Differences in proteome response to cold acclimation in *Zoysia japonica* cultivars with different levels of freeze tolerance

J.M. Brown[1], H. McCamy P. Holloway[1], Michelle DaCosta[2], Rachael P. Bernstein[2], Jefferson Lu[2], Tan D. Tuong[3], Aaron J. Patton[4], Jeffrey C. Dunne[1], Consuelo Arellano[5], David P. Livingston[3], and Susana R. Milla-Lewis[1]

[1] J.M. Brown, H.M.P. Holloway, J.C. Dunne, and S.R. Milla-Lewis, Department of Crop and Soil Sciences, Campus Box 7620, North Carolina State University, Raleigh, NC, 27695-7620; [2]R.P. Bernstein, J. Lu, M. DaCosta, Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA 01003; [3]T.D. Tuong and D.P. Livingston, Department of Agriculture and Department of Crop and Soil Sciences, North Carolina State University, Raleigh, NC 27695; [4] A.J. Patton, Department of Horticulture and Landscape Architecture, Purdue University, 625 Agriculture Mall Drive, West Lafayette, IN 47907; [5] C. Arellano, Department of Statistics, Campus Box 8203, North Carolina State University, Raleigh, NC 27695-8203

Received: _____ * Corresponding author email: susana_milla-lewis@ncsu.edu

ABSTRACT

Zoysiagrasses (*Zoysia* spp.) are warm season turfgrasses primarily grown in the southern and transition zones of the United States. An understanding of the physiological changes that zoysiagrasses undergo during cold acclimation may shed light on physiological phenotypic traits useful in selection of freeze tolerant genotypes. We investigated the relationship between cold acclimation, protein expression, and freeze tolerance in cold-acclimated (CA) and non-acclimated (NCA) plants of *Zoysia japonica* Steud. cultivars 'Meyer' (freeze-tolerant) and 'Victoria' (freeze-susceptible). Freeze tolerance was assessed using chambers reaching -6, -8, -10, and -12ºC. Additionally, meristematic tissues from the grass crowns of 'Meyer' and 'Victoria' were harvested for proteomic analysis after a four week cold acclimation period. Freeze testing indicated that cold acclimation accounted for a 1.9-fold increase in plant survival compared to the non-acclimation treatment. Overall, proteomic analysis identified 62 protein spots having at least a twofold change in abundance under cold acclimation. Nine and 22 unique protein spots were identified for Meyer and Victoria, respectively, with increased abundance (up-regulated) or decreased abundance (down-regulated). In addition, 23 shared protein spots were found among the two cultivars having differential expression in response to cold acclimation. In Meyer, protein response to cold acclimation was primarily upregulated, while in Victoria, protein response was primarily downregulated. These cold acclimation responsive proteins were found to be involved primarily in transcription, metabolism, protein destination and storage, and energy production. As identified through MALDI-TOF/TOF mass spectrometry followed matching of protein homologues against the NCBI Arabidopsis database, major proteins of interest for their association with cold acclimation were LEA 3, MAPK, SOD, GAST1, Phytochrome A, ATP synthase, AGP, PLD, and

PSII. Further investigation of these proteins and their functional categories may contribute to increase our understanding of the differences in freezing tolerance among zoysiagrass germplasm.

INTRODUCTION

Zoysiagrasses (*Zoysia* spp.) are warm season perennial turfgrasses primarily grown in the warm-humid and transitional climatic zones of the United States. They are geographically constrained to these regions because of their limited winter hardiness. Zoysiagrasses are popular for their low maintenance requirements as well as their slow growth habit, high shoot density, and tolerance to many abiotic stresses such as drought and shade [1]. These attributes make zoysiagrass especially well-suited for home lawns, landscapes, and golf courses and creates a demand for more freeze tolerant cultivars that can be used in the colder climates of the Northern US.

Varying levels of winter injury have been observed among zoysiagrass genotypes. The two most popular species of zoysiagrass in the U.S., *Zoysia japonica* Steud. and *Zoysia matrella* (L.) Merr., have significantly different levels of winter injury and spring green-up as first reported by Forbes and Ferguson [2]. Both in the field and freeze chamber studies, *Z. japonica* genotypes show more average freeze tolerance than *Z. matrella* genotypes [3, 4, 5]. Patton and Reicher [5] reported that the LT_{50}s for *Z. japonica* commercial cultivars range from -9.5ºC to -11.5ºC for 'Victoria' and 'Meyer', respectively. Additionally, LT_{50}s have been found to differ depending on the origin of the tested material and freeze testing protocol [3, 4, 5, 6].

Cold acclimation refers to the natural physiological process that takes place in a plant when it is exposed to low but above freezing temperatures. Studies in zoysiagrass [4, 5, 6], as well as

saltgrass (*Distichlis spicata* (L.) Greene) [7], buffalograss (*Bouteloua dactyloides* (Nutt.) Engelm.) [8], bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burt-Davy) [9, 10] and St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) [11, 12, 13] have indicated that warm-season grasses respond well to cold acclimation suffering less injury from freezing when acclimated. Hinton et al., [4] found that across nine different zoysiagrass cultivars with varying LT_{50}s, samples collected in the winter (after cold acclimation) were consistently more freeze tolerant in controlled environment freezing tests than samples collected in the spring (non-acclimated/de-acclimated).

Although field evaluation of freeze tolerance provides the most realistic indication of how a cultivar will perform through winter, variable environmental conditions across year and location can make consistent and reproducible winter stress very difficult to attain [14]. Controlled environment freeze tests may be a more cost-effective and efficient way to assess cold acclimation and freeze tolerance of turfgrass species and generally corresponded well with field screenings [8, 14, 15]. This constancy demonstrates that plants most likely undergo similar physiological changes during cold acclimation, freezing, and deacclimation in temperature controlled chambers as in the field. In zoysiagrass, freezing chambers have been successfully used to evaluate the low temperature tolerance of a variety of genotypes [3, 4, 5, 16].

Our understanding of the physiological changes that zoysiagrass undergoes during cold acclimation has been somewhat limited. Zhang et al. [17], investigated response to cold acclimation in zoysiagrass cultivars 'Meyer' and ‘Cavalier' to identify changes in hormones and dehydrin expression. Most notably, they found an increase in abscisic acid and dehydrin levels, and a decrease in cytokinin levels in Meyer compared to Cavalier, which may play a role in winter survival and freeze tolerance. Patton et al. [18, 19] compared levels of compounds suspected to be

92 associated with freeze tolerance such as carbohydrates, organic acids, and soluble proteins at
93 various points in the cold acclimation process. The study found that carbohydrates such as glucose
94 and sucrose increase in stolons during seasonal temperature decreases in zoysiagrass, similar to
95 what is known to occur in other warm season turfgrasses such as saltgrass (*Distichlis spicata* var.
96 stricta (L.) Greene) [7], centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) [20] and
97 buffalograss (*Buchloe dactyloides* (Nutt.) Englem.) [21]. However, a comparison between LT_{50}
98 and sucrose concentration in CA grasses revealed no relationship with freezing tolerance. Despite
99 this result, evidence suggested that genotypes with poor freezing tolerance produce more sucrose
100 during acclimation than genotypes with high freeze tolerance, and that total reducing sugars such
101 as glucose likely play a role in total freezing tolerance. Proline concentration is known to increase
102 dramatically during cold acclimation due to an uptake in synthesis with freeze tolerance improving
103 with increased proline [19]. Santarius [22] found that proline protects membranes using
104 hydrophobic interactions, osmotic adjustment, and decreased water potential, but it is less efficient
105 as temperatures decline. Lastly, Zhang et al. [6] evaluated changes in lipid composition during
106 cold acclimation, but found no consistent relationship between lipid classes and zoysiagrass
107 freezing tolerance.

108 Comprehensive studies of the proteins involved in cold acclimation as well as their
109 functions may lead to more accurate and efficient selection of associated genes in zoysiagrass.
110 One such study, by Xuan et al. [23] performed quantitative analysis of protein accumulation in
111 stolon tissues collected from two species, *Z. japonica* cv. Meyer and *Z. matrella* cv. Diamond,
112 during cold acclimation. Forty-five proteins with a two-fold change in expression were
113 functionally identified as playing a role in key functional protein classes such as redox
114 homeostasis, signal transduction, photosynthesis, and energy metabolism among others. A close

115 examination of the proteins involved suggests that a greater energy supply is needed to respond to
116 cold stress in zoysiagrass stolons. Meyer's ability to respond to cold stress most likely is related to
117 its increased ROS scavenging ability, photosynthesis, protein synthesis, proteolysis, and its greater
118 energy reserves. However, it may be relevant to note that the LT_{50}s of the two cultivars in this
119 study was determined through electrolyte leakage, a method which tends to overestimate the LT_{50},
120 rather than whole plant survival. Furthermore, their use of stolons rather than meristematic tissues
121 from the grass crown may lead to different inferences as a result of the specialization of plant
122 tissues. To identify genomic regions which play a role in freezing tolerance, Holloway et al. [24]
123 generated a high density linkage map of *Zoysia japonica*. Used in conjunction with phenotypic
124 data on winter injury, establishment, and turf quality, this map allowed for the identification of
125 seven winter injury associated QTL on chromosomes 8, 11, and 13. A BLAST study into these
126 QTL of interest identified coding regions for proteins associated with abiotic stress tolerance in
127 other species. Proteins of interest were associated with signal transduction pathways such as the
128 mitogen-activated protein kinase pathway, as well as DRE family protein coding regions, both of
129 which are linked to abiotic stress response and mitigation. These findings provided a compelling
130 case for which proteins and pathways play a role in freeze tolerance.

131 Furthering our understanding of the physiological changes that zoysiagrass undergoes
132 during cold acclimation may shed light on phenotypic traits useful in selection of more freeze
133 tolerant genotypes. To investigate the relationship between cold acclimation, protein expression,
134 and freeze tolerance in zoysiagrasses, *Z. japonica* cultivars with a reported differing level of
135 freeze susceptibility and previously studied quantitative trait loci (QTL) associated with winter
136 hardiness [24] were investigated here. The objectives of this study were to (i) compare the effects

of cold acclimation treatments on freeze tolerance in these cultivars and (ii) to identify proteins associated with cold acclimation and freezing tolerance.

MATERIALS AND METHODS

Plant materials and growing conditions

Commercial cultivars 'Meyer' (*Z. japonica*, [25]) and 'Victoria' (*Z. japonica*, [26]) were selected for evaluation due to their reported range in freeze susceptibility (acclimated LT_{50} of -11.5°C and -9.5°C, respectively) [4, 5]. In the fall of 2015, cultivars were collected from research plots at the Lake Wheeler Turfgrass Field Laboratory (Raleigh, NC) and established in 24.5 cm x 50.8 cm plastic trays (Hummert International, Earth City, MO) containing Fafard P3 potting mix (Conrad Fafard Inc., Agawam, MA) in the greenhouse. In spring 2016, plants were vegetatively propagated into 2.5-cm-diameter, 12-cm-deep Ray Leach cone-tainers (Stuewe & Sons, Inc., Corvallis, OR) in USGA grade sand [27] using a single stolon or rhizome containing root, node, and shoot material according to Patton and Reicher [5]. Plants were allowed to establish for at least six weeks in the greenhouse at 27±2°C.

Acclimation treatments

After establishment in the greenhouse, plants of each genotype were randomly arranged in a EGC walk-in chamber made by Environmental Growth Chambers (510 East Washington St. Chagrin, OH 44022) and cold acclimated (CA) for four weeks. The growth chamber used a consistent light emitting diode (LED) and temperatures of 8/2°C day/night cycles with a 10-h photoperiod of 300 µmol $m^{-2}s^{-1}$ photosynthetically active radiation [9]. Non acclimated (NA) plants of both genotypes remained in the greenhouse at 27°C during this four-week period.

Evaluation of freezing tolerance

160 After acclimation treatments, intact plants in cone-tainers were evaluated for freeze tolerance
161 based on whole plant survival. Immediately before placement in the freezer, NA plants were
162 randomized with the CA plants. Based on the capacity of the freeze chambers, plants were arranged
163 in each rack in a randomized complete block design with two replications. Each experimental unit
164 consisted of 10 cone-tainers for each of the genotypes by acclimation treatment combinations.
165 Based on reported LT_{50}s [4, 5] and a pilot study in the spring of 2015, temperatures of -8°C, -9°C,
166 -10°C, and -11°C were selected for this study. Each rack was placed in a plastic bag to minimize
167 desiccation during freezing [11, 12] and placed into upright Kenmore refrigerators modified with
168 cooling fans and FE Micro-controllers PXR4 (Price's Scientific Services, Inc.). In the freezers,
169 plants were initially held at -3°C for 15 h to remove latent heat from the soil. The temperature was
170 then decreased at a rate of -1°C h^{-1} until reaching the target temperature, which was maintained
171 for 3 h. Plants were thawed at a rate of 2°C h^{-1} until reaching the target temperature of 3°C. All
172 trays were subsequently removed from plastic bags and placed in a walk-in growth chamber at
173 3°C for 24 h. Finally, plants were allowed to come to room temperature (22°C) overnight before
174 being returned to the greenhouse. This experiment was repeated for a total of three runs. The
175 second run of freeze tests was discarded because of a malfunction in the commercial freezers so
176 only data from runs one and three were used in the analysis.

177 Plants were evaluated weekly for survival for four weeks after freezing. Survival data was
178 taken on a binary scale of 0 (death) to 1 (survival) evaluating each individual plant for the presence
179 of green tissue [15]. Survival data collected four weeks after freezing was evaluated with a logistic
180 regression analysis. This method uses binary data to determine the probability of survival against
181 the temperature gradient and estimate LT_{50}s according to Dunne et al. [15]. The LOGISTIC

procedure in SAS version 9.4 (SAS Institute; Cary, NC) with a stepwise selection method generates a model and maximum likelihood estimates that were used to calculate odds ratios, survival probabilities, and LT_{50}s. Regression models were created to evaluate each cultivar by acclimation treatment combination as well as acclimation treatments with cultivars pooled. Temperature was treated as a continuous variable to account for variability in the commercial freezers. Non-acclimated Victoria was used as a reference variable for the generation of the logistic regression model of cultivar by acclimation treatment because it had the lowest reported LT_{50}. In the model of acclimation treatments alone, the non-acclimated treatment was considered a reference variable.

Proteomic analysis

After acclimation treatments, sixteen cone-tainers of each cultivar × treatment combination for each of three reps were randomly selected and destructively sampled for proteomic analysis. Meristematic tissues from the grass crowns was harvested with less than 2.5 cm of root and shoot tissue remaining, and bulked for each cultivar-treatment within each run. Fresh tissues were immediately frozen in liquid nitrogen, held at -80°C until completion of the experiment, and then lyophilized (Genesis Pilot, SP Scientific, Warminster, PA). Lyophilized tissues were then ground into a fine powder (Retsch Mill Mixer MM 400, Haan, Germany) to aide in protein extraction.

Proteins were extracted from approximately 0.5 g lyophilized tissues using the trichloroacetic acid (TCA)/acetone/phenol method previously described by Xu et al. [28]. Total protein concentration was quantified according to the methods of Bradford [29] with bovine serum albumin as a standard. Separation of proteins based on first- dimension isoelectric focusing (IEF) was done by loading 300 ug of protein dissolved in rehydration buffer (8M Urea, 2% Chaps, 50mM dithiothreitol (DTT) 0.2% (w/v) 3/10 ampholytes, and trace amounts of Bromophenol Blue) onto immobilized pH

gradient (IPG) strips (pH 3-10, linear gradient, 11 cm), which were then focused in a Protean i12 IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA). Second- dimension sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) was done on 12.5% Tris-HCL Precast Gels using a Criterion electrophoresis cell (Bio-Rad Laboratories, Hercules CA, USA), run at 80V for 20 min before switching to 100V for approximately 2 h. Gels containing protein spots were then stained using the ProteoSilver Plus Silver Stain Kit (Sigma-Aldrich, MO, USA) according to manufacturer instructions. Three technical replicates for each biological replicate were used for 2-DE, resulting in a total of nine gels per cultivar-treatment combination.

The 2-DE gels were scanned using a GE Typhoon FLA 9500 Phosphorimager (GE Healthcare, PA, USA). Scanned images were aligned and spot intensity was quantified using Melanie 8 (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland). Spot intensity was used to determine fold change between the non-acclimated and acclimated samples within each cultivar. Differences in protein expression between the cultivars and cold acclimation treatments were analyzed using ANOVA, and spots with a minimum of 2.0-fold difference in abundance (upwards or downwards) between cultivar, treatment, or interaction were selected. Spots that showed consistent changes throughout replicates were considered for spot excision, and selected spots were manually excised. The silver stained protein spots were de-stained using the ProteoSilver Plus Stain Kit (Sigma-Aldrich, MO, USA), and in-gel enzymatic digestion was done overnight at 37^0C using Trypsin Singles, Proteomics Grade (Sigma-Aldrich, MO, USA) following manufacturer instructions for preparation of reagents.

The sample peptide solutions were analyzed using a MALDI-TOF/TOF mass spectrometer (Bruker UltrafleXtreme, Coventry, UK). The observed MS peaks obtained were compared using MASCOT. The NCBI non-redundant protein database using the Viridiplantae filter was used to

obtain the most likely protein match based on a peptide mass fingerprinting, and only the top hits were used and a score threshold of 40 was used as a cut off. The search parameters were as follows: trypsin enzyme, fixed modification of cysteine as carbamidomethylated, peptide charge state of +1, peptide tolerance of 0.5 daltons, and up to 1 missed cleavage. For automatic, high throughput functional annotation, the closest Arabidopsis thaliana (AT) match was run through Blast 2 Go. For functional characterization, protein homologues were identified against the NCBI Arabidopsis database. Further functional information was gathered from the Arabidopsis Information Resource database (TAIR). Proteins were classified into categories based on their function [30].

RESULTS AND DISCUSSION

Freezing tests. Treatment, temperature and the interaction between them had significant type III effects based on the Wald x^2 in the logistic regression model (Table 1). In the model that evaluated each cultivar by treatment combination, cultivar was not a significant effect ($p = 0.6486$) (Table 1a). However, when cultivar was excluded from the modes (Table 1b), acclimation treatment and temperature were both significant ($p<0.0001$).

Table 1. a) Type III analysis of stepwise-selection logistic regression model used to estimate LT_{50} values for freeze tests of zoysiagrass cultivars under controlled environmental conditions: a) with and b) without cultivar included in the model.

Effect	DF	Wald Chi-Square†	Pr > ChiSq
a)			
Acclimation Treatment	1	0.6272	0.4284
Temperature	1	88.5819	<0.0001

Cultivar	1	0.2077	0.6486
Cultivar*Acclimation Treatment	1	4.6298	0.0314
b)			
Acclimation Treatment	1	66.9812	<0.0001
Temperature	1	141.1069	<0.0001

[†] Confident interval displacement diagnostic for model a = 0.89 and for model b = 0.88 indicating both models show high concordance and predictability with the data collected.

Maximum likelihood estimates generated from the logistic model were used to assess the survivability of each acclimation treatment or cultivar by acclimation treatment combination in relation to the reference variable by way of the LT_{50} (Table 2). Because NA Victoria was used as a reference variable, all estimates show differences in freezing tolerance in relation to it. The mean separation test indicated that cultivars within acclimation treatments were significantly different ($p < 0.05$). The cold acclimation treatment altered Meyer and Victoria's LT_{50}s by -2.66ºC and -1.50ºC, respectively (Figure 1). Fitness of both these models showed high concordance and predictability with the data collected (Tables 1 and 2). Through modeling of acclimation treatments alone, CA plants were determined to be approximately 1.9-fold more likely to survive than NA plants (Table 2). Similar studies in zoysiagrass [4], St. Augustinegrass [11, 12, 13], bermudagrass [10], buffalograss [8] and saltgrass [7] have also reported that plants demonstrated better freeze tolerance when subjected to cold acclimation. Our results of a nearly two-fold increase supports these findings. Moreover, CA Meyer was significantly different from CA Victoria, but NA cultivars were similar to each other (Figure 1).

Table 2. Maximum likelihood estimates used to calculate survival probabilities and LT_{50} values in freeze tests of zoysiagrass under controlled environmental conditions: a) excluding cultivar from the model and b) for each cultivar by acclimation treatment combination.

Parameter	Estimate†	Wald Chi-Square††	Pr > ChiSq
a)			
Intercept	7.6470	130.9546	<0.0001
Cold Acclimation Treatment	1.8934	66.9812	<0.0001
Temperature	0.8685	141.1069	<0.0001
b)			
Intercept	7.7784	124.5539	<0.0001
Meyer Acclimated	2.6222	58.3716	<0.0001
Meyer Non-Acclimated	0.1241	0.1860	0.6663
Victoria Acclimated	1.4277	22.1395	<0.0001
Temperature	0.8902	140.8128	<0.0001

† The reference variable used for the estimation of the logistic regression model was a) the non-acclimated treatment and b) Victoria Non-Acclimated. Temperature was treated as a continuous variable for both models.

†† Confident interval displacement diagnostic for model a = 0.84 and for model b = 0.87 indicating both models show high concordance and predictability with the data collected.

Figure 1. Probability of zoysiagrass survival in controlled environment freeze tests across temperature curves for each cultivar by acclimation treatment combination where MAC: acclimated 'Meyer' with an LT50 of -11.56°C, MNA: non-acclimated 'Meyer' with an LT50 of -8.89°C, VAC: acclimated 'Victoria' with an LT50 of -10.30°C, and VNA: non-acclimated 'Victoria' with an LT50 of -8.75°C. LT50 values occur at probability 0.50.

Patton et al., [5] observed that specific carbohydrates, proline, and certain proteins in CA zoysiagrass were correlated with freeze tolerance in thirteen genotypes. A proteomic response to

cold acclimation was also observed in velvet bentgrass (*Agrotosis canina*) [31] where CA plants were not only more freeze tolerant but showed thirteen different protein spots that were differentially expressed in CA versus NA plants. Further analysis of metabolic processes that occur during cold acclimation and how they differ between these cultivars, may explain differences in freeze tolerance between *Z. japonica* genotypes.

Protein abundance: Variation in the abundance of specific proteins was assessed in response to acclimation treatment by way of 2-DE SDS-PAGE. The non-acclimated meristematic tissue samples of each cultivar were used as the baseline to control for variation between the treatments. A total of 471 protein spots were detected on the 2-DE gels. From those, 62 spots were statistically significant ($p \leq 0.05$) and exhibited a minimum of a two-fold change in response to cold acclimation for at least one cultivar (Table 3). There were significant differences in expression of these 62 proteins for Meyer and Victoria. In Meyer, 35 spots were higher in abundance in response to cold acclimation (12, 17, 37, 40, 44, 56, 61, 62, 64, 80, 90, 124, 162, 185, 187, 191, 201, 234, 243, 272, 293, 311, 321, 340, 348, 366, 371, 401, 403, 414, 424, 438, 443, 466, 468), while five spots were lower in abundance (86, 87, 223, 240, 261). In Victoria, 46 spots were found to be in lower abundance in response to cold acclimation (17, 40, 44, 61, 62, 80, 90, 104, 114, 116, 131, 142, 148, 162, 169, 184, 185, 187, 191, 193, 201, 216, 223, 238, 240, 243, 261, 265, 270, 285, 292, 293, 300, 310, 311, 317, 321, 340, 348, 366, 371, 375, 401, 424, 438, 467), and seven were in higher abundance (37, 59, 75, 86, 87, 403, 468) (Figure 2). When comparing the 31 protein spots identified in both Meyer and Victoria, 23 were found to increase during cold acclimation in Meyer but decrease in abundance in Victoria. Moreover, two proteins were significantly increased for Victoria, but decreased for Meyer in response to cold acclimation. Protein spots that were unique to each cultivar were identified in order to attempt to elucidate their role in cold acclimation.

307

308

309 **Table 3:** Functional data of the hypothetical proteins matching to each identified protein spot
310 number as determined through Blast 2 Go. The protein name, fold change and direction of the
311 change were determined and listed with the accession and Glycemic Index (GI) number.

		Meyer		Victoria			
Spot	**Protein Name**	**Direction**	**Fold Change**	**Direction**	**Fold Change**	**Accession**	**GI**
12	nudix hydrolase homolog 2	↑	2.51			NP_568687	18422823
17	ovate family protein 14	↑	2.68	↓	-2.15	NP_178114	15220029
37	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	↑	2.47	↑	2.42	NP_850171	30685043
40	No Match	↑	2	↓	-2.83	-	-
44	Subtilisin-like serine endopeptidase family protein [Arabidopsis thaliana]	↑	2.02	↓	-3.68	NP_565309	22325457
56	NAD(P)-binding Rossmann-fold superfamily protein [Arabidopsis thaliana]	↑	2.08			NP_196567	30682984
59	GAST1 protein homolog 4			↑	2.05	NP_197027	15242249
61	No Match	↑	2	↓	-5.38	-	-
62	ADPGLC-PPase large subunit [Arabidopsis thaliana]	↑	3.73	↓	-2.99	NP_174089	15217670
64	Late embryogenesis abundant protein 3 (LEA3)	↑	6.7			-	-
75	ovate family protein 5 [Arabidopsis thaliana]			↑	2.19	NP_193618	22328779
80	phospholipase D beta 1 [Arabidopsis thaliana]	↑	2.03	↓	-2.67	NP_565963	30688872
86	AAA-type ATPase family protein	↓	-7.14	↑	1.96	NP_567238	30679158
87	No Match	↓	-2.22	↑	4.39	-	-
90	No Match	↑	2.38	↓	-2.72	-	-
104	Carbohydrate-binding X8 domain superfamily protein			↓	-2.09	NP_181895	30689458
114	pentatricopeptide (PPR) repeat-containing protein [Arabidopsis thaliana]			↓	-2.81	NP_199470	15237467
116	RGA-like 2			↓	-2.64	NP_186995	15228553
124	transmembrane protein, putative (DUF506) [Arabidopsis thaliana]	↑	2.56			NP_683503	22330691
131	Auxin-responsive GH3 family protein			↓	-2.05	NP_179101	15226032
142	No Match			↓	-4.08		
148	phytochrome A [Arabidopsis thaliana]			↓	-3.21	NP_172428	15217562
162	No Match	↑	2.02	↓	-3.09	-	-
169	ATPase, F1 complex, alpha subunit protein [Arabidopsis thaliana]			↓	-1.92	NP_178788.1	15226092
184	maturase K (chloroplast) [Arabidopsis thaliana]			↓	-3.01	NP_051040.2	126022795
185	photosystem II protein I (chloroplast) [Arabidopsis thaliana]	↑	2.46	↓	-3.62	NP_051043	7525017
187	No Match	↑	2.21	↓	-2.86	-	-

191	SHK1 binding protein 1 [Arabidopsis thaliana]	↑	1.92	↓	-2.48	NP_194841	30688918
193	Nucleotidylyl transferase superfamily protein			↓	-2.68	NP_850070	30682913
201	No Match	↑	1.82	↓	-2.13	-	-
216	Hypoxanthine-guanine phosphoribosyltransferase [Arabidopsis thaliana]			↓	-2.72	NP_177320	15217571
223	zinc finger protein 6 [Arabidopsis thaliana]	↓	-2.08	↓	-4.44	NP_176873	15219811
234	No Match	↑	2.32			-	-
238	cyclopropyl isomerase			↓	-2.52	NP_568727	18423143
240	orange protein	↓	-1.59	↓	-1.94	NP_200975	15240312
243	Fumarylacetoacetate (FAA) hydrolase family	↑	1.8	↓	-2.0	NP_193329	15234757
261	Pyridoxal phosphate phosphatase-related protein	↓	-1.61	↓	-3.89	NP_173213	79346258
265	RNI-like superfamily protein			↓	-4.61	NP_197725	15237286
270	galacturonosyltransferase 6			↓	-3.58	NP_563771	18390688
272	manganese superoxide dismutase 1 [Arabidopsis thaliana]	↑	1.84			NP_187703	15228407
285	cation/H+ exchanger 20 [Arabidopsis thaliana]			↓	-2.24	NP_190940	15231867
292	No Match			↓	-5.05	-	-
293	pentatricopeptide repeat-containing protein	↑	1.86	↓	-3.07	-	-
300	Serine protease inhibitor (SERPIN) family protein			↓	-3.47	NP_175202	15220298
310	isopentenyltransferase 3			↓	-3.55	NP_567138	18412615
311	Acyl-CoA N-acyltransferases (NAT) superfamily protein	↑	2.22	↓	-1.74	NP_180763	15225174
317	Leucine-rich repeat protein kinase family protein			↓	-5.52	NP_178291	15226361
321	No Match	↑	1.91	↓	-3.51	-	-
340	No Match	↑	3.11	↓	-3.33	-	-
348	maturase K (chloroplast) [Arabidopsis thaliana]	↑	1.81	↓	-2.60	XP_002949552	1.26E+08
366	No Match	↑	2.27	↓	-2.08	-	-
371	indoleacetic acid-induced protein 16	↑	1.98	↓	-3.15	NP_187124	15229343
375	Protein kinase superfamily protein [Arabidopsis thaliana]			↓	-2.08	NP_850360	186507320
401	alpha/beta-Hydrolases superfamily protein	↑	2	↓	-1.80	NP_565437	18398716
403	Ribosomal protein S26e family protein [Arabidopsis thaliana]	↑	1.91	↑	2.60	NP_181591	15226715
414	general regulatory factor 2	↑	2.99			NP_565176	18411901
424	RNA polymerase beta subunit (chloroplast) [Arabidopsis thaliana]	↑	2.55	↓	-2.60	NP_051051	7525025
438	Zincin-like metalloproteases family protein	↑	2.3	↓	-2.44	NP_199967	79534875
443	MAP kinase 10	↑	3.01			NP_191538	15231753
466	Nucleotidyltransferase family protein	↑	3.14			NP_568798	18423551
467	ribosomal protein L15 [Arabidopsis thaliana]			↓	-1.77	NP_189221	15231867

468	NB-ARC domain-containing disease resistance protein	↑	2.46	↑	2.282	NP_190237	15231449

312
313

314 **Figure 2:** Number of protein spots identified in nodes of zoysiagrass cultivars 'Meyer' (freeze
315 tolerant) and 'Victoria' (freeze susceptible) acclimated at 8/2°C (day/night) for four weeks,
316 against a non-acclimated baseline.

317
318 In Meyer, eight unique spots were identified with at least a two-fold change in response to
319 CA, all of which were upregulated (Table 3). These proteins were associated with late
320 embryogenesis abundant protein 3, nudix hydrolase homolog 2, putative transmembrane protein
321 (DUF506), manganese superoxide dismutase 1, general regulatory factor 2, Rossmann-fold
322 NAD(P)(+)-binding protein, general regulatory factor 2, MAP kinase 10, and
323 nucleotidyltransferase family protein. Among proteins that were upregulated in response to CA
324 in Meyer, compared to the NA baseline, three proteins were identified as being of interest: Late
325 embryogenesis abundant protein 3 (LEA3), with a 6.7 fold change during CA (one of the highest
326 changes observed) has been found to be functionally associated with disease and defense, and
327 serves as a chaperone protein, to protect proteins and membranes particularly in situations of
328 dehydration such as drought and cold stress [32]. This is consistent with previous reports of
329 increased production of dehydrins, soluble proteins in the LEA family, in freeze tolerant
330 zoysiagrass cultivars [17, 18]. Second, manganese superoxide dismutase 1 (SOD), with an almost
331 two fold increase, is another disease and defense related protein which assists with reducing
332 oxidative stress, an important factor during cold acclimation when metabolism is slowing yet light
333 levels are still high [33]. Third, MAP Kinase 10, or mitogen-activated protein kinase
334 (MAPK/MPK), exhibited a three-fold increase. This protein plays a crucial role in signal
335 transduction pathways for several biotic and abiotic stresses, and also serves to regulate C-repeat

binding factor (CBF) gene expression during cold stress signaling in which the expression levels of CBFs determine the levels of cold-regulated (COR) gene expression and freezing tolerance [34, 35, 36].

In Victoria, there were 20 identifiable, unique spots with at least a two-fold change, two of which were upregulated in response to CA, and 18 of which were downregulated (Table 3). Of these 20 spots, the seven proteins with the largest change in abundance were identified to be homologous with known proteins in online databases (Table 3). These predicted protein domains included Phytochrome A, GAST1 protein homolog 4, ovate family protein 5, leucine-rich repeat protein kinase family protein, galacturonosyltransferase 6, isopentenyltransferase 3, Maturase K (MatK), and RNA like superfamily protein. A few proteins of interest were identified through the differential expression in the NA vs CA treatments in Victoria. GAST1 protein homolog 4, an upregulated protein associated with cell growth and division, which serves as a GA-responsive protein [37]; Phytochrome A, with more than a 3-fold decrease, is a signal transduction related protein that plays a critical role in the cold response by sensing the changes in light conditions, and has been shown to promote *CBF* gene expression 9*CBF1*, a gene encoding AP2 domain-containing transcriptional activator that binds to the low-temperature-responsive element CCGAC and induces the expression of some cold-regulated genes, increasing plant freezing tolerance and subsequent cold tolerance [38]; ATPase F1 complex, alpha subunit protein, a down regulated energy related protein that extrudes protons from cells of plants to generate electrochemical proton gradients, which has a major role in providing the energy for secondary active transport across the plasma membrane, as well as playing a role in adaptation of plants to changing conditions, particularly stress [39].

Expression of the numerous shared proteins between Meyer and Victoria were determined to be of interest particularly if there was significant differential regulation of the proteins between the two cultivars as a response to CA. The shared cold responsive proteins of interest were: ADP-glucose pyrophosphorylase large subunit (upregulated in Meyer and downregulated in Victoria), a key metabolism related enzyme in the starch biosynthesis pathway [40]; Phospholipase D beta 1 (PLD) (upregulated in Meyer and downregulated in Victoria) which functions in metabolic processes, was implicated in the activation of cold response and may play an important, positive role in freezing tolerance [41, 42]; and Photosystem II protein I (PSII) (upregulated in Meyer and downregulated in Victoria), an energy related protein that could point to a better ability to keep photosynthesis active and therefore support energy needed for cold acclimation [43]. Glucose-1-phosphate adenylyltransferase large subunit (AGP), with a greater than three-fold upregulation in Meyer, and a greater than two-fold downregulation in Victoria, functions in the process of starch metabolism due to the significant role it plays in temperature induced yield loss, such as serving as a rate limiting step in starch biosynthesis under elevated temperatures in maize [44].

Potential functions. Due to the relative lack of genetic information on turfgrasses, proteins of interest were identified based on homology with *Arabidopsis thaliana*, as well as through supporting data on their relation to abiotic stress tolerances associated with freezing. In the present study, 62 spots showed at least a two-fold change from the control treatment. The identified protein spots determined through mass spectrometry were sorted into categories/families according to their associated functions via the TAIR database. Out of the 62 identified protein spots, only 49 could be characterized by way of category/family and/or homologous protein. Proteins that were unnamed but classified were still included. However, proteins that could not be associated with a category/family were discarded from further analysis. Some overlap was present between the

categories/families, but proteins were sorted according to their strongest match. Transcription and metabolism related proteins accounted for 31% and 21% of the total proteins, respectively. Energy related, and protein destination and storage related proteins made up 10% of the total identified proteins. Signal transduction related proteins consisted of four individuals (8%). The remaining categories of proteins were disease/defense and protein synthesis (3 proteins or 6% each), cell structure (two proteins or 4%), and cell growth/division and secondary metabolism (one protein or 2% each) (Figure 3).

Figure 3: Functional categories (and their corresponding relative distributions) of proteins with a two-fold change or greater in response to cold acclimation.

Transcription. Transcription related proteins were primarily upregulated in Meyer and downregulated in Victoria, though there were a few exceptions. S-adenosyl-L-methionine-dependent methyltransferase super family was upregulated for both cultivars at a similar fold change. Zinc finger protein 6 was downregulated for both, but with a greater fold change for Victoria. The ovate family protein 5 transcriptional repressor was upregulated in Victoria. Todaka et al. [45] conducted a study in rice to investigate a large number of stress-responsive genes related to gene expression regulation, such as protein kinases and transcription factors, and direct plant protection against stress, such as metabolic enzymes, aquaporin, and late embryogenesis abundant proteins. Transcription factors play important roles in signaling cascades that lead to abiotic stress responses, by regulating the expression of downstream target genes via the utilization of cis-elements in the promoter regions of target genes under stress conditions. The dehydration-responsive element binding protein 1/C-repeat factor (DREB1/CBF) regulon has been identified

404 in Arabidopsis to function in cold stress response, particularly in the accumulation of proline,
405 regulation of stress-responsive gene expression, induction of transcription factor expression [46],
406 and regulation of ABA responsive gene expression [47]. Overexpression of DREB1-type genes
407 led to enhanced expression of stress inducible genes, including cold and drought stress, and
408 elevated tolerance levels, an indicator that the DREB1 regulon may be one of the master regulatory
409 systems for abiotic stress responses [45]. Therefore, a major fold change in transcription related
410 proteins related to these regulatory systems might indicate a key effect response to cold stress.

411 Metabolism. Metabolism related proteins, such as phosopholipase D and glucose-1-
412 phosphate adenylyltransferase were upregulated in CA Meyer, and downregulated in CA Victoria.
413 Sugars are utilized by plants to counteract stressors in their natural environment. As a response to
414 evolutionary pressures in stressful environments, there was a diversification of sugar structures
415 and functions, most importantly the ability to modulate the expression of genes related to abiotic
416 and biotic stress response systems through sugar/starch metabolism. Soluble sugars, as well as
417 amino acids, polyamines, and polyols are all known to contribute to the development of cold stress
418 tolerance mechanisms [48]. Saccharides can stabilize phospholipid membrane composition to
419 maintain fluidity under cold stress conditions [49]. Nitrogen reserves in the form of increased
420 abundance of certain amino acids, may function to improve overwintering and regrowth ability in
421 plants [50]. The differential regulation of glucose-1-phosphate adenylyltransferase, supports the
422 importance of starch metabolism. In addition to being the main storage carbohydrate in plants,
423 starch provides a steady supply of energy when photosynthesis is not possible, resists water loss
424 in stomatal pores, and aids cell differentiation. Under abiotic stresses, starches are remobilized to
425 provide energy when photosynthesis becomes limited. Fructan, an important storage
426 carbohydrates, is known to stabilize cell membranes to prevent water leakage during freezing [51].

427 Other sugars and metabolite derivatives support growth under stress conditions, as well as mitigate
428 some of the negative effects of the stress by activating stress response cascades with signaling
429 sugars [52].

430 Protein destination and storage. Protein destination and storage related proteins were
431 upregulated in Meyer and downregulated in Victoria. Serpins play a critical role in the control of
432 proteolysis, through inhibition of proteinases. Proteolysis is in turn crucial for plant stress
433 response, defense, growth, and development. Silverman et al. [53] purports that cell lysis as a result
434 of cold stress may release proteinases and damage cells. Therefore, plant serpins may act in a
435 manner similar to mammalian serpins to protect cells from proteinases. The Shk1 kinase binding
436 protein 1 (SKB1) is a floral initiator that responds to salt stress by suppressing transcription of
437 flowering locus c (FLC) and several stress responsive genes [54]. FLC is also modulated by a
438 flowering autonomous pathway gene (FLV) when cold temperatures are sensed to initiate cold
439 response [55]. The upregulation of destination and storage related proteins in freeze-tolerant Meyer
440 may serve to mediate and increase response time to damage caused by abiotic stress.

441 Energy. Proteins related to energy were differentially expressed between the two cultivars
442 under the different acclimation treatments. Pyridoxal phosphate phosphatase-related protein was
443 downregulated for both Meyer and Victoria CA cultivars compared to non-acclimated, with a
444 greater fold change for Victoria. Meanwhile, ATP synthase had a two-fold downward change in
445 Victoria only. The AAA-type ATPase family protein had a greater than two-fold downward
446 change in Meyer, and a two-fold upward change in Victoria. Conversely, photosystem II protein I
447 (chloroplast) had a greater than two-fold upward change in Meyer, and a greater than two-fold
448 downward change in Victoria. Proteins related to photosynthesis, such as ATPases, are sensitive
449 to changes in the environment, particularly those which result in a change in photoperiod, such as

450 the changing of seasons into winter. This sensitivity is due to the need to balance light energy
451 absorption with energy consumed by plant metabolism during the process of photosynthesis.
452 Additionally, low temperatures have been found to heighten the disparity between light absorption
453 and metabolic needs, making it necessary to adjust photosynthesis systems to maintain balanced
454 energy flow. There is supporting evidence that the processes which interact with photosynthesis,
455 particularly during cold acclimation, involve several pathways leading to the regulation of
456 acclimation to cold temperatures, including photosynthetic redox, acclimation, and sugar signaling
457 pathways [56].

458 Protein synthesis. Proteins related to protein synthesis were upregulated in Meyer, and
459 primarily downregulated in Victoria, with the exception of spot 403: ribosomal protein S26e
460 family protein, which was upregulated in Victoria. The decrease in ribosomal proteins overall may
461 play a role in translation, ribosome assembly, and proper function of mRNA under low-
462 temperature conditions [57]. A study in tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.
463 syn. *Festuca arundinacea* Schreb.] demonstrated that the introduction of the
464 isopentenyltransferase (IPT) gene enhanced cold tolerance, as well as tillering ability and
465 chlorophyll a and b levels [58]. This gene is purportedly responsible for inhibited apical
466 dominance, delayed senescence, and increased chlorophyll and secondary metabolite levels [59].
467 Research in peanut (*Arachis hypogaea* L.) [60] and sugarcane (*Saccharum* spp. L.) [61] support
468 the ability of IPT to delay stress induced plant senescence, resulting in enhanced drought and cold
469 tolerance. Synthesis related proteins such as IPT likely play an important role in generating cold
470 acclimation induced proteins.

471 Signal transduction. Signal transduction related proteins were upregulated in Meyer, but
472 downregulated in Victoria. Additionally, a higher number of significant changes in signal

transduction related proteins occurred in Victoria at a 3:1 ratio. Research in *Arabidopsis thaliana* identified calcium as the most common signaling factor for cells to translate external stimuli into a biochemical response. In the presence of cold stress, calcium levels are elevated, and are correlated to the activation of certain chilling sensitive genes [62]. A decrease in endogenous levels of IAA-amido synthetase, as observed in Victoria, was shown to enhance drought tolerance in rice in conjugation with TLD1, as well as influence an increase in LEA gene expression [63]. A decrease in free IAA led to enhanced ROS scavenging and increased cold response and membrane permeability as well, leading to increased cold tolerance [64]. MAP kinase 10, the highly upregulated protein observed in Meyer, is known in several species, including *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*), as a major player in plant stress signaling. The MAPK cascade is a major pathway for multiple plant stress responses through its involvement in mediating oxidative stress, as well as its interaction with several other important signaling pathways [65]. Signal transduction related proteins may be of particular interest in breeding due to their key roles in stress response pathways.

Disease and defense. While a small percentage of proteins related to disease and defense showed a significant fold change, all identifiable proteins showed two-fold or greater upregulation. The two proteins of particular interest in Meyer were LEA3 and SOD. Kobayashi et al., 2004 observed that LEA proteins are a major downstream group involved in the ABA-dependent and independent signaling pathways for freezing tolerance in wheat and may be able to be further enhanced for increased response. Genes in the LEA family commonly encode highly hydrophilic proteins, which positively correlate with greater freezing tolerance when overexpressed. Additionally, in a similar manner to cold acclimation, light conditions influenced the accumulation of cold responsive LEA proteins. Transcriptional processes were enhanced by light and suppressed

by darkness in the Kobayashi et al. [66] study. SODs operate as a first line of defense against reactive oxygen species, which are increased under stress conditions. Exposure to oxidative stressors results in an increase in SODs, and induced overexpression of SODs has led to increased protection against specific stresses, though results have been mixed due to the complexity of an associated scavenging pathway [33]. However, some attempts to overexpress SOD have been successful in increasing stress response [67], and may be utilized in future breeding efforts.

Other proteins. Cell structure, cell growth and division, and secondary metabolism related proteins made up the smallest percentage of the significant fold changes between the two cultivars. The plasma membrane is the primary site of freezing injury, and maintaining an intact membrane can act as an effective barrier to the formation of ice crystals [68]. The 2.5 upward fold change of a transmembrane cell structure related protein in Meyer may indicate a role in the maintenance of this membrane for protection against freezing injury. GAST1 protein homolog, associated with cell growth and division, is involved in numerous biological processes, including elongation, flowering, seed development and light signaling, flowering and stem growth, shoot elongation and flower transition. Rubinovich and Weiss [37] proposed that any sort of higher growth/elongation during the cold acclimation period could potentially interfere with proper cold acclimation, such as using photosynthates towards growth rather than protection. Little information exists on the pentatricopeptide repeat contain protein, other than it may be a product of partial degradation [69].

In comparison to Xuan et al. [23], their study found more differential expression between cold acclimated Meyer (*Zoysia japonica*) compared to Diamond (*Zoysia matrella*), whereas our study indicated a greater change in abundance in Victoria compared to Meyer. The use of cultivars of different species in their study might provide some explanation into this difference in results. Some of the differential expression observed in that study might be attributed to inherent

519 differences between the species rather than actual differences in freeze response. They found that
520 energy and metabolism were the largest functional categories at 23% and 21% respectively, as
521 well as photosynthesis (13%), signal transduction (13%), and redox homeostasis and defense
522 response (14%). While we also found these categories to be important, we had 31% of identified
523 proteins from meristematic crown tissue as falling into the transcriptional category, in comparison
524 with the 2% of stolon proteins associated with transcription by Xuan et al. The amount of
525 transcription related activity may be significantly higher in crown tissue compared to stolon tissue,
526 and therefore may be more involved in meristematic tissue growth compared to stolon growth.
527 There was some overlap in identified proteins, particularly MAP kinase, Photosystem II, and
528 phosphatase related proteins. Although there were several different proteins and pathways found
529 through proteomic analysis, their study came to a similar conclusion that signal transduction and
530 photosynthesis may explain some of the differences in freeze tolerance between Meyer and less
531 freeze tolerant cultivars such as Diamond or Victoria. ADP glucose pyrophosphorylase
532 (ADPGLC-PPase) and other similar ATPases were identified as potentially playing an important
533 role in carbohydrate metabolism under cold stress by Xuan et al. [23], and was also observed in
534 our study to be significantly upregulated in Meyer and downregulated in Victoria.

535 The study by Holloway et al. [24] identified 146 proteins that were potentially associated
536 with abiotic stress response related pathways, a number which corresponded to the putative winter
537 hardiness QTL that had been identified through SNP-based linkage mapping. Some overlap
538 between those previously identified winter injury stress associated proteins and the cold
539 acclimation associated proteins in this study were found. Leucine-rich repeat receptor-like protein
540 kinase and pentatricopeptide repeat family protein are among the most notable corresponding
541 proteins, as well as several related kinases and auxin related proteins. The presence of these

542 proteins in both studies strengthens their association with cold stress response. Further analysis
543 will be of great importance to confirming the findings of relevant cold acclimation associated
544 proteins in different tissues of *Zoysia* spp.

545 **Overall cultivar differences.** The uniformity in freeze tolerance of NA cultivars suggests that the
546 physiological changes that occur during cold acclimation may be the separating factor between
547 freeze tolerances of zoysiagrass cultivars. The large disparity in response to cold acclimation in
548 Meyer and Victoria is of particular interest requiring further study. The large number of down-
549 regulated proteins observed in Victoria in response to cold acclimation might indicate a differential
550 way in which freeze tolerant and freeze sensitive cultivars physiologically respond to cold
551 stimulus. The upregulation of proteins in freeze tolerant Meyer may drive the acclimation process,
552 while the downregulation of proteins in freeze sensitive Victoria may be the result of shutting
553 down processes to conserve energy. Victoria was originally selected by its developer for its ability
554 to perform well under California growing conditions of drought and high temperatures with the
555 ability to remain green during the onset of winter dormancy [26]. Conversely, Meyer zoysiagrass
556 originated from Korea and was selected in Virginia and Maryland for its survival in the Northeast
557 and Midwest despite its "disadvantage" of poor winter color [25, 1]. Under stress conditions,
558 reduced photosynthesis can limit carbon supply and biomass production, resulting in reduced
559 growth and fitness of the plant. However, plants that utilize starch remobilization can avoid these
560 dangers by providing an alternate source of energy and carbon to survive and even mitigate the
561 effects of a stressful environment [52]. The high level of downregulation in unique protein spots
562 in Victoria, may be a contributing factor of reducing photosynthesis, and therefore sensitivity to
563 temperatures possibly allowing for improved fall color but reduced cold acclimation. Meyer adapts
564 to cold temperatures by upregulating protein expression, and has shown higher levels of starch

565 metabolizing proteins that may help with remobilization. However, further investigation is needed
566 to verify these hypotheses.

567 Freeze tolerance is believed to be correlated with the accumulation of soluble proteins,
568 notably by a general increase in winter and subsequent decline in spring [68]. However, as
569 observed in this study, it is not always an increase in protein accumulation during cold acclimation,
570 but rather about overall quantitative changes that occur during cold acclimation which contribute
571 to freezing tolerance. Although the largest percentage of identified proteins were functionally
572 related to transcription and metabolism, the relative importance of these proteins to cold
573 acclimation and freezing tolerance is still being researched. Based on the functional data behind
574 homologous proteins in related species, the major proteins of interest for their association with
575 cold acclimation are LEA 3, MAPK, SOD, GAST1, Phytochrome A, ATP synthase, AGP, PLD,
576 and PSII. The diversity in the metabolic pathways these proteins follow further emphasizes the
577 complexity of abiotic stress response systems. The differential reactions between Meyer and
578 Victoria to cold acclimation highlight the need for greater depth of knowledge on the mechanisms
579 behind cold and freeze stress response, particularly the metabolic differences. Determining the
580 nature of protein composition changes in the crown tissue of zoysiagrass genotypes following cold
581 acclimation will aid in the development of new breeding strategies to produce cultivars with
582 increased freezing tolerance.

583

584 **Acknowledgements:** This research was supported in part with funding provided by the NC State
585 University Plant Breeding Consortium, the NC State University Center for Turfgrass
586 Environmental Research and Education, and the United States Golf Association.

587 **REFERENCES**

588 1. Patton, A.J., B.M. Schwartz, and K.E. Kenworthy. 2017. Zoysiagrass (Zoysia spp.) history,
589 utilization, and improvement in the United States: A Review. Crop Science. 57:S-37–S-72.
590 doi:10.2135/cropsci2017.02.0074
591 2. Forbes I, Ferguson M. Observations on the Zoysia grasses. The Greenkeepers' Reporter,
592 1947;15(2):7-9.
593 3. Dunn, J, Bughrara, S, Warmund, M and Fresenburg, B. Low Temperature Tolerance of
594 Zoysiagrasses. HortScience, 1999;34(1):96-99.
595 4. Hinton J, Livingston D, Miller G, Peacock C, Tuong T. Freeze Tolerance of Nine
596 Zoysiagrass Cultivars Using Natural Cold Acclimation and Freeze Chambers. HortScience.
597 2012;47(1):112-115.
598 5. Patton, A and Reicher, Z. Zoysiagrass Species and Genotypes Differ in Their Winter Injury
599 and Freeze Tolerance. Crop Science, 2007;47(4):1619.
600 6. Zhang, Q., Fry, J., Rajashekar, C., Bremer, D., and Engelke, M. 2009. Membrane polar lipid
601 changes in zoysiagrass rhizomes and their potential role in freezing tolerance. Journal of the
602 American Society for Horticultural Science 134:322-328.
603 7. Shahba M, Qian Y, Hughes H, Koski A, Christensen D. Relationships of Soluble
604 Carbohydrates and Freeze Tolerance in Saltgrass. Crop Science. 2003;43(6):2148.
605 8. Qian Y, Ball S, Tan Z, Koski A, Wilhelm S. Freezing Tolerance of Six Cultivars of
606 Buffalograss. Crop Science. 2001;41(4):1174.
607 9. Anderson J, Taliaferro C, Martin D. Evaluating Freeze Tolerance of Bermudagrass in a
608 Controlled Environment. HortScience. 1993;28(9):955.

609 10. Anderson J, Taliaferro C, Martin D. Longer Exposure Durations Increase Freeze Damage to
610 Turf Bermudagrasses. Crop Science. 2003;43(3):973.
611 11. Kimball J, Tuong T, Arellano C, Livingston III D, Milla-Lewis S. Assessing freeze-tolerance
612 in St. Augustinegrass: temperature response and evaluation methods. Euphytica.
613 2017;213(5).
614 12. Kimball J, Tuong T, Arellano C, Livingston D, Milla-Lewis S. Assessing Freeze Tolerance
615 in St. Augustinegrass: II. Acclimation Treatment Effects. Euphytica. 2017;213(12).
616 13. Kimball J, Tuong T, Arellano C, Livingston D, Milla-Lewis S. Linkage Analysis and
617 Identification of Quantitative Trait Loci Associated with Freeze Tolerance and Turf Quality
618 Traits in St. Augustinegrass. Molecular Breeding. 2018;38(5).
619 14. Anderson J, Taliaferro C, Martin D. Freeze Tolerance of Bermudagrasses. Crop Science.
620 2002;42(3):975.
621 15. Dunne J, Tuong T, Livingston III D, Milla-Lewis S. Field and Laboratory Evaluation of
622 African Bermudagrass Germplasm for Freezing Tolerance. Proc. Amer. Soc. Agron. Intl.
623 Ann. Mtg., Phoenix, AZ. Nov 6-9, 2016.
624 16. Holloway H. Genomic Approaches for Improving Freeze Tolerance in Zoysiagrass M.SC.
625 Thesis, North Carolina State University; 2016. Available from:
626 http://www.lib.ncsu.edu/resolver/1840.20/33610
627 17. Zhang X, Shang C, Liu Y, Hu G, Harich K, Ervin E. Hormone and Dehydrin Expression
628 Responses to Cold Acclimation in Two Zoysiagrass Cultivars with Contrasting Freezing
629 Tolerance. itsrj. 2017;13(1):547.
630 18. Patton A, Cunningham S, Volenec J, Reicher Z. Differences in Freeze Tolerance of
631 Zoysiagrasses: I. Role of Proteins. Crop Science. 2007;47(5):2162.

632 19. Patton A, Cunningham S, Volenec J, Reicher Z. Differences in Freeze Tolerance of
633 Zoysiagrasses: II. Carbohydrate and Proline Accumulation. Crop Science. 2007;47(5):2170.
634 20. Cai Q, Wang S, Cui Z, Sun J, Ishii Y. Changes in Freezing Tolerance and its Relationship
635 with the Contents of Carbohydrates and Proline in Overwintering Centipedegrass
636 (Eremochloa ophiuroides(Munro) Hack.). Plant Production Science. 2004;7(4):421-426.
637 21. Ball S, Qian Y, Stushnoff C. Soluble Carbohydrates in Two Buffalograss Cultivars with
638 Contrasting Freezing Tolerance. Journal of the American Society for Horticultural Science.
639 2002;127(1):45-49.
640 22. Santarius K. Freezing of Isolated Thylakoid Membranes in Complex Media. VIII.
641 Differential Cryoprotection by Sucrose, Proline and Glycerol. Physiologia Plantarum.
642 1992;84(1):87-93.
643 23. Xuan J, Song Y, Zhang H, Liu J, Guo Z, Hua Y. Comparative Proteomic Analysis of the
644 Stolon Cold Stress Response between the C4 Perennial Grass Species *Zoysia japonica* and
645 *Zoysia matrella*. PLoS ONE. 2013;8(9):e75705.
646 24. Holloway H, Yu X, Dunne J, Schwartz B, Patton A, Arellano C et al. A SNP-based high-
647 density linkage map of zoysiagrass (*Zoysia japonica* Steud.) and its use for the identification
648 of QTL associated with winter hardiness. Molecular Breeding. 2017;38(1).
649 25. Grau F and Radko A. Meyer (Z-52) *Zoysia*. USGA J. Turf Manage. 1951;4(6): 30-31.
650 26. Gibeault V, Leonard M, Youngner V. Zoysiagrass plant named ‘Victoria. U.S. Plant Patent
651 9,135. Date issued: 9 May, 1995.
652 27. Hatwiger C. Help your bunkers make the grade. USGA Green Section Record. 1998;36(6),
653 1-4. http://turf.lib.msu.edu/1990s/1998/981101.pd

654 28. Xu C, Xu Y, Huang B. Protein Extraction for Two-Dimensional Gel Electrophoresis of
655 Proteomic Profiling in Turfgrass. Crop Science. 2008;48(4):1608.
656 29. Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of
657 Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry. 1976;72(1-
658 2):248-254.
659 30. Bevan M, Bancroft I, Bent E, Love K, & al,e. Analysis of 1.9 mb of Contiguous Sequence
660 from Chromosome 4 of *Arabidopsis thaliana*. Nature, 1998;391(6666), 485-8.
661 doi:http://dx.doi.org/10.1038/35140
662 31. Espevig T, DaCosta M, Hoffman L, Aamlid T, Tronsmo A, Clarke B et al. Freezing
663 Tolerance and Carbohydrate Changes of Two Species during Cold Acclimation. Crop
664 Science. 2011;51(3):1188.
665 32. Ingram J, Bartels D. The Molecular Basis of Dehydration Tolerance in Plants. Annual
666 Review of Plant Physiology and Plant Molecular Biology. 1996;47(1):377-403.
667 33. Alscher R, Erturk N, Heath L. Role of Superoxide Dismutases (SODs) in Controlling
668 Oxidative Stress in Plants. Journal of Experimental Botany. 2002;53(372):1331-1341.
669 34. Liu S, Jiang Y. Identification of Differentially Expressed Genes under Drought Stress in
670 Perennial Ryegrass. Physiologia Plantarum. 2010;139:375–387.
671 35. Sangwan V, Orvar B, Beyerly J, Hirt H, Dhindsa R. Opposite Changes in Membrane Fluidity
672 Mimic Cold and Heat Stress Activation of Distinct Plant MAP Kinase Pathways. The Plant
673 Journal. 2002;31(5):629-638.
674 36. Gilmour S, Sebolt A, Salazar M, Everard J, Thomashow M. Overexpression of the
675 Arabidopsis CBF3 Transcriptional Activator Mimics Multiple Biochemical Changes
676 Associated with Cold Acclimation. Plant Physiology. 2000;124(4):1854-1865.

677 37. Rubinovich L, Weiss D. The Arabidopsis Cysteine-rich Protein GASA4 Promotes GA
678 Responses and Exhibits Redox Activity in Bacteria and in Planta. The Plant Journal.
679 2010;64(6):1018-1027.
680 38. Wang F, Guo Z, Li H, Wang M, Onac E, Zhou J et al. Phytochrome A and B Function
681 Antagonistically to Regulate Cold Tolerance via Abscisic Acid-Dependent Jasmonate
682 Signaling. Plant Physiology. 2015;170(1):459-471.
683 39. Muzi C, Camoni L, Visconti S, Aducci P. Cold stress affects H -ATPase and phospholipase
684 D activity in Arabidopsis. Plant Physiology and Biochemistry. 2016;108, 328–336.
685 40. Vantourout P, Radojkovic C, Lichtenstein L, Pons V, Champagne E, & Martinez L. Ecto-F1-
686 ATPase: A Moonlighting Protein Complex and an Unexpected ApoA-I Receptor. World
687 Journal of Gastroenterology. 2010;16(47), 5925–5935.
688 http://doi.org/10.3748/wjg.v16.i47.5925
689 41. Singh A, Pandey A, Baranwal V, Kapoor S, Pandey G. Comprehensive Expression Analysis
690 of Rice Phospholipase D Gene Family During Abiotic Stresses and Development. Plant
691 Signaling & Behavior. 2012;7(7):847-855.
692 42. Ruelland E. Activation of Phospholipases C and D Is an Early Response to a Cold Exposure
693 in Arabidopsis Suspension Cells. Plant Physiology. 2002;130(2):999-1007.
694 43. Rapacz M. Regulation of Frost Resistance During Cold De-acclimation and Re-acclimation
695 in Oilseed Rape. A Possible Role of PSII Redox State. Physiologia Plantarum.
696 2002;115(2):236-243.
697 44. Greene T, Hannah L. Enhanced Stability of Maize Endosperm ADP-Glucose
698 Pyrophosphorylase is Gained Through Mutants that Alter Subunit Interactions. Proceedings
699 of the National Academy of Sciences. 1998;95(22):13342-13347.

700 45. Todaka D, Nakashima K, Shinozaki K, Yamaguchi-Shinozaki K. Toward Understanding
701 Transcriptional Regulatory Networks in Abiotic Stress Responses and Tolerance in Rice.
702 Rice. 2012;5(1):6.
703 46. Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M et al. Functional Analysis of
704 Rice DREB1/CBF-type Transcription Factors Involved in Cold-Responsive Gene Expression
705 in Transgenic Rice. Plant and Cell Physiology. 2006;47(1):141-153.
706 47. Wang Q, Guan Y, Wu Y, Chen H, Chen F, Chu C. Overexpression of a Rice OsDREB1F
707 Gene Increases Salt, Drought, and Low Temperature Tolerance in Both Arabidopsis and
708 Rice. Plant Molecular Biology. 2008;67(6):589-602.
709 48. Tarkowski Ł, Van den Ende W. Cold Tolerance Triggered by Soluble Sugars: a Multifaceted
710 Countermeasure. Frontiers in Plant Science. 2015;6.
711 49. Ruelland E, Collin S. Chilling Stress. In: Wallingford UK, CABI Publishing, Shabala S,
712 editor. Plant Stress Physiology. 2011. pp. 94–118 10.1079/9781845939953.0094
713 50. Volenec J, Ourry A, Joern B. A Role for Nitrogen Reserves in Forage Regrowth and Stress
714 Tolerance. Physiologia Plantarum. 1996;97(1):185-193.
715 51. Livingston D, Hincha D, Heyer A. Fructan and its Relationship to Abiotic Stress Tolerance
716 in Plants. Cellular and Molecular Life Sciences. 2009;66(13):2007-2023.
717 52. Thalmann M, Santelia D. Starch as a Determinant of Plant Fitness Under Abiotic Stress. New
718 Phytologist. 2017;214(3):943-951.
719 53. Silverman G, Whisstock J, Askew D, Pak S, Luke C, Cataltepe S et al. Human Clade B
720 Serpins (ov-serpins) Belong to a Cohort of Evolutionarily Dispersed Intracellular Proteinase
721 Inhibitor Clades that Protect Cells from Promiscuous Proteolysis. Cellular and Molecular
722 Life Sciences (CMLS). 2004;61(3):301-325.

723 54. Zhang Z, Zhang S, Zhang Y, Wang X, Li D, Li Q et al. Arabidopsis Floral Initiator SKB1
724 Confers High Salt Tolerance by Regulating Transcription and Pre-mRNA Splicing through
725 Altering Histone H4R3 and Small Nuclear Ribonucleoprotein LSM4 Methylation. The Plant
726 Cell. 2011;23(1):396-411.
727 55. Blázquez M, Ahn J, Weigel D. A Thermosensory Pathway Controlling Flowering Time in
728 *Arabidopsis thaliana*. Nature Genetics. 2003;33(2):168-171.
729 56. Ensminger I, Busch F, Huner N. Photostasis and Cold Acclimation: Sensing Low
730 Temperature Through Photosynthesis. Physiologia Plantarum. 2006;126(1):28-44.
731 57. Kim K, Park S, Chung Y, Chung C, Kim J, Lee J. Molecular Cloning of Low-Temperature-
732 Inducible Ribosomal Proteins from Soybean. Journal of Experimental Botany.
733 2004;55(399):1153-1155.
734 58. Hu Y, Jia W, Wang J, Zhang Y, Yang L, Lin Z. Transgenic Tall Fescue Containing the
735 *Agrobacterium tumefaciens* IPT Gene Shows Enhanced Cold Tolerance. Plant Cell Reports.
736 2004;23(10-11):705-709.
737 59. Li Y, Hagen G, Guilfoyle T. Altered Morphology in Transgenic Tobacco Plants that
738 Overproduce Cytokinins in Specific Tissues and Organs. Developmental Biology.
739 1992;153(2):386-395.
740 60. Qin H, Gu Q, Zhang J, Sun L, Kuppu S, Zhang Y et al. Regulated Expression of an
741 Isopentenyltransferase Gene (IPT) in Peanut Significantly Improves Drought Tolerance and
742 Increases Yield Under Field Conditions. Plant and Cell Physiology. 2011;52(11):1904-1914.
743 61. Belintani N, Guerzoni J, Moreira R, Vieira L. Improving Low-Temperature Tolerance in
744 Sugarcane by Expressing the IPT Gene Under a Cold Inducible Promoter. Biologia
745 Plantarum. 2011.

746 62. Knight M. Signal Transduction Leading to Low–Temperature Tolerance in *Arabidopsis*
747 *thaliana*. Philosophical Transactions of the Royal Society of London Series B: Biological
748 Sciences. 2002;357(1423):871-875.
749 63. Zhang S, Li C, Cao J, Zhang Y, Zhang S, Xia Y et al. Altered Architecture and Enhanced
750 Drought Tolerance in Rice via the Down-Regulation of Indole-3-Acetic Acid by
751 TLD1/OsGH3.13 Activation. Plant Physiology. 2009;151(4):1889-1901.
752 64. Du H, Wu N, Fu J, Wang S, Li X, Xiao J et al. A GH3 Family Member, OsGH3-2,
753 Modulates Auxin and Abscisic Acid Levels and Differentially Affects Drought and Cold
754 Tolerance in Rice. Journal of Experimental Botany. 2012;63(18):6467-6480.
755 65. Nakagami H, Pitzschke A, Hirt H. Emerging MAP Kinase Pathways in Plant Stress
756 Signalling. Trends in Plant Science. 2005;10(7):339-346.
757 66. Kobayashi F, Takumi S, Nakata M, Ohno R, Nakamura T, Nakamura C. Comparative Study
758 of the Expression Profiles of the Cor/Lea Gene Family in Two Wheat Cultivars with
759 Contrasting Levels of Freezing Tolerance. Physiologia Plantarum. 2004;120(4):585-594.
760 67. Perl A, Perl-Treves R, Galili S, Aviv D, Shalgi E, Malkin S et al. Enhanced Oxidative-Stress
761 Defense in Transgenic Potato Expressing Tomato Cu,Zn Superoxide Dismutases. Theoretical
762 and Applied Genetics. 1993;85(5):568-576.
763 68. Guy C. Cold Acclimation And Freezing Stress Tolerance: Role Of Protein Metabolism.
764 Annual Review of Plant Physiology and Plant Molecular Biology. 1990;41(1):187-223.
765 69. Yan S, Zhang Q, Tang Z, Su W, Sun W. Comparative Proteomic Analysis Provides New
766 Insights into Chilling Stress Responses in Rice. Molecular & Cellular Proteomics.
767 2005;5(3):484-496.
768

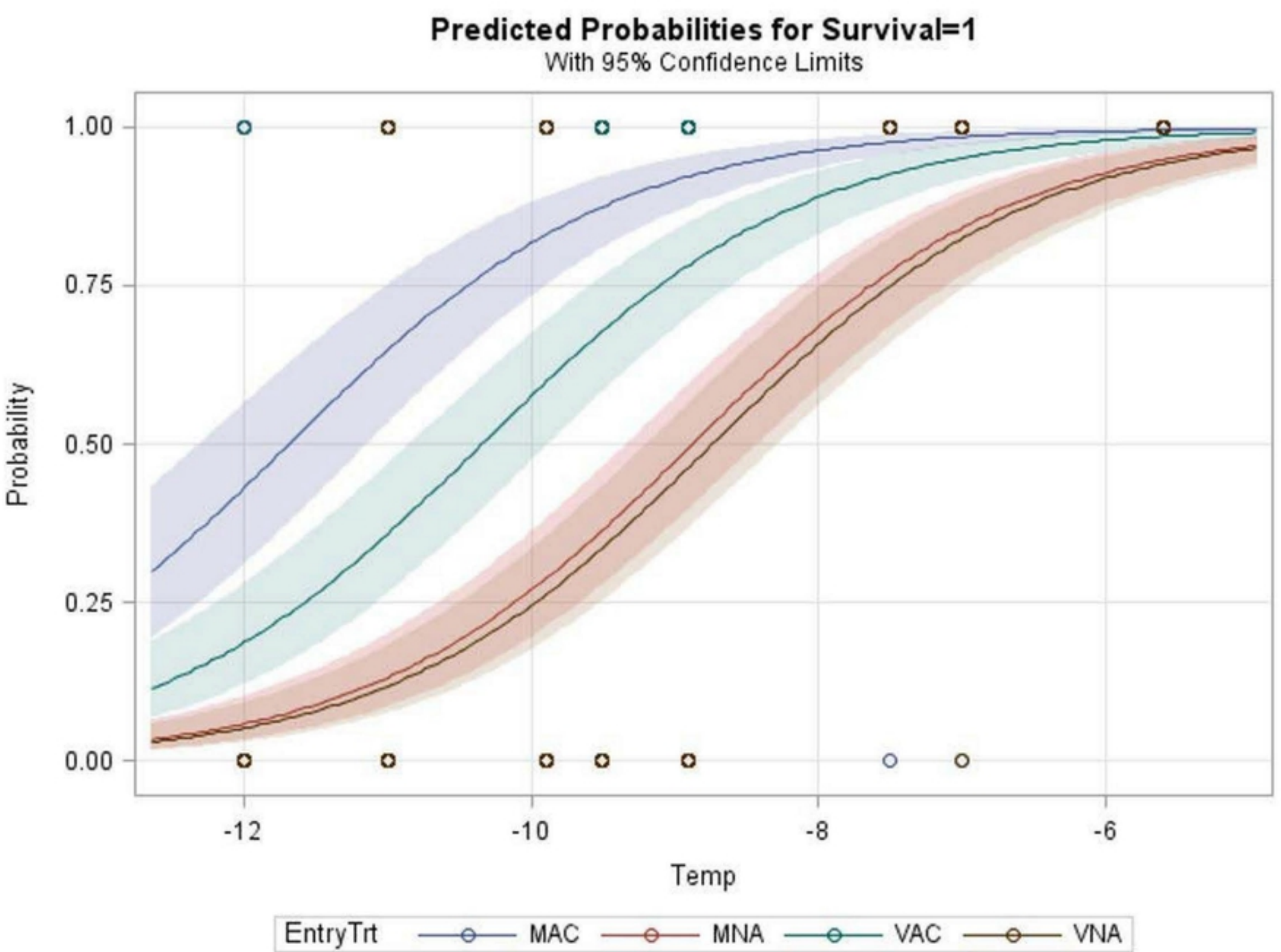
Predicted Probabilities for Survival=1
With 95% Confidence Limits
1.00
0.75
0.50
0.25
0.00
Probability
-12
-10
-8
-6
Temp
EntryTrt
MAC
MNA
VAC
VNA

Figure 1

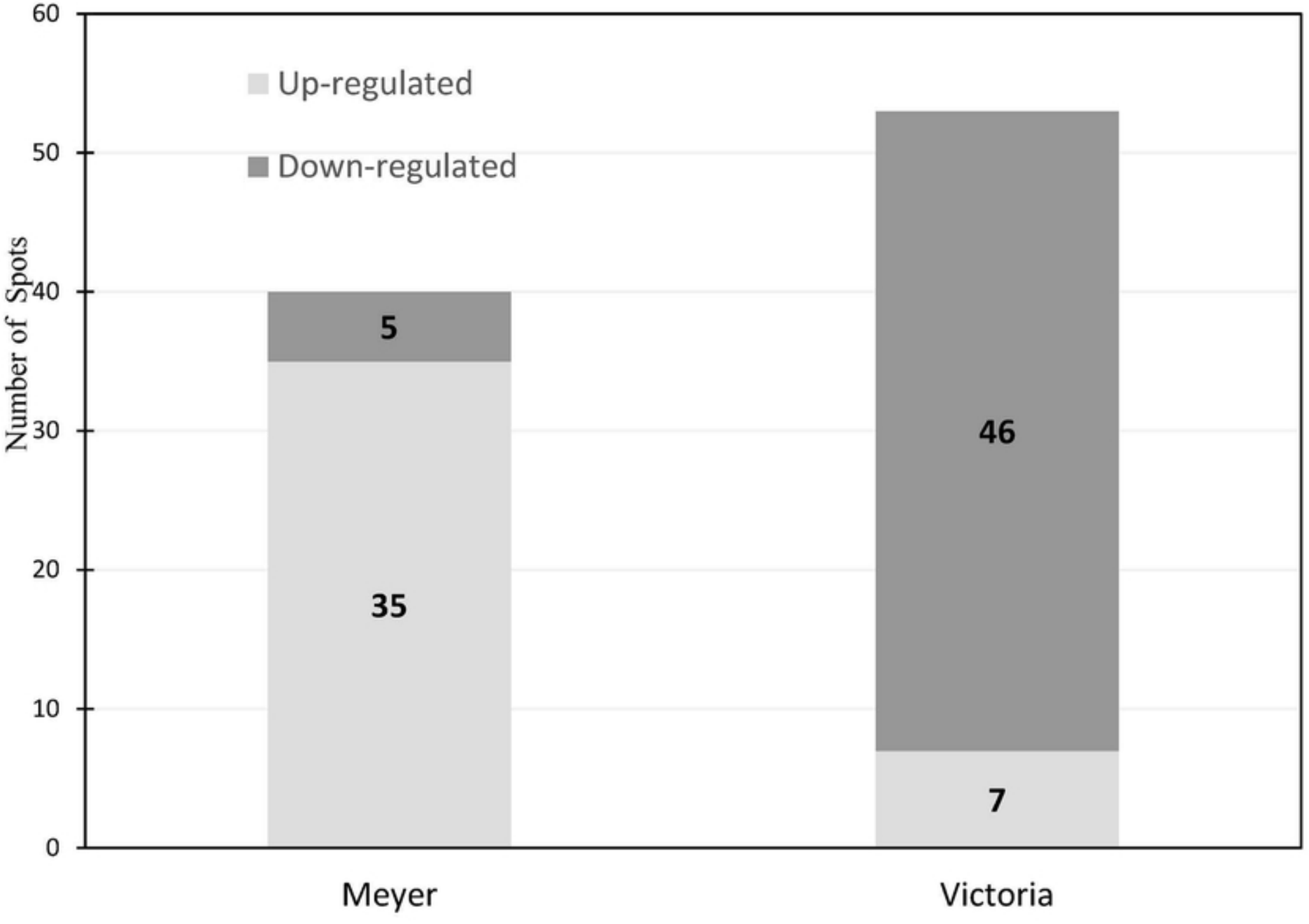
60
50
40
30
20
10
0
Number of Spots
Up-regulated
Down-regulated
5
35
46
7
Meyer
Victoria

Figure 2

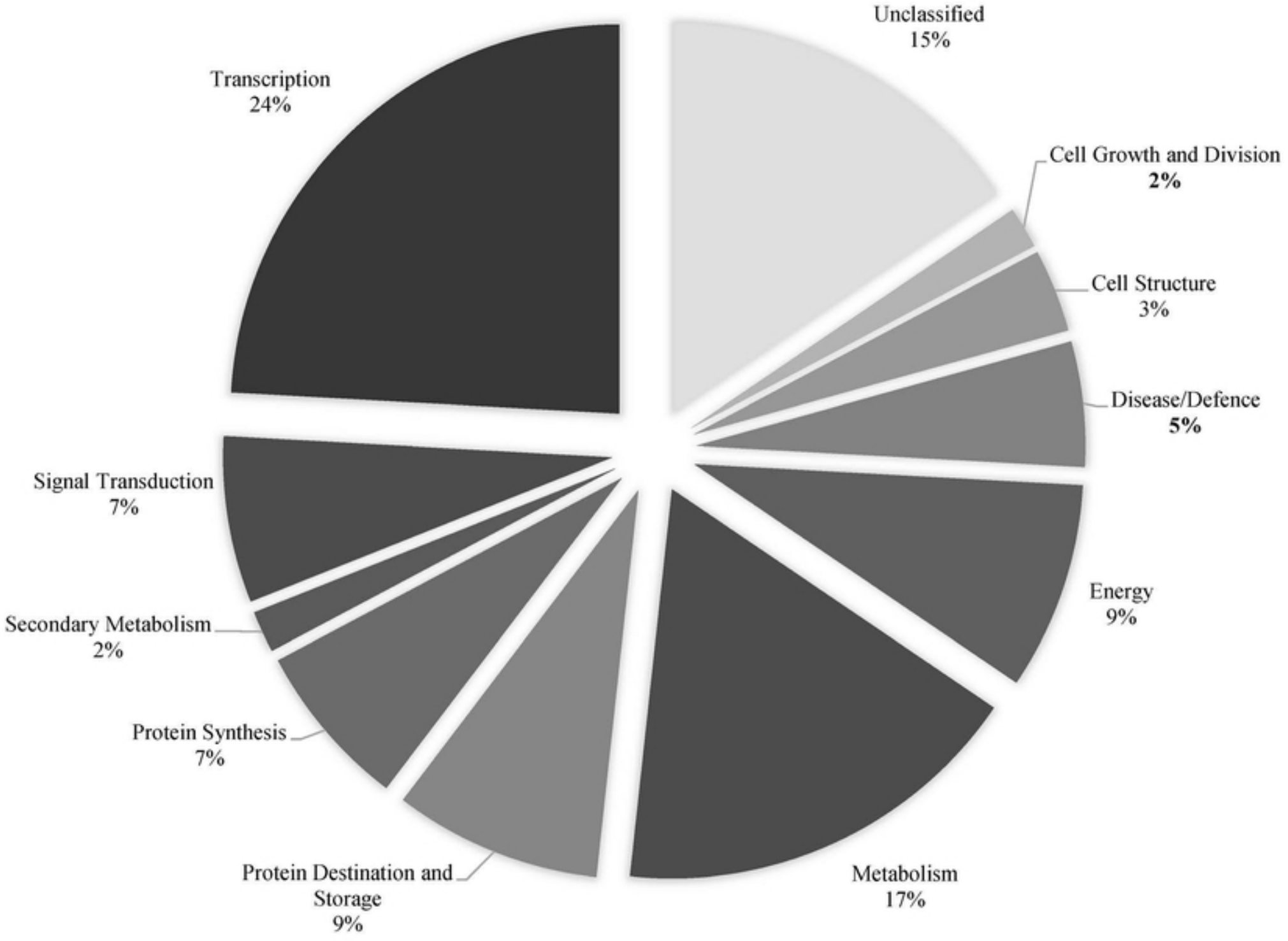
Unclassified
15%
Cell Growth and Division
2%
Cell Structure
3%
Disease/Defence
5%
Energy
9%
Metabolism
17%
Protein Destination and
Storage
9%
Protein Synthesis
7%
Secondary Metabolism
2%
Signal Transduction
7%
Transcription
24%

Figure 3