Functional Annotation of Transcripts Obtained from Fall-Grown Tall Fescue as Influenced by Endophyte Infection

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Abstract

Tall fescue is one of the primary sources of forage for livestock. It grows well in the marginal soils of the temperate zones. It hosts a fungal endophyte (*Epichloë coenophiala*), which helps the plants to tolerate abiotic and biotic stresses. The genetics and biology underlying mechanism of freezing stress tolerance of tall fescue is still unknown, due to its complex genetic background and outbreeding modes of pollination, limited genomic, and transcriptomic resources. The aim of this study was to identify differentially expressed genes (DEGs) in two tissues between novel endophyte-positive (E+) and endophyte-free (E-) tall fescue genotypes at three diurnal time points; in the morning (-3.0 to 0.5°C), afternoon (11 to 12°C), and evening (12 – 10°C) in the field environment, by exploring the transcriptional landscape via RNA sequencing. For the first time, we generated 226,054 and 224,376 transcripts from E+ and E- Texoma MaxQ II tall fescue, respectively by *de novo* assembly. The upregulated transcripts were detected fewer than the downregulated ones in both tissues (S: 803 up and 878 down; L: 783 up and 846 down) under the freezing temperatures in the morning. By Gene Ontology enrichment analysis, 10 GO terms were found only under the freezing stress in the morning. Metabolic pathway and biosynthesis of secondary metabolites genes showed lowest number of DEGs under morning freezing stress and highest number in evening cold condition by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. The DEGs expressed under morning stress condition and the nine candidate genes that we identified using GO analysis, might be the possible route underlying cold tolerance in tall fescue.

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season perennial grass species in the temperate zone worldwide. They grow well in the transition zone of the United States, where cool- and warm-season grasses are cultivated successfully. Tall fescue is highly productive and provides quality forage. Thereby, it is grown for pasture, hay, and silage and being used as a primary source of herbage protein of livestock feed. Tall fescue can grow in a wide range of temperatures between 4–35°C with an optimum 20–25°C. They can tolerate extreme cold stress, which includes chilling (0–12°C) and/or freezing (< 0°C) temperatures for a short period during fall. The forage production of tall fescue is reduced during January and February, and growth resumes when temperature rebound to ≥ 12°C in spring.

Increasing evidence suggests that a wild-type endophyte (*Epichloë coenophiala*) living in the intercellular space helps tall fescue plant to fight against abiotic and biotic stresses2–7. However, the endophyte produces alkaloids that are harmful to grazing animal8. A novel endophyte strain, AR584, purified from tall fescue originating from Morocco by AgResearch, New Zealand9 that provides similar benefits to the tall fescue plants, and at the same time it is not harmful to the grazing animal10–12. To elucidate the genetic relationship between novel endophyte and tall fescue, genome resources for the *Festuca* grasses and their endophytes are needed.

The genome size of hexaploid tall fescue is ~ 6 Gb that makes genome sequencing studies critical13, though a draft genome sequence of a related diploid perennial ryegrass (*Lolium perenne*) is available14. Next-generation sequencing of mRNA (RNA-seq) is a powerful method to evaluate transcriptional responses into biotic and abiotic stresses without a reference genome. RNA-seq offers the opportunity to identify transcripts, by mapping RNA-seq reads onto a genome, or by first assembling the reads *de novo* into contigs and then mapping the contigs onto the transcriptome for organisms without reference genome15. Transcriptome analyses were used to identify differentially expressed genes (DEGs) responsible for the expression of traits within contrasting plant materials. The first leaf transcriptome was developed on four different tropical guinea grass (*Panicum maximum* Jacq.) genotypes. *De novo* transcriptome assemblies from two buffalograss (*Bouteloua dactyloides* Nutt. Columbus) cultivars17, multiple tissues from two highly inbred perennial ryegrass (*Lolium perenne* L.) genotypes18, and four *Lolium-Festuca* species19 were also reported.

By comparing the transcriptome profiles of two tall fescue genotypes (heat tolerant and heat sensitive), candidate genes response to plant heat tolerance were identified20. In order to identify DEGs under lead (Pb) stress, the leaf transcriptome of two tall fescue cultivars (Pb tolerant and Pb sensitive) were compared21. To investigate the molecular mechanism of tall fescue adaptability to cadmium (Cd) stress, candidate genes were reported by comparing the root and leaf transcriptome with or without Cd treatments22. Dinkins et al.23 analyzed tall fescue transcriptomes and compared DEGs in different tissues of endophyte-positive (E+) and
endophyte-free (E-) clones. To study the effect of endophyte on drought tolerance, endophyte (AR584)-infected contrasting tall fescue genotypes (water stress tolerant and susceptible)\textsuperscript{24} harboring different endophyte strains\textsuperscript{25} were analyzed to identify DEGs.

To improve our understanding on cold/freezing responsive genes, comparisons of transcripts under different temperatures were conducted in Kentucky bluegrass (\textit{Poa pratensis} L.)\textsuperscript{26}, zoysiagrass (\textit{Zoysia} spp. Willd.), sheepgrass (\textit{Leymus chinensis})\textsuperscript{27}. However, it is not known how novel endophyte responded under extreme cold stress and maintain their symbiotic relationship. Studies on transcriptomic analyses have revealed the mechanism of cold tolerance without the presence of endophyte in winter rapeseed (\textit{Brassica rapa} L.)\textsuperscript{28} and \textit{Lotus japonicus}\textsuperscript{29}. It is therefore important for grass breeders to understand whether the tall fescue alone has the ability to withstand under freezing condition, or needs support from the novel endophyte to survive under extreme cold stress. To address the above questions, we analyzed transcripts from pseudostem (S) and leaf (L) tissues of E+ and E- Texoma MaxQ II tall fescue genotypes at three different time points in a day from a natural field environment using RNA-seq. Tissue type, cellular conditions, and environmental factors all guided transcript profiles that may influence regulatory events such as splicing and the expression of genes or their isoforms\textsuperscript{30}. These findings encouraged us to use two tissue types utilizing E+ and E- tall fescue for transcriptome analysis to monitor changes in plant gene expression under cold stress in the natural field environment by considering genetic and environmental interaction, evaluating plant responses, and endophyte's influence on the host responses. The aims of this study were to: (i) investigate genome-wide transcriptomic profile of E+ and E- Texoma tall fescue, (ii) identify DEGs in two tissues under three time points, and (iii) identify candidate gene(s) responsible for cold tolerance under freezing condition in the natural field environment. It could provide us useful information on how the endophyte influences genes and their regulatory pathways associated with cold/freezing response in tall fescue.

\textbf{Results}

\textbf{Sequencing and \textit{de novo} assembly of the transcriptome}

After the quality assessment and data filtered, a total of 553.8 million high quality paired-end reads were identified in the E+ (18) samples and 484.8 million in the E- (18) samples (Table 1 and Supplementary Figure S1). The filtered reads were \textit{de novo} assembled into 5,520,386 and 5,133,272 contigs in the E+ and E- samples, respectively for downstream analysis. From these contigs, we identified unique transcripts varied from 186,653 to 200,380 in the E+ samples and from 188,468 to 194,606 in the E- samples. Overall, we identified a total of 226,054 transcripts in the E+ samples and 224,376 transcripts in the E- samples. The length of these transcripts varied from 177-27,968 bp and the N50 varied from 1,288-1,326. Finally, we found 234,883 transcripts from all the samples collected in this study (Table 1). In addition, the result showed about 5.18% and 0.95% more transcripts were expressed in endophyte-infected S and L, respectively over endophyte-free S and L under freezing temperatures in the morning.

\textbf{Identification of orthologue genes from other plant species}

Among the combined transcripts (234,883), about 13.5% got hit to switchgrass (31,780), rice (31,622), and \textit{Arabidopsis} (31,604) genomes. A total of 5919, 4476, and 4002 orthologue genes were identified in switchgrass, rice, and \textit{Arabidopsis}, respectively (Supplementary Figure S2). Due to lack of well annotated tall fescue genome, the function of the majority fescue transcripts remain unknown when compared to the reference genomes of the related species.

\textbf{Analysis of DEGs between different cold stress and E+/E- Texoma tall fescue}

Differential gene expression in E+ tall fescue was analyzed relative to E- tissues under three different time points under natural field environment. A total of 5,757 significant DEGs (\textit{p}-value ≤ 0.05) with log2 fold change (FC) ≤ -5 and ≥5 were identified at least in one of the six following comparisons: E+MS vs. E-MS, E+ML vs. E-ML, E+NS vs. E-NS, E+NL vs. E-NL E+ES vs. E-ES, and E+EL vs. E-EL (Figure 1 and Supplementary Table S1). Analysis of DEGs showed that higher number of transcripts were expressed in S than L tissues at each time point (Figure 1). The upregulated transcripts were detected fewer than the downregulated ones in both tissues under the freezing (-3°C to 0.5°C) temperatures (S: 803 up and 878 down; L: 783 up and 846 down) in the morning. At afternoon
(11°C to 12°C), fewer upregulated (678) than downregulated (928) transcripts were identified in S tissues, but reverse scenario (688 up- vs. 633 down-regulated) was observed in L tissues. In contrast to the afternoon, in the evening (12°C to 10°C), more upregulated (1,309) than downregulated (916) transcripts were identified in S tissues, and fewer upregulated (590) than downregulated (705) transcripts were detected in L tissues (Figure 1).

The specific- and overlapping DEGs among the comparisons were visualized in DiVenn (Figure 2). The result showed 463 DEGs were specific to E+NS vs. E-NS, 321 were specific to E+NL vs. E-NL, 961 were specific to E+ES vs. E-ES, and 470 were specific to E+EL vs. E-EL under normal cold condition in the afternoon and evening time. Among the morning time expressed transcripts, 97 DEGs were common between S (E+MS vs. E-MS) and L (E+ML vs. E-ML), of which 42 were upregulated in one but downregulated in other comparison (Figure 2). In addition, there were 556 DEGs were specific to E+MS vs. E-MS and 529 were specific to E+ML vs. E-ML, totaling of 1,085 were significantly up- and down-regulated in the morning freezing conditions, and were not expressed in the normal cold condition in the afternoon and evening time.

The DEGs were used for linkage hierarchical clustering analysis (Figure 3). We observed a distinct pattern of gene expression at transcriptional level under different time points. Cluster analysis showed that some genes upregulated in the morning were downregulated in the afternoon and evening time or vice versa. Heat map also showed that the expression profiles of the majority genes were different between the S and L tissues in all the time points (Figure 3). This result indicates that tall fescue responded to the stress conditions in time- and tissue-specific manners.

**Gene Ontology analysis of DEGs**

Out of 5,757 significant DEGs, 1,099 got hit to 732 rice genes in the six comparisons and were used for GO analysis (Supplementary Table S2). We obtained 98 significant GO terms of three major categories, such as biological process (BP), molecular function (MF) and cellular component (CC) (Figure 4, Supplementary Table S3). Some of the identified GO terms were often contained common genes. Since our key objective was to investigate influence of novel endophyte on freezing tolerance, our primary interest was on the GO terms associated with samples collected under the freezing temperatures (-3°C to 0.5°C) in the morning. In E+MS vs. E-MS samples, 38 GO terms under BP, 31 under MF, and 11 under CC categories were identified. The most significant GO terms involved are ‘protein complex biogenesis’ and ‘protein complex assembly’ under BP; ‘nucleotide binding’ under MF; ‘membrane coat’ and ‘coated membrane’ under CC (Supplementary Figure S3). There were 86 genes under the ‘nucleotide binding’ category (Figure 4), of which four genes: *LOC_Os04g58410.1* (tall fescue gene ID: Fa.36660.1), *LOC_Os03g51030.1* (Fa.8356.1), *LOC_Os03g51030.2* (Fa.8921.1 and Fa.10647.1), *LOC_Os03g54084.1* (Fa.6944.1), are involved in GO:0006974 (cellular response to DNA damage stimulus), GO:0033554 (cellular response to stress), GO:0071214 (cellular response to abiotic stimulus), GO:0071478 (cellular response to radiation), and GO:0071482 (cellular response to light stimulus) (Table 2).

Similarly, in E+ML vs. E-ML, GO analysis detected 21 GO terms under BP, 30 under MF, and nine under CC categories. The most significant GO terms involved are ‘cellular amino acid metabolic process’, ‘cellular amine metabolic process’ and ‘cellular nitrogen compound metabolic process’ under BP; ‘pyrophosphatase activity’, ‘nucleoside-triphosphatase activity’, ‘hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides’, ‘hydrolase-activity, acting on acid anhydrides’ and ‘nucleotide binding’ under MF; and ‘membrane part’ under CC category (Supplementary Figure S4). There were 32 genes under each of the GO terms ‘pyrophosphatase activity’, ‘nucleoside-triphosphatase activity’, ‘hydrolase activity’ and ‘membrane part’, and the other 78 genes under the ‘nucleotide binding’ category (Figure 4), of which three genes, *LOC_Os04g32560.2* (Fa.47164.1), *LOC_Os12g12850.2* (Fa.23585.2) and *LOC_Os02g57530.2* (Fa.32995.1) are involved in GO:0033554 (cellular response to stress), GO:0006974 (response to DNA damage stimulus), GO:0071369 (cellular response to ethylene stimulus) and GO:0071495 (cellular response to endogenous stimulus) (Table 2).

There were three significant GO terms ‘amino acid activation’, ‘tRNA aminoacylation’ and ‘tRNA aminoacylation for protein translation’ under BP, four GO terms ‘protein transporter activity’, ‘aminoacyl-tRNA ligase activity’, ‘ligase activity forming carbon-oxygen bonds’ and ‘ligase activity forming aminoacyl-tRNA and related compounds’ under MF and two GO terms ‘membrane coat’ and ‘coated membrane’ under CC, which were enriched only E+MS vs. E-MS under the freezing stress (-3°C to 0.5°C) in the M (Figure 4). Most of the genes associated with the nine GO categories above were related to ligase activity, kinase activity, binding, signaling, and transporter activity. Interestingly, there was only one molecular function GO term ‘lyase activity’, which was
significantly enriched in E+ML vs. E-ML during the freezing stress in the morning than other cold stresses. There were 12 genes under ‘lyase activity’ category, of which one gene, LOC_Os04g37920.1 (Fa.63716.1) was identified in GO:0033554 (cellular response to stress) and GO:0006974 (response to DNA damage stimulus).

In this study, we identified nine DEGs specifically expressed during the freezing stress in the morning than the cold stress at afternoon or in the evening, of which five DEGs (Fa.8356.1, Fa.8921.1, Fa.10647.1, Fa.6944.1 and Fa.36660.1) were upregulated (6-22 fold) and two (Fa.63716.1, Fa.32995.1) were downregulated (8-27 fold) in E+MS vs. E-MS, and the rest two (Fa.47164.1 and Fa.23585.2) were upregulated (21-38 fold) in E+ML vs. E-ML (Table 2).

KEGG pathway analysis of DEGs

We identified 85 and 90 KEGG pathways at E+MS vs. E-MS and E+ML vs. E-ML, respectively under freezing stress in the morning. Similarly, 81 and 71 pathways were identified at E+NS vs. E-NS and E+NL vs. E-NL, respectively under afternoon cold condition, and 86 pathways were identified in both E+ES vs. E-ES and E+EL vs. E-EL under evening cold period (Supplementary Table S4). Top 10 KEGG pathways of each six comparisons under freezing and cold conditions were shown in Figure 5. Among the top 10 enriched pathways, ‘metabolic pathways (dosa01100)’, ‘biosynthesis of secondary metabolites (dosa01110)’, and ‘carbon metabolism (dosa01200)’ were contained maximum number of DEGs. Under different cold conditions, metabolic pathway contained lowest number (50) of DEGs at morning freezing stress (E+MS vs. E-MS) and maximum number (80) at evening cold condition (E+ES vs. E-ES). Similarly, biosynthesis of secondary metabolites contained lowest number (24) of DEGs at morning freezing stress (E+MS vs. E-MS) and maximum number (45) at evening cold condition (E+ES vs. E-ES) (Figure 5).

Validation of DEGs using qRT-PCR

To verify the DEGs identified by pairwise comparison between E+ and E- tissues, eight DEGs, including seven upregulated and one downregulated genes in E+MS vs. E-MS were evaluated using qRT-PCR method. The expression levels of the selected DEGs detected with qRT-PCR were consistent with the expression levels calculated from RNA-Seq data (Supplementary Figure S5), indicating the RNA-seq data were reliable in this study.

Discussion

The objectives of this study were to characterize the tall fescue transcriptome, and to identify genes that were differentially expressed due to the presence of a fungal symbiont under freezing condition. Due to lack of a well-annotated tall fescue reference genome, we generated 36 de novo assemblies, consist of S and L tissues of E+ and E- Texoma tall fescue genotypes at three time points and three replications. Individual assembly were performed to keep right track of the DEGs in the E+ and E- Texoma tall fescue under different level of cold condition in the field as well as to know the transcript abundance in the individual samples.

Our transcriptome assemblies result in 234,883 transcripts, which may constitute important transcriptomic resources for understanding cold tolerance mechanism of this allohexaploid forage species. In a previous transcriptome study, de novo assembly obtained 199,399 contigs from novel endophyte (AR584) infected two tall fescue genotypes under water stress condition in a greenhouse study using the Illumina Genome Analyzer Iix system. Recently, Dinkins et al. generated transcriptome resources from two tall fescue genotypes infected with common toxic endophyte (CTE), one with non-toxic strain (NTE19) and the other with hybrid endophyte species (FaTG-4) under water deficit condition in the greenhouse, and assembled against a tall fescue TF153K transcriptome assembly developed by Dinkins et al.. Both studies are performed in controlled condition in the greenhouse, but we performed this transcriptomic study of AR584 infected Texoma tall fescue at natural field environment. The field condition is always more variable than that of the controlled growth chamber, due to the direct effect of sunlight, day length, soil microbial community, and genotype-environment interaction but can provide more naturalistic outcome.

By comparing the transcript abundance within E+ and E- Texoma tall fescue, we observed that the number of unique transcripts were higher in S than that of L tissue in all three different temperatures. More importantly, our results showed that novel endophyte had positive influence on gene expression over E- tall fescue under freezing temperatures in the morning (E + MS: 200,380 > E-MS: 190,514; E + ML: 190,568 > E-ML: 188,760) when the plants were in stress (Table 1). The number of unique transcripts were almost
similar in S tissues, but slightly different in L tissues between E+ and E- tall fescue in the afternoon and evening temperature (10–12°C). Thus, we speculated that the plant does not need support from endophyte under normal cold condition, but does need assistance to survive under freezing temperatures by altering their gene expression. Although Dinkins et al.23 reported that the presence and/or absence of endophyte do not change global expression, more number of transcripts obtained in E+ over E- samples in all tissues examined under three temperatures conditions in this study (Table 1) might be due to endophyte's response. DiVenn showed that 1,085 DEGs were specifically expressed in the morning freezing conditions might play a key role to maintain symbiotic relation between a novel endophyte (AR584) and its host tall fescue under morning freezing stress.

Under morning freezing temperatures, plants triggered genes in response to extreme cold stress that was evidenced in GO analyses where 10 GO terms (three under BP, five under MF, and two under CC category) were only found in the morning time (Fig. 4). However, we did not able to analyze all the DEGs due to lack of available information in tall fescue genome and orthologous genes in related species. The genes expressed at the morning freezing conditions may be a possible route to tolerate the cold stress. This study would be very useful to develop hypothesis that can bring further understanding of underlying genetics of cold tolerance in tall fescue. Mahmood et al.32 reported that the data obtained from transcriptome study can be the starting point to formulate hypothesis to dig genetics of ergot resistance.

Light plays an important role in cold acclimation by accelerating the expression of cold regulated genes (CORs) in different species and phytochromes have been identified as important factors in the transcriptional control of CORs33. Three types of phytochromes such as phytochrome A (phyA), phytochrome B (phyB), and phytochrome C (phyC) were identified in the flowering plants34 that respond to radiation in the environment. In tomato, phyA perceived far-red light to positively and phyB perceived red light to negatively regulate cold tolerance through abscisic-acid dependent jasmonate signaling35. In rice, He et al.36 reported that phyB negatively controls cold tolerance by regulating OsDREB1 gene expression through phytochrome interacting factor-like protein OsPIL16. Woods et al.37 reported phyC as an essential light receptor for photoperiodic flowering in a temperate grass model Brachypodium distachyon.

In this study, we identified four transcripts (Fa.8356.1, Fa.8921.1 and Fa.10647.1 orthologs of rice phyA gene, and Fa.6944.1 ortholog of rice phyC gene) only upregulated in S under morning freezing temperatures (Table 2 and Supplementary Table S2) could be a possible route for extreme cold tolerance in tall fescue. We performed qRT-PCR experiment on S samples collected under freezing stress in the morning to validate RNA-seq data and observed that Fa.6944.1, Fa.8356.1, and Fa.10647.1 genes were upregulated in S under freezing stress in the morning (Supplementary Figure S5). The ATP-dependent Clp protease ATP-binding subunit clpA homolog CD4B, chloroplast precursor, putatively expressed (LOC_Os04g32560.2/Fa.47164.1 and LOC_Os12g12850.2/Fa.23585.2) were upregulated in L tissues under morning freezing temperature. Okuzaki et al.38 reported that the acidic domain of the chloroplast ribonucleoprotein 31A (CP31A) is essential for cold-tolerance in Arabidopsis. Under extreme low temperatures, plant growth and development was ceased and thus plant adjusted to different physiological and biochemical processes in response to cold stress. Exogenous ethylene level altered under cold stress. In this study, we identified a gene Fa.32995.1, ortholog of rice ethylene receptor, was downregulated under morning freezing temperature. Ethylene levels are negatively correlated with cold tolerance in Medicago truncatula39, but positively affect cold tolerance of tomato (Lycopersicon esculentum)40.

**Conclusion**

This study represents the first transcriptome analysis of E+ and E- Texoma tall fescue under freezing and chilling temperatures in the natural field environment. We generated 234,883 unique transcripts from 36 de novo assemblies. A total of 5,757 DEGs were identified between E+ and E- samples under three diurnal temperature conditions, of which 1085 were only up- or down-regulated under freezing temperatures in the morning. We were not able to analyze all the genes expressed differentially in two tissues under three temperature conditions, due to lack of available information in related species. Using GO analysis, nine candidate genes were identified from E+ vs. E-samples collected during morning freezing temperature that might help to understand the endophyte influence on the genetic basis of freezing tolerance in tall fescue. Moreover, the transcriptomic resources generated in this study would serve as valuable resources for grass breeders and to the research community for further structural annotation of tall fescue genome.
Methods

Plant material

Novel endophyte (AR584) positive (E+) and endophyte-free (E-) tall fescue genotypes of cv. Texoma MaxQ II (referred as “Texoma”) (Pennington, USA, https://www.pennington.com/) were developed at the Noble Research Institute, Ardmore, Oklahoma, USA. Texoma MaxQ II is a commercial cultivar freely available for cultivation in USA. The E+ and E- plants were transplanted in the field for seed production via open pollination among them. Since the endophyte does not transmit through pollen, seeds were harvested from the E+ and E- mother plants separately. The seeds obtained from the E+ and E- Texoma genotypes were sown in rows in the experimental farm located at Dupy (Latitude: 34°17'12.106"N, Longitude: 96°59'36.608"W), Gene Autry, Oklahoma. Before collecting samples for transcriptome analyses, we collected S tissues separately in ice-cold 15 mL falcon tubes from 15 genotypes of each E+ and E- from three random rows of the plot to test their endophyte status. In an earlier study, Takach et al. confirmed that endophyte is residing in the S, not in L, in Texoma tall fescue. The S samples were freeze-dried and ground separately in the presence of liquid N\textsubscript{2} using mortar and pestle. Genomic DNA was extracted using MagAttract 96 DNA Plant Core Kit (QIAGEN Cat. No. 67163, Hilden, Germany) according to the manufacturer’s recommendation. PCR amplifications were performed using primers described in\textsuperscript{42} to confirm endophyte status of the Texoma tall fescue genotypes.

After confirming E+ and E- status through PCR amplification, equal volume of S and L tissues were collected on December 10, 2018 under freezing/cold temperatures from the field at: morning between 7:40 (-3°C) to 9:00 am (0.5°C), afternoon between 1:15 (11°C) to 2:15 pm (12°C), and evening between 4:45 (12°C) to 5:45 pm (10°C) (Supplementary Figure S6). The 12 samples with three replicates that were collected from the E+ and E- tall fescue at morning, afternoon, and evening were referred as E+MS (endophyte positive, morning, pseudostem), E+ML (endophyte positive, morning, leaf blade), E+NS (endophyte positive, afternoon, pseudostem), E+NL (endophyte positive, afternoon, leaf blade), E+ES (endophyte positive, evening, pseudostem), E+EL (endophyte positive, evening, leaf blade), E-MS (endophyte-free, morning, pseudostem), E-ML (endophyte-free, morning, leaf blade), E-NS (endophyte-free, afternoon, pseudostem), E-NL (endophyte-free, afternoon, leaf blade), E-ES (endophyte-free, evening, pseudostem), and E-EL (endophyte-free, evening, leaf blade). In each sample, L/S tissue from 10 genotypes was pooled in a 15 mL tube and immediately frozen in liquid N\textsubscript{2}. After arrival to the laboratory, the samples were stored at -80°C until processing.

RNA extraction and sequencing

The total RNA of each of 36 samples (Supplementary Figure S1) was isolated from approximately 100 mg ground tissues using a Spectrum\textsuperscript{TM} Plant Total RNA Kit (Sigma, Cat. No. STRN250, St. Louis, USA) according to the manufacturer recommendation. The RNA quality was measured in Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA) using Agilent RNA 6000 Nano Kit (Agilent, 5067-1511), and RNA was quantified using Qubit® RNA BR (Broad-Range) Assay Kit (Life Technologies, Cat. No. Q10211, Carlsbad, USA). Then the RNA samples were treated with TURBO DNA-free Kit (Invitrogen, Cat. No. AM1907, Carlsbad, USA) following their protocol (https://assets.fishersci.com/TFS-Assets/LSG/manuals/1907M_turbodnafree_UG.pdf). RNA samples were then cleaned using the RNeasy MinElute Cleanup Kit (Qiagen, Cat. No. 74204, Hilden, Germany) according to the manufacturer protocol (https://www.qiagen.com).

RNA-seq libraries were prepared using TruSeq Stranded mRNA Sample Preparation Kits (Illumina, Cat. No. 20020594). Briefly, mRNA was purified from one microgram of total RNA, fragmented, and converted to double-stranded DNA for sequencing. Individual libraries were uniquely indexed using TruSeq RNA CD Indexes (Illumina, Cat. No. 20019792), and pooled in equimolar ratio. The pooled libraries were sequenced on an Illumina NovaSeq 6000 150PE Sequencing system.

Quality assessment and assembly of the RNA-seq reads

The raw reads of 36 samples (Figure 1) were quality trimmed to remove any low quality bases and primer/adapter sequences before performing the assembly using the Trimmomatic (v. 0.36) using default settings\textsuperscript{43}. Reads less than 30 bases long after trimming were discarded, along with their mate pair. Endophyte-derived reads were identified by mapping the trimmed reads to the \textit{Epichloë coenophiala} transcriptome\textsuperscript{44} (http://csbio-l.csr.uky.edu/ec/) and successfully mapped reads were excluded from further
analysis. The trimmed and filtered reads from each sample were independently de novo assembled using the software Trinity (v. 2.8.5) with default parameters. These assemblies were then combined by randomly selecting one as a starting transcriptome and then iteratively aligning the transcriptome with each assembly, identifying that assembly's novel transcripts, and adding those transcripts to the combined transcriptome. Each sample was then mapped to the combined transcriptome using HISAT2 (v. 2.0.5) (https://daehwankimlab.github.io/hisat2/) with 24 threads and the default mapping parameters. The expressed transcripts in each sample were quantified using the StringTie (v. 1.2.4) with the default assembly parameters to produce more complete and accurate reconstructions of transcripts and better estimates of their expression levels.

**Identification of differentially expressed genes**

To identify genes which expressed under different temperature condition during morning, afternoon, and evening time with or without the presence of endophyte, pairwise differential gene expression testing was performed using DESeq2 with default parameters setting. DESeq2 method was used for differential read counts per gene in RNA-seq, using shrinkage estimation for dispersions and fold changes to improve stability of estimates across experimental conditions. A log2 FC ≤ -5 and ≥ 5 and adjusted p-value ≤ 0.05 were used to determine the significant differences in differential gene expression between two samples. The DEGs with log2 FC – and + sign indicates downregulated and upregulated genes, respectively.

**Hierarchical clustering of differentially expressed genes**

Hierarchical clustering analysis of DEGs from the six comparisons was constructed using the function heatmap.2 in the R package gplots in R Studio.

**Visualization of differentially expressed genes**

The DEGs that were biologically significant were visualized using the web-based software DiVenn. The red and blue nodes represent up- and down-regulated genes, respectively. The yellow nodes represent upregulated in one dataset but downregulated in the other dataset.

**Identification of orthologous genes using tall fescue transcripts**

As annotation of tall fescue genome is not available till May 30, 2021, the complete and accurate tall fescue transcripts were aligned against the switchgrass non-redundant protein sequences in Phytozome v13 database (https://phytozome.jgi.doe.gov/pz/portal.html) using BLASTX searches to identify best matched switchgrass orthologues. Using switchgrass orthologues, we also obtained rice and Arabidopsis orthologues of tall fescue transcripts from the Phytozome database.

**Gene Ontology analysis of differentially expressed genes**

We performed GO enrichment analysis using orthologue genes of rice (Supplementary Table S2) to identify their involvement in BP, MF, and CC categories. To study the influence of endophyte, the DEGs between E+MS vs. E-MS, E+ML vs. E-ML, E+NS vs. E-NS, E+NL vs. E-NL, E+ES vs. E-ES and E+EL vs. E-EL were used for GO enrichment analysis. The rice orthologues of the tall fescue DEGs were used as input data to perform GO analysis using singular enrichment analysis (SEA) tool against Oryza sativa japonica annotation of the web-based AgriGO v2.0 with modified statistical parameter settings: statistical test method, Fisher; multi_test adjustment method, Yekutieli (FDR under dependency); significance level, 0.01; and minimum number of mapping entries, 10. MSU7.0 gene ID (TIGR) of rice orthologues was used as reference during SEA analysis.

**KEGG pathway enrichment analysis**
The KEGG pathway enrichment analysis was performed using KOBAS 3.0 on the basis of Fisher's exact test with Benjamini and Hochberg (1995) FDR corrected p-value <0.05. The top most significant pathways based on FDR corrected p-values in all six comparisons were presented.

Validation of RNA-seq by quantitative real time PCR (qRT-PCR)

Eight DEGs were selected for validation using qRT-PCR. Total RNA treated with TURBO DNA-free Kit (Invitrogen, Cat. No. AM1907, Carlsbad, USA) was used to synthesize the first-strand cDNA using SuperScript III Reverse Transcriptase (RT) kit (Invitrogen, Carlsbad, CA, USA, Cat. no.: 18080044) following the manufacturer protocol (https://www.thermosher.com). Briefly, for each RNA sample, the following components was combined in a PCR tube on ice to a volume of 12 µL containing 5 µL DNase-free RNA (200 ng/µL), 1 µL 50 mM oligo (dT)20, 1 µL 10 mM dNTPs and 5 µL RNase/DNase-free water. The reaction mixtures were incubated at 65°C for 5 min and then placed on ice for 2 min. The first-strand cDNA synthesis master mix was prepared on ice by adding 4 µL 5x First Strand buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT Recombinant RNase Inhibitor (40 units/µL) and 1 µL SuperScript III RT (200 units/µL). The first-strand cDNA synthesis master mix was mixed properly by gentle vortex and was added into the pre-incubated RNA and oligo tube. The mixture was mixed by pipetting up and down and incubated at 50°C for 60 min. The reaction was terminated at 70°C for 15 min and cooled on ice. The cDNA was stored at -20°C.

qRT-PCR reactions were prepared in an optical 384-well plate in a volume of 10 µL containing 2 µL of the forward and reverse primer (1 µM/µL), 5 µL of 2x Sigma KiCqStart SYBR Green qPCR Ready-Mix (Cat no.: KCQS01), 1 µL molecular biology grade water, and 2 µL cDNA (1:20). qRT-PCR amplifications were performed on QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific, Singapore) using a protocol of 2-step PCR cycle (an initial denaturation of cDNA at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 45 sec) and a 3-step of melting curve analysis (95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec). Experiments were performed with three technical replicates of each S tissues of E+ and E- tall fescue collected under freezing temperature in the morning. Gene expression was quantified using the 2^-ΔΔCT method. The tall fescue Actin gene was used as an internal reference gene. Primers used to amplify 53-63 bp of the genes were designed using Primer Express software (v3.0.1) (Thermo Fisher Scientific) (Supplementary Table S5) and synthesized by Sigma-Aldrich, MO, USA.

Declarations

The authors hereby declared that all methods were performed in accordance with the relevant guidelines and regulations of the Noble Research Institute, LLC. Plant materials used (Texoma MaxQ II) is a publicly available commercial cultivar of tall fescue.

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Authors' contributions

MSI and MCS conceived the study. MSI conducted the experiment. MSI and NK analyzed RNA-seq data, TK prepared the library for RNA-seq, MSI and GL prepared RNA for sequencing and performed qRT-PCR, MSI wrote the manuscript, MCS critically revised the manuscript. All the authors read and approve the manuscript for publication.

Competing interests

The authors declare no competing interest with others.

Data availability

The raw sequence reads were deposited in the NCBI's Sequence Read Archive (SRA) with accession number "PRJNA734807".
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Tables

Table 1: Assembly and annotation of the transcriptome data obtained from short read Illumina sequencing of Texoma E+ and Texoma E- tissues under cold stress in the natural field environment.

| Parameters                      | Texoma E+                | Texoma E-                | Combined                |
|--------------------------------|--------------------------|--------------------------|-------------------------|
|                                | Time| Pseudostem | Leaf blade | Pooled | Pseudostem | Leaf blade | Pooled |
| Filtered paired-end reads*     | M   | 137,172,033| 62,692,906 | 553,802,405 | 71,139,077| 78,140,815| 484,814,092 |
|                                | N   | 87,040,508 | 71,783,216 | 92,460,683 | 73,609,080| 74,046,686| 148,605,766 |
|                                | E   | 86,632,321 | 108,481,421 | 95,417,751 | 74,046,686| 74,046,686| 148,605,766 |
| No of contigs after Trinity assembly* | M   | 1,125,143 | 780,760 | 5,520,386 | 817,647| 841,846| 5,133,272 |
|                                | N   | 929,259 | 769,068 | 906,924 | 799,442| 837,857| 1,637,305 |
|                                | E   | 941,726 | 974,430 | 930,388 | 837,857| 837,857| 1,637,305 |
| No of unique transcript        | M   | 200,380 | 190,568 | 226,054 | 190,514| 188,760| 224,376 |
|                                | N   | 193,981 | 186,653 | 193,370 | 188,468| 190,710| 382,878 |
|                                | E   | 194,588 | 193,295 | 194,606 | 188,468| 190,710| 382,878 |
| Transcript length (bp)         | M   | 177-16,616 | 179-15,501 | 177-16,616 | 177-16,644| 178-15,236| 177-27,968 |
|                                | N   | 178-15,651 | 177-15,604 | 177-15,596 | 178-16,026| 179-27,968| 177-27,968 |
|                                | E   | 179-15,718 | 178-15,966 | 177-15,590 | 179-27,968| 177-27,968| 177-27,968 |
| N50                            | M   | 1,316 | 1,288 | 1,288-1,338 | 1,317 | 1,303 | 1,300-1,326 |
|                                | N   | 1,318 | 1,338 | 1,318 | 1,326| 1,300 | 1,304 |
|                                | E   | 1,317 | 1,319 | 1,300 | 1,304| 1,300 | 1,304 |

M, morning; N, noon; and E, evening
Data presented as sum of three biological replicates

Table 2: List of nine candidate DEGs in tall fescue expressed under freezing temperatures in the morning. The expression values (log2 fold change) in each comparison, important GO terms and rice orthologs were provided.
| Gene ID   | log2 FC* | Important GO terms                                                                 | Rice orthologs |
|----------|---------|-----------------------------------------------------------------------------------|----------------|
|          | TE+MS vs. TE-MS | TE+ML vs. TE-ML | TE+NS vs. TE-NS | TE+NL vs. TE-NL | TE+ES vs. TE-ES | TE+EL vs. TE-EL |
| Lyase activity |          |                                                                                 |                |
| Fa.63716.1 | -26.87 | GO:0033554 cellular response to stress | LOC_Os04g37920.1 |
|           |         | GO:0006974 response to DNA damage stimulus |                |
| Nucleotide binding |         |                                                                                 |                |
| Fa.32995.1 | -7.69  | GO:0071369 cellular response to ethylene stimulus | LOC_Os02g57530.2 |
|           |         | GO:0071495 cellular response to endogenous stimulus |                |
| Fa.8356.1  | +22.11 | GO:0071478 cellular response to radiation | LOC_Os03g51030.1 |
|           |         | GO:0071482 cellular response to light stimulus |                |
|           |         | GO:0071214 cellular response to abiotic stimulus |                |
| Fa.8921.1  | +21.68 | GO:0071478 cellular response to radiation | LOC_Os03g51030.2 |
|           |         | GO:0071482 cellular response to light stimulus |                |
| Fa.10647.1 | +22.42 | GO:0071214 cellular response to abiotic stimulus |                |
|           |         | GO:0071482 cellular response to light stimulus |                |
| Fa.6944.1  | +7.46  | GO:0071478 cellular response to radiation | LOC_Os03g54084.1 |
|           |         | GO:0071482 cellular response to light stimulus |                |
|           |         | GO:0071214 cellular response to abiotic stimulus |                |
| Fa.47164.1 | +37.57 | GO:0006974 response to DNA damage stimulus | LOC_Os04g32560.2 |
|           |         | GO:0033554 cellular response to stress |                |
| Fa.36660.1 | +6.56  | GO:0006974 response to DNA damage stimulus | LOC_Os04g58410.1 |
|           |         | GO:0033554 cellular response to stress |                |
| Fa.23585.2 | +20.74 | GO:0006974 response to DNA damage stimulus | LOC_Os12g12850.2 |
|           |         | GO:0033554 cellular response to stress | |
Pyrophosphatase activity/nucleoside-triphosphatase activity/hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides/ hydrolase-activity, acting on acid anhydrides

| Gene ID | Log2 Fold Change | Description | Gene ID |
|---------|-----------------|-------------|---------|
| Fa.47164.1 | +37.57 | GO:0006974 response to DNA damage stimulus | LOC_Os04g32560.2 |
| Fa.47164.1 | -37.57 | GO:0033554 cellular response to stress | |
| Fa.23585.2 | +20.74 | GO:0006974 response to DNA damage stimulus | LOC_Os12g12850.2 |
| Fa.23585.2 | -22.57 | GO:0033554 cellular response to stress | |
| LOC_Os04g32560.2 | | | |
| LOC_Os12g12850.2 | | | |
| Membrane part |
| Fa.32995.1 | -7.69 | GO:0071369 cellular response to ethylene stimulus | LOC_Os02g57530.2 |
| Fa.32995.1 | | GO:0071495 cellular response to endogenous stimulus | |

*+, upregulated; -, downregulated; FC, fold change

**Figures**

![Graph showing differentially expressed genes between E+ and E- tissues under cold stress](image)

**Figure 1**

Identification of differentially expressed genes between E+ and E- tissues under cold stress. Samples were: E+MS (endophyte positive, morning, pseudostem), E+ML (endophyte positive, morning, leaf blade), E+NS (endophyte positive, afternoon, pseudostem), E+NL (endophyte positive, afternoon, leaf blade), E+ES (endophyte positive, evening, pseudostem), E+EL (endophyte positive, evening, leaf blade), E-MS (endophyte-free, morning, pseudostem), E-ML (endophyte-free, morning, leaf blade), E-NS (endophyte-free, afternoon, pseudostem), E-NS (endophyte-free, afternoon, leaf blade), E-ES (endophyte-free, evening, pseudostem), E-EL (endophyte-free, evening, leaf blade), E-NS (endophyte-free, evening, pseudostem), E-NS (endophyte-free, evening, leaf blade).
(endophyte-free, afternoon, pseudostem), E-NL (endophyte-free, afternoon, leaf blade), E-ES (endophyte-free, evening, pseudostem),
and E-EL (endophyte-free, evening, leaf blade).

Figure 2
Visualization of differential gene expression pattern among E+MS/E-MS, E+ML/E-ML, E+NS/E-NS, E+NL/E-NL, E+ES/E-ES and
E+EL/E-EL using DiVenn program. The red and blue nodes represent up- and downregulated genes, respectively. The yellow node
represent upregulated in one dataset but downregulated in the other dataset. Abbreviations of the samples are the same as in
Figure 1.
Figure 3

Hierarchical clustering analysis of all differentially expressed genes from six comparisons. Each column represents a different sample with subject to endophyte status, cold stress and tissue type. Red represent upregulated; blue, downregulated; and white, no change. Abbreviations of the samples are the same as in Figure 1.
Figure 4

Significant GO terms of the differentially expressed genes of rice orthologues. Significantly ($p \leq 0.01$) enriched GO terms of A- biological process (BP), B- molecular function (MF) and C- cellular component (CC) are shown in x-axis and the number of genes of each GO term are displayed in y-axis. The GO terms for the biological process are (a) amine metabolic process, (b) carbohydrate metabolic process, (c) carbohydrate biosynthetic process, (d) small molecule metabolic process, (e) oxoacid metabolic process, (f) organic acid metabolic process, (g) carboxylic acid metabolic process, (h) cellular ketone metabolic process, (i) cellular amino acid metabolic process, (j) cellular carbohydrate metabolic process, (k) cellular nitrogen compound metabolic process, (l) cellular amine metabolic process, (m) cellular amino acid and derivative metabolic process, (n) protein complex assembly, (o) protein complex biogenesis, (p) macromolecule localization, (q) localization, (r) tRNA metabolic process, (s) amino acid activation, (t) tRNA aminoacylation, (u) transport, (v) establishment of localization, (w) translation, (x) tRNA aminoacylation for protein translation, (y) cellular macromolecule localization, (z) cellular protein localization, (aa) ncRNA metabolic process, (ab) vesicle-mediated transport, (ac) intracellular transport, (ad) catabolic process, (ae) cellular catabolic process, (af) macromolecular complex assembly, (ag) cellular component assembly, (ah) protein localization, (ai) macromolecular complex subunit organization, (aj) establishment of protein localization, (ak) intracellular protein transport, (al) protein transport, (am) ion transport, (an) cation transport, (ao) establishment of localization in cell, (ap) cellular localization, (aq) cellular protein metabolic process, (ar) cellular component biogenesis. The GO terms for the molecular function are: (a) protein tyrosine kinase activity, (b) nucleoside-triphosphatase activity, (c) pyrophosphatase activity, (d) hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, (e) hydrolase activity, acting on acid anhydrides, (f) ATPase activity, (g) ATPase activity, coupled, (h) ATPase activity, coupled to transmembrane movement of substances, (i) ATPase activity, coupled to movement of substances, (j) hydrolase activity, acting on acid anhydrides,
catalyzing transmembrane movement of substances, (k) adenyl nucleotide binding, (l) catalytic activity, (m) purine nucleoside binding, (n) nucleoside binding, (o) nucleotide binding, (p) purine nucleotide binding, (q) purine ribonucleotide binding, (r) ribonucleotide binding, (s) P-P-bond-hydrolysis-driven transmembrane transporter activity, (t) primary active transmembrane transporter activity, (u) transporter activity, (v) ATP binding, (w) adenyl ribonucleotide binding, (x) substrate-specific transporter activity, (y) oxidoreductase activity, (z) active transmembrane transporter activity, (aa) ligase activity, (ab) protein transporter activity, (ac) transmembrane transporter activity, (ad) cofactor binding, (ae) coenzyme binding, (af) aminoacyl-tRNA ligase activity, (ag) ligase activity, forming carbon-oxygen bonds, (ah) ligase activity, forming aminoacyl-tRNA and related compounds, (ai) transferase activity, transferring hexosyl groups, (aj) ion transmembrane transporter activity, (ak) lyase activity, (al) hydrolase activity, hydrolyzing O-glycosyl compounds, (am) substrate-specific transmembrane transporter activity, (an) hydrolase activity, acting on glycosyl bonds, (ao) transferase activity, transferring glycosyl groups. The GO terms for the cellular component are: (a) cell, (b) cell part, (c) cytoplasm, (d) cytoplasmic part, (e) membrane coat, (f) coated membrane, (g) membrane, (h) membrane part, (i) protein complex, (j) macromolecular complex, (k) integral to membrane, (l) intrinsic to membrane, (m) intracellular. Abbreviations of the samples are the same as in Figure 1.

Figure 5

The distribution of differentially expressed genes in KEGG pathways. The top 10 enriched KEGG pathways based on FDR-corrected p-value (p < 0.05) in each six comparisons were displayed in y-axis and the number of DEGs under each KEGG pathways were displayed in x-axis. Abbreviations of the samples are the same as in Figure 1.

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