Chapter 2
Evaluation of Perennial Ryegrass Association Mapping Population for Freezing Tolerance Traits

A Aleliūnas, G Statkevičiūtė, K Jonavičienė, V Kemešytė and G Brazauskas

2.1 Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important agricultural cool-season grass species in temperate climate areas throughout the world, grown for forage with a high nutritive value. It is also widely used as a turf grass species with rapid establishment rate and excellent tolerance to traffic. These properties make the perennial ryegrass economically the most important species within the genus *Lolium* (Humphreys et al. 2006). Despite its many superior properties perennial ryegrass is sensitive to abiotic stresses. It exhibits poor winter survival under harsh and cold winters (Hulke et al. 2008), thus limiting its cultivation in certain areas. It was shown that perennial ryegrass responds to cold-acclimation, which increases its freezing tolerance (Ebdon et al. 2002). Cold acclimation is a multigenic quantitative trait associated with many physiological and biochemical changes within the plant cell (Hannah et al. 2005). Increase of water soluble sugar and proline concentration, as well as alteration of lipid composition and significant differentiation in profiles of protein accumulation is observed during cold acclimation in perennial ryegrass (Hoffman et al. 2010; Bocian et al. 2011). Proline acts as a free radical scavenger (Kaul et al. 2008), as osmoprotectant (Yoshiba et al. 1997), or as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995). The assumption was made that proline accumulation in plants under stress conditions has a protective function; however, correlation between proline accumulation and abiotic stress tolerance in plants is not always apparent (Szabados and Savouré 2010). Some data indicate that overproduction of proline in plants stabilises the cell membrane thereby preventing electrolyte leakage (Hayat et al. 2012). Moreover, low temperature can induce cell membrane lipid peroxidation (Campos et al. 2003), thus proline, as a free radical scavenger, could have an effect on membrane stability

G. Brazauskas (✉) · A. Aleliūnas · G. Statkevičiūtė · K. Jonavičienė · V. Kemešytė
Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, Instituto a. 1, Akademija, 8344 Kėdainiai distr, Lithuania
e-mail: gintaras@lzi.lt

© Springer International Publishing Switzerland 2015

H. Budak, G. Spangenberg (eds.), *Molecular Breeding of Forage and Turf,*
DOI 10.1007/978-3-319-08714-6_2

maintenance. However, a lack of close association between freezing tolerance and proline content in perennial ryegrass and other species has been reported (Hoffman et al. 2010; Thomas and James 1993; Dionne et al. 2001). Cell membrane deterioration is the primary freeze-induced damage in plants, caused by cell dehydration, as it occurs when extracellular ice forms during freezing (Pearce 2001). Water potential of ice depends on the temperature, hence, the lower the temperature is the greater the damage is caused by the dehydration. Extracellular ice formation can also obstruct gas and solute exchange, while growing crystals can cause cell lysis (John et al. 2009). Freezing tolerant organisms have developed diverse mechanisms to survive subfreezing temperatures (Zachariassen and Kristiansen 2000) by minimising the damage caused by ice formation. Antifreeze proteins (AFPs) are considered to be one of the mechanisms to reduce freezing induced damage in plants and other kingdoms. Ice recrystallization activity (IRI) of AFPs is more common in freeze tolerant organisms such as plants (Middleton et al. 2012). As the common freeze-induced damage is cell membrane physical deteriorations, one of the most popular methods to measure the extent of the membrane injury is electrolyte leakage assay. This technique has been applied to quantify damages to cell membranes in various abiotic stress conditions (Bajji et al. 2001). The objective of this study was to evaluate perennial ryegrass association mapping population for variation in freezing tolerance related traits. This included estimation of winter survival of 210 perennial ryegrass accessions in the field, and further investigation of plant survival, electrolyte leakage and proline content in a subset of 76 genotypes under controlled-environment conditions.

2.2 Methods

2.2.1 Plant Material and DNA Extraction

Perennial ryegrass mapping panel consisted of 94 cultivars and 116 ecotypes. The ecotype group comprised 37 accessions from Lithuania, 3 accessions from Latvia, 2 from Poland, 9 accessions from Kaliningrad region (Russian Federation), 3 accessions from Slovakia and 62 accessions from Ukraine (Statkevičiūtė et al. 2014). All ecotype accessions were collected in their natural habitats. Cultivar group consisted of 89 cultivars developed in Europe, 4 in the USA and 1 in Japan. Plants were established from seeds and propagated in the greenhouse. Ploidy level of accessions was determined by Partec PA (Partec Gmbh, Germany) flow cytometer, and tetraploid plants were discarded from further 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 using a NanoDrop® 2000 (Thermo Fisher Scientific, USA) spectrophotometer.

2.2.2 Field Winter Survival Analysis

Ramet-based field collection of 210 perennial ryegrass accessions was established in the experimental fields of the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry (Akademija, Lithuania) in 2013. Single seed per accession was used for plant establishment in the greenhouse. Four ramets of each genotype were planted at 50 × 50 cm distances, using a randomised complete block design. Winter survival was evaluated in spring 2014 by visual scoring using a scale ranging from 1 (dead) to 9 (no visible damage) as described by Tyler et al. (1987).

2.2.3 Freezing Tolerance Testing Under Controlled Environment

Freezing tolerance test under controlled environment conditions was performed on a subset of 76 perennial ryegrass genotypes. Twenty days prior cold-acclimation plants were moved into phytotron, maintaining +20 °C, 450 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and 80% relative air humidity. Plants were cold-acclimated for 15 days prior the freezing test under controlled environment conditions at +5 °C, maintaining 200 µmol m^{-2} s^{-1} PAR, 12/12 h photoperiod and 80% relative air humidity. After cold-acclimation period plants were divided into individual tillers, while leaves and roots were trimmed to 2 cm. Tillers were placed into test tubes of 10 ml, containing 1 ml of deionised water and 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 lowered from +5 °C to the desired treatment temperature at a rate of 1.2 °C h^{-1}. As temperature reached −8 °C and −12 °C test tubes of our examined genotypes were removed from the chamber and thawed on ice overnight at +4 °C. Later plants were replanted in cell trays and placed back to phytotron, while maintaining +20 °C, 450 µmol m^{-2} s^{-1} PAR and 80% relative air humidity. Plant survival was scored after 3-week recovery period. Regrown plants were scored as survivors.

2.2.4 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, while leaves and roots were trimmed. Tillers were washed with deionised water, placed into glass test tubes containing 1 ml of deionised water and agitated for 15 min. Then they were washed again and wiped with paper towel. Four tillers per accession were placed into individual test tubes, containing 1 ml of deionised water and covered with parafilm. In total, 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. Prepared samples were subjected to freezing. When the target temperature of −8 °C and −12 °C was reached, racks were removed from freezing chamber and thawed

on ice at +4 °C and 20 ml of deionised water was added into each tube. The tubes were shaken overnight at 120 rpm and then initial conductivity (C_{ini}) was measured by YSI 3100 (YSI Incorporated, USA) conductivity meter with YSI 3253 Glass Dip Cell. The total conductivity (C_{tot}) was measured after autoclaving the samples at 120 °C for 15 min. Electrolyte leakage (EL) was computed as $EL = (C_{ini})/(C_{tot}) \times 100$.

2.2.5 Proline Content Evaluation

Free proline content was determined in cold-acclimated plants prior to freezing application. Upper leaves were collected for each accession and immediately transferred to +70 °C preheated incubator and dried 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.

2.2.6 Molecular Profiling by AFLP

AFLP profiling was used for the assessment of genetic diversity among the perennial ryegrass accessions. Genomic DNA (500 ng) was digested with Fast Digest® *Taq*I and *Ase*I restriction endonucleases (Thermo Scientific, Lithuania). *Taq*I adapter was prepared by combining 25 µg oligo_T_top and 22 µg oligo_T_bottom (Table 2.1) and deionised water was added to total volume of 100 µl. Prepared solution was incubated at 95 °C for 5 min and then slowly cooled for 2 h. *Ase*I adapter was prepared in the same manner, except 2.6 µg of oligo_A_top and 2 µg of oligo_A_bottom were used. The adapters were added to the digested DNA mixture (as described above) to final concentrations of 50 pM (*TaqI* adapter) and 5 pM

Table 2.1 List of the oligos for AFLP analyses

Oligo ID	Oligo sequence
oligo_A_bottom	5'- TAGGTACGCAGTC
oligo_A_top	5'- CTCGTAGACTGCGTACC
oligo_T_top	5'- GACGATGAGTCCTGAC
oligo_T_bottom	5'- CGGTCAGGACTCAT
oligo_Pre_amp_F	5'- CTCGTAGACTGCGTACCTAAT
oligo_Pre_amp_R	5'- GACGATGAGTCCTGACCGA
IR800Ase + ACG	5'- GACTGCGTACCTAATACG
IR800Ase + ACC	5'- GACTGCGTACCTAATACC
Taq + CAC	5'- GATGAGTCCTGACCGACAC
Taq + CTA	5'- GATGAGTCCTGACCGACTA
Taq + CAA	5'- GATGAGTCCTGACCGACAA

(*Ase*I adapter) and ligated to restricted genomic DNA fragments. Preamplification was carried out using 10 µl tenfold diluted ligation mixture, 0.5 µM oligo_Pre_amp_F and 0.5 µM oligo_Pre_amp_R preamplification primers and 1x DreamTaq PCR Master Mix (Thermo Scientific, Lithuania) in 50 µl total volume using following PCR program with 20 cycles of each: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. Selective amplification was performed on 5 µl 20-fold diluted preamplification PCR product, as PCR mixture consisted of 1x DreamTaq PCR buffer, 0.25 mM dNTP, 1.25 units of DreamTaq DNA polymerase, 0.05 µM IRDye®800-labelled forward primer and 0.3 µM reverse primer. Total volume of the mixture was adjusted to 10 µl by adding deionised water. Three primer combinations, harbouring three selective bases were used for selective amplification in AFLP: IR800AseACG + TaqCAC, IR800AseACC + TaqCTA and IR800AseACC + TaqCAA (Table 2.1). PCR program for selective amplification included 12 cycles of each: 94 °C for 30 s, initial annealing temperature of 65 °C (with decrement of −0.7 °C each cycle) for 30 s, 72 °C for 1 min, followed by 24 cycles with 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min. PCR products were separated on 6.5% KB$_{PLUS}$ Gel Matrix (LI-COR, Inc., USA) and detected by the DNA Analyzer 4300 (LI-COR, Inc., USA). Fragments were scored using SAGA Generation 2 software (LI-COR, Inc., USA). AFLP analysis was conducted in duplicate to check for the consistency between replicates.

2.2.7 Population Structure and Relative Kinship

An admixture model in STRUCTURE software package (Pritchard et al. 2000) was used to infer population structure. 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.

2.2.8 Statistic Analysis

Analysis of variance (ANOVA) with *post hoc* Tukey HSD test and Spearman's rank correlation coefficients were calculated using STATISTICA 7 (StatSoft, Inc.).

2.3 Results

Winter survival under field conditions was evaluated for 210 perennial ryegrass genotypes. Analysis of variance revealed that one replicate block in our field collection differed significantly ($p < 0.05$) from the other three in winter survival, so it was excluded from further analyses. ANOVA *post hoc* Tukey HSD test on winter

survival revealed significant ($p < 0.0001$) differences between ecotype and cultivar groups. Plants within ecotype group showed better winter survival performance compared to cultivar group. The mean winter survival score was 4.71 and 2.76 for ecotype and cultivar groups, respectively. In general, perennial ryegrass accessions demonstrated good winter survival with only 41 plant out of 630 being lost due to poor overwintering. A subset of 76 perennial ryegrass genotypes was further examined for freezing tolerance in controlled-environment experiment. Freezing tolerance was defined as percentage of tiller survival (PTS) in controlled freezing test. PTS ranged from 0 to 100 % (mean 55.76 % ± 3.67 %) and from 0 to 91.67 % (mean 9.99 % ± 2.01 %) at −8 °C and −12 °C, respectively. Twenty five freeze-susceptible (PTS at −8 °C < 44 %) and 5 freeze-tolerant (PTS at −12 °C > 44 %) genotypes were identified in this subset. While significant differences in freezing tolerance were observed between individual genotypes, ANOVA *post hoc* Tukey HSD test revealed no differences in PTS between ecotype and cultivar groups at both tested temperatures. Investigated perennial ryegrass accessions significantly ($p < 0.001$) differed in proline content (PC), as well as in electrolyte leakage (EL) (Table 2.2). PC ranged from 0.47 to 6.15 mg g^{-1} DW, while EL varied from 6.09 to 25.80 % (mean

Table 2.2 Electrolyte leakage (EL) at −8 °C and −12 °C, percentage tiller survival (PTS) at −8 °C and −12 °C, proline content (PC) and winter survival (FWS) variation among perennial ryegrass accessions

Trait	Ecotypes				Cultivars			
	Mean	Min	Max	SD[a]	Mean	Min	Max	SD[a]
EL −8 °C	14.27	6.09	25.80	5.23	15.83	7.82	25.06	4.82
EL −12°C	38.91	22.27	57.06	9.14	43.09	23.85	63.51	11.48
PTS −8°C	53.46	0.00	100.00	5.32	58.75	0.00	100.00	4.80
PTS −12°C	10.30	0.00	91.67	3.15	9.60	0.00	53.33	2.62
PC	2.55	0.47	6.15	1.36	2.52	0.51	5.84	1.34
FWS	4.71	0.00	9.00	2.77	2.76	0.00	9.00	2.36

[a] Standard deviation

Table 2.3 Phenotypic correlation between electrolyte leakage (EL) at −8 °C and −12 °C, percentage tiller survival (PTS) at −8 °C and −12 °C, proline content (PC) and field winter survival (FWS)

Trait	EL −8°C	EL −12°C	PTS −8°C	PTS −12°C	PC
	rho[a]	rho	rho	rho	rho
EL −12°C	0.43***	–	–	–	–
PTS −8°C	−0.40***	−0.21 ns	–	–	–
PTS −12°C	−0.14 ns[b]	−0.49***	0.46***	–	–
PC	−0.06 ns	0.02 ns	−0.20 ns	0.03 ns	–
FWS	−0.21 ns	−0.48***	0.01 ns	0.03 ns	−0.06 ns

*** Significant ($p < 0.001$)

[a] Spearman's rank correlation coefficient

[b] Non significant ($p > 0.05$)

15.17%±0.58%) at −8 °C and from 22.27 to 63.51% (mean 41.10%±1.23%) at −12 °C between individual accessions. No significant ($p>0.05$) differences were detected in PC between ecotype and cultivar groups. ANOVA *post hoc* Tukey HSD test also revealed no significant ($p>0.05$) differences in EL at −8 °C between ecotype and cultivar groups, however difference in EL at −12 °C between the groups was significant ($p<0.05$), averaging 43.94%±1.94% and 39.09%±1.42% for cultivar and ecotype groups, respectively. No significant ($p>0.05$) differences in EL between plants in ecotype group regarding to the place of origin at both tested freezing temperatures was detected. Spearman's rank correlation coefficients between investigated individual traits were calculated (Table 2.3). Significant moderate negative correlations were observed between EL at −12 °C and PTS at −12 °C (rho=−0.49, $p<0.001$), as well as EL at −8 °C negatively correlated with PTS at −8 °C (rho=−0.40, $p<0.001$). Moderate positive correlation (rho=0.43, $p<0.001$) was found between EL at −8 °C and EL at −12 °C, as well as PTS at −8 °C and PTS at −12 °C (rho=0.46, $p<0.001$). There was no correlation between PC and any other examined trait. No significant ($p>0.05$) correlation was observed between winter survival and freezing-tolerance under controlled environment conditions. Population structure and kinship was assessed with AFLP markers. AFLP markers are known to show random distribution across genomes which makes them markers of choice for population structure examination. AFLP analysis scored a total of 227 bands. Majority of the bands were polymorphic (98.67%). Inbreeding coefficient was low with a value of 0.068. Methodology of Evano et al. (2005) was used to define the number of subpopulations. The Q value matrix with k=2 was generated, which indicates two clusters in our perennial ryegrass panel. The STRUCTURE analysis revealed that most ecotype accessions from Ukraine fall into the subpopulation 1 while ecotypes from Lithuania, Latvia and Kaliningrad region correspond to subpopulation 2. AFLP data showed remarkable heterogeneity among cultivars with accessions nearly-equally distributed between both subpopulations. No obvious kinship was found in perennial ryegrass panel. Pairwise kinship estimates were low with 49.49% of the values equal to zero, while 82.71% were lower than 0.05.

2.4 Discussion

Winter survival is a complex trait. Although freezing-tolerance of the plants is considered as the major component of winter survival (Teutonico et al. 1995), the absence of significant correlation between PTS under controlled-environment conditions and FWS under field conditions in this experiment indicates the existence of other major factors contributing to winter survival performance of the perennial ryegrass. Furthermore, the absence of correlation between the traits could reflect the differences in cold-acclimation, as relatively long and warm autumn in 2013 could have had an impact on the physiological condition of the plants in the field. In general, ecotypes demonstrated better FWS performance than cultivars. It indicates that the genetic potential for freezing tolerance has not been fully exploited in perennial ryegrass cultivars. A subset of 76 genotypes was screened for direct and indirect freezing tolerance

parameters in controlled-environment experiment. EL assay was used to assess cell membrane damage and very low variation was observed between replicates using this method (data not shown). No differentiation in freezing tolerance parameters between ecotype and cultivar groups was detected, except for EL at −12 °C. EL is considered as indirect freezing tolerance parameter (Espevig et al. 2011). Ebdon et al. (2002) revealed that LT_{50} derived from EL curves correlate with winter survival estimates for both hardened and non-hardened perennial ryegrass plants, nonetheless we found only moderate negative correlations between EL and PTS at both tested temperatures. Most studies use LT_{50} or EL_{50} values to define plant freezing tolerance and EL. Estimation of these values are labour-intensive and time-consuming, thus problematic for numerous sample sizes. A freezing test at fixed-point temperature was chosen to screen a larger number of samples in the current population. Cell membrane stability is maintained by the accumulation of solutes, such as free proline, during cold acclimation (Zhang et al. 2010). Substantial variation for free proline content was observed in our perennial ryegrass panel; however, no correlation could be detected with other freezing tolerance parameters. While some studies indicate, that proline plays a role in cell protection against detrimental effects during freezing (Xin and Browse 2000), it's role is still controversial. Although significantly higher proline content in freezing tolerant accessions of perennial ryegrass as compared to freezing-susceptible genotypes was detected after 14 days of cold-acclimation, the differences were not apparent a week later (Hoffman et al. 2010). Thomas and James (Thomas and James 1993) compared freezing tolerance of compost- and sand-grown perennial ryegrass. They found the latter to be as hardy as grown in compost, despite having a tenth fraction of the proline content. Those findings altogether make evidence that there is no strong association between proline content and freezing tolerance.

AFLP markers revealed two subpopulations in the population of 210 genotypes and low kinship between individuals. High variation for freezing tolerance and minimum kinship between genotypes indicate the suitability of the population for association mapping of freezing tolerance related traits, while existence of two subpopulations implements the use of population structure controlled model for marker-trait association analysis.

Acknowledgments This study was funded by the Research Council of Lithuania, grant No. MIP-032/2012 (FUMAG).

References

Ábrahám E, Hourton-Cabassa C, Erdei L, Szabados L (2010) Methods for determination of proline in plants. In: Sunkar R (ed) Plant stress tolerance. Humana Press, New York, pp 322–324

Bajji M, Kinet JM, Lutts S (2001) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul 00:1–10

Bocian A, Kosmala A, Rapacz M, Jurczyk B, Marczak Ł, Zwierzykowski Z. (2011) Differences in leaf proteome response to cold acclimation between *Lolium perenne* plants with distinct levels of frost tolerance. J Plant Physiol 168:1271–1279

Campos PS, Quartin V, Ramalho JC, Nunes MA (2003) Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea sp.* plants. J Plant Physiol 160:283–292

Dionne J, Castonguay Y, Nadeau P, Desjardins Y (2001) Amino acid and protein changes during cold acclimation of green-type annual bluegrass (*Poa annua* L.) ecotypes. Crop Sci 41:1862–1870

Ebdon JS, Gagne RA, Manley RC (2002) Comparative cold tolerance in diverse turf quality genotypes of perennial ryegrass. Hort Sci 37:826–830

Espevig T, Dacosta M, Hoffman L, Aamlid TS, Tronsmo AM, Clarke BB, Huang B (2011) Freezing tolerance and carbohydrate changes of two agrostis species during cold acclimation. Crop Sci 51:1188–1197

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14:2611–2620

Hannah MA, Heyer AG, Hincha DK (2005) A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. PLoS Genet 1:e26

Hardy OJ, Vekemans X (2002) SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol Ecol Notes 2:618–620

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant Signal Behav 7:1456–1466

Hoffman L, DaCosta M, Ebdon JS, Watkins E (2010) Physiological changes during cold acclimation of perennial ryegrass accessions differing in freeze tolerance. Crop Sci 50:1037–1047

Hulke BS, Watkins E, Wyse DL, Ehlke NJ (2008) Freezing tolerance of selected perennial ryegrass (*Lolium perenne* L.) accessions and its association with field winterhardiness and turf traits. Euphytica 163:131–141

Humphreys MW, Yadav RS, Cairns AJ, Turner LB, Humphreys J, Skøt L (2006) A changing climate for grassland research. New Phytol 169:9–26

John UP, Polotnianka RM, Sivakumaran KA, Chew O, Mackin L, Kuiper MJ, Talbot JP, Nugent GD, Mautord J, Schrauf GE, Spangenberg GC (2009) Ice recrystallization inhibition proteins (IRIPs) and freeze tolerance in the cryophilic Antarctic hair grass *Deschampsia antarctica* E. Desv. Plant Cell Environ 32:336–348

Kaul S, Sharma SS, Mehta IK (2008) Free radical scavenging potential of L-proline: evidence from in vitro assays. Amino Acids 34:315–320

Lassner MW, Peterson P, Yoder JI (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol Biol Rep 7:116–128

Middleton AJ, Marshall CB, Faucher F, Bar-Dolev M, Braslavsky I, Campbell RL, Walker VK, Davies PL (2012) Antifreeze protein from freeze-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. J Mol Biol 416:713–724

Pearce SR (2001) Plant Freezing and Damage. Ann Bot 87:417–424

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Srinivas V, Balasubramanian D (1995) Proline is a protein-compatible hydrotrope. Langmuir 11:2830–2833

Statkevičiūtė G, Aleliūnas A, Kemešytė V, Pašakinskienė I, Brazauskas G (2014) AFLP analysis of genetic diversity in an association mapping panel of *Lolium perenne* L. In: Sokolović D, Huyghe C, Radović J (eds) Quantitative traits breeding for multifunctional grasslands and turf. Springer, Netherlands

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15:89–97

Teutonico RA, Yandell B, Satagopan JM, Ferreira ME, Palta JP, Osborn TC (1995) Genetic analysis and mapping of genes controlling freezing tolerance in oilseedBrassica. Mol Breed 1:329–339

Thomas H, James AR (1993) Freezing tolerance and solute changes in contrasting genotypes of *Lolium perenne* L. acclimated to cold and drought. Ann Bot 72:249–254

Tyler BF, Hayes JD, Davies WE (eds) (1987) Collection, characterization and utilization of genetic resources of temperate forage grass and clover. IBPGR, Rome, pp 1–65

Xin Z, Browse J (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. Plant Cell Environ 23:893–902

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 38:1095–1102

Zachariassen KE, Kristiansen E (2000) Ice nucleation and antinucleation in nature. Cryobiology 41:257–279

Zhang C, Fei SZ, Arora R, Hannapel DJ (2010) Ice recrystallization inhibition proteins of perennial ryegrass enhance freezing tolerance. Planta 232:155–164

http://www.springer.com/978-3-319-08713-9

Molecular Breeding of Forage and Turf
The Proceedings of the 8th International Symposium on the Molecular Breeding of Forage and Turf
Budak, H.; Spangenberg, G. (Eds.)
2015, XIII, 194 p. 53 illus., 26 illus. in color., Hardcover
ISBN: 978-3-319-08713-9