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The objectives of this research conducted by Iowa State University scientists were to (1) determine the effect of the length of cold hardening on freezing tolerance in perennial ryegrass; (2) compare the response of annual and perennial ryegrass to cold hardening; (3) identify genes of which their expression levels are either increased, significantly suppressed, or stayed the same in response to cold treatment by either expressed sequence tag (EST) analysis or microarray using a barley gene chip; and (4) isolate antifreeze proteins genes from perennial ryegrass genes and assess their functions in the model species of *Arabidopsis*.

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PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 350 projects at a cost of $29 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

Editor

Jeff Nus, Ph.D.
1032 Rogers Place
Lawrence, KS 66049
jnus@usga.org
(785) 832-2300
(785) 832-9265 (fax)

Research Director

Michael P. Kenna, Ph.D.
P.O. Box 2227
Stillwater, OK 74076
mkenna@usga.org
(405) 743-3900
(405) 743-3910 (fax)

Identification of Genes Associated with Cold Hardening in Perennial Ryegrass

Shui-zhang Fei and Chunzhen Zhang

SUMMARY

Cold hardening dramatically increases freezing tolerance in many temperate plant species. An understanding of the mechanism that regulates cold hardening is important for cultivar development of perennial ryegrass which lacks adequate freezing tolerance. This research investigated varied responses of annual and perennial ryegrass to low temperature and the duration of cold hardening on freezing tolerance in perennial ryegrass. A systems biology approach was taken to identify genetic elements (genes) that are responsive to cold hardening. From this research we have found that:

- A 14-day cold hardening at 4^{o} C is sufficient to induce maximum freezing tolerance in mature perennial ryegrass plants. Further exposure to low temperatures above zero does not increase freezing tolerance.
- While perennial ryegrass has the capacity of cold hardening, annual ryegrass does not have such capacity.
- Survey of gene expression by using expressed sequence tags (ESTs) or microarray revealed that many genes are highly responsive during cold hardening; their expressions are either highly increased or significantly suppressed, suggesting their potential roles in cold hardening.
- Two ice recrystallization inhibition protein genes, also referred to as antifreeze protein genes, were isolated from perennial ryegrass. Transfer of these two genes into the model plant species of *Arabidopsis* improved survival of the transgenic plants carrying the genes following a freeze treatment, suggesting that these genes play important roles in freezing tolerance in perennial ryegrass.

Perennial ryegrass is an important turfgrass known for its rapid establishment rate, good wear and traffic tolerance, as well as its low mowing tolerance. However, its lack of winter hardiness often results in winterkill following a severe winter (Figure 1). Improving perennial ryegrass cultivars with enhanced winter hardiness is critically important to extending its area of adaptation. Many temperate plant species can increase freezing tolerance significantly through a period of exposure to nonfreezing low temperatures, a phenomenon known as cold acclimation or cold hardening (9).

Cold hardening occurs naturally when temperature drops and photoperiod becomes shorter in the fall, and it plays a critical role in rendering freezing tolerance to plants. During cold hardening, plants may undergo a number of biochemical or physiological changes including lipid composition changes to maintain cell membrane fluidity, synthesis of cyroprotectants and nonstructural carbohydrates, and production of antioxidants or protein chaperones, among others. These changes are the results of the expression of a subset of genes in response to cold. Identification and characterization of these genes will help elucidate the mechanism that regulates cold hardening and eventually facilitate development of cold hardy perennial ryegrass cultivars.

The development of high-throughput DNA sequencing has made it possible to identify this subset of genes by analysis of either expressed sequence tags (ESTs) or gene chip (microarray) in a high-throughput fashion.

Figure 1. Winterkill of perennial ryegrass following a severe winter in 2007-2008 in Ames, Iowa.

SHUI-ZHANG FEI, Ph.D., Assistant Professor; and CHUNZHEN ZHANG, Graduate Student; Department of Horticulture, Iowa State University, Ames, IA

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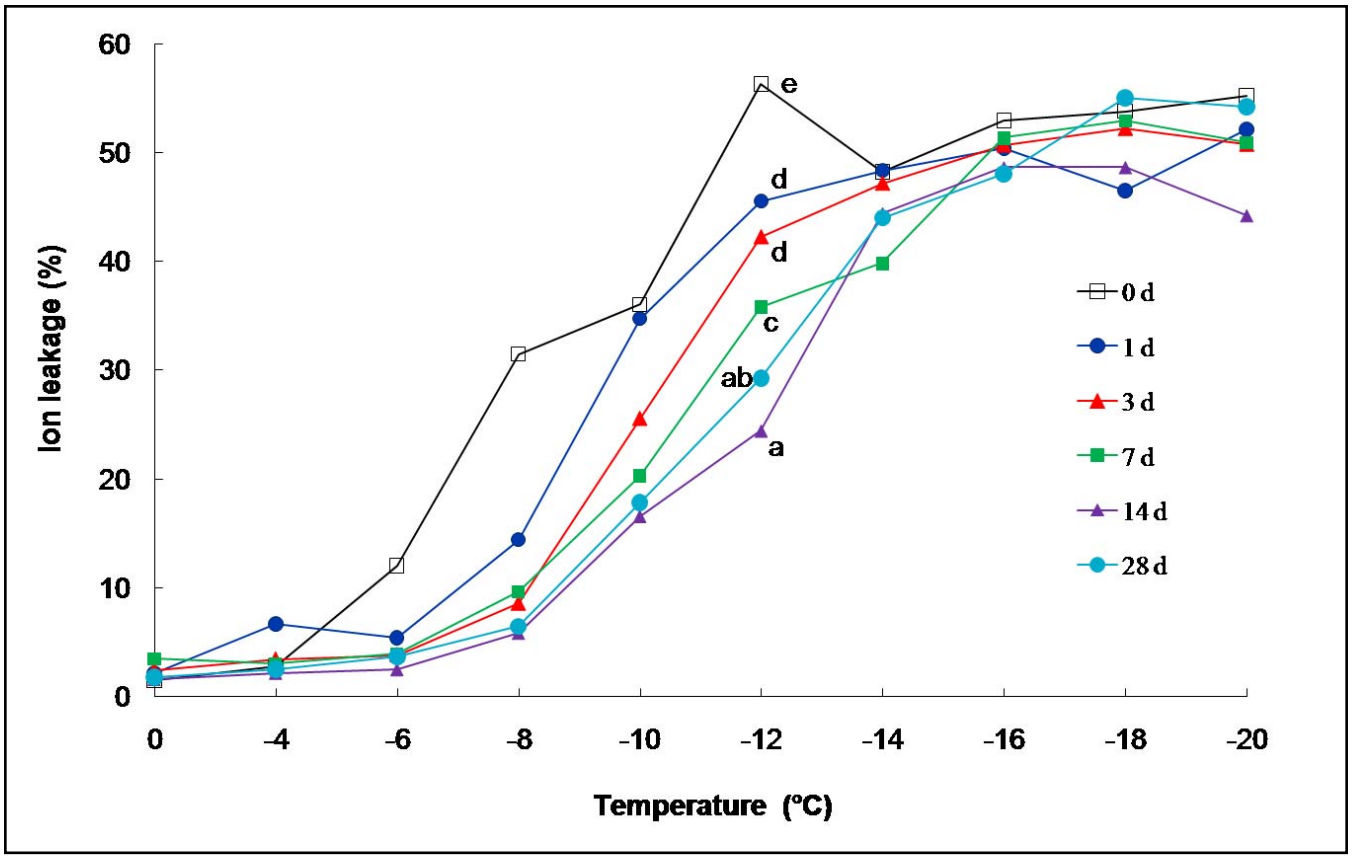

Figure 2. The effect of the length of cold hardening on freezing tolerance in perennial ryegrass. Ion leakage induced by freezing in leaf tissues of perennial ryegrass cv. 'Caddyshack'. Mature plants were cold-hardened at 4 °C for 0, 1, 3, 7, 14, or 28 days and leaf segments were subjected to freezing treatment. Ion leakage was measured at 0°C, and -4 to -26 °C with an interval of 2°C. Only results from -4 to -20 °C are shown. An F-test was done for the ion leakages at -12 °C. Data points with a same letter are not statistically different.

Instead of studying a single gene at a time, both EST and microarray analyses can obtain a global view of what genes are expressed or suppressed by determining the relative abundance of the expressed forms of these genes. Both methods can be used to compare the relative abundance of each gene in stressed and nonstressed plants at the same time. Such comparative analysis can reveal the patterns of gene expression by examining the expression levels of these genes (either up- or down-regulated) in response to a particular stress (1). Information generated from such studies provides important insights into the roles that certain genes may play in important signal transduction cascade that leads to stress responses (2).

The objectives of this research were to (1) determine the effect of the length of cold hardening on freezing tolerance in perennial ryegrass; (2) compare the response of annual and perennial ryegrass to cold hardening; (3) identify genes of which their expression levels are either increased, significantly suppressed, or stayed the same in response to cold treatment by either EST analysis or microarray using a barley gene chip; and (4) isolate antifreeze proteins genes from perennial ryegrass genes and assess their functions in the model species of *Arabidopsis*.

A Two-week Long Cold Hardening Develops Maximum Freeze Tolerance in Perennial Ryegrass

To determine the effect of the length of cold hardening on freezing tolerance in perennial ryegrass, plants were propagated vegetatively from a single mature tiller (cv. Caddyshack) in pots containing Sun Gro Hort soil mix in a greenhouse. Four-week-old plants were placed into

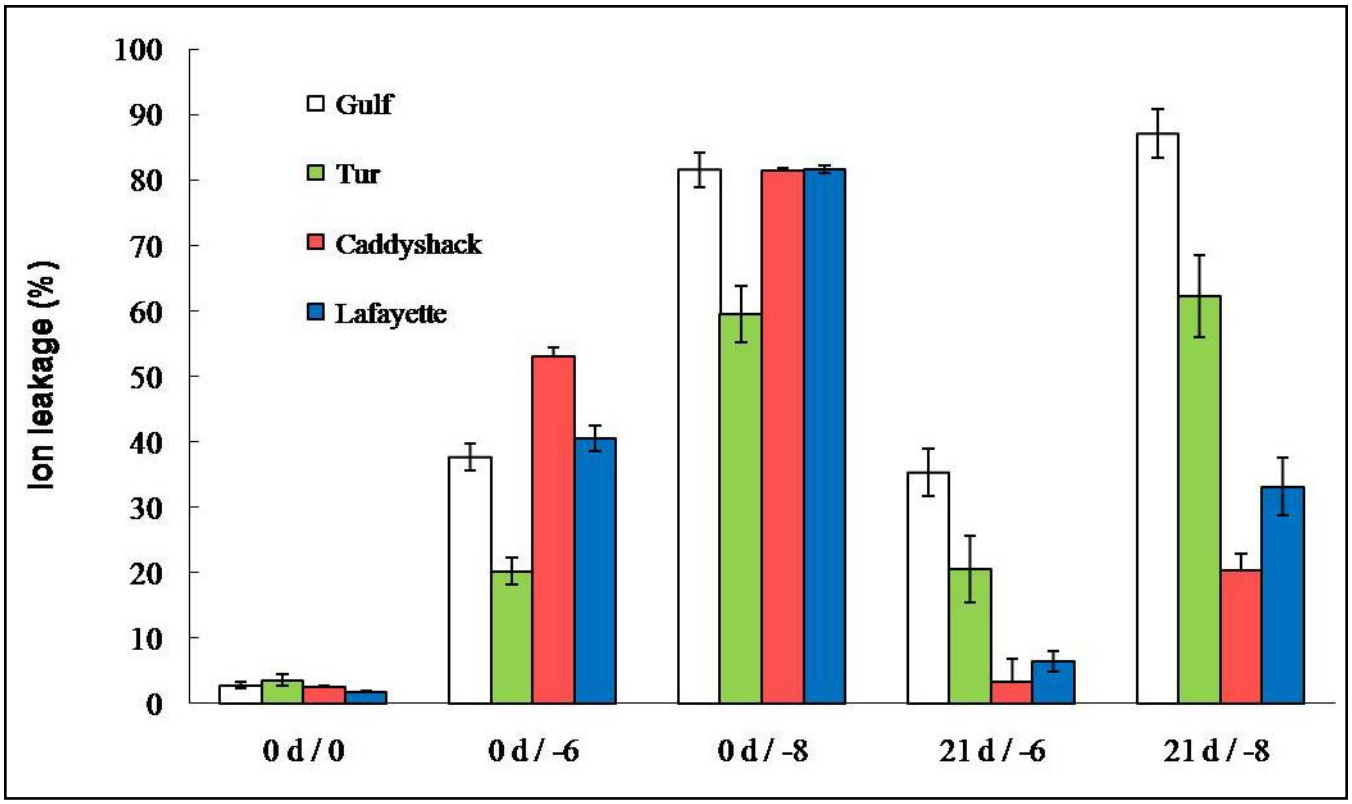

Figure 3 Cold hardening on freezing tolerance in annual and perennial ryegrass. Ion leakage in leaves from young seedlings of perennial ryegrass cvs. 'Caddyshack' and 'Lafayette' and annual ryegrass cvs. 'Gulf' and 'Tur' was measured. Seven-week-old plants were subject to cold hardening, and detached leaf segments were subjected to freezing treatments. Ion leakage was measured at 0, and -4 to -14 °C with an interval of 2°C, and results from 0, -6 and -8 °C are shown. Error bars represent the standard errors of three replications.

growth chambers at 23° C and a 16/8 hour (day/night) photoperiod with an irradiance of 280 ± 30 µmol m^{-2} s^{-1} for two weeks for conditioning. Plants were then placed into growth chambers at 4° C and an 8/16 hour (day/night) photoperiod with 100 ±20 µmol m^{-2} s^{-1} irradiance for cold hardening.

Leaves from mature plants that were cold hardened for 0 (control), 1, 3, 7, 14, or 28 days were used for freezing tolerance assessment. Ion leakage, which measures the degree of cell membrane damage, was measured for each of the treated plants by subjecting detached leaves to a freeze-thaw regime in a glycol bath, followed by measuring electrolyte leakage with a conductance meter as described by Xiong et al. (10). Ion leakage induced by freezing (-6 to -14° C) decreased continuously as the length of cold hardening increased until 14 days and leveled off after that (Figure 2).

These results indicate that maximum freezing tolerance can be achieved by exposing perennial ryegrass plants to 4° C for as short as two weeks. Further increase in the length of cold hardening does not improve freezing tolerance. However, for field grown plants, additional freezing tolerance may be acquired if plants are exposed to subzero temperatures as was reported in cereal crops in which cold hardening occurs in two distinct phases with the first phase occurring at a relatively high temperature followed by a second phase occurring at a lower temperature (6, 7).

Cold Hardening Occurs in Perennial Ryegrass but Not in Annual Ryegrass

Seven-week-old seedlings of the perennial ryegrass (*Lolium perenne* L. cvs. Caddyshack and Lafayette) and the annual ryegrass (*Lolium multiflorum* Lam. cvs. Gulf and Tur) were subjected to 0 (control), 2, 7, 14, or 21 days of cold hardening. Ion leakages continue to drop as the length of cold hardening increased for both cultivars of perenni-

Highly up-regulated	NA	CA	Highly down-regulated	NA	CA	Not affected	NA	CA
COR/LEA proteins	1	15	Lectin/high light protein	81	2	Rubisco activase	12	10
Stress responsive proteins	3	16	Phosphoribulokinase	7	1	Photosystem I subunit/center	7	8
Thioredoxin protein	1	7	NB-LRR containing protein	6	1	Fructose-bisphosphate aldolase	8	9
Inorganic pyrophosphatase	1	6	Receptor protein kinase	5	1	Ubiquitin-protein ligase	11	9
ABC-type transport system	1	5	ADP-ribosylation factor	5	1	Metallothioneine	13	11
Ribulose-1,5-bisphosphate carboxylase	2	9	Cytochrome b	5	1	Chlorophyll a/b-binding	12	10
Alanine aminotransferase	1	4	WD-40 associated protein	5	1			
Ice recrystallization inhibition protein (IRIP)	0	3	Protein kinase Xa21	10	2			
Osmotic/dehydration responsive proteins	4	10	Drm3, auxin-repressed protein	6	0			
			14-3-3 binding factor	5	0			
			ATPase family	5	0			
			Oxygen-evolving enhancer 3-1	5	0			
			Photosystem II 10-kDa polypeptide	113	66			

Table 1. Putative proteins encoded by the most abundant transcripts in the nonacclimated (NA) and cold-acclimated (CA) *Lolium perenne* cv. Caddyshack.

al ryegrass, but not for the annual ryegrass cultivars, indicating that perennial ryegrass has the ability to be cold hardened, whereas annual ryegrass does not have such ability. This explains why annual ryegrass is sensitive to even a short period of freezing or frost stress during late fall or early spring (8).

Cold hardened 'Caddyshack' resulted in less ion leakage than did the cold hardened 'Lafayette' from -4 to -10° C (Figure 3), indicating that 'Caddyshack', an improved turf-type cultivar has much better freezing tolerance than 'Lafayette', an unimproved forage-type cultivar. No differences were observed at the -14° C testing temperature among all experimental entries, at which point all cultivars showed an ion leakage of 80 to 90% which is lethal for both annual and perennial ryegrass regardless of cold treatments. Seedlings of 'Caddyshack' and 'Lafayette' acquired less freezing tolerance with the same length of the acclimation period than mature plants at 4° C. Unlike mature plants, however, longer period of acclimation beyond 14 days further increased freezing tolerance for seedlings.

Cold Hardening Leads to Significant Changes in Gene Expression Profiles in Perennial Ryegrass

Over 60 EST groups representing various genes were found to be either increased or decreased by a factor of three or more in cold hardened plants than in the non-hardened plants. An example of these genes is listed in Table 1 (11). Our results suggest that increased expression of cold-regulated (COR), dehydration-responsive, and ice recrystallization inhibition (IRI) protein genes, and suppression of photosynthesis-related genes such as the photosystem II 10-kDa polypeptide (chloroplast) and respiration-related genes are important to increasing freezing tolerance in perennial ryegrass.

Expression analysis of each of the 23 selected genes using polymerase chain reaction (PCR)-based approach confirmed that 19 of them exhibited expression patterns consistent with the EST abundance analysis. Microarray analysis using a barley gene chip also revealed similar gene expression pattern in response to cold as the EST analysis (Figure 4), suggesting barley

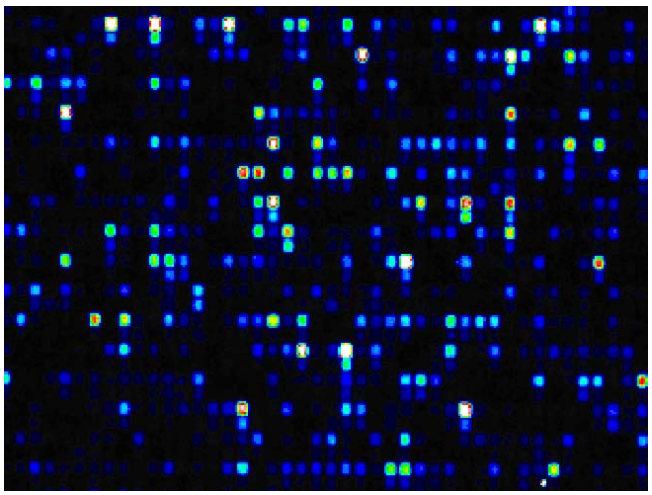

Figure 4. GeneChip® Barley1 genome array image after hybridization. Colors of the dots represent different hybridization signal intensities, from the strongest to weakest: white, red, green, blue and black.

Figure 5. Transgenic *Arabidopsis* carrying ice crystallization inhibition protein genes isolated from perennial ryegrass showed improved survival than the control plants. All plants were treated at -4º C for 70 minutes followed by recovery at 23º C. WT- wild type, EV-empty vector, LpIRI1 and LpIRI2 are transgenic plants carrying the ryegrass ice recrystallization inhibition protein gene 1 and 2, respectively.

genome array can be used effectively in probing gene expression profiles in perennial ryegrass. For example, genes encoding for cold hardening / COR protein/ low temperature and salt responsive protein (including dehydrins), stress responsive proteins (all kind of stress responsive proteins), and ice recrystallization inhibition protein 1 were all identified as highly expressed in both EST analysis and microarray with barley genome array.

Anti-Freeze Protein Genes Isolated from Perennial Ryegrass Enhanced Freeze Tolerance in the Model Species of *Arabidopsis*

Two ice recrystallization inhibition (IRI) protein genes, LpIRI1 and LpIRI2 were isolated from perennial ryegrass. Ice recrystallization inhibition proteins putatively binds to ice crystals and inhibit their growth, thus prevent the cell membrane from being damaged by growing ice crystals (3). Both genes were transferred to the model plant species of *Arabidopsis* and were overexpressed. Transgenic *Arabidopsis* plants expressing the perennial ryegrass LpIRI genes showed improved survival following a freeze test at -4° C even without the cold hardening process, compared to control plants (Figure 5). This result indicates that the perennial ryegrass IRI genes function in the model species by protecting plants from being injured by freezing.

Practical Implications

The current study provided direct evidences that while perennial ryegrass has the ability to cold acclimate, annual ryegrass does not have such ability. EST analysis and microarray studies shed lights on what biochemical or physiological pathways are likely changed during the cold hardening process. Genes identified to be involved in cold hardening and development of freezing tolerance can be used as direct molecular markers for marker-assisted selection in developing cold hardy perennial ryegrass cultivars.

Genes that enhance freezing tolerance also tend to enhance drought and salt tolerance (4, 5). Therefore, the identification of genes related to freezing tolerance may also have practical implication for tolerance to other stresses.

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Literature Cited

1. Fei, Z., X. Tang, R. M. Alba, J. A. White , C. M. Ronning, G. B. Martin, S. D. Tanksley, and J. J. Giovannoni. 2004. Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. *Plant J.* 40:47-59.

2. Fowler, S., and M. F. Thomashow. 2002. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14:1675-1690.

3. Griffith, M., M. Antikainen, W. C. Hon, M. K. Pihakaski, W. Yu, J. U. Chun, and D. S. C. Yang. 1997. Antifreeze proteins in winter rye. *Physiol. Plant.* 100:327-332.

4. Jaglo-Ottosen, K. R., Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. 1998. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* 280:104-106.

5. Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10:1391-1406.

6. Livingston, D. P., III. 1996. The second phase of cold hardening: Freezing tolerance and fructan isomer changes in winter cereal crowns. *Crop Sci.* 36:1568-1573.

7. Olien, C. R. 1984. An adaptive response of rye to freezing. *Crop Sci.* 24:51-54.

8. Redfearn, D. D. , B. C. Venuto, W. D. Pitman, D. C. Blouin, and M. W. Alison. 2005. Multilocation annual ryegrass cultivar performance over a twelve-year period. *Crop Sci.* 45:2388-2393.

9. Thomashow, M. F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu Rev. Plant Physiol. Plant Mol. Biol.* 50:571-599.

10. Xiong Y, S. Fei, E. C. Brummer, R. Arora, R. E. Barker, G. Jung, and S.E. Warnke. 2007. QTL analysis of winter hardiness in an annual × perennial ryegrass interspecific hybrid population. *Molecular Breed.* 19:125-136.

11. Zhang C, S. Fei , S. Warnke, L. Li, and D. Hannapel. 2009. Identification of genes associated with cold acclimation in perennial ryegrass. *J. Plant Physiol.* (In press).