Comparative transcriptome profiling provides insights into different photosynthetic responses in two tall fescue cultivars under low-light stress

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Abstract

Background

Tall fescue (*Festuca arundinacea* Schreb) is a primary cool-season forage and turfgrass, low-light (LL) stress is a primary limiting factor for turfgrass growth. Studies on two tall fescue cultivars, Arid 3 and Airlie, showed that Airlie is more susceptible to LL stress than Arid 3. However, the underlying susceptibility mechanism is obscure.

Results

In this study, we investigated the physiological and transcriptional changes of two tall fescue cultivars under LL stress. The two cultivars differed in growth characteristics, chlorophyll contents, photosynthetic gas exchange, and chlorophyll fluorescence. A total of 136925 unigenes were obtained from our RNA-sequencing. There were 24944 and 30816 differentially expressed genes (DEGs) between LL stress and the control in Arid 3 and Airlie, respectively. Meanwhile, there were 20599 DEGs detected between Arid 3 and Airlie under the control, and under LL stress the DEGs between two cultivars were 20783. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis suggested that the DEGs are mainly involved in 'cell prap' and 'glutathione metabolism'. These results indicated that LL stress affects the expression of phytochrome-interacting factor 5 (*PIF5*), constitutively photomorphogenic 1 (*COP1*), phytochrome A (*PhyA*), and phytochrome B (*PhyB*) in tall fescue, which are vital transcription factors. Genes related to chlorophyll metabolism and photosynthesis were also affected by LL stress, and were significantly differentially expressed between the two cultivars.

Conclusions

This study reveals two tall fescue cultivars develop different physiological and transcriptional changes to cope with LL stress, and provides an insight into different photosynthetic responses in two tall fescue cultivars responds to LL stress.

1. Background

Turfgrass is the primary vegetative ground cover in the landscape. It effectively reduces the environmental problems and improves the quality of our life. However, low-light (LL) stress is a common environmental factor for turf deterioration [1]. It is estimated that as much as 50% of the turfgrass grown in China is subjected to varying degrees of LL stress [2]. LL stress causes numerous morphological and physiological changes to turfgrass. Leaves and leaf cuticles become thinner in shady areas, stems become taller and slender, the root biomass decreases, and the leaf color becomes lighter [3, 4]. In addition, LL stress restricts the rate of photosynthesis, resulting in decreased turfgrass growth [5, 6]. LL stress also destroys the chloroplast ultrastructure, and reduces the activity of Rubisco [7]. However, plants have developed intricate mechanisms for adapting to biotic and abiotic stresses. The physiological growth of plants changes under LL stress. In order to adapt to LL stress, leaves adjust the shape of epidermal cells and palisade tissue to

increase the ability of light to passage through leaf epidermis to the mesophyll [8]. Besides, LL stress increases the ratio of Chl *b* to Chl *a*, and plants produce more LHC π to obtain sufficient light [9]. Interestingly, turfgrass species and cultivars are variedly adapted to shade. Thus, a better understanding of the gene expressing profiles of turfgrass under LL stress is imperative to elucidate the mechanisms of turfgrass resistance to LL stress. So far, the LL stress-response mechanism in tall fescue remains unknown.

Tall fescue (*Festuca arundinacea* Schreb) is a primary cool-season forage and turfgrass, which has been widely used in temperate zones. As a hexaploidy outcrossing grass, tall fescue has better tolerance to various abiotic stresses. Notably, certain turfgrass species and cultivars have excellent growth and development under LL stress due to their higher light energy utilization or less light needs than intolerant species and cultivars [1]. In our preliminary experiments, two cultivars of tall fescue, Arid 3 and Airlie, exhibited specific LL stress tolerance. Under LL stress, Arid 3 had an excellent growth and a higher photosynthetic rate than Airlie, suggesting that Arid 3 is better adapted to LL than Airlie [10, 11]. However, the molecular mechanism of LL stress response in the two cultivars (Arid 3 and Airlie) remains unknown. This study aimed to elucidate the physiological and molecular mechanisms by which two tall fescue cultivars respond to LL stress. Profiling LL tolerant cultivars and identifying genes involved in LL stress responses greatly contributes to the study of stress tolerance mechanisms.

RNA-Seq technology, a powerful high-throughput method for investigating gene expression and regulatory networks in various plant species [12, 13], has been widely applied in plants lacking a genome sequence. Several RNA-Seq studies on Kentucky Bluegrass (*Poa pratensis*) [14], Manila Grass (*Zoysia matrella*) [15], and Japanese Lawn grass (*Zoysia japonica*) [16] focused on transcriptional changes in response to abiotic stress and associated pathways. In tall fescue, RNA-Seq was used to reveal resistance responses to cadmium (Cd) induced stress [17], investigate the transcriptome under water stress [18], explore two cultivars in response to plumbum (Pb)-induced stress [19], and analyze for thermotolerance [20]. However, no such analysis has addressed the effect of LL stress on tall fescue growth. So, this study aims to unravel the molecular mechanisms of tall fescue's response to LL stress. An RNA-seq study was performed to reveal the potential genes and networks involved in LL stress responses of two tall fescue cultivars.

2. Results

2.1. Morphology and physiology parameters under LL stress

Measurements of growth, chlorophyll contents, and gas exchange parameters were performed in the two cultivars under LL stress and the control. The tiller number and total dry weight of the two cultivars were significantly reduced after LL treatment. Notably, obvious difference in tiller number between Arid 3 and Airlie were also observed, as demonstrated by that Arid 3 produced more tillers than Airlie under LL stress (Fig. 1).

Contents of Chl *a*, Chl *b*, Chl *a* + *b*, and carotenoid decreased remarkably in the two cultivars under LL stress compared to the control. As expected, the content of detected photosynthetic pigments was more abundant in Arid 3 compared to Airlie under LL stress. In particular, the content of Chl *b* in Airlie was significantly decreased (39.1%) in Airlie under LL stress compared to Arid 3 (Fig. 1). LL treatment also reduced net photosynthetic rate (*P_n*), stomatal conductance (*G_s*), intercellular CO₂ concentration (*C_i*), and transpiration

rate (Tr) in Arid 3 and Airlie significantly compared with respective the control. Under LL stress, the value of Pn , Gs , and Tr values were 30.6%, 40.4%, and 29.5% higher in Arid 3 than them in Airlie, respectively (Fig. 2).

Chlorophyll fluorescence characteristics were measured to elucidate changes in the photosynthetic capacity of the two cultivars under LL stress. Values of F_v/F_m and Φ_{PSII} in Arid 3 decreased under LL stress compared to the control, but the difference between LL stress and the control was not significant. However, the chlorophyll fluorescence traits (F_v/F_m , Φ_{PSII} , q_p , and ETR) in Airlie remarkably reduced under LL stress compared with the control. Moreover, Arid 3 displayed a better chlorophyll fluorescence than Airlie under LL stress (Fig. 2). Collectively, this results suggested that Arid 3 possesses higher photosynthetic capacity under LL stress than Airlie, which may explain Arid 3 is more tolerant to LL than Airlie.

2.2. The sequencing data from transcriptome analysis

To understand the molecular mechanism underlying LL responses of tall fescue, RNA-sequencing was performed on two cultivars under LL stress and the control using Illumina HiSeq 2500 platform. Statistical analysis and counts of raw reads cleaned reads (after removing low-quality reads) and reads containing poly-N or adapter sequences are listed in Additional file 1. Phred quality score (Q-score) was used to assess the accuracy of sequencing. The clean Q30 base rate of all samples was above 93%, to ensure that a high quality of transcriptome sequencing data for further analysis (Additional file 1).

De novo assembly was performed to generate transcripts from our RNA-Seq data using the Trinity tool, since the tall fescue genome is not yet sequenced. A total of 423,008 transcripts and 136,925 unigenes were obtained from the sequencing data. The minimum and maximum lengths of transcripts were 201 bp and 13,340 bp, consistent with the unigenes (Additional file 2). The length of most transcripts and unigenes were concentrated between 200 to 400 bp (Additional file 3). The KOG analysis identified 25 categories and functions of unigenes. The 'general function prediction only' was the largest category (8105 genes), followed by 'signal transduction mechanisms' (3192 genes) and 'posttranslational modification, protein turnover, chaperones' (3182 genes) (Fig. 3A). Altogether, the unigenes identified in this study contribute to the data of transcriptomes responsive to LL stress in tall fescue, and provide useful information for other turfgrass and forage grass.

2.3. DEGs identification in two tall fescue cultivars under LL stress and the control

A total number of 24944 DEGs were identified in Arid 3 between LL stress and the control, whereas more DEGs were found in Airlie. For Arid 3, the number of up-regulated genes was higher than that of down-regulated genes. However, more than half of DEGs in Airlie were down-regulated (Table 2; Fig. 4). A total of 20783 DEGs were identified between the two cultivars under LL stress; over half were up-regulated. To understand which DEGs account for the LL response in tall fescue, we performed a composition analysis with DEGs identified in the two cultivars under LL stress and the control. There were considerable overlaps and distinctions in DEGs between two cultivars, and LL stress and the control. Airlie had 9269, and Arid 3 had 6403 specific DEGs after LL treatment, while the two cultivars shared 5254 DEGs (Fig. 3B; Fig. 3C). The overlapping differences between the two cultivars under LL stress suggest that they may have shared, but

different molecular mechanisms to cope with LL stress. The genes differentially expressed among samples were also shown in the heatmap (Fig. 5).

2.4. Gene Ontology categorization and KEGG pathway analysis

GO enrichment analysis of DEGs was performed under three categories, including cellular components, biological processes, and molecular functions (Fig. 6A; Fig. 6B). The DEGs in both cultivars under LL stress and the control displayed similar GO enrichment patterns, but different gene numbers. Most of the DEGs in both cultivars were involved in 'cellular process', 'metabolic process', 'cell part', 'organelle', 'binding', and 'catalytic activity'. For Arid 3, the proportion of up-regulated DEGs was higher than that of down-regulated DEGs in these abundant categories. However, The DEGs of Airlie distributed in these abundant categories showed an opposite trend.

The KEGG pathway analysis was performed to unravel the signaling pathway involved in LL response of two cultivars. It showed that 'glutathione metabolism', 'plant-pathogen interaction', 'alpha-Linolenic acid metabolism', and 'phenylpropanoid biosynthesis' were significantly enriched in Arid 3 and Airlie after LL treatment ($P < 0.05$). Apart from the co-enriched pathways, other pathways related to metabolism were also significantly enriched in Airlie. These include 'phenylalanine', 'tyrosine and tryptophan biosynthesis', 'linoleic acid metabolism', 'flavonoid biosynthesis', 'galactose metabolism', 'plant hormone signal transduction', 'amino sugar and nucleotide sugar metabolism', 'photosynthesis', 'starch and sucrose metabolism', 'flavone and flavonol biosynthesis', 'MAPK signaling pathway-plant', 'biosynthesis of amino acids, cutin, suberine', 'wax biosynthesis', and 'carotenoid biosynthesis'.

Among the metabolic pathways enriched in Airlie, the largest number of annotated DEGs were involved in 'plant-pathogen interaction' (72 up and 134 down), 'plant hormone signal transduction' (55 up and 89 down), 'biosynthesis of amino acids' (28 up and 113 down), 'phenylpropanoid biosynthesis' (27 up and 89 down). In Arid 3, 'plant-pathogen interaction' (111 up and 55 down) had the largest number of annotated DEGs, followed by 'protein processing in endoplasmic reticulum' (96 up and 14 down), 'phenylpropanoid biosynthesis' (46 up and 46 down), and 'glutathione metabolism' (47 up and 18 down) (Fig. 6C; Fig. 6D).

2.5. Differential expression of genes involved in photosynthetic procession

The expression of genes associated with chlorophyll metabolic pathways and photosynthetic pathway was obviously influenced by LL stress in two cultivars. In Arid 3, genes that encode for four important enzymes, glutamyl-tRNA synthetase (GluTR, EC:6.1.1.17), divinyl chlorophyllide a 8-vinyl-reductase (DVR, EC:1.3.1.75), protochlorophyllide oxidoreductase (POR, EC:1.3.1.33), and chlorophyll *a* oxygenase (CAO, EC:1.14.13.122) these four enzymes were down regulated. While genes encoding for chlorophyll synthase (CHLG, EC:2.5.1.62 2.5.1.133), a key enzyme for transforming Chlide to chlorophyll, was considerably up regulated after LL treatment. Surprisingly, the expression level of these genes decreased in Airlie under the LL stress, except for CHLG, which was not influenced by LL treatment (Fig. 7; Additional file 4).

In photosystem II, four genes encoding for photosystem II oxygen-evolving enhancer protein 2 (PsbP), photosystem II oxygen-evolving enhancer protein 3 (PsbQ), photosystem II PsbW protein (PsbW) and

photosystem II 13 kDa protein (Psb28) had a decreased expression level in Arid 3 after LL treatment. Arid 3 had four down-regulated genes of photosystem II, but almost all genes in Airlie's photosystem II were down regulated after LL treatment, which involved in ten genes (Fig. 8; Additional file 5). Similar results are reported for genes involved in electron transport chain and F-type ATPase, which were down-regulated in LL-treated Airlie. By contrast, only two genes in LL-treated Arid 3 were down regulated, included ferredoxin (PetF) and ferredoxin-NADP⁺ reductase (PetH). Expression of the chlorophyll a/b-binding protein, well known as an important component of the light-harvesting chlorophyll protein complex I and II, was examined in Arid 3 and Airlie. There were 18 up-regulated and 15 down-regulated genes in Arid 3 after LL treatment, whereas Airlie had only one up-regulated and 26 genes down-regulated (Additional file 6). These results indicate that in Airlie, photosynthesis process is strongly inhibited by LL treatment, while Arid 3 has better photosynthetic capacity under LL stress. This is an agreement with the better photosynthetic capacity in Arid 3 than in Airlie under LL stress.

The expression of several genes changed after LL treatment. The expression of *FaPhyA* remarkably increased in LL treated Arid 3 and Airlie compared with the control. Under LL stress, the expression level of *FaPhyA* in Arid 3 was almost twice as much as it in Airlie. For gene *FaPhyB*, under LL stress, the expression increased in Arid 3, while it decreased in Airlie when compared with the control. And the expression level in *FaPhyB* was much higher than in Airlie under LL stress. *FaPIF5* was up-regulated in both Arid 3 and Airlie, but its expression was more substantial in Airlie. The expression of *FaCOP1* in Airlie remained stable with LL treatment, but remarkably decreased in Arid 3 (Fig. 9).

2.6. Validation of RNA-seq data using qRT-PCR

Ten DEGs were randomly selected for expression analysis using qRT-PCR. Each cDNA preparation was normalized using *FaActin* and *Faα-Tubulin* as internal controls. The qRT-PCR results of the ten selected genes showed consistent expression patterns with the RNA-seq data, confirming the reliability of the Illumina sequencing results. Moreover, expression profiling of the ten DEGs through qRT-PCR and RNA-seq results showed high correlation coefficients value of 0.8835 (Fig. 10A) and 0.8812 (Fig. 10B) in Arid 3 and Airlie, respectively.

3. Discussion

When subjected to LL stress, plants utilize two different adaptation strategies: shade avoidance and shade tolerance [21, 22]. In this study, LL tolerant cultivar Arid 3 displayed better performance under LL stress than LL sensitive cultivar Airlie. The most notable features are that Arid 3 had relatively higher biomass and produced more tillers than Airlie under LL stress. Tiller is one of the important characteristics of turfgrass, which directly affects trampling resistance and the turf quality. Moreover, more tillers mean higher stem density and biomass content of fescue [23]. Chlorophyll has a significant response to LL stress; it traps light energy and drives electron transfer process, playing a central role in plant photosynthesis [24]. The content of Chl *b* in Arid 3 was observably higher than in Airlie under LL stress (Fig. 1). Under LL stress, the content of chlorophyll in cells decreases significantly, especially chlorophyll *b* of *Scenedesmus dimorphus* [25]. However, some plant species produce high levels of Chl *b* to improve light-catching ability under LL stress, as well as

increase the content of light-catching pigment complexes and the activity of photophosphorylation [26]. The synthesis of chlorophyll is regulated by many factors, including the stimulation of photoreceptors by external light [27]. The transformation from glutamic acid (Glu) to chlorophyll is catalyzed by many enzymes, such as GluTR, uroporphyrinogen III synthase (UROS), uroporphyrinogen III decarboxylase (UROD), chlorophyll specific synthase (MgCh), DVR, POR, CAO, and CHLG. The expression levels of genes encoding these key enzymes differed between Arid 3 and Airlie (Fig. 5). GluTR is the key enzyme in the biosynthesis of porphyrin compounds, regulated by biological rhythm, light, and end products [28]. As one of the three known light-dependent enzymes in the chlorophyll biosynthesis pathway, POR catalyses the reduction of photosensitizer and substrate protochlorophyllide to form the pigment chlorophyllide. The active site of POR promotes the light-driven reduction of protochlorophyllide through the transfer of local hydride and long-distance proton [29]. The different expression levels of genes encoding for these key enzymes in Arid 3 and Airlie under LL stress may explain the varied chlorophyll contents between the two cultivars. The gene encoding for chlorophyll degradation enzyme pheophorbide, a monooxygenase (PAO), was down-regulated after LL treatment. The down-regulation may be a self-protection mechanism of plants when subjected to LL stress. The expression level of *FaCHLI*, the gene related to chlorophyll biosynthesis, was higher in Arid 3 than Airlie under LL stress, which is an important evidence that Arid 3 produced more Chl than Airlie under LL stress (Fig. 7).

Photosynthesis and light are two closely related, primary processes in all plants [30]. LL stress decreases photosynthetic and transpiration rates, as reported in seashore paspalum and bermudagrass [31]. In this study, GO and KEGG enrichment analysis of Arid 3 and Airlie suggested obvious changes in photosynthesis in the two cultivars under LL stress (Fig. 6). Under the LL stress, the values of F_v/F_m and Φ_{PSII} remarkably decreased in Airlie, but the decrease in Arid 3 was not substantial. Consistent with other stresses, the degree of opening of reaction centers of PSII also declined under LL stress [32]. Compared to control, tall fescue under LL stress broke the maintenance of photochemical health due to lower Φ_{PSII} activity. Due to LL stress, several proteins of PSII, including the PsbP, PsbQ, PsbR, PsbW, Psb27, and Psb28, were down-regulated in Arid 3, more than in Airlie. The different effects of LL on PSII proteins of Arid 3 and Airlie imply a stronger photosynthetic capacity of Arid 3 (Fig. 6). Chlorophyll fluorescence provides valuable information for the study of light in the PSII photochemical reaction. The results of this study showed that LL stress inhibited F_v/F_m and Φ_{PSII} in tall fescue, suggesting that electronic produced from PSII shift from chlorophyll was limited by PQ [33]. The F_v/F_m , Φ_{PSII} , q_p , and ETR indicators were higher in Arid 3 than in Airlie under LL stress, suggesting that Arid 3 had high PSII maximal quantum yield and electron transfer efficiency.

Interestingly, the value of F_v/F_m and Φ_{PSII} in Arid 3 only changed slightly under LL stress compared with control, while in Airlie, these two indicators were substantially reduced. The q_p value represents the open ratio of PSII reaction centers. A rise in q_p value accounts for the increased capacity for electron transport, but q_p value decreases under abiotic stress [34, 35]. This study showed that q_p significantly decreased under LL stress, although the q_p value in Arid 3 maintained a remarkably higher level than in Airlie (Fig. 1), demonstrating that Arid 3 mitigates the damage of the PSII reaction center and maintains a higher ETR between electron acceptors and providers compared with Airlie. LL stress also decreased the expression of genes encoding for PetF, PetH, and PetJ, which are related to photosynthetic electron transport. Moreover, the

decreased levels of these genes in Arid 3 were lower than in Airlie. These results could explain why Arid 3 maintained a higher ETR than Airlie under LL.

Phytochrome is a widely studied family of photoreceptors that respond to red and far-red light in *Arabidopsis thaliana* [36, 37], which involves in many physiological processes, such as seed germination, seedling development, photosynthesis, and shading reaction [38]. LL stress, defined as the reduced photosynthetically active radiation (PAR) and changed red/far-red light ratio, involves phytochrome A and phytochrome B [39, 40]. Phytochrome A (*PhyA*) is the only photoreceptor capable of sensing far-red light and responding to this signal in plants [41]. In this study, the expression levels of *FaPhyA* were significantly up-regulated in both cultivars, and at higher levels in Arid 3 than in Airlie (Fig. 7). Studies have shown that *PhyA* plays an important role under LL stress, inhibiting the negative regulatory factor phytochrome-interacting factors (*PIFs*) in the photomorphogenesis process [40]. *PIFs*, a branch of the superfamily of basic helix-loop helix transcription factors, are key inhibitors of photomorphogenesis [42]. *PIF4* plays a role in the phytochrome signaling pathway [43]. *PIFs* interact with abscisic acid (ABA) receptors to regulate ABA signal transduction under LL and inhibits plant photomorphogenesis [44]. *FaPIF5* was up-regulated in two cultivars under LL stress. However, the expression level in Airlie was higher than that in Arid 3, indicating that Arid 3 inclined to photomorphogenesis, while Airlie was inclined to skotomorphogenesis under LL stress. *PhyB* functions as red-light receptors and accumulates in large amounts under the induction of red light [45].

Phytochrome A (*PhyB*) degrades *PIF5* through phosphorylation and then promotes photomorphogenesis; On the one hand, *MYB30* inhibits this degradation process, on the other hand, it directly induces the expression of *PIF5* [46]. This study showed that the expression of *FaPhyB* was significantly decreased in Arid 3, but up-regulated in Airlie. The differential expression of the positive regulatory factor *FaPhyB* in photomorphogenesis may explain shade avoidance and shade tolerance strategies utilized by the two cultivars. Constitutively photomorphogenic 1 (*COP1*), an E3 ubiquitin ligase, is a protein complex that acts as a central inhibitor in photomorphogenesis [47]. *COP1* is believed to be a central switch of global light-responsive gene expression in *Arabidopsis thaliana* by disrupting *HY5* and other transcriptional regulators critical to light morphogenesis in the dark [48, 49]. It was found that (Fig. 7) the expression of *FaCOP1* decreased remarkably in Arid 3 under LL stress compared to control, but little change was observed in Airlie. Under red light, red receptor *PhyB* enters the nucleus, where it inhibits the activity of *COP1* and promotes its degradation. *PhyB* also promotes the degradation of *PIFs*, thus promoting photomorphogenesis [50]. Synthesis of *COP1* is induced by far-red light and inhibited by *PhyA*, indicating the positive regulatory role of *COP1* in photomorphogenesis [51].

Except for *FaPIF5* and *FaCOP1*, both *FaPhyA* and *FaPhyB* played positive roles in regulating photomorphogenesis of tall fescue cultivars Arid 3 and Airlie under LL stress and the control. *FaPIF5*, *FaCOP1*, *FaPhyA*, and *FaPhyB* inter-regulated with each other to jointly promote the photomorphogenesis of Arid 3 and the skotomorphogenesis of Airlie, which explains the better performance of Arid 3 under LL stress. However, the functional mechanisms of these transcription factors in tall fescue remains to be further explored.

4. Conclusion

This study revealed gene numbers and expression levels caused by cultivar and LL stress-dependent differences in Arid 3 and Airlie. Photosynthetic physiology of tall fescue under LL stress is associated with several genes and pathways, such as phytochrome genes, chlorophyll metabolism, photosystem II, and photosynthetic electron transport. Additionally, the two cultivars had contrasting photosynthetic capacity under LL stress, reflected in the transcriptome profile. This study firstly provides a understanding of LL responses in tall fescue at the photosynthetic physiological and molecular level and highlights the importance of utilizing RNA-seq technology to determine the genes responsible for LL responses.

5. Methods

5.1. Plant materials and treatments

Seeds of tall fescue (*Festuca arundinacea* Schreb. cvs. Arid 3 and Airlie) were obtained from Beijing Clover Seed & Turf CO., China. The seeds were disinfected using 0.1% (w/v) sodium hypochlorite and rinsed thrice with distilled. At 12 d after germination, seedlings of morphologically uniform size were selected and transferred to black plastic pots (9 cm diameter × 15 cm depth) filled with sterile quartz sand (sterilized at 180°C for 2 h). Each pot contained six plants. At 24 d, all the plants were divided into four treatment groups; ACK (Arid 3 Control), ALL (Arid 3 LL), VCK (Airlie Control), and VLL (Airlie LL). Each group had 15 pots, and all were treated with distilled water. All grouped plants were grown in an artificial climate cabinet set at 25/20°C temperature and 60/50% relative humid humidity, day and night. The Light/ dark cycle was 16/8 h, and the photosynthetic photon flux density was 200 and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for control and LL stress groups, respectively. After 14 d of treatments, the second leaf of each plant was sampled and immediately frozen in liquid nitrogen and then stored at -80°C until use.

5.2. Low-light phenotyping and measurement of photosynthetic parameters

The tiller numbers of each plant were counted individually. Total dry weight contains aboveground dry weight and belowground dry weight, were determined after drying at 80°C for 72 h in drying.

Chlorophyll (Chl) and carotenoid contents were determined as previously using the modified method of Arnon [52]. In brief, approximately 0.1 g of fresh leaves were soaked in a solutions of 10 mL acetone (80%) and 5 mL ethyl alcohol (95%) for 24 h in the dark. The absorbance of the supernatant was measured at 440, 663, and 645 nm using a spectrophotometer. The contents were calculated using the formula described by Arnon [52].

Net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r) of the second fully expanded leaf from the top were monitored by open gas exchange system (Li-6400, LICOR, Inc, Lincon, NE). All samples were illuminated for 30 min with 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of saturated photosynthetic photon flux density using a 6400-02B LED light source [53].

Chl fluorescence was measured by portable pulse-amplitude-modulated fluorometer (PAM 2500, Walz, Effeltrich, Germany) with PamWin software as described by Oliveira and Peñuelas [54] on the same leaf. Four

standard parameters including, maximal quantum yield of photosystemII photochemistry (F_v/F_m) = $[(F_m - F_0)/F_m]$, effective quantum yield of photosystemII photochemistry (Φ_{PSII}) = $[(F_m' - F_s)/F_m']$, the relative electron transport rate (ETR) = $(\Phi_{PSII} \times PPFD \times 0.5)$, and photochemical quenching coefficient (q_p) = $[\Delta F'/(F_m' - F_0')]$ were computed. For ETR and q_p calculation, 0.5 is a partition coefficient of energy between PSI and PSII.

5.3. Total RNA extraction and sequencing

The second leaves of plants from the four groups were collected after 14 d under LL treatment and immediately frozen in liquid nitrogen for total RNA extraction. Three replicates were applied for each treatment of each cultivar. Total RNA was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit (TAKARA, Dalian) according to the manufacturer's instructions. The quality of RNA samples was determined with kaiaoK5500®Spectrophotometer (Kaiao, Beijing, China) and RNA Nano 6000 Assay Kit.

The cDNA libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit Illumina® (#E7530L, NEB, USA), following the manufacturer's protocol, by performing with a series of procedures, including oligo (dT) enrichment, RNA fragmentation, random hexamer-primed cDNA synthesis, poly (A) and adapter addition and PCR amplification. The cDNA libraries were sequenced on an Illumina HiSeq™ platform (Annoroad Gene Technology Co. Ltd. Beijing, China) to generate 150 bp paired-end reads were generated.

High-quality clean reads were obtain after filtering the low-quality and primer/adaptor contaminated reads with NGSQC ToolKit. The constructed library was sequenced with Illumina platform, and the sequencing strategy was PE150. The original offline sequences obtained by Illumina platform sequencing were processed by removing low-quality sequences, removing joints and other processes to obtain high-quality sequences.

5.4. Analysis of RNA sequencing data

The Trinity software was used for *de novo* assembly of tall fescue transcriptome. Busco evaluation was performed on all transcripts, and for integrity assessment, Unigene was assembled by Trinity. Meanwhile, other assessments, including accuracy and uniformity, were performed to verify the assembly results. The Trinotate (Trinotate Release v3.0.2) integration was used to obtain comprehensive functional annotation results. Functional annotations from the metabolic pathway and KOG annotations [55] were obtained to classify the functional characteristics.

5.5. Gene expression profiling

Differentially expressed genes (DEGs) between two cultivars, and the LL stress and the control were determined as $\log_2 \geq 1$ and $q \leq 0.05$ [56]. Gene Ontology (GO) analysis (<http://geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.kegg.jp/>) enrichment classification were carried out using the DEG datasets. Heatmaps were generated using the Heatmapper Plus tool of the BAR (Bio-Analytic Resource for Plant Biology) software. (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

5.6. Quantitative real-time PCR validation

Total RNA (~ 5 ug) was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (TAKARA, Dalian, China). After determining the quality of RNA by ultraviolet spectrophotometer and nucleic acid gel electrophoresis,

cDNA was reverse-transcribed using the Superscript First-Strand Synthesis System (Invitrogen, USA) following the manufacturers's instructions. Quantitative real-time PCR (qRT-PCR) was conducted using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711) with the protocol on QuantStudio®5 real-time PCR system (Life Technologies, Waltham, MA, USA). Three replicates of each gene were analyzed alongside two internal reference genes, *FaActin* and *Faa-Tubulin*. The primers used for qRT-PCR were designed by Primer Premier v 5.0 and are listed in Table 1.

5.7. Statistical Analysis

The experiments were designed in a completely randomized design with three biological replicates. All of the data were verified for normality and homoscedasticity by the Shapiro-Wilk test and Levene's test. Normal distributions and homoscedasticity tests were performed with Fisher's protected least significant difference (LSD) at a 0.05 significance level using SPSS 22 software (IBM Corp., Chicago, IL, USA). All figures were drawn using the SigmaPlot software (v 12.5, SYSTAT Software Inc., Richmond, CA, USA).

Declarations

Acknowledgments

Not applicable.

Authors' contributions

SL, QL, TL and XY conceived the research. SL and HG designed and conducted the pot experiment. SL, XL, XY, BL, SG and SW performed the laboratory analysis. SL and QL analyzed the data and wrote the manuscript with contributions from all other authors.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that no competing interests exist

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Tables

Table.1 The primers for qRT-PCR.

Gene Name	Forward Primer	Reserve Primer
<i>FaActin</i>	TCTTACCGAGAGAGGTTACTCC	CCAGCTCCTGTTCATAGTCAAG
<i>Faα-Tubulin</i>	ATGCTTTCGTCTTATGCCC	CTCTTGTTTTTGATGGTTGC
<i>FaPhyA</i> (TRINITY_DN36090_c0_g1)	AGCAAACCCTAGTGCCTTAT	TACCCAGTGTTGATGACCC
<i>FaPhyB</i> (TRINITY_DN30068_c1_g1)	AGACGACCACGAACCTGCTG	CAGCGTCTCGTTCAGGATCAT
<i>FaCOP1</i> (TRINITY_DN32337_c1_g1)	GAGGATTACTCGGCTCGTCGT	CTCAGCGAGTTCGAGGACGA
<i>FaPIF5</i> (TRINITY_DN39380_c1_g1)	TGCCTCACTGCAACAAGAGC	CCATCCCAGTTGTCATCCAC
<i>FaSPA</i> (TRINITY_DN39424_c0_g2)	TTGCTGGTTATCATCGTTTGT	TTGTCGGATTATCTGTTACAG
<i>FaCHLG</i> (TRINITY_DN39470_c0_g1)	AACAACGAAGCCGAAGGTC	CTTGATTCCGAGCAACTGG
<i>FaCHLI</i> (TRINITY_DN30755_c0_g1)	CTCGGCACAGACCTTGGAT	AATCGTTCCGCCAGTCCTA
<i>FaCB21-1</i> (TRINITY_DN14521_c0_g2)	TACATTCTTCACTGCCTTGCC	GTCTTCTCCAGCGCCATAAA
<i>FaPPL1</i> (TRINITY_DN26772_c0_g1)	TGGGATAAGATGAAGGAAAGG	AACGAGGATAATCAGCACAGG
<i>FaCB21</i> (TRINITY_DN14521_c0_g1)	GCAGTGTCCCACCCGTAGT	CAAGCCCGTGTCATCAGG

Table.2 Number of up-regulated and down-regulated genes between LL-treated and control samples of RNA sequencing results of tall fescue Arid 3 and Airlie ($P<0.05$).

Name	ALL_ACK	VLL_VCK
Up	12,503	14,696
Down	12,441	16,120
Total	24,944	30,816

ACK, Arid 3 control; ALL, Arid 3 treated by LL stress; VCK, Airlie control; VLL, Airlie treated by LL stress.
 ALL_ACK, ALL vs ACK; VLL_VCK, VLL vs VCK.

Figures

Figure 1

Effects of LL stress. The changes on morphological changes(A), total dry weight (B), tiller numbers (C), content of Chl a (D), the content of Chl b (E), the content of Chl a+b (F), the content of Car (G). ACK (Arid 3

control), ALL (Arid 3 treated by LL stress), VCK (Airlie control), and VLL (Airlie treated by LL stress). Each value is the mean±SD of five independent experiments (n=5). Different letters indicate significant differences at P < 0.05, according to Duncan's multiple range tests.

Figure 2
Effects of LL stress on gas exchange and chlorophyll fluorescence parameters. Pn (A), Gs (B), Ci (C), Tr (D), Fv/Fm (E), ΦPSII (F), qp (G), ETR (H).

Figure 3

KOG annotation and classification. The x-axis represents 25 groups of KOG, and the y-axis indicates the percentage of the number of genes annotation under the group in the total number of genes annotation (A). The x-axis shows the fold-change, and the y-axis shows statistical significance of the differences. Significantly Up and Down-regulated genes are filtered ($|\log_2(\text{Fold Change})| > 1$, $P < 0.05$) and highlighted in blue and yellow dots, respectively. Venn diagram for differentially expressed genes between different comparisons (B). The differentially expressed genes count (C).

Figure 4

Volcano plot for differentially expressed genes. The Volcano plot between ALL and ACK (A), VLL-VCK (B).

Figure 5

Heatmap of genes differentially expressed. The Heatmap between ALL and ACK (A), VLL and VCK (B). Blue color represents low expressed genes and red color represents highly expressed genes. Cluster analysis was done using the $\log_2(\text{FPKM} + 1)$ value from larger to smaller.

Figure 6

Gene Ontology classification. The classification of ALL-ACK (A), VLL-VCK (B). Red color represents up-regulated genes and green color represents down-regulated genes. KEGG pathway enrichment analysis of ALL and ACK (C), VLL and VCK (D). The size of each dot indicates the number of DEGs enriched in the pathway, the color closer to red indicated the higher the enrichment degree.

Figure 7

The expression of genes encoding important enzymes in chlorophyll metabolic pathway between ALL-ACK and VLL-VCK. Blue color represents down-regulate and red color represents up-regulate. The darker the gradient, the larger the value of the gradient.

Figure 8

Photosynthesis-related gene expression based on the KEGG pathway analysis. Blue represents the down-regulation. The darker the gradient, the larger the value of the gradient.

Figure 9

Relative expression level of some genes on tall fescue Arid 3 and Airle.

Figure 10

Correlation of the RNA-Seq and qRT-PCR data. The assay was conducted with ten DEGs (transcripts from the VLL vs VCK (A) and ALL VS ACK (B) comparisons).

Supplementary Files

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