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Gene expression differs in codominant prairie grasses under drought

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Abstract

Grasslands of the central United States are expected to experience severe droughts and other climate extremes in the future, yet we know little about how these grasses will respond in terms of gene expression. We compared gene expression in *Andropogon gerardii* and *Sorghastrum nutans*, two closely related codominant C_4 grasses responsible for the majority of ecosystem function, using RNA-seq. We compared Trinity assemblies within each species to determine annotated functions of transcripts responding to drought. Subsequently, we compared homologous annotated gene-groups across the two species using cross-species meta-level analysis and functional clustering based on key terms. The majority of variation was found between species, as opposed to between drought and watered treatments. However, there is evidence for differential responses; *Andropogon* allocated gene expression differently compared to *Sorghastrum*, suggesting *Andropogon* focuses on stress alleviation (such as oxygen radical scavenging) rather than prevention. In contrast, *Sorghastrum* may employ a drought avoidance strategy by modulating osmotic response, especially with hormonal regulation. We found *Sorghastrum* tended to be more sensitive within 10 key gene-groups related to stress, abscisic acid and trichomes, suggesting gene expression may mechanistically parallel sensitivity at the physiological level. Our findings corroborate phenotypic and physiological differences in the field and may help explain the phenotypic mechanisms of these two species in the tallgrass prairie community under future drought scenarios.

KEYWORDS
Andropogon gerardii, C4 grass, comparative expression, dominant species, RNA-seq, *Sorghastrum nutans*

1 | INTRODUCTION

Droughts plague many ecosystems, including the central United States, and are expected to increase in frequency and severity (Wuebbles et al., 2014). Through manipulative experiments, ecologists have learned what we may expect under these scenarios (Fay, Carlisle, Knapp, Blair, & Collins, 2003; Hoover, Knapp, & Smith, 2014b; Smith, 2011). Yet, detecting stress within individuals and in ecological communities remains a problem of scale (Levin, 1992), and selecting responses and tools that are most appropriate for measuring drought stress remains a challenge. Ecologists have grown increasingly interested in pairing the molecular responses to drought, such as gene expression and metabolite analysis, with morphological and physiological data (Lovell et al., 2016) in order to reveal underlying mechanisms. An understanding of gene expression is a critical hurdle revealing these stress response mechanisms (Johnson et al., 2014; Leakey et al., 2009; Meyer et al., 2014; Swarbreck et al., 2011). Despite this, ecologists have generated few resources for studying gene expression in nonmodel plants, such as the native codominant tallgrass prairie species *Andropogon gerardii* (big

bluestem) and *Sorghastrum nutans* (Indiangrass) of the central United States.

High aboveground biomass (Smith & Knapp, 2003), C4 photosynthetic capacity and community codominance describe both *Andropogon* and *Sorghastrum*, which are similar in appearance and traits (Forrestel, Donoghue, & Smith, 2015). Yet, these two grasses differ in their physiological responses to stress, with these differential responses ultimately cascading to affect ecosystem functioning (Hoover, Knapp, & Smith, 2014a). Specifically, *Sorghastrum* is more sensitive overall to both soil moisture and temperature, while *Andropogon* is primarily responsive to temperature (Nippert, Fay, Carlisle, Knapp, & Smith, 2009). Under severe water limitation, *Sorghastrum* suffers a greater loss of function (Hoover et al., 2014a) and grows more gradually than *Andropogon* (Nippert et al., 2011). Trichome morphology also differs substantially in these species and could be related to drought prevention, particularly in *Andropogon* (Olsen, Caudle, Johnson, Baer, & Maricle, 2013). These findings suggest that *Andropogon* and *Sorghastrum* differ in physiological mechanisms for coping with stress, particularly with respect to avoidance vs. tolerance of climate stress. Investigating gene expression responses in these codominant grasses will improve our understanding of drought response through molecular mechanisms.

Andropogon and *Sorghastrum* cope with and respond to temperature and water stress differently at the genomic level (Smith, Hoffman, & Avolio, 2016; Travers et al., 2007, 2010), reflecting differences in their ecophysiological performance in the field (Hoover et al., 2014a; Nippert et al., 2009, 2011). However, these previous studies utilized heterologous hybridization to the maize (*Zea mays*) cDNA microarray; it is unclear how many *Andropogon* and *Sorghastrum* genes are not detected due to nucleotide dissimilarity to maize probes. Although one RNA-seq transcriptomic resource exists for the *Andropogon* genus (Raithel et al., 2016), high genetic diversity in the *A. gerardii* species alone (Avolio, Chang, & Smith, 2011) may lead to failed transcript alignments. Next-generation resources are completely lacking for *Sorghastrum*. Therefore, the goal of this study was to conduct the first RNA-seq analysis of *Andropogon* and *Sorghastrum* to more rigorously assess molecular mechanisms of differential response of these species under drought.

We manipulated water availability via soil moisture drying for these two grass species to provide two controlled conditions: drought vs. watered. We aimed to (i) characterize transcriptome homology of *Andropogon* and *Sorghastrum*, (ii) determine differential expression within *Andropogon* and *Sorghastrum* under drought conditions and (iii) compare differential expression responses among homologous gene-groups in the two species. Based on ecophysiology in these species and previous microarray research (Smith et al., 2016; Travers et al., 2007, 2010), we expected expression under drought and watered treatments to differ for both species. Specifically, *Sorghastrum* would have more extreme differences in gene expression between treatments and more genes overall experiencing significant change. We expected enriched stress alleviation expression within *Andropogon* (such as heat shock or oxygen scavenging proteins). Conversely, we expected *Sorghastrum* to exhibit enriched expression of osmotic regulatory processes like aquaporin production, abscisic acid (ABA) production or stomatal regulation. Thus, we expected differential expression responses among homologous gene-groups to encompass these categories. We also expected trichome-related gene-groups to to be more sensitive within *Andropogon*, in agreement with morphological differences between the two species. This study provides the first next-generation sequencing resource for comparing these two ecologically important grasses and enhances our depth of knowledge of the molecular phenotype, response mechanisms and ecosystem genetics in the tallgrass prairie.

2 | MATERIALS AND METHODS

2.1 | Study species

Andropogon gerardii and *Sorghastrum nutans* are the most common grass species of the tallgrass prairie ecosystem of eastern Kansas. Both are self-incompatible polyploid species, where *Andropogon* is typically hexaploid (Keeler, 1990) and *Sorghastrum* is typically tetraploid (Riley & Vogel, 1982) (these cytotypes are used in this study). Both reproduce primarily through asexual tiller formation and experience comparatively low local recruitment from seed germination. Both have large genomes; hexaploid *Andropogon* contains approximately seven gigabases, while tetraploid *Sorghastrum* contains approximately six gigabases (Delaney & Baack, 2012; Keeler, Kwankin, Barnes, & Galbraith, 1987). Both species persist during experimental rainfall manipulation, drought and heat waves (Avolio & Smith, 2013; Hoover et al., 2014b), helping prevent loss of ecosystem function under extreme climatic events in the tallgrass prairie (Smith, 2011).

2.2 | Plant material, RNA extraction and sequencing

Common genotypes of both species were collected from the lowlands of Konza Prairie Biological Station in Manhattan, KS, and were clonally propagated in tissue culture (cultured by SMK Plants, Billings, MT). Tissue culture resulted in plants of approximately the same size and phenological stage. Once rooted, we transplanted plantlets to Premier pro-mix HP (Griffin Greenhouse Supplies, Inc., Tewksbury, MA). These plant clones adapted to 70% relative humidity and light irradiance of 37.5 μmol m^{-2} s^{-1} in growth chambers for three weeks. We then transferred plants to the greenhouse with a 28°C daytime temperature of 28°C, 22°C night temperature and 16-hr photoperiod. We initiated drought treatment after one month of greenhouse acclimation. At this stage, plants were under 30 cm in height and were not root bound or elongating for flower production. Nondroughted plants were kept at field capacity, which is approximately 30% volumetric water content (VWC) in similar experiments using the same media (Fig. S1). Water was withheld in an uncontrolled drydown for six days to produce an extreme drought. While this was a relatively short and extreme drought, soils within native tallgrass prairie are known to dry down quickly, especially late in the growing season (August) (Nippert & Knapp,

2007). Although droughted and nondroughted treatments were performed distinctly, uncontrolled drydowns may produce significant variation (Lovell et al., 2016) and may be an important source of error in this study. In other words, substantially lower VWC in one pot could lead to incorrect conclusions about more extreme expression responses. After six days of drought, we collected newly emerged leaves from two biological replicates within the watered treatment (water not withheld) and drought treatment ($n = 2$, with two treatments and two species). We immediately flash-frozen leaves in liquid nitrogen and stored tissue at −80°C prior to RNA extraction.

We extracted RNA from leaf tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified RNA with the RNeasy kit (Qiagen, Valencia, CA) after on-column DNase treatment (Qiagen). Eight total cDNA libraries were multiplexed and sequenced on the Illumina HiSeq 2000 platform to generate 72 bp paired-end reads. Library construction, sequencing and demultiplexing were performed at the Yale Center for Genome Analysis.

2.3 | De novo assembly

We examined raw data FastQC reports for anomalies using the iPlant Collaborative HTProcess pipeline, after which we trimmed sequences to suitable length and quality using TRIMMOMATIC (version 0.32) (see Appendix S1). Trimmed reads were assembled in TRINITY (version 2.1.1) (Haas et al., 2013) using the Colorado State University Correns cluster, creating an assembly for each species. We chose the Trinity pipeline because it has been shown to work well with polyploid species, including nonmodel grasses (Bushman, Amundsen, Warnke, Robins, & Johnson, 2016). To evaluate the quality of the Trinity assemblies, we used (i) Trinity's built-in evaluation scripts, (ii) read realignment percentage using BOWTIE 2 (version 2.2.7) and (iii) Samtools scripts.

2.4 | Differential expression within species

Using two Trinity transcript assemblies as references (one for each species), we realigned reads using BOWTIE 2. We then used RSEM (version 1.2.28) to count expression of each transcript (transcript per million, TPM) (Li & Dewey, 2011). In other analyses, Trinity transcripts may be known as genes or contigs. In our analysis, we retained all isoforms of transcripts produced by Trinity. Although there was weak coverage for many transcripts, we retained all transcripts so as not to discard any genes with potential ecological relevance. We calculated differential expression across treatments by comparing negative binomial distributions of transcript counts within EDGER (EDGER version 3.16.4) (Robinson, McCarthy, & Smyth, 2010) using Fisher's exact tests for each transcript. After normalizing across sample coverage using trimmed mean of M-values (TMM), we filtered results for a false discovery rate of <0.05 (Benjamini–Hochberg method) and minimum log fold change of >1 (2× difference in expression). All statistical analyses were performed using R (version 3.3.0, R Core Team 2016).

2.5 | Homology and annotation

We used the BLAST+ tool BLASTN to compare the assembled *Andropogon* and *Sorghastrum* transcriptomes to existing cDNA resources. We downloaded cDNA for *Arabidopsis thaliana*, *Brachypodium distachyon*, *Hordeum vulgare* (barley), *Leersia perrieri* (cutgrass), *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), *Setaria italica* (foxtail millet), *Sorghum bicolor* (sorghum), *Triticum aestivum* (wheat) and *Zea mays* (maize) from Ensembl genomes (Kersey et al., 2015). Other species were downloaded from the Joint Genome Institute Genome Portal (Nordberg et al., 2014). We used an e-value of e-10 to parameterize all BLASTN searches. Homology percentages were determined by calculating the average percentage of identical matches.

We used the TRINOTATE (version 3.0.1) (Haas et al., 2013) pipeline to annotate transcripts in the assembled transcriptomes to known genes. First, we used TRANSDECODER (version 2.1.0) to extract potential protein-coding regions within long open reading frames (ORFs). We simultaneously aligned these predicted protein regions and Trinity assembled transcriptomes for each species to the SWISS-PROT database using BLASTX (The UniProt Consortium, 2015) and PFAM (Finn et al., 2015) database using BLASTP. Specific releases of SWISS-PROT and PFAM were required for use with TRINOTATE (see Appendix S1). HMMER (version 3.1b2), SIGNALP (version 4.1) and TMHMM (version 2.0c) were used to further predict and identify protein domains. Gene GO annotations (The Gene Ontology Consortium, 2015) were also retrieved from the SWISS-PROT database. Finally, we downloaded the transcription factor databases for *Zea mays* and *Sorghum bicolor* (Charoensawan, Wilson, & Teichmann, 2010). We searched these databases using PFAM annotations above.

Following annotation, we combined count matrices produced by RSEM with annotations compiled by TRINOTATE. Transcripts with significantly different expression between drought and watered treatments were examined for Gene Ontology (GO) category enrichment using GOSEQ (version 1.24.0) (Young, Wakefield, Smyth, & Oshlack, 2010) with a *P*-value cut-off of $P < .05$. We also searched for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (using KEGG Mapper—Pathway Search against *Z. mays*) (Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016), and presence of transcription factors. REVIGO was used to reduce and visually assess GO enrichment categories (Supek, Bošnjak, Škunca, & Šmuc, 2011). We also contrasted the top 400 highly expressed transcripts (average TMM across samples) against the remaining transcripts for both species (Table S4).

2.6 | Comparative expression between species

We used SWISS-PROT annotations to compare orthologous transcripts between *Andropogon* and *Sorghastrum*, hereafter referred to as gene-groups. We chose this approach as opposed to direct sequence comparison for leniency; *Andropogon* and *Sorghastrum* transcripts were able to align to different parts of the same SWISS-PROT annotated gene and different splice variants could be combined as long as they

matched the same annotation. Similar annotation-based comparisons have been used in other studies (Cui & LoVerso, 2015). While using homology to model organisms to compare species is useful (Sudmant, Alexis, & Burge, 2015), our indirect comparison approach eliminates any transcripts lacking model species annotations (Rowley et al., 2011). Moreover, neither direct nor indirect alignment can account for neofunctionalization, even within species (Duan et al., 2016).

To examine species differences outside of treatment, we compared gene-groups within drought samples separately from watered samples. For this broader analysis, we summed count expression within each unique gene-group and normalized across samples using the TMM method. Comparisons were made using Fisher's exact test within EDGER with a false discovery rate of <0.05 and minimum log fold change of >1 (2× difference in expression). We analysed gene-groups for GO category enrichment, KEGG enrichment and transcription factors (as above). We then determined differentially expressed gene-groups across *Andropogon* and *Sorghastrum* with respect to treatment using the meta-level analytic method described in Kristiansson et al. (2013). This technique is powerful for deducing species by environment interactions where multiple orthologs and paralogs are found within each gene-group, as would be expected comparing species of different ploidies. We filtered these results for *P*-values < .05 and false discovery rate <.05.

2.7 | Functional annotation clustering

We clustered all gene-groups from the above analysis by filtering out keywords "stress," "heat shock," "trichome," "abscisic acid," "stomata" and "aquaporin" from all possible annotations. These categories were determined a priori due to the physiological and morphological differences between *Andropogon* and *Sorghastrum*.

3 | RESULTS

3.1 | De novo assembly and differential expression

After trimming, we retained 5.33×10^7 total reads for *Andropogon*, representing 73% of original reads. For *Sorghastrum*, many more reads were trimmed out, leaving 3.44×10^7 reads (50%). All samples were between 50 and 52% GC with all reads between 40 and 64 base pairs (Table S1). Trinity de novo transcriptome assembly produced 64,930 transcripts for *Andropogon* with an N50 = 789. *Sorghastrum* had 47,807 transcripts assembled with an N50 = 764. High-quality transcriptomes typically observe realignment rates between 70% and 80% using BOWTIE 2; our realignments met these criteria, with rates between 75% and 85% (Table 1).

In *Andropogon*, 106 total transcripts differed significantly between the treatments, with 52 highly expressed under the drought compared to the watered treatment and 54 transcripts highly expressed under the watered treatment (Figure 1a). *Sorghastrum* regulated slightly fewer transcripts: 85 total transcripts differed, with 39 highly expressed only during drought and 46 only expressed when watered (Figure 1b). When we regressed the representative subset of transcripts against their coverage using RSEM, *Andropogon* expressed an estimated 7,300 transcripts, while *Sorghastrum* expressed an estimated 8,419 transcripts (Fig. S2). The discrepancy between the Trinity transcripts and the transcripts aligned using RSEM indicates many lowly expressed transcripts or transcripts with low coverage. Generally, *Andropogon* retained more gene isoforms which could be of interest for further studies investigating alternative splicing.

TABLE 1 Summary of transcriptome assembly and quality checks in both species. Proper pairing indicates both paired-end reads aligned together

		Andropogon gerardii	*Sorghastrum nutans*
Assembly	Total Trinity "genes"	35,656	29,155
	Total Trinity "transcripts"	64,930	47,807
	Per cent GC	49.25	49.2
	Transcript N50	789	764
	Median transcript length	522	514
	Average transcript length	684.96	670.36
	Total assembled bases	44,474,391	32,047,794
QC	Average BOWTIE 2 realignment	83%	80%
	Average proper pairing	77%	74%

3.2 | Homology and annotation within species

Andropogon gerardii and *Sorghastrum* generally showed the same order of relatedness to all other species' transcriptomes at the nucleotide level. The two grasses were most related to other Andropogoneae like *Sorghum bicolor* (93.6%–93.8%) and *Zea mays* (91.4%–91.6%). More distant relationships were observed with other C_4 (~89%) and C_3 (~86%) Poales. Unsurprisingly, eudicots and one distantly related monocot (*Zostera marina*) showed the least homology (78.8%–80.9%) (Figure 2). Similar order of relatedness was obtained at the protein level (Fig. S3a). We matched 81% and 84% of transcripts to annotations or conserved domains for *Andropogon* and *Sorghastrum*, respectively. Transmembrane helices (TmHMM) represented 8% of transcripts in *Andropogon*, but *Sorghastrum* mapped more (9%), which could correspond to *Sorghastrum* greater water response or transport. *Andropogon* had far greater transcription factor expression (8%) vs. *Sorghastrum* (3%), which could be reflective of greater heat response. Overall, 42% of *Andropogon* transcripts and 45% of *Sorghastrum* transcripts mapped to conserved protein domains (Pfam) and 1.5% of transcripts in both species mapped to signal peptide cleavage sites (SignalP).

Gene Ontology (GO) enrichment of differentially expressed transcripts varied between the two species (Figure 3). Enriched biological processes upregulated under drought in *Andropogon* included dhurrin biosynthesis (plant defence), amino acid salvage, response to stimulus, abscission and misfolded protein catabolic processes. In

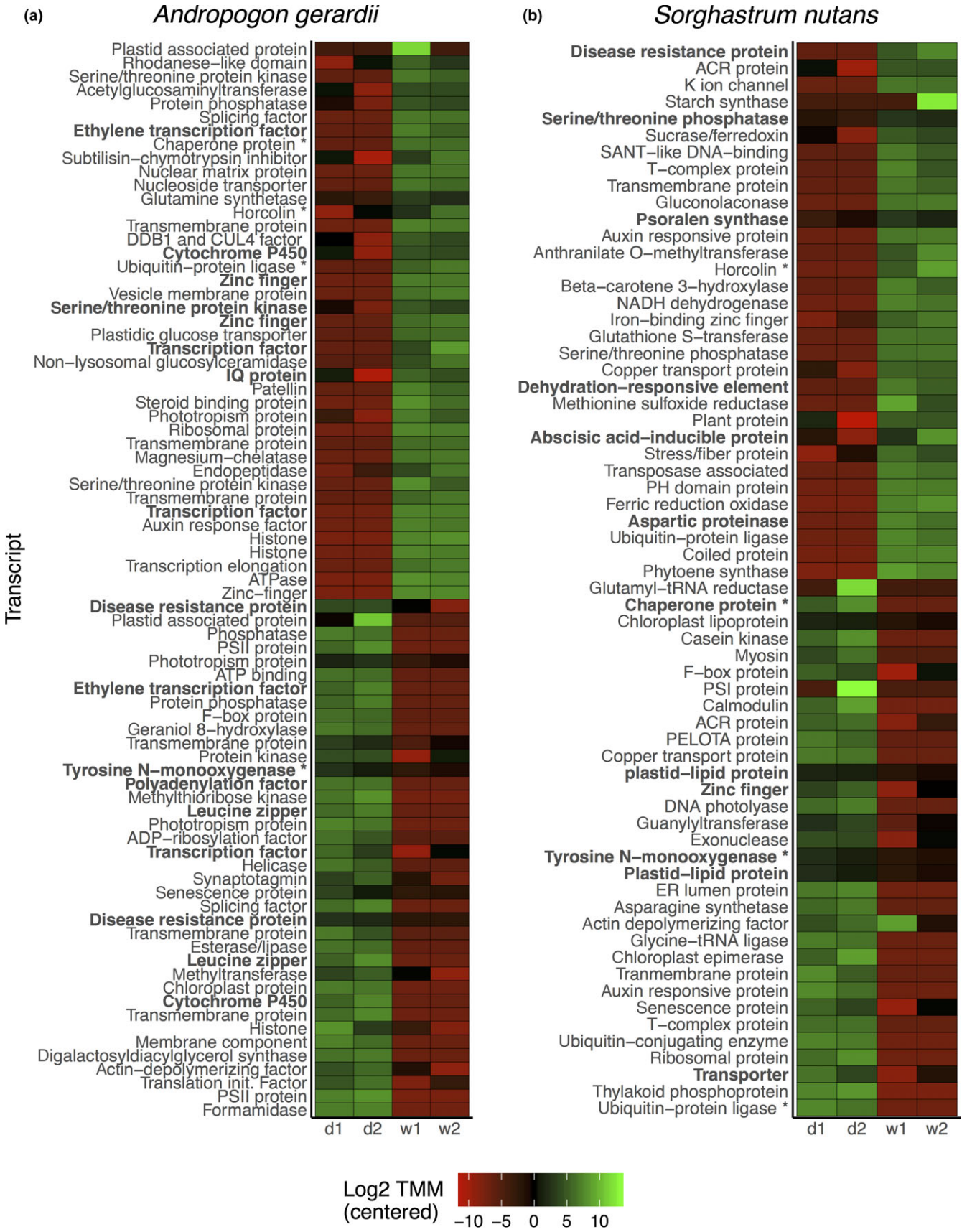

FIGURE 1 Transcripts differentially expressed in (a) *Andropogon* and (b) *Sorghastrum*. Drought samples are indicated "d1" or "d2," while watered samples are indicated "w1" or "w2." Units are log2 TMM (trimmed mean of M-values). Bold transcripts map to known transcription factors. Transcripts with asterisk (*) are found in both species

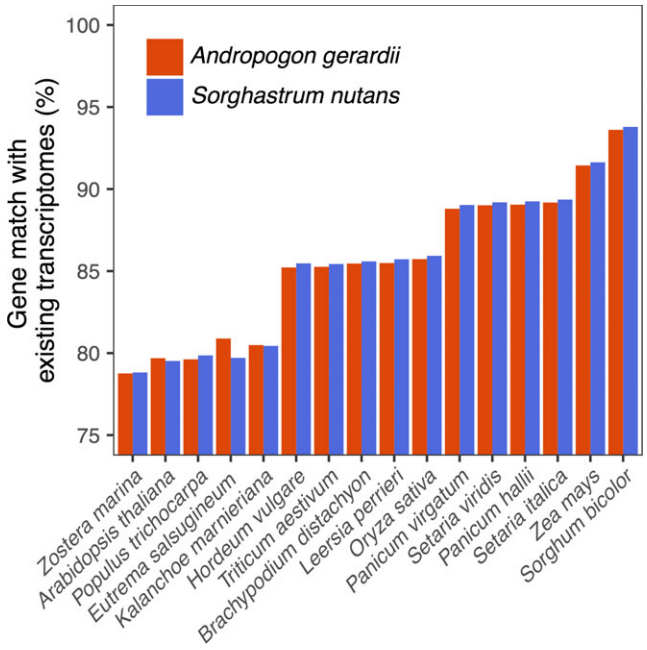

FIGURE 2 Homology of both species to known transcriptomes at a per gene basis. Per cent match refers to average percentage of identical matches at the nucleotide level (BLASTN)

Sorghastrum, dhurrin biosynthesis was also enriched, but response to osmotic stress, hypersalinity response and response to cytokinin were also represented. *Andropogon* molecular functions were enriched in tetrapyrrole (chlorophyll) binding, oxygenase activity and transcription factor activity under drought, while *Sorghastrum* transcripts were not.

Using KEGG, we found that for *Andropogon* drought-upregulated transcripts most represented metabolic pathways, glycerolipid metabolism (stored energy), mRNA surveillance pathway (e.g., degradation of aberrant transcripts) and spliceosome. Transcripts upregulated under the watered treatment also included metabolic pathways and spliceosome, but with more emphasis on amino acid synthesis (Table S3). In *Sorghastrum*, drought transcripts were strongly enriched in plant hormone signal transduction, followed by ubiquitin-mediated proteolysis (protein degradation), and RNA degradation. For watered transcripts, plant hormone signal transduction was also most represented (Table S3). Using homology to known sequences, we found *Andropogon* differentially regulated 17 transcription factors, while *Sorghastrum* showed differentially expression of 12 (Figure 1). Only four similarly annotated transcripts were found in both species.

3.3 | Comparative expression between species

Using count data across all shared annotated transcripts, we saw that species separated dramatically in multidimensional space (Figure 4). Drought and watered samples were first compared separately to focus on species differences. Within the drought treatment, *Andropogon* and *Sorghastrum* differed significantly across 4,032 gene-groups. *Andropogon* upregulated gene-groups represented transferase activity, transcription factor activity, flavonoid synthesis and metabolism, glycosylation and vesicle GO terms compared to *Sorghastrum*. In contrast, *Sorghastrum* gene-groups were most represented by DNA-complexes, regulation and response to stress and hormones, and RNA and compound binding (Table S5). Metabolic pathways differed in the two species: *Andropogon* gene-groups represented a greater proportion of secondary metabolite biosynthesis, plant hormone signal transduction, citrate cycle and amino/nucleotide sugar metabolism pathways (Table S6). *Sorghastrum* gene-groups represented a greater proportion of fatty acid degradation, nucleotide excision repair, mismatch repair and thiamine metabolism pathways (Table S6). Significantly upregulated gene-groups in *Andropogon* matched 321 known transcription factors vs. 213 transcription factors in *Sorghastrum*.

Compared to the drought treatment, watered plants of the two species diverged significantly across 4,929 gene-groups. *Andropogon* watered gene-groups were enriched in cellular and stem cell proliferation, binding activity, flavonoid synthesis and metabolism, and regulation of biological processes. In contrast, *Sorghastrum* watered upregulated gene-groups represented primary, nitrogen and aromatic compound metabolism, negative regulation of transcription, ion binding activity and nucleoplasm (Table S5). Metabolic pathways were largely similar to those represented in droughted plants. *Andropogon* gene-groups were additionally enriched in the MAPK signalling pathway and glycerolipid metabolism (Table S6). *Sorghastrum* watered gene-groups also represented carbon fixation and photosynthesis (Table S5). Transcription factor matches stayed consistent across treatments, with only one fewer transcription factor upregulated in *Andropogon* (320) and three fewer transcription factors upregulated in *Sorghastrum* (210).

We examined differential responses between *Andropogon* and *Sorghastrum* accounting for multiple orthologs and paralogs in a meta-level analysis. Of 11,878 gene-groups, we found significantly different responses in 83 (Figure 5) indicating a small but present species × environment interaction overall. These gene-groups were enriched in response to stimulus, response to oxidative stress, membranes and plastoglobule, tetrapyrrole binding and ER retention sequence binding GO terms (Figure 6). Of the 83 significantly changing gene-groups, 17 mapped to known transcription factors.

3.4 | Functional annotation clusters

Within differentially expressed gene-groups from the meta-level analysis, 10 genes matched the functional annotation clusters. These included seven stress gene-groups, one trichome group and two ABA groups. *Sorghastrum* appeared more sensitive in five of the seven stress groups and both ABA groups (MSRB1_ORYSJ, USPA-L_ARATH, SALT_ORYSJ, NDUS4_ARATH, TIL_ARATH, CUT1B_ARATH and GRPA_MAIZE, Figure 7). *Andropogon* appeared more sensitive in one stress group and the trichome group (AFG1_YEAST and MYO17_ARATH, Figure 7).

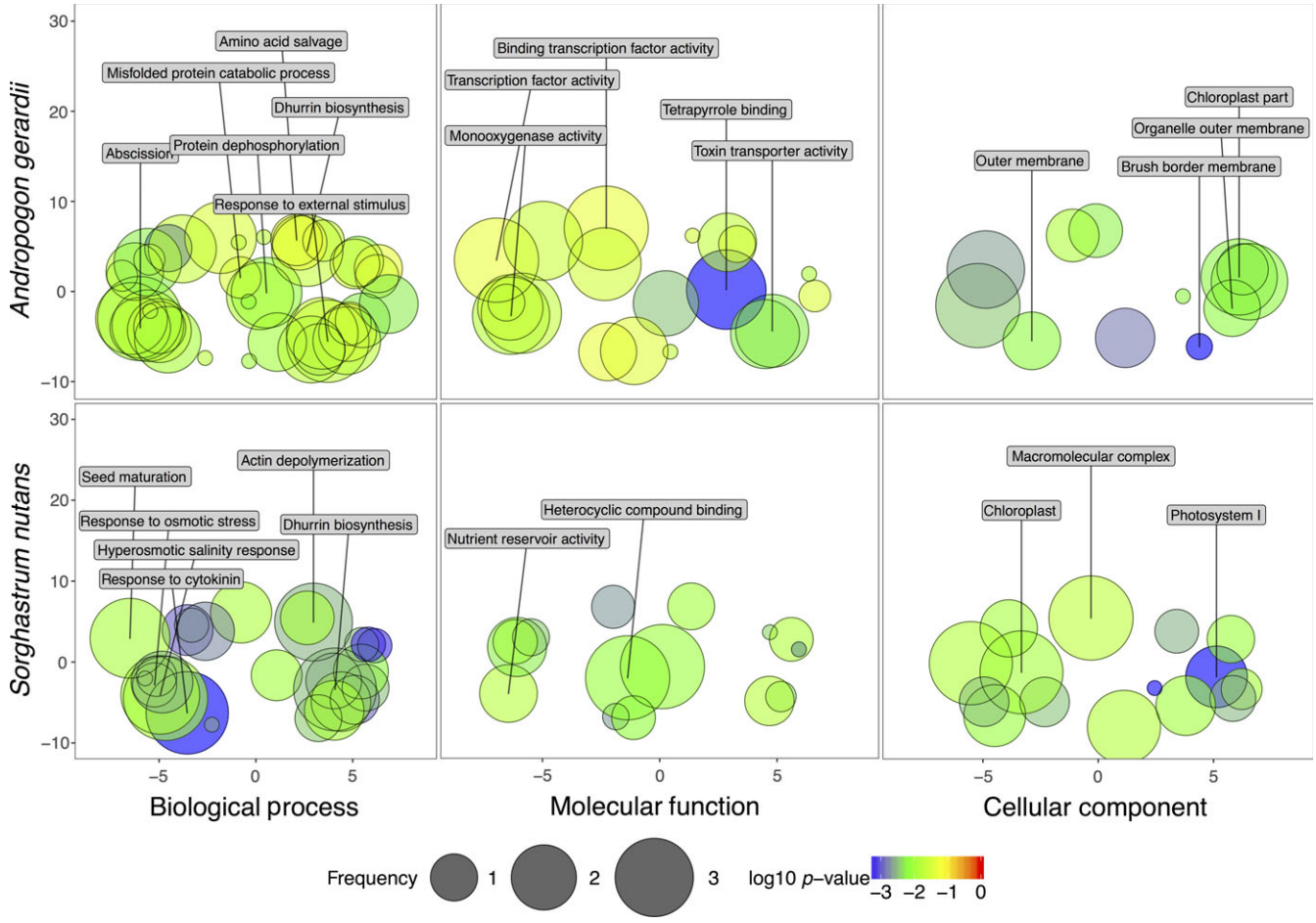

FIGURE 3 Gene Ontology (GO) enrichment in drought-upregulated transcripts within *Andropogon* and *Sorghastrum*. Colour represents significance of overrepresentation of a specific GO term within the drought-upregulated transcripts. Size represents the relative natural log scale frequency of the cluster of transcripts. Horizontal and vertical axes represent semantic space (SimRel similarity measure). Full GO enrichment data are available in Supporting Information (Table S2)

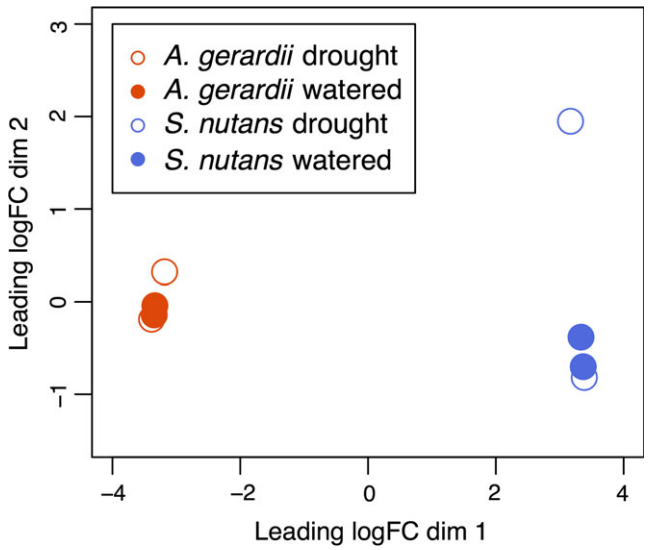

FIGURE 4 Nonmetric multidimensional scaling based on annotated genes shared between *Andropogon* and *Sorghastrum*. Euclidean distances are based on log2 fold change differences in all genes among samples using transcript counts. Stress = 0.0657

4 | DISCUSSION

In this study, we assembled the first publicly available and comparative transcriptomes for the tallgrass species *Andropogon gerardii* and *Sorghastrum nutans* as a means to understand molecular phenotypes and pathways. Average realignment of reads back to the transcriptome was >80%, and 50% of the transcriptome consisted of transcripts longer than 700 bp (N50) for both species. Both metrics indicate a successful assembly, despite our use of short sequencing reads. These assemblies will be particularly useful for researchers performing gene expression work on these species as less sequencing depth is needed when a reference transcriptome is available.

Both species shared between 85% and 86% homology (BLASTN alignment) with C_3 monocot species, but between 89% and 94% with C_4 monocots, indicating an important divergence in gene structure. *Andropogon* showed almost consistently lower similarity to model species compared to *Sorghastrum*, which could be indicative of more unique gene changes in *Andropogon*. As *Andropogon* is hexaploid, it is possible that more paralogous genes have been able to sub- or neofunctionalize and could benefit from paralog network analysis (Gallagher, Grover, Hu, & Wendel, 2016).

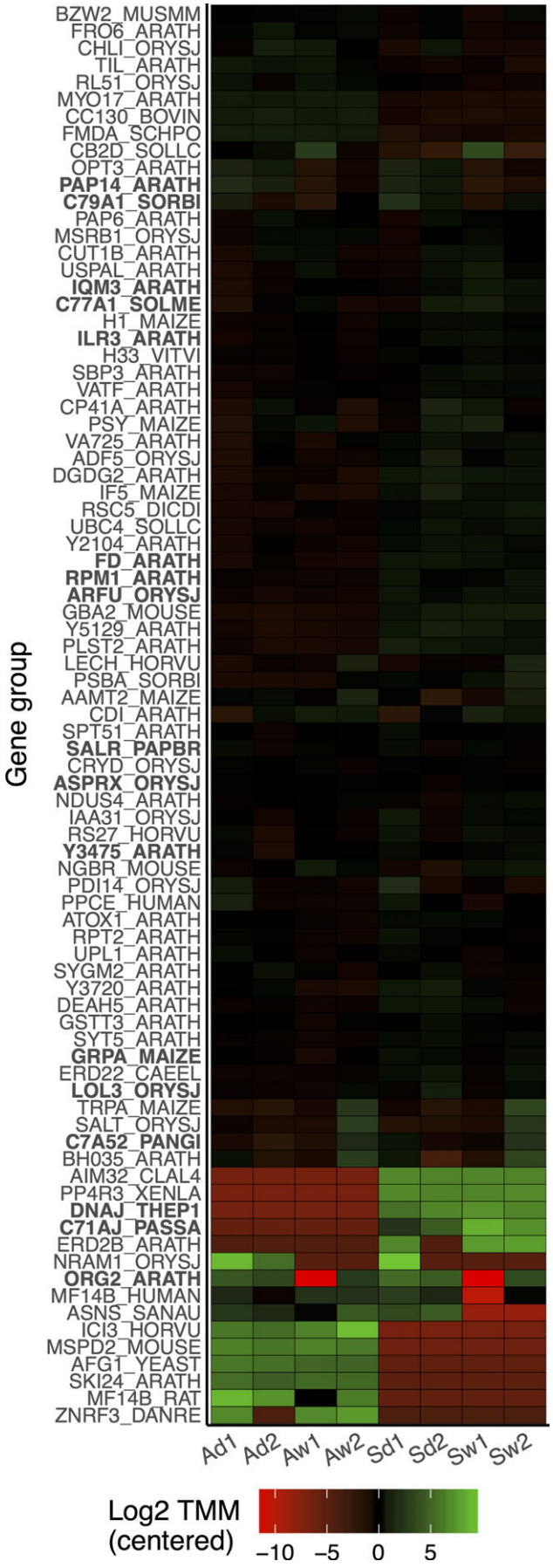

FIGURE 5 Differentially expressed gene-groups representing species by environment interaction between *Andropogon* and *Sorghastrum*. Units are log2 TMM (trimmed mean of M-values). Bold transcripts map to known transcription factors

Although *Andropogon* expressed fewer estimated genes in the transcriptome, it also had more lowly expressed genes and a greater percentage matching known transcription factors. *Andropogon* may only appear less sensitive (fewer genes expressed), while in reality a greater diversity of gene expression could occur via downstream regulation. Greater ploidy and gene duplication have also been previously connected to more important expression differences in regulatory elements like transcription factors and ribosomal proteins (Roulin et al., 2013). *Sorghastrum* mapped a slightly greater percentage of transmembrane helices, lending support to previous findings on the importance of aquaporins (Smith et al., 2016). Overall, 17% and 15% of the transcriptomes of *Andropogon* and *Sorghastrum*, respectively, were not annotated, indicating many thousands of transcripts that are unique to either or both species and warrant exploration.

Within species, relatively few transcripts were differentially expressed with the watered treatment. Dhurrin biosynthesis (plant defence) was enriched in the drought treatment for both *Andropogon* and *Sorghastrum*. Although the metabolism of plant defences may seem an unusual response to drought stress, changing dhurrin content in sorghum has been linked to regulation of pre- vs. postflowering senescence (Burke et al., 2013). Other categories were unsurprising given the two species' differing physiology. *Andropogon* emphasized misfolded protein and amino acid salvage under drought, which could reflect alleviation of the symptoms of drought stress with greater sensitivity to temperature-induced misfolding. Aberrant mRNA surveillance and spliceosome regulation pathways are also implicated in drought tolerance (Lee et al., 2015; Lu et al., 2016). On the other hand, *Sorghastrum* upregulated transcripts related to osmotic and salinity stress, which could reflect its greater sensitivity to soil water content (Nippert et al., 2009). Cytokinin response and general plant hormone signal transduction enrichment by *Sorghastrum* could indicate a greater emphasis on stomatal regulation through the cytokinin antagonistic relationship with ABA (Pinheiro & Chaves, 2011). Overall, these annotations suggest *Andropogon* may have modified regulatory elements and tolerates stress symptoms under drought, while *Sorghastrum* avoided stress by focusing on water use. This also corroborates physiological data on *Andropogon*'s sustained activity under drought (Hoover et al., 2014a; Knapp, 1985). Like the transcriptome, differentially expressed genes in *Andropogon* also contained more transcription factors, which are widely implicated in drought resistance (Baldoni, Genga, & Cominelli, 2015).

By comparing the annotations of gene-groups in *Andropogon* and *Sorghastrum*, we may reveal strategic differences between these two grasses outside of treatment. Nearly 23% more gene-groups differed between the two species under watered conditions than under

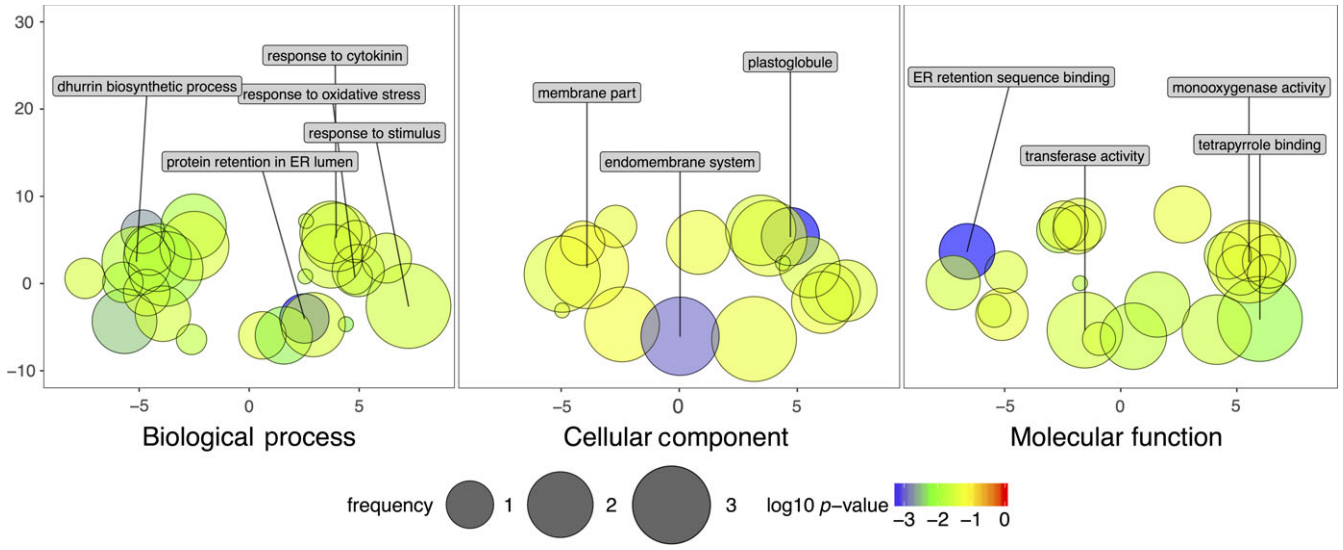

FIGURE 6 Gene Ontology (GO) enrichment for differentially expressed genes between *Andropogon* and *Sorghastrum* (genes showing a species × treatment interaction). Colour represents the significance of overrepresentation of a specific GO term within these transcripts upregulated under drought. Size represents the relative natural log scale frequency of the cluster of transcripts. Horizontal and vertical axes represent semantic space (SimRel similarity measure)

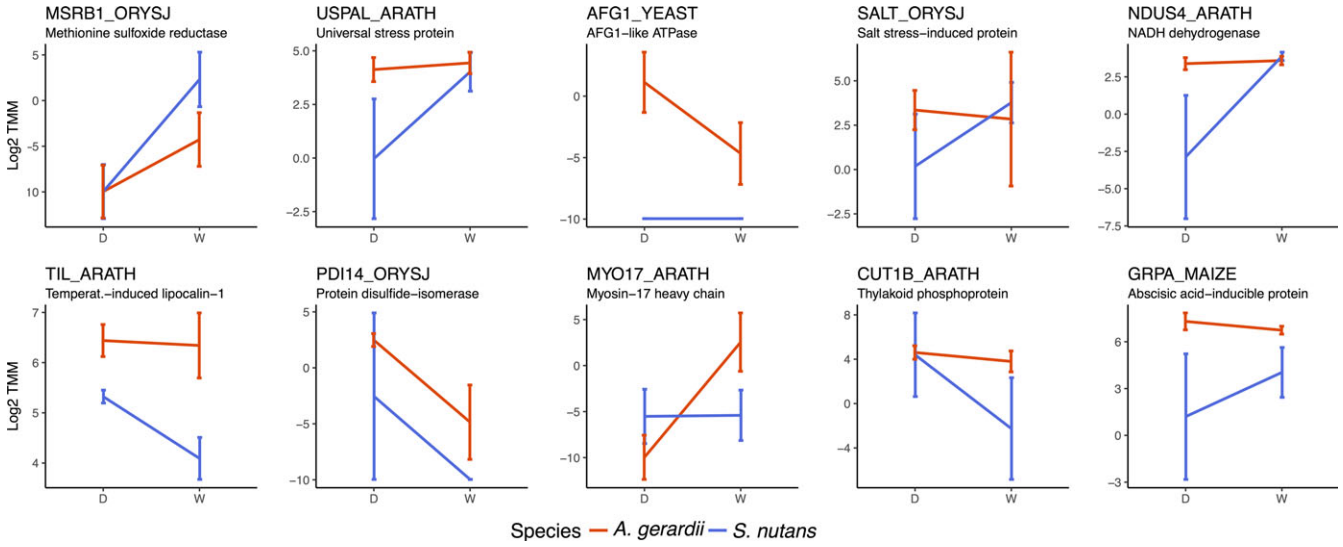

FIGURE 7 Expression change for significant species by environment interaction gene-groups. Figures depict the average expression of the top two most significant paralogs from each gene-group. Bars represent standard error. MSRB1_ORYSJ, USPAL_ARATH, AFG1_YEAST, SALT_ORYSJ, NDUS4_ARATH, TIL_ARATH and PDI14_ORYSJ belong to the stress category; MYO17_ARATH belongs to the trichome category; CUT1B_ARATH and GRPA_MAIZE belong to the ABA category

drought, suggesting that the two species may converge on more similar molecular function when stressed. Only watered *Sorghastrum* upregulated carbon fixation and photosynthesis, supporting the notion that *Sorghastrum* is more sensitive in terms of carbon allocation. Greater numbers of transcription factors in *Andropogon* under both drought and watered conditions suggest enhanced plasticity to maintain more constant carbon acquisition. Compared to *Sorghastrum*, *Andropogon* gene-groups were highly represented by transcription factor activity, flavonoid metabolism and glycosylation (i.e., heat response, Jiang et al., 2015), meaning that this species may divert more resources towards antioxidant and regulatory processes compared to *Sorghastrum*. In contrast, *Sorghastrum* gene-groups were represented by RNA binding (e.g., in response to ABA, Ambrosone et al., 2015) and regulation and response to stress and hormones. Investing greater gene expression dedicated to hormonal signalling could be the mechanism by which *Sorghastrum* is more sensitive to water availability (Nippert et al., 2009; Silletti & Knapp, 2001). Meanwhile, *Andropogon*'s lack of sensitivity could be the result of greater emphasis on stress alleviation vs. prevention. This difference in allocation of gene expression and strategy could portend

community shifts, especially greater variability in production, under future climate change scenarios. On the other hand, *Andropogon* showed more metabolic pathway hits tied to hormonal regulation and response compared to *Sorghastrum*. This could indicate that despite no expression enrichment of these gene-groups, signalling pathways may be more complex in *Andropogon* and involve more modulators (e.g., MicroRNAs, Ding, Tao, & Zhu, 2013).

When we performed a meta-level analysis across species and treatment, we found 83 differentially expressed gene-groups, including 10 within the previously mentioned functional annotation clusters. These 83 genes were enriched in response to oxidative stress (including tetrapyrrole binding) as well as membranes and plastoglobule, which are tied to osmotic stress. As we suspected, the key differences between *Andropogon* and *Sorghastrum* stress response are likely to reflect the abiotic physiological sensitivities (Nippert et al., 2009).

Focusing on functional clusters of gene-groups may add clarity when comparing responses to complex stressors like drought. Selecting clusters *a priori* according to suspected relevance also prevents bias when data abound. When we explored the 10 differing gene-groups matching our functional annotation clusters, we saw greater sensitivity by *Andropogon* with MYO17_ARATH (trichome) and AFG1_YEAST (stress) revealed through steeper slope across treatments (Falconer, 1990). Greater sensitivity of MYO17 in *Andropogon* is unsurprising considering trichomes are visibly more abundant on this species in the field and trichome density is known to vary across populations along a precipitation gradient (Olsen et al., 2013). Trichomes help plants avoid dehydration by increasing the boundary layer, thus reducing transpiration (Schreuder, Brewer, & Heine, 2001) and could allow less regulation of osmotic balance in *Andropogon*. AFG1 acts as a chaperone degrading misfolded proteins in yeast and responds to oxidative stress in *Arabidopsis* (Al Ameri, 2015); it could reflect greater regulation of *Andropogon* gene-groups to alleviate stress. More gene-groups showed greater sensitivity within *Sorghastrum*, however. Of these, one was linked to osmotic stress (SALT_ORYSJ), three with general stress (USPAL_ARATH, NSUS4_ARATH and TIL_ARATH), two with ABA response (CUT1B_ARATH and GRPA_MAIZE) and only one with oxidative stress (MSRB1_ORYSJ) (Abo-Ogiala et al., 2014; Alvarez, Roy Choudhury, & Pandey, 2014; Dinakar, Vishwakarma, Raghavendra, & Padmasree, 2016; Kline, Barrett-Wilt, & Sussman, 2010; Liu et al., 2014; Roy & Nandi, 2017; Udawat, Jha, Sinha, Mishra, & Jha, 2016; Zhang, Zhao, Xiao, Zheng, & Yue, 2014). The more sensitive response by these gene-groups in *Sorghastrum* could indicate greater stress response by this species, especially for gene-groups related to water management.

Our results and conclusions would be incomplete without a discussion of sample size in this study. The number of replicates per species and treatment is small ($n = 2$), though not uninformative within ecology (Lemoine et al., 2016). Therefore, despite best efforts to correct for type I and II error, we are likely to have missed or incorrectly characterized expression. The number of differentially expressed genes within species is small compared to other studies (e.g., Bushman et al., 2016; Dong, Fan, Deng, Xu, & Zhao, 2014; Meyer et al., 2014; Wilkins, Waldron, Nahal, Provart, & Campbell, 2009), indicating that our power to detect differential expression was low. Although care was taken to minimize differences among plants, we performed an uncontrolled drydown which often leads to variation in water content (Lovell et al., 2016) and has somewhat limited applicability to field studies. As is seen in Figure 4, there is variation among droughted plants. Uncontrolled drydowns may lead to variation in VWC and could have produced more extreme responses in one droughted pot for *Sorghastrum*. This must be taken as a caveat for the analyses comparing the two species. Thus, our results should be considered as preliminary evidence for differences in these species.

Overall, variation between these two species far outweighed plasticity in gene expression under different water conditions. This could certainly stem from our limited sample size as well as the particular type of stress we invoked. Previous research revealing greater sensitivity in *Sorghastrum* (e.g., Hoover et al., 2014b; Nippert et al., 2009; Smith et al., 2016) is corroborated by this study, but specific mechanisms are likely much more complex. For example, *Andropogon* may actually exhibit greater sensitivity through regulatory elements like transcription factors and may be more sensitive with regard to oxidative stress and reactive oxygen species scavenging. Most results from this study suggest a more passive drought tolerance strategy by *Andropogon* vs. an active drought avoidance strategy by *Sorghastrum*. However, this study only accounts for one genotype and a single time point for each species; in the field, plasticity emerging from genotypic diversity and temporal variation may provide more resistance and resilience to drought and other stressors (Avolio, Beaulieu, & Smith, 2013; Avolio & Smith, 2013). Going forward, studies involving these two species should take into account differences in gene expression, including differences in stress, ABA and trichome gene-groups as well as differences in metabolic pathway allocation. The transcriptome resources generated in this study will also serve as templates for future exploration of the molecular phenotype in these two ecological important grasses, especially as studies using RNA-seq become more important in ecology.

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AUTHOR CONTRIBUTIONS

A.M.H. performed all analyses, produced all figures, wrote the first draft of this manuscript and edited all subsequent drafts. M.D.S. planned the study, guided sample and library preparation, and edited later drafts of this manuscript.

DATA ACCESSIBILITY

All raw data, assembled transcriptomes, differentially expressed data, comparative expression data sets: Dryad (https://doi.org/10.5061/dryad.5bk4c). R scripts, perl scripts, general information and RNA-seq user guide: http://www.avahoffman.com/resources.html

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SUPPORTING INFORMATION

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