Dissection of physiological, transcriptional, and metabolic traits in two tall fescue genotypes with contrasting drought tolerance

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Tall fescue is an important cool-season perennial forage grass that forms mutualistic symbioses with fungal endophytes. Physiological, biochemical and transcriptional comparisons were made between two tall fescue genotypes with contrasting drought tolerance (tolerant, T400, and sensitive, S279), either with or without endophyte (*Epichloë coenophiala*). Drought stress was applied by withholding watering until plants reached mild, moderate and severe stresses. Physiological characterization showed that T400 had narrower, thicker leaves, and lower leaf conductance under well-watered conditions, compared to S279. After severe drought and recovery, endophytic T400 had greater shoot and root biomass than other plant types. Under drought, leaf osmotic pressure increased much more in T400 than S279, consistent with accumulation of metabolites/osmolytes, especially proline. Gene Ontology enrichment analysis indicated that T400 had more active organic acid metabolism than S279 under drought, and implicated the role of endophyte in stimulating protein metabolism. Transcription factor (TF) binding motif enrichment analysis of the promoters of drought up-regulated genes point to important regulatory roles for bZIPs and bHLHs in controlling such genes, with the core binding motif (C/G)ACGTG being identified. A much larger variance was observed in TF binding motif enrichment in the promoters of drought down-regulated genes.

Key words: tall fescue, drought, endophyte, proline, bZIP, bHLH, promoter, transcriptome

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a cool season perennial grass. It is the most widely planted forage crop in the United States and covers almost 14 million ha (Dinkins et al., 2019; Sleper and West, 1996). Genetically it is an allohexaploid (2n = 6x = 42) cross-pollinated species. Tall fescue evolved under a Medditerranean climate of hot, dry summers and cool, wet winters and, thus, performs well in the transitional zone of the United States, which includes a combination of cool/humid, cool/arid, warm/humid and warm/arid geographic zones. Tall fescue generally has better drought tolerance and/or avoidance mechanisms than other cool-season perennial grasses such as ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa pratensis* L.; Huang and Gao 2000; Sheffer et al., 1987).

The persistence and performance of tall fescue is enhanced by symbiotic association with the fungal endophyte *Epichloë coenophiala* under various stress conditions, including drought (Pedersen et al., 1990; Bouton et al., 1993; Takach et al., 2014; Malinowski and Belesky, 2020). Endophytes enhance tall fescue tillering, root growth, aboveground biomass production, ability to absorb mineral phosphate from soil, osmotic adjustment, nitrogen utilization, and anti-nematode activity (Assuero et al., 2002; Dinkins et al., 2019; Elmi et al., 2000; Panaccione et al., 2006). Thus, endophyte-infected tall fescue has great potential as a forage crop in regions affected by episodic drought, amongst other environmental challenges.

Drought is the most important environmental factor limiting agriculture (Farooq et al., 2012). Plant responses to drought stress are complex and vary over space and time. Lack of water is perceived, in part, by membrane sensors in the root, which trigger systemic signaling pathways that affect gene expression throughout the plant. Plants have evolved diverse strategies to survive periods of drought, including developmental escape and avoidance, and biochemical tolerance (Fang & Xiong 2015; Hirayama & Shinozaki 2010; Meena & Kaur 2019).

Drought resilience involves multiple traits, each typically controlled by multiple genes, which presents a major challenge for researchers and plant breeders interested in the underlying mechanisms and harnessing them to increase crop drought tolerance.

Previous studies have identified common transcriptional responses to drought in various species, including induction of genes involved in transcriptional regulation, photosynthesis, hormone especially ABA metabolism, antioxidant biosynthesis, and metabolism of carbohydrate, amino acids, and fatty acids (Benny et al., 2019; Egea et al., 2018; Wang et al., 2017). Changes in both primary and secondary metabolites are associated with drought responses. Previous studies also point to important roles of osmolytes (e.g., trehalose, fructan and proline) as osmoprotectants under drought stress, among which, the importance of proline has been confirmed by various genetic studies (reviewed in Kaur & Asthir 2017; Meena & Kaur 2019).

Despite the importance of tall fescue as a primary forage species and of drought as a key limitation on forage production, the molecular and genetic mechanisms of drought tolerance in tall fescue remain largely unknown. Previous studies have explored physiological, biochemical and root developmental aspects of drought responses in tall fescue (Chen et al., 2018; Ebrahimiyan et al., 2013; Pirnajmedin et al., 2015; Saha et al., 2015; Sarmast et al., 2015; Sun et al., 2013b) and the influence of endophytes on stress tolerance (Nagabhyru et al., 2013). Only a few studies identified over/under-expressed transcripts responsive to drought stress (Dinkins et al., 2019; Talukder et al., 2015). A systems study on the drought tolerance mechanism and the role of endophyte in tall fescue drought responses is still lacking.

Here, we investigated the physiological, biochemical, and transcriptional responses to drought stress of two tall fescue genotypes contrasting in drought tolerance, which were selected based on field and preliminary greenhouse experiments. We also determined the impact of symbiosis with the endophyte, *E. coenophiala* on drought responses in the two plant genotypes. We aimed to understand the systems mechanisms underlying the variance in tall fescue drought tolerance and the role of endophyte in this process.

Materials and Methods

Plant material

Tall fescue plants were propagated by tillers and planted in tall plastic cones (35 x 7 cm, D60L, Stuewe and Sons., Inc., https://www.stuewe.com). The soil was a mixture of metromix 360 and common sand (v/v = 2/1). At planting, two tillers were planted in each pot. The soil water content was monitored with EC-5 soil sensors (https://www.metergroup.com/). For uniformity, the top edge of the soil sensor was 20 cm to the soil upper surface in each pot.

Drought treatment and plant sampling

Three weeks after being planted into the soil, ¾ of the plants were subjected to water withholding (drought-stressed) and ¼ plants remained well-watered (control). Drought-stressed plants were harvested when the soil volumetric water content (VWC) reached 10% (mild-stressed DrtA), 5% (moderately-stressed, DrtB), and 1% (severely-stressed, DrtC), respectively. The VWC of well-watered plants (Ctl) were maintained at ~30%. To minimize variance, all samples were harvested between 1 pm to 2 pm each day. At harvest, the shoots and roots were collected separately and then frozen in liquid nitrogen immediately. The tissues were stored at -80°C until being ground in liquid nitrogen for RNA purification (RNAseq) and metabolite analysis (GC-MS). For tissue collection to be used in quantification of the leaf osmotic potential, shoot/root dry weight and other physiological parameters, a separate drought experiment was performed.

Drought and re-watering experiment

In a separate experiment from above, two tillers of each of the four plant types were planted in a three-gallon plastic pot, two centimeters away from the edges avoiding the center of the pot, in random orders. A total of four pots and eight tillers of each plant type were used. When the soil VWC decreased to less than 1% and all the leaves lost chlorophyll and dried out, each pot was re-watered and the plants were allowed to re-grow for 20 days. At the end of re-growth, shoots and roots were harvested separately and dried completely in a 55°C oven for dry weight quantification.

Leaf size and specific leaf weight

The size of the youngest fully-expanded leaf of a tall fescue plant was measured with a Li-3000A portable area meter (Li-Cor, https://www.licor.com/). After area measurement,

the leaf was completely dried in a 55°C oven and then the dry weight was quantified using a lab balance. The leaf specific weight was calculated by dividing the leaf area by the dry weight.

Guard-cell density

The youngest fully-expanded leaf of a tall fescue plant at harvest was collected and nail polish imprints were made of the middle session of the leaf, avoiding the edges. The imprints were subsequently observed and photographed under a microscope (Nikon TE300) at 100X. Stomata density was counted from photos.

Leaf conductance

Leaf conductance was measured with the SC-1 Leaf Porometer (METER Group, Inc, https://www.metergroup.com/) on the youngest fully-expanded leaf. Each leaf was measured twice at the middle session and the average value was used.

***In-vivo* leaf chlorophyll measurement**

In-vivo leaf chlorophyll content was measured with a Chlorophyll Meter SPAD-502plus (Spectrum Technologies, http://www.specmeters.com/) on the youngest fully-expanded leaf. Each leaflet was measured two times at the middle session and the average reading was used. The leaf edges were avoided at all measurements.

Leaf osmotic potential

The middle section (1 cm) of the youngest fully-expanded leaf was sampled and then fully hydrated in sterile and de-ionized water in a 2 ml Eppendorf tube for 48 hours at 4°C. Next, the fully-hydrated leaves were tap-dried on a filter paper to remove surface water, and then stored at -80°C for over 24 hours in a 0.65 ml Eppendorf tube. At the end of storage, a hole was punched at the bottom of the 0.65 ml Eppendorf tube and then it was placed inside a 1.5 ml Eppendorf tube, being centrifuged at 12,000 rpm for 10 min at 4°C to collect the leaf sap. The molal concentration of the leaf sap was measured at room temperature with a Wescor EliTechGroup Vapro 5600 Vapor Pressure Osmometer. Osmotic potential was calculated using the formula “OP= iCRT”, where i = ionization

constant, C= Molal concentration (mole/ kg), R= pressure constant (0.0831 liter bar/ mole °K), T= temperature °K (273 + °C).

Transcriptome analysis with RNAseq

Total RNA was isolated with the Spectrum™ Plant Total RNA Kit (Sigma) and then treated with TURBO DNA-free™ Kit (Invitrogen) to remove DNA molecules. RNeasy MinElute Cleanup Kit (Qiagen) was used to further clean the DNase-treated RNA samples. The quality of RNA samples was monitored by bio-analyzer analysis using Agilent RNA 6000 Nano Kit (Agilent).

RNA samples were quantified using Qubit® RNA BR (Broad-Range) Assay Kit (Life Technologies). RNA-seq libraries were prepared using TruSeq Stranded mRNA Sample Prep kits (Illumina). Individual libraries were uniquely indexed using TruSeq RNA Single Indexes (Illumina), and pooled in equimolar ratio. The pooled libraries were sequenced on a Hiseq4000 system (Illumina).

All sequences were first quality trimmed using a custom Perl script which removed low quality bases (quality score < 30). Each sample was then *de novo* assembled with Trinity version 2.2.0 (https://github.com/trinityrnaseq/trinityrnaseq). These independent assemblies were then merged and aligned with HISAT2 version 2.0.5 (http://ccb.jhu.edu/software/hisat2/index.shtml). The aligned reads were assembled and quantified using Stringtie version 1.2.4 (http://www.ccb.jhu.edu/software/stringtie/). For the purpose of obtaining functional annotations, the merged transcript set was aligned with the reference proteomes of Arabidopsis (TAIR 10), *Medicago truncatula* (IMGAG v4.0), and rice (MSU version 7). Finally, all transcripts were filtered by at least 500bp long, FPKM (Fragments Per Kilobase of exon per Million reads) > 1 in at least one sample, and majority of non-zero FPKM > 1. The normalized and filtered FPKM values were used in further analyses.

Metabolite analysis with GC-MS

Metabolite analysis of polar and non-polar metabolites were conducted following the procedure in Kang et al., (2011). Data analysis was performed using software MS-DIAL (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/).

Proline biochemical assay

Proline content was analyzed with a biochemical assay following Bates et al (Bates et al., 1973) and Hamid et al (Hamid et al., 2003). Proline concentration was determined using a standard curve generated using L-proline.

Statistical analysis

For phenotypic and leaf osmotic pressure data, significant analysis was performed in R with package “agricolae”. Two-way analysis of variance (ANOVA) (aov) was performed first and then Duncan's New Multiple Range Test was conducted for p value calculations. For GC-MS and RNAseq data, significant analysis was performed by calculating p values with student’s t test (two tails assuming equal variance) in excel. False discovery rate (FDR) adjusted p values (p_{adj}) were calculated in R using function “fdr”.

Bioinformatic analysis

For having the best annotations, all bioinformatic analyses were performed using the closest *Arabidopsis thaliana* or rice (promoter motif enrichment) orthologs of corresponding tall fescue transcripts. List of drought regulatory gene illustration was performed using DiVenn 2.0 (https://divenn.noble.org/; Sun et al., 2019). GO Enrichment analysis was performed in AgriGO v2 (http://systemsbiology.cau.edu.cn/agriGOv2/; Tian et al., 2017). Transcription factor binding site enrichment in the promoters was performed in ShinyGO v0.61 (http://bioinformatics.sdstate.edu/go; Ge et al., 2020) using rice orthologs and Pscan (http://159.149.160.88/pscan/) using Arabidopsis orthologs of corresponding tall fescue genes. Compared to ShinyGO, Pscan provides a more complete list of all enriched transcription factor (TF) binding motifs, and more information about the TFs, e.g. matrix ID, which can be easily connected to the JASPAR database. However, Pscan can only analyze Arabidopsis genes among all plant species, further analysis was therefore performed in ShinyGO using rice homologs, for rice being evolutionarily closer to Tall Fescue than Arabidopsis with relatively high-quality genome annotations. Classes of the transcription factors were assigned according to JASPAR2018 database (http://jaspar.genereg.net/).

Results

Physiological characterization of drought adaptation traits

Before performing drought stress experiments, we compared the shoot, root, and leaf phenotypes of the drought-tolerant tall fescue genotype, T400, and the drought-sensitive genotype, S279 under well-watered conditions, with (E+) or without endophyte (E-). Shoot dry weights of the four plant-endophyte combinations (T400E+, T400E-, S279E+, S279E-) were similar (Figure 1a). Interestingly, S279E+ invested significantly more in root growth than S279E- (Figure 1b, Figure S1). On the other hand, no significant difference was observed between T400E+ and T400E- in either shoot or root biomass (Figure 1a, 1b).

Leaf size and thickness of well-watered plants were then compared. T400 had relatively long, narrow, and thick leaves, whereas S279 leaves were shorter, wider, and thinner (Figure 1 c, d, e, f, g). The area of each leaf was similar among all plant-endophyte combinations (Figure 1c). The difference between E+ and E- was not significant. Stomatal density on the abaxial side of leaves was similar among different plant types (Figure S2), while leaf conductance was significantly higher in S279 compared to T400 (Figure 1h).

Drought stress was applied by withholding water. In a preliminary experiment, when soil volumetric water content (VWC) reached 10% (mild stress, DrtA), leaf gaseous water conductance decreased by 53% in S279E+ plants (274.1±39.8 to 113.5±17.8 mmol m^{-2} s^{-1}, n=4), although they appeared visibly similar to the well-watered controls (Figure 2, DrtA). Leaf rolling was first evident at soil VWC of 5% (moderate stress, DrtB) and reached an extreme at 1% soil VWC (severe stress, DrtC; Figure 2). Under well-watered conditions, T400E+ had the highest leaf chlorophyll content with 48.5 SPAD units, which was 18.2% higher than that of S279E+ with the lowest chlorophyll content. In addition, endophyte infection significantly reduced leaf chlorophyll content by 12% in S279, but did not cause significant changes in T400 (Figure 3a). Under severe drought stress, leaf chlorophyll content significantly decreased in S279E- but not in other plant types (Figure 3a). T400 and S279 had similar leaf osmotic potential at well-watered conditions (Figure 3b). Under severe drought stress, the leaf osmotic potential of T400 was 19% (E+) to 24% (E-) higher than that of well-watered controls, whereas no significant difference was observed in S279E+/- between drought and well-watered conditions (Figure 3b). After

severe drought stress, all plant types had similar shoot biomass, while S279E- had smaller root biomass than S279E+, which was similar to T400E+/- (Figure S3).

In a separate experiment, the four tall fescue plant types were planted together in three-gallon pots and the shoot/root biomass was measured after severe drought stress (< 1% soil VWC) and recovery (Figure 4). After 20 days of recovery and re-growth, T400E+ had much larger shoot and root biomass compared to other plant types, especially root biomass, which was nearly twice that of other plant types. No significant difference was observed between S279E+ and S279E-, either in shoot or root (Figure 4b, c).

Transcriptomic and metabolomic analyses of well-watered and drought-stressed tall fescue plants

To gain insight into possible molecular and biochemical mechanisms underlying the contrasting physiological and developmental responses to drought stress between T400 and S279, and between E+ and E-, we performed RNAseq and GC-MS analyses to examine transcriptomic and metabolomic changes, respectively. Under severe drought stress, a larger number of polar metabolites accumulated in T400 shoots (22 metabolites) compared to S279 (9 metabolites), especially organic acids (Table 1). In the root, metabolite accumulation and depletion patterns were similar for S279E- and T400E+/-, while S279E+ roots appeared to be unique with the majority of metabolites decreasing in abundance compared to the control (Table 1). Among all polar metabolites, proline and trehalose exhibited the greatest increase in relative abundance in response to drought in both roots and shoots of T400E+/- and S279E+/-. Because proline accumulated to much higher levels in T400 than S279 under severe drought, we further quantified levels of proline and analyzed transcripts of genes involved in proline biosynthesis and degradation (RNAseq results), under all stress conditions. Using a quantitative biochemical assay, proline levels were found to increase under drought stress in both shoots and roots, with the highest levels observed in severely-stressed plants (DrtC, Table 2). Severely-stressed shoots of tolerant plants with endophyte, T400E+, had the highest proline levels (8.16 mg/g dry weight) among all samples. Transcript levels of the major proline biosynthetic enzyme, delta-1-Pyrroline-5-carboxylate synthetase (P5CS), but not delta1-Pyrroline-5-carboxylate reductase (P5CR), mirrored the levels of proline,

in both shoots and roots. In contrast, transcripts of proline dehydrogenase (PRODH), which mediates proline degradation, decreased with drought intensity, with the lowest levels under severe drought stress (Tables 2, S1).

RNA-seq analysis was carried out for plants with or without endophyte exposed to different levels of drought stress, to identify genes and associated biological processes affected by drought. Under drought stress, there were generally more down-regulated than up-regulated genes, and more differentially expressed genes (DEGs) in the roots than shoots (Figures 5a, d, e). Severely drought-stressed T400E+ plants had the largest number of DEGs among all treatments, in both shoots and roots (Figure 5a). Comparing T400 and S279 (Figure 5b), severely drought-stressed T400E+ also had the most number of DEGs compared with S279E+, with up to 1,273 down-regulated DEGs in the shoot (Figures 5d, e). The difference in transcript regulation between T400 and S279 was minimal under moderate stress (DrtB) (Figure 5b). Numbers of DEGs between E+ and E- plants were much smaller compared to that between T400 and S279, showing generally higher transcript levels in E+ than E- plants, especially in the shoot (Figure 5c). The endophyte effect on root gene expression was very small in both T400 and S279 (Figure 5c).

Gene Ontology (GO) enrichment analysis was performed on drought-regulated genes, which revealed that the following processes were induced under drought stress in all plant types: response to abiotic stresses (temperature, heat, high light, desiccation, salinity, cold, oxidative) and catabolism of organic acids, amino acids, cofactors, porphyrin-containing compounds, and tetrapyrrole/chlorophyll. In contrast, genes associated with photosynthesis, biotic stress response (chitin), growth (response to nitrogen), receptor signaling pathways, phosphorylation and phosphate metabolism were substantially repressed under drought conditions (Table S2).

Next, GO enrichment analyses were performed on genes that were differentially expressed in T400 and S279, and between E+ and E-. When looking at the GO enrichment of genes that were differentially expressed in T400E- and S279E-, which presumably reflect intrinsic genetic differences between T400 and S279, five categories of genes were found to be enriched in the shoot, but none in the root (Table 3). Enriched

genes involved in (programmed) cell death were generally more highly expressed in T400 shoots than in S279, under both well-watered and stressed conditions. On the other hand, enriched genes related to response to chitin and nitrogen compound typically had lower expression levels in T400 shoots than in S279 under well-watered conditions (Table 3). When comparing E+ and E- treatments, the presence of endophyte affected T400 and S279 in similar ways under drought stress, primarily by stimulating gene expression related to protein and nitrogen compound metabolism in the shoot (Table 3). However, under well-watered conditions, similar endophyte effects on protein and nitrogen compound metabolism were observed only in T400 and not in S279. On the other hand, biotic stress responsive genes (chitin) and genes responding to nitrogen compounds (growth) were expressed at higher levels in E+ than E- roots in S279 under drought stress (Table 3).

When combining the genotype and endophyte effects and comparing between T400E+ and S279E+, we found that genes involved in degradation of organic acids and amino acids were enriched among the genes that had higher expression levels in drought-stressed T400E+ than S279E+ in the shoot, but no significant category enrichment was identified in up-regulated genes (T400E+/S279E+) in roots (Table 3). In contrast, strong enrichment was observed in the shoot down-regulated genes (T400E+/S279E+), many in categories related to photosynthesis activity that responded to drought stress, i.e. light reaction, porphyrin-containing compound biosynthesis/metabolism, tetrapyrrole biosynthesis/metabolism, photosynthetic electron transport, plastid organization, and chlorophyll biosynthesis (Table 3).

Transcription factor binding motif analysis of putative orthologs of drought responsive genes in tall fescue

In further analyzing the transcriptomic data, we were interested in identifying DNA sequence motifs that might be involved in control of gene expression during drought, via TF binding, as well as potential difference in these motifs between T400 and S279. For the lack of reference genome sequence in tall fescue, we took advantage of genome sequences of Arabidopsis and rice, and used these to examine conserved TF binding motifs in putative orthologs of the tall fescue drought-responsive genes using both the

Pscan Web Interface (Arabidopsis orthologs, 1000 bp), and ShinyGO v0.61 (rice orthologs, 600 bp).

In both analyzes, substantial enrichment of bZIP and bHLH transcription factor binding sites were found in the promotors of Arabidopsis and rice orthologs of the tall fescue genes that were significantly up regulated by drought stress (2 fold, $p_{adj} < 0.05$; Tables 4, S3, S5). Furthermore, all of the enriched bZIP and bHLH TF binding sites contain the consensus motif "(C/G)ACGTG" (Tables 4, S3, S5), and belong to the JASPAR Plantae CORE cluster 3 of plant TFs that are classified based on core binding motifs (Tables 4, S3). To determine whether this motif was over-represented in drought-responsive genes of other plant species, we analyzed published gene expression data from *Medicago truncatula* (Zhang et al., 2018) and alfalfa (Kang et al., 2011), which yielded similar results (Tables 4, S3).

In contrast to up-regulated genes, the TF binding motif enrichments in drought stress down-regulated genes were much more diversified, with bHLH, C2H2 zinc finger factors, CG-1 domain, homeo domain factors, NAC/NAM, helix-turn-helix, and WRKY on the top list (Tables S4, S6). The top identified consensus motif was "GTCAA" for WRKYs (cluster 5) (Table S4, S6). No clear and consistent consensus motifs were identified in other TF families. Overall, the patterns of promoter binding motif enrichment of drought stress repressed genes were similar among tall fescue, *M. truncatula*, and alfalfa (Tables S4).

Discussion

Physiological, molecular, and biochemical bases and endophyte effects for contrasting drought tolerance in tall fescue genotypes

Plant drought adaptation and resistance include three main strategies: drought escape, drought avoidance, and drought tolerance (Aslam et al., 2015; Levitt 2015). Drought escape refers to plants that alter their life cycle by either entering dormancy or flowering early when faced with drought stress (Kramer 2015). Drought avoidance is related to a plant's ability to maintain high water potential under water limitation, mostly by reducing

leaf transpiration and/or enhanced root growth (Levitt 2015). In contrast, plant drought-tolerance is primarily related to maintaining water uptake by accumulating osmolites under drought stress (Levitt 2015). Earlier studies indicate that tall fescue uses all three strategies to survive drought stress. It is well known that Mediterranean tall fescue can enter summer dormancy in dry and hot environments, which is a typical mechanism of drought escape (Volaire & Norton 2006). Under drought, tall fescue plants tend to develop deeper roots and larger root systems, an important mechanism for drought survival that was shown repeatedly to be associated with drought tolerance among different varieties (Carrow 1996; Huang & Fry, 1998; Pirnajmedin et al., 2015). Past studies also showed that drought tolerant tall fescue cultivars contain higher protein and soluble carbohydrate content, and lower H_2O_2 content than sensitive ones (Rohollahi et al., 2018). For osmotic adjustment, multiple studies reported sharp increase of proline in tall fescue leaves under drought stress (Ebrahimiyan et al., 2013a; Pirnajmedin et al., 2017; Rohollahi et al., 2018; Sarmast et al., 2015). The role of other osmolytes such as sugar alcohols were much less studied (Bacon 1993).

In the current study, we compared drought responses of two contrasting tall fescue genotypes and found that the drought tolerant genotype, T400, showed morphological and physiological characteristics related to drought avoidance and are typical for plants that are adapted to dry environments, e.g. small, narrow, but thick leaves, and relatively lower leaf conductance compared to the sensitive genotype, S279 (Figure1). This phenomenon has been reported broadly in grasses and other plant species, and these plants are generally called “water savers” (Kang et al., 2011; Maricle et al., 2007; Polania et al 2016). Although leaf traits of E+ and E- plants were similar in both T400 and S279, endophyte symbiosis affected plant biomass differently in T400 and S279. Under both well-watered (Figure 1) and drought (Figure S3) conditions, S279E+ plants had significantly higher root biomass but similar shoot biomass compared to S279E-. In T400, endophyte infection did not promote root growth significantly under either conditions (Figures 1, S3). However, after severe drought stress and recovery, T400E+ had much larger shoot and root biomass than T400E-, revealing a delayed effect of endophyte during drought and recovery.

In earlier studies, endophyte symbiosis has been shown to promote plant growth and improve drought resistance (Feng et al., 2006; Khan et al., 2014). In tall fescue, endophyte presence was reported to increase shoot biomass, tiller numbers, and survival under field drought stress, while the benefit was not noticeable during wet years (West et al., 1993). In another study using three tall fescue genotypes and multiple endophyte species, significant genotype x endophyte interactions ($p<0.001$) were observed for tiller density and shoot dry weight per area, indicating the promoting effect of endophyte on plant growth is association-specific (Elbersen and West 1996). Similar tall fescue cultivar x endophyte interaction was found in a separate study with elite cultivars infected with elite endophytes performing the best, and endophyte was more important in conferring resistance than difference between cultivars (Hume & Sewell 2014). Therefore, the interaction between specific tall fescue and endophyte genotype appears to be important for the outcome. Here, we demonstrate that T400 and S279 responded to the same endophyte infection differently at the levels of phenology, physiology, molecular and biochemistry, and endophyte infection is crucial in enabling drought tolerance in T400, as discussed further below.

At molecular level, GO enrichment analysis revealed that genes related to photosynthesis were expressed at lower levels in T400E+ than in S279E+ (Table 3) under drought stress, consistent with a conservative strategy of T400E+ with respect to photosynthesis and linked transpiration. However, despite the drop in photosynthesis, reflected by the decline in biomass under drought stress (Figures 1, S3), plants accumulated osmolytes especially proline, apparently via increased synthesis (Tables 1, 2, S1). Under both well-watered and drought stressed conditions, T400E+ was much more active in protein biosynthesis and metabolism than T400E- (Table 3). Together, these observations may explain why T400E+ had the largest root and shoot biomass after severe drought stress and recovery (Figure 4). Our study confirms that the presence of endophyte has a positive effect on root growth and drought stress tolerance, as reported earlier in tall fescue (Arachevaleta et al., 1989; Bacon 1993; West et al., 1993). In addition, T400 and S279 responded to endophyte differently in multiple levels (Figures 1, 3, 4; Table 1), presumably due to plant genotype-specific reactions to endophyte infection as reported earlier in tall fescue (Elbersen & West 1996; Hume & Sewell 2014).

As mentioned above, we observed a significant difference between plant genotypes in leaf osmotic pressure changes during drought, with T400 having a much larger leaf osmotic pressure increase under drought stress compared to S279 (Figure 3b). Higher leaf osmotic pressure indicates stronger osmotic adjustment and more osmolite accumulation, which is crucial for surviving drought stress and has been reported in tall fescue (West et al., 1990). Compared with drought-adaptive phenotypic changes, e.g. smaller and thinker leaves, and lower stomatal density, osmotic adjustment is inducible and temporary. Therefore, it generally has less negative effect on growth and is more cost-effective to plants (Johnson et al., 1993; McCree 1986). Metabolite profiling confirmed greater accumulation of specific metabolites under severe drought stress in T400 than in S279 shoots (Table 1), especially organic acids. Consistent with this, GO enrichment analysis revealed genes involved in amino acid and organic acid catabolism amongst those with higher expression levels in T400 than S279 under drought stress (Table 3).

Among all metabolites detected, proline accumulated much more in T400 than in S279 under severe drought stress, in both roots and shoots, and both E+ and E- (Tables 1, 2). In T400E+, proline content increased from 0.16 to 8.16 mg/g DW in the shoot, equivalent to a change in osmotic potential of 69.5 mmol/kg, explaining much of the leaf osmotic pressure increase under drought stress (Figure 3b). Transcript levels of one of the two proline biosynthetic enzymes, P5CS, mirrored those of proline content, consistent with P5CS being a rate-limiting enzyme in proline biosynthesis (Delauney & Verma 1993). Early studies demonstrated that over-expression of P5CS in multiple plant species promotes proline biosynthesis and improves drought tolerance (Amini et al., 2015; Kavi Kishor et al., 1995; Yamchi et al., 2007; Vendruscolo et al., 2007). Similar association between proline accumulation, P5CS induction, and genotype drought sensitivity was reported in rice (Choudhary et al., 2005), *Brassica juncea* (Phutela et al., 2000), and wheat (Maghsoudi et al., 2018). However, proline accumulation was found not to be associated with genotype drought tolerance in Arabidopsis (Marín‐de la Rosa et al., 2019), alfalfa (Kang et al., 2011), and Tibetan hulless barley (Deng et al., 2013). Increased proline content does not necessarily associate with improved drought tolerance either (Pospisilova et al., 2011). Therefore, while proline is undoubtedly an important

443 drought osmolite in plants, it may not be a universal marker for plant drought tolerance.
444 In tall fescue, we observed contrasting patterns of proline accumulation associated with
445 drought tolerance in the two genotypes, with more proline accumulated in the tolerant
446 genotype. An earlier study in tall fescue obtained similar results with tolerant cultivar 'Van
447 Gogh' accumulating 32% more leaf proline than the sensitive cultivar 'AST7002' under
448 drought (Man et al., 2011). In the future, it would be interesting to expand this study to
449 more genotypes and test the potential role of proline as a biochemical signature in
450 screening for drought tolerance in tall fescue.

451 **Potential master regulatory roles of bZIP and bHLH transcription factors in drought**
452 **stress responses in tall fescue**

453 Plants possess a large number of TF genes and families. For example, Arabidopsis
454 contains about 2000 TFs in over 60 TF families (Hong 2016). Under drought stress, a
455 large number of TFs are either up- or down-regulated (Joshi et al., 2016; Kaur & Asthir
456 2017; Leng & Zhao 2020). Numerous studies generated transgenic plants with altered
457 expression of TFs to improve drought tolerance (Joshi et al., 2016; Kang et al., 2016;
458 Leng & Zhao 2020; Nadeem et al., 2019). Among all TFs, AP2/ERF, AREB/ABF, bZIP,
459 NAC, NF-Y, WRKY, and Zinc finger proteins are the major families that have been
460 investigated in plant drought stress studies (Joshi et al., 2016; Leng & Zhao 2020).
461 However, it remains unclear on the relative importance of various TF families in regulating
462 gene expression under drought. In the current study, analysis of putative TF binding
463 motifs in the promoters of Arabidopsis and rice orthologs of drought-induced genes in tall
464 fescue, revealed a significant enrichment of bZIP or bHLH TF binding motifs (Tables 4,
465 S3, S5). Furthermore, all of these motifs belonged to just one JASPAR cluster, cluster 3,
466 with core binding motif (C/G)ACGTG, which contains the same ACGT core as the abscisic
467 acid (ABA)-response element (ABRE), PyACGTGG/TC (Nakashima et al., 2014; Singh &
468 Laxmi 2015). In contrast, binding sites for the ABA-independent TFs DREB (AP2/ERF
469 domain) and zinc finger homeodomain (ZFHD) that have also been implicated in drought
470 responses/tolerance (Phuong et al., 2015; Kaur & Asthir 2017; Leng & Zhao 2020) ranked
471 much lower in the motif enrichment list (Table S3). Similar results were obtained from our
472 analysis of TFs induced by gradual drought stress in alfalfa and *M. truncatula* (Table 4,

S3). Taken together, these results point to the dominate role of ABA-dependent up-regulation of gene expression during gradual soil drought stress.

Earlier studies demonstrated that the highly conserved G-box motif, CACGTG, is bound by bZIPs and bHLHs in plants, either as homodimers or heterodimers (Ezer et al., 2017b). It is well-known that bZIPs mediate ABA-dependent drought responsive pathways (Banerjee & Roychoudhury 2017; Shinozaki & Yamaguchi-Shinozaki 2000), and numerous studies have explored their functions in plants (Gahlaut et al., 2016; Joshi et al., 2016; Rabara et al., 2014). Interaction between bZIPs and bHLHs has been predicted and explored in plants and other organisms (Chow et al., 2008; De Jong 2013; Ezer et al., 2017a; Kuras et al., 1997). In Arabidopsis, it was found that bZIPs and bHLHs could form transcriptional modules to integrate light and reactive oxygen species signaling (Chen et al., 2013). Therefore, it would be interesting to explore the potential role of bHLH–bZIP interactions in plant drought responses. Although bHLHs and bZIPs each belong to several clusters in JASPAR CORE Plants clustering (Table S3, S4), only cluster 3 has binding motifs enriched in drought up-regulated genes. This information may aid in selecting promising TFs to generate drought-tolerant transgenic plants. It also implies that the binding motif analysis of TFs may be more biologically relevant than family classifications based on protein/nucleotide sequences and conserved domains

Besides focusing on drought up-regulated genes, we also studied TF binding motif enrichment in drought down-regulated genes, which have been much less studied (Huang et al., 2008). In contrast to drought up-regulated genes, much greater variance in TF binding motifs was found in drought down-regulated genes with much larger enrichment p values (Table S4, S6). In addition, we observed specificity associated with tissue types as well as with stress severity. For example, WRKY (cluster 5) binding motifs were enriched preferentially in the promoters of down-regulated genes in mildly-stressed shoots, but less so in moderately and severely-stressed shoots (Table S4, S6). To our knowledge, this phenomena has not been reported previously and it will be interesting to find out how plants coordinate down-regulation of gene expression under drought stress, and the importance of this.

Finally, the current analysis of promoter TF binding motif was performed on orthologs of genes that responded to gradual soil drought stress that lasted over ten days, in contrast to many other studies that applied rapidair/soil dehydration stress or PEG/mannitol treatment (e.g. Abdel-Ghany et al., 2020; Haake et al., 2002; Huang et al., 2019; Wu et al., 2019). It would be of particular interest to perform similar TF promoter binding motif enrichment analysis in other plant species that undergo either similar or accelerated drought stresses to draw a more general conclusion.

Summary

In summary, gradual soil drought stress was applied to two tall fescue genotypes (T400 and S279) with contrasting drought tolerance, either with or without endophyte symbiosis. Physiological and biochemical analysis indicate that T400 (tolerant genotype) utilizes both drought escape and drought tolerance strategies to confer greater drought tolerance than S279 (sensitive genotype), for example, thicker and narrower leaves, lower transpiration, and more osmoticum especially proline accumulation under drought stress. Metabolite analysis with GC-MS identified common and unique metabolites altered by drought stress in T400 and S279, with or without endophyte symbiosis. GO enrichment analysis of transcriptome changes revealed that the drought tolerant genotype, T400, repressed more genes related to photosynthesis and induced more genes related to organic acid and amino acid metabolism than the sensitive genotype. GO enrichment analysis also highlighted the role of endophyte in stimulating protein biosynthesis and metabolism in both genotypes. Finally, promoter transcription factor binding motif enrichment analysis of up-regulated genes by drought stress implies the master regulatory role of bZIP and bHLH transcription factors, with core binding motif ACGTG, which was conserved in tall fescue, *M. truncatula*, and alfalfa, and the potential role of bHLH–bZIP interactions in plant drought responses was speculated.

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Table 1. Polar metabolite accumulation in severely drought-stressed (DrtC) compared to well-watered plants (Ctl). Log2 fold changes (FC) of metabolites are shown, with up- and down-regulated metabolites colored in red and blue, respectively. FCs less than 1.5 (-0.85 < log_2ratio < 0.85) are not shown. All FCs having p values less than 0.1 are in bold and FCs with p < 0.05 are in bold and underlined, n=3.

		DrtC/Ctl (shoot)				DrtC/Ctl (root)			
Group	Metabolite ID	S279E+	S279E-	T400E+	T400E-	S279E+	S279E-	T400E+	T400E-
Amino acid	Alanine	**-2.15**	-1.36			**-1.93**	1.71	1.55	
	Isoleucine			1.99	**1.78**	**-1.63**		2.78	1.15
	Phenylalanine	**-3.03**	-1.33	-0.79		**-2.79**			-1.10
	Proline		2.98	**4.27**	**4.26**	0.95	**2.15**	**7.48**	**5.59**
	Serine	**-1.69**				**-2.60**	0.74	1.13	1.25
	Valine	**-1.58**	-1.13	0.73	**1.09**	-1.16	1.23	1.72	1.60
Organic acid	4-Aminobutyric acid	-0.96	-1.53	2.26	2.27		2.50	2.67	1.45
	Aconitic Acid	-0.81	**-5.19**	-0.69		-3.44	1.37	1.25	3.27
	Arabonic acid	**-3.91**	**-3.69**	-0.85			2.57	1.89	**3.63**
	Ascorbic acid	-2.99	-2.70		0.74	-0.86			
	Aspartic Acid	**-1.12**	-2.46				2.65	2.24	1.75
	Caffeic acid	-1.48	-0.90		0.72	-2.25	-2.92	**-2.54**	-3.56
	Chlorogenic acid		-2.17	**-1.15**	**-1.80**	-0.76	-0.85		-0.61
	Citric Acid	-2.67	-2.22	**-1.93**	**-2.32**	-3.63	-2.20	**-1.63**	-1.54
	D-Galactonic acid	0.65	-3.50			-0.99	2.16	**4.56**	-1.86
	Dicrotalic acid	**-2.35**	-1.86	**-1.03**	-1.13	-1.60			-0.94
	Gluconic acid		-1.11	2.07		**-2.50**	-2.89	-0.83	-0.70
	Glutamic acid	**-3.56**	**-2.54**	-0.69		-1.39	**2.98**	**-2.54**	
	Glyceric acid	**-1.35**	-2.60	**-2.16**		**-2.03**		2.78	1.76
	Glycolic acid	-0.60	-1.07			-1.67		2.82	
	Lactobionic acid	-1.84	-5.26	**-5.10**	-4.03	-4.88	-2.45	**-4.30**	-3.68
	Maleic acid	**-1.88**	-2.60	2.07	1.14	**-1.68**	1.12	**2.06**	-1.14
	Malic acid	**-4.12**	-1.16			0.97	1.05	**2.79**	**3.04**
	Mesaconic acid	**-2.30**	-1.79	0.76	1.96	-0.79	1.12	1.57	
	Octanoic acid		-1.82	1.32	**2.69**	-0.85	**2.64**	1.26	
	Oxalic Acid	**-1.66**	**-2.65**	-0.80		0.68	2.80	**2.23**	**1.66**
	Palmitic acid	1.82	**-4.35**	**-2.58**	-2.54		0.71	1.13	-1.74
	Phosphoric acid	**-1.42**	-1.45	-0.93	-1.00	-0.93	-1.81	**-1.63**	-3.05
	Pyroglutamic acid	**-2.48**	-1.12		**1.66**		3.03	2.40	**2.91**
	Quininic acid	-1.35	-1.30			-0.94	1.02	1.06	
	Shikimic acid	**-3.30**	-2.28	-1.45	**-1.98**		-3.72	**-1.55**	-4.75
	Threonic acid	-2.77					2.83	2.40	1.76
	trans-Vaccenic acid	-3.21	-1.35			-1.46	-0.76	-1.92	-1.30
Sugar	Ribose	-1.80	-1.63	-1.31		**-1.21**		-0.80	
	Galactose	0.70			1.09	1.77		3.91	**1.69**
	Maltose	**-1.09**	-1.65		-1.58	**3.69**	4.57	**3.28**	**2.43**
	Glucose	**1.64**	2.36	1.85	**3.62**	**1.91**		0.96	2.15
	Fructose	1.80	0.64	**1.82**	**2.65**			-0.79	-0.81
	Lactose	**-0.96**	-1.37			-1.95	**-2.92**	**-3.36**	-3.06
	Maltose	**-2.13**	-2.98	-1.07			**3.97**	**3.82**	**1.60**
	Maltotriose	**-3.85**	-3.05	**-3.00**	**-2.72**	-2.52	-0.86		-1.33
	Raffinose	**-3.23**	-2.12	**-1.91**	**-2.19**	-2.09		0.64	-1.64
	Sorbose	1.56		0.98	**1.74**		-0.64	-0.60	
	Sucrose				-0.83	-0.79	1.37		-0.79
	Trehalose	2.11	2.85	**2.87**	4.56	**1.73**	**7.22**	**6.02**	**6.56**
Sugar alcohol	Galactinol	-1.20	-1.42			0.98	1.95	1.41	
	Maltitol	-3.23	-3.18	**-3.86**	**-5.05**	-1.06	**-3.98**		
	Myo-Inositol	**-2.19**	-2.38	**-0.91**	**-1.80**				-1.09
	Palatinitol	**-2.04**	-1.69	**-2.22**	-1.22	-5.61	-0.74		
	Ribitol		-0.70	1.06		-1.23	**-1.60**	**-0.75**	**-1.36**
Others	Cholesterol		**-2.56**	2.98	**-0.83**	-2.68	-1.15	-3.43	
	Coniferyl alcohol	-1.15	-1.65	0.70	3.00	-1.27	1.98	1.12	
	D-Ribono-1,4-lactone	-0.93	-2.09	1.53		-0.99			1.77
	Ethanolamine	1.06	2.28	**2.41**	0.98	-0.60	5.30	**5.18**	**4.57**
	Glucose-6-phosphate	-5.11	-3.27	-1.02		**-1.01**		-0.72	-0.91
	Glucuronolactone	**-3.08**	-4.01		**2.03**	-0.66	**2.80**	2.48	
	Glycerol	-1.91	-1.96	-0.79		-1.64		0.81	0.63
	Mercaptoethanol	**-3.99**	-2.75	**-1.48**	-1.23	**-3.56**	-1.12	-1.54	**-2.91**
	Methyl tetradecanoate		-0.64		-0.77		-0.83	**-0.92**	-1.05

Table 2. Accumulation of proline and transcript (FPKM) changes of major proline biosynthesis and degradation genes under drought stress. Average values of three replicates are shown. Nucleotide sequence of each transcript and annotation details (rice, wheat, Arabidopsis, and *M. truncatula*) are in Table S1. FPKM, Fragments Per Kilobase of exon per Million reads. P5CS, delta-1-Pyrroline-5-carboxylate synthetase; P5CR, delta1-Pyrroline-5-carboxylate synthase; PRODH, proline dehydrogenase. Intensities of colors indicate relative abundance of proline or transcripts (highlighted separately).

			Shoot_Ctl				Shoot_DrtA				Shoot_DrtB				Shoot_DrtC				Root_Ctl				Root_DrtA				Root_DrtB				Root_DrtC			
			S279		T400		S279		T400		S279		T400		S279		T400		S279		T400		S279		T400		S279		T400		S279		T400	
			E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-	E+	E-
Metabolite (proline)	GC-MS		23	9	37	9	3	10	44	2	1	8	28	15	26	73	709	168	3	2	2	3	4	34	9	4	8	10	12	26	7	10	385	128
	Chemical assay (mg/g)		0.1	0.1	0.2	0.1									0.6	0.9	8.2	4.5	0.1	0.1	0.1	0.1									0.5	0.8	4.0	1.6
Transcripts related to proline metabolism (RNAseq/RPKM)	P5CS	MSTRG.157030	6	6	15	15	6	19	17	12	11	14	22	27	19	30	93	41	7	8	17	16	13	17	27	23	14	16	25	29	20	32	53	35
		MSTRG.168651	8	8	8	9	7	17	12	9	14	15	14	18	21	33	73	37	9	8	10	10	13	17	18	15	15	17	17	19	23	33	38	25
		MSTRG.15185	2	3	3	2	3	9	11	5	5	6	10	18	12	11	96	50	2	2	1	1	5	5	9	6	5	4	7	12	8	11	28	15
		MSTRG.160931	6	6	5	6	5	15	9	6	10	11	11	15	16	24	66	30	6	6	7	6	10	11	13	11	11	12	12	16	17	24	30	21
		MSTRG.154369	3	4	7	5	11	20	43	22	26	28	62	76	59	54	284	209	2	2	2	2	8	9	21	13	12	8	20	36	20	25	82	53
		MSTRG.188412	1	1	1	1	1	2	3	2	1	1	4	3	2	2	14	9	1	1	1	1	1	1	3	2	2	2	3	3	1	2	5	3
		MSTRG.53009	2	3	6	3	3	10	17	7	7	9	18	20	17	18	119	67	2	2	1	1	6	4	12	6	5	5	8	17	9	13	38	21
		MSTRG.98685	5	5	4	4	5	10	5	4	9	10	6	9	14	23	34	17	5	6	5	5	9	11	9	7	10	10	10	13	14	20	25	16
		MSTRG.102487	5	5	9	7	4	11	10	8	8	10	13	16	14	19	83	38	7	6	11	9	11	14	19	16	12	13	14	17	20	28	32	23
		MSTRG.181050	2	3	4	4	2	6	5	4	4	5	6	9	7	11	33	14	3	3	5	5	5	7	8	7	6	6	7	8	8	13	15	11
		MSTRG.34622	7	7	7	7	6	18	14	8	14	15	20	23	23	32	138	59	7	7	10	10	13	16	19	17	14	16	14	17	22	33	33	24
		MSTRG.117130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	0	5	0	0	0	1	0	0
		MSTRG.67251	3	4	10	5	8	18	33	17	17	21	43	53	44	41	216	151	2	3	2	2	7	6	16	10	7	6	14	26	13	17	51	29
		MSTRG.205271	4	4	6	4	7	15	46	19	12	14	44	80	28	26	340	244	3	3	2	2	6	7	22	12	7	6	20	38	11	15	89	43
		MSTRG.182094	3	3	9	8	5	17	22	13	10	12	29	31	24	25	145	85	3	3	8	7	8	10	20	16	8	9	26	35	11	19	71	42
		MSTRG.222130	5	6	12	6	14	33	57	30	28	32	76	79	73	61	386	272	2	2	2	1	8	8	22	17	10	8	21	32	18	22	70	42
	P5CR	MSTRG.124244	3	3	3	3	4	5	5	3	5	6	6	6	8	9	20	14	3	3	2	3	4	5	4	3	4	5	3	3	6	7	5	4
		MSTRG.164792	5	5	5	5	5	6	11	6	7	7	11	12	9	10	37	28	7	7	5	5	9	7	8	7	9	8	9	10	9	12	15	12
		MSTRG.151010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	11	0	0	0	9	0	0	0	1	0	0
		MSTRG.177524	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	10	0	0	0	9	0	0	0	1	0	0
	PRODH	MSTRG.110129	18	18	11	14	19	12	6	8	9	11	5	6	3	3	1	2	26	26	27	19	17	4	6	6	11	5	18	7	12	4	4	5
		MSTRG.193388	3	0	3	0	3	0	2	0	2	0	2	0	2	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		MSTRG.59111	3	0	3	0	3	0	2	0	3	0	1	0	2	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
		MSTRG.188184	15	16	6	8	14	10	3	4	8	10	3	3	3	3	1	1	17	18	11	10	11	3	3	3	8	3	6	3	7	2	1	2
		MSTRG.227060	23	25	15	18	23	14	7	9	11	13	6	7	3	4	2	2	35	34	28	20	20	5	7	7	14	6	17	7	16	5	4	5
		MSTRG.84443	14	16	10	11	15	10	5	6	6	8	4	3	2	2	1	1	20	17	17	13	13	3	4	5	7	3	8	4	9	2	2	3
		MSTRG.4066	33	31	19	20	29	21	10	13	17	18	7	8	5	7	2	3	44	38	33	27	24	7	9	8	16	7	17	7	19	6	4	6

Table 3. GO enrichment of differentially expressed genes (DEGs) between T400 and S279 and between endophytic (E+) and non-endophytic (E-) plants with FCs > 2 and p_{adj} < 0.05. Comparisons were made directly between plant types for stressed plants. Values are enrichment false discovery rates (FDRs) with cutoff threshold of 0.01. Categories without significant FDRs are not shown. Up, up-regulated; down, down-regulated; Ctl: control, well-watered; Drt, drought (DrtA, DrtB, and DrC combined).

		400/279						E+/E-			
		Shoot					Root	Shoot			Root
		Up			Down		Down	Up			Up
		Ctl	Drt		Ctl	Drt	Drt	Ctl	Drt		
GO term	Description	E-	E+	E-	E-	E+	E+	T400	S279	T400	S279
GO:0008219	cell death	1.9E-03		7.6E-04							
GO:0012501	programmed cell death	9.0E-03		2.9E-03							
GO:0010200	response to chitin				7.1E-07		2.3E-13				2.5E-06
GO:0010243	response to organonitrogen compound				3.4E-06		6.9E-13				6.0E-06
GO:1901698	response to nitrogen compound				2.7E-04		3.5E-09				1.2E-04
GO:1901700	response to oxygen-containing compound										1.0E-02
GO:0006468	protein phosphorylation						2.0E-03				
GO:0009063	cellular amino acid catabolic process		2.0E-05								
GO:0046395	carboxylic acid catabolic process		2.0E-05								
GO:0016054	organic acid catabolic process		8.6E-05								
GO:1901565	organonitrogen compound catabolic process		7.6E-04								
GO:0044282	small molecule catabolic process		1.7E-03								
GO:0006552	leucine catabolic process		1.7E-03								
GO:0015979	photosynthesis					4.8E-09					
GO:0019684	photosynthesis, light reaction					1.8E-06					
GO:0006779	porphyrin-containing compound biosynthetic process					2.7E-04					
GO:0033014	tetrapyrrole biosynthetic process					3.4E-04					
GO:0033013	tetrapyrrole metabolic process					6.7E-04					
GO:0051186	cofactor metabolic process					6.7E-04					
GO:0006778	porphyrin-containing compound metabolic process					6.7E-04					
GO:0009773	photosynthetic electron transport in photosystem I					3.1E-03					
GO:0009657	plastid organization					3.5E-03					
GO:0015995	chlorophyll biosynthetic process					9.3E-03					
GO:0043043	peptide biosynthetic process							1.7E-09	8.3E-06	6.4E-09	
GO:0043604	amide biosynthetic process							1.7E-09	8.3E-06	6.4E-09	
GO:0006518	peptide metabolic process							1.7E-09	8.3E-06	6.4E-09	
GO:0006412	translation							1.7E-09	8.3E-06	6.4E-09	
GO:1901566	organonitrogen compound biosynthetic process							1.0E-10	8.9E-06	6.4E-09	
GO:0043603	cellular amide metabolic process							2.2E-09	8.9E-06	7.1E-09	
GO:1901564	organonitrogen compound metabolic process							3.3E-10	2.2E-05	7.1E-09	
GO:0042254	ribosome biogenesis							5.0E-04	5.1E-03	3.3E-05	
GO:0044267	cellular protein metabolic process							7.9E-04		1.3E-05	
GO:0022613	ribonucleoprotein complex biogenesis							1.2E-03		9.6E-05	
GO:0019538	protein metabolic process							3.2E-03		9.6E-05	
GO:0006807	nitrogen compound metabolic process							2.6E-03		3.4E-03	
GO:0044271	cellular nitrogen compound biosynthetic process							2.6E-03		9.0E-03	
GO:0034641	cellular nitrogen compound metabolic process							6.7E-03		9.8E-03	
GO:0044249	cellular biosynthetic process							2.2E-03			
GO:0009058	biosynthetic process							2.6E-03			
GO:1901576	organic substance biosynthetic process							4.3E-03			

Table 4. Transcription factor binding site enrichment in the promoters of up-regulated genes in tall fescue, *M. truncatula* (Zhang et al. 2018), and alfalfa (Kang et al. 2011). The analysis was performed using corresponding Arabidopsis orthologs in Pscan. Differentially drought-regulated genes with FCs > 2 and p_{adj} < 0.05 were used in the analysis. The lowest enrichment p value of each category is ranked as number one, and ranks equal or less than 20 are highlighted in each category. Binding motifs/transcription factors that have a p-value rank of 20 or less in any of the categories are listed. A complete list of all TFs with binding enrichment p value < 0.05 is in Table S3. Similar results generated using corresponding rice orthologs of tall fescue genes are presented in Table S4 (up-regulated genes) and S5 (down-regulated genes).

Up-regulated genes				All (T400, S279, E+, E-)				T400E-_ST						T400E-_RT						*M. truncatula*				Alfalfa				
	JASPAR			ST		RT		DrtA/Ctl		DrtB/Ctl		DrtC/Ctl		DrtA/Ctl		DrtB/Ctl		DrtC/Ctl		ST		RT		ST		RT		
TF_Name	ID	Cluster	Class	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	p	rank	Consensus
ABI5	MA0931.1	3	bZIP	2E-50	1	1E-45	1	6E-19	4	2E-15	1	1E-30	1	1E-33	2	5E-38	1	1E-37	2	6E-32	1	4E-44	1	2E-37	1	8E-53	1	MCACGTGKCV
bZIP28	MA1344.1	3	bZIP	9E-48	2	8E-44	2	1E-15	9	2E-12	8	3E-24	5	5E-24	13	1E-29	5	4E-36	4	2E-29	2	3E-38	2	7E-34	3	2E-45	3	dwwgrtsACGTGKCa
bZIP68	MA0968.1	3	bZIP	3E-47	3	4E-42	4	4E-20	1	6E-15	2	2E-27	3	4E-27	6	5E-33	3	1E-38	1	6E-26	5	5E-33	7	2E-34	2	5E-43	5	DWWKSTSACGTGGCA
ABF2	MA0941.1	3	bZIP	1E-46	4	2E-39	6	1E-16	6	1E-12	6	2E-28	2	6E-30	3	8E-29	7	1E-32	7	2E-26	4	5E-36	3	6E-33	4	3E-44	4	AMCACGTGTYRTG
AREB3	MA1338.1	3	bZIP	2E-43	5	7E-42	5	3E-14	13	3E-12	9	3E-21	9	5E-29	4	5E-30	4	7E-35	5	6E-27	3	2E-35	4	3E-31	5	6E-50	2	wwwggwsACGTGKCA
HY5	MA0551.1	3	bZIP	5E-43	6	2E-42	3	3E-17	5	8E-14	5	2E-21	8	5E-39	1	1E-34	2	2E-36	3	2E-22	9	1E-34	6	3E-28	7	6E-42	7	WWTGMCACGTGKCAWW
OJ1058_F05.8	MA1033.1	3	bZIP	6E-43	7	1E-33	9	2E-16	8	2E-12	7	8E-24	6	7E-29	5	3E-29	6	2E-29	8	1E-22	8	1E-28	10	6E-27	10	4E-32	14	MCACGTGK
bZIP16	MA1349.1	3	bZIP	2E-41	8	3E-39	7	3E-14	14	2E-11	11	2E-20	12	4E-24	12	1E-28	8	9E-33	6	2E-23	7	2E-30	8	2E-30	6	2E-42	6	dwwksysACGTGGCA
PIF7	MA1364.1	3	bHLH	5E-40	9	3E-32	12	1E-16	7	9E-12	10	1E-19	13	3E-25	8	6E-25	12	2E-27	11	3E-20	15	2E-27	13	1E-24	12	6E-34	11	swkrrwsCCACGTGg
PIF4	MA0561.1	3	bHLH	2E-39	10	6E-33	10	3E-15	10	3E-10	14	5E-21	10	3E-26	7	3E-25	11	3E-26	13	3E-21	12	5E-29	9	2E-26	11	6E-37	10	CACGTGsc
PIF1	MA0552.1	3	bHLH	6E-39	11	2E-32	11	6E-15	11	2E-09	17	1E-19	14	1E-24	10	6E-27	10	2E-26	12	3E-24	6	3E-35	5	1E-24	13	7E-40	8	rkrrggwCACGTGg
SPT	MA1061.1	3	bHLH	2E-38	12	2E-30	13	1E-19	2	8E-14	4	5E-25	4	5E-25	9	8E-28	9	2E-28	10	1E-21	10	9E-26	15	2E-27	8	7E-32	15	GCACGTGSG
GBF3	MA1351.1	3	bZIP	2E-37	13	1E-34	8	4E-12	21	1E-09	16	3E-19	16	1E-18	30	7E-25	14	6E-29	9	2E-20	14	5E-27	14	5E-27	9	3E-37	9	DWWKSTSACGTGGCA
bHLH31	MA1359.1	3	bHLH	2E-37	14	3E-29	14	2E-13	15	4E-10	15	2E-17	21	1E-22	15	8E-23	17	1E-21	21	3E-19	21	6E-28	11	1E-23	15	2E-33	12	GWGWWWSVCACGTGYCWCMWS
UNE10	MA1074.1	3	bHLH	8E-37	15	1E-26	20	1E-12	18	2E-09	18	2E-19	15	4E-20	21	2E-21	18	2E-22	19	3E-21	13	1E-24	16	3E-20	24	3E-27	19	KCACGTGG
PIF3	MA0560.1	3	bHLH	3E-36	16	2E-28	15	1E-14	12	4E-11	12	2E-18	18	2E-22	16	3E-23	16	2E-23	17	6E-16	31	3E-21	28	6E-21	21	7E-25	26	YCACGTGGCH
EmBP-1	MA0128.1	3	bZIP	6E-35	17	7E-27	19	5E-19	3	1E-14	3	1E-21	7	5E-23	14	7E-25	13	3E-24	15	9E-20	17	1E-23	17	9E-21	22	2E-27	18	CCACGTSW
BEE2	MA0956.1	3	bHLH	1E-33	18	5E-23	26	1E-12	19	1E-08	23	5E-18	19	5E-19	27	6E-20	25	8E-20	23	2E-18	23	4E-22	23	8E-23	17	8E-27	22	HGCACGTGCD
PHYPADRAFT_48267	MA1021.1	3	bHLH	2E-33	19	2E-23	25	2E-12	20	9E-09	22	6E-18	20	3E-19	25	2E-19	27	1E-19	25	2E-18	24	2E-23	18	1E-19	27	2E-25	25	GCACGTGG
PHYPADRAFT_143875	MA0988.1	3	bHLH	4E-33	20	9E-25	23	6E-13	17	5E-09	19	6E-19	17	1E-20	20	9E-21	21	8E-20	24	1E-18	22	3E-21	27	5E-22	18	7E-27	20	HGCACGTGCD
PHYPADRAFT_72483	MA1011.1	3	bHLH	8E-33	21	1E-25	22	2E-11	24	1E-08	24	1E-15	26	1E-20	19	9E-21	20	1E-21	20	4E-20	16	3E-22	22	7E-20	26	7E-25	27	RKCACGTGMY
BZR2	MA0549.1	3	unknown	7E-32	23	1E-27	17	4E-13	16	9E-09	21	2E-20	11	1E-24	11	3E-24	15	2E-24	14	2E-21	11	2E-27	12	1E-23	16	3E-32	13	BSMCACGTGYG
bZIP3	MA1340.1	3	bZIP	4E-31	24	1E-27	18	1E-09	29	1E-06	33	4E-17	23	1E-13	43	5E-20	24	2E-23	16	9E-17	28	2E-21	26	5E-22	19	1E-30	16	dwwGmTGACGTGGCa
BIM1	MA0964.1	3	bHLH	1E-30	25	6E-22	28	3E-11	25	8E-09	20	2E-14	29	3E-19	26	9E-20	26	1E-19	26	2E-19	19	4E-21	29	2E-19	28	4E-24	29	bcaCGTGmyv
BIM3	MA0966.1	3	bHLH	3E-30	26	8E-22	29	5E-11	26	1E-07	29	4E-15	27	1E-16	34	7E-18	30	3E-18	30	1E-19	18	5E-23	19	8E-24	14	2E-26	23	RGCACGTGCY
BIM2	MA0965.1	3	bHLH	1E-28	28	2E-21	31	7E-10	28	8E-07	30	3E-14	31	2E-17	33	1E-17	31	2E-18	28	2E-19	20	2E-22	21	2E-21	20	9E-26	24	SKCACGTGMS
BEH3/AT4G18890	MA1333.1	3	Other	1E-26	33	1E-23	24	7E-09	37	1E-06	34	3E-17	22	8E-22	18	3E-21	19	2E-18	29	5E-16	30	1E-22	20	1E-20	23	2E-28	17	YCACACGTGYR
ABF1	MA0570.1	3	unknown	6E-25	36	5E-28	16	1E-07	43	9E-11	13	2E-12	39	4E-22	17	2E-20	22	1E-22	18	1E-11	48	2E-21	25	1E-15	43	3E-24	28	aCACGTGkCAww

Figure legends

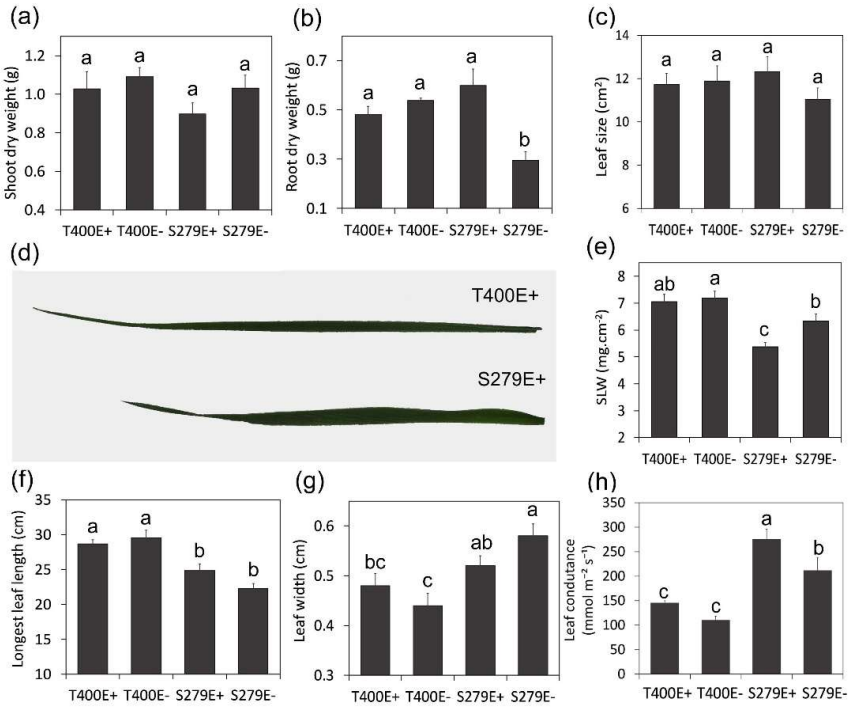
Figure 1. Biomass and leaf traits of well-watered T400 and S279 plants. (a) Shoot dry weight, (b) root dry weight, (c) leaf size, (d) leaves of T400E+ and S279E+, (e) specific leaf weight (SLW), (f) leaf length, (g) leaf width, and (h) leaf conductance. Data were collected on four-week-old plants after one cut back. Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.

Figure 2. Well-watered (Ctl), mild-stressed (DrtA, soil VWC~10%), moderately-stressed (DrtB, soil VWC~5%), and severely-stressed (DrtC, soil VWC~1%) tall fescure plants (S279E+) at harvest.

Figure 3. Leaf chlorophyll content (a) and leaf sap osmotic potential (b) of well-watered and severely drought-stressed tall fescue plants (soil VWC ~1%). Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.

Figure 4 Tall fescue plants (a), shoot (b) and root (c) dry weight after severe drought stress (soil VWC < 1%) and recovering. Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs. Image of plants in a pot is shown in a.

Figure 5. Numbers of differentially expressed genes (DEGs) (FC > 2, $p_{adj} < 0.05$) that were regulated by drought stress (a), between T400 and S279 (b), and between E+ and E- (c). Severe drought stress (DrtC) regulated genes in shoots (d) and roots (e) are illustrated by Divenn. Red denotes up-regulated genes; blue denotes down-regulated genes, and yellow denotes up- or down-regulated genes.



857

858 **Figure 1.** Biomass and leaf traits of well-watered T400 and S279 plants. (a) Shoot dry
859 weight, (b) root dry weight, (c) leaf size, (d) leaves of T400E+ and S279E+, (e) specific
860 leaf weight (SLW), (f) leaf length, (g) leaf width, and (h) leaf conductance. Data were
861 collected on four-week-old plants after one cut back. Different letters indicate significant
862 difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.

863

864 **Figure 2.** Well-watered (Ctl), mild-stressed (DrtA, soil VWC~10%), moderately-stressed
865 (DrtB, soil VWC~5%), and severely-stressed (DrtC, soil VWC~1%) tall fescure plants
866 (S279E+) at harvest.

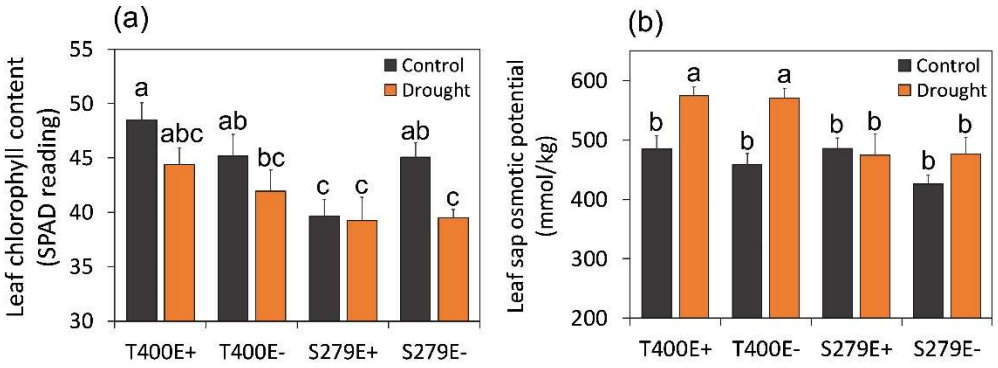


Figure 3. Leaf chlorophyll content (a) and leaf sap osmotic potential (b) of well-watered and severely drought-stressed tall fescue plants (soil VWC ~1%). Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.

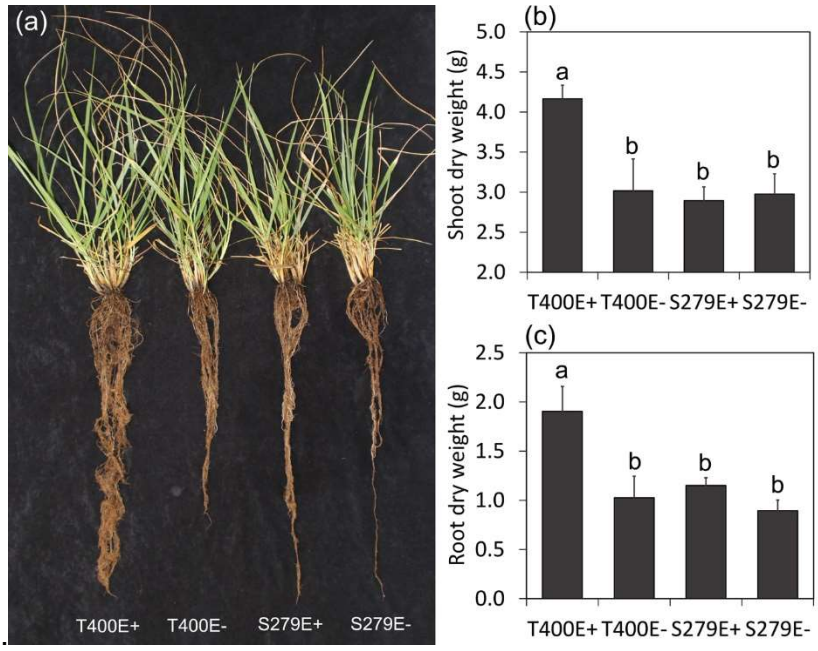


Figure 4 Tall fescue plants (a), shoot (b) and root (c) dry weight after severe drought stress (soil VWC < 1%) and recovering. Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs. Image of plants in a pot is shown in a.

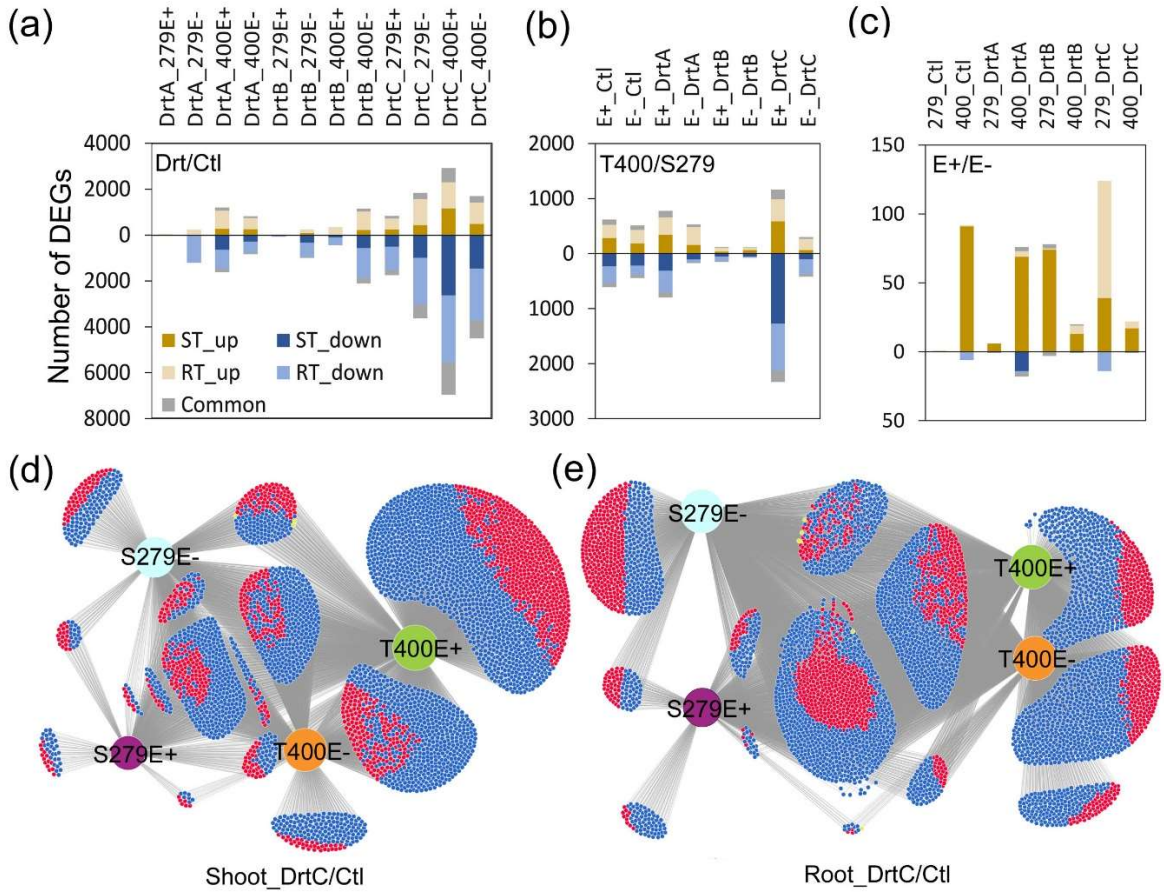


Figure 5. Numbers of differentially expressed genes (DEGs) (FC > 2, p_{adj} < 0.05) that were regulated by drought stress (a), between T400 and S279 (b), and between E+ and E- (c). Severe drought stress (DrtC) regulated genes in shoots (d) and roots (e) are illustrated by Divenn. Red denotes up-regulated genes; blue denotes down-regulated genes, and yellow denotes up- or down-regulated genes.

Supporting Information:

Figure S1. Well-watered T400E+, T400E-, S279E+, and S279E- plants, from the left to the right.

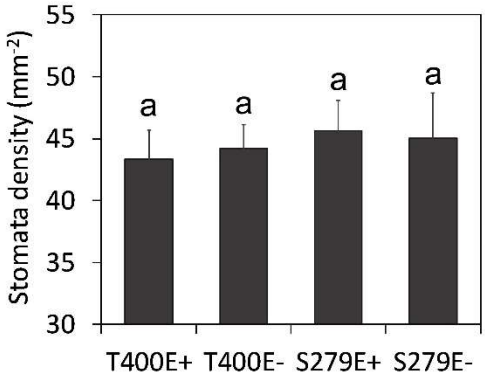
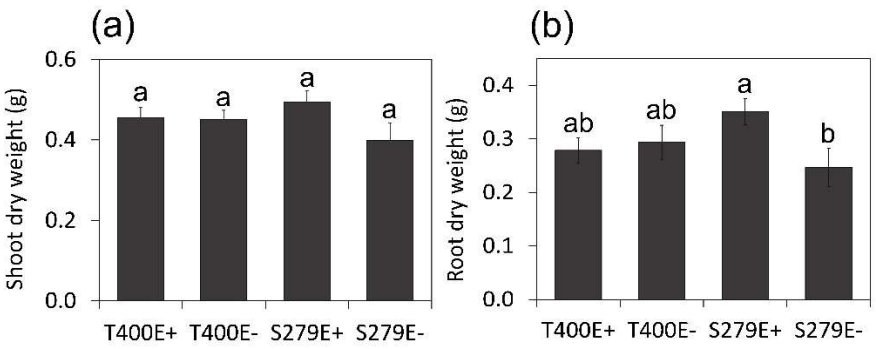


Figure S2. Stomata density on the leaf abaxial side of well-watered tall fescue plants. Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.



FigureS3. Shoot (a) and root (b) biomass of severely drought-stressed plants (DrtC). Different letters indicate significant difference at $p < 0.05$ (Duncan's Test), n=5, error bars are SEs.

Table S1. Transcript (FPKM) changes, annotations, and sequences of major proline biosynthesis and degradation genes under drought stress.

Table S2. GO enrichment of differentially expressed genes (DEGs) between drought-stressed and well-watered plants with FCs > 2 and $p_{adj} < 0.05$.

Table S3. Transcription factor binding site enrichment in the promoters of up-regulated genes under gradual drought stress in tall fescue, *M. truncatula*, and alfalfa. The analysis was performed using corresponding Arabidopsis orthologs.

Table S4. Transcription factor binding site enrichment in the promoters of down-regulated genes under gradual drought stress in tall fescue, *M. truncatula*, and alfalfa. The analysis was performed using corresponding Arabidopsis orthologs.

Table S5. Transcription factor binding site enrichment in the promoters of up-regulated genes in tall fescue. The analysis was performed using corresponding rice orthologs.

Table S6. Transcription factor binding site enrichment in the promoters of down-regulated genes in tall fescue. The analysis was performed using corresponding rice orthologs.