrix to depict the position of each library within genetic space (Fig. 1D). Among experiments with multiple experimental factors (excluding the 2012 shelter), the first PCA axis delineated treatment differences only in the greenhouse experiment. Diurnal patterns were the strongest drivers of gene expression variation in the cylinder experiment, but were secondary to the drought treatment in the greenhouse (Fig. 1C). In the 2013-14 shelters, differential expression across years—and to a lesser degree, sites—dominated (Fig. 1C-D). Like in the cylinders, drought effects seemed to contribute a small proportion of expression variation in the 2013-14 shelters. In the 2012 shelter experiment, PCA analyses clearly clustered the two wetter treatments ("wet" and "75%") away from the drier treatments (Fig. 1C); however, gene expression variation within treatments was considerable (Fig. 1D). These results demonstrated that differential expression to drought stress was strongest in the greenhouse and least observable in the field. Consistent with the multivariate analysis of gene expression variation (Fig. 1C-D), we observed many more genes with significant drought-induced differential expression in the greenhouse than the field (Table 1).

Phenotypic and gene expression assays are as precise in the field as in the greenhouse. We observed ~5500 more differentially expressed genes (between wet and dry treatments) in the greenhouse and cylinders than we did across the shelter experiments (Table 1). Here, we qualified differential expression as any case where the FDR-corrected P-value of the linear model exceeded $\alpha = 0.05$. It is important to note that sample sizes were not identical across experiments (Table 1), which may alter the power to detect differential expression. To determine if our results were biased by sample size inconsistencies, we subsampled individuals in each experiment to generate rarefaction curves. These analyses demonstrated that the observed differences among experiments were not artifacts of statistical power (Fig. S1). This massive difference in signal was not mirrored in the physiology data, where predawn Ψ_{leaf} values varied strongly between treatments and were of similar magnitude in the greenhouse and the field (Table 2). Furthermore, when both experiments were fit in a single model, there was only a marginally significant

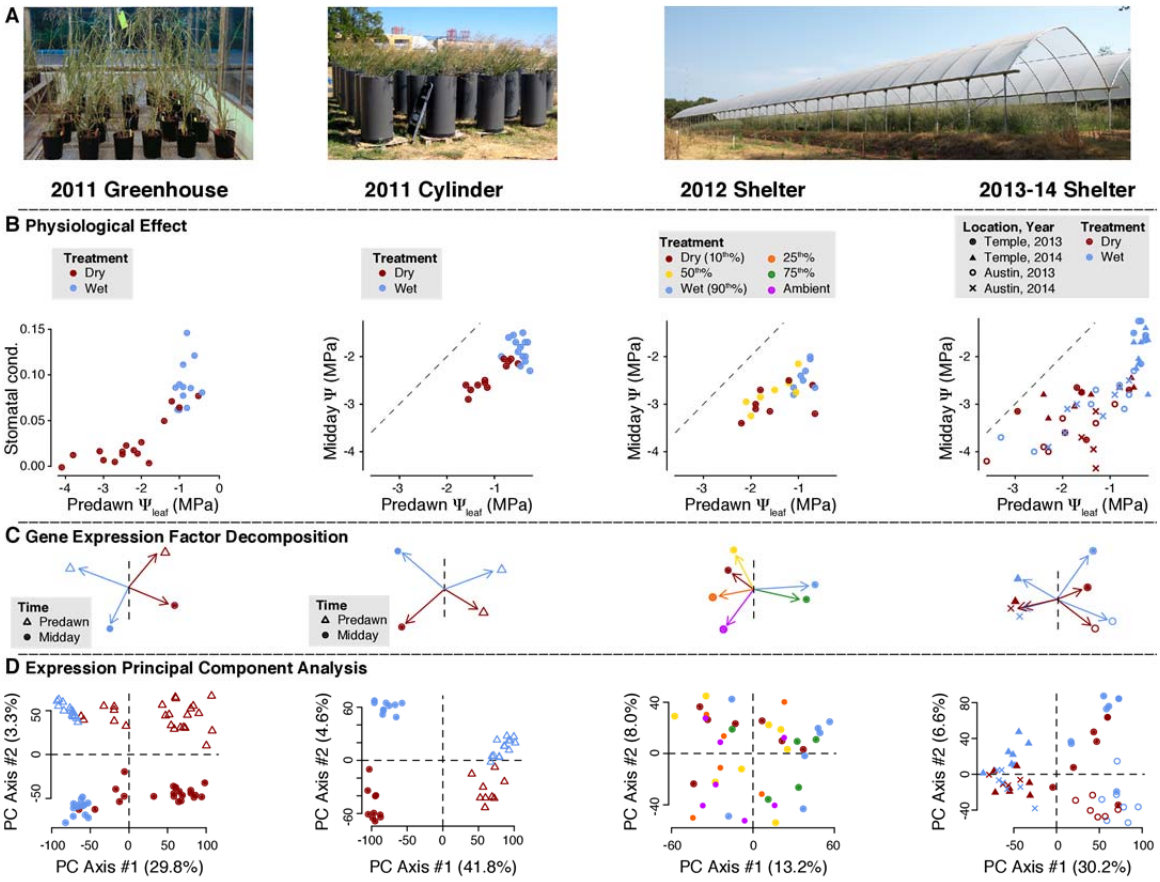

Figure 1. Physiological and gene expression responses to drought across three experiments. Replicates of the AP13 switchgrass genotype were grown in three separate experiments (A). Predawn leaf water potential (Ψ_{leaf} , MPa) was assessed for each plant. Midday Ψ_{leaf} measurements were paired with tissue collection for RNA for all experiments except the greenhouse, where stomatal conductance (g_s) was assayed at midday instead of Ψ_{leaf}. These midday and predawn measures are plotted with independent scales for the greenhouse and remaining experiments (B). The expression matrices for genes with a significant effect of any experimental factors (time of sample collection, location and year) were used to conduct principal component analysis (PCA) decompositions (C). The length and direction of the vectors indicates the strength of each experimental level. A vector perpendicular to the 1st PCA axis is plotted as a dashed line. Finally, the principal component score for the transposed expression count matrix is plotted and grouped by the experimental factors (D). The percent variance explained by the 1st two PCA axes accompanies the axis labels. Note that in the 2012 shelter experiment expression was assayed across six treatments, but physiological phenotypes were only measured in the wet, mean and dry treatments. Levels of replication for each experiment can be found in Table 1.

treatment-by-experiment interaction ($F = 2.48$, $df = 4$, $P = 0.06$). This indicates that drought treatments elicited Ψ_{leaf} responses in the same direction and with similar effect sizes across experiments.

When comparing treatments, Ψ_{leaf} was more variable among droughted plants than those in wet conditions (Fig. 1B). The coefficient of variation (*cv*) within experiments and treatments confirms this observation (Fig. 2A); *cv* of dry treatment predawn Ψ_{leaf} was on average 58% greater than that of the wet treatment. Increased variability of Ψ_{leaf} in drought plants may be due to the physical properties of drying soils. Plants perceive soil moisture as total soil water potential (Ψ_{soil}); however, as soils dry, Ψ_{soil} exponentially declines. We modeled the progression of soil moisture decline from observed values of soil volumetric water content and Ψ_{soil} in the

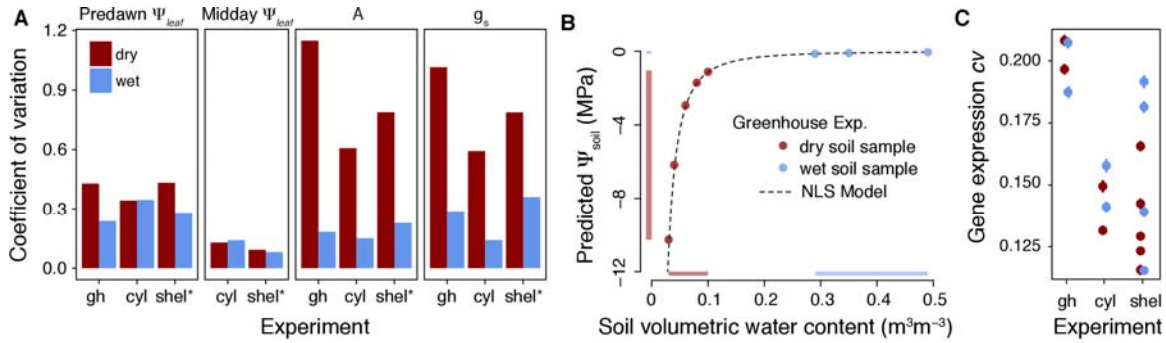

Figure 2. Physiological, soil and normalized expression variability across experiments and treatments. Coefficients of variation (*cv* = standard deviation / mean) were calculated for each physiological and gene expression phenotype. Raw *cv* for each physiological phenotype is plotted in panel A. To understand the relationships between Ψ_{soil} and soil volumetric water content, we conducted soil moisture release curves for the greenhouse potting soil where a soil sample was progressively dried and volumetric water content and Ψ_{soil} were repeatedly measured; the range of observations for each treatment are presented by the marginal line segments (B). Finally, mean (± SE) *cv* across all expression traits is plotted (C). The experiments are abbreviated as shelter ("shel"), greenhouse ("gh") and cylinder ("cyl").

greenhouse (Fig. 2B). The range of Ψ_{soil} predictions in the dry treatment (10.3-1.1 -MPa) was much greater than in the wet treatment (0.14-0.05 -MPa) despite a narrower range of volumetric water content measurements (see marginal line segments in Fig. 2B). As such, implementing a consistent drought treatment in terms of Ψ_{soil} was difficult, even in the greenhouse experiment. Our physiology data clearly mirrors the soil water potential measures: there is much more variability in the drought treatment than in well-watered conditions across all experiments (Fig. 2A).

We examined the variability of our measurements of physiology and expression and (surprisingly) found that those taken in the field were less variable than those in the greenhouse. In 3/4 of the physiological phenotypes, *cv* in the drought treatments was greatest in the greenhouse and lowest in the cylinders. The *cv* of the fourth physiological phenotype, predawn Ψ_{leaf}, was nearly identical in the greenhouse (0.33), cylinder (0.34) and shelter (0.36) experiments (Fig. 2A). The *cv* among normalized expression phenotypes largely recapitulated the physiology data (Fig. 2C): the greenhouse experiment produced the most variable data, while the large cylinders displayed the least. The field planted individuals had similarly variable expression phenotypes as those in the cylinders (Fig. 2C).

The effects of drought treatments are modulated by time of sampling. Time of sampling (predawn and midday) in the greenhouse and cylinder experiments had a substantial effect on the expression patterns of many genes (Fig. 1C-D); however, these effects varied across experiments. For example, in the cylinder experiment, 47% (11880) of all genes differentially responded to sampling time, compared to only 30% (5278 genes) in the greenhouse (Fig. 3A-D). These results revealed reduced diurnal expression regulation in the greenhouse, compared to plants grown in the field in cylinders.

In addition to additive time-of-sampling effects, we detected complex interactions between drought treatments and sampling time. Drought treatment effects were directly modulated by time of sampling (treatment-by-time interactions in the linear model) in 8.4% (2125) and 4.6% (805) of genes in the cylinder and greenhouse experiments. While expression patterns were generally conserved between predawn and midday sampling in the greenhouse (Fig. 3A-B), the effect of the drought treatment strengthened from predawn to midday in the cylinder experiment (Fig. 3C-D). Indeed, 67% of drought-responsive genes at midday were significantly differentially expressed in the same direction in the predawn sampling in the greenhouse (Fig. 3E), but only 30% of genes

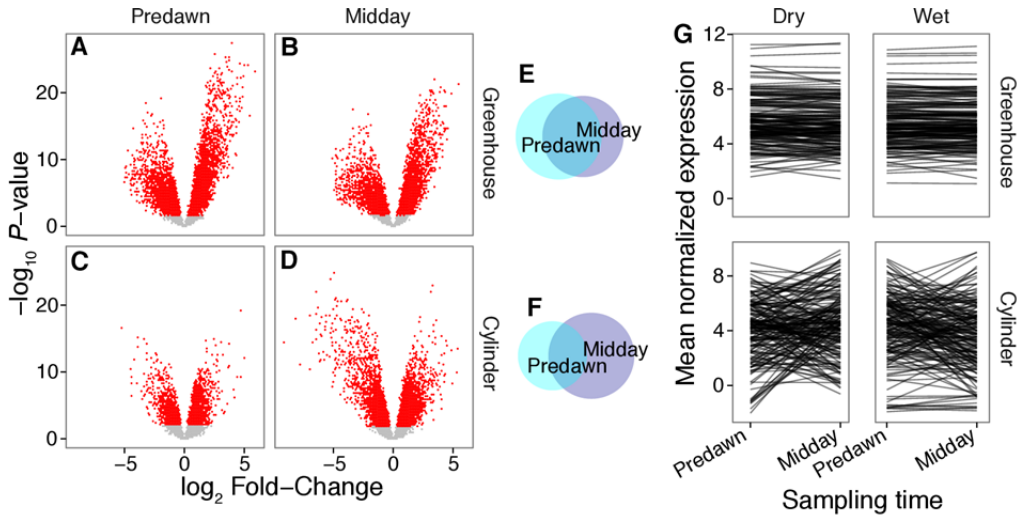

Figure 3. Differential gene expression due to soil water deficit is affected by the time of sampling. Differential expression between treatments was characterized via "volcano" plots, where the $\log_2$ fold change of treatment contrasts is plotted on the horizontal axis and the *P*-value of the associated test is on the vertical (A-D). Points were colored by whether the FDR-corrected *P*-value exceeded $\alpha = 0.05$ threshold. The total number of significant genes for each of the four contrasts were plotted in Euler diagrams (E-F), where disc size is proportional to the number of genes that were significant for each treatment contrast in the greenhouse (E) and cylinder experiment (F). The corresponding number of differentially expressed genes can be found in Table 1. To visualize the treatment*time interactions that make up these differential responses, we plotted mean normalized expression values for each of the top 100 treatment*time genes from the cylinder and greenhouse (G).

followed this pattern in the cylinders (Fig. 3F). Interestingly, the effect sizes of drought treatments were similar (mean = 2.4% smaller) at midday vs. predawn in the greenhouse but 35% greater in the cylinders (Fig. 3A-F). Such diurnal-by-treatment interactions were much stronger in the cylinders than the greenhouse. Specifically, there tended to be much stronger differential expression across sampling times in wet treatments than dry (Fig. 3G-H, Fig. S2). This effect was strongest in the greenhouse, where the drought treatment was extreme (Fig. 1B). Indeed, in the greenhouse, >1.7x more genes were diurnal-regulated in wet than in dry conditions (4,544 vs. 2,548, respectively, Fig. S2). It is possible that the extreme nature of the drought treatment in the greenhouse caused the cessation of diurnal gene regulation.

Finally, we also detected a small influence of the precise time at which plants were sampled (i.e., the order that a leaf was harvested for RNA extraction) on gene expression in the field conditions (Fig. S3). The order of sampling was a significant predictor of Ψ_{leaf} variation in 2013-14 (r^2 = 0.12, $P = 0.002$), but not 2012 (r^2=0.01; $P > 0.1$). Likewise, 21 genes differentially correlated with sampling order in 2012, but 125 did so in 2013-2014.

Paired gene expression and physiology permits inference of drought effects in variable environments. So far we have presented statistical tests between discrete watering treatments; however, due to environmental heterogeneity within soil moisture treatments (Fig. 1B), it may be more powerful and biologically relevant to look at associations between a metric of stress (e.g., Ψ_{leaf}) and physiological or gene expression phenotypic responses. Therefore, we augmented our previous comparisons with regressions of gene expression against Ψ_{leaf} measurements for all three of our experiments. In the cylinder experiment, the effects of treatment were largely recapitulated

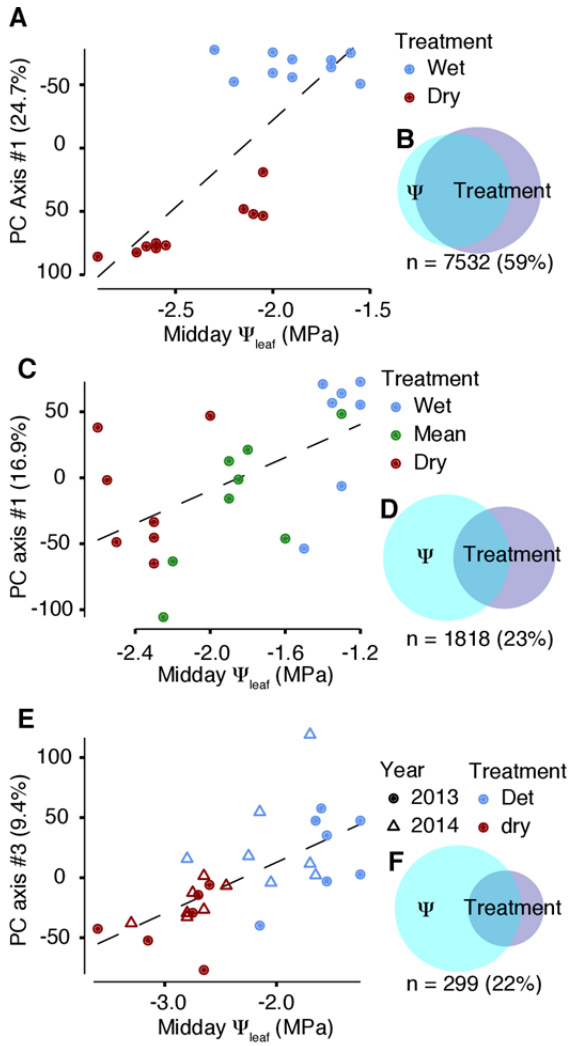

Figure 4. Gene expression variation associated with leaf water potential. The cylinders, and 2012 and 2013-14 shelter experiments represent the experiments that have paired midday Ψ_{leaf} and expression assays. Principal components (PC) from the complete gene expression matrix were calculated. Of the top three PC axes, the one which is most strongly explained by midday Ψ_{leaf} is plotted. A paired Euler diagram displaying the total amount of genes differentially expressed due to treatment accompany the PCA- plots for the cylinder (A-B), 2012 experiment (C-D) and 2013-14 shelter (E-F) experiments. The total number of genes presented can be found in Table 3.

by regressing leaf water potential on expression data. For example, within the midday sampling 83% of genes significantly associated with water potential were also detected by a contrast between treatments (Fig 4A-B, Table 3). This consistency reflected the strong experimental effects observed during the midday harvest (Fig. 1B, Fig. 4A).

In contrast, the regression approach and factorial treatment contrasts produced different outcomes in the shelters: fitting midday Ψ_{leaf} across treatments in 2012 increased the number of significantly differentially expressed genes by 70% (1510 total genes compared to 887). Additionally, 965 genes responded across the gradient of midday Ψ_{leaf}, 623 (85.7%) of which were significant only when Ψ_{leaf} was the predictor (Table 3). In these field experiments where a

more continuous range of precipitation treatments were applied, significant gene expression across drought stress intensities was not fully captured by treatment of precipitation levels as factorial variable.

The increased power of midday Ψ_{leaf} relative to discrete treatment variables was even more pronounced in the 2013-2014 experiments, where 1758 genes were significantly differentially expressed across Ψ_{leaf} but only 727 genes were differentially expressed across treatments (a 142% increase, Fig. 4e-f). It is possible that this across site effect was due to differential site characteristics, including soil quality and nutrient availability, and not drought per se. To further examine differences between the Temple and Austin shelter experiments, we split the 2013-14 dataset by site and reanalyzed differential expression. Within sites, we observed differential expression across drought treatments among 665 genes in Temple, but only 3 in Austin. However, water potential explained differential expression of 309 (1.1x) and 160 (53x) additional genes in Temple and Austin respectively (Table 3). These results indicate that utilizing measurements of physiological variation can account for expression variation that is not predicted by treatment factors alone.

Leaf-level physiological responses, but not differential gene expression, are highly correlated across experiments. The majority of genomic studies of drought have been conducted in highly controlled laboratory or greenhouse settings, which are intended to elucidate the patterns and processes of drought responses in the field. Here, we find broadly different characteristics of drought response in the shelters from those in either cylinder or greenhouse conditions. For example, across all differentially expressed genes the absolute effect size (mean absolute $\log_2$ fold change) was 75% -148% greater between treatments in the greenhouse than in the 2012, 2013-2014 shelter experiments and the cylinder experiment; furthermore, more genes significantly responded to the drought treatments of the greenhouse (6597 genes, treatment main effect, $\alpha = 0.05$) and cylinders (4489) than either the 2012 (887) or 2013-2014 shelters (752). Combined with our physiological response data, these results indicated that field-grown plants were responding to drought in a similar manner to potted greenhouse plants among some phenotypes, but altered expression values at a much lower rate.

Such weaker responses could be at the gene-level, in which case the direction and effect size of differential expression in the greenhouse should be highly predictive of that in the field. Alternatively, entirely different genetic responses may be present in the field. In many cases, we found strong correlations between expression in the field and the greenhouse, but r^2 between $\log_2$ fold changes in the greenhouse and field never exceeded 0.35 (Fig. S4), indicating that much of the drought responsive expression in the field was not predicted by that in the greenhouse.

While greenhouse responses to drought did not entirely predict those in the field, drought-responsive genes in the field were very clearly differentially expressed in the greenhouse. Of the 716 genes that were differentially expressed in the field and quantifiable in the greenhouse, 549 (76.7%) were also differentially expressed in the greenhouse, representing a highly significant enrichment of overlapping genes (*odds ratio* = 3.1, $P < 0.0001$, Table S1). Furthermore, despite relatively weak predictive power across experiments, the genes found in each experiment were much more likely to be found in other experiments than would be expected by chance. In the extreme case, the overlap between significant genes in the 2012 and 2013-2014 shelter experiments is 7.1x greater than the null expectation (Table S1). Combined, these results provide a potentially surprising result: the relatively uncontrolled field environment offered the strictest test of differential expression.

Genes and gene functions related to drought. While $>10^4$ genes displayed phenotypic plasticity to drought, only 546 were differentially expressed in three or more of our experiments (84 were found in all experiments). Interestingly, with few exceptions, these "core" genes were

consistently up- or down-regulated across all experiments (Fig. S5). Among this set, 460 genes were homologs of annotated *Arabidopsis thaliana* genes. However, of the 84 genes that were drought-responsive in all experiments, nine had no annotation, six were annotated only by protein domain/motif or general process and two were annotated as "Protein of unknown function (PUF)". These data suggest that the majority of drought-responsive genes belong to a well-known group of genes with specific assigned functions in plant biology.

As expected, we detected a significant enrichment of genes related to responses to stress and water ($P < 0.0001$) among the core set of genes (Table S2). These genes included homologs of dehydrins, LEA-type proteins, aquaporins, ascorbate peroxidase and other genes related to reactive oxygen species (ROS) detoxification, and abscisic acid (ABA)-responsive phosphatases and transcription factors. LEA proteins, dehydrin and aquaporins are induced by drought and involved in desiccation tolerance (Table S3 and references therein). Among the ABA responsive gene families were PP2C genes (ABI1-2) four members of the AP2 family of transcription factors including DREB1-2, and an ABA-responsive element-binding element (AREB). In addition, we detected genes coding glutathione S-transferase, L-ascorbate peroxidase, ascorbate oxidoreductase and other genes involved in reactive oxygen radical detoxification. Reactive oxygen species (ROS) are known to accumulate during many biotic and abiotic stresses, and defense against ROS appears to be a common mechanism during drought (Table S3 and references therein). Interestingly, two homologues of NCED9, a key enzyme in ABA biosynthesis, were also present in this core set of drought responsive genes (Table S3 and references therein).

Other GO drought-related annotation categories were enriched in the core set of genes, including photosynthesis (which was the most highly enriched GO term, $P = 9.1 \times 10^{-9}$) and several annotations related to oxidation-reduction status. We detected 7 genes encoding light-harvesting complex II and many others related to C_4 photosynthesis including two alanine aminotransferases, a phosphoenolpyruvate carboxykinase, and two malate dehydrogenases. Additionally, P5CS, which encodes a key enzyme in the biosynthesis of the osmoregulator proline, was also present in the identified list of "core" drought-responsive switchgrass genes.

In the cylinder and greenhouse experiments we confirmed that genes affected by time of sampling (predawn vs. midday) were enriched for GO terms related to circadian, or light-responsive annotations as photosynthesis and "regulation of circadian rhythm" annotations were some of the most overrepresented categories (Table S2).

DISCUSSION

To test the consistency of physiological and gene expression plasticity to soil moisture variation, we exposed clones of a single switchgrass genotype to drought treatments in the greenhouse and field. Pairing physiological measurements with detailed analyses of the genes that respond to drought revealed similar physiological responses, but qualitatively different patterns of molecular plasticity in the field than the controlled edaphic environments of the greenhouse and cylinders. For example, plants grown in small pots in the greenhouse displayed similar leaf water potential plasticity to plants grown in the field but ~10x more differentially expressed genes. Combined, these data indicated that many fewer differentially expressed genes were responsible for similar physiological plasticity in the field than the greenhouse.

Comparison of soil water deficit manipulations across experiments. Soil moisture manipulation experiments are generally practiced as "dry-downs" where watering is limited or ceased and potted plants experience attenuated soil moisture. Such experiments are the basis of much of the molecular understanding of drought physiology. Alternatively, ecologists and agronomists often test drought physiological responses through precipitation exclusion (or irrigation supplementation) treatments that persist through much of the growing season, or even across years. Both approaches may be ecologically realistic. For example, many annual species grow and reproduce with the water remaining from a single rainfall event, mimicking the progressive dry-down approach. However, crop breeders or climate change biologists may seek to understand how fitness and yield can be maintained during relatively dry growing seasons across drought years. This type of "press" drought treatment imposed in the field is known to elicit different physiological, gene expression (Barker et al., 2005), and ultimately community-scale, responses relative to "pulse" droughts such as those imposed by our cylinder and greenhouse experiments (Hoover et al., 2015; Hoover and Rogers, 2016).

The analyses presented here contrast not only the dry-down and field-scale approaches, but also different climactic (air temperature, vapor pressure deficit, photosynthetic photon flux density (PPFD), etc.) and edaphic (soil water retention, particle size, biotic interactions, etc.) characteristics. For example, the highly controlled aerial environment of the greenhouse elicited weaker diurnal air temperature and vapor pressure deficit progressions compared to plants grown in larger cylinders in the field (Fig. 3, Meyer et al. (2014)). Consequentially, diurnal patterns of gene expression dominated drought-responsive expression profiles in the cylinders, but were significantly weaker in the greenhouse, especially with regard to drought-responsive genes. Similar patterns, which have been observed among physiological characters, may be attributable to light-responsive stress pathways including phytochemical quenching cycles (Chaves et al., 2003; Mishra et al., 2012). Such temporal variation is intentionally dampened in controlled environments, such as the greenhouse or growth chamber. However, many drought responsive genes (e.g., photosystem I-II and other light-responsive pathways) are expected to modulate expression between predawn and midday conditions. As such, the cylinder experiment offers a compromise. While permitting tight control of the soil environment by using a common soil medium and uniform drying, the cylinders exposed plants to natural varying climatic features like temperature, VPD, and changing photoperiod cycles. Our results demonstrated that the realistic aerial environment of the cylinders increased statistical power to define the diurnal-by-drought interactions that are critical to soil moisture deficit responses (Fig. 4).

In the shelters, irrigation manipulations were implemented at the plot level over the course of multiple years. Such long timescales provided time for plants to acclimate not only to the drought treatments, but also to the edaphic characteristics of each site. Indeed, among-site differences were a major driving variable in the 2013-2014 shelter experiment. Most plants in Austin exhibited lower (more negative) midday Ψ_{leaf} than any plants in Temple (Fig. 1B). Despite similar irrigation levels, both wet and dry treatments in Austin (2014) clustered with the Temple (2014)

dry treatment (Fig. 1C). It is likely that plants grown in the shallow, rocky-clay soil of the Austin site (~0.2 m depth) experienced substantial water deficits even in the wettest irrigation treatments, while deeper soils at the Temple site (~5 m depth) would have provided much greater buffering capacity against drought. As such, it is not necessarily surprising that we did not observe many significantly drought-induced gene expression responses at the Austin site.

Synthesis of drought responses across experiments: What factors led to physiological, but not expression plasticity in the field? Field experimentation is typically thought to require greater replication because increased environmental heterogeneity of field conditions (e.g., variation in soils, microclimate, timing of sampling, etc.) may produce more variable measurements. Our analyses generally rejected this hypothesis and demonstrated that similar ranges of environmental, physiological and molecular heterogeneity existed within treatments across greenhouse and field sites. Instead, other factors besides residual variation must be driving the difference in gene expression—but not physiological plasticity among experiments—including (1) different mechanisms of drought responses, where short term treatments elicit different gene expression responses than long term droughts; (2) plant morphological characteristics, such as the ability to buffer soil water variation through tissue capacitance (water storage); (3) edaphic and climactic variation, such as stronger soil water potential gradients and temporally variable vapor pressure deficit in the field; or (4) physiological acclimatization.

It is possible that plants in the field have acclimated, following an initial drought responsive phase in which gene expression was similar to that of the plants studied in the greenhouse (Chaves et al., 2003). For example, the development of a larger root system, may permit greater soil water foraging (e.g. Comas et al., 2013). Such acclimation responses are driven by the expression of many genes (e.g. Werner et al., 2010) and may be initiated at the time of exposure to drought. Over time, expression of such genes would no longer be required, as the necessary structures would already be in place (Maseda and Fernández, 2006). However, while acclimation may play a large role in differential physiology across experiments it does not fully explain the massive disconnect between physiological and gene expression plasticity we observed. In concert with acclimation, different genetic pathways may modulate plant responses to press and pulse droughts. One interesting possibility is that the ecologically unrealistic shock imposed by the high rate of soil moisture reductions in the dry-down experiments may elicit programmed cell death and other shock responsive processes that are not drought-related per se. Such effects may be less important in larger field-grown plants if they are better able to buffer some of these extreme stress effects because of greater water storage and access to soil water (Maseda and Fernández, 2006). Genes that respond across different types of drought experiments seem most likely to offer the clearest picture of molecular responses to drought, as they may be less likely to represent treatment specific or shock-induced responses.

Defining drought-responsive genes across experiments. We expected to find similar gene expression responses across experiments because several evolutionarily-conserved (Rabbani et al., 2003) pathways are responsible for drought acclimatization, including: cell signaling, transport and communication, plant hormone metabolism, photosynthesis, and carbohydrate biosynthesis (Schafleitner et al., 2007). However, the types of genes that responded to drought treatments varied considerably across experiments (Table S3). This observation is not necessarily surprising, given the physiological differences between greenhouse- and field-grown plants (Chaves et al., 2003; Mittler, 2006; Mishra et al., 2012). For example, photosynthesis and energy production genes dominated the greenhouse experiment, whereas metabolism biosynthesis and stress response genes were less abundant. Similar observations have previously been reported in sunflower (Rengel et al., 2012) among other species. In contrast, longer term drought treatments disproportionally induced genes involved in other biological processes such as membrane biogenesis, redox mechanisms, cellular biosynthesis and metabolism (Table 4, Table S3, Table

S4, Des Marais et al. (2012)). In addition, while many transcription factors or genes related to DNA metabolism were previously found in the greenhouse dataset alone (Meyer et al., 2014) very few of them were identified as significant across all four drought experiments (Table 4, Table S3). It is possible that changes in transcription factor expression levels resulted in substantial but short-term effects in plants that experienced sudden drought in a greenhouse setting. By contrast, these effects may have been dampened in well-established and acclimatized plants in the field.

While transcriptional drought responses of AP13 plants varied substantially among different experiments, we detected a set of "core" responsive genes that were enriched in all four (84 genes) or 3/4 (n = 546) experiments (Table S3). This list of genes provided a set of candidate pathways necessary to confer water deficit responses in switchgrass. Homologs of many of these genes have been documented as drought related in other species. Included in this list were dehydrin (Lopez et al., 2003) and DnaJ chaperones, which are induced under drought in many plants and may contribute to better performance under water stress (Seki et al., 2002; Nguyen et al., 2004). We found homologs of two NCED9 loci, which are key enzymes in the abscisic acid (ABA) biosynthesis pathway (Lefebvre et al., 2006) and a broad range of ABA responsive enzymes and transcription factors. These included homologs of AREB, two PP2C genes (ABI1-2) and several AP2-binding transcription factors (DREB1-2). Interestingly, AREB transcription factors have been implicated in drought-responsive trans-regulatory divergence in *P. hallii*, a close relative of switchgrass (Lovell et al., 2016). Furthermore, ABI1-2, are known to be transcriptionally up regulated in response to ABA and control responses to drought, heat shock and oxidative stress (Vranová et al., 2000; Merlot et al., 2001; Schweighofer et al., 2004; Schafleitner et al., 2007). *DREB* (drought responsive element binding proteins) and other members of the AP-2 binding gene family represent some of the best documented regulators of ABA dependent and independent drought responsive transcription regulatory elements (Liu et al., 1998).

It is well documented that reduced transpiration, which accompanies drought acclimatization, may result in increased leaf temperature, light damage and a need for transcriptional responses to both heat (Bogeat-Triboulot et al., 2007; Swarbreck et al., 2011) and reactive oxygen species (ROS) stress (Smirnoff, 1993; Schafleitner et al., 2007). Interestingly, we observed many genes annotated to these abiotic stress responses, including heat shock-responsive transcription factors, which corroborates the previously proposed link between thermal defense and drought response in plants (Feder and Hofmann, 2003; Meyer et al., 2014). In addition, we detected genes coding for glutathione S-transferase, L-ascorbate peroxidase, ascorbate oxidoreductase and others involved in active oxygen radical detoxification.

Finally, we found genes from many of the *a priori* drought-responsive candidate groups; however, there were a few notable sets of drought-acclimatization genes that did not appear in our lists. Transcription factor families identified in the "core" set of drought responsive genes include members of the MYB-like, zinc finger, CCAAT-binding factors, Nuclear Factor Y (NF-Y), and MADS box. However, despite the apparent abundance of various types of transcription factors, the total number of identified genes and gene families with DNA binding activity was much smaller than detected previously in other studies (Schafleitner et al., 2007; Meyer et al., 2014). Specifically, we did not observe members of WRKY, NAM, TAF, SCARECROW, NAC, and CPP1 (among others) families of transcription factors (Meyer:2014eg; Rizhsky et al., 2002; Seki et al., 2002). It is possible that many of these transcription factors are currently mis- or un-annotated due to the very preliminary nature of the *Panicum virgatum* genome assembly (84% of the core genes were annotated). However these genes may be more critical in early signaling processes and would not be expected to be differentially responsive over term acclimation response in the field.

Discussion of best practices in field-scale physiological genomics. We found that despite strong physiological and soil moisture differences, plants in the field adjusted many fewer genes than potted plants in the greenhouse or plants grown in cylinders. It is clear that press droughts cause qualitatively different patterns of expression than dry-downs. These genetic differences between sustained and shock drought stress responses offer a challenge, but also a unique opportunity to study physiological diversity in the field. In our experiments we encountered several significant barriers that are important to experiments addressing these differences: particularly important are (1) the effect size of soil water deficit treatment differences, (2) fine-scale temporal expression differences across sampling, and (3) among-site variation.

In the 2012 shelter experiment, although we applied 6 different water treatments, clustering significant genes by expression profile similarity clearly differentiated plants under wet (and to a lesser extent 75th percentile) water treatment from all other treatment levels. In fact, at the whole transcriptome level we observed very weak differences in gene expression among mean, dry, ambient and 25th percentile water treatment conditions. Thus, under our experimental conditions in the field the observed 2012 differences in gene expression could have been captured by applying just two experimental water treatments: wet and dry. This result indicates that fewer, more distinct treatments with stronger within-treatment replication will result in more statistical power when using the experimental treatment as a factorial predictor. However, the stress gradient present across the six treatment levels proved useful as it provided a broader distribution of water potentials and improved power to detect gene expression plasticity to Ψ_{leaf}.

Time of sampling was an important factor across all experiments, ranging from within days to across years. While this is clear from contrasts between predawn and midday sampling, where >10k genes were differentially expressed, we also found subtle differences between expression patterns at the beginning and end of sampling in any given experiment. For example, the 2012 shelter data presented here comes from a larger experiment with >400 individuals in total. Overall sample collection for all plants took 2 hours, from 11 am to 1 pm. Many genes showed linear changes in expression over the sampling period of 2 hours (Fig. S3). These data clearly demonstrated that sampling order (and likely other micro-variation factors) could affect gene expression in the field. However, the small effect size (only 21 significant genes) observed in our experiments indicates that carefully planned and carried out experimental design (a narrow and consistent enough sampling window) can produce stable estimates of treatment effects that are not confounded with time-of-sampling micro-variation artifacts. We corrected for the time of sampling by using the spatial and temporal block in which each individual was sampled as a random effect; where spatial and temporal blocking factors do not covary, correcting for sampling time alone can improve statistical power to define differentially expressed genes (Lovell et al., 2016).

Across experiments, we paired leaf water potential with gene expression assays. The use of physiology as a covariate for assessment of differential gene expression permitted inference of effects across harvests, years, sites, and even experiments. Combined, these results support the expectation that leaf water potential serves as a powerful proxy for the degree of drought stress experienced by individual plants. Since most plants strive to avoid the effects of drought by maintaining leaf water potential homeostasis, this variable may be a strong predictor of the perceived stress of the local environment. Whereas leaf water potential certainly confers greater power to detect differential expression and assessment of across-site drought response, using this variable as a predictor does not permit causal inference (Jones, 2007). Genes that are correlated with leaf water status may either respond to such decreases in water potential or may have led to the reduction of water potential in the first place (e.g. Fu et al., 2000). For example, ABA sensitive genes in guard cells both cause variation in leaf water potential through stomatal regulation and respond directly to water potential (e.g. Tardieu and Davies, 1992; Speirs et al.,

2013). Therefore, inference regarding leaf water potential as a predictor should be interpreted carefully, possibly corroborated with comparisons among treatments. The use of other metrics of plant water status, like absolute or relative water content, may also be useful in assessing the molecular impact of water-deficit treatments (Maseda and Fernández, 2006; Jones, 2007).

Despite the long-term nature of our field-scale droughts, our measures of drought response are a snapshot, taken at a single time point when we perceived drought to have reached a critical point (in the greenhouse and cylinders) or when field conditions were optimal for sampling (in the shelter). Additionally, there may be subtle circadian-by-treatment interactions, which would not be captured by discrete predawn and midday sampling. However, by combining leaf physiology and gene expression measurements at these sampling points we endeavored to gain a synthetic picture of how drought was affecting individuals at a whole-plant scale. While we present single time-point analyses, repeated sampling across natural progressions of soil wetting/drying might be especially illuminating.

Conclusions. A major goal of modern plant biology is to better understand abiotic stress responses to improve crop plants—especially in the face of climate change (Ahuja et al., 2010; Tuteja and Gill, 2013). To do so requires that our fundamental understanding of physiology and molecular processes translate from controlled greenhouse and laboratory experiments into the field. Methods to emulate field-like conditions in the laboratory or greenhouse settings have been developed as an alternative to traditional soil water dry-downs (Harb et al., 2010); however, even factorial combinations of stressors (Suzuki et al., 2014)) may fail to capture the complex interplay of environmental variables experienced in the field. Thus, it is crucial to relate findings of field studies with those performed under controlled conditions, but only a few studies comparing physiological traits and gene expression data in drought treatments in both field and greenhouse conditions have been published (but see Rengel et al., 2012; Marchand et al., 2013).

Our study demonstrates how genes and phenotypic traits differentially respond to soil water deficit across greenhouse and field trials that impose different severity and duration of drought treatments. We find that the bulk of differentially regulated genes in the field are also found in the greenhouse. This indicates that the molecular and functional understanding of field grown plants is mirrored in the laboratory. However, a mechanistic understanding of how plants achieve similar physiological responses to drought across laboratory, greenhouse and field experiments—while regulating expression of different and generally fewer genes—remains to be developed. A collection of studies both linking controlled experiments to the field and exploiting natural precipitation and drying (e.g. Kudoh, 2015) will likely provide critical steps towards achieving this goal. Finally, biological replication is critical to detecting physiological and gene expression variation in the field. New high-throughput tools for measuring both relevant physiological and genome-wide expression phenotypes, such as TAG-Seq and tractor-mounted imaging, may provide an excellent avenue with which to achieve the necessary replication to compare field and laboratory physiological genomic studies.

MATERIALS AND METHODS

Overview of Experiments. Here, we present results from three separate experiments. Experimental design and conditions for the shelter and greenhouse experiments have been published previously (Aspinwall et al., 2013; Meyer et al., 2014). Relevant details are briefly reiterated below and expanded in the online supplementary material (Table S4). The levels of replication for each experiment are presented in Table 1.

2011 Greenhouse Experiment: Plants were grown in 3.78 L pots at the University of Texas Brackenridge Field Laboratory (Austin, TX) in the greenhouse with mean daytime air

temperature of 30°C and relative humidity of 65% (Meyer et al., 2014). Abundant watering was applied for the first 45 days of growth followed by complete withdrawal of water for the subsequent 14 days (experimental dry-down treatment group) or continued abundant watering (control group).

2011 Cylinder Experiment: Plants were grown outside at the University of Texas J.J. Pickle Research Facility and experienced natural lighting, photoperiod, humidity and temperature changes. At sampling, the maximum air temperature was 37°C and relative humidity was 13%. Plants were grown in 1.22 m tall cylinders constructed from 0.61 m diameter, grey schedule 40 polyvinyl chloride pipe with a wall thickness of 13 mm. Cylinders were arranged in 5 × 6 grid, spaced 1.2 m center-to-center and filled with Ranch Rose Mix (Geo Growers, Austin, TX). For the drought treatment, water was withheld from plants for 18 days while the control treatment continued to receive irrigation. The dry-down began on 21-August. Plants were phenotyped and tissue was sampled on 8-9 September 2011.

2012-2014 Shelter Experiment: This experiment was designed to test the effects of multiple climatically realistic levels of precipitation and soil moisture on the drought responses of plants (Aspinwall et al., 2013). The treatments represented five sets of historical (87-year record) rainfall patterns (Aspinwall et al., 2016): the 10 driest years ("dry"), 25th, 50th, and 75th percentiles and the 10 wettest years ("wet") at each site (Table 2). An ambient precipitation treatment applied amounts falling at the site immediately after they occurred. The pattern of watering events in these treatments was produced using the stochastic weather generator, LARS-WG 5.5 (Semenov, 2007), calibrated with an 87-year precipitation record (Aspinwall et al., 2016). Such climatically relevant drought treatments provide a proxy for the stresses experienced over the recent history and short-term future climactic scenarios at these sites (Mearns et al., 2013; Knapp et al., 2015). Due to the drought that occurred during the 2012 growing season, the ambient treatment clustered closely with the driest treatments. While gene expression was collected for all treatments, physiology was only paired with the "dry", "mean" and "wet" treatments. In 2013 and 2014, expression data was only assayed for the wet and dry treatments.

Physiological Measurements. In each experiment, predawn Ψ_{leaf} was measured with a Scholander-type pressure chamber (PMS 1000, PMS Instruments Company, Oregon, USA) at approximately 05:00 hrs local time on the uppermost fully-expanded leaf of a tiller representative of the canopy. For collection, the leaf was excised from the tiller with a sharp pair of scissors slightly above the ligule and sealed in a ziplock bag to prevent transpirational water loss until measurement (<5 min). Midday Ψ_{leaf} was determined following the same protocol between 13:00 and 15:00 h local time.

Leaf net photosynthesis (A, μmol $m^{-2}s^{-1}$) and stomatal conductance to water vapor (g_s, mmol $m^{-2}s^{-1}$) were measured using portable photosynthesis systems (LI-6400XT, LI-COR, Inc., Nebraska, USA) on two adjacent uppermost fully-expanded leaves from two separate tillers representative of the canopy. In each experiment, measurements of A and g_s occurred between 11:00 and 14:30 h local time. In the greenhouse and rainout shelter experiments leaves measured for photosynthesis were subsequently measured for midday Ψ_{leaf}. In the cylinder experiment water status was determined for similar but independently sampled leaves.

Leaf tissue collection and RNA sequencing. Tissue for RNA was collected from two leaves similar to those subjected to physiological measures as follows: (1) two tillers that were representative of the canopy were chosen, (2) the uppermost fully expanded leaves from each tiller were excised at the ligule, (3) 2 cm of the proximal portion of the excised leaf were separated from the midrib, and (4) both leaf samples were combined in a single 2 ml Eppendorf tube loaded with three stainless steel beads, immediately frozen in liquid nitrogen and transported on dry ice to the laboratory. Tissue was homogenized with a Geno/Grinder 2000. RNA was

extracted with the standard Trizol protocol and treated with DNase I to remove contaminating genomic DNA. RNA-Seq library samples were prepared using a modified version of the TAG-seq protocol (Meyer et al. (2011); Supplementary methods, 1.3). In short, purified 3' RNA was amplified and tagged prior to sequencing on the Illumina HiSeq platform. Prepared libraries were submitted to the Genomic Sequencing and Analysis Facility (University of Texas, Austin, USA) aiming to obtain 5 million reads per sample. RNA sequencing for the greenhouse experiment was accomplished on the SOLiD platform and described in detail in (Meyer et al., 2014).

Differences in library construction and sequencing chemistry between SOLiD and Illumina systems have been implicated in producing variation in transcriptomic profiles (Tariq et al., 2011) with library construction expected to feature heavily in observed qualitative and quantitative differences (Linsen et al., 2009). Nonetheless, correlations across such samples remain reasonable (i.e., global transcriptomic profiles remain largely intact) (Tyakht et al., 2014), and within-protocol (between sample) fold change estimates are thought to be robust to platform biases (i.e., differential expression analysis within a uniform protocol remains a viable assay (Toedling et al., 2012)). Platform biases may influence the power of differential expression tests at the gene level (and subsequently bias our comparisons of significant genes across experimental designs) through their impact on gene level sequencing depth. However, it is known that power in gene expression count contexts is primarily driven by sample size and secondarily by sequencing depth (Ching et al., 2014). We compared differences in the power to detect differential expression among sequencing technologies and experiments (which have slightly different levels of replication). To do so, we conducted "significance curves", where the total number of individuals in each experiment was rarefied, allowing direct comparison of the power of an experiment when replication is identical (Fig. S1).

Bioinformatic analysis of RNA-seq data. Shelter, cylinder (Illumina) and greenhouse (SOLiD) data was processed into fastq format and poly-A tail and known TAG-Seq 5' adapter sequence was removed using cutadapt (Martin, 2011). The trimmed sequences were subsequently aligned to the *P. virgatum* V2.0 reference (http://phytozome.jgi.doe.gov, Goodstein et al. (2012)). Base space reads were aligned with BWA-mem (Li and Durbin, 2009) SOLiD were aligned with the Bowtie color space aligner (Langmead et al., 2009). Hits to genes based on the *P. virgatum* V2.1 annotation were assessed using the "union" mode of htseq-count (Anders et al., 2015). Multiple alignments were utilized in the sam files and non-uniquely mapping reads were excluded (see supplementary materials). Library preparation and sequencing effort resulted in generally similar levels of saturation of the transcriptome (Figure S6, Supplementary material).

Statistical Analysis. The LIMMA R package (Ritchie et al., 2015) was used to conduct all statistical analyses pertaining to gene expression assays and GO annotations. Various model specifications can be found in the online supplementary material, and the functions used to streamline our analyses have been written into an R package (github.com/jtlovell/limmaDE2). For each model, normalization factors were calculated to scale libraries by total counts after first excluding any genes with mean expression < 5 raw counts. These factors were used as a covariate in the "voom" normalization procedure. In addition to the normalized counts, where possible, we also used either a spatial variable or repeated measures as a blocking variable in the linear model. Gene-wise statistics were calculated via generalized least squares linear models and subsequent empirical Bayes procedures to infer variance structure across genes. *P*-values were FDR corrected via the Benjamini-Hochberg method via the R function, "p.adjust" (Benjamini and Hochberg, 1995). Both GO and gene overlap enrichments were inferred via Fisher's tests.

Statistical analyses of physiological data were treated similarly to expression counts. We tested the effects of drought treatments, while controlling for spatial and temporal sampling variation in mixed effects linear models implemented in the R lme4 package (see supplementary material;

675 Bates et al. (2014)). Type III SS tests were calculated with the lmerTest package (Kuznetsova et
676 al., 2013).

677 Multivariate tests of gene expression were conducted via principal component analyses (PCA) of
678 normalized expression matrices in R. To determine the relative importance of each experimental
679 factor, we culled the expression matrix to genes that were significantly differentially expressed in
680 any factor. We subsequently applied an ANOVA decomposition of variance via PCA from the
681 LIMMA fitted linear model (Fresno et al., 2014) on the culled expression matrix.

682 **Accession numbers:** RNA-seq data analyzed here have been deposited in the short read archive
683 under BioProject ID: PRJNA322529. Accession numbers and metadata are presented in Table S5.

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694

695 SUPPLEMENTAL DATA

696

697 Supplementary Methods: Additional physiological methods, Additional RNA extraction methods,

698 Additional bioinformatics methods, and Additional statistical methods

699 Supplemental Appendix: Model specifications

700 Supplemental Table 1: ANOVA TypeIII statistics from models fitting experimental treatments to
701 physiological response variables.

702 Supplemental Table 2: Significance and odds ratios of significantly differentially expressed gene
703 overlaps.

704 Supplemental Table 3: GO enrichment across experiments (see separate file).

705 Supplemental Table 4: Gene lists and annotations of genes differentially expressed in 3 or 4 of the
706 experiments with homologs related to drought (see separate file).

707 Supplemental Table 5: Climactic conditions during harvest

708 Supplemental Figure 1. The number of significant genes detected per fixed replication level.

709 Supplemental Figure 2: The differential impact of diurnal patterns on gene expression in the wet
710 and dry treatments of the shelter experiment.

711 Supplemental Figure 3: The physiological and gene expression effects of the order of sample
712 collection.

713 Supplemental Figure 4: Pairwise expression correlations.

714 Supplemental Figure 5. Conserved expression across all experiments.

715 Supplemental Figure 6. Rarefaction analysis of library sequencing depth.

716
717
718
719 **FIGURE CAPTIONS**
720
721 **Figure 1. Physiological and gene expression responses to drought across three experiments.**
722 Replicates of the AP13 switchgrass genotype were grown in three separate experiments (A).
723 Predawn leaf water potential (Ψ_{leaf} , MPa) was assessed for each plant. Midday Ψ_{leaf}
724 measurements were paired with tissue collection for RNA for all experiments except the
725 greenhouse, where stomatal conductance (g_s) was assayed at midday instead of Ψ_{leaf}. These
726 midday and predawn measures are plotted with independent scales for the greenhouse and
727 remaining experiments (B). The expression matrices for genes with a significant effect of any
728 experimental factors (time of sample collection, location and year) were used to conduct principal
729 component analysis (PCA) decompositions (C). The length and direction of the vectors indicates
730 the strength of each experimental level. A vector perpendicular to the 1st PCA axis is plotted as a
731 dashed line. Finally, the principal component score for the transposed expression count matrix is
732 plotted and grouped by the experimental factors (D). The percent variance explained by the 1st
733 two PCA axes accompanies the axis labels. Note that in the 2012 shelter experiment expression
734 was assayed across six treatments, but physiological phenotypes were only measured in the wet,
735 mean and dry treatments. Levels of replication for each experiment can be found in Table 1.
736
737 **Figure 2. Physiological, soil and normalized expression variability across experiments and**
738 **treatments**. Coefficients of variation (*cv* = standard deviation / mean) were calculated for each
739 physiological and gene expression phenotype. Raw *cv* for each physiological phenotype is plotted
740 in panel A. To understand the relationships between Ψ_{soil} and soil volumetric water content, we
741 conducted soil moisture release curves for the greenhouse potting soil where a soil sample was
742 progressively dried and volumetric water content and Ψ_{soil} were repeatedly measured; the range
743 of observations for each treatment are presented by the marginal line segments (B). Finally, mean
744 (± SE) *cv* across all expression traits is plotted (C). The experiments are abbreviated as shelter
745 ("shel"), greenhouse ("gh") and cylinder ("cyl").
746
747 **Figure 3. Differential gene expression due to soil water deficit is affected by the time of**
748 **sampling.** Differential expression between treatments was characterized via "volcano" plots,
749 where the $\log_2$ fold change of treatment contrasts is plotted on the horizontal axis and the *P*-value
750 of the associated test is on the vertical (A-D). Points were colored by whether the FDR-corrected
751 *P*-value exceeded $\alpha = 0.05$ threshold. The total number of significant genes for each of the four
752 contrasts were plotted in Euler diagrams (E-F), where disc size is proportional to the number of
753 genes that were significant for each treatment contrast in the greenhouse (E) and cylinder
754 experiment (F). The corresponding number of differentially expressed genes can be found in
755 Table 1. To visualize the treatment*time interactions that make up these differential responses,
756 we plotted mean normalized expression values for each of the top 100 treatment*time genes from
757 the cylinder and greenhouse (G).
758
759 **Figure 4. Gene expression variation associated with leaf water potential.** The cylinders, and
760 2012 and 2013-14 shelter experiments represent the experiments that have paired midday Ψ_{leaf}
761 and expression assays. Principal components (PC) from the complete gene expression matrix
762 were calculated. Of the top three PC axes, the one which is most strongly explained by
763 midday Ψ_{leaf} is plotted. A paired Euler diagram displaying the total amount of genes differentially

764 expressed due to treatment accompany the PCA- plots for the cylinder (A-B), 2012 experiment
765 (C-D) and 2013-14 shelter (E-F) experiments. The total number of genes presented can be found
766 in Table 3.

TABLES:

Table 1. Summary of experiments and the effects of drought treatments. Sample sizes (*n*) and mean leaf water potentials are displayed for each treatment and experiment. Accompanying each experimental factor is the number of differentially expressed genes (*n* DE) due to the wet-dry treatment contrast therein. *Treatment replication in 2012: dry = 7; 25th = 6; mean = 8; ambient = 7; 75th = 5; wet = 7.

Experiment	Location	Leaf taken at	*n* Wet	*n* Dry	$\Psi_{\square\square t}$	Ψ_{dry}	Ψ Diff.	*n* DE
"Greenhouse"	Greenhouse	Predawn	14	24	-0.89	-2.42	1.53	6623
		Midday	13	21	NA	NA	NA	5918
"Cylinder"	1.4m^3 cylinders	Predawn	14	9	-0.45	-1.09	0.64	3285
		Midday	10	10	-1.88	-2.43	0.55	5745
"2012 Shelter"	Field	Midday	*	*	-1.32	-2.36	1.04	887
"2013-14"	Field	Austin, 2013	7	6	-3.03	-3.63	0.6	0
		Austin, 2014	7	4	-3.1	-3.79	0.69	4
		Temple, 2013	7	6	-1.57	-2.91	1.34	319
		Temple, 2014	7	7	-2.04	-2.77	0.73	154

Table 2: ANOVA statistics from models fitting experimental treatments to physiological response variables. Time effects were not estimable for conductance (g_s) and photosynthetic rate (*A*), which were only measured at midday in the cylinder and greenhouse experiments. Timing of Ψ_{leaf} measurements are defined by subscripts. TypeIII *F*-statistics and *P*-values are presented along with absolute effect size, which is the proportion of differences between group means and the overall mean. Only a subset of all absolute effect sizes are presented and are indicated by: * Predawn, ** Midday; `Temple 2013, ``Austin 2013; ^ Wet vs. Dry, ^^ Mean vs. Dry

Experiment	TypeIII Term	Phenotype	*df*	Abs. effect size	*F*	*P*
cylinder	Treatment	g_s	1	0.99	78.85	<0.001
cylinder	Treatment	*A*	1	0.92	60.70	<0.001
cylinder	Treatment	Ψ	1	*0.91, **0.93	79.72	<0.001
cylinder	Time	Ψ	1		273.9	<0.001
cylinder	Treatment:Time	Ψ	1		0.268	0.6080
greenhouse	Treatment	g_s	1	0.81	43.24	<0.001
greenhouse	Treatment	*A*	1	0.88	80.20	<0.001
greenhouse	Treatment	$\Psi_{predawn}$	1	0.72	24.65	<0.001
shelter 2012	Treatment	g_s	2	^1.09, ^^0.17	19.66	<0.001
shelter 2012	Treatment	*A*	2	^2.10, ^^0.52	19.43	<0.001
shelter 2012	Treatment	$\Psi_{\square\square\square\square\square\square}$	2	^0.20, ^^0.08	36.98	<0.001
shelter 2012	Treatment	$\Psi_{predawn}$	2	^0.48, ^^0.01	10.88	0.0010
shelter 2013-14	Treatment	$\Psi_{\square\square\square\square\square\square}$	1	`0.48, ``0.22	49.89	<0.001
shelter 2013-14	Location	$\Psi_{\square\square\square\square\square\square}$	1		17.85	0.0063
shelter 2013-14	Year	$\Psi_{\square\square\square\square\square\square}$	1		2.545	0.1185
shelter 2013-14	Treatment:Location	$\Psi_{\square\square\square\square\square\square}$	1		4.710	0.0357
shelter 2013-14	Treatment	$\Psi_{predawn}$	1	`1.21, ``0.56	20.48	<0.001
shelter 2013-14	Location	$\Psi_{predawn}$	1		1.682	0.2425
shelter 2013-14	Year	$\Psi_{predawn}$	1		1.680	0.2025
shelter 2013-14	Treatment:Location	$\Psi_{predawn}$	1		7.149	0.0107

Table 3. The number of significant genes in each experiment. Treatment ("Trt.") and Ψ_{leaf} at midday sampling were characterized for 2012, 2013-14 and cylinder experiments. The number of significant genes were determined ($\alpha = 0.05$) for two models: 1) ~ treatment, 2) ~ Ψ_{leaf}. For consistency, these models were only fit at midday for the cylinder and greenhouse data and within each site for the 2013-14 shelters.

Experiment	Trt.	Ψ_{leaf}	% Ψ_{leaf} not Trt.
2012	887	965	85.7%
2013-14	727	1758	177.7%
- Temple	665	309	11.0%
- Austin	3	160	5300.0%
Cylinder at midday	6365	5323	14.3%
Greenhouse at midday	5584	-	-

Table 4. List of "core" switchgrass genes enriched in ≥ ¾ experiments with known homologues involved in drought response. The number of genes found in the core list is presented. For specific genes, references and additional information, see Table S3.

Functional category	Predicted gene function	n genes
Water stress response	Dehydrins	4
	LEA proteins	3
	Aquaporin	1
Cell rescue, abiotic stress response and senescence	DnaJ-like molecular chaperones	4
	Other chaperones	6
	Senescence	2
	Response to biotic and abiotic stress	3
	chitinase	1
	multidrug resistance	1
ROS detoxification	Glutathione S-transferase	2
	L-ascorbate peroxidase	1
	ascorbate oxidoreductase	1
ABA response pathway	Protein phosphatase 2C	13
	AREB factor	1
	NCED9	2
	ABA/WDS induced protein	5
Transcription factors	Homeobox family	4
	AP2 domain	4
	Heat shock responsive TF	1
	MYB family	4
	Zink finger family	10
	CCAAT-binding factor	1
	MADS box	2
Cell signaling,	Protein kinases	23
	Osmotic stress potassium transporter	1
	GTP-binding	2
	Ca2+-binding transmembrane protein	1
C4 Photosynthesis	Auxin response	2
	Light harvesting	7
	Alanine aminotransferase	2
	Phosphoenolpyruvate carboxykinase	1
	Malate dehydrogenase	3
Metabolism	Proline biosynthesis	1
	Sucrose synthase	2
	β-amylase	3

802
803

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