Association of single nucleotide polymorphisms in *LpIRI1* gene with freezing tolerance traits in perennial ryegrass

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Abstract

Perennial ryegrass is an important agricultural species, however, susceptible to winterkill. Freezing injury is caused primarily by ice formation. The LpIRI1 protein has the potential to inhibit ice recrystallization, thus minimize the damage. An association study was conducted using single nucleotide polymorphisms obtained through allele sequencing of the *LpIRI1* gene and phenotypic data were collected using two phenotyping platforms in a perennial ryegrass association mapping population of 76 diverse genotypes. Winter survival (FWS) was evaluated under field conditions, while tiller survival (PTS) and electrolyte leakage (EL) at -8 °C and -12 °C were determined under controlled-environment conditions. Proline content (PC) in cold-acclimated plants was measured prior the freezing test. Significant variation in FWS, PTS, EL and PC was observed among genotypes in our panel. EL and PTS revealed significant negative correlations at -8 °C (r_s = -0.40) and -12 °C (r_s = -0.49). PC, however, did not show significant correlations with any of the measured traits, while FWS was correlated (r_s = -0.48) with EL at -12 °C. The *LpIRI1* gene was found to be highly polymorphic with an average SNP frequency of 1 SNP per 16 bp. Association analysis revealed two non-synonymous SNPs being associated with increased EL, both being located in the *LpIRI1* leucine-rich repeat. The results indicate that allelic variation in the *LpIRI1* gene plays an important role in the cell membrane integrity of perennial ryegrass during freezing, and can be exploited for developing more freezing tolerant cultivars.

Key words: Association mapping, electrolyte leakage, proline, winter survival

Introduction

Perennial ryegrass (*Lolium perenne* L.) is a cool-season grass species of paramount economic importance used both for turf and forage. It is a key species for temperate pasture agriculture due to superior digestibility, grazing tolerance as well as adequate seed production (Wilkins 1991; Forster et al. 2008), while the rapid establishment rate and excellent tolerance to traffic make perennial ryegrass one of the most extensively used grass species for turf or amenity purposes (Wang et al. 2001). Despite many superior properties perennial ryegrass is sensitive to abiotic stresses. It exhibits poor performance under drought conditions and is susceptible to freezing temperatures. Poor winter survival under harsh winters limits it's cultivation at northern latitudes (Nekrošas and Kemešytė 2007; Hulke et al. 2008). Substantial genetic variation has been found for traits relevant for adaptation in wild perennial ryegrass populations, e.g., for freezing tolerance (Wilkins 1991; Hulke et al. 2007). Cold acclimation or exposure to low non-lethal temperatures can significantly increase freezing tolerance in perennial ryegrass (Ebdon et al. 2002). During cold-acclimation gene expression patterns change rapidly (Ciannamea et al. 2006; Zhang et al. 2009; Dinari et al. 2013) which leads to differential profiles of proteins, compatible sugars, and proline accumulation among cold-acclimated perennial ryegrass plants (Bocian et al. 2011; Lee et al. 2012). Various functions have been attributed to free proline in plants: it may act as free radical scavenger (Alia et al. 2001; Kaul et al. 2008), an osmoprotectant (Yoshiba et al. 1997), or a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995). Furthermore, proline was shown to act like a signal molecule triggering a process essential for plant recovery from stresses (Szabados and Savouré 2009).

The primary site of freezing injury in plant cells is located in cellular membranes, especially cell plasma (Yoshida 1984; Uemura et al. 1995) and chloroplast membranes (Thalhammer et al. 2014). Cell membrane damage is caused by cell dehydration, which occurs when extracellular ice forms during freezing, thus drawing the water from the cell until water potential equalises between the compartments (Pearce 2001). When dehydration exceeds the dehydration-tolerance of the cell, cell membrane damage occurs. The extent of the damage can be measured by an electrolyte leakage (EL) assay. EL is assumed to reflect extensive disruption of the membrane by evaluating the ratio of electrolyte leakage of freeze-treated tissue and total electrolyte content within the tissue by disrupting cell membranes (Bajji et al. 2001).

Overwintering plants produce antifreeze proteins (AFPs), which increase freezing tolerance by modifying the growth of ice crystals and minimising the damage caused by ice formation (Atici and Nalbantoglu 2003). Ice recrystallization is the process where larger ice crystals grow at the expense of smaller crystals. However, when IRIPs (Ice recrystallization inhibition proteins) are present under freezing conditions, ice recrystallization is inhibited, forcing new small crystals to form rather than increasing the crystal size. Four genes coding AFP proteins with putative IRI activity, *LpIRI1* to *LpIRI4*, were identified in perennial ryegrass. However, *LpIRI2* gene has undergone deletion of LRR and is considered to be a pseudogene (Sandve et al. 2008). Protein modelling and functional protein studies of *LpIRI1* have shown that NxVxG/NxVxxG repeat motifs form a beta-roll with a beta-sheet ice-binding face that can bind to ice and is responsible for ice

recrystallization inhibitory action *in vitro* (Middleton et al. 2009). *FpIRI1,* an orthologue of *LpIRI1* gene, was also mapped to a quantitative trait locus (QTL) for freezing tolerance in meadow fescue (Alm et al., 2011). Furthermore, Arabidopsis plants expressing *L. perenne* IRIP genes were shown to display improved cell membrane stability during freezing and increased freezing tolerance (Zhang et al. 2010). Despite the impressive progress made in understanding of IRIP gene function, the importance of IRIPs in determining freezing tolerance in forage grasses and its utility in breeding programs have yet to be demonstrated.

Association mapping which is based on linkage disequilibrium (LD) is a powerful tool for genetically dissecting complex traits, such as freezing tolerance. Association mapping exploits historical recombination events among chromosome segments. Recent advances in sequencing technologies enabled characterization of genetic variation on a genome-wide scale, even in species that have little or no pre-existing genomic resources. However, re-sequencing of the complete perennial ryegrass genome (estimated to be 2.69 Gb, Bennett and Leitch 2011) on a large scale is still relatively costly and various strategies, e.g., genotyping by sequencing (GBS) (Elshire et al. 2011) of single genotypes or population-based genome wide allele frequency fingerprints (GWAFFs) (Byrne et al. 2013), have been developed to reduce the complexity. Alternatively, re-sequencing of candidate genes is an option of choice in species with rapidly decaying LD. Rapid LD decay within 300-2000 bp has been reported in perennial ryegrass (Xing et al. 2007, Brazauskas et al. 2011), and several studies have already demostrated the feasibility of the candidate gene-based association mapping approach in perennial ryegrass. A candidate gene, *FLOWERING LOCUS T*, was found to be associated with changes in flowering time across a range of populations (Skøt et al. 2011). Significant associations were also identified between *LpLEA3* and *LpFeSOD* genes and leaf water content, as well as a *LpCytCu-ZnSOD* gene and chlorophyll fluorescence under drought conditions (Yu et al. 2013). Furthermore, one major and several minor quantitative trait loci (QTL) were discovered in *LpCBFIIIc* for freezing tolerance in perennial ryegrass (Hulke et al. 2012).

The objectives of this study were: i) to evaluate perennial ryegrass association mapping population for variation in the freezing tolerance traits under controlled environment and in the field; ii) to identify *LpIRI1* sequence variants significantly associated with freezing tolerance traits; iii) to discuss the applicability of identified SNPs in molecular breeding of perennial ryegrass for freezing tolerance.

Materials and methods

Plant material and DNA extraction

Perennial ryegrass association panel of 33 cultivars and 43 ecotypes was established (Online Resource 1). The ecotype group comprised 17 genotypes from Lithuania, 1 accession from Latvia, 5 genotypes from Kaliningrad region (Russian Federation) and 20 genotypes from Ukraine. All ecotype genotypes were collected in their natural habitats. Cultivar group consisted of 31 cultivars developed in Europe and 2 in the USA. Plants were established from seeds and propagated in the greenhouse. The diploid ploidy level of genotypes was confirmed by Partec PA (Partec Gmbh, Germany) flow cytometry. One plant per accession was used to make

corresponding clone collection. The same clones were used both in field trials and in freezing tests under controlled climate condition, as well as for EL assays and proline content analyses. Approximately 100 mg of pathogen-free leaf material of each genotype was harvested for genomic DNA extraction. DNA was isolated using a cetyltrimethyl ammonium bromide (CTAB) method according to the modified protocol of Lassner et al. (1989), supplemented with an additional chloroform wash step and RNA digestion with ribonuclease A. DNA concentration was quantified with a NanoDrop® 2000 (Thermo Fisher Scientific, USA) spectrophotometer.

Field overwintering scoring

A field collection of 76 perennial ryegrass genotypes was established in the experimental fields of the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry (Akademija, Lithuania) in 2013. The soil of the experimental site is classified as *Endocalcari–Epihypogleyic Cambisols* (CMg-p-w-can). A single kernel per accession was used for plant establishment in the greenhouse and then cloned into four ramets. Each ramet was planted at 50 x 50 cm distances using a randomised complete block design in July of 2013. Winter survival was evaluated by visual scoring using a scale ranging from 1 (dead) to 9 (no visible damage) as described by Tyler et al. (1987) in April of 2014. Autumn and early winter of 2013/2014 season were unusually warm (Fig. 1). Freezing temperatures were first observed in mid-January, 2014. Maximal snow cover of 3 cm and frozen ground depth of 32 cm was reached at the end of January. Shortly afterwards air temperatures started to rise and snow cover melted off. Thawing of the frozen ground lasted until mid-February of 2014 (Fig. 1).

Freezing tolerance testing under controlled-environment

Freezing tolerance tests under controlled-environment conditions were performed on the same perennial ryegrass panel as in the field collection. Freezing tolerance was defined as percentage of tiller survival (PTS). Twenty days prior cold-acclimation plants were moved from the greenhouse into the growth chamber PlantMaster (CLF Plant Climatics GmbH, Germany) set to 20 °C, 450 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 16/8 h photoperiod and 80 % relative air humidity. Plants were cold-acclimated for 15 days prior the freezing test under controlled environment conditions at 5 °C, 200 µmol m^{-2} s^{-1} PAR, 12/12 h photoperiod and 80 % relative air humidity. Cold-acclimated plants were divided into individual tillers with leaves and roots trimmed to 2 cm. Twelve tillers of each genotype were used for each target temperature. Tillers were placed into test tubes of 10 ml, containing 1 ml of deionised water, the roots were submerged in water and thereafter subjected to freezing. Freezing tests were conducted in a walk-in freezing chamber PE 2412UY-LX (Angelantoni Industrie S.p.A, Italy) in the dark. Temperature was decreased gradually from 5 °C to -12 °C at a rate of 1.2 °C h^{-1}. As temperature reached target temperatures of -8 °C and -12 °C, tillers were removed from the chamber and thawed on ice overnight at 4 °C. Later plants were replanted in cell trays containing soil substrate and moved to the growth chamber PlantMaster (CLF PlantClimatics GmbH,

Germany) set to 20 °C, 450 µmol m^{-2} s^{-1} PAR, 16/8 h photoperiod and 80 % relative air humidity. Plant survival was scored after a 3-week recovery period. Regrown plants were scored as survivors.

Electrolyte leakage assay

Membrane integrity of the plant cells was tested by measuring electrolyte leakage (EL) at -8 °C and -12 °C temperatures. Cold-acclimated plants were divided into ramets of single tillers with leaves and roots trimmed to 2 cm. Tillers were washed with deionized water, placed into glass test tubes containing 1 ml of deionized water and agitated for 15 min. Then they were washed again and wiped with paper towel.

A total of twelve tillers per genotype divided into 3 replicates were used for EL assay at each targed temperature. Each replicate contained four tillers, which were placed into individual test tubes containing 1 ml of deionized water and covered with parafilm. Overall, three replicates per accession were prepared for each target temperature and randomly arranged in the test tube rack.

Sample preparation work was conducted at 5 °C temperature. Freezing tests were performed under the same conditions as in the freezing tolerance experiment. When the target temperature of -8 °C or -12 °C was reached racks were removed from the freezing chamber and thawed on ice at 4 °C. Twenty ml of deionized water was added into each tube. The tubes were shaken overnight at 120 rpm and then initial conductivity (C_{ini}) was measured with YSI 3100 conductivity meter with YSI 3253 Glass Dip Cell (YSI Incorporated, USA). The total conductivity (C_{tot}) was measured after autoclaving the samples at 120 °C for 15 min. Electrolyte leakage (EL) was estimated as $EL = (C_{ini}) / (C_{tot}) \times 100$.

Proline content evaluation

Free proline content was determined in cold-acclimated plants prior to freezing application. Flag leaves were collected from each accession and immediately dried at 70 °C until the final weight of dry sample became stable. Dried leaves were ground with Retsch MM 400 (Retsch GmbH, Germany) mixer mill. Proline content was determined by the ninhydrin-based colorimetric assay (Ábrahám et al. 2010) assessing three replicates per accession.

Population structure and relative kinship

An admixture model in the STRUCTURE software package (Pritchard et al. 2000) was used to infer population structure from the AFLP dataset of 227 markers generated in previous study using the same perennial ryegrass panel (Statkevičiūtė et al. 2014). Ten independent simulations were run by setting the number of populations (k) from 1 to 10, with 100 000 burn-in time and 100 000 iterations of Markov chain convergence for each run. The subpopulation number was determined using methodology developed by Evanno et al. (2005). Pairwise kinship matrix was calculated by SPAGeDi1-4c (Hardy and Vekemans 2002) using AFLP dataset. All negative kinship values between individuals were set to zero.

***LpIRI1* gene amplification and sequencing**

Primers with sequences 5‘-GCTGTTGCTGCTCTTCTT-3‘ (forward primer) and 5‘-ATAACCCGATTGTCCCCA-3‘ (reverse primer) were used for the amplification of *LpIRI1* gene. The primers were based on *LpIRI1* gene sequence (EU680848). Amplified *LpIRI1* fragments were cloned using CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer’s protocol. Five bacterial colonies were isolated for each perennial ryegrass accession and subsequent plasmid extraction was carried out using GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific, Lithuania). Isolated plasmids were sequenced at GATC Biotech (GATC Biotech AG, Germany). Sequences were aligned using MEGA6 software (Tamura et al. 2013) and trimmed to *LpIRI1* gene open reading frame (ORF) length.

Marker–trait association analysis and linkage disequilibrium

SNP calling was performed with TASSEL 3 (Bradbury et al. 2007). SNPs with minor allele frequency of less than 5 % (MAF ≤ 5 %) were excluded from further analyses while continuous Indels were treated as a single polymorphic site. Marker-trait associations were further estimated using TASSEL 3 mixed linear model (MLM) function. SNPs with nominal p-value < 0.05 were further tested for statistical significance of phenotypic differentiation between allelic variants of SNPs using ANOVA. Linkage disequilibrium (LD) plot was generated with TASSEL 3.

Statistical analysis

Analysis of variance (ANOVA) with *post hoc* Unequal N HSD test and Spearman's rank correlation coefficients (r_s) were calculated with STATISTICA 7 (StatSoft, Inc.). Mean ± SE (standard error of mean) were used to describe the variability of measurements. Repeatability estimates were calculated using PLABSTAT version 3A (Utz 1998) for each investigated trait in controlled experiment and for winter survival under field conditions.

Results

Population structure and kinship

Population structure and pairwise kinship coefficients were determined using AFLP markers. AFLP analysis yielded a total of 227 bands of which 98.6 % were polymorphic. The maximum ΔK value was observed for k = 2, thus a Q-value matrix with k = 2 was generated for subsequent association analyses. Most of the ecotypes collected in Lithuania, Latvia, and the Kaliningrad region were assigned to subpopulation 1 (Sb_1) while subpopulation 2 (Sb_2) was mainly composed of the ecotypes collected in Ukraine (Fig. 2). A remarkable heterogeneity was observed among cultivars with genotypes being nearly-equally distributed between both Sb_1 and Sb_2 subpopulations. There was no obvious kinship in the perennial ryegrass panel. Pairwise kinship estimates were low with 49.5 % of the values being equal to zero and 82.7 % being lower than 0.05, while only 3.9 % and 0.06 % of the pairwise kinship estimates were higher than 0.1 and 0.2, respectively.

Variation for freezing tolerance traits

Substantial variation for freezing tolerance parameters was observed among perennial ryegrass genotypes (Fig. 3). Fourteen genotypes showed poor winter survival (average winter survival score less than 2), while six genotypes exhibited superior winter survival performace with a mean score of 8 or more. The same panel comprised of 76 perennial ryegrass genotypes was examined for freezing tolerance, measured as plant tiller survival (PTS), electrolyte leakage (EL) under controlled environment at fixed-temperature points of -8 °C and -12 °C (Online Resource 2). Proline content (PC) for cold-acclimated plants was also evaluated. Highest repeatabilities among all examined traits were observed for proline content (91.36), electrolyte leakage at -8 °C (71.81) and -12 °C (73.66). Significant differences in freezing tolerance were observed among genotypes under controlled experiment conditions. PTS ranged from 0.00 to 100.00 % (mean 53.05 ± 3.58 %) and from 0.00 to 91.67 % (mean 9.99 ± 2.01 %) at -8 °C and -12 °C, respectively. EL varied from 6.09 to 25.80 % at -8 °C and from 22.27 to 63.51 % at -12 °C. Genotypes differed significantly ($p \leq 0.001$) in proline content (PC) of cold-acclimated plants, while PC ranged from 473.3 to 6955.9 µg g^{-1} dry weight. No significant differences in examined traits were observed between ecotype and cultivar group, except for winter survival under field conditions where ecotypes demonstrated significantly ($p = 0.007$) higher winter survival with mean winter survival score of 3.39 and 4.42 for cultivar and ecotype groups, respectively. Comparison of subpopulations revealed no significant ($p \geq 0.05$) differences in examined freezing tolerance traits nor cold-acclimated plant proline content between Sb_1 and Sb_2. Moderate correlations were identified between individual traits (Table 1). Highly significant negative correlations were observed between EL and PTS at both -8 °C ($r_s = -0.40$, $p \leq 0.001$) and -12 °C ($r_s = -0.49$, $p \leq 0.001$). Similarly positive correlations were observed for EL ($r_s = 0.43$, $p \leq 0.001$) and PTS ($r_s = 0.46$, $p \leq 0.001$) measurements at two temperatures of -8 °C and -12 °C. No correlation was revealed between PC and any other examined trait. While analysing field winter survival data, the only significant correlation ($r_s = -0.48$, $p \leq 0.001$) was observed between FWS and EL at -12 °C (Table 1).

LpIRI1 gene polymorphism and association mapping

A total of 380 *LpIRI1* gene sequences from 76 genotypes were obtained. Sequence alignment revealed the gene to be highly polymorphic. Fifty two single nucleotide polymorphisms (SNPs) were found within *LpIRI1* open reading frame (ORF) after minor allele (MAF ≤5 %) exclusion, resulting in an average SNP frequency of 1 SNP per 16 bp. An insertion-deletion (Indel) polymorphism of 15 bp was further detected in the coding sequence of *LpIRI1* within our perennial ryegrass panel. The average LD within the gene was $r^2 = 0.61$. Two strong LD blocks were identified. The first LD block corresponded to the Indel spanning from position 397 to 411 (mean $r^2 = 1$), while the second LD block spanned 130 bp from SNP position 461 to 591 (mean r^2 within LD block = 0.74) (Online Resource 3). A total of 52 SNPs and 1 Indel along with phenotypic traits were further used for marker-trait association analysis. Three SNPs at positions 322, 369, and 726 were found to be significantly ($p \leq 0.05$) associated with EL at both temperatures of -8 °C and -12 °C (Fig. 4). The majority

(68.42 %) of genotypes were homozygous for these SNPs and were defined as wild-type (*Wt*) alleles while the rest were assigned to the four groups of recombinants (*Rec-1* to *Rec-4*). No genotypes with homozygous mutant alleles of these SNPs were detected in the panel. The strongest association was detected between SNP at position 369 with EL at -8 °C and EL at -12 °C showing nominal p-values of 0.007 and 0.014, respectively. SNPs at positions 322 and 369 were identified as being non-synonymous causing amino acid substitutions from serine to threonine at amino acid residue 108 and from histidine to glutamine at amino acid residue 123, respectively. SNP at position 726 is synonymous. It was significantly ($p \leq 0.05$) more frequent in genotypes with mutant alleles at 322 and/or 369 positions than in genotypes harbouring *Wt* alleles at those positions. Significant differences for EL were found at -8 °C between *Wt* and recombinant genotypes at position 322 ($p \leq 0.01$), while allelic variants at locus 369 significantly differed in EL at both tested temperatures of -8 °C ($p \leq 0.01$) and -12 °C ($p \leq 0.01$). Differences in EL at -8 °C between allelic variants at SNP position 726 were not significant ($p \geq 0.05$). The Indel polymorphism had no significant effect on EL.

Discussion

Winter survival is a complex trait determined by the ability of plants to respond to a combination of frost, ice-encasement, snow cover, anoxia and desiccation. Freezing tolerance is a major component that generally explains most of the variation in winter survival (Sandve et al. 2011). Various methods can provide important information about freezing damage and tolerance in plants. As cell membrane damage is the primary site of freezing injury, the extent of it can be measured by EL assays as an indirect freezing tolerance measurement (Espevig et al. 2011). We found a moderate negative correlation between EL and PTS at both tested temperatures with correlation coefficients being higher at -12 °C than at -8 °C. This indicates, that optimization of the freeze temperature applied might further improve the prediction accuracy of the test. Furthermore, EL at -12 °C showed a moderate negative correlation with winter survival in the field. Similarly, Xiong et al. (2007) reported good correspondence between electrolyte leakage of naturally acclimated plants and plant survival in the field for two winters in an annual × perennial ryegrass interspecific hybrid population. However, this relationship is not always apparent, especially when cold-acclimation under natural and controlled environment differs substantially. Alternative estimation of LT_{50} and EL_{50} values is labour-intensive and time-consuming, thus problematic for larger sample sizes in breeding programs while high-throughput screening for freezing tolerance using a fixed-temperature point would substantially reduce the workload. Numerous physiological, biochemical, and molecular changes occur in plants during cold-acclimation, enhancing cell membrane stability and plant survival at sub-zero temperatures (Lissarre et al. 2010; Zhang et al. 2010). Reports indicate free proline to accumulate in plant tissues during cold-acclimation and to protect cells against detrimental effects during freezing (Xin and Browse 2000; Zhao et al. 2009). Moreover, low temperatures induce cell membrane lipid peroxidation. Thus proline might enhance membrane stability as a free radical scavenger (Campos et al. 2003). Recently, overproduction of proline in plants was reported to stabilise cell membranes (Hayat et al. 2012). Perennial ryegrass genotypes varied significantly for freezing tolerance

parameters in this study while substantial variation in free proline content was also observed. However, no significant correlations between free proline content and EL, or between free proline content and any other tested trait were detected. It seems that a direct correlation between proline accumulation and abiotic stress tolerance in plants is not always apparent (Szabados and Savouré 2009).

Substantial variation for freezing tolerance traits, the absence of clustering between ecotype and cultivar groups along with the absence of obvious kinship between individual genotypes make this perennial ryegrass panel particularly suitable for the association analysis of the genetic factors governing freezing tolerance. An AFP gene, *LpIRI1*, was selected as a candidate gene to search for allelic variants putatively associated with FT in perennial ryegrass, as various AFPs possessing IRI activity were shown to protect cells from freeze-induced damage in a number of plant species (Zhang et al. 2010). Allele sequencing revealed high level of nucleotide diversity in *LpIRI1*. Perennial ryegrass is a self-incompatible outcrossing species and high levels of nucleotide diversity are expected to be maintained throughout ryegrass genome as was shown for drought tolerance genes with 1 SNP per 11 bp to 1 SNP per 75 bp detected depending on the gene (Yu et al. 2013). Fifty-two SNPs identified in *LpIRI1* gene along with 6 examined phenotypic traits were subjected to the genetic association analysis. Three significant marker-trait associations were detected between EL and *LpIRI1* SNPs at positions 322, 369 and 731. This association seems straightforward, as ice recrystallization inhibition proteins play a direct role in membrane structure stabilisation under freezing by interfering with the migration of ice boundaries and thus inhibiting ice crystal growth (John et al. 2009). Indel affected 5 amino acid residues of the LpIRI1 protein spanning amino acid positions from 133 to 138 and, to our knowledge, doesn't cover any functional domain of the protein. The Indel polymorphism was not associated with any of the examined trait. Two significant SNPs at position 322 and 369 changed amino acid residues of the LpIRI1 protein, while the third SNP at position 731 was synonymous and had no effect on the putative protein structure. Both non-synonymous mutations occurred at the Leucine-rich repeat of the LpIRI1 protein. Leucine-rich repeats (LRR) are ligand interaction domains found in various types of proteins. The ubiquity of the domain may be due to its ability to interact with a wide range of substrates (Helft et al. 2011). LRR are particularly important for protein-protein interactions (Bella et al. 2008) and mechanism of ice-recrystalisation inhibition might be guided by the presence of LRR and IRI patterns in protein sequences (Muthukumaran et al. 2011). Single mutation in LRR can impair the function of the protein and it's interaction with other proteins (Warren et al. 1998). According to some reports LRR alone is capable, at least partly, to exhibit antifreeze activity (Meyer et al. 1999). Recombinant genotypes of SNPs at positions 322, 369 and 726 showed increased EL at -8 °C, whereas recombinant genotype at SNP position 369 demonstrated an increase in EL at both -8 °C and -12 °C in comparison to the wild type genotypes. An increase in EL indicates that mutant alleles had a negative effect on the ice recrystallization inhibition activity of the LpIRI1 and reduced the freezing tolerance of the plant. Similarly an introduction of single steric point mutation in LpIBP, Ice-binding protein from the same anti-freeze protein family, reduced ice recrystallization inhibition and thermal hysteresis activities of the protein by up to 90 % in perennial ryegrass (Middleton et al. 2012). Such mutations should be under natural selection pressure as carrier genotypes would be eliminated, especially at northern latitudes, due to the poor winter

survival. This is further supported by the fact that homozygous genotypes for mutant alleles were absent in our perennial ryegrass panel. We propose *LpIRI1* SNP at position 369 as a putative functional marker applicable in high-throughput perennial ryegrass genotype screening for enhanced freezing tolerance.

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Fig. 1. Snow cover, frozen ground, air temperature and thawing during winter of 2013-2014 in the experimental field. Each data point represents average value per 10-day period

Fig. 2. Genetic relatedness of perennial ryegrass genotypes used for association mapping as defined by the STRUCTURE. Two subpopulations indicated as Sb1 and Sb2. Y-axis represents membership coefficient (Q), while vertical bars on X-axis represents individuals (cult = cultivars, LT = ecotypes from Lithuania, LV = ecotypes from Latvia, RU = ecotypes from Kaliningrad region, UA = ecotypes from Ukraine)

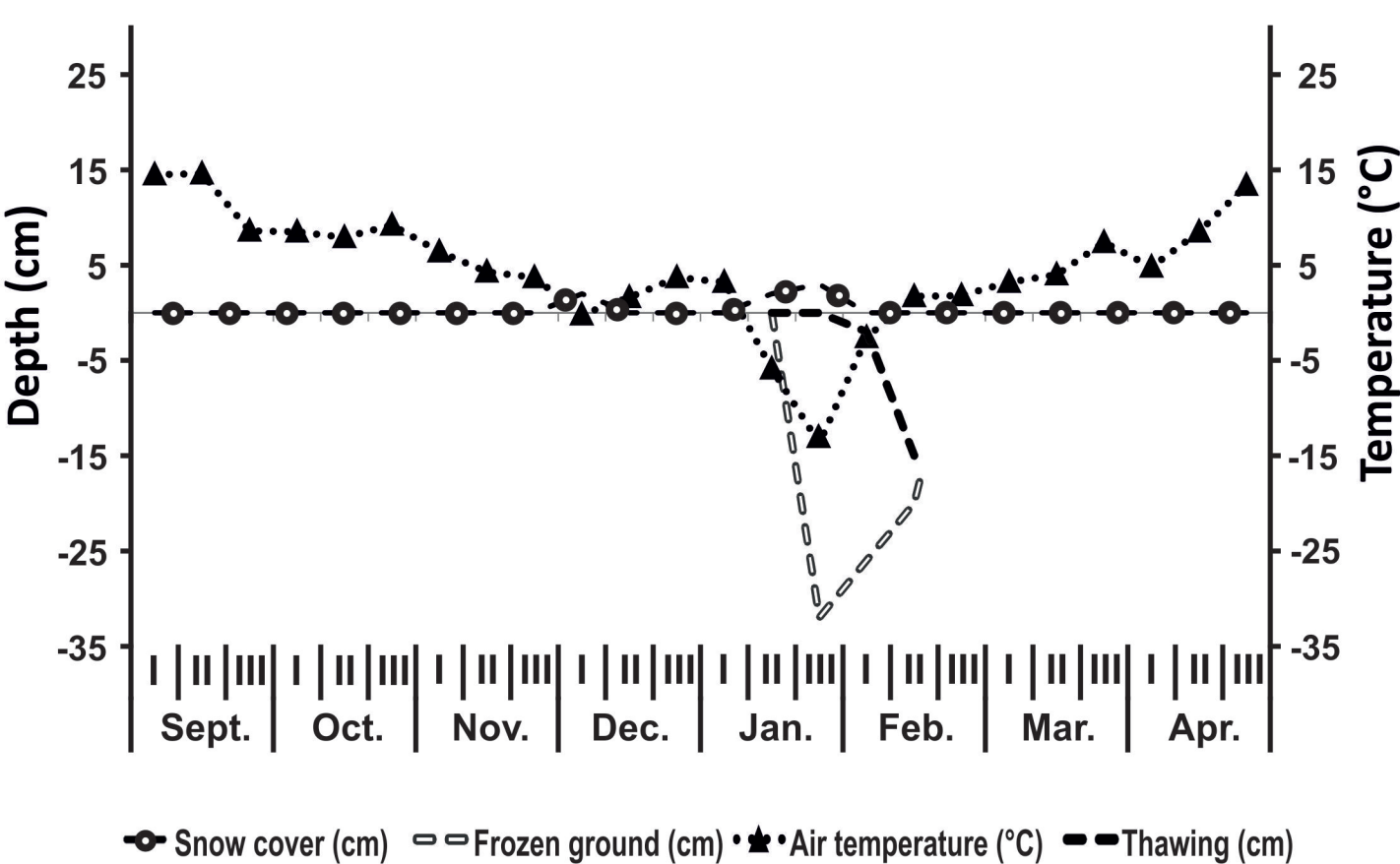
Fig. 3. Percentage of tiller survival (PTS), electrolyte leakage (EL), proline content (PC) and field winter survival (FWS) variation among 76 perennial ryegrass genotypes

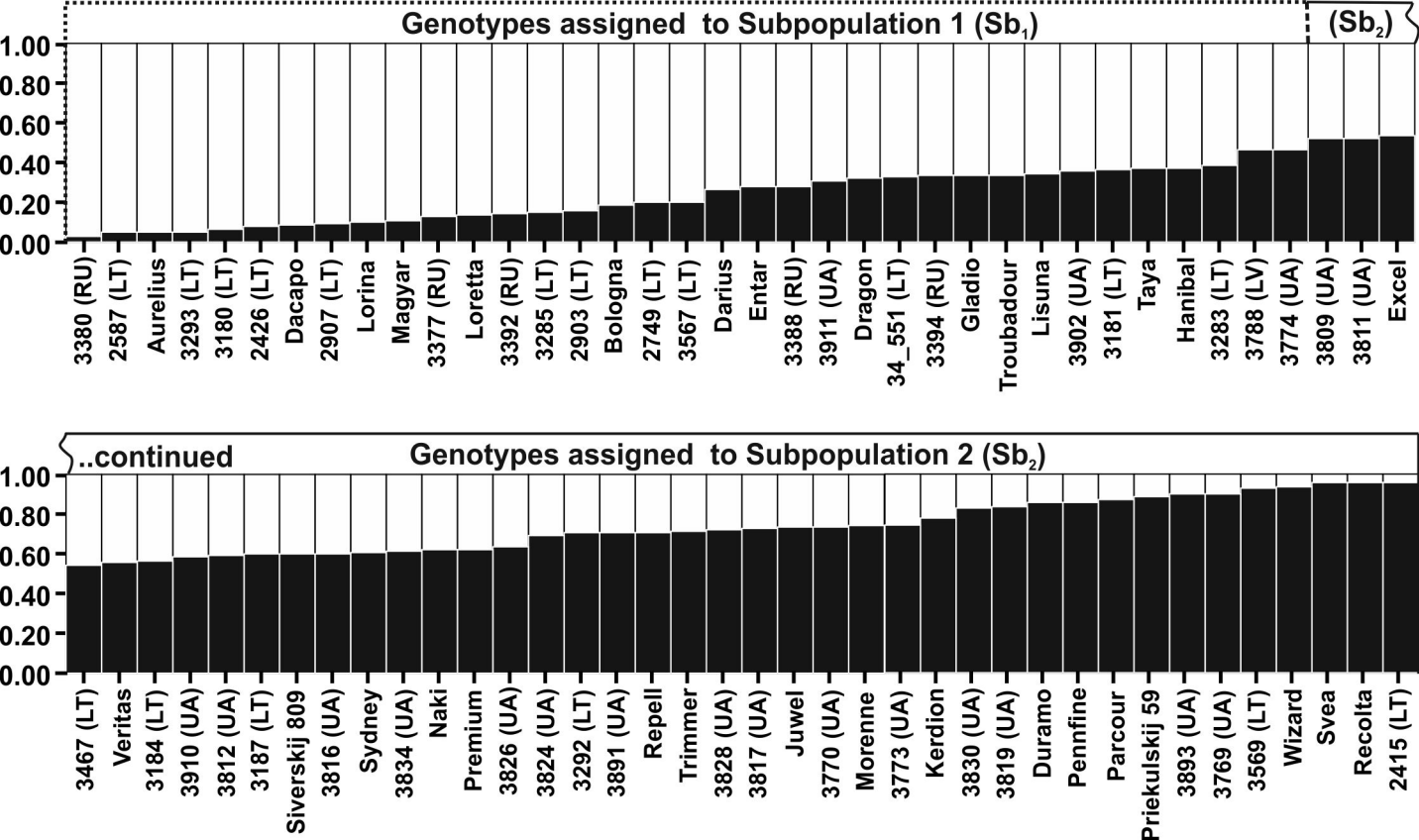
Fig. 4. Marker-trait association and variant alleles of *LpIRI1* gene. (A) Schematic structure of the *LpIRI1* gene. The relative positions of the significant SNPs yielded by marker-trait association analysis are shown within the gene (ATG - start codon; TAA - stop codon). *Wt* represents wild-type genotypes of analysed locuses, while *Rec* defines recombinant genotypes. Amino acid substitutions are indicated in bold for missense mutation loci. (B) Genotype analysis of the *LpIRI1* gene. Electrolyte leakage (EL) at -8 °C and -12 °C against significant SNP markers in *LpIRI1* gene. The error bars represent the standard deviation of measurements

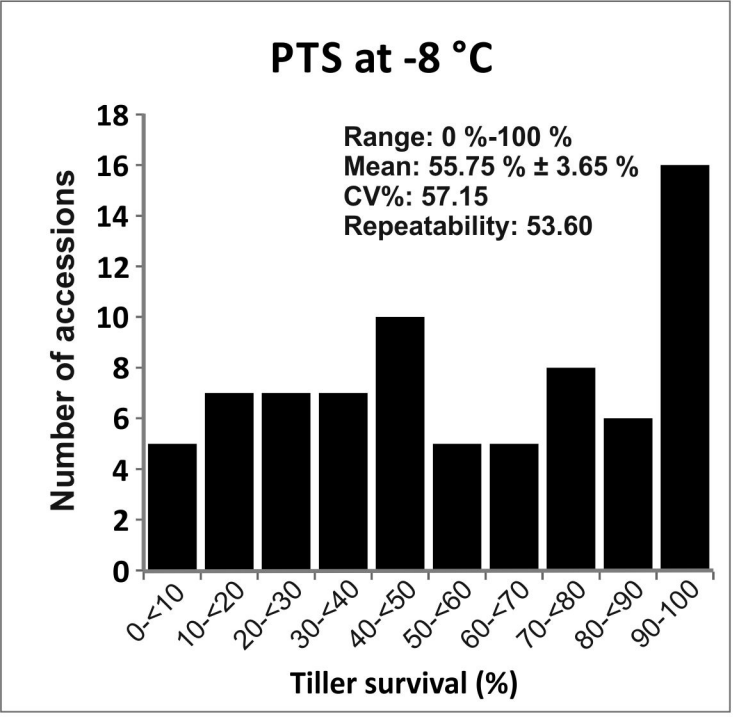
Table 1. Phenotypic Spearman rank correlation between electrolyte leakage (EL), percentage of tiller survival (PTS), cold-acclimated plant proline content (PC) and field winter survival (FWS)

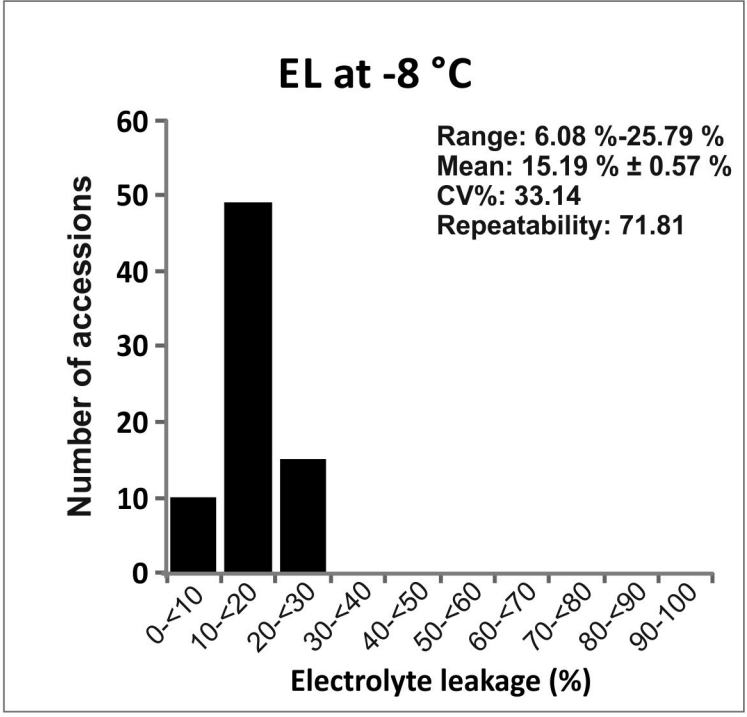
Trait	EL at -8 °C (%)	EL at -12 °C (%)	PTS at -8 °C (%)	PTS at -12 °C (%)	PC (µg $^{-1}$ DW)
EL at -8 °C (%)	-				
EL at -12 °C (%)	0.43***	-			
PTS at-8 °C (%)	-0.40***	-0.21 ^{ns}	-		
PTS at -12 °C (%)	-0.14 ^{ns}	-0.49***	0.46***	-	
PC (µg g $^{-1}$ DW)	-0.06 ^{ns}	0.02 ^{ns}	-0.20 ^{ns}	0.03 ^{ns}	-
FWS (score)	-0.21 ^{ns}	-0.48***	0.01 ^{ns}	0.03 ^{ns}	-0.06 ^{ns}

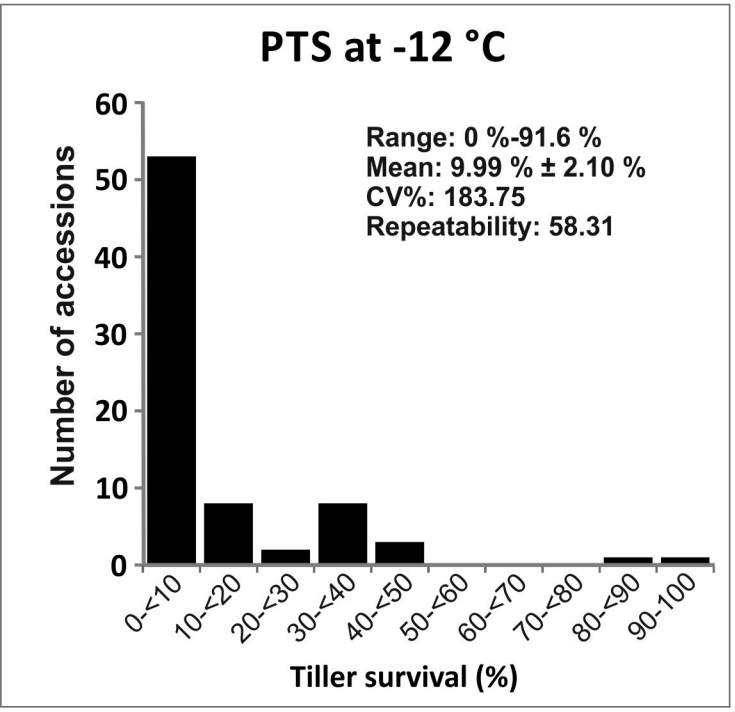
^{ns} Not significant at the 0.05 probability level
*** Significant at 0.001 probability level

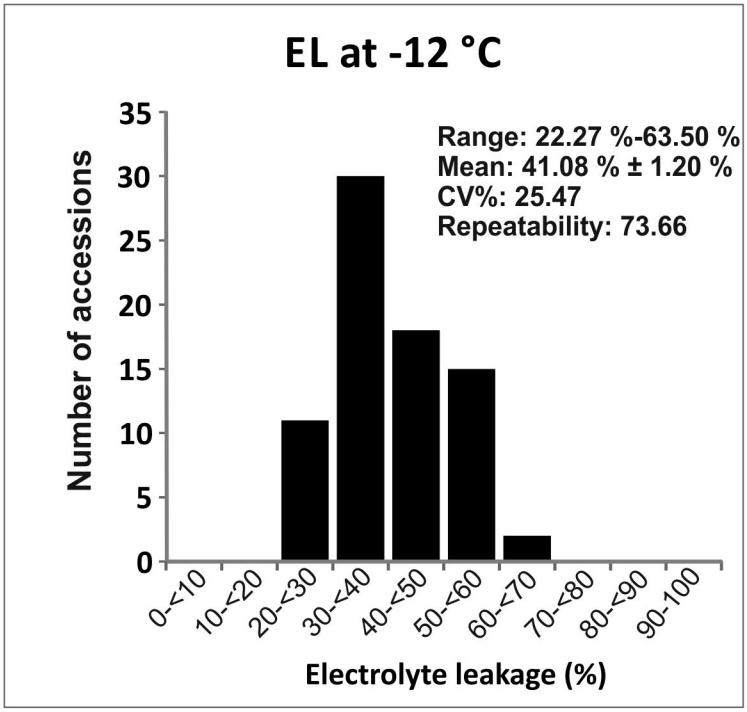

25
15
5
-5
-15
-25
-35
Depth (cm)
Temperature (°C)
I II III
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
Snow cover (cm)
Frozen ground (cm)
Air temperature (°C)
Thawing (cm)

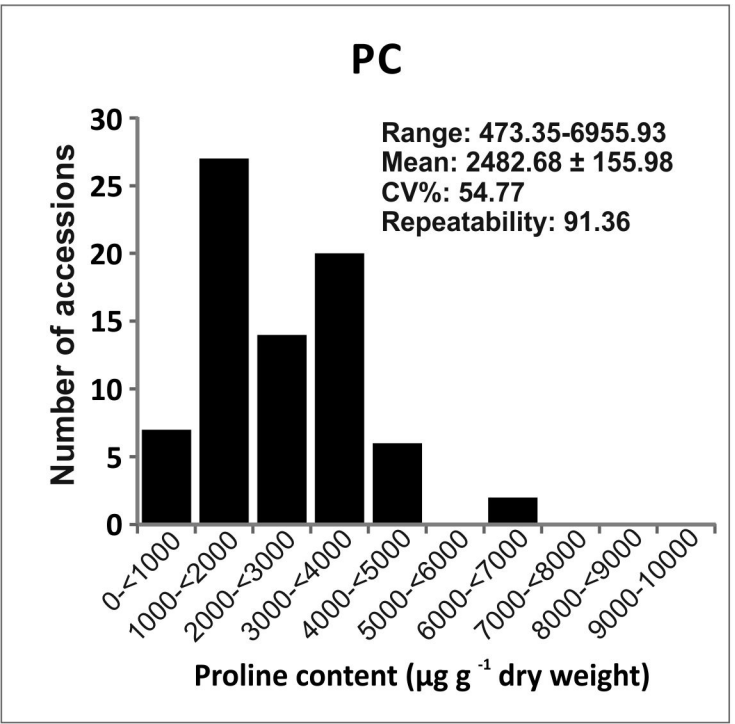

Genotypes assigned to Subpopulation 1 (Sb1)
(Sb2)
1.00
0.80
0.60
0.40
0.20
0.00
3380 (RU)
2587 (LT)
Aurelius
3293 (LT)
3180 (LT)
2426 (LT)
Dacapo
2907 (LT)
Lorina
Magyar
3377 (RU)
Loretta
3392 (RU)
3285 (LT)
2903 (LT)
Bologna
2749 (LT)
3567 (LT)
Darius
Entar
3388 (RU)
3911 (UA)
Dragon
34_551 (LT)
3394 (RU)
Gladio
Troubadour
Lisuna
3902 (UA)
3181 (LT)
Taya
Hanibal
3283 (LT)
3788 (LV)
3774 (UA)
3809 (UA)
3811 (UA)
Excel
..continued
Genotypes assigned to Subpopulation 2 (Sb2)
1.00
0.80
0.60
0.40
0.20
0.00
3467 (LT)
Veritas
3184 (LT)
3910 (UA)
3812 (UA)
3187 (LT)
Siverskij 809
3816 (UA)
Sydney
3834 (UA)
Naki
Premium
3826 (UA)
3824 (UA)
3292 (LT)
3891 (UA)
Repell
Trimmer
3828 (UA)
3817 (UA)
Juwel
3770 (UA)
Morenne
3773 (UA)
Kerdion
3830 (UA)
3819 (UA)
Duramo
Pennfine
Parcour
Priekulskij 59
3893 (UA)
3769 (UA)
3569 (LT)
Wizard
Svea
Recolta
2415 (LT)

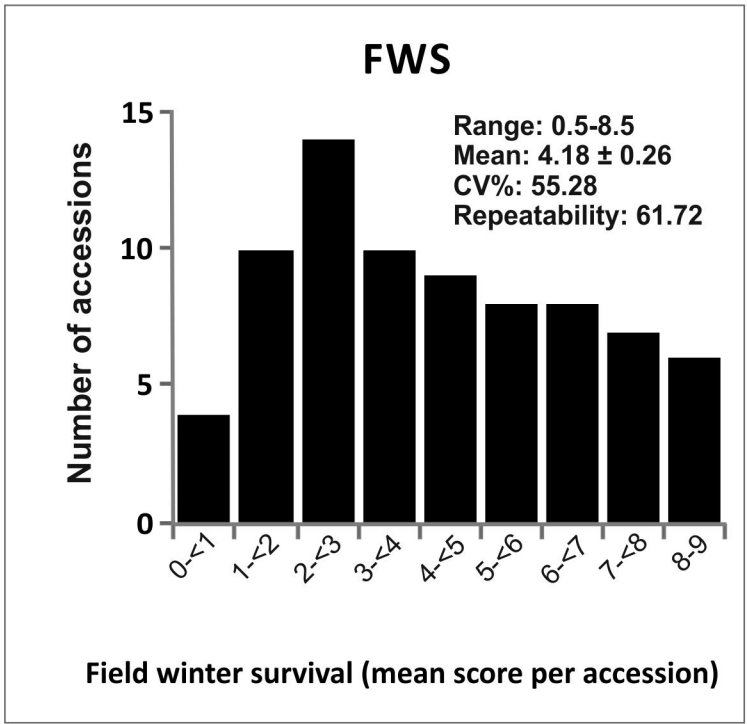

PTS at -8 °C
Range: 0 %-100 %
Mean: 55.75 % ± 3.65 %
CV%: 57.15
Repeatability: 53.60
Number of accessions
0
2
4
6
8
10
12
14
16
18
0-<10
10-<20
20-<30
30-<40
40-<50
50-<60
60-<70
70-<80
80-<90
90-100
Tiller survival (%)


EL at -8 °C
Range: 6.08 %-25.79 %
Mean: 15.19 % ± 0.57 %
CV%: 33.14
Repeatability: 71.81
Number of accessions
0
10
20
30
40
50
60
0-<10
10-<20
20-<30
30-<40
40-<50
50-<60
60-<70
70-<80
80-<90
90-100
Electrolyte leakage (%)


PTS at -12 °C
Range: 0 %-91.6 %
Mean: 9.99 % ± 2.10 %
CV%: 183.75
Repeatability: 58.31
Number of accessions
0
10
20
30
40
50
60
0-<10
10-<20
20-<30
30-<40
40-<50
50-<60
60-<70
70-<80
80-<90
90-100
Tiller survival (%)


EL at -12 °C
Range: 22.27 %-63.50 %
Mean: 41.08 % ± 1.20 %
CV%: 25.47
Repeatability: 73.66
Number of accessions
0
5
10
15
20
25
30
35
0-<10
10-<20
20-<30
30-<40
40-<50
50-<60
60-<70
70-<80
80-<90
90-100
Electrolyte leakage (%)


PC
Range: 473.35-6955.93
Mean: 2482.68 ± 155.98
CV%: 54.77
Repeatability: 91.36
Number of accessions
0
5
10
15
20
25
30
0-<1000
1000-<2000
2000-<3000
3000-<4000
4000-<5000
5000-<6000
6000-<7000
7000-<8000
8000-<9000
9000-10000
Proline content (µg g -1 dry weight)


FWS
Range: 0.5-8.5
Mean: 4.18 ± 0.26
CV%: 55.28
Repeatability: 61.72
Number of accessions
0
5
10
15
0-<1
1-<2
2-<3
3-<4
4-<5
5-<6
6-<7
7-<8
8-9
Field winter survival (mean score per accession)

(A)

missense mut.

missense mut.

synonymous mut.

SNP at position 322:

Homozygous Wt	NLANNRLVGTIP **S/S** WIGELD
Recombinant genotype	NLANNRLVGTIP **S/T** WIGELD

SNP at position 369:

Homozygous Wt	GELDHLLYLDLS **H/H** NSLVGE
Recombinant genotype	GELDHLLYLDLS **H/Q** NSLVGE

(B)

Genotype	Number of access.	Locus 322	Locus 369	Locus 726	EL at -8 °C (%)	EL at -12 °C (%)
Wt	52	T:T	T:T	C:C	14.0 ± 0.63	39.4 ± 1.38
Rec-1	5	A:T	G:T	C:C	18.1 ± 2.48	46.9 ± 4.08
Rec-2	4	T:T	T:T	C:T	16.8 ± 2.89	42.4 ± 5.57
Rec-3	6	A:T	T:T	C:T	14.8 ± 1.88	34.8 ± 3.47
Rec-4	8	A:T	G:T	C:T	19.7 ± 1.75	50.3 ± 3.79