Transcriptome Analysis and Differential Expression in Tall Fescue Harboring Different Endophyte Strains in Response to Water Deficit

Randy D. Dinkins*, Padmaja Nagabhyru, Carolyn A. Young, Charles P. West, and Christopher L. Schardl

ABSTRACT

Two tall fescue [Lolium arundinaceum (Schreb.) Darbysh. = Schedonorus arundinaceus (Schreb.) Dumort. = Festuca arundinacea var. arundinacea Schreb.] plant genotypes with an Epichloë coenophiala (Morgan-Jones & W. Gams) C.W. Bacon & Schardl common toxic endophyte (CTE), one with a nontoxic strain (NTE19) and one with another Epichloë species (FaTG-4) were evaluated and compared with their respective endophyte-free clones for responses to water-deficit stress in the greenhouse. One of the plant genotypes (P27) showed a positive effect of its CTE strain on tiller production after stress and resumed watering. In transcriptome analysis of the pseudostems (leaf sheath whorls), differentially expressed genes (DEGs) were defined as having at least twofold expression difference and false discovery rate (FDR) < 0.05 in comparisons of water treatment (stressed or watered), endophyte presence or absence, or both. Stress affected 38% of the plant transcripts including those for the expected stress-response pathways. The DEGs affected by endophyte in stressed plants were unique to individual plant genotypes. In unstressed plants, endophyte presence tended to reduce expression of genes putatively for defense against fungi, but in unstressed P27 endophyte presence there was enhanced expression of dehydrin and heat shock protein genes. Our results indicated subtle and variable effects of endophytes on tall fescue gene expression; where the endophyte confers protection, its effects on plant gene expression may help prime the plant for stress resistance.

Core Ideas

- RNA-seq was performed on four tall fescue clone pairs.
- Three Epichloë coenophiala strains were evaluated.
- Gene expression was compared for stressed and unstressed plants.
- Differentially expressed unigenes were identified.
- Few positive endophyte effects on stress tolerance were observed.

Tall fescue (Banfi et al., 2017) is one of the most abundant cultivated pasture grasses in the United States where it is especially adaptable to the entire C3–C4 plant transition zone (Buckner and Bush, 1979; Hoveland, 2009) occupying over 15 million ha in the United States. Although tall fescue possesses many desirable traits, namely stand longevity and good nutritive value, it has, nevertheless, been shown to have significant negative effects on animal production, causing symptoms such as poor livestock weight gain and reproductive performance (Waller, 2009), collectively referred to as fescue

Abbreviations: CTE, common toxic endophyte; DEG, differentially expressed gene; E+, endophyte-harboring; E−, endophyte-free; FDR, false discovery rate; GO, gene ontology; NTE, nontoxic endophyte; RNA-seq, RNA sequencing; ROS, reactive oxygen species.


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toxicosis. Tall fescue and many other grasses belonging to the subfamily Poöideae form mutualistic beneficial symbioses with endophytic fungi belonging to genus *Epichloë* (Clavicipitaceae). Beginning in the late 1970s, researchers identified symbiotic *E. coenophiala* [= *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin = *Acremonium coenophialum* (Morgan-Jones and Gams)] as the cause of fescue toxicosis.

Many of the naturally occurring *E. coenophiala* strains produce alkaloids of various classes—namely lolines, peramine, indole-diterpenes, and ergot alkaloids—of which some ergot alkaloids and some indole-diterpenes are toxic to mammals (Bacon et al., 1977; Bacon, 1995; Finch et al., 2018; McLeay et al., 1999; Takach and Young, 2014). Although it is feasible to eliminate the endophyte, because tall fescue does not require it to grow, it has been demonstrated that tall fescue lacking *E. coenophiala* does not persist as well as plants that harbor the endophyte (Bouton et al., 1993). With approximately $1 billion annual economic losses to US livestock as a result of fescue toxicosis (Allen and Segarra, 2001), multiple efforts have been undertaken to identify nontoxic endophyte (NTE) strains to replace the ergot alkaloid-producing CTE strains that were usually present in early cultivar releases. Thus, several new cultivars have been released harboring NTE strains of *E. coenophiala* that appear to have no negative effects on livestock performance (Beck et al., 2008; Bouton et al., 2002; Hopkins et al., 2010).

There is an apparent host–fungal, species-specific compatibility in grass–*Epichloë* symbioses, many of which persist indefinitely through maternal line dissemination in host seeds (Scharld & M.R. Siegel, 2004). In tall fescue, *E. coenophiala* is asexual and disseminated only via seeds (Bacon and Siegel, 1988; Welty et al., 1986). Most of the asexual *Epichloë* species are interspecific hybrids from different ancestral *Epichloë* species (Tsai et al., 1994; Takach and Young, 2014), although the CTE strain is a three-species hybrid derived from *E. festucae* (Leuchtm., Scharld & M.R. Siegel), *Epichloë poae* Tadych, K.V. Ambrose, F.C. Belanger et J.F. White, and an unidentified species belonging to the *Lolium*-associated endophyte clade of *Epichloë* (Tsai et al., 1994).

Much remains to be learned about the mechanisms by which the benefits of the endophyte are realized by the tall fescue plant and how the endophyte and plant coordinate growth and physiological interactions. In the related perennial ryegrass (*L. perenne* L.)–*E. festucae* symbiosis, for example, deletion of endophyte genes for components of the fungal NADPH oxidase complex has been shown to result in an altered phenotype in symbio, such as increased proliferation and branching, which is usually associated with detrimental effects on the plant (Eaton et al., 2010; Takemoto et al., 2011; Tanaka et al., 2006). The importance of fungal regulatory genes and the beneficial effects of the endophytes suggest that grass–*Epichloë* signal exchanges may be discernible in transcriptome studies. Indeed, several transcriptome studies of perennial ryegrass with *E. festucae* strain Fl1 (Dupont et al., 2015; Johnson et al., 2003), perennial ryegrass with *E. festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C.W. Bacon & Schardl (Schmid et al., 2017), and tall fescue with *E. coenophiala* (Dinkins et al., 2010, 2017) and others (Ambrose and Belanger 2012) have indicated endophyte effects on host gene expression relevant to stress tolerance and responses to fungi.

Symbiosis with *E. coenophiala* appears to be crucial for tall fescue to persist in regions such as the “fescue belt” of the United States that are subject to episodic drought stress (Belesky and West 2009; Bouton et al., 1993; West et al., 1993). Research has demonstrated that this is partially, but perhaps not entirely, a result of increased tillering, root growth, aboveground biomass accumulation, ability to acquire mineral phosphate from soil, osmotic adjustment, nitrogen utilization, and antinematode activity (Elmi et al., 2000; Assuero et al., 2002; Arachevaleta et al., 1989; Panaccione et al., 2006; West et al., 1993). As new cultivars are deployed with different *Epichloë* strains, it is critical to address questions regarding endophyte roles in persistence under stress conditions. The physiological, molecular, and metabolomic effects of water-deficit stress are well documented in many plant species, including tall fescue (Elmi and West, 1995; Nagabhyru et al., 2013; Talukder et al., 2015), but the endophyte effects on tall fescue responses to stress have not yet been evaluated at the level of the transcriptome. In this study we prepared four pairs of tall fescue clones in which one clone possesses and the other lacks CTE or NTE strains or another species—FalTG-4 (Takach and Young 2014)—and we used these clone pairs for transcriptome analysis (RNA sequencing [RNA-seq]) to monitor changes in plant gene expression on the imposition of water deficit in the greenhouse, evaluating plant responses, and how the endophytes influence the host responses.

### MATERIALS AND METHODS

#### Biological Materials

Tall fescue is obligately outcrossing. Therefore to control for host genotype differences, pairs of endophyte-harbor (E+) and endophyte-free (E−) tall fescue clones were generated from four original plants, of which two harbored the CTE strain and two that harbored different non-ergot alkaloid-producing NTE strains. Plants P27CTE+ and P46CTE+ were derived from locally obtained KY31 seeds harboring *E. coenophiala* strain CTE-1 (Young et al., 2014). Two plants with NTE strains were obtained by inoculation of tall fescue cultivar KY31 E− seedlings with different fungal strains from *L. arundinaceum* plants collected in Morocco (West et al., 1998), one of which was a strain (designated NTE-19) of *E. coenophiala*, and the other was a strain (designated FaTG-4–16) of the *E. typhina* × *Epichloë* sp. hybrid species FaTG-4 (Takach and Young, 2014). Plant P12NTE+ with strain NTE-19 lacked ergot alkaloids, and plant P15FaTG4+ with strain FaTG4-16 lacked both ergot alkaloids and loline alkaloids. The E− clones were generated from each of the E+ plants by fungicide treatment as previously described for tall fescue...
(Nagabhyru et al., 2013). Thus, the four E+ and E− clone pairs were designated P12 for P12NT+E− and P12E−, P15 for P15FaTG4+E− and P15E−, P27 for P27CT+E− and P27E−, and P46 for P46CT+E− and P46E−. Each clone pair was propagated for several tillering and division cycles in the greenhouse over >1 yr prior to conducting experiments with continual monitoring for endophyte presence or absence using tissue print immunoblot (Hill and Agee, 1994) and polymerase chain reaction (Dombrowski et al., 2006). In routine tests (Dinkins et al., 2017), the respective Epichloë strains were the only endophytes detected although we recognize that fungicide treatments may cause other, unknown effects on the microbiome.

Water Deficit and Watered Control Treatments
Ramets consisting of three tillers of similar size were planted into 8.5- by 8.5-cm square pots in sand and grown in the greenhouse for 4 wk, during which they were watered twice daily using an overhead sprinkler to mist 4 min each cycle and supplemented with Peters Peat Lite Special 20–10–20 (JR Peters Inc., Allentown PA, USA) at 100 mg L−1 N to allow for plant growth and retillering. Water deficit was imposed by terminating watering on day zero and stress imposed for 2 d. All the tillers from three pots of E+ and E− clones for each clone pair after 2 d of water withholding along with three replicates of each watered control were collected for RNA isolation. Random leaves were selected from each pot prior to harvest and water potential was tested using a Scholander-type pressure chamber (Model 600; PMS Instrument Company). Plant samples were harvested between 9:30 and 11:30 h. Each pot was cleaned of sand, and the whorls of vegetative leaf sheaths (pseudostems) were separated and immediately frozen in liquid nitrogen for tissue processing and RNA extraction. Pseudostem segments were selected for RNA isolation and quantitation of differential expression in the present experiment because this is where the highest number of fungal RNA-seq reads were previously observed in vegetative tall fescue (Dinkins et al., 2017). A second set of pots treated in the same manner was used to evaluate the recovery from the stress treatment. Tillers were counted from three to five pots of each E+ and E− clone from each clone pair at the same time as those used for RNA isolation (controls and stressed), whereupon the pots were returned to the normal watering regime and number of tillers counted again after 30 d following rewatering. Because of limited space, the water withholding experiment for each clone pair was done at different times during the year so that analysis of tiller recovery was done within each clone pair and not between plant or endophyte genotypes.

Analysis of RNA Sequencing Data
Reads were filtered to remove low quality reads using the CLCbio Trim tool (CLC Genomics Workbench, Qiagen Aarhus A/S), trim quality score set to 0.05, the six 5′ terminal nucleotides removed, and ambiguous set to minimum to maximum 2. Reads <30 bases were discarded. To remove the endophyte and tall fescue chloroplast reads, the filtered reads were mapped to the E. coenophiala e4163 and e19 strains genome assemblies (www.endophyte.uky.edu; Schardl et al., 2013b) and the tall fescue chloroplast sequence (GenBank: KM974751.1 [Saarela et al., 2015]) with minimum length fraction set to 0.9 and minimum similarity fraction set to 0.95. The unmapped reads were then mapped to tall fescue TF153K transcriptome assembly containing 153,321 unigenes (Dinkins et al., 2017) with minimum length fraction set to 0.9 and minimum similarity fraction set to 0.8. Reads were normalized by the Quantile method in CLC Genomics Workbench, and only unigenes that had at least one clone–endophyte–treatment combination with a minimum of 20 average mapped reads were used for differential expression analysis.

The read number for each unigene was transformed by adding one (1.0) to eliminate zeros, and then normalized using the trimmed mean of M-values method (Robinson and Oshlack, 2010) using JMP Genomics 8.0 (SAS Institute, 2013). The normalized data were log transformed (log2) and principal component and correlation analysis was done using the JMP Basic RNA-Seq Workflow. Analysis of variance (ANOVA) was based on a mixed model for a split-plot design using a log2-normalized distribution for the corrected read counts. The experimental design was set as a split plot with four main treatments consisting of the individual plant–strain combinations and two treatments (stress vs. control), and in the plant comparisons, the endophyte strain (E+ or E−) was included with three replicates each that consisted of individual pots. Based on the error estimated by ANOVA for a given transcript, contrast statements were used to make comparisons between two means. A FDR multiple testing method at P < 0.05 and a twofold difference were chosen as criteria for significance for each comparison.
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Gene Ontology Pathway Annotations

Biological processes and molecular function gene ontology (GO) terms were annotated to the TF153K tall fescue assembly based on the Arabidopsis gene annotations as described in Dinkins et al. (2017). Gene lists associated with DEGs from the various treatments were compared with the TF153K unigene lists to identify overrepresented pathways using the custom input tool at the web-based agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) using the Fisher statistical test method, Bonferroni multitest adjustment method, and a cut-off of $10^{-5}$ (Tian et al., 2017).

RESULTS

Plant Tillering Before and After Stress

Water potential measurements of the control plants were similar for the E+ and E− plants under the control conditions in the 0.5- to 1.0-MPa range. Following the 2-d water-withholding (stress) treatment the 4 MPa limit of the pressure chamber was exceeded, indicating substantial water deficit.

Both before and after stress treatments, unstressed (watered) clones of P15 and P27 tillered more prolifically and had finer tillers similar to turf-type tall fescue, whereas P12 and P46 produced fewer but thicker tillers similar to forage-type tall fescues (Fig. 1; Supplemental Fig. S1). Although experiments were conducted at different dates, the phenotypic difference in tiller numbers and morphology was typical of these plants. Plants exhibited a slight decline in the number of tillers after 2-d stress treatment vs. their watered controls and only P27 was shown to be significant ($P > 0.05$), and in each experiment the presence or absence of the endophyte was not a significant factor ($P > 0.05$) (Fig. 1; Supplemental File S1).

In experiments with different clone pairs, we observed different effects of stress and endophyte on tiller numbers after the 30-d recovery (Fig. 1). For two, P12 and P15, the stressed clones had slightly lower but statistically insignificant reductions in average tiller numbers and insignificant effects of their respective NTE and FaTG-4 endophytes. In contrast, clone pairs P27 and P46 showed dramatic effects of stress, but stark differences from each other in the effects of their CTE endophytes on recovery. After the recovery period, their unstressed E− clones averaged more than twice the number of tillers as their stressed E− clones. This difference was largely absent for the P27CTE+ clones, indicating a significant benefit of the endophyte. However, P46 showed no such benefit of its endophyte. Thus, the P27 clone pair presented the best opportunity to reveal the basis for endophyte enhancement of resistance to water deficit stress.

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Plant Gene Expression

Following removal of the reads that mapped to the E. coenophiala and tall fescue chloroplast genome (Supplemental Table S1), ~80% of the remaining reads from each library mapped onto the TF153K tall fescue assembly (Table 1). A correlation of >96% was observed between the three replicates within each treatment (Supplemental Table S2). One replicate of control P12E− returned only 5.4 million reads, with a data distribution significantly different from the others, so that replicate was omitted from further analysis. Only TF153K unigenes that had a minimum of 20 normalized average mapped reads in at least one clone–endophyte–treatment combination were retained, giving a final number of 77,055 unigenes for analysis in JMP Genomics. When the four experiments were combined, the majority of the variance was due to the differences between the four plant genotypes ($G$) (41.4%), where $G$ is defined here to include plant genotype, endophyte genotype, and any environmental effect because different clone pairs were tested on different dates in the greenhouse. Water stress treatment ($W$) contributed roughly 39% of the variance, $G \times W$ contributed 7.0%, and endophyte status ($E$) plus $E \times G$ or $W$ totaled 3.0% of the variance (Fig. 2A), leaving a 9.4% residual effect. Overall, significant differences in plant unigene
expression between the four genotypes were observed where expression was most similar between P27 (±CTE) and P15 (±FaTG-4), and P12 (±NTE) was the most different from others (Fig. 2B).

Endophyte Effects on Unstressed Plant Gene Expression

In unstressed plants, no endophyte-affected DEGs were common to all four genotypes, and only two were significant DEGs in more than one clone pair (Fig. 3). Endophyte effects on P27 gene expression were of particular interest because P27 was the only plant to show a protective effect of endophyte based on tillering during recovery. Notably, the 54 E+ > E− DEGs in unstressed P27 included several that were functionally annotated for stress tolerance including two dehydrin unigenes and 22, 23.2, and 70 kDa heat shock protein unigenes (Supplemental Table S3). A separate analysis of the P27 clone pair gave similar results. Clone pair P27 with FDR = 0.1 gave nearly the same set of E+ > E− as well as E− > E+ DEGs including all of those described below. In this analysis, E− clones grouped separately from E+ clones in both stressed and unstressed conditions (Fig. 4).

Of all E+ > E− DEGs that were significant in one of the clone pairs, 53 trended similarly but were not significant or were not twofold different in the other clones (Fig. 5; Supplemental Table S3). No GO pathway was associated with the DEGs. BLASTX matches for all of these 53 unigenes were found in the Grass Phytozome database and databases of Brachypodium distachyon (L.) Beauv., sorghum [Sorghum bicolor (L.) Moench], rice (Oryza sativa L.), and perennial ryegrass, although many of the genes fell into the uncharacterized category. Unigenes that were expressed at higher levels in the E+ plants encoded two putative sugar transporter proteins, three protein components of the elongation factor complex, a dehydrin protein, and a phosphate transporter (Supplemental Table S6).

Fig. 2. Principle component analysis, heat map, and dendrogram correlation of tall fescue endophyte-harboring and endophyte-free clones subjected to stress. (A) Principle component analysis. (B) Heat map and dendrogram. G, genotypes, including plant genotypes (P27, P46, P15, and P12), respective endophyte genotypes (CTE, CTE, NTE, and FaTG-4) and environmental effects due to different dates for each experiment; E, endophyte presence (E+) or absence (E−); W, water treatment, either unstressed control or stressed by withholding water for 2 d.
Fig. 3. (A) Volcano plots and (B,C) Venn diagrams of differential expression profiles comparing differentially expressed plant genes (DEGs) due to the presence or absence of the endophyte in the four tall fescue clones under unstressed (watered) conditions. (A) The vertical dashed lines in the volcano plots represent twofold changes (axis expressed as log2 values) and the horizontal dashed lines show the log10(P-value) used to call significant differences in JMP Genomics using a false discovery rate (FDR) at P < 0.05. The number of DEGs for each clone is presented for (B) E− > E+ plants and (C) E+ > E− plants based on the significance shown in the volcano plots.

Fig. 4. Results of principle component and clustering analysis of gene expression of P27CTE+ and P27E− clones of tall fescue in unstressed and stressed conditions. (A) Variance components. (B) Principle component 3-D analysis plot. (C) Heat map with correlation dendrogram. W, water treatment, either unstressed control or stress by withholding water for 2 d; E, endophyte presence (E+) or absence (E−); R, residual.
Fig. 5. Differential expression profiles of genes that appeared to be similarly regulated in the endophyte-free vs. endophyte-harboring plants under control and stress conditions in the four tall fescue clones. The heat maps reflecting relative expression levels were (A) expression profiles of genes where E− > E+ under control conditions, (B) E+ > E− in control conditions, (C) E− > E+ in the water withholding condition, and (D) E+ > E− under water withholding condition. Differential expression is presented by the color legend and represents log2 scale. Unigene designations are presented for frames (B), (C), and (D), but not (A) because of the high number. BlastX matches for the unigenes are provided in the supplemental tables.
DEGs of unstressed P27 had biological process GO terms GO:0009407 (toxin catabolic process), GO:0060416 (response to growth stimulus), GO:0006749 (glutathione metabolic process) and GO:0009704 (de-etiolation). Most of the unigenes associated with the GO terms GO:0006612 (protein targeting to membrane) and GO:0050832 (defense response to fungus) were also included in the GO term GO:0010200 (response to chitin). The majority of the unigenes associated with GO:0010200 (response to chitin) and GO:0050832 (defense response to fungus) appear to be transcription factor genes. Expression of these unigenes trended similarly but not statistically significant ($P > 0.05$) in the P15 clone pair (Fig. 6B).

The biological process term GO:0009686 (gibberellin biosynthetic process) also contained DEGs that were significantly different in both the P27E− and P27CTE+ and P15E− and P15FaTG4+ clone pairs where the unigene expression was twofold or more greater in the E− plants than the E+ plants (Fig. 5). Most of these unigenes were also found to be twofold higher in the E− plants for the other clones tested but not statistically significant.

**Plant Differentially Expressed Genes Resulting from Water Treatment**

Approximately one third of the TF153K transcripts were differentially expressed in at least one of the four genotypes as a result of water treatment (Fig. 7, 8). In the instances where differences were not declared statistically significant or were slightly less than twofold different in individual clones, the trends were similar in the other clones (Fig. 9). Clone pair P12E− and P12NTE+ (which, based on tiller recovery, appeared to be less affected than others by the stress) had the lowest total number of DEGs so its inclusion lowered the total number of DEGs common to all clone pairs.

Using the GO annotations associated with our TF153K assembly unigenes (Dinkins et al., 2017), GO terms associated with the unstressed control > stress and stress > control were similar for both E− and E+ plants where all DEGs were significant across all clones (Supplemental Table S5). Biological process GO terms significantly enriched in the stress > control DEGs included many previously identified GO terms associated with stress in other species (Dugas et al., 2011; Liu and Jiang, 2010; Yue et al., 2008; Zhao et al., 2016), including water stress and deprivation response (GO:0009414; GO:0009415), oxidative stress (GO:0009738), metabolic process (GO:006560), disaccharide biosynthetic process (GO:0046351), hyperosmotic salinity response (GO:0009651; GO:0042538), seed dormancy (GO:0010162), germination (GO:0010029), response to heat stress (GO:0009408), and acclimation (GO:0015286). All putative unigenes identified for proline biosynthetic enzymes (GO:0006561) $\Delta 1$-pyrroline-5-carboxylate synthases 1 and 2 (P5CS1 and P5CS2) were differentially overrepresented in all four clones.
Fig. 7. (A) Volcano plots and (B,C) Venn diagrams comparing differentially expressed plant genes (DEGs) for control and stress in endophyte-free tall fescue clones. (A) The vertical dashed lines in the volcano plots for each clone represent twofold changes (axis expressed as log_2 values) and the horizontal dashed lines show the log_{10}(P-value) used to call significant differences in JMP Genomics using a false discovery rate (FDR) at P < 0.05. The number of DEGs for each clone is presented in (B) for expression in unstressed clones greater than in stressed clones and (C) expression in stressed clones greater than in unstressed clones based on the significance shown in the volcano plots.

Fig. 8. (A) Volcano plots and (B,C) Venn diagrams comparing differentially expressed plant genes (DEGs) for control and stress in endophyte-harboring tall fescue clones. (A) The vertical dashed lines in the volcano plots for each clone represent twofold changes (axis expressed as log_2 values) and the horizontal dashed lines show the log_{10}(P-value) used to call significant differences in JMP Genomics using a false discovery rate (FDR) at P < 0.05. The number of DEGs for each clone is presented in (B) for expression in unstressed clones greater than in stressed clones and (C) expression in stressed clones greater than in unstressed clones based on the significance shown in the volcano plots.
A large number of biological process GO terms significantly enriched in the unstressed control > stress DEGs included those associated with unstressed growth and development and metabolism. Significant biological process GO terms included the following: cell division (GO:0051301), ribosome biogenesis (GO:0042254), translation (GO:0006412), chloroplast organization (GO:0044085), isopentenyl diphosphate biosynthetic process (GO:0009240), glyceraldehyde-3-phosphate metabolic process (GO:0019682), cell cycle process (GO:0022402), DNA-dependent DNA replication (GO:0006261), and others associated with growth and development (Supplemental Table S6).

**Endophyte Effects on Stressed Plant Gene Expression**

Under the stressed conditions, all but nine DEGs were specific to individual genotypes under either the E− > E+ or E+ > E− endophyte status (Fig. 10A,B). No pathway appeared to be differentially affected, as no GO terms were significant in any of the DEG comparison within any of the clone pairs. Unigenes that were found to be differentially expressed in the E− > E+ under control conditions were observed to be much more variable in regard to differential expression under the stress conditions (Supplemental Table S7). Of the 107 DEGs observed in the combined clone pair comparisons, only 19 were found to trend across all clone pairs where the endophyte-free plants had higher expression than the endophyte harboring plants (Fig. 5C). Similarly, only 27 of 161 of the putative unigenes were DEGs where E+ plants had higher expression than the E− plants and only one unigene (BLASTX to translation initiation factor 5A) was also a DEG under control conditions (Fig. 5D; Supplemental Table S8).

**DISCUSSION**

The interactions of tall fescue plants and other grasses with endophytic *Epichloë* strains have been described as mutualistic symbioses, and in addition to alkaloids that deter herbivores (Clay et al., 2005; Schardl et al., 2013a) the endophytes can confer several benefits including enhanced drought tolerance (reviewed in Schardl et al., 2004). Reports have indicated endophyte-enhanced persistence of tall fescue in the field under stress conditions (Bouton et al., 1993; Clay and Holah, 1999; West et al., 1993), although others have shown little benefit to the plant (Brosi et al., 2011; Saikkonen et al., 1998, 2016), so such benefits depend on environmental conditions, plant genotype, and fungal genotype (Saikkonen et al., 2006). Our study tested recovery from water deficit stress along with transcriptional responses of tall fescue plants to different symbiotic *Epichloë* strains under unstressed and stressed conditions. Because tall fescue is obligately outcrossing, our tests controlled for host genotypes by using four different pairs of clones with and without the endophyte. These clone pairs differed considerably in their responses to stress and, interestingly, only one (P27) showed a substantial and significant endophyte benefit as measured by tillering during a 30-d recovery. In stark contrast, another clone pair (P46) with a similar CTE also showed a strong stress effect but no effect of its endophyte on recovery. Thus, especially in the case of the P27 clone pair, the effects of endophyte on host transcription may reveal important genes for endophyte-enhanced drought tolerance. For example, in unstressed P27 the effect of the endophyte on expression of a dehydrin gene (which trended similarly in other clone pairs)
suggests possible priming of the plant for enhanced resistance to the onset of water deficit.

Previous studies have suggested that the presence of the endophyte alters plant transcription under unstressed conditions (Ambrose and Belanger, 2012; Dinkins et al., 2010; Dupont et al., 2015; Johnson et al., 2003; Schmid et al., 2017). We have also shown that for one of the clone pairs presented here (P46E− and P46CTE+) under unstressed conditions, the presence of the endophyte has minimal effect on plant transcription when different tissues were tested (Dinkins et al., 2017). In the present study, the highest number of DEGs between the E− and E+ plants was observed in unstressed clones, particularly in the P27E− and P27CTE+ and P15E− and P15 FTaTG4+ comparisons. Furthermore, most of those were E− > E+ DEGs including unigenes encoding putative transcription factors involved in fungal and defense response, glutathione-S-transferases, toxin catabolic pathway, de- etiolation, proline transport, and the early steps of gibberellin biosynthesis. We have previously documented the higher expression in several WRKY transcription factors in leaf tissues of E− plants vs. E+ plants (Dinkins et al., 2017), though, as we pointed out, those WRKY factors may enhance or depress resistance to different pathogenic fungi. Also noteworthy was the differential expression of a number of unigenes encoding glutathione-S-transferase proteins, which can be involved in regulation of drought and salt stress responses, hormone regulation, and flavonoid metabolism (Moons, 2005). It is presently unknown why expression of these glutathione-S-transferase proteins would be higher in E− plants, but it is interesting to speculate that the endophyte may aid in the control of plant derived reactive oxygen species (ROS) (superoxides and peroxides) thereby repressing the expression of these genes under nonstress conditions.

Gene ontology pathway annotation of E+ > E− DEGs across all clone pairs did not indicate any significant stress-response pathway, although even in unstressed plants some of those DEGs might aid in stress responses. In particular, we can speculate that genes encoding dehydrin and heat shock proteins (protein chaperones), which were identified as DEGs in unstressed P27 (Supplemental Table S3), may help prime the plant to better tolerate water deficit stress (Hanin et al., 2014; Wang et al., 2004).

In previous experiments with tall fescue clone pairs, we observed metabolite changes associated with endophyte-enhanced tolerance of water stress similar to that imposed in this study (Nagabhryru et al., 2013). These changes included increases in free hexoses, sugar alcohols, and amino acids. We also observed that those E+ plants with endophytes that produce loline (P27CTE+, P46CTE+, and P12NTE+) increased loline alkaloid levels on a dry-mass basis on withholding water. A similar experiment with the P27 clone pair gave similar results with respect to plant and endophyte metabolites, and the same enhancement of stress tolerance as documented herein (data not shown). Conceivably, some or all of these metabolic changes contribute to endophyte-enhanced
stress tolerance by increasing osmotic protectants and perhaps also protecting against ROS (Gill and Tuteja, 2010). However, among the plant DEGs we describe, only proline transporters are obviously connected with these metabolites. Furthermore, the endophyte’s loline genes showed no major changes, and many were slightly reduced in expression in the stressed plants (data not shown). This was not surprising, however, considering our previous evidence that levels of the amino acid precursors, and not gene expression, regulate loline alkaloid levels in the related symbiotic system, *L. pratense*–*E. siegeli* (Zhang et al., 2009). The elevated metabolites under stress, further augmented in endophyte-symbiotic plants, probably contribute to plant tolerance of water deficit (Nagabhyru et al., 2013). Mechanisms of endophyte-associated augmentation of sugars, sugar alcohols, and amino acids appear not to be due to effects on plant gene transcription but simply may be due to their biosynthesis by the endophyte.

The response of tall fescue subjected to the stress treatments—both with and without endophyte—is similar to that previously described in other plant species (Dugas et al., 2011; Liu and Jiang, 2010; Yue et al., 2008; Zhao et al., 2016). Roughly one-third of the plant transcriptome was differentially expressed over the four genotypes in response to the stress treatment. Gene ontology terms associated with increased expression in the stressed plants included genes in pathways comprising response to heat, oxidative stress, water stress, osmotic stress, as well as increases in the biosynthesis of metabolites (trehalose) and amino acids (proline). The increase in transcription of genes involved in the biosynthesis of proline are hallmark indicators of stress, as is the increase of trehalose in plants, and this was observed in our stress treatment and in others using tall fescue (Delauney and Verma, 1993; Fernandez et al., 2010; Nagabhyru et al., 2013; Verbruggen and Hermans, 2008).

Although some putative stress-response genes, such as dehydrin, were among the DEGs affected by endophyte, particularly in P27, no pathway was identified as significantly regulated, up or down, in any of the clones under the stress conditions when comparisons were done between the E+ and E− plants. It is possible that the transcriptional regulation by the presence of the fungus in response to imposed stress is more subtle than the criteria used to determine the DEGs in the present study. In a number of instances where differences were observed in one clone, similar trends in expression profiles were observed in the other clones but were not declared significant. However, in most instances where expression was observed in a clone pair, the expression was observed in both the E+ and E− clones. It is clear that there are large differences in gene expression as well as differences in stress responses between tall fescue genotypes (Huang and Fry, 1998; Huang et al., 1998; West et al., 1990). It may be that the high genetic diversity in tall fescue may obscure the more subtle transcriptional changes in its interaction with the endophyte especially considering that endophyte effects on tiller recovery varied across clone pairs. It is also possible that the endophytes signal such changes but that the effect on plant gene expression is too low (less than twofold) or inconsistent under the stress condition or time of its imposition selected for this study. Day two was selected for molecular analysis because our previous work suggested that as stress was initiated, osmoregulatory metabolites, such as proline, trehalose, and other sugars and sugar alcohols, tended to be higher in E+ plants (Nagabhyru et al., 2013). In the present study, however, we saw no indication of corresponding endophyte effects on plant gene transcription. It may be that levels of these metabolites are regulated at a different stage (i.e., post-transcriptionally) or by the combined effects of the plant and endophyte. Other compounds have been proposed to act as signals between the endophyte and the plant including auxins (De Battista et al., 1990; Torres et al., 2012) and the fungal response to ROS (Tanaka et al., 2006; Scott and Eaton, 2008), and these might also aid in priming the plant for stress responses.

In summary, our results indicate overall that in some tall fescue–endophyte interactions the endophyte has a small but potentially important effect on the plant transcription that may aid the plant in stress resistance including repression of some genes involved in fungal and defense response and priming of some genes that may enhance drought tolerance. However, we also observed considerable variation in endophyte effects on recovery from the stress and the plant transcriptome, which seems likely to be because of variation both in plant and endophyte genotypes and perhaps slightly different greenhouse conditions for each of the tests. Challenges for the future are to tease apart the host and endophyte genotype and environmental effects, as well as roles of particular genes and metabolites, and to assess effects under different conditions or time periods of stress.

**Supplemental Information Available**

Supplemental information is available with the online version of this manuscript.

**Conflict of Interest Disclosure**

The authors declare that there is no conflict of interest.

**Author Contributions**

RDD, PN, and CLS designed experiments; RDD, PN, and CAY performed experiments; RDD and CPW contributed new materials; RDD, PN, CAY, and CLS analyzed data; RDD, PN, CAY, CPW, and CLS contributed to writing of the manuscript.

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