



Candidate gene association mapping for winter survival and spring regrowth in perennial ryegrass

Xiaoqing Yu^a, Paula M. Pijut^b, Stephen Byrne^c, Torben Asp^c, Guihua Bai^d, Yiwei Jiang^{e,*}

^a Department of Agronomy, Iowa State University, Ames, IA 50011, USA

^b USDA-Forest Service, Northern Research Station, Hardwood Tree Improvement and Regeneration Center, West Lafayette, IN 47907, USA

^c Department of Molecular Biology and Genetics, Faculty of Science and Technology, Research Centre Flakkebjerg, Aarhus University, Forsøgsvej 1, Slagelse 4200, Denmark

^d USDA-ARS Hard Winter Wheat Genetics Research Unit, Manhattan, KS 66506, USA

^e Department of Agronomy, Purdue University, West Lafayette, IN 47907-2054, USA

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ABSTRACT

Perennial ryegrass (*Lolium perenne* L.) is a widely cultivated cool-season grass species because of its high quality for forage and turf. Susceptibility to freezing damage limits its further use in temperate zones. The objective of this study was to identify candidate genes significantly associated with winter survival and spring regrowth in a global collection of 192 perennial ryegrass accessions. Significant differences in winter survival (WS), percentage of canopy green cover (CGC), chlorophyll index (Chl), and normalized difference vegetation index (NDVI) were found among accessions. After controlling population structure, *LpLEA3* encoding a late embryogenesis abundant group 3 protein and *LpCAT* encoding a catalase were associated with CGC and Chl, while *LpMnSOD* encoding a magnesium superoxide dismutase and *LpChl Cu-ZnSOD* encoding a chlorophyll copper–zinc superoxide dismutase were associated with NDVI or Chl. Significant association was also discovered between C-repeat binding factor *LpCBF1b* and WS. Three sequence variations identified in *LpCAT*, *LpMnSOD*, and *LpChl Cu-ZnSOD* were synonymous substitutions, whereas one pair of adjacent single nucleotide polymorphisms (SNPs) in *LpLEA3* and one SNP in *LpCBF1b* resulted in amino acid change. The results demonstrated that allelic variation in *LpLEA3* and *LpCBF1b* was closely related to winter survival and spring regrowth in perennial ryegrass.

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1. Introduction

Perennial ryegrass (*Lolium perenne* L.) ($2n=2x=14$) is a cool-season grass species from the family *Poaceae* with a self-incompatible and outcrossing nature [1]. Native to Europe, Asia, and Northern Africa, it is one of the most important perennial grasses worldwide [2]. Widely cultivated in the temperate regions, perennial ryegrass has the highest forage quality of all cool-season grasses for feeding dairy cattle and sheep and is also a primary turf species with rapid growth and establishment [3]. Growing perennial ryegrass also benefits ecosystems by improving carbon sequestration, and soil formation, protection, and nutrient cycling [4]. However, perennial ryegrass used in cultivation is susceptible to extreme cold temperatures because of its relatively poor winter hardiness, which limits expanding the use of this grass in temperate zones. Genetic mechanisms of winter hardiness in perennial

grasses are not well understood, owing to the quantitative trait of winter hardiness as well as the complex genetic nature and genomes of most popular perennial grasses. Exploration of genetic mechanisms underlying winter survival and spring regrowth is valuable for breeding programs aimed at improving winter hardiness of perennial grasses.

Winter hardiness is a complex trait, which is controlled by multiple genes involved in different pathways or physiological processes. The up-regulation of cold-regulated and dehydration-responsive ice-recrystallization-inhibition genes, and down-regulation of photosynthesis and respiration-related genes were found in cold-acclimatized perennial ryegrass, but not in non-acclimatized plants, suggesting a role of these genes in freezing tolerance [5]. Candidate genes involved in water movement across the membrane, cellular dehydration, and antioxidant metabolism may also facilitate winter hardiness of the plants. For example, cold stress induced a gradual increase in root osmotic hydraulic conductivity of rice (*Oryza sativa* L.), which accompanied a coordinated up-regulation of root aquaporin gene expression especially *OsPIP2;5* under low root temperature, indicating that root water

* Corresponding author. Tel.: +1 765 494 0651; fax: +1 765 496 2926.

E-mail address: yjiang@purdue.edu (Y. Jiang).

uptake function during the cold acclimation process was possibly regulated through aquaporins [6]. When a gene encoding late embryogenesis abundant (LEA) protein in barley (*Hordeum vulgare* L.) (HVA1) was introduced into mulberry (*Morus alba*), the mulberry plants showed increased cold tolerance [7]. A novel LEA protein in wheat (WC116) was induced during cold acclimation and contributed to freezing tolerance in winter wheat (*Triticum aestivum* L.) [8]. Transcriptome profiling analysis revealed that genes encoding antioxidant enzyme such as catalase, peroxidase, and Cu–Zn superoxide dismutase was induced by cold stress [9,10]. At the protein level, the freezing-tolerant cultivar of strawberry (*Fragaria ananassa*) [11] and zoysiagrass (*Zoysia japonica*) [12] had more stress-responsive proteins including those antioxidant and detoxification enzymes than the freezing-sensitive cultivars. Induction of cold-regulated proteins such as LEA, antifreeze proteins, and detoxification enzymes was common across some plant species [5,13], suggesting a protective role of these proteins in freezing tolerance.

C-repeat-binding factor (CBF)-dehydration-responsive element-binding factor (DREB) is an important pathway that regulates cold acclimation. CBF genes appeared to be ubiquitous in plant species [14–17]. It was estimated that up to 20% of cold-induced transcriptional changes were involved in CBF1–3 in *Arabidopsis thaliana* [18]. A large cold responsive CBF3 subfamily was identified in purple false brome (*Brachypodium distachyon*), while CBF4 homologs were absent from the genome [19]. In perennial ryegrass, Tamura and Yamada [14] found 10 putatively distinct CBF genes that were similar to either the *HvCBF3* or *HvCBF4* subgroups in barley, and some of these genes were responsive to cold treatment. Overexpression of CBF genes from *Arabidopsis*, rice, wheat, and perennial ryegrass induced strong expression of stress-responsive genes in transgenic *Arabidopsis* plants, resulting in increased tolerance to freezing stresses [20–22]. Similarly, when two genes from winter wheat (*TaCBF14* and *TaCBF15*) were introduced into spring barley, transgenic plants were able to survive freezing temperatures several degrees lower than that which proved lethal for the wild-type spring barley [17]. In addition, the deletion of nine CBF genes in tetraploid wheat was associated with significant reductions in survival after exposure to freezing temperatures [23]. The results indicated a regulatory function of CBF in freezing tolerance.

The study of natural variation of winter hardiness holds great potential to dissect the genetic network controlling freezing tolerance [16,24–26]. Analysis of gene and trait association in a natural population allows identification of SNPs associated with freezing tolerance and winter survival traits. For example, through candidate-gene association analysis, two SNPs in *ScCBF15* and one in *ScCBF12* were significantly associated with frost tolerance in 201 genotypes from five Eastern and Middle European winter rye populations [16]. One major quantitative trait locus (QTL) in *LpCBFIIIc* was associated with freezing tolerance in 109 perennial ryegrass plants with the majority of the plants possessing the superior allele [25]. Through genome-wide association analysis, *FR-H1* and *FR-H2* controlling low temperature tolerance QTL were identified, which explained 25% of the phenotypic variation of winter hardiness in barley [24]. In *Arabidopsis*, natural variation of CBF genes was a major cause of divergence in freezing tolerance in four populations [27]. McKhann et al. [15] demonstrated that the Versailles core collection of 48 *Arabidopsis* accessions varied largely in freezing tolerance, polymorphism in the CBF genes as well as expressions of CBF and cold-regulated gene (*COR*), but CBF or *COR* gene expression was not closely correlated with freezing tolerance. The results suggested that a complexity of mechanisms underlying natural variation of freezing tolerance, and the CBF genes alone, cannot explain all differences in phenotype.

Although a large number of genes have been identified in the process of cold acclimation and freezing tolerance, little is

known about whether allelic diversities of candidate genes contribute to winter survival and spring regrowth in perennial grass species. Given the fact that functional genes involved in dehydration, antioxidant, water movement across membranes, as well as regulatory genes such as CBF may influence winter survival of the plants, thus this study was designed to identify associations between candidate genes and winter survival related traits in a global collection of perennial ryegrass accessions. Perennial ryegrass is a diploid grass with more genetic and genomic information available for this species [28–33] than for any other major economically important perennial forage and turf grass species. The knowledge gained from this study will benefit the genetic improvement of winter hardiness in perennial ryegrass, and will also be valuable for investigation of other major cool-season perennial grass species with more complex genomes.

2. Materials and methods

2.1. Plant materials

The experiment was conducted at the Pinney Purdue Agriculture Center (PPAC) (Wanatah, IN, USA; 41°26' N and 86°54' W) and Turfgrass Research and Diagnostic Center (TRDC) at Purdue University (West Lafayette, IN, USA; 40°25' N and 86°54' W). A global collection of 192 accessions were used in the study, representing a wide range of ecotype diversity (Fig. 1, Supplemental Table S1). The panel included 72 wild, 66 cultivated, and 54 accessions with uncertain pedigree according to germplasm bank classification [32]. All the accessions were confirmed as diploid by flow cytometry [34]. Each accession was vegetatively propagated multiple times by tillers in a greenhouse to maintain genetic uniformity. The grasses were planted in the field at PPAC and TRDC in August 2008. Each location had three replications for each accession with the same genotype across locations. Soil type was sandy loam in PPAC and silt loam in TRDC. The maintenance of plants in the field during the study followed the routine practices for perennial ryegrass. The air temperatures of the two locations were shown in Supplemental Fig. S1.

2.2. Phenotypic traits

Phenotypic traits were recorded in two locations and years as well as different dates within a particular year. Specifically, winter survival (WS) was rated visually on April 25 at PPAC and April 27 at TRDC in 2009 using a 1–4 scale in which 1, 2, 3, and 4 represented completely dead plants without recovery, low, moderate, or high recovery of green tissues, respectively (Fig. 2). This observation served as a preliminary assessment of freezing tolerance and winter survival for the mapping population. The surviving plants were well established the second year after planting, and measurements focused only on traits related to spring regrowth. Percentage of canopy green cover (CGC), chlorophyll index (Chl), and normalized difference vegetation index (NDVI) parameters were recorded as indicators of spring regrowth on March 12, April 5, and April 23, 2011 at TRDC and April 12 and May 24, 2011 at PPAC. Canopy green coverage was rated visually as percentage of grass recovered after winter with zero for no green tissue coverage and 100% for complete green tissue coverage. A FieldScout CM 1000 Chlorophyll Meter (Spectrum Technologies, Inc., Plainfield, IL, USA) was used to measure Chl by setting wavelengths at 700 nm and 840 nm to estimate the quantity of chlorophyll in the leaves. This “point-and-shoot” technology instantly measures reflectance at these two wavelengths at the canopy level, and provides fast and reliable measurements of chlorophyll. Measurement distance between the meter and grass canopy was around 100 cm.

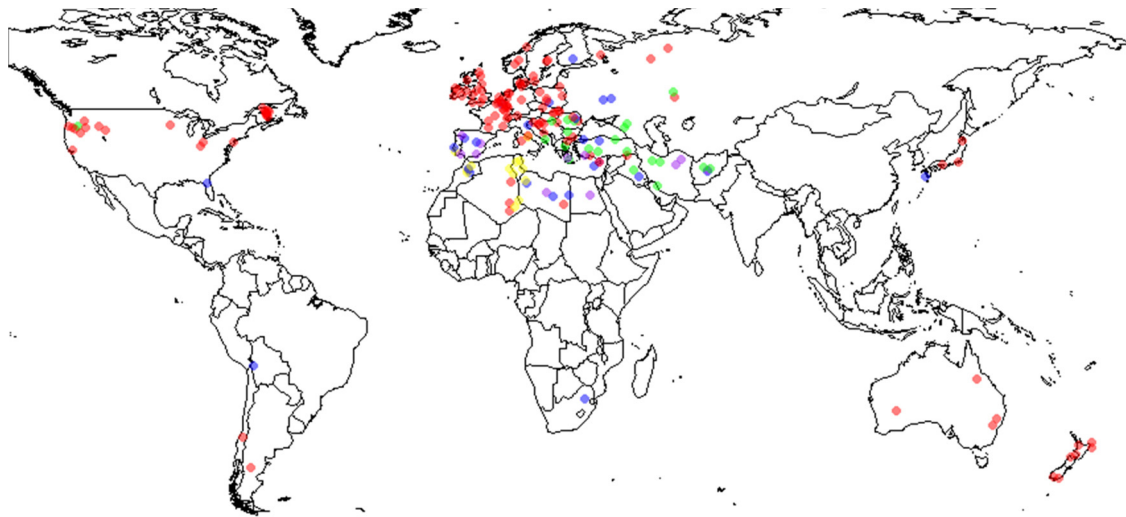


Fig. 1. Germplasm origin and genetic relationships among worldwide perennial ryegrass accessions. Accessions are color-coded by subgroup type identified previously based on population structure [32]. Accessions with the same color belong to the same subgroup. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

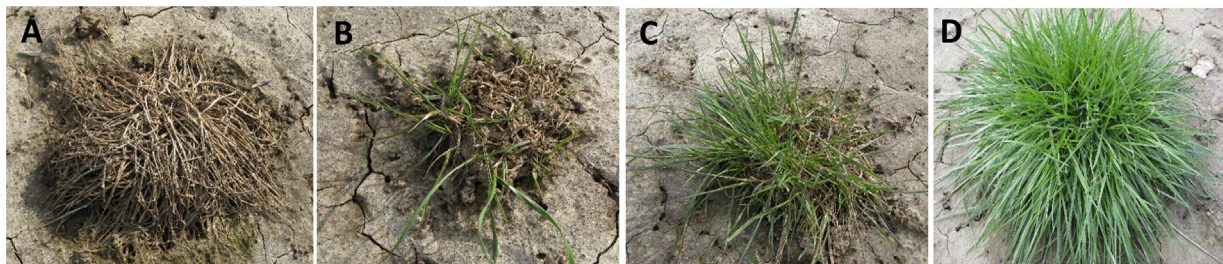


Fig. 2. Differential capacity of winter survival and recovery of perennial ryegrass accessions in early spring. (A) Dead, no survival; (B) low recovery; (C) moderate recovery; (D) high recovery.

Canopy reflectance was measured using a Crop Circle TM ACS-210 Plant Canopy Reflectance Sensor (Holland Scientific Inc., Lincoln, NE, USA). The crop circle radiometer was held at a height of 100 cm above the canopy. Reflectance at near infrared (NIR) wavelength 800 nm (R880) and at red wavelength 650 nm (R650) was collected, and NDVI was calculated as $(R880 - R650)/(R880 + R650)$. The chlorophyll meter or radiometer was paused after data was collected each time, prior to the next plant, to avoid noisy background signals from the soil. However, data points on bare soil from both meters were also recorded for necessary correction of the data collected from the canopy. If the accession was completely dead after winter and no dead tissue remained on the ground, the values of Chl and NDVI, equally to the bare soil, were assigned to those accessions.

2.3. Repeatability of phenotypic traits

Analysis of variance (ANOVA) was calculated using SAS PROC GLM [35] with both accessions and environment as fixed effects, and genotype $\times$ environment as random factors. To examine consistence and accuracy of phenotypic data, repeatability (r) of phenotypic traits across different locations, years, and testing dates were calculated using SAS PROC MIXED. The r was calculated as: $r = \sigma_g^2 / (\sigma_g^2 + \sigma_{ge}^2 / e + \sigma_e^2 / re)$, where σ_g^2 , σ_e^2 , σ_{ge}^2 represent Type III SS for genotype (G), environment (E), and $G \times E$, respectively; 'e' is the degree of freedom of environment and 're' is the degree of freedom of replication $\times$ E, where E was a testing location at a specific date. Winter survival rating data used for the analysis were from two environments: PPAC and TRDC in 2009, while parameters

of CGC, Chl, and NDVI were evaluated at five testing dates in 2011 at PPAC and TRDC.

2.4. Population structure and model testing

Population structure (Q) was determined using STRUCTURE 2.3.1 [36] and pairwise relative kinship (K) was determined using SPAGeDi [37], using 109 simple sequence repeat (SSR) markers in the 192 accessions of perennial ryegrass [32]. Model testing with the 109 SSR markers was conducted to assess the effect of population structure and relative kinship on various traits in the mapping panels. Following the previously recommended procedures [38,39] simple linear model, Q (considering population structure), K (considering relative kinship), and Q + K models were tested with subpopulation membership percentage as fixed covariates and kinship as a random effect. The best fit model was determined for each trait based on the value of Bayesian Information Criterion (BIC). The lower BIC indicated a better model fit. The effects of population structure on the phenotypic variation of target traits were assessed by multiple regressions using the SAS program [35].

2.5. Gene sequencing, SNP identification and association analysis

Eighteen candidate genes in the functions of antioxidant (*LpCAT*, *LpChl* Cu-ZnSOD, *LpCyt* Cu-ZnSOD, *LpGPX*, *LpMnSOD*, *LpFeSOD*, *LpAPX*, *LpMDAR*, *LpDHAR*, and *LpGR*), kinase (*LpMAPK*), dehydration (*LpLEA3*), aquaporin (*LpPIP1* and *LpTIP1*) and transcription factor (*LpCBF1b*, *LpCBF3b*, *LpCBF3c*, and *LpCB4b*) were sequenced in the

Table 1
Analysis of variance of winter survival rating (WS), percentage of canopy green cover (CGC), chlorophyll index (Chl), normalized difference vegetation index (NDVI), and repeatability (r) of these traits in 192 perennial ryegrass accessions.

	WS	CGC	Chl	NDVI
Accession (A)	1031.7***	616,563***	1,130,907***	7.1***
df ^a	191	191	191	187
F	19.1	6.65	4.32	3.03
Environment (E)	51.3***	314,061***	1,238,733***	14.0***
A × E	137.9***	269,226	1,105,818***	7.6
r	0.87	0.74	0.82	0.67

^a df, degree of freedom.

Significant effects shown as ** $P < 0.01$; *** $P < 0.001$.

Table 2
Range and mean values of winter survival rating (WS), canopy green cover (CGC), chlorophyll index (Chl), normalized difference vegetation index (NDVI), and percentage of phenotypic variation explained by population structure (R^2) in 192 perennial ryegrass accessions.

Trait	Minimum	Maximum	Mean	Std ^a	R^2	p^b
WS	1	4	3.38	0.95	0.721	<0.001
CGC (%)	0	79	31.5	18.0	0.016	0.077
Chl	62	212	100.4	24.0	0.002	0.537
NDVI	0.21	0.67	0.33	0.07	0.058	0.0008

^a Standard deviation.

^b Significant for R^2 .

perennial ryegrass population used for this study. The procedures of primer design, gene sequencing, and SNP identification of genes other than *CBF* family were reported previously [32]. Based on gene expression patterns of *CBF* in perennial ryegrass [14], four *CBF* genes were selected for sequencing. Primers were designed based on perennial ryegrass sequences available in gene bank, including accession AB258393 for *LpCBF1b*, AB258396 for *LpCBF3b*, AB258397 for *LpCBF3c*, and AB258399 for *LpCBF4b*. Since *CBF* genes contain no introns [25], genomic DNA was used as polymerase chain reaction (PCR) amplification template for synthesis and sequencing of these genes. Briefly, SNPs were identified using the NovoSNP program 3.0.1 Microsoft Windows Platform version [40]. Rare SNP was excluded for SNP counting when the total non-major allele counting was <5%.

2.6. Association analysis

Quantile–quantile (Q–Q) plots of F -test value for model comparisons of simple linear (S), Q, K and Q+K model across all traits were examined for verifying the best fit model for association analysis. Associations between candidate genes and traits of WS, CGC, Chl and NDVI were analyzed using GLM in the TASSEL 2.1 software [41]. Each SNP was considered as a fixed effect to test the association between the SNP and phenotype. Minor alleles with frequency <5% were removed prior to association analysis. Associations were only considered to be significant at a P -value lower than the $P_{\text{threshold}}$ -value in the association mapping panel. The $P_{\text{threshold}}$ -value for each individual candidate gene was calculated using $P_{\text{threshold}} = 0.01/N$, where N was the number of SNPs in a candidate gene.

2.7. Sequence analysis for functional substitution(s)

Reference full-length genes, either from perennial ryegrass or closely related species, were used to map the position of significantly associated SNPs identified from the association analysis. Full-length *LpLEA3*, *LpMnSOD*, and *LpCAT* genes from de novo perennial ryegrass genome assembly [33] were used to map SNP positions in these genes. Barley full-length *Chl Cu-ZnSOD* gene (NCBI accession: JQ364454.1) was used for anchoring SNPs in *LpChl Cu-ZnSOD*. Perennial ryegrass full-length *CBF* genes available in NCBI were used for anchoring SNPs in *LpCBF1b*, *LpCBF3b*, *LpCBF3c*, and *LpCBF4b*. Only genes that showed significant associations with traits

were analyzed for putative functional amino acid variations. DNA sequences were aligned using ClustalW2 online service [42]. Amino acid sequences of each peptide were directly inferred using BlastX in NCBI and matched with orthologous proteins in perennial ryegrass and other plant species.

3. Results

3.1. Trait variation

Significant accession or environment effects on WS, CGC, Chl, and NDVI were observed and the interaction between accession and environment was found for WS and Chl in perennial ryegrass (Table 1). The overall repeatability for WS, Chl, CGC, and NDVI among and across the environments was 0.87, 0.74, 0.82, and 0.67, respectively (Table 1), indicating that winter recovery for accessions was consistent across locations. Therefore, a grand mean of individual trait for each accession was generated for marker-trait association. The variations in phenotypic traits were largely found in accessions across environments, ranging from 1 to 4 for WS, 0–79% for CGC, 62–212 for Chl, and 0.21–0.67 for NDVI, respectively (Table 2). Averaged across two locations and sampling dates, approximately 10% of the accessions exhibited overall good or poor winter survival, respectively (Supplemental Table S1). Thirty-one accessions in TRDC and six accessions in PPAC were dead without recovery in spring 2009. The distribution patterns of CGC, Chl, and NDVI across accessions shifted with the sampling dates. For example, at the early stage of winter recovery (March 12, 2011 at TRDC), approximately 86% of the accessions had Chl ranging from 60 to 90; 3 weeks after (April 5, 2011), approximately 77% of the accessions showed Chl values in a range of 70–110 with several greater than 140; while on April 23, 2011, a wider range of Chl value from 90 to 340 was noted among the population, with 50% of the accessions having a value from 90 to 140 (Fig. 3). At PPAC, recovery of accessions was slower than that of TRDC. The overall Chl distribution patterns for the two testing dates (April 12, 2011 and May 24, 2011) at PPAC were similar to those of two testing dates (March 12, 2011 and April 23, 2011) at TRDC, respectively (Fig. 3). The WS was correlated with NDVI, while Chl was highly correlated with CGC ($r = 0.86$) or NDVI ($r = 0.79$), and CGC was highly correlated with NDVI ($r = 0.73$) among all accessions across the environments (Table 3).

Table 3

Pearson correlation coefficients among winter survival rating (WS), chlorophyll index (Chl), percentage of canopy green cover (CGC), and normalized difference vegetation index (NDVI) in 192 perennial ryegrass accessions.

Traits	WS	Chl	CGC	NDVI
WS	1			
Chl	0.09	1		
CGC	0.16*	0.86***	1	
NDVI	0.30***	0.79***	0.73***	1

Significant effects shown as * $P < 0.01$, *** $P < 0.001$.
N = 192.

Table 4

Five population structures (groups) differing in winter survival rating (WS), percentage of canopy green cover (CGC), chlorophyll index (Chl), and normalized difference vegetation index (NDVI) in 192 perennial ryegrass accessions.

Group ^a	N ^a	WS	CGC (%)	Chl	NDVI
1	121	3.90a	31.5a	99ab	0.338a
2	21	3.86a	36.5a	107a	0.339a
3	25	2.26b	38.4a	112a	0.339a
4	13	1.99b	27.0ab	95ab	0.302ab
5	12	1.41c	14.7b	85b	0.265b

^a Group and number of accessions (N) within each group were identified previously [32].

3.2. Population structure and model testing

Five population structures (groups) were identified previously in the mapping panel with no obvious kinship [32]. Group one to five (G1–G5) contained 121, 21, 25, 13, and 12 accessions, respectively [32]. Phenotypic variations explained by population structure were 0.2% for Chl, 1.6% for CGC, 5.8% for NDVI, and 72.1% for WS, respectively; with an average of 19.9% (Table 2). Significant differences in WS were observed among the five groups except between G1 and G2 and between G3 and G4 (Table 4). The higher WS values in G1 and G2, and lower WS values in G5 indicated that WS was associated with population structure. G5 had the lowest values for all traits, compared to the other four groups. To reduce possible false-positive signals, various models were compared. The model implemented with Q exhibited the smallest BIC values for WS, CGC, Chl, and NDVI compared to other models (Table 5). Therefore, the Q model was selected as the best-fit model for association analysis of genes and traits in this study, which was also confirmed by Q–Q plots of *F*-test value (Supplemental Fig. S2). Q–Q plots of *F*-tests verified the adequate control of false positives for the Q model because the deviation of the observed *F*-statistics for SNP markers from the expected value was much smaller than that of the S and K model (Supplemental Fig. S2).

Table 5

Goodness of fit of four models explaining phenotypic variation of winter survival rating (WS), percentage of canopy green cover (CGC), chlorophyll index (Chl), and normalized difference vegetation index (NDVI) in 192 perennial ryegrass accessions.

	WS	CGC	Chl	NDVI
Simple linear (S)	532.5 ^a	778.5	887.7	441.8
Structure (Q)	204.0	762.8	868.6	417.5
Kinship (K)	329.1	783.7	892.8	440.9
Q + K	209.3	768	873.9	422.8

^a Bayesian information criterion (BIC) values; smaller was better.

3.3. Association between genes and traits

A total of 346 SNPs were previously identified from 14 candidate genes involved in dehydration, antioxidant, water movement across membranes, and signal transduction in perennial ryegrass [32]. An additional 59 SNPs were found in four *LpCBF* genes. One hundred and five significant associations of these genes with all traits were found by using the simple linear model, including 33 associations of *LpCBF1b* and *LpCBF3b* with WS, and 72 associations of other genes with various traits (Supplemental Fig. S3). After controlling population structure, seven SNPs from five genes (*LpMnSOD*, *LpCAT*, *LpLEA3*, *LpChl* Cu-ZnSOD, and *LpCBF1b*) were

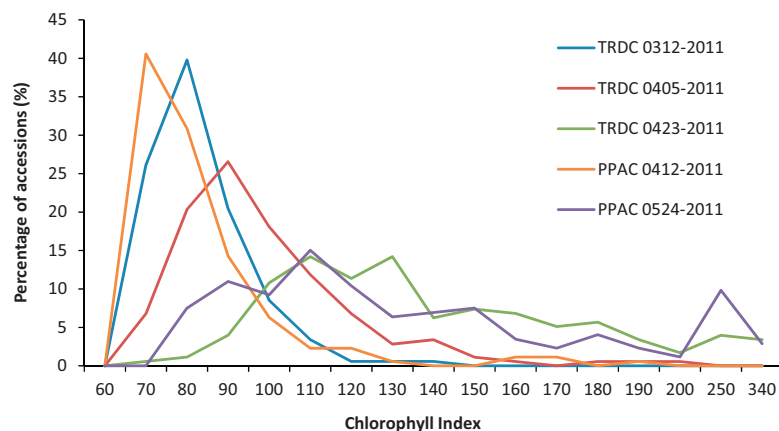


Fig. 3. The patterns of distribution in chlorophyll index data collected from different dates and locations in 192 perennial ryegrasses accessions. TRDC, Turfgrass Research and Diagnostic Center at West Lafayette, IN, USA; PPAC, Pinney Purdue Agriculture Center at Wanatah, IN, USA. 0312-2011 indicates March 12, 2011; same meaning for the other numbers.

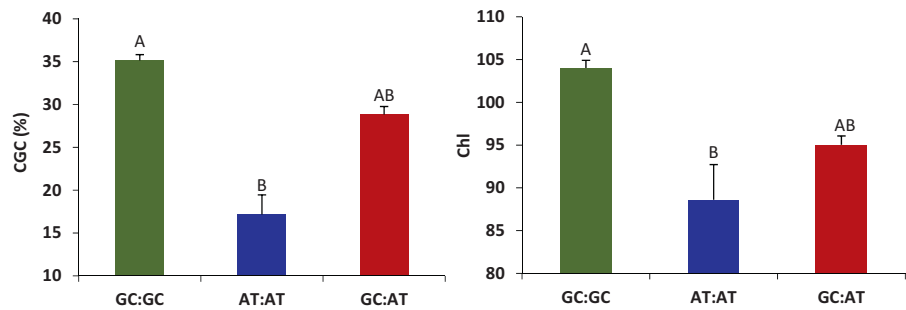


Fig. 4. Allelic variations in *LpLEA3* associated with percentage of canopy green coverage (CGC) and chlorophyll index (Chl) in diverse perennial ryegrass accessions. Columns with the same letter were not significantly different at $P < 0.05$.

Table 6
Association of candidate genes with percentage of canopy green cover (CGC), chlorophyll index (Chl), and normalized difference vegetation index (NDVI) in 192 perennial ryegrass accessions.

Trait	Putative gene	SNP (bp)	P-value	R ² Marker
NDVI	<i>LpMnSOD</i>	210	4.21×10^{-4}	0.062
CGC	<i>LpCAT</i>	1074	9.07×10^{-5}	0.085
Chl	<i>LpCAT</i>	1074	1.58×10^{-4}	0.081
CGC	<i>LpLEA3</i>	247	1.95×10^{-4}	0.085
CGC	<i>LpLEA3</i>	248	1.95×10^{-4}	0.085
CGC	<i>LpLEA3</i>	282	2.58×10^{-4}	0.081
Chl	<i>LpLEA3</i>	282	2.66×10^{-4}	0.078
Chl	<i>LpLEA3</i>	247	2.73×10^{-4}	0.079
Chl	<i>LpLEA3</i>	248	2.73×10^{-4}	0.079
NDVI	<i>LpChl C-ZnSOD</i>	531	4.73×10^{-6}	0.114
Chl	<i>LpChl Cu-ZnSOD</i>	531	1.61×10^{-4}	0.080
WS	<i>LpCBF1b</i>	440	3.05×10^{-4}	0.023

Table 7
Amino acid substitution in the loci from candidate genes significantly associated with phenotypic traits.

Putative gene	SNP (bp)	Nucleotides	AA residual
<i>LpMnSOD</i>	210	CTC → CTT	L/L
<i>LpCAT</i>	1074	CCA → CCG	P/P
<i>LpLEA3</i>	247–248	GCG → ATG	A/M
<i>LpLEA3</i>	282	GCC → GCT	A/A
<i>LpChl Cu-ZnSOD</i>	531	GGG → GGA	G/G
<i>LpCBF1b</i>	440	CAT → CCT	H/P

(Fig. 4). For *LpCBF1b*, the mean value of WS was 1.9, 2.2, and 3.6 for the accessions carrying homozygous G:G, heterozygous T:G and homozygous T:T at position 440 bp, respectively (Fig. 5).

3.4. Functional sequence variations in the candidate genes

The predicted functional amino acid substitutions were analyzed for significant SNPs identified in *LpLEA3*, *LpMnSOD*, *LpChl Cu-ZnSOD*, *LpCAT*, and *LpCBF1b* (Table 7). Of these sequence variations, two neighboring SNPs at position 247 bp and 248 bp of *LpLEA3* were two nucleotide substitutions from GCG to ATG, resulting in one amino acid change from alanine (A) to methionine (M) (Fig. 6). One SNP at position 440 bp of *LpCBF1b* showed one nucleotide substitution from CAT to CCT, causing one amino acid change from histidine (H) to proline (P) (Table 7, Supplemental Fig. S4). The nucleotide changes in the other three genes were synonymous and caused no changes in amino acids (Table 7).

4. Discussion

Winter hardiness of perennial grass species is critical for survival of the plants. In general, the ability for regrowth in the spring following a severe winter indicates the degree of winter hardiness of turf and forage grasses in fields [43]. Previous research demonstrated that perennial ryegrass germplasm varied significantly in winter hardiness and freezing tolerance [25,44,45] assessed by turf quality, seedling vigor, tiller survival, early spring growth, and survival after freezing. Perennial ryegrass cultivars that were able to turn green quickly in the early spring most likely survive the next winter [46]. In addition, plant canopy related measurements such as canopy reflectance has been widely used for field-based high-throughput phenotyping of crops subjected to abiotic stresses [43,47,48]. In this study, visual ratings were used to assess the ability of the plants to survive winter, while Chl, CGC, and NDVI evaluated plant growth after winter in a global collection of diverse perennial ryegrass germplasm. The high correlation of Chl with CGC and NDVI as well as between CGC and NDVI suggested that these parameters were appropriate for a rapid and reliable assessment of canopy characteristics for indicating spring regrowth of perennial grasses. Correlations between WS and NDVI and between WS and

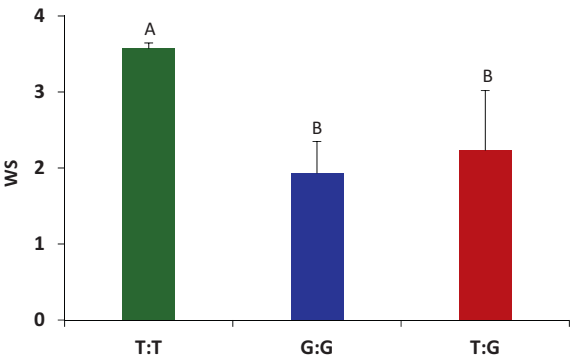


Fig. 5. Allelic variations in *LpCBF1b* associated with winter survival (WS) in diverse perennial ryegrass accessions. Columns with the same letter were not significantly different at $P < 0.05$.

significantly associated with CGC, Chl, NDVI, or WS. These markers explained 7.8% of phenotypic variation on average, ranging from 2.3% to 11.4% (Table 6). The SNP at position 210 bp in *LpMnSOD* was associated with NDVI; SNPs at position 1074 bp in *LpCAT* and at positions 247 bp, 248 bp, and 282 bp in *LpLEA3* were associated with CGC and Chl; and SNP at position 531 bp in *LpChl Cu-ZnSOD* was associated with NDVI and Chl (Table 6). One SNP at position 440 bp in *LpCBF1b* was significantly associated with WS (Table 6), while no associations were identified between *LpCBF3c* and *LpCBF4b* with any of the traits by using either the simple linear or Q implemented model.

Sequence variations and phenotypic differences of different alleles of *LpLEA3* and *LpCBF1b* were compared (Figs. 4 and 5). For *LpLEA3*, the mean Chl and CGC values were significantly higher in the accessions carrying homozygous GC than the accessions carrying homozygous AT at SNP positions 247 bp and 248 bp, while the heterozygous accessions carrying the GC:AT was in between

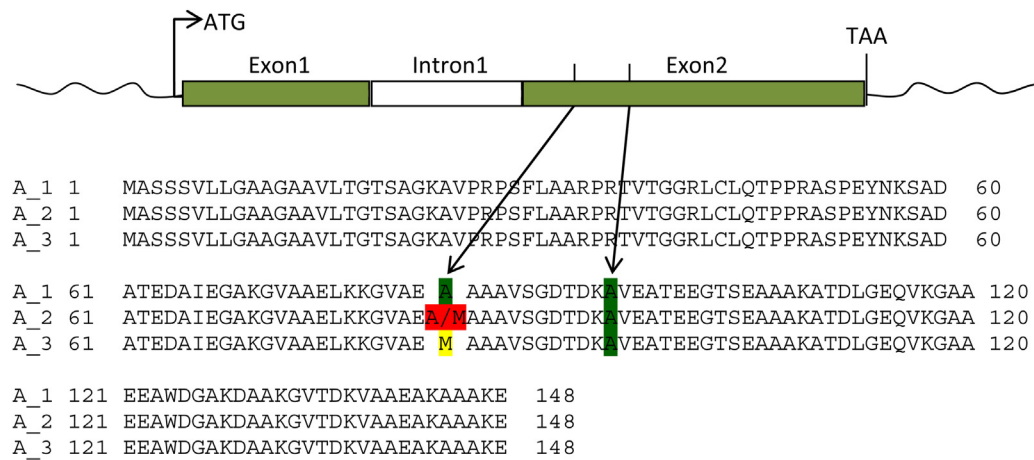


Fig. 6. Amino acid substitution caused by the significantly associated single nucleotide polymorphisms (SNPs) in *LpLEA3*. The positions of these two significant SNPs at 247–248 bp and 282 bp were shown using the full-length of *LpLEA3* gene sequence as reference. ATG, start codon; TAA, stop codon; arrow line points to amino acid substitutions in *LpLEA3* among different genotypes. A.1, A.2, and A.3 indicate allele1, allele2, and allele3, respectively.

CGC were also found, but were less strong compared to those correlations among other parameters. The high overall repeatability for WS, Chl, CGC, and NDVI among and across the environments indicated that winter survival and regrowth of accessions were consistent across locations.

Population structure usually exists in natural populations because of local adaptation or diversifying selection and familial relatedness from recent co-ancestry. Analysis of population structure in the mapping panel used in this study identified five groups (G1–G5), but no obvious kinship was observed [32]. Many accessions in the G4 and G5 group that did not survive winter were mainly from Northern Africa and Southern Europe with mild winter temperatures. The trait of WS was strongly affected by population structure (Table 4), suggesting that structured WS may be related to geographical origin of the accessions. Therefore, breeding for improved winter hardiness in perennial ryegrass may need to avoid using some accessions in G4 and G5 as the parents. The effects of population structure on phenotypic traits have been examined in annual or perennial species. Tang et al. [31] demonstrated that population structures generally accounted for approximately 11% of phenotypic variations in perennial ryegrass exposed to high salinity stress. Population structure also accounted for 16% to 31% of growth and yield trait variation in durum wheat [49]. In rice, an average of 22% phenotypic variation of 25 agronomic traits such as starch quality, grain color, nutritional quality, and antioxidant were explained by population structure [50]. Population structure often results in spurious correlations between markers and traits [38,51]. To reduce possible false-positive signals, various models were compared. As a result, the model implemented with Q was the best-fit model, exhibiting the smallest BIC values for WS, CGC, Chl, and NDVI compared to other models (Table 5). Across all genes with traits in this study, approximately 89% significant associations were eliminated by implementing population structured model, compared to the simple linear model. In maize (*Zea mays* L.), the number of significant SNPs was reduced by 30% for male flowering time and by 90% for thousand-kernel weight after controlling population structure [52]. The results demonstrated an increased accuracy of association results by controlling population structure.

The *CBF* genes, which were rapidly induced in response to low temperature, encode transcriptional activators that control the expression of genes containing the C-repeat-dehydration responsive element DNA regulatory element in their promoters [53].

Plants belonging to *Poaceae* (the grasses) contain *CBFs* that have been classified into ten groups, the members of which share a common phylogenetic origin and similar structural characteristics [54]. In perennial ryegrass, ten novel putative *CBF* cDNAs were isolated from cold-treated leaf tissue and the expression of six *LpCBF* genes was rapidly induced in response to low temperature, but varied under a long time period of cold treatment [14]. Hulke et al. [25] discovered that one significant locus in *LpCBF3c* was associated with freezing tolerance of 109 perennial ryegrass plants and no significant loci were found in *LpCBF3b*. Li et al. [16] reported that nine out of 12 *CBF* genes were significantly ($P < 0.05$) associated with frost tolerance in 201 winter rye genotypes, and two significant SNPs in *ScCBF15* and one in *ScCBF12* led to amino acid exchanges. Our results in the field support these observations by demonstrating a significant association between *LpCBF1b* and winter survival in a global collection of perennial ryegrass germplasm. This significant SNP at 440 bp caused an amino acid change (histidine to proline), suggesting a role of the genes in winter hardiness of perennial ryegrass.

Members of the *CBF* gene family were not the only key factors in the cold responsive network [9,55,56]. Investigation of other candidate genes involved in winter survival and spring regrowth is necessary in illustrating mechanisms underlying various traits. LEA proteins are a family of hyper-hydrophilic proteins that accumulate in response to cellular dehydration. LEA proteins can act as protectors of macro-molecules or cellular structures to prevent protein aggregation during water deficit [57], and unfavorable structural changes caused by dehydration were thought to be associated with dehydration and cold tolerance [58,59]. A wheat WCI16 protein with unknown function, but sharing common features with LEA proteins, exhibited in vitro cryoprotection of both proteins and DNA during environmental stresses; moreover, heterologous expression of WCI16 in *Arabidopsis thaliana* plants conferred freezing tolerance [8]. Perennial ryegrass *LpLEA3* is a homolog of *Lcs19*, a cold-stimulated gene identified in Italian ryegrass (*Lolium multiflorum* Lam.) [60]. The presence of *Lcs19* homologs was found in temperate grass species, but not in tropical grass species [60], suggesting that *LEA3* was a candidate gene associated with winter hardiness of temperate grasses. In this study, the significantly associated SNP in *LEA3* caused one amino acid change from alanine to methionine in perennial ryegrass. It indicated that *LEA3* might be involved in increasing capacity of early recovery and spring regrowth of perennial ryegrass.

Antioxidant metabolism plays a role in abiotic stress tolerance by scavenging and decomposing reactive oxygen species. In perennial ryegrass, approximately 10% of differentially expressed genes were associated with functions of detoxification under drought stress [61]. Overexpression of the antioxidant genes such as *MnSOD*, *FeSOD*, or *Chl Cu-ZnSOD* enhanced cold tolerance or winter survival of tobacco (*Nicotiana tabacum* L.) [62] and alfalfa (*Medicago sativa* L.) [63]. However, antioxidant responses of plants to low temperature or cold acclimation were not consistent. The elevated antioxidant enzyme activities contributed to increased survival at low temperatures in wheat [64], but cold acclimation led to decreased activities of antioxidant enzymes in perennial ryegrass and bermudagrass (*Cynodon dactylon*) under controlled environments [65,66]. In general, maintaining a high level of antioxidative enzyme activities can benefit plant survival from stress by increasing the capacity against oxidative damage. Since antioxidant metabolism pathways include several key enzymes, differential trends in activities of certain antioxidant enzymes occurred in plants exposed to various stresses including low temperatures [67,68]. This may indicate a co-ordination of different antioxidant mechanisms during cold acclimation. The variation of antioxidant metabolism in response to low temperature may be a result of differences in species, cultivars, stage of growth, and the condition and duration of cold acclimation in the experiment. In this study, significant associations of *LpMnSOD*, *LpCAT*, and *LpChl Cu-ZnSOD* with phenotypic traits suggested a positive relationship between antioxidant and spring regrowth of perennial ryegrass at the population level, although associated SNPs in these genes did not lead to amino acid substitutions. In maize, among the analyzed genome-wide one-million SNPs, only 4% of casual trait-associated SNPs (TASs) were nonsynonymous and resulted in functional amino acid substitution [69]. Thus, it appears that synonymous substitution was not rare in association mapping results.

Significant allelic effects (2.3–11.4%) were found in association results of genes with traits in this study. Similarly, 6.5–9.7% of allelic effects were noted in association of candidate genes with drought tolerance traits in perennial ryegrass [32]. Since responses of the plants to abiotic stress are rather complex involving numerous genes, it is likely that some key genes had small allele effects on stress tolerance. In maize, SNPs from *lg1* and *lg2* explained the leaf angle difference by −0.8 degree and −1.13 degree [70], demonstrating that genetic architecture of the leaf traits was dominated by small allele effects. These allelic effects indicate that association mapping analysis of complex quantitative traits in out-crossing species could be challenging and that larger populations will be needed to uncover alleles with smaller effects [32].

5. Conclusions

LpCBF1b was associated with winter survival, while *LpLEA3*, *LpMnSOD*, *LpCAT*, and *LpChl Cu-ZnSOD* were mainly associated with traits related to plant regrowth in the spring. In particular, one functional amino acid substitution occurred in *LpLEA3* and in *LpCBF1b*, suggesting that allelic diversities of these two genes may contribute to variable capacity of winter survival or regrowth after winter in perennial ryegrass population. This discovery illustrated an important genetic mechanism underlying winter hardiness and recovery in perennial grass species. The results will be valuable for further molecular studies of gene function in perennial ryegrass or other perennial grass species with a more complex genome. The enhancement of winter hardiness is one of the most important tasks facing breeders of perennial grasses. Therefore, examination of functional and regulatory genes involved in winter survival and regrowth after winter is of central importance. Further research is needed to

conduct genome-wide association of winter survival related traits in perennial grasses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2015.03.003>.

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