Proteomic Responses during Cold Acclimation in Association with Freezing Tolerance of Velvet Bentgrass

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ABSTRACT. Cold acclimation improves freezing tolerance in various plants, including perennial grass species. The objectives of this study were to determine protein changes in crowns of velvet bentgrass (Agrostis canina) during cold acclimation in association with freezing tolerance. Treatments consisted of: 1) nonacclimated (NA) plants maintained at 18/12 °C (day/night); 2) plants acclimated at a constant 2 °C for 4 weeks with a 10-hour photoperiod (A4 (cold acclimation)); and 3) plants acclimated at a constant 2 °C for 4 weeks with additional subzero acclimation (SZA) at a constant –2 °C for 2 weeks (A4 + SZA2). Exposing plants to A4 significantly increased freezing tolerance, but additional SZA had no further beneficial effects on freezing tolerance, as demonstrated by the lethal temperature for 50% of the test population (LT50). Thirteen protein spots with increased abundance (up-regulated) or with decreased abundance (down-regulated) during cold acclimation were identified for biological functions. Proteins up-regulated after cold acclimation (A4 or A4 + SZA2) included methionine synthase, serine hydroxymethyltransferase, aconitase, UDP-D-glucuronate decarboxylase, and putative glycine-rich protein. Cold acclimation-responsive proteins involved in amino acid metabolism, energy production, stress defense, and secondary metabolism could contribute to the improved freezing tolerance induced by cold acclimation in velvet bentgrass.

Winter injury in field environments is a common problem for perennial grass species used as forage or turf in cool climatic regions. Winter injury may be caused by different stresses such as the duration and magnitude of low temperature exposure, lack of oxygen resulting from ice encasement, and/or susceptibility to low-temperature fungal diseases (Bertrand et al., 2003, 2011; Humphreys and Eagles, 1988). Although specific resistance mechanisms may vary among these different stresses, it has been demonstrated that acquisition of freezing tolerance is a major component of winterhardiness of perennial grasses (Hulke et al., 2008; Humphreys and Eagles, 1988; Xiong and Fei, 2006).

Freezing tolerance is dependent on a period of acclimation to low temperatures that alter various metabolic processes to physiologically precondition plants for subsequent freezing stress (Guy, 1999; Thomashow, 1999). Two consecutive stages of cold acclimation have been suggested in winter cereals and temperate grass species (Tumanov, 1940). The first acclimation phase occurs at temperatures above freezing (≥2 to 5 °C) and contributes to accumulation of cryoprotectants, including antifreeze proteins, reserve carbohydrates, and alterations in phospholipid and fatty acid composition, which enhance cellular stability when freezing occurs (Dionne et al., 2001a, 2001b; Guy, 1999; Hoffman et al., 2010; Livingston, 1991; Rajashekar, 2006; Thomashow, 1999; Tronsmo et al., 1993). The second phase of acclimation occurs at temperatures below freezing (2 to 5 °C). It is commonly referred to as SZA and leads to acquisition of additional freezing tolerance in some plant species, including perennial grasses (Herman et al., 2006; Livingston, 1996; Tumanov, 1940). Exposure to subfreezing temperatures is commonly associated with ice formation in the winter environment.
apoplast and dehydration of plant cells (Steponkus and Lynch, 1989). The high intracellular concentration of solutes helps to reduce significant loss of intracellular water and prevent ice formation inside cells (Herman et al., 2006). For winter wheat (Triticum aestivum), the completion of SZA occurred within a shorter period of time compared with acclimation at above-freezing temperatures (Tumanov, 1940). Herman et al. (2006) also demonstrated that morphological changes in crowns of winter wheat occurred within a few hours of exposure to SZA, whereas maximal acclimation was achieved after 3 d of SZA. In general, experimental evidence regarding structural, biochemical, and metabolic changes during SZA in winter cereals is restricted to few studies (Herman et al., 2006; Livingston, 1996), and little is known in general regarding mechanisms of SZA in perennial grasses.

Mechanisms underlying cold acclimation in perennial grasses can be revealed using novel genomic and proteomics approaches. The identification of cold-regulated (COR) proteins may provide additional information about numerous metabolic processes and their activities under cold acclimation. Changes in COR proteins have been observed in response to decreasing temperatures, including late embryogenesis abundant (LEA) proteins, antifreeze proteins (AFP), heat-shock proteins, detoxification enzymes, and ice recrystallization inhibition proteins (Close et al., 1989; Griffith and Yaish, 2004; Guy, 1999; Iansikà et al., 2010; Sandve et al., 2008; Zhang et al., 2010). Many of these proteins function in direct protection from freezing-induced cellular dehydration and mechanical damage. For example, the specific family of LEA proteins known as dehydrins have been widely reported to function as chaperones to help stabilize membranes and proteins under conditions of dehydration, including cold and drought (Close, 1996). In addition, AFP have been reported to adhere and inhibit the growth of ice crystals (Duman and Olsen, 1993; Griffith et al., 1997), inhibit ice recrystallization (Sandve et al., 2008), and protect thylakoid membranes against freeze–thaw damage (Sieg et al., 1996).

The synthesis and expression of specific COR proteins during cold acclimation may vary according to plant species or genotype such that certain proteins differentially accumulate between freezing-tolerant and freezing-sensitive plants (Dionne et al., 2001b; Patton et al., 2007; Perrras and Sarhan, 1989; Puhakainen et al., 2004). For example, Kosmala et al. (2009) found that 41 proteins were differentially accumulated between freezing-tolerant and freezing-sensitive meadow fescue (Festuca pratensis) plants with differences in protein abundance detected as early as 2 d of cold acclimation at 4 °C. In addition, the abundance of COR proteins has also been shown to vary based on exposure to different temperatures and durations of cold acclimation (Amme et al., 2006; Bocian et al., 2011; Kosmala et al., 2009). In a study evaluating winter wheat at different stages of cold acclimation, Herman et al. (2006) identified significant changes in protein abundance (either increased and decreased abundance) when plants were shifted from a cold acclimation regime of 3 °C to a SZA regime of –3 °C. To date, this is one of the few published studies on the proteomic effects of SZA in relation to freezing tolerance of plants. Therefore, additional research is needed to understand how the regulation of specific proteins may be involved at different stages of cold acclimation, including SZA, and contribute to improved freezing tolerance in perennial grass species.

Among perennial grasses used as turf, Agrostis species demonstrate better winter survival capacity compared with other turfgrasses (Aamilid et al., 2006; Tompkins et al., 2000). In an evaluation of cool-season grasses under controlled freezing conditions, Gusta et al. (1980) reported that creeping bentgrass (Agrostis stolonifera) was the most freezing-tolerant, surviving temperatures below –30 °C, whereas Kentucky bluegrass (Poa pratensis) and perennial ryegrass (Lolium perenne) exhibited significantly higher killing temperatures (–21 to –30 °C and –5 to –15 °C, respectively). Recent studies have also demonstrated potential differences in winter survival capacity among different Agrostis species. In cultivar evaluation trials at two locations in Norway from 2003 to 2006, velvet bentgrass was found to exhibit better winter survival and turf quality characteristics compared with creeping bentgrass (Aamilid et al., 2006; Molteberg et al., 2008). Based on appropriate cultivar selection, there is also evidence to suggest the potential use of velvet bentgrass as an alternative Agrostis species for reduced input environments (Brilman, 2003; Chakraborty et al., 2006; DaCosta and Huang, 2006; Koeritz and Stier, 2009). As a result of potential expanded use of this winter-hardy species in northern climates, additional information is required to understand the metabolic processes involved in cold acclimation and freezing tolerance of velvet bentgrass. Therefore, the objectives of our study were to determine protein changes in response to different stages of cold acclimation and to identify specific proteins associated with freezing tolerance of velvet bentgrass.

Materials and Methods

Plant material and growing conditions. Detailed information regarding plant material and experimental procedures was described previously (Espevig et al., 2011). Briefly, mature sods of ‘Greenwich’ velvet bentgrass were taken from field plots at Rutgers University (North Brunswick, NJ) and transplanted into polyvinyl chloride tubes filled with sand. Plants were maintained in a growth chamber at 18/12 °C (day/night temperatures) with a 16-h photoperiod and photosynthetic photon flux (PPF) of 500 μmol·m⁻²·s⁻¹ for 5 weeks. Plants were irrigated daily, hand-clipped to 3-mm height three times per week, and fertilized once per week with 100 mL of a complete Hoagland’s nutrient solution (Hoagland and Arnon, 1950).

Acclimation treatments. The experiment consisted of three treatments: 1) NA plants maintained at 18/12 °C (day/night) with a 10-h photoperiod and PPF of 500 μmol·m⁻²·s⁻¹; 2) plants acclimated at a constant 2 °C for 4 weeks with a 10-h photoperiod and PPF of 250 μmol·m⁻²·s⁻¹ (A4); and 3) plants acclimated at a constant 2 °C for 4 weeks with an additional SZA at a constant –2 °C for 2 weeks (A4 + SZA2). Because it has previously been shown that there is no light requirement for SZA (Le et al., 2008), this acclimation treatment was conducted in darkness. The durations of the acclimation treatments were selected based on previous studies and reported to be sufficient to induce freezing tolerance in grasses (Dionne et al., 2001a, 2001b; Hoffman et al., 2010). After each acclimation treatment, plants were harvested for freeze tests and protein analyses as described subsequently.

Determination of freezing tolerance. After each acclimation treatment, intact plants with leaves and roots were used for determination of freezing tolerance based on whole plant survival. Plants exposed to SZA were thawed overnight at 4 °C to facilitate sampling as described by Dionne et al. (2001a). For
each test temperature, 10 groups of plants (four to five plants per group) were washed free of soil, wrapped in a moistened paper towel to ensure ice nucleation, and placed into a freezer bag according to the methods previously described by Espevig et al. (2011). Four replicates were used for each test temperature. During harvest, the bags were temporarily stored at 4 °C until all plant material had been sampled. Freezing tests were conducted using a programmable freeze chamber (SciTemp Corp., Adrian, MI). The freezer was cooled in a stepwise fashion at a rate of 2 °C h⁻¹ to the desired temperature and held at the each test temperature (4, –6, –9, –12, –15, –18, and –21 °C) for 2 h. Bags were removed from the freezer after each test temperature and thawed at 4 °C for a minimum of 12 h.

After thawing, tillers were re-planted into cell trays filled with a commercial potting medium of Pro-Mix (Premier Horticulture, Québec, Canada) and placed in a greenhouse at ≈20 °C. Recovery was assessed after a 3-week regrowth period and percent whole plant survival (WPS) was calculated as:

\[
\text{WPS} = \left( \frac{\text{number of plants survived}}{\text{total number of plants}} \right) \times 100
\]

The lethal temperature for 50% of the test population (LT₅₀) was determined mathematically by curve fitting percent survival to temperature using a four-parameter sigmoid model (Sigma Plot Version 4.0; Systat Software, Chicago, IL) as previously described by Ebdon et al. (2002).

**Proteomic Analysis.** After each acclimation treatment, ≈0.5 g (fresh weight) of crown tissues (including stem bases) were harvested from each of three replicates, immediately frozen in liquid nitrogen, and stored at –80 °C for protein analysis. Plant crown tissues used for proteomic analysis were separate from those whole plants used for whole-plant survival assessment (LT₃₀) but were collected from the same pots (replicates).

Protein extraction was performed according to the trichloroacetic acid (TCA)/acetone method described by Xu et al. (2008). Briefly, crowns were homogenized with liquid nitrogen and incubated with 10 mL of precipitation solution (10% TCA and 0.07% 2-mercaptoethanol in acetone) overnight at –20 °C. The solution was centrifuged at 10,062 g, for 15 min at 4 °C, the pellets were rinsed twice with ice-cold 0.07% 2-mercaptoethanol in acetone to remove pigments and lipids, then vacuum-dried, resuspended in rehydration solution (8 M urea, 2 M thiourea, 2% 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte), and sonicated to provide transition of proteins from the pellet to the solution. The solution was then centrifuged at 10,062 g, for 15 min at 4 °C, the supernatants were removed, and protein concentrations were determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

First-dimension [isoelectric focusing (IEF)] and second-dimension [sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)] separation of proteins were performed according to a procedure described by Xu et al. (2008). Briefly, immobilized pH gradient (IPG) strips (pH 3.0–10.0, linear gradient, 13 cm) were filled with 250 μL rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 1% pharmalyte, and 0.002% bromophenol blue) containing 200 μg of proteins and rehydrated at room temperature in IPGPhor apparatus (GE Healthcare, Piscataway, NJ) at 50 V for 12 h. After IEF for a total 94.5 kVh, IPG strips were denatured with 10 mL of equilibration buffer (50 mM tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate, and 0.002% bromophenol blue) containing 1% DTT for 20 min. Then the strips were incubated twice with 10 mL of the same buffer containing 2.5% iodoacetamide for 15 min. The second-dimension electrophoresis was performed on 12.5% SDS–polyacrylamide gel using an electrophoresis unit (Hoefer SE 600 Ruby; GE Healthcare). The gels were stained with Coomassie brilliant blue-250 (Newsholme et al., 2000) and scanned using a Personal Densitometer (GE Healthcare). Protein abundance was the normalized spot volume (area × intensity) on scanned protein gels using Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC). Data were subjected to analysis of variance to test the effects of acclimation treatment. Significant differences in protein abundance between acclimation treatments were separated by Fisher’s least significant difference (LSD) test at the 0.05 P level.

Protein digestion and identification were performed as described previously (Xu et al., 2008). The gel spots were excised and washed with 30% acetonitrile in 50 mM ammonium bicarbonate before DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 μL of 1% trifluoroacetic acid followed by C₁₈ Zip Tip (Millipore, Billerica, MA) desalting. For the mass spectrometry (MS) analysis, the peptides were mixed with 7 mg/mL²⁻¹ α-cyano-4-hydroxy-cinnamic acid matrix in a 1:1 ratio and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4800 MALDI time-of-flight (TOF)/TOF analyzer (Applied Biosystems, Framingham, MA). Mass spectra (MS; m/z 880–3200) were acquired in positive ion reflector mode. For subsequent MS/MS sequencing analysis in 1 kV mode, 25 most intense ions were selected. Protein identification was performed by searching the combined MS and MS/MS spectra against the green plant NCBI nonredundant protein sequence database using a local MASCOT search engine (Version 1.9; Matrix Science, London, U.K.) on a Global Proteome Server (Version 3.5; Applied Biosystems). A protein containing at least two unique peptides with confidence interval values no less than 95% was considered being identified.

**Experimental design and statistical analyses.** The experimental design was completely randomized with three treatments and four replicates per treatment (pots) for freezing tolerance determination (LT₅₀ based on whole plant survival) and three replicates per treatment (pots) for protein analysis. Analysis of variance for LT₅₀ was performed using the software packages STATISTICA (Version 6.0; StatSoft, Tulsa, OK) (Hill and Lewicki, 2007). Significant differences among treatments were identified by Fisher’s LSD test at the 0.05 P level.

**Results and Discussion**

Acclimation of plants for 4 weeks at 2 °C resulted in an increase in freezing tolerance, as determined using LT₅₀ assessments, which consisted of –14.5 °C for A4 and –8.6 °C for NA (Table 1). In some studies with annual crops and grass species, SZA was shown to improve freezing tolerance compared with acclimation at low, above-freezing temperatures only (Dionne et al., 2001a; Herman et al., 2006; Livingston, 1996; Tumanov, 1940). However, in our study, additional SZA did not result in enhanced freezing tolerance because the LT₅₀ of plants exposed to A4 and A4 + SZA2 was not significantly different. As previously discussed by Espevig et al. (2011), the lack of additional benefits with SZA on freezing tolerance in our study could be the result of the long duration (4 weeks) of the cold acclimation period before the subzero temperature treatment.
The sufficiency of artificial acclimation effects on freezing tolerance may depend on plant species and temperature (Huner et al., 1993; Tepperman et al., 2001) as well as the duration of SZA (Herman et al., 2006; Vágújfalvi et al., 1999; Veisz and Sutka, 1989). Vágújfalvi et al. (1999) and Veisz and Sutka (1989) reported a negative effect of prolonged period of cold acclimation at 2 °C (from 7 to 8 weeks or from 5 to 6 weeks) on freezing tolerance in wheat. In addition, Herman et al. (2006) reported a negative effect of prolonged SZA on winter wheat survival when crowns were maintained at −3 °C for periods longer than 3 d. In contrast, Dionne et al. (2001a) reported that prolonged SZA at −2 °C for 2 weeks improved freezing tolerance of annual bluegrass (Poa annua) relative to plants acclimated at 2 °C. Differences between our study and that of Dionne et al. (2001a) may be the result of interspecific differences in freezing tolerance, because velvet bentgrass and other Agrostis species exhibit greater winterhardiness compared with annual bluegrass (Aamlid et al., 2006; Tompkins et al., 2000). However, additional research is necessary to examine the effects of artificial SZA on changes in freezing tolerance compared with that encountered under natural field conditions.

Currently, there are very few reports on protein changes in crown tissues in grasses in response to cold acclimation. In this study, among 375 separated protein spots from crown tissues in velvet bentgrass (Fig. 1), ≈86 spots exhibited response to at least one acclimation treatment. Nineteen protein spots that were differentially expressed among NA, A4, and A4 + SZA2 plants were identified using mass spectrometry. Among them, 13 spots were identified with putative biological functions and six spots with unknown functions. The abundance level of the six unknown proteins is shown in Figure 2. Spots 14, 15, 16, 17, and 18 exhibited accumulation under both A4 and A4 + SZA2 treatments. Spot 19 had increased abundance at A4 + SZA2 treatment but did not change at A4 treatment compared with the non-acclimated treatment. Because the functions of these proteins were unknown, their involvement in cold acclimation and freezing tolerance could not be further discussed. The 13 identified proteins were divided into four functional categories described by Bevan et al. (1998), including metabolism, energy, stress defense, and secondary metabolism (Table 2). The association of these proteins with cold acclimation-induced freezing tolerance is discussed subsequently based on their biological functions. There were two proteins with two or more spots with different pl and molecular weight (methionine synthase and cytosolic glyceraldehyde-3-phosphate dehydrogenase). Multiple spots for a single protein are commonly found on two-dimensional (2D) gels (Giavalisco et al., 2005; Sarnighausen et al., 2004). Several factors may be responsible for this phenomenon. The migration of proteins on a 2D-PAGE gel is very sensitive to small structural differences. These spots might be different isoforms derived from different genes of a multigene family. The complex genome of velvet bentgrass is expected to be different isoforms derived from different genes of a multigene family. The complex genome of velvet bentgrass is expected to contain multiple copies of many genes, and the distinct biological properties might be the result of amino acid sequence differences in the different isoforms. Alternatively, one gene product may undergo different co- and/or post-translational modifications that affect its pl and/or molecular weight.

**Metabolism.** The proteins associated with metabolism that exhibited significant responses to cold acclimation treatments included methionine synthase (spots 1–3) and serine hydroxymethyltransferase (spot 4) (Table 2). The abundance of both proteins increased in response to A4 or A4 + SZA2 treatments, but these proteins did not exhibit significant differences in the abundance level between the two acclimation treatments. Quantitative changes in amino acids in plants in response to cold acclimation have been previously reported, but their specific role in freezing tolerance remains unclear (Bertrand et al., 2011; Dionne et al., 2001b; Naidu et al., 1991). In addition to possible direct roles in conferring freezing tolerance, increased availability of specific amino acids may serve as nitrogen reserves to improve overwintering capacity and spring regrowth of plants (Volenc et al., 1996) and also serve as precursors for defense compounds involved in resistance to low temperature fungi (Bertrand et al., 2011).

In our study, the increased abundance of both serine hydroxymethyltransferase and methionine synthase in response to cold acclimation suggested a cold-induced increase or activation of amino acid metabolism. The major reaction catalyzed by serine hydroxymethyltransferase is the interconversion of serine and glycine, resulting in generation of one-carbon units for the biosynthesis of many organic compounds including methionine, nucleotides, thymidylate, and choline.

### Table 1. Effect of cold acclimation on freezing tolerance of velvet bentgrass expressed as mean lethal temperatures for 50% of test population (LT50).

| Acclimation treatment | LT50 (°C) | P  
|----------------------|----------|------
| NA                   | −8.6 b  |
| A4                   | −14.8 a  |
| A4 + SZA2            | −14.7 a  |
| P                    | <0.001   |

The same letter indicates no significant difference among treatments based on Fisher’s protected least significant difference test.

NA = nonacclimated plants maintained at 18/12 °C (day/night); A4 = plants acclimated at a constant 2 °C for 4 weeks; A4 + SZA2 = plants acclimated at a constant 2 °C for 4 weeks with an additional subzero acclimation at a constant −2 °C for 2 weeks.
Increased availability of glycine would also be essential for glutathione synthesis. Glutathione has been shown to increase in plants in response to low temperature and is thought to play a key role in the reduction of hydrogen peroxide concentration in plants during cold acclimation (Kocsy et al., 2001). In other studies, serine hydroxymethyltransferase was also shown to be induced in response to cold treatment in leaves of arabidopsis [Arabidopsis thaliana (Byun et al., 2009)] and sunflower [Helianthus annuus (Balbuena et al., 2011)] and was reported to play a role in protecting plants from oxidative stress in rice (Oryza sativa) during drought (Ali and Komatsu, 2006). The induction of methionine synthase in response to low temperature has been reported in other plant species (Machida et al., 2008; Renault et al., 2009; Rorat et al., 1997). Methionine synthase catalyzes the terminal step of methionine biosynthesis, which then serves as an important starting point in the biosynthesis of important metabolites including polyamines and ethylene (Ravanel et al., 1998; Wang et al., 2002). In general, polyamines are reported to play an important role in stabilization of lipids and proteins as well as cell signaling and regulation of plant hormones including abscisic acid (Alcazar et al., 2010; Kramer and Wang, 1990). An increase in polyamine synthesis has been observed in response to cold acclimation for several plant species with an increase in freezing tolerance associated with an increase in key polyamines such as putrescine, spermidine, and spermine (Alcazar et al., 2011). Ethylene was reported to increase protein production in the apoplast and induce antifreeze activity in non-acclimated winter ryegrass [Phleum pratense (Bertrand et al., 2003)]. There is limited information regarding the physiological role of cytoplasmic aconitase in plants. The enzyme appears to be involved in the glyoxylate cycle (Courtois-Verniquet and Douce, 1993), which converts lipid to sucrose. Mitochondrial aconitase catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle (Verniquet et al., 1991). A more recent study by Moeder et al. (2007) demonstrated a possible role for aconitase in mediating plant responses to oxidative stress. Thus, additional research is necessary to understand how the up-regulation of aconitase could be associated with conferring freezing tolerance in grasses.

**Energy.** Some proteins in this category showed differential responses to the two acclimation treatments (Table 2). The abundance of aconitase (spot 5) increased significantly under both cold acclimation treatments compared with the non-acclimated control but did not differ between the two acclimation treatments. The abundance of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large and small subunits (spots 6 and 7, respectively) and glyceraldehydes-3-phosphate dehydrogenase (spots 8 and 9) decreased significantly in response to A4 or A4 + SZA2 treatment but was not significantly different between the two acclimation treatments. Fructose–biphosphate aldolase (spot 10) exhibited a significant decline in its abundance in response to the A4 + SZA2 treatment but did not change during the A4 treatment. The abundance of fructose–biphosphate aldolase was significantly higher in A4 treatment than A4 + SZA2 treatment.

Because photosynthesis and respiration are temperature-dependent processes (Guy, 1999; Huner et al., 1993; Klimeov, 2009), significant changes in enzymes and other proteins involved in carbon metabolism in response to cold acclimation have been reported (Herman et al., 2006; Kosmala et al., 2009; Rapacz et al., 2008). Kosmala et al. (2009) showed that significant changes in leaf proteins of meadow fescue occurred within 2 d after exposure of plants to cold acclimation, and most of the regulated proteins were involved in photosynthesis. In the current study, we found significant down-regulation of Rubisco large and small subunits in response to cold acclimation. Similarly, Herman et al. (2006) reported down-regulation of Rubisco in leaves of winter wheat. Hahn and Walbot (1989) also showed ≈90% reduction in the synthesis for the small Rubisco subunit and 80% for the large Rubisco subunit in leaves of rice.

Fructose–biphosphate aldolase was down-regulated only after SZA in our study. The metabolic function of cytosolic fructose–biphosphate aldolase is cleavage of fructose 1,6-biphosphate (F–1,6-BP) into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis. In addition, cytosolic glyceraldehyde-3-phosphate dehydrogenase, which catalyzes the subsequent conversion of glyceraldehyde 3-phosphate to glyceral 1,3-biphosphate, was down-regulated in response to A4. Taken together, the data suggest that adaptation of velvet bentgrass to prolonged cold acclimation involved a down-regulation of respiration, which could reduce the potential for metabolism of cryoprotective solutes and improve over-wintering capacity. In a previous study, winter wheat cultivars exhibiting lower respiration rates and carbohydrate consumption during winter months had significantly improved survival compared with cultivars with higher rates of winter respiration (Sagisaka et al., 1991). Lower winter respiration rates have also been associated with improved winter survival for timothy [Phleum pratense (Bertrand et al., 2003)].

There is limited information regarding the physiological role of cytoplasmic aconitase in plants. The enzyme appears to be involved in the glyoxylate cycle (Courtois-Verniquet and Douce, 1993), which converts lipid to sucrose. Mitochondrial aconitase catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle (Verniquet et al., 1991). A more recent study by Moeder et al. (2007) demonstrated a possible role for aconitase in mediating plant responses to oxidative stress. Thus, additional research is necessary to understand how the up-regulation of aconitase could be associated with conferring freezing tolerance in grasses.

**Stress defense.** Two proteins involved in stress defense were found to be responsive to cold acclimation treatments (Table 2). A putative glycine-rich protein (spot 11) exhibited an
increase in abundance after A4 treatment but then decreased back to similar levels as the non-acclimated control in response to A4 + SZA2 treatment. The abundance of a putative peroxidase (spot 12) was significantly lower in response to both A4 or A4 + SZA2 treatment. The abundance of a putative peroxidase might be expected to confer better freezing tolerance by cold and is capable of binding nucleic acids, were found in generating pathways of plants, only one putative peroxidase was identified in our study. Therefore, additional proteomic analyses will be necessary to better understand the regulation of oxidative stress during the acquisition of freezing tolerance in grasses.

A putative glycine-rich protein was up-regulated in response to cold acclimation, but its production was found to return to the same level as in non-acclimated plants after SZA. This protein had two-sequence similarity with cold shock protein (CSP) from wheat (Karlson et al., 2002). Plant CSPs with high sequence similarity to bacterial CSP, which is up-regulated by cold and is capable of binding nucleic acids, were found in barley (Hordeum vulgare) [blt 801] (Dunn et al., 1996) and winter wheat [WCSPI (Karlson et al., 2002)]. Chaikam and Karlson (2008) showed that CSP in rice (O.sativa) was more highly expressed in reproductive tissues and tissues with high meristematic activity. Similar to our study, Karlson et al. (2002)

### Table 2. Relative abundance of cold-regulated proteins from crowns of velvet bentgrass divided into functional groups according to the classification in Bevan et al. (1998).

| Spot no. | Protein name (species) | Accession | Protein molecular wt (kDa) | Protein isolectric point | Avg normalized values |
|----------|------------------------|-----------|----------------------------|--------------------------|----------------------|
| 1        | Methionine synthase (Hordeum vulgare) | gi|8134570 | 84.8 | 6.1 |
| 2        | Methionine synthase (H. vulgare) | gi|8134570 | 84.8 | 6.10 |
| 3        | Methionine synthase (H. vulgare) | gi|50897038 | 84.5 | 5.68 |
| 4        | Serine hydroxymethyl-transferase (Arabidopsis thaliana) | gi|11762130 | 51.8 | 7.12 |
| 5        | Aconitase (Lycopersicon pennellii) | gi|29027432 | 98.1 | 6.07 |
| 6        | Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Agrostis capillaries) | gi|41056380 | 51.8 | 6.13 |
| 7        | Rubisco small subunit (Avena clauda) | gi|6573202 | 18.8 | 8.60 |
| 8        | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Oryza sativa ssp. indica) | gi|120680 | 36.5 | 6.67 |
| 9        | Glyceraldehyde-3-phosphate dehydrogenase cytosolic (O. sativa ssp. indica) | gi|120668 | 33.2 | 6.20 |
| 10       | Fructose-bisphosphate aldolase, cytosolic (O. sativa ssp. japonica) | gi|218157 | 38.7 | 6.56 |
| 11       | Putative glycine-rich protein (Triticum aestivum) | gi|40363759 | 19.2 | 5.63 |
| 12       | Putative peroxidase (O. sativa ssp. japonica) | gi|115436084 | 37.9 | 6.46 |
| 13       | UDP-D-glucuronate decarboxylase (H. vulgare) | gi|50659026 | 38.9 | 7.10 |

*NA = nonacclimated plants maintained at 18/12 °C (day/night); A4 = plants acclimated at a constant 2 °C for 4 weeks; A4 + SZA2 = plants acclimated at a constant 2 °C for 4 weeks with an additional subzero acclimation at a constant –2 °C for 2 weeks. Columns in each graph followed by same letter indicate no significant difference based on Fisher’s protected least significant difference test.
observed that the level of WCSPI was low in NA plants, but then it was gradually increased during 18 d of cold acclimation.

**Secondary metabolism.** One protein involved in secondary metabolism, the enzyme UDP-D-glucuronate decarboxylase (spot 13), was found to increase in abundance in response to A4. In response to A4 + SZA2, however, the abundance of UDP-D-glucuronate decarboxylase was lower than the abundance at A4 and did not differ from the non-acclimated control.

The enzyme UDP-D-glucuronate decarboxylase catalyzes conversion of UDP-D-glucuronate into UDP-D-xyllose. In general, there is an increase in the activity of enzymes involving in the synthesis of polysaccharides entering the pectin matrix of cell wall during periods of cell division and cell elongation (Dalessandro and Northcote, 1977). Along with the plasma membrane, the cell wall serves as a barrier for the propagation of extracellular ice and maintains the cell integrity during desiccation caused by freezing (Yamada et al., 2002). Taken together, the increased abundance of UDP-D-glucuronate decarboxylase in our study may suggest changes in the composition of the cell wall matrix during cold acclimation and/or induced cell division for the purpose of reduction of cell size. However, additional research is necessary to understand how changes in cell wall mechanical behavior are related to acquisition of freezing tolerance.

In summary, acclimation of plants for 4 weeks at 2 °C significantly increased freezing tolerance in velvet bentgrass, whereas additional SZA did not provide additional freezing tolerance compared with prolonged cold acclimation at above-zero temperatures. The cold acclimation-induced freezing tolerance could be mainly associated the cold acclimation-responsive (up-regulated or down-regulated) proteins involved in amino acid metabolism, energy, stress defense, and secondary metabolism in velvet bentgrass. The expression pattern and specific biochemical functions involved in freezing tolerance for those cold acclimation-responsive proteins deserves further confirmation using Western blotting and additional molecular analyses.

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