Chlorophyll-associated genes and SSR markers linked to genetic variations in heat tolerance of perennial ryegrass

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Abstract

Identification of genetic diversity in heat tolerance and associated markers is of great importance for improving heat tolerance in cool-season grass species. In this study, 98 accessions of perennial ryegrass (Lolium perenne L.) were subjected to heat stress (35/30 , day/night) or optimal growth temperature (25/20) for 24 d in growth chambers. Overall heat tolerance of those accessions was ranked by principal component analysis (PCA) based on eight growth and physiological traits. Among these traits, chlorophyll (Chl) content had the highest correlation coefficient (0.864) with the PCA ranking of heat tolerance, indicating it was the most closely linked parameter to heat tolerance. And expressions of four Chl catabolic genes (CCGs) were negatively correlated with PCA ranking of heat. Furthermore, simple sequence repeat (SSR) markers were identified that significantly associated with Chl content and other heat tolerance-related traits. Together, the result highlighted the importance of Chl catabolism in heat tolerance of cool-season grasses. Chl content, heat-associated CCG genes and their associated SSR markers could be used as reliable trait or molecular markers in the breeding program of perennial ryegrass toward better heat tolerance.

Abbreviations: Chl, chlorophyll; TQ, turf quality; Fv/Fm, photochemical efficiency; RWC, leaf relative water content; Pn, photosynthetic rate; WUE, water use efficiency; RA, root activity; LW, leaf width; PH, maximum plant height; PCA, principal component analysis; HSI, heat stress index; SSR, simple sequence repeat; CCGs, Chl catabolic genes.

INTRODUCTION

Improving heat tolerance is among major efforts of breeding improvement in cool-season grass species (Xu et al., 2018). The available germplasm collections with large genetic variability and a wide range of heat-tolerance levels are important breeding materials for grass breeding program (Jespersen et al., 2017; Krans et al., 2000; Minner et al., 1983). While lack of genetic variability and limited knowledge of physiological and molecular factors underlying heat stress tolerance restrict the efficiency on identification of heat-tolerant cultivars.

Genetic diversity among germplasms with varied levels of stress tolerance can be evaluated using phenotypic traits and molecular markers (Cao et al., 2015; Li et al., 2017; Varshney et al., 2010). Phenotypic analysis

focuses on growth, morphological, and physiological parameters among germplasm and provides information regarding genetic diversity, homogeneity, and stability (Li et al., 2017; Ober et al., 2005). Since abiotic stress adversely affect a multitude of morphological and physiological processes, a number of distinct morphological and physiological characteristics have been used as marker traits to evaluate genetic variations in plant stress tolerance, such as root activity (RA), photochemical efficiency (Fv/Fm), photosynthetic rate (Pn), water use efficiency (WUE), chlorophyll (Chl) content, and leaf relative water content (RWC) (Larkindale et al., 2005; Li et al., 2017; Shah et al., 2011; Zhang et al., 2017). The selection of germplasm based on physiological traits is an efficient approach in breeding for improved stress tolerance in various crop species (Paolo et al., 1998; Rana et al., 2002). In the case of cool-season grass breeding for heat tolerance, it is pivotal to firstly identify phenotypic and/or physiological traits closely correlated with heat tolerance, which traits can be used as markers for the rational development of improved germplasm (Jespersen et al., 2017; Mondal et al., 2015).

Leaf senescence characterized by loss of chlorophyll is one hallmark of heat stress damages in cool-season grass species. Our previous studies found that leaf senescence induced by heat stress was mainly due to heat accelerated chlorophyll (Chl) catabolism rather than attenuated Chl biosynthesis in one cool-season grass, creeping bentgrass (*Agrostis stolonifera*) (Jespersen et al., 2016) and reducing Chl catabolic rate by suppressing a Chl catabolic gene (*PPH*) in perennial ryegrass (*Lolium perenne* L.) delayed heat-induced leaf senescence (Zhang et al., 2019). Selecting for stay-green traits by controlling chlorophyll loss or leaf senescence is of great significance for developing heat-tolerant cool-season grass cultivars using leaf senescence-related parameters for heat tolerance assessment.

Molecular markers linked to phenotypic and physiological traits have been developed to understand the genetic diversity and to predict desirable traits of a given germplasm or breeding materials (Li et al., 2017). To date, molecular makers associated with several important agronomic traits of perennial ryegrass have been developed, including crown rust resistance (Dumsday et al., 2003), drought tolerance (Yu et al., 2013), winter survival and spring re-growth (Yu et al., 2015), submergence (Yu et al., 2011), and salinity tolerance (Tang et al., 2013). For examples, Yu *et al.* (2011) evaluated the submergence tolerance of 99 diverse perennial ryegrass accessions using 109 simple sequence repeat (SSR) markers, and identified 15 pairs of SSR markers associated with alterations of several morphological and physiological traits (e.g. leaf color, Fv/Fm, maximum plant height, and relative growth rate). Tang *et al.* (2013) analyzed the genetic diversity of 56 perennial ryegrass accessions of different origins using 66 SSR makers, and found that population structure influenced phenotypic traits, and allelic variation in *LpNHX1* might explain the variation of salinity tolerance of perennial ryegrass.

Perennial ryegrass (*Lolium perenne* L.), native to Europe, Asia, and Northern Africa, is the most widely cultivated perennial cool-season grass in the temperate regions worldwide for its turf and forage purposes (Altpeter et al., 2000; Chastain et al., 2015), but it is one of the most heat sensitive grass species. Despite of the diversity of perennial ryegrass to various other stresses and diseases, as discussed above, neither the diversity of heat tolerance among perennial ryegrass germplasm collections was quantitatively measured, nor the molecular markers associated with heat tolerance been developed for this species. The objectives of this study were to determine genetic variations in heat tolerance associated with phenotypic and physiological traits and to identify molecular markers associated with heat tolerance in a diverse collection of perennial ryegrass.

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

A collection of 98 perennial ryegrass accessions was obtained from United States Department of Agriculture's National Plant Germplasm System (USDA-GRIN), including 37 wild, 33 cultivated, and 28 with uncertain pedigree accessions (Table 1). A single seed of each germplasm was sown in plastic pots (13 cm diameter and 13 cm height) filled with fritted clay and maintained in a greenhouse at Nanjing Agricultural University, Jiang Su, China. Each accession was propagated using tillers to generate stock plants. In this experiment,

tillers from stock plants of each accession were transplanted into eight pots (with each pot has 10 tillers) and maintained in a growth chamber controlled at 25/20 (day/night temperature), 70% relative humidity, a photoperiod of 16 h, and photosynthetic active radiation of 750 μmol $m^{-2}s^{-1}$ for 60 d. Plants were maintained at a height of 12 cm by weekly mowing and fertilized weekly with half-strength Hoagland's nutrient solution (Hoagland & Arnon, 1950).

2.2 Temperature treatments

Plants in four pots (four replicates) for each accession were exposed to normal growth temperature of 25/20 (day/night) or heat stress at 35/30 (day/night) in growth chambers. Each temperature treatment was repeated in two growth chambers. Plants were subjected to those temperature conditions for 24 d. The plants received regular water and fertilization during the treatment period.

2.3 Phenotypic and physiological measurements

At 24 d of heat stress treatment, growth and physiological parameters, including leaf width (LW), maximum plant height (PH), turf quality (TQ), photochemical efficiency (Fv/Fm), chlorophyll (Chl) content, leaf relative water content (RWC), photosynthetic rate (Pn), water use efficiency (WUE), and root activity (RA) were measured. For LW analysis, 10 mature leaves of each accession were measured using a digital caliper. PH was measured manually as the length from the base to the top of each plant. TQ was visually rated using a 1–9 scale based on plant color, shoot density and uniformity to assess the overall plant health and vigor (1 represents brown and dead plant, and 9 represents the best plant in all these quality components) (Turgeon, 1991). Fv/Fm was determined using a fluorescence meter (Dynamax, Houston, TX, USA), described previously by Oxborough and Baker (1997). Chl content was measured according to the method by Arnon (1949). In brief, about 0.1 g of leaves were soaked in 10 ml dimethyl sulfoxide and maintained in the dark for 72 h, and then the absorbance of extracts at 663 nm and 645 nm were measured using a spectrophotometer (Spectronic in Instruments, Rochester, NY, USA). For RWC quantification, about 0.1 g fresh leaves were detached and immediately weighed as the fresh weight (FW), then soaked in distilled water and maintained at 4 °C in the dark for 24 h and weighted as turgid weight (TW). Leaf samples were then placed in an oven at 80 °C for 72 h prior to being weighted for dry weight (DW). The RWC was calculated as formula: (FW-DW)/(TW-DW) ×100% (Flexas et al., 2006). Pn and WUE were measured according to the method described by Burgess and Huang (2014) using a portable photosynthesis system (Li-COR6400, LI-COR Inc., Lincoln, NE, USA). RA was measured using 2,3,5-triphenyl tetrazolium chloride (TTC) reduction method (Comas et al., 2000). The TTC reduction assay was performed following the method of Steponkus and Lanphear (1967) with minor modifications. In brief, approximately 0.5 g root tips (0-50 mm) were excised and washed three times with distilled water and transferred to 10 ml TTC solution (0.4% TTC in 0.1 M sodium phosphate buffer, pH 7.0). After 3 h incubation at 37 °C, TTC solution was removed and root segments were washed with distilled water. The roots were cut into 1 cm segments and incubated overnight at room temperature in 10 ml of 95% ethanol. The reduction of TTC was expressed as the absorbance of the extraction solution at 485 nm.

2.4 Ranking of overall heat tolerance

Heat stress index (HSI) was used to evaluate the overall heat tolerance for 98 accessions integrating multiple growth and physiological traits according to the following formula (Bouslama and Shapaugh, 1984), which was expressed as percentage of control, HSI = (value of parameter under heat stress condition) / (value of parameter under control condition) ×100. Principal component analysis (PCA) was used to rank heat tolerance of 98 accession, following the method used in ranking of drought and salinity tolerance for different grass genotypes as described in Liu*et al.* (2015) and Tang *et al.* (2013). PCA ranking value for each accession was calculated using the formula: PCA rank value $=\sum_{j=1}^{n}$ [PCj × contribution of PCj (%)] $j = 1,\ 2,\ 3,\ \ldots, n$.(Zhu et al., 2014). In this formula, 'PCj' represents the value of principal component j and 'contribution of PCj (%)' represents the variance in response to stress treatment that principal component$j$ could explain. When the total contribution of first several PCs was higher than 85%, these PCs were selected for PCA rank calculation (Huang et al., 2017). Relative heat tolerance of 98 perennial ryegrass accessions was subsequently ranked

according to their PCA ranking values.

2.5 Genetic diversity and association analysis

A total of 66 pairs of publicly available SSR primers of nuclear DNA distributed on seven chromosomes (Supplementary materials 1) were used to genotype the 98 perennial ryegrass accessions (Tang et al., 2013). PCR reaction was performed in a 10 μl reaction volume with 60 ng DNA, 1.0 unit of *Taq* DNA Polymerase, 1×PCR buffer, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 0.05 μM forward tailed primer, 0.05 μM fluorescent labeled M13 primer, and 0.1 μM reverse primer. All PCR reactions were carried out in a Bio-Rad thermocycler (Bio-Rad Inc., Hercules, CA, USA) using a touch-down program described by Yu *et al.* (2013). PCR products were separated in an ABI 3730 DNA Sequencer (Applied Biosystem, Inc., Foster City, CA, USA). Alleles were identified by GeneMarker 1.6 software (SoftGenetics, LLC, State College, PA, USA). The allelic bands with at least 2 bp differences were considered as polymorphic among accessions. All confirmed polymorphic alleles were used for cluster analysis.

Nei's genetic distance among accessions was estimated by the "Phylogeny" function of PowerMarker software version 3.25 based on the SSR marker data (Nei, 1973). The Nei's genetic distance was then used for cluster analysis and generation of Neighbour Joining (N-J) dendrogram by FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) (Zhang et al., 2019). Association analysis of SSR markers with heat tolerance traits was conducted using TASSEL 5.0 software along with the general liner model (GLM) procedure (Bradbury et al., 2007). P [?] 0.01 was used to identify significant associations.

2.6 Gene expression analysis

The Qiagen RNA Extraction Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA form perennial ryegrass leaves following the manufacturer's instructions. The first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY, USA). The qRT-PCR reaction was performed using the Roche LightCycler® 480 II Real-Time PCR System (Roche Molecular Systems, Inc., Branchburg, NJ, USA). All PCR reactions were performed in a 20 μl reaction volume with four biological replicates. Primers used for qRT-PCR analysis were listed in supplemental table 1 and *LpeIHF4A*was used as a reference gene (Huang et al., 2014).

2.7 Data analysis

Data from all samples were subjected to analysis of variance (ANOVA) using SAS 9.3 software (SAS Institute Inc., Cary, NC, USA) as shown in Table 2. Treatment means were separated using Fisher's protected LSD test at a 0.05 probability level as shown in Table 5 and Figure 5. Pearson correlation analysis between physiological parameters was performed using the Bivariate Correlations program in software SPSS for Windows (Version 12, SPSS Inc., Chicago, IL) as shown in Table 4. Heatmap (Figure 1) and principal component analysis (PCA) (Figure 2) of the morphological and physiological parameters were performed using R statistical software (R3.5.2 by R Development Core Team). Correlation between CCGs' expression levels and PCA ranking values was analyzed using linear regression analysis program in Microsoft Office Excel 2013 software (Microsoft Corporation) (Figure 5 & 6).

RESULTS

3.1 Evaluation of heat tolerance in 98 accessions of perennial ryegrass

A total of six physiological traits (WUE, Pn, RA, Chl content, RWC, and Fv/Fm) and three phenotypic traits (TQ, LW, and PH) were used to evaluate heat tolerance of 98 perennial ryegrass accessions. Effects of heat stress treatment, genotype, and interaction of these two factors were all significant (p [?] 0.05) for WUE, Pn, RA, Chl, RWC, Fv/Fm, and TQ. Only genotypic variations were significant for LW and PH (Table 2).

The data for physiological and phenotypic traits of 98 accessions exposed to heat stress or optimum temperature (control) conditions were used to plot a heatmap (Figure 1), in which the hierarchical cluster was clearly grouped into two distinct sub-clusters: accessions under the control condition (sub-cluster 'a') and

those under the heat stress (sub-cluster 'b' as shown in Figure 1). Accordingly, nine traits used to evaluate heat tolerance were grouped into three sub-clusters: sub-cluster I included WUE, Pn, and RA, where their values were lower under heat stress than those under control condition; sub-cluster II included Chl content, TQ, RWC, and Fv/Fm, and their values were also lower under heat stress; sub-cluster III, consisting of the two morphological traits, PH and LW, were not responsive to temperature effects (Figure 1). Therefore, seven traits, WUE, Pn, RA, Chl content, RWC, and Fv/Fm, and TQ were used to evaluate heat tolerance in the following analyses.

3.2 Ranking of overall heat tolerance for 98 accessions of perennial ryegrass

The PCA analysis of variations in heat tolerance of 98 accessions based on HSI identified a total of seven principal components (PC 1-7). The sum of the first PCs (PC1 to PC3) explained 86.3% of the total variance, among which the 1st, 2ndand 3rd PCs were the major ones explaining 54.3%, 23.3% and 8.7% of the variance among 98 ryegrass accessions, respectively (Supplementary Table 2). Based on the PCA results, the following formulas were developed (details of the formula were shown in supplementary Table 2 and supplementary Table 3): PC1 value = 0.915×TQ + 0.791×Fv/Fm + 0.858×Chl content + 0.369×Pn + 0.243×WUE + 0.887×RWC + 0.789×RA; and PC2 value = (-0.186)xTQ + (-0.298)xFv/Fm + 0.003xChl content + 0.813xPn + 0.900xWUE + (-0.172)xRWC + 0.049xRA; and (3) PC3 value = 0.096xTQ + 0.366xFv/Fm + (-0.255)xChl content + 0.312xPn + (-0.079)xWUE + 0.173xRWC + (-0.517)xRA, and PCA ranking value = (54.34%xPC1) + (23.25%xPC2) + (8.69%xPC3).

According to PCA results, we clustered 98 ryegrass accessions into two groups:Group-i consisted of 49 accessions with PCA rank value at the top half of all accessions, while the rest 49 accessions clustered to group-ii (Figure 2). Heat tolerance of 98 accessions were then ranked according to their PCA ranking values based on the HIS of seven parameters (WUE, Pn, RA, Chl content, RWC, and Fv/Fm, and TQ), with accessions 598892, 275660, 516605, 231565, and 598443 ranked as the top five accessions for heat tolerance and 311075, 538976, 278773, 610950, and 321681 ranked as the most heat-sensitive accessions (Table 3). Phenotypes of the top and least five heat tolerant ryegrass accessions were shown in Figure 3 that plants of the top-rated accessions had more green leaves or greener leaves while the most heat-sensitive accessions had more yellow or less green leaves.

To understand contributions of different traits to heat tolerance in perennial ryegrass, the relationships between PCA ranking based on HSI for overall heat tolerance and each physiological/phenotypic trait was determined using Pearson correlation analysis. As shown in Table 4, the PCA ranking was significantly correlated with all seven traits used in the evaluation ($p < 0.05$), among which ranking values of PCA and Chl content had the largest correlation coefficient (r= 0.864). And ranking values of TQ, RWC, and Fv/Fm also showed high correlation coefficients with those of PCA ($r = 0.840$, 0.833, and 0.715, respectively). Values of Pn and WUE had low correlation coefficients with those of PCA (r = 0.432 and 0.309, respectively), although their correlations were statistically significant as well ($p < 0.05$) (Table 4).

3.3 Transcript levels of chlorophyll-catabolic genes correlated to heat tolerance in perennial ryegrass

Results of Pearson correlation analysis indicated that heat tolerance ranking based o PCA and Chl content had the largest correlation coefficient (Table 4), indicating leaf senescence characterized by Chl loss was mostly associated with overall heat tolerance in perennial ryegrass. To confirm the contribution of Chl catabolism to heat-tolerant accessions of perennial ryegrass, we further analyzed whether there was a correlation between transcription of four Chl catabolic genes (*CCG* s, including *LpNYC1* , *LpNOL* , *LpSGR* , and*LpPPH*) and the PCA ranking values of heat tolerance of 98 ryegrass accessions. As shown in Figure 5, relative expression levels of*CCG* s were significantly higher in heat-sensitive accessions than those in heat-tolerant accessions. The relationship between the relative expression levels of *CCG* s and PCA ranking values were further analyzed using linear regression analysis and Pearson correlation coefficient analysis. As shown in Figure 6, relative expression levels of *LpNYC1* , *LpNOL* , *LpSGR* , and *LpPPH* had strong positive correlations to PCA ranking values with R^2=0.943, 0.878, 0.814, and 0.896, respectively.

3.4 Classification of 98 accessions of perennial ryegrass based on SSR markers

Genotypic diversity within the selected ryegrass accessions was estimated using 66 pairs of SSR molecular markers. The SSR analysis yielded 864 polymorphic bands in total, with an average of 13 and a range of 3 to 26 bands *per* pair of primers (Supplementary material 2). The resultant polymorphism information content (PIC) values varied from 0.16 to 0.93, with an average of 0.70; while the gene diversity index (Di) values ranged 0.16 to 0.94, with an average of 0.72 (Supplementary material 2), confirming that the selected accessions represented a diverse genetic pool of perennial ryegrass germplasm. An N-J dendrogram was constructed based on the SSR results, clustering the 98 ryegrass accessions into three groups: Cluster A, B, and C consisting of 14, 10, and 74 ryegrass accessions, respectively (Figure 4).

Values of PCA ranking of heat tolerance and physiological traits were averaged across ryegrass accessions in each phylogenetic cluster (Table 5), and the results showed that averaged PCA ranking values of accessions in cluster C (146.2) were significantly higher than those in clusters A and B (123.9 and 127.9, respectively), suggesting that accessions in clusters A and B were less heat tolerant than those in cluster C. Similar difference was also observed for Chl content, WUE, and RA for genotype ranking of heat tolerance in each phylogenetic cluster (Table 5).

3.5 SSR markers associated with physiological traits in heat tolerance

The associations between the 66 SSR markers and the seven physiological traits were further analyzed using a general linear model (GLM) in TASSEL. As shown in Figure 7 and Table 6, a total of 30 associations were identified between the SSR markers and the relative values of Chl content, Fv/Fm, Pn, WUE, and RA at R^2>0.05 (p <0.01). We found that two markers M144 and rv0941, located on chromosome 4, were associated with Chl content. The markers Lp165, rv0941, DLF008, B3C10, B3B8, and B5E1, located on chromosomes 3, 4, 5, and 7, were associated with Fv/Fm. The marker rv0985-1, located on chromosome 6, was associated with Pn. Thirteen markers, including PRG, PR10, M4213, 25ca1, LPSSRH01A07, rv0985-1, rv0005, rv1133, LPSSRH02C11, rv0663, B3B7, LpHCA16B2, and PR37, located on chromosomes 1, 3, 4, 6, and 7, were associated with WUE. The markers M844, LPSSRH01A07, B3B7, B1A10, LpSSR100, LP194, rv0757, and LPSSRH02C11, located on chromosomes 1, 3, and 5, were associated with RA. No association was identified between SSR markers and TQ or RWC (Figure 7).

DISCUSSION

Plant heat tolerance is a complex trait that could be attributed by many phenotypic and physiological factors, including those parameters examined in this study, such as Fv/Fm, Pn, Chl content, WUE, and RWC, and root activity (Barnabas et al., 2010; Jiang & Huang, 2001; Kahan et al., 2014; Larkindale et al., 2005; Shah et al., 2011; Zhang et al., 2019). Therefore, PCA integrating all those parameters was used to rank heat tolerance among 98 accessions of perennial ryegrass, with 598892, 275660, 516605, 231565, and 598443 ranked as the top five heat-tolerant accessions and 311075, 538976, 278773, 610950, and 321681 as the least heat-tolerant accessions. Similar strategy has been successfully adopted in stress tolerance evaluation in soybean (*Glycine max* . L.) and switchgrass (*Panicum virgatum* L.) (Bouslama & Shapaugh, 1984; Liu et al., 2015; Wojcik-Jagła et al., 2013). Liu *et al.* (2015) have used PCA to analyze drought tolerance of 49 switchgrass accessions by integrating values of seven physiological traits and four morphological traits. Heat tolerance ranking was further associated with specific growth and physiological traits in this study.

Through Pearson correlation analysis, Chl content was found to be most closely linked to overall heat tolerance ranking based on PCA, followed by TQ, RWC, and Fv/Fm, while Pn and WUE had low correlation coefficients with those of PCA ranking for heat tolerance in 98 accessions of perennial ryegrass. These results suggested that Chl content or stay-green trait could be used as the major selection criterion for a large population of perennial ryegrass germplasm for elite heat tolerance, although TQ, RWC, and Fv/Fm were also found to be good indicators of heat tolerance.

Leaf senescence and Chl loss is the most remarkable phenotype of cool-season grass species after prolonged heat stress. Jespersen*et al* . (2016) showed that heat-induced Chl decline was mainly due to accelerated Chl

degradation rather than attenuated Chl biosynthesis in *Agrostis* species, suggesting that Chl catabolism, rather than Chl biosynthesis, was the dominant mechanisms associated with heat tolerance in perennial grass species. Our previously work have found that the expression levels of three Chl catabolic genes, including*LpNYC1* , *LpNOL* , and *LpPPH* , were significant lower in a heat-tolerant genotype of perennial ryegrass than heat-sensitive genotype under heat stress. Moreover, the expression levels of these CCGs were negatively correlated to ROS level, while positively correlated to activities of ROS scavenging enzymes (Zhang et al., 2019). In the current study, we found that the transcript levels of four CCGs, including *LpNYC1* , *LpNOL* , *LpSGR* , and *LpPPH* , were strongly correlated to heat tolerance ranking, indicating that these CCGs could be used as marker genes to select for heat-tolerant plants with less Chl loss or stay-green traits in perennial ryegrass, and potentially applicable for other cool-season grass species as well. Thus, we considered that Chl content and Chl catabolic genes were reliable physiological trait and marker genes for evaluation of heat tolerance in perennial ryegrass accessions.

Understanding the genetic structure and phenotypic diversity is the basis of parental selection for trait improvement in breeding, such as for heat tolerance in this study. Barre *et al.* (2017) classified 213 perennial ryegrass accessions into three clusters according to their vegetative and reproductive investment traits (e.g. leaf growth parameters, tillering parameters, heading data and reproductive investment). SSRs derived from expressed sequence tags (ESTs) are most widely used marker system because they might be linked to known genes, lower cost for development, and having higher transferability among related species (Wang et al., 2018). In this study, the 98 accessions of perennial ryegrass were classified into three clusters according to clustering analysis using 66 pairs of SSR markers. The same sets of SSR markers have also been used to understand genetic diversity of various perennial ryegrass germplasm and identify alleles contributing to plant tolerance to salt, drought, submergence, and winter stress, as well as spring re-growth (Tang et al., 2013; Yu et al., 2011; Yu et al., 2013). Physiological traits positively correlated to heat tolerance, including Chl content, WUE, and RA had significantly greater levels in accessions in cluster C than those in cluster A and B based on SSR marker classification, suggesting that accessions in cluster C were more heat tolerant than those in cluster A and B, and variations in heat tolerance were related to their genetic structure in the population of perennial ryegrass examined in this study. Furthermore, the most heat-sensitive and -tolerant ryegrass accessions were identified in this study that could be used as base material for ryegrass breeding in the future.

SSR markers linked with important agronomic traits, e.g. crown rust resistance (Dumsday et a., 2003), submergence (Yu et al., 2011), and heading date (King et al., 2008) in perennial ryegrass have been identified. However, no genic SSR markers that are associated with heat tolerance in perennial ryegrass have been reported so far. In the present study, twenty-five SSRs linked to heat tolerance-related traits were detected in perennial ryegrass. Two markers LPSSRH01A07 and LPSSRH02C11, both located on chromosome 4, which were reported to be associated with relative growth rate under submergence stress (Yu et al., 2011), were correlated with WUE and RA in this study, suggesting a QTL in chromosome 4 could also affect heat tolerance. It is interesting to find that three markers M4213, B3B7, and PR37, located on chromosome 1 with 6 cM apart, were linked to WUE. In addition, Lp165, DLF008, and B3C10 located on chromosome 7 with 12cM apart, were associated with Fv/Fm. Thus, it is most likely that a QTL for WUE and Fv/Fm located at chromosome 1 and 7, respectively. The results indicate that at least three candidate QTLs located in these chromosome positions play an important role in heat tolerance of perennial ryegrass.

CONCLUSION

In summary, heat tolerance varied widely among 98 accessions of perennial ryegrass examined in this study. The most and least heat-tolerant accessions were identified, and accessions in cluster C were relatively more heat tolerant than those in cluster A and B. Chl content and CCGs were reliable physiological trait and marker genes for heat stress tolerance assessment in perennial ryegrass. Furthermore, SSR markers associated with Chl content, Fv/Fm, Pn, WUE, and RA were also identified. The result highlighted the importance of Chl catabolism, either with a regulatory role in heat tolerance or as merely a passive indicator of heat-stress imposed damage, in heat tolerance of cool-season grasses. Such knowledge is of significance for heat-tolerance

breeding of perennial ryegrass and for further studies on heat tolerance mechanisms in perennial ryegrass as well as in other cool-season grass species.

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CONFLICT OF INTEREST

There is no conflict of interest.

Table 1. Accession number (PI No.), origin, status, and genetic clusters of 98 perennial ryegrass accessions.

Accession No.	Origin	Status[a]	Status[a]	Cluster	Accession No.	Origin	Status[a]
287855	Spain	Spain	U	A	284823	Australia	Australia
231606	Portugal	Portugal	U	A	231604	Portugal	Portugal
610950	Morocco	Morocco	W	A	516605	Yugoslavia	Yugoslavi
220178	Afghanistan	Afghanistan	W	A	371952	Bulgaria	Bulgaria
265340	Portugal	Portugal	U	A	440474	Former USSR	Former U
319018	Spain	Spain	U	A	265351	Chile	Chile
410155	South Africa	South Africa	W	A	231576	Algeria	Algeria
577251	Morocco	Morocco	W	A	418726	France	France
231566	Libya	Libya	U	A	403868	Canada	Canada
287849	Spain	Spain	U	A	231580	Algeria	Algeria
239730	Egypt	Egypt	U	A	634205	USA	USA
206376	Cyprus	Cyprus	U	A	198070	Sweden	Sweden
268333	Former USSR	Former USSR	W	A	321681	France	France
423136	Spain	Spain	W	A	266293	Netherlands	Netherlan
182857	Czech Republic	Czech Republic	U	B	578763	USA	USA
538976	Russia	Russia	C	B	321397	Czech Republic	Czech Re
197270	Finland	Finland	CD	B	303020	Sweden	Sweden
547390	Iran	Iran	W	B	231595	Morocco	Morocco
204085	Cyprus	Cyprus	U	B	418721	Belgium	Belgium
619474	Romania	Romania	CD	B	418722	Luxembourg	Luxembo
306292	Bolivia	Bolivia	U	B	418741	France	France
277848	Cyprus	Cyprus	U	B	619003	Norway	Norway
231567	Libya	Libya	U	B	251224	Yugoslavia	Yugoslavi
598911	Tunisia	Tunisia	W	B	628717	Bulgaria	Bulgaria
598839	Morocco	Morocco	W	C	290368	Hungary	Hungary
418712	Yugoslavia	Yugoslavia	U	C	376878	New Zealand	New Zeal
277846	Yugoslavia	Yugoslavia	U	C	303037	Sweden	Sweden
610802	Norway	Norway	W	C	420126	Japan	Japan
231565	Libya	Libya	U	C	204880	Turkey	Turkey
225825	Denmark	Denmark	U	C	265344	Ireland	Ireland
204879	Turkey	Turkey	W	C	237187	Netherlands	Netherlan
265342	Ireland	Ireland	C	C	577254	Luxembourg	Luxembo
303026	France	France	C	C	274637	Poland	Poland

Accession No.	Origin	Status[a]	Status[a]	Cluster	Accession No.	Origin	Status[a]
275660	Australia	Australia	CD	C	298091	Hungary	Hungary
418723	Luxembourg	Luxembourg	W	C	285101	Australia	Australia
577273	Turkey	Turkey	W	C	505842	Former USSR	Former U
577269	Norway	Norway	W	C	632542	Hungary	Hungary
238938	New Zealand	New Zealand	U	C	610965	Italy	Italy
267059	Poland	Poland	U	C	202451	Argentina	Argentina
303027	Denmark	Denmark	C	C	231605	France	France
317452	Afghanistan	Afghanistan	W	C	284826	Australia	Australia
403907	Canada	Canada	C	C	502412	Russia	Russia
278773	Canada	Canada	C	C	418714	Italy	Italy
254898	Iraq	Iraq	W	C	598434	Italy	Italy
267058	Poland	Poland	U	C	610926	Tunisia	Tunisia
418736	Switzerland	Switzerland	W	C	420128	Japan	Japan
598443	Switzerland	Switzerland	W	C	418707	Romania	Romania
600878	USA	USA	C	C	598892	Tunisia	Tunisia
303028	Denmark	Denmark	C	C	311075	Romania	Romania

Status[a]: Improvement status obtained from USDA germplasm bank. U: uncertain; W: wild; C: cultivar; CD: cultivated.

Table 2. Effects of treatments and genotypes, and the interaction effect between temperature and genotype on phenotypic traits. Abbreviations: Turf quality (TQ), photochemical efficiency (Fv/Fm), chlorophyll content (Chl, mg/g DW), photosynthesis rate (Pn, μmol $CO_2/m^2/s$), water use efficiency (WUE, μmol CO_2/mmol H_2O), leaf relative water content (RWC, %), root activity (RA, mg/g·h), leaf width (LW, cm), and maximum plant height (PH, cm).

	Physiological traits	Physiological traits	Physiological traits	Physiological traits
	TQ	**Fv/Fm**	**Chl**	**Pn**
Treatment	***	***	***	***
Genotype	**	*	**	**
Treatment **X** Genotype	***	**	***	***

Table 3. Three major components (PC1, PC2, and PC3) and PCA ranking values of each ryegrass genotypes.

Accession No.	PC1	PC2	PC3	PCA	Rank	Accession No.	PC1	PC2	PC3	PCA	Rank
598892	393.1	27.7	18.3	221.5	1	265351	264.5	-17.7	26.1	141.8	50
275660	380.9	19.9	36.8	214.7	2	231606	270.5	-36.4	38.7	141.7	51
516605	362.2	21.3	33.9	204.6	3	287849	253.4	-0.3	38.3	140.9	52
231565	362.9	-7.7	6.1	195.8	4	628717	262.1	-25.2	44.5	140.3	53
598443	334.6	41.0	45.5	195.2	5	265340	249.5	6.3	35.1	140.0	54
303026	341.0	14.0	22.0	190.3	6	547390	250.0	-0.3	32.3	138.5	55
182857	339.7	11.2	21.2	188.9	7	418726	253.7	-10.7	35.8	138.4	56
610802	354.5	-24.0	11.2	187.9	8	231566	258.5	-26.8	33.7	137.1	57
265342	338.0	-13.1	30.1	183.1	9	619474	249.5	-9.0	37.8	136.7	58
277846	340.0	-20.1	28.6	182.4	10	610965	252.9	-15.3	29.6	136.3	59
598839	297.3	74.4	26.3	181.1	11	420126	258.9	-32.6	32.8	135.8	60
251224	316.0	18.1	45.1	179.7	12	303020	243.0	-2.4	38.1	134.7	61

Accession No.	PC1	PC2	PC3	PCA	Rank	Accession No.	PC1	PC2	PC3	PCA	Rank
577254	308.8	28.8	46.4	178.4	13	284823	256.5	-38.2	29.9	133.0	62
231605	319.8	-10.1	27.8	173.7	14	440474	213.3	39.4	41.8	128.7	63
231604	310.2	-0.9	26.3	170.5	15	376878	229.7	1.3	38.3	128.4	64
418712	287.4	34.0	37.4	167.2	16	268333	228.8	2.5	41.3	128.4	65
267059	282.7	35.6	47.0	165.9	17	317452	216.5	26.0	43.7	127.4	66
303028	299.3	-1.6	42.2	165.8	18	254898	232.3	-6.3	29.2	127.2	67
266293	299.4	0.5	34.8	165.7	19	231595	231.4	-11.9	35.5	126.0	68
418736	283.7	24.0	46.7	163.7	20	198070	232.2	-13.3	32.1	125.8	69
371952	309.1	-27.4	23.7	163.5	21	502412	223.8	-4.0	36.9	123.8	70
418722	285.6	13.3	44.7	162.1	22	420128	227.8	-15.1	33.2	123.1	71
267058	299.3	-20.7	34.5	160.7	23	418707	225.9	-22.1	35.0	120.6	72
225825	298.2	-22.6	30.4	159.3	24	423136	232.0	-39.1	28.0	119.3	73
197270	275.9	17.1	44.7	157.7	25	577273	205.2	17.6	36.7	118.7	74
600878	300.1	-35.5	24.4	156.8	26	319018	226.4	-30.7	31.2	118.5	75
619003	274.2	18.5	41.3	156.8	27	277848	223.9	-25.5	30.3	118.3	76
238938	280.3	3.3	44.0	156.8	28	303027	198.7	31.1	32.5	118.0	77
418723	280.4	5.3	35.0	156.5	29	410155	227.3	-42.9	40.1	116.9	78
505842	288.4	-14.5	37.9	156.5	30	202451	208.2	-1.7	34.7	115.7	79
237187	286.6	-11.2	36.1	156.2	31	265344	215.1	-26.0	26.8	113.1	80
274637	296.0	-31.8	32.4	156.1	32	204879	211.2	-21.6	34.0	112.6	81
610926	298.0	-37.5	30.7	155.7	33	577269	213.7	-31.2	30.3	111.4	82
285101	278.3	-0.7	47.8	155.1	34	231576	212.9	-28.9	28.3	111.3	83
418741	272.9	13.7	30.5	154.0	35	632542	196.6	6.1	34.9	111.2	84
287855	271.6	12.8	41.2	154.0	36	403868	202.6	-12.9	30.5	109.6	85
598911	260.7	34.2	51.5	154.0	37	231580	200.5	-9.1	29.5	109.3	86
303037	292.7	-32.7	25.9	153.6	38	321397	204.9	-16.6	20.7	109.2	87
204880	277.1	-6.3	41.9	152.6	39	239730	200.8	-17.9	30.5	107.5	88
284826	272.7	0.8	46.3	152.3	40	598434	204.3	-27.5	33.3	107.4	89
418721	269.0	8.4	43.3	151.8	41	418714	182.0	19.1	36.9	106.5	90
578763	254.7	31.2	38.2	148.9	42	634205	185.5	13.0	26.8	106.1	91
403907	270.9	-13.5	34.6	146.9	43	206376	179.0	21.7	33.9	105.2	92
298091	271.8	-17.4	35.7	146.6	44	577251	188.7	-23.6	32.0	99.7	93
303032	276.5	-27.6	30.5	146.4	45	311075	173.4	1.0	28.9	96.9	94
231567	277.3	-35.4	32.7	145.2	46	538976	159.4	19.0	20.7	92.8	95
290368	277.4	-40.1	33.5	144.2	47	278773	165.0	-18.2	29.3	87.9	96
220178	266.0	-17.1	29.6	143.0	48	610950	153.5	4.6	36.4	87.6	97
204085	256.0	0.0	40.4	142.5	49	321681	127.6	-1.4	23.1	70.9	98

Table 4. Pearson correlation coefficients analysis among the TQ, Fv/Fm, Chl content, Pn, WUE, RWC, RA, and PCA values.

	PCA rank	TQ	Fv/Fm	Chl	Pn	WUE	RWC	RA
PCA	1							
TQ	0.840^{***}	1						
Fv/Fm	0.715^{***}	0.755^{***}	1					
Chl	0.864^{***}	0.828^{***}	0.693^{***}	1				
Pn	0.432^{***}	0.136	0.097	0.115	1			
WUE	0.309^{**}	-0.005	-0.044	0.021	0.795^{***}	1		

	PCA rank	TQ	Fv/Fm	Chl	Pn	WUE	RWC	RA
RWC	0.833^{***}	0.871^{***}	0.727^{***}	0.817^{***}	0.107	-0.028	1	
RA	0.595^{***}	0.566^{***}	0.466^{***}	0.546^{***}	0.066	-0.061	0.518^{***}	1

** and *** indicate significance at$P < 0.01$ and $P < 0.001$, respectively.

Table 5. Average values of TQ, Fv/Fm, Chl content, Pn, WUE, RWC, RA, and PCA of perennial ryegrass accessions classified in three phylogenetic clusters.

Cluster	TQ	Fv/Fm	Chl	Pn	WUE	RWC	RA	PCA rank
A	4.41	0.65	6.11b	3.76	0.67b	57.91b	219.50b	123.9b
B	4.97	0.64	7.03b	3.83	0.65b	60.440ab	223.91b	127.9b
C	5.05	0.65	7.72a	3.77	0.77a	63.44a	234.71a	146.2a

Table 6.Association of SSR markers with the heat stress index of TQ, Fv/Fm, Chl content, Pn, WUE, RWC, and RA.

Trait	Locus	Chromosome No.	Position (cM)	P value	R^2
Fv/Fm	LP165	7	66	2.85E-06	0.67
Fv/Fm	rv0941	4	20.3	6.57E-05	0.47
Fv/Fm	DLF008	7	77	4.74E-04	0.49
Fv/Fm	B3C10	7	80	3.68E-03	0.29
Fv/Fm	B3B8	3	70	8.18E-03	0.48
Fv/Fm	B5E1	5	NA	9.20E-03	0.19
Chl	M144	4	56	5.21E-03	0.52
Chl	rv0941	4	20.3	5.60E-03	0.37
Pn	rv0985-1	6	51.7	8.92E-03	0.64
WUE	PRG	4	119	3.98E-06	0.53
WUE	PR10	NA	NA	4.50E-06	0.47
WUE	M4213	1	46	1.78E-04	0.44
WUE	25ca1	3	91.1	2.25E-04	0.56
WUE	LPSSRH01A07	3	NA	3.34E-04	0.33
WUE	rv0985-1	6	51.7	5.99E-04	0.70
WUE	rv0005	7	0	4.28E-03	0.75
WUE	rv1133	3	27.8	4.52E-03	0.17
WUE	LPSSRH02C11	3	NA	4.97E-03	0.37
WUE	rv0663	7	6.7	6.88E-03	0.31
WUE	B3B7	1	49	7.87E-03	0.29
WUE	LpHCA16B2	4	0	8.79E-03	0.48
WUE	PR37	1	52	9.62E-03	0.48
RA	M844	NA	NA	3.01E-06	0.60
RA	LPSSRH01A07	3	NA	1.05E-04	0.35
RA	B3B7	1	49	7.47E-04	0.35
RA	B1A10	3	74	9.35E-04	0.63
RA	LpSSR100	3	62	2.60E-03	0.21
RA	LP194	NA	NA	3.12E-03	0.47
RA	rv0757	5	77.7	4.87E-03	0.72
RA	LPSSRH02C11	3	NA	7.88E-03	0.36

FIGURE LEGENDS

FIGURE 1. Heatmap and hierarchical clustering for physiological and morphological parameters under control and heat stress conditions in 98 ryegrass accessions after 24 d of treatment. Abbreviations: WUE, water use efficiency; Pn, photosynthesis rate; RA, root activity; Chl, chlorophyll content; RWC, leaf relative water content; Fv/Fm, photochemical efficiency; PH, maximum plant height; LW, leaf width.

FIGURE 2. Principal component analysis biplot of the heat stress index (HSI) of 98 ryegrass accessions. *Arrows* represent physiological and morphological traits with various lengths based on the impact of each trait on the separation of accessions. Accessions marked with green color in group i and red color in group ii are the ten most and least heat tolerant genotypes, respectively. Abbreviations: WUE, water use efficiency; Pn, photosynthesis rate; RA, root activity; Chl, chlorophyll content; RWC, leaf relative water content; Fv/Fm, photochemical efficiency; PH, maximum plant height; LW, leaf width.

FIGURE 3. Phenotypes of the five most and least heat tolerant ryegrass accessions. Pictures were taken after 24 d of treatment. Bar in each photo represents 6.5 cm in length.

FIGURE 4. Neighbour-joining tree of 98 perennial ryegrass accessions based on SSR markers.

FIGURE 5. Relative expression levels of four CCGs of the five most and least heat-tolerant ryegrass accessions. Relative expression levels of *LpNYC1* (A), *LpNOL* (B), *LpSGR* (C), and*LpPPH* (D). Represented data were means and standard error (n=4).

FIGURE 6. Correlation between relative expression levels of four CCGs and PCA ranking values. Different spots in A–D indicate the gene relative expression levels and PCA ranking values of each accessions.

FIGURE 7. Manhattan plots of the general linear model (GLM) for association analysis between SSR markers and each physiological trait. The $-\log_{10}(P$ -values) from each SRR markers are plotted against seven heat tolerance-related traits, including TQ, Fv/Fm, Chl content, Pn, WUE, RWC, and RA.

SUPPLEMENTARY DATA

Supplementary table 1. Primers used for gene expression analysis.

Supplementary table 2. Eigenvectors and percentage of accumulated contribution ratios of each principal component.

Supplementary table 3. Principle component analysis of eight physiological traits across 98 perennial ryegrass accessions using heat stress index (HSI) after 24 days of treatment.

Supplementary materials 1. The linkage group, physical distance, and primer sequences of 66 SSRs used in this study.

Supplementary materials 2. The major allele frequency, polymorphic bands, polymorphism information content, and gene diversity index of 66 pairs of SSR used for genetic diversity analysis in 98 ryegrass accessions.

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