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ARTICLE

Transcriptional responses to water stress and recovery in a drought-tolerant fescue wild grass (*Festuca ovina*; Poaceae)

Fan Qiu, Seton Bachle, Ryan Estes, Melvin R. Duvall, Jesse B. Nippert, and Mark C. Ungerer

Abstract: Water stress associated with drought-like conditions is a major factor limiting plant growth and impacts productivity of natural plant communities and agricultural crops. Molecular responses of plants to water stress have been studied most extensively in model species and crops, few of which have evolved natural drought tolerance. In the current study, we examined physiological and transcriptomic responses at multiple timepoints during increasing water stress and following initial recovery from stress in a drought-tolerant C_3 species, *Festuca ovina*. Results demonstrated non-linear transcriptomic changes during increasing stress, but largely linear declines in physiological measurements during this same period. Transcription factors represented approximately 12.7% of all differentially expressed genes. In total, 117 *F. ovina* homologs of previously identified and molecularly characterized drought-responsive plant genes were identified. This information will be valuable for further investigations of the molecular mechanisms involved in drought tolerance in C_3 plants.

Key words: *Festuca ovina*, RNA-seq, water stress, transcription factor, drought tolerant.

Résumé : Le stress hydrique associé à des conditions de sécheresse est un facteur important limitant la croissance des plantes et affectant la productivité au sein de communautés végétales naturelles et chez les espèces cultivées. Les réponses moléculaires des plantes au stress hydrique ont été étudiées le plus chez des espèces modèles et des espèces cultivées, dont certaines ont acquis une tolérance naturelle à la sécheresse. Dans le présent travail, les auteurs examinent les réponses physiologiques et transcriptomiques à différents stades temporels suite à l'imposition d'un stress hydrique croissant et après rétablissement post-stress chez une espèce C_3 tolérante à la sécheresse, *Festuca ovina*. Les résultats ont montré une évolution non-linéaire des réponses transcriptomiques en réponse à l'accroissement du stress, alors que les déclins physiologiques étaient largement linéaires au cours des mêmes périodes. Les facteurs de transcription constituaient environ 12,7 % des gènes montrant une expression différentielle. Chez *F. ovina*, les auteurs ont identifié 117 homologues de gènes précédemment connus au plan moléculaire pour réagir à la sécheresse. Cette information sera utile pour des futurs travaux des mécanismes moléculaires impliqués dans la tolérance à la sécheresse chez les plantes C_3. [Traduit par la Rédaction]

Mots-clés : *Festuca ovina*, RNA-seq, stress hydrique, facteur de transcription, tolérance à la sécheresse.

Introduction

Drought is a major environmental stress for plants and negatively affects growth and reproduction. Drought events cause yield loss in crop systems (Brevedan and Egli 2003; van Asten et al. 2011) and negatively impact productivity of natural plant communities. Given increases in the frequency and severity of drought events associated with global climate change (IPCC 2007; Walter et al. 2011; Farooq et al. 2012, and references within), understanding the physiological and molecular responses of plants to this important environmental stressor is an important goal of plant biology research.

Water stress associated with drought-like conditions actuates multiple physiological responses in plants (Chaves et al. 2003; Fang and Xiong 2015; Reddy et al. 2004; Reyer et al. 2013). Water stress typically leads to decreased leaf water potential (Ψ), increased cellular oxidative stress, and, under prolonged periods and (or) severe conditions, hydraulic failure and death (McDowell 2011). A common response to water stress is closure of stomata to conserve existing water supplies. Stomatal closure leads

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to increases in leaf temperature and reduced photosynthetic capacity, impacting oxidative stress, carbon assimilation, and growth (Fang and Xiong 2015; Farooq et al. 2012).

Molecular and physiological responses to water stress are governed largely by gene expression changes (Zhang et al. 2018). Considerable effort has been put forth in identifying and characterizing drought-responsive genes in plants, with much of this work performed in model systems such as *Arabidopsis thaliana* and *Oryza sativa* (rice). One of the most useful genetic resources to date is DroughtDB, a database assembled by Alter et al. (2015) consisting of 199 genes identified from 38 plant species that have been characterized molecularly to have roles in drought tolerance. DroughtDB is thus a powerful resource for characterizing specific transcriptomic responses across plant lineages.

The development of high-throughput sequencing technology has made it possible to obtain a high depth of sequencing to sufficiently cover the transcriptome of non-model higher plants (da Fonseca et al. 2016; Garg and Jain 2013). Studies of wild plant species with natural drought tolerance will facilitate better understanding of transcriptomic responses associated with the physiological and molecular mechanisms through which plants cope with limited water availability associated with drought-like conditions. Such knowledge will increase our understanding of the evolution of natural drought tolerance in plants as well as aid research efforts to develop more drought-tolerant crops.

In the current study, we examine transcriptional responses of a cool season, C_3 perennial dwarf bunchgrass, *Festuca ovina* L., at multiple timepoints during increasing water stress and during initial recovery following re-watering. *Festuca ovina* possesses anatomical features characteristic of natural drought tolerance such as acicular leaves and a dense root structure (Ogle et al. 2010). Previous studies indicate moderate natural drought tolerance of this species (Khoshkholghsima and Rohollahi 2015; Tarakanovas et al. 2008). We focus our analyses both on global patterns of differential expression across timepoints and on specific pathways/gene categories of known importance for plant responses to water stress, informed in part by the DroughtDB resource.

Materials and methods

Plant materials, growing conditions, and dry-down

Seeds of *F. ovina* (Accession# 595178, a diploid population (Qiu et al. 2020)) utilized in this study were acquired from the United States Department of Agriculture (USDA) National Plant Germplasm System (https://www.ars-grin.gov/npgs/). The taxonomic identity of the seed accession was genetically verified. A plastid genome (plastome) sequence was determined using methods described in Orton et al. (2019) (GenBank accession MH569081). Pairwise comparison of this plastome against an existing one in GenBank (NC_019649.1) from the same species indicated 132 821 identical sites (99.3%) as expected in conspecific accessions. Seeds were germinated on moist filter paper in Petri dishes and then transferred to 10 cm plastic pots containing a mixture (2:1) of Metro-mix 360: all-purpose sand with soil collected from a nearby tallgrass prairie added at 10% by volume with a total soil volume of 600 mL. Plants were grown in the Kansas State University greenhouse facility under a daily light cycle of 16 h light: 8 h dark, with supplemental lighting consisting of six lighting fixtures using 400 W high pressure sodium bulbs. Watering was conducted daily or as needed. Plants were grown for 131 days prior to initiation of the dry-down experiment and thus were well-established at the time of the experiment, though still pre-reproductive and with multiple leaves per plant, as is typical for bunchgrasses (Fig. S1[1]). Ten individuals were well watered on Day 0 of the experimental dry-down, and water was withheld until photosynthetic rates reached 0 μmol $m^{-2}s^{-1}$, after which re-watering was performed and recovery monitored the following day. Physiological measures were made daily (dry-down: Days 1–7; recovery: Day 8) and leaf samples were harvested for transcriptomic analyses on Days 1, 3, 5, 7, and 8 for three of the 10 biological replicates.

Physiological responses during drought and recovery

Physiological measurements were collected each day between 11:00 and 14:00 CDT. Because *F. ovina* leaves are acicular, 4–5 leaves were grouped laterally to increase surface area for physiological measurements. We also marked leaves for repeated sampling to ensure consistent measurements throughout the dry-down period. Photosynthesis and stomatal conductance were measured with a LI-6400 system (LiCOR, Inc., Lincoln, NE, USA) equipped with an LED light source (CO_2 concentration at 400 μmol mol^{-1}, light intensity within the cuvette at 2000 μmol $m^{-2}s^{-1}$ and relative humidity at ambient levels, ∼35%). Measurements from the LI-6400 were logged when gas exchange measurements were stable for 2 min.

RNA extraction, library construction, and sequencing

Three of 10 biological replicates utilized for physiological analyses were selected for transcriptomic analyses, with these same three individuals sampled repeatedly across the experimental timecourse. A single, full-length leaf was sampled from each plant at each timepoint. Plant size, coupled with the bunchgrass architecture of *F. ovina* (Fig. S1[1]) resulted in removal of only a small fraction of total biomass at each harvesting episode. All harvested leaves were flash-frozen in liquid nitrogen and stored at −70 °C prior to RNA extraction.

[1]Supplementary data are available with the article at https://doi.org/10.1139/gen-2020-0055.

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer instructions. Total RNA was purified to avoid genomic DNA contamination using a RNeasy Mini Kit and an on-column DNase I digestion (Qiagen, Valencia, CA). The quality and quantity of the total RNA were examined using Agilent Tape Station (Agilent Technologies, Santa Clara, CA) and a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA per sample was utilized for library preparation and sequencing on an Illumina HiSeq2500 platform, generating 2×100 bp paired-end reads. Library preparation was performed following the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina Inc., San Diego, CA). Library construction and sequencing were performed at the University of Kansas Genome Sequencing Core Facility, Lawrence, KS, USA (http://gsc.drupal.ku.edu/).

Sequence assembly and differential gene expression

Raw sequence reads were trimmed and filtered using Trimmomatic V0.35 (Bolger et al. 2014) according to the following criteria: (1) adapters and barcodes removed, (2) reads <40 bases removed, (3) bases trimmed from read ends if quality <30, and (4) read ends trimmed while mean quality <30 in a 5 bp sliding window. The trimmed reads from samples from all timepoints were combined and used for de novo assembly, which was performed with Trinity V2.2.0 (Grabherr et al. 2011) with default parameters except to keep contigs with length ≥300 bp. Following assembly, CD-HIT V4.6.8 (Fu et al. 2012) was used to obtain distinct sequences. The following parameters were used in CD-HIT analysis: (1) sequence identity threshold: 0.95, and (2) alignment coverage for the shorter sequence: 0.9. These non-redundant sequences were used for the downstream analysis.

The de novo transcriptomic assembly was used as a reference for read mapping of all samples. Trimmed reads were aligned to the reference assembly using Bowtie V1.1.2 (Langmead et al. 2009) and transcript and gene abundance was estimated with RSEM V1.2.28 (Li and Dewey 2011) and PERL script align_and_estimate_abundance.pl from Trinity (Haas et al. 2013). Samples collected on Day 1 were used as controls to identify differentially expressed genes (DEGs) for the remainder of sampling timepoints (i.e., Day 3, 5, 7, and 8). edgeR V3.3 (Robinson et al. 2010) was used to determine DEGs with a false discovery rate (FDR) < 0.05 and $\log_2$ fold change of 2.

Functional annotation and enrichment test

The final contigs (i.e., those of ≥300 bp) from the de novo assembly were compared to the entire UNIPROT and NCBI non-redundant (nr) databases with the BLASTX tool of BLAST v2.2.31 (Altschul et al. 1990) and an e-value cutoff of 10^{-5}. Contigs with significant BLAST hits were annotated with the GO terms of their top matches using BLAST2GO V3.0.6 (Conesa et al. 2005). To identify putative transcriptional factors (TFs) differentially expressed during water stress, all contigs were used in BLAST similarity search against the plantTFDB 4.0 (Jin et al. 2017) with an E-value cutoff of 10^{-5}. To determine potential overlap between identified DEGs in the current study and previously identified and molecularly characterized plant drought-responsive genes, all DEGs in the current study were used in BLAST similarity search against the DroughtDB (Alter et al. 2015), using three different E-value cutoffs: 10^{-5}, 10^{-10}, and 10^{-20}. GO Enrichment analysis were then performed for DEGs at each timepoint compared to the control with the Fisher's exact test of BLAST2GO. The significance level for these tests was set to $\alpha = 0.05$ after correcting for the FDR due to multiple testing with the Benjamini and Hochberg method (Benjamini and Hochberg 1995).

The Kyoto Encyclopedia of Genes and Genome (KEGG, http://www.genome.jp/kegg/) was used to generate the photosynthetic pathway overview of DEGs expressed in response to water stress at different timepoints. DEGs were assigned to functional categories based on sequence similarity with annotated reference sequences from the KEGG GENES database (Kanehisa and Goto 2000).

Availability of data

Raw sequence reads are available from the NCBI Short Read Archive (SRA) with project #PRJNA531147 (https://www.ncbi.nlm.nih.gov/bioproject/531147).

Results

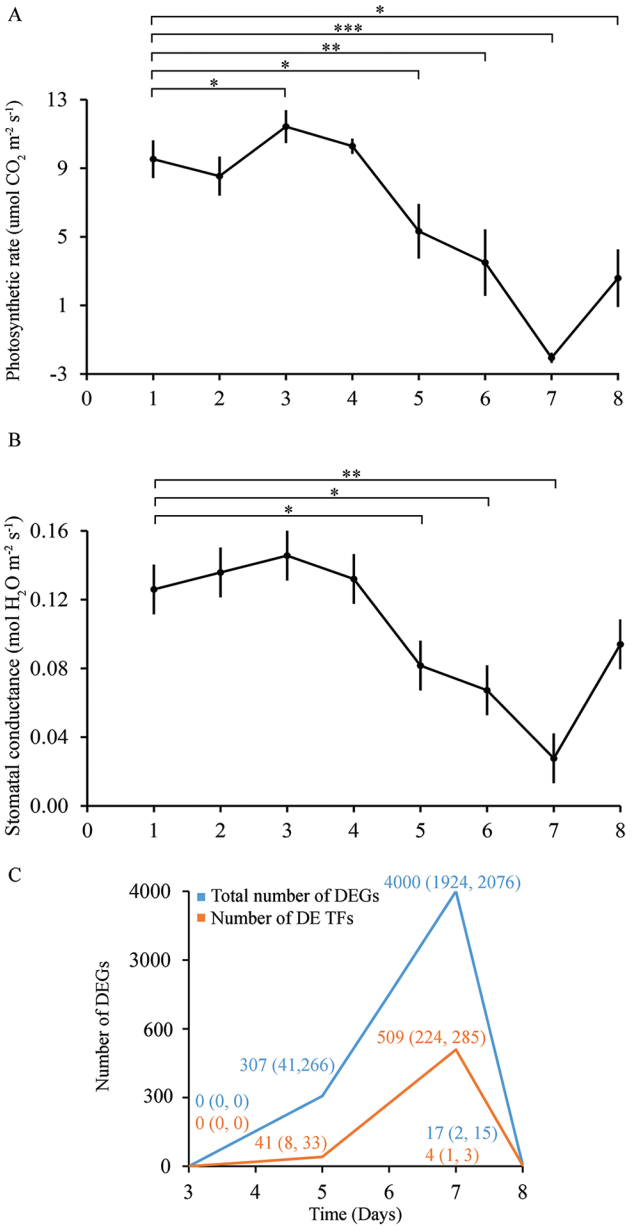
Physiological responses to water stress

The dry-down manipulation impacted *F. ovina* leaf-level physiological processes. Average photosynthetic rate (A_n) remained stable for the first four days of the experiment, with the exception of a slight increase on Day 3 ($p = 0.045$, Student's *t*-test, Fig. 1A), after which it declined linearly until Day 7 when for most plants, A_n was at or near zero and most individuals exhibited signs of wilting (Fig. S1[1]). The day following re-watering (Day 8), A_n increased to an average of 23% of its initial Day 1 value (Fig. 1A), but remained significantly lower than the mean Day 1 value ($p = 0.047$). The response of stomatal conductance (Fig. 1B) was qualitatively similar to that observed for photosynthetic rate, though a significant increase was not detected on Day 3 and average stomatal conductance on the day following re-watering (i.e., Day 8) was not significantly different from Day 1.

RNA sequencing and de novo transcriptome assembly

Transcriptome sequencing generated 26.4–39.7 M raw reads per sample (Table S1[1]). After trimming, the number of reads was reduced by ~16% on average across samples and read lengths were reduced from their original size of 101 bases to a mean of 94.6–95.8 bases (Table S1[1]). These trimmed, high-quality reads were used for de novo assembly in Trinity and generated 119 628 non-redundant contigs with a mean length and mean N50 length of 934 and 1371 bp, respectively (Table S2[1]). The

Fig. 1. Responses of (A) photosynthetic rate, (B) stomatal conductance, and (C) gene expression during drought stress and recovery. In panels A and B, vertical bars associated with symbols represent 1 SE of the mean and horizontal lines with asterisks indicate significant differences compared to Day 1 values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-tests). In panel C, the three numbers associated with each timepoint indicate, from left to right, the total number of differentially expressed genes (DEGs) or DE transcription factors (TFs), the number of down-regulated genes or TFs, and the number of up-regulated genes or TFs.



total number of contigs in this transcriptome assembly likely is higher than the true number of protein-coding genes in the *F. ovina* genome and may result from non-coding RNAs as well as assembly fragmentation attributable to factors such as polymorphism, sequence repeats, sequencing errors, and lowly expressed genes with inadequate sequencing depth (see https://informatics.fas.harvard.edu/best-practices-for-de-novo-transcriptome-assembly-with-trinity.html).

Functional annotation of the *F. ovina* transcriptome

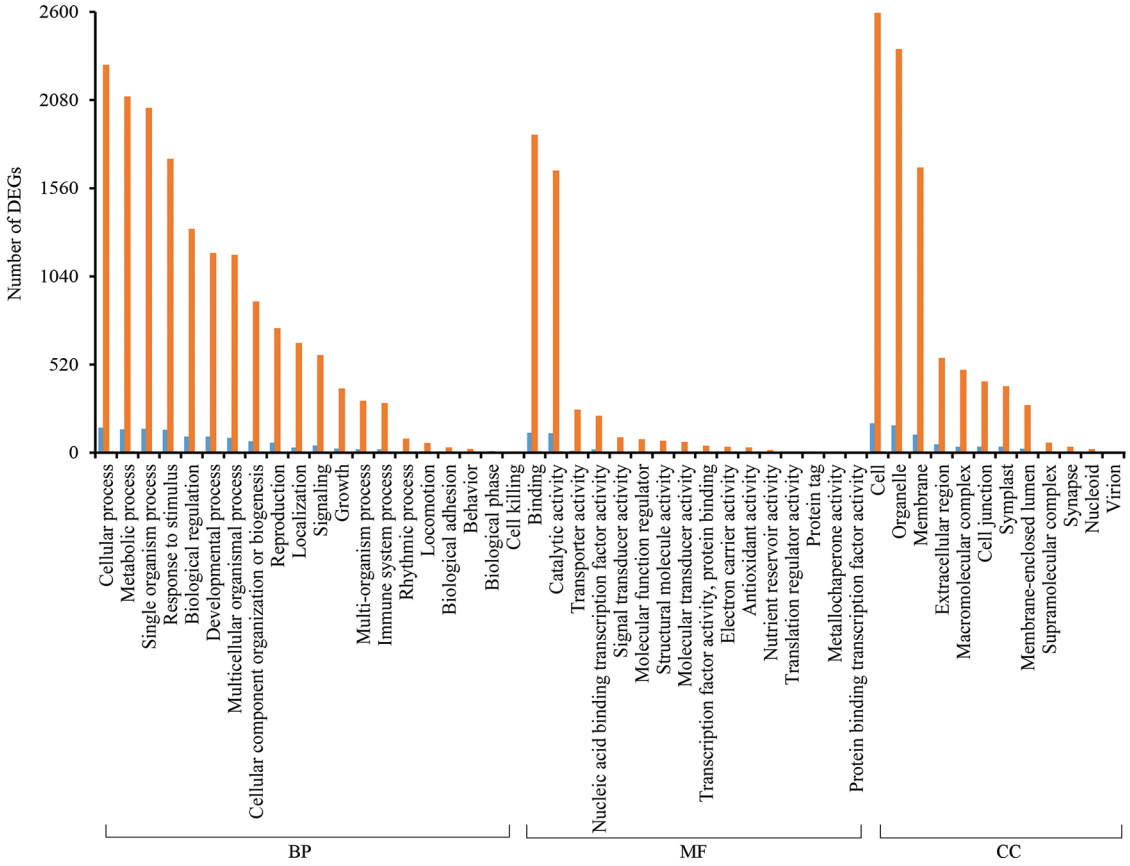
Of the 119 628 contigs, 51 174 (42.8%) had at least one significant blast hit in the NCBI nr and Swiss-Prot databases (E-value $< 10^{-5}$) (Table S2[1]). Surprisingly, a large number of the assembled contigs (57.2%) had no significant BLASTX hits to any known protein sequences. These contigs may represent non-coding RNAs with potential biological importance in plants (Li et al. 2014; Lu et al. 2016; Wang et al. 2015). Future genome sequencing and non-coding RNA information would improve our current annotation in this species. Using BLAST2GO (Conesa et al. 2005), gene ontology (GO) functional annotations were obtained for the assembled contigs. In total, 38 904 contigs (32.5%) were identified and assigned to 49 GO terms (Table S3[1]; Fig. S2[1]).

Differentially expressed genes (DEGs) and functional classification

Trimmed reads were mapped to the assembled non-redundant contigs to quantify gene expression changes during dry-down on Days 3, 5, and 7 and the day following re-watering (Day 8), using Day 1 samples as controls. The number of DEGs increased nonlinearly with increasing physiological stress, with zero DEGs observed at Day 3, 307 DEGs (41 down-regulated and 266 up-regulated) observed at Day 5, and 4000 DEGs (1924 down-regulated and 2076 up-regulated) observed at Day 7 (Fig. 1C). Following re-watering on Day 8, global gene expression largely reverted to pre-stress levels, with only 17 genes differentially expressed (2 down-regulated and 15 up-regulated). Eleven of these 17 genes (2 down-regulated and 9 up-regulated) also were differentially expressed on Day 7, but none of these were differentially expressed on Day 5. GO term analysis revealed the functional categories for these DEGs. Under the category Biological Process (BP), Cellular process was the largest group, followed by Metabolic process and Single organism process (Fig. 2). For the category Molecular Function (MF), DEGs were mostly assigned to Binding and Catalytic activity. For the category Cellular Component (CC), the largest number of DEGs were assigned to Cell, followed by Organelle and Membrane (Fig. 2). An annotated database of all DEGs detected in this study is presented in Table S4[1].

TFs were among the mostly common identified DEGs in the current study (Fig. 1C), with approximately 12.7% of all identified DEGs being TFs belonging to 46 families based on classification in plantTFDB (Jin et al. 2017) (Table S5[1]). The DE TFs detected in the current study showed patterns of both up- and down-regulation, with the most common up-regulated TF families including ERF (9.4%), E2F-DP (9.1%), C2H2 (8.2%), and NAC (7.5%) (Fig. 3A) and the most abundant down-regulated TF

Fig. 2. Gene ontology of differentially expressed genes (DEGs) on Day 5 (blue) and Day 7 (orange). BP: Biological Process; MF: Molecular Function; CC: Cellular Component.



families including MYB-related (10.8%), bHLH (8.6%), EF2-DP (7.3%), C2H2 (6.0%), and WRKY (6.0%) (Fig. 3B). More TFs were differentially expressed at Day 7 (509 TFs: 224 down-regulated, 285 up-regulated) versus at Day 5 (41 TFs: 8 down-regulated, 33 up-regulated) (Fig. 1C) though TFs represent a similar fraction of the total number of DEGs at these two timepoints (13.4% and 12.7% at Day 5 and Day 7, respectively). A larger fraction of DE TFs were up- versus down-regulated at Day 5 (80.5% versus 19.5%) compared to Day 7 (66% versus 34%).

On Day 5, average fold changes for the 33 up-regulated and 8 down-regulated TFs were 51.1× and 41.2×, respectively. On Day 7, average fold change for the 285 up-regulated and 224 down-regulated TFs were 36.5× and 32.3×, respectively. The three TFs (TRINITY_DN51695_c1_g3, TRINITY_DN59022_c1_g1, and TRINITY_DN57801_c0_g1) with highest up-regulation on Day 5 exhibiting fold changes of 141.8×, 123.5×, and 123.1× and belong to families bHLH, C2H2, and MYB-related, respectively. These TFs remained the most highly up-regulated on Day 7, with fold changes increasing to 206.7×, 172.5×, and 161.3×, respectively. On Day 8, two of these TFs (TRINITY_DN51695_c1_g3 and TRINITY_DN57801_c0_g1, belonging to bHLH and MYB-related families) remained significantly up-regulated but with fold changes decreasing to 106.8× and 77.7×, respectively. A more comprehensive description of individual TFs exhibiting highest levels of up- and down-regulation during stress and recovery can be found in Table S6[1].

GO enrichment tests were used to identify enriched functional categories of DEGs at different sampling timepoints during dry-down and following recovery. While hundreds of GO terms were found enriched, some of the most significant based on FDR criteria include, for up-regulated genes: response to stress (GO: 0006950), response to abiotic stress (GO:0009628), response to water (GO:0009415), and response to abscisic acid (GO:0009737), all of which were over-represented on both Day 5 and Day 7, with response to hormone (GO:0009725) only over-represented at Day 7. Genes in these categories include late embryogenesis abundant (LEA) proteins, peroxidases, transporters, protein kinases, and TFs involved in ABA-dependent and ABA-independent pathways. For down-regulated genes, cell wall organization or biogenesis (GO:0071554), multidimensional cell growth (GO:0009825), and cell wall organization (GO:0071555) were over-represented both on Day 5 and Day 7, while GO terms enriched in photosynthesis (GO:0015979), photosynthesis, light reaction (GO:0019684), and photosynthesis,

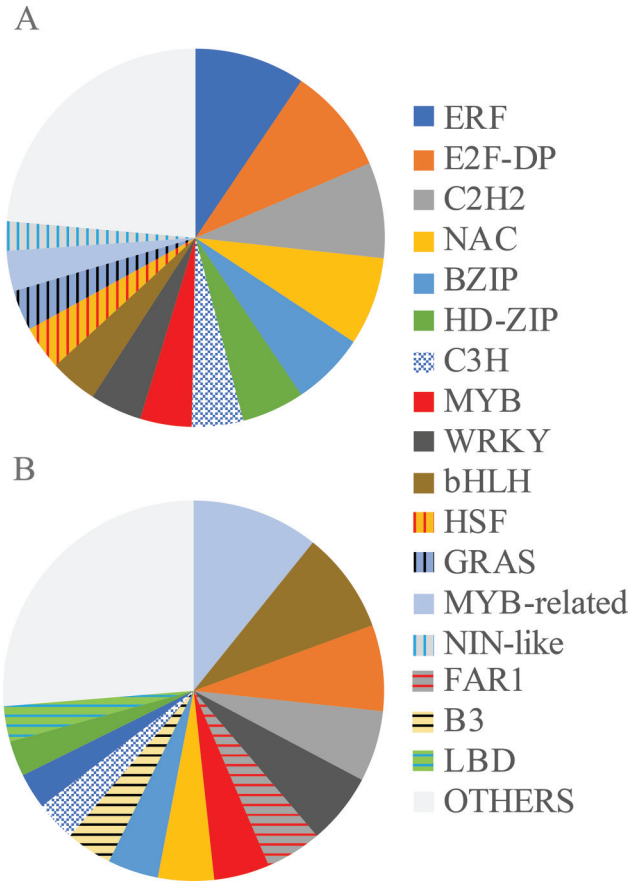
dark reaction (GO:0019685) were only found at Day 7. Down-regulated genes enriched in photosynthesis are members of the Photosystem II reaction core (PS II-RC) complex, Photosystem I reaction center (PS I-RC) subunits, the Cytocrome b6/f complex, Photosynthetic electron transport, and F-type ATPase. No enriched GO terms were found on Day 8 (recovery).

Differentially expressed homologs of molecularly characterized drought tolerance plant genes

To determine potential overlap of DEGs identified in the current study with previously identified and molecularly characterized plant genes involved in drought tolerance, a BLAST similarity search was performed for the 4012 DEGs identified in the current study against the Drought Stress Gene Database (DroughtDB) (Alter et al. 2015). This database consists of 199 previously identified and molecularly characterized drought stress genes from 38 plant species. These genes are categorized into groupings of Molecular Adaptation and Physiological Adaptation, and levels of subcategorization within. BLAST analyses conducted using E-value cutoffs of 10^{-5}, 10^{-10}, and 10^{-20} yielded at least one significant hit for 122, 117, and 96 genes in this database, respectively, corresponding to 605, 499, and 334 DEGs. The majority of the 4012 DEGs identified in this study thus did not have significant BLAST hits to the DroughtDB. Analyses based on an E-value cutoff of 10^{-10} (117 genes) indicated representation in all categories and subcategories of Molecular Adaptation and Physiological Adaptation of the DroughtDB (Fig. 4). Gene functional/pathway categories in DroughtDB with the highest representation of DEGs include Enzymes for osmolyte biosynthesis and Osmolyte protection, with significant BLAST hits for 8 of 10 and 9 and 11 genes in those categories, respectively. The single largest subcategory in DroughtDB is Gene expression, and BLAST analyses (E-value cutoff 10^{-10}) yielded significant hits to 42 of 72 genes in this category (Fig. 4). A majority of the genes in this category are TFs (n = 62), and 38 of these 62 genes were detected with significant BLAST hits, consistent with our findings that TFs represent one of the most abundant categories of DEGs in *F. ovina* in response to water stress.

GO analysis showed that response to abscisic acid (GO:0009737) was over-represented under water stress, with 6 of 11 genes identified in the associated DroughtDB category, Hormone signaling (Fig. 4). One of these, 9-*cis*-epoxycarotenoid dioxygenase (NCED), is an enzyme involved in ABA biosynthesis which regulates the rate-limiting step in the ABA biosynthesis pathway. Five copies of NCED were detected in the transcriptome assembly, with two copies significantly up-regulated on Day 5 and all five copies significantly up-regulated on Day 7. None of the five copies remained up-regulated on Day 8. Pairwise nucleotide sequence comparisons of these *F. ovina* NCED copies yielded p-distances of 0.112–0.583. Phylogenetic analysis of these NCED copies together with five identified copies in each of the *Arabidopsis thaliana* and *Oryza sativa* genomes failed to show patterns of copy orthology across species, with sequences largely grouping by species of origin (Fig. 5). A listing of all 117 genes in the DroughtDB with significant BLAST hits and corresponding *F. ovina* contig indicators is presented in Table S7[1].

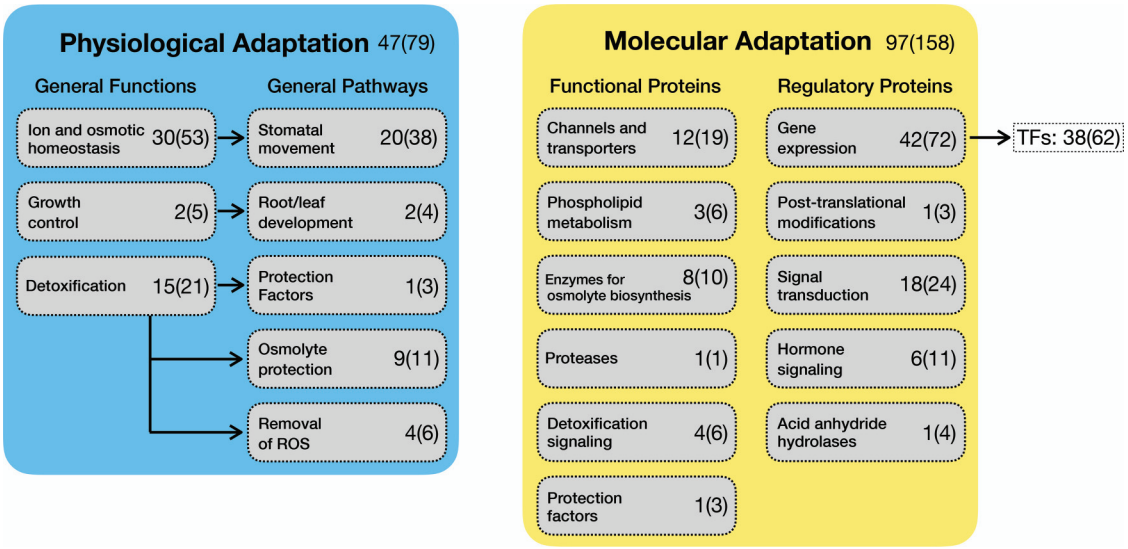
Fig. 3. Top 14 (A) up-regulated and (B) down-regulated differentially expressed transcription factor (TF) families based on Day 5 and Day 7 combined. Pie slices with vertical lines in panel A indicate TF families not represented in the top 14 families in panel B, and slices with horizontal lines in panel B represent TF families not represented in the top 14 families in panel A.



Discussion

Drought is an important abiotic stress that can severely impact plant development and productivity. Plants respond to drought-like conditions through numerous physiological, biochemical, and transcriptomic pathways. While responses of plants to water stress have been heavily studied (Chaves et al. 2003; Fang and Xiong 2015; Farooq et al. 2012; Hong et al. 2016; McDowell 2011; Reddy et al. 2004; Reyer et al. 2013; Yu et al. 2016; Zhang et al. 2018), much of our current understanding is based on responses in model

Fig. 4. Major blocks and subcategories of the DroughtDB (Alter et al. 2015) with representation of *Festuca ovina* homologs identified in the current study. The number of genes in DroughtDB for each block/subcategory is indicated in parentheses, with the numbers preceding parentheses indicating the number of those genes with at least 1 BLAST hit (E-value cutoff: 10^{-10}) based on BLAST analyses of all differentially expressed genes (DEGs) identified in the current study to the DroughtDB. The vast majority of the 4012 DEGs identified in this study did not have significant BLAST hits to the DroughtDB. This figure is redrawn with modification from figure 1 in Alter et al. (2015). A listing of *F. ovina* contigs represented in this figure is provided in Table S7[1].



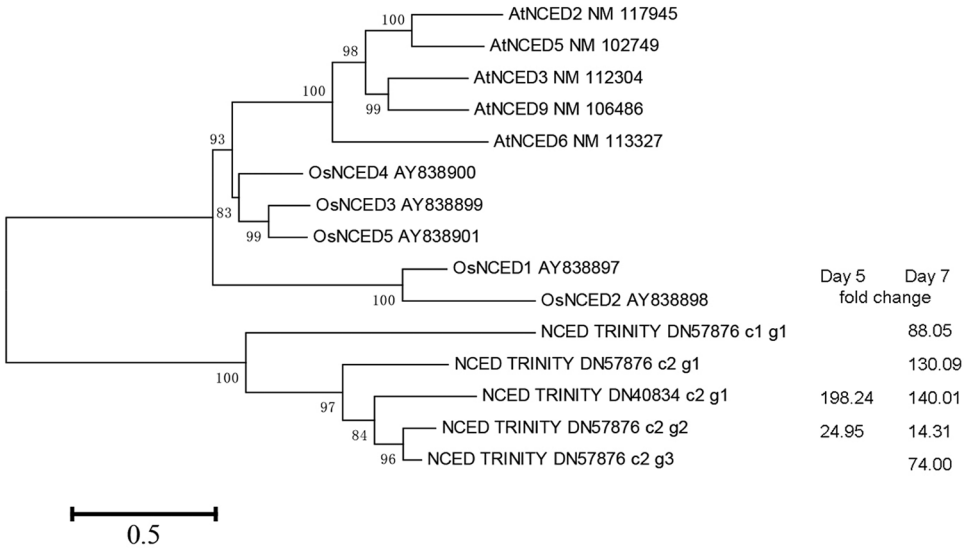
plant systems and economically important crops, few of which are classified as drought tolerant or drought resistant. In the current study, we examined physiological and transcriptomic responses during increasing water stress in a non-model, drought-resistant C_3 grass species, *Festuca ovina*. Our experiment consisted of daily measurements of photosynthesis and stomatal conductance to assess plant physiological responses to increasing water stress and sampling of leaf material for analyses of transcriptomic changes at five timepoints, including a recovery stage one day following re-watering. This multi-timepoint monitoring indicated non-linear transcriptomic changes, but a largely linear decline in physiological performance during increased water stress. These results imply that plants reach certain thresholds at which gene expression changes abruptly, both during increasing water stress and once that stress is relieved.

Analyses of DEGs and GO enrichment

In general, fewer unique DEGs (4012) were detected in *F. ovina* in response to water stress and recovery compared to studies of other less tolerant plant species, where typically 1.5 to 2.6 fold more DEGs have been identified under similar experimental conditions (Meyer et al. 2014; Muthusamy et al. 2016; Wu et al. 2014; Zhang et al. 2018). For example, 10 180 DEGs were found in switchgrass between high drought stress and control conditions (Meyer et al. 2014), whereas only 4000 DEGs were detected at the highest stress timepoint in the current study (Fig. 1C); a similar pattern of fewer DEGs associated with higher drought tolerance also has been observed in comparisons of drought-tolerant versus sensitive cultivars of banana (i.e., 8112 vs.10 537 DEGs, respectively) (Muthusamy et al. 2016). These results suggest that plants with greater drought tolerance may respond to water limitation with less pronounced gene expression changes. Another notable result of the current study is the rapidity with which the transcriptome reverted to pre-stress-like conditions following re-watering. For example, only 17 DEGs were detected in the current study one day following re-watering, whereas 2780 DEGs were still detected at a similar recovery timepoint in maize (Zhang et al. 2018). Higher numbers of TFs also were detected in the current study (n = 510), versus other studies where comparable data are available (e.g., n = 160 and 415 DE TFs) (Muthusamy et al. 2016; Wu et al. 2014). Transcriptional reprogramming is central in the response to water stress in plants, though it is currently unclear if higher numbers of DE TFs contribute to elevated drought tolerance in *F. ovina*. In the following sections, we elaborate on responses of *F. ovina* to water stress, highlighting comparisons with studies in other plant systems.

The transcriptional responses observed in *F. ovina* are representative of those documented more generally in plants and include expression changes for key categories of stress-responsive genes (Fig. 4). Noteworthy and well-characterized examples include genes involved in transcriptional regulation and genes encoding functional

Fig. 5. Midpoint-rooted Maximum Likelihood tree of the five NCED copies obtained from the *Festuca ovina* transcriptome assembly together with the five NCED copies from each of the *Arabidopsis thaliana* (At) and *Oryza sativa* (Os) genomes. All sequences used in the analysis represent mRNA and numbers at tree nodes represent bootstrap support levels. Numbers to the right of contig IDs (*F. ovina* contig IDs only) represent expression fold changes at Day 5 and Day 7 of the experiment.



proteins such as LEA proteins and enzymatic antioxidants that protect cellular membranes and other proteins. LEA proteins are a group of highly hydrophilic proteins that are formed during the process of seed development (Shao et al. 2005). They can protect other proteins to avoid damage due to the accumulated high concentrations of ions under water stress conditions, preventing dehydration of plant tissues as well as regulate expression of other genes (Fang and Xiong 2015).

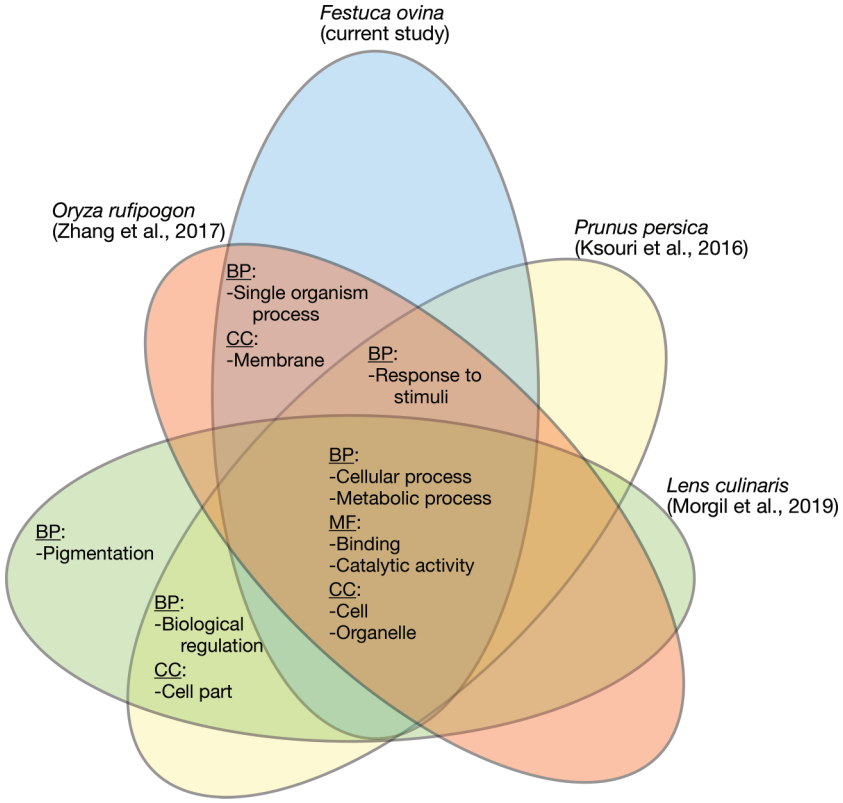
Previous studies have identified that reactive oxygen species (ROS) accumulate in plants under water stress (Ahmad et al. 2016; Hayano-Kanashiro et al. 2009; Selote et al. 2004). Biological processes such as oxidation/reduction, oxidoreductase activity, and transferase activity are known to be activated in plants in response to ROS accumulation (Hayano-Kanashiro et al. 2009; Min et al. 2016). Our GO analysis showed that oxidation-reduction process (GO:0055114) was enriched on Day 5 and Day 7, and oxidoreductase activity (GO:0016491), response to oxidative stress (GO:0006979), and transferase activity (GO:0016772) were enriched on Day 7. No enriched categories were detected one day after re-watering, indicating a rapid attenuation of these stress-response pathways.

Analysis of transcription factors

Among the 4012 unique DEGs identified across all timepoints in this study, 510 (12.7%) were TFs, confirming an important role for this category of genes in governing the broader molecular responses during water stress in the fescue grass. Members of 46 TF families were differentially expressed during water stress (Table S5[1]), though a majority of these TFs belong to a limited set of TF families such as ERF, E2F-DP, C2H2, NAC, MYB-related, bHLH, and WRKY. Many of these TF families have been shown to be responsive to water stress in other plant systems (Davey et al. 2009; Mizoi et al. 2012; Sakuraba et al. 2015; Singh and Laxmi 2015) and control independent, stress-response pathways. For example, AREB/ABF (ABA-responsive element binding protein/ABA-responsive element binding factor) are bZIP TFs that regulate ABA-dependent gene expression. It has been demonstrated that they act as major TFs under abiotic stress conditions in *Arabidopsis* (Fujita et al. 2011, 2013). In *F. ovina*, one AREB/ABF TF was up-regulated 72.99-fold on Day 7. Overexpression of an *A. thaliana* AREB/ABF TF was shown to improve drought tolerance in transgenic *A. thaliana* plants as well as in rice and soybean (Barbosa et al. 2013; Fujita et al. 2005; Oh et al. 2005).

Dehydration-responsive element binding proteins (DREBs) belong to the ERF family of TFs. DREBs consist of two subclasses: DREB1/CBF and DREB2, which are induced by cold and dehydration, respectively. These TFs respond to abiotic stresses by regulating downstream genes involved in stress responses that contain a core DRE sequence (i.e., a *cis*-acting element) in their promoters. Transgenic rice plants overexpressing the DREB1 genes showed improved tolerance to drought (Ito et al. 2006) and overexpression of DREB2 improved drought tolerance in *Arabidopsis* and soybean (Engels et al. 2013; Sakuma et al. 2006). In our study, we found one copy of DREB1 and three copies of DREB2 (two copies of DREB2b and one copy of DREB2c) up-regulated

Fig. 6. Venn diagram illustrating shared GO terms among the four most abundant categories in Biological Process (BP), three most abundant categories in Cellular Component (CC), and two most abundant categories in Molecular Function (MF) for the current study (*Festuca ovina,* blue) compared with studies of *Oryza rufipogon* (red), *Prunus persica* (yellow), and *Lens culinaris* (green). Details of studies included in this comparison are provided in Table S8[1].



during water stress. Phylogenetic analysis of these *F. ovina* DREB contigs with DREB sequences from the *A. thaliana* (At) and *O. sativa* (Os) genomes revealed that the *F. ovina* DREB1 contig groups related most closely with At and Os DREB1s, whereas *F. ovina* DREB2 contigs form a monophyletic group more closely related to At, Os, and *F. ovina* DREB1 sequences than At and Os DREB2s (Fig. S3A[1]).

The NAC TF family is another important group involved in the tolerance of plants to abiotic stress, especially to drought and high salinity (Hu et al. 2006; Redillas et al. 2012). More than 100 NAC genes have been identified in *Arabidopsis* and rice (Nakashima et al. 2012). The stress-responsive NAC TFs can bind to the NAC recognition sequence (CACG core) and play important roles in the control of environmental stress tolerance. Studies have shown stress-responsive NAC genes can improve drought tolerance when overexpressed (Hu et al. 2006; Nakashima et al. 2007; Takasaki et al. 2010; Zhu et al. 2014). In soybean, for example, nine NAC copies were induced by dehydration stress with differential induction levels in both shoot and root (Tran et al. 2009). Our results identified 24 putative NAC copies that were significantly overexpressed under drought (average fold change 25.7; Fig. S3B[1]).

Abscisic acid pathway and NCED genes

Abscisic acid (ABA) is a critical phytohormone for plant growth and development, and production of ABA increases under abiotic stress conditions such as drought. ABA plays an important role in integrating stress signals and controlling downstream stress responses. Under water stress, increased ABA levels in leaves influences stomatal closure to reduce water loss and activate many stress-related genes, thus enabling plants to respond to this important abiotic stress (Sah et al. 2016). 9-*cis*-epoxycarotenoid dioxygenase (NCED) encodes a protein product that regulates the rate-limiting step in the ABA biosynthesis pathway (Vishwakarma et al. 2017). Overexpression of NCED has been demonstrated under water stress conditions in maize, tomato, bean, *Arabidopsis*, and cowpea (Burbidge et al. 1999; Iuchi et al. 2000, 2001; Qin and Zeevaart 1999; Tan et al. 1997). Our results identified five copies of NCED, two of which were up-regulated on Day 5 (average fold change = 111.6; range = 25.0–198.2), and all five of which were up-regulated on Day 7 (average fold change = 89.3; range = 14.3–140.0). Copy number variation of NCED genes is found in other plant species: *Arabidopsis* (five copies) (Tan et al. 2003), rice (five copies) (Saika et al. 2007), avocado (three copies) (Chernys and Zeevaart 2000), and orange (two copies) (Rodrigo et al. 2006). Interestingly, however,

only a single copy appears drought-inducible in each of these other plant species, such as AtNCED3 in *Arabidopsis* (Tan et al. 2003), OsNCED3 in rice (Hwang et al. 2010), PaNCED1 in avocado (Chernys and Zeevaart 2000), and CsNCED1 in orange (Rodrigo et al. 2006). Differential expression of all five copies in *F. ovina* in response to water stress is noteworthy and may represent an adaptive mechanism to water limitation in this drought-tolerant grass species. Phylogenetic analysis of *F. ovina* NCED contigs together with *A. thaliana* and *O. sativa* NCEDs was not able to elucidate patterns of copy orthology across species (Fig. 5). Indeed, this analysis suggests copies within species all tend to be more similar to one another than any comparison across species. Phylogenetic analysis based on NCED amino acid sequences yielded similar results (data not shown).

Comparison with other plant transcriptomic studies of drought

Increasing numbers of studies examining plant transcriptomic responses to water limitation and drought-like conditions are enabling researchers to compare responses across species, yielding general insights into this important abiotic stress response and providing knowledge for crop improvement research. Toward this goal, we compared results from *F. ovina* with published reports examining transcriptomic responses for six domesticated crop species subjected to water limitation (Ksouri et al. 2016; Liu et al. 2015; Morgil et al. 2019; Singh et al. 2017; Tang et al. 2017; Zhang et al. 2017). These studies include *Triticum aestivum*, *Oryza rufipogon*, and *Setaria italica* (all three of which are domesticated grass species), as well as *Prunus persica* and *Lens culinaris* (2 studies). Details relating to these studies, sampling timepoints, and criteria for DEG identification are provided in Table S8[1]. While in-depth comparison across all studies is beyond the scope of the current report, two interesting patterns were revealed. First, the total numbers of DEGs detected across these studies is highly variable, ranging across studies and sampling timepoints from 307 (*F. ovina*) to 11 231 (*O. rufipogon*) (Fig. S4[1]). Interpretation of this variation is admittedly difficult given the inability to control across studies for important factors such as plant developmental stage, how stress was implemented, and severity of physiological stress when tissue was sampled. It is interesting to note, however, that in instances when tolerant and sensitive accessions were compared in the same study, tolerant accessions tended to have fewer DEGs. Second, and despite the variable numbers of DEGs detected across these studies, similar and overlapping patterns of GO enrichment were revealed when the most abundant categories of GO terms were compared across studies for which similar data were available. For example, of the four studies compared in this way (i.e., *F. ovina* (our study), *O. rufipogon* (Zhang et al. 2017), *P. persica* (Ksouri et al. 2016), and *L. culinaris* (Morgil et al. 2019)), the most abundant categories for Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) were highly overlapping, with 6 of these 12 abundant GO terms shared by all four species, another GO term shared by three species, and 4 GO terms shared by two species groups separately (Fig. 6). These results indicate considerable overlap in induced pathways despite highly dissimilar numbers of DEGs in response to stress.

Conclusions

We performed a comprehensive multi-timepoint investigation of physiological and transcriptomic responses to increasing water stress and recovery in a drought-tolerant C_3 grass species. Physiological measures demonstrated expected declines in gas exchange and photosynthetic parameters with increasing stress, with RNA-seq analysis demonstrating pronounced patterns of down-regulation of genes involved in photosynthesis, but only at more severe levels of stress. More general analyses of differential gene expression showed many TF families were sensitive to water stress and duplicated genes involved in a rate-limiting step of the ABA pathway were all significantly up-regulated. Results from our multi-timepoint sampling strategy showed clear evidence of non-linear relationships between physiological and transcriptomic responses both during increasing water stress and recovery. *Festuca ovina* homologs of genes in DroughtDB are presented. Collectively, these findings may serve as a useful resource for future investigations in stress-response research and drought-tolerance improvement efforts in C_3 crops.

Author contributions

F.Q., J.B.N., and M.C.U. planned and designed the research; F.Q., S.B., R.E., M.R.D., J.B.N., and M.C.U. performed experiments; F.Q. and S.B. analyzed the data; F.Q. and M.C.U. wrote the manuscript; and S.B., M.R.D., and J.B.N. provided comments on drafts of the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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