TURFGRASS AND ENVIRONMENTAL RESEARCH ONLINE
USING SCIENCE TO BENEFIT GOLF

Research at Oklahoma State University seeks to extend the current understanding of cold tolerance to the global genetic level using microarray analysis of gene expression under cold acclimating conditions. Under cold treatment conditions, only about 13% of the genes examined responded in some way to cold treatment. Of the 586 differentially expressed genes, only 97 showed any similarity to known genes in the National Center for Biotechnology Information data base.

Volume 11, Number 1
January 1, 2012

PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 400 projects at a cost of $31 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

Editor

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

Research Director

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

Gene Expression in Cold Acclimating Bermudagrass Crown Tissues

Michael Anderson, Kalpalatha Melmaiee, Sathya Elavarthi, and Arron Guenzi

SUMMARY

Bermudagrass is a premier forage and turfgrass that is grown throughout the southern parts of the United States. This grass is susceptible to damage due to cold temperatures. Here for the first time, we look at genes in bermudagrass crowns that respond to cold temperature treatments using microarray analysis. Results to date include:

- Surveyed over 4,589 genes for changes in gene expression.
- Only 586 or 13% of all genes responded to cold temperatures.
- Of those that responded, only 97 or 17% were identifiable.
- Many more genes were suppressed than enhanced.
- Many more genes changed their response in resistant 'MSU' than susceptible 'Zebra'.
- More genes responded to cold temperatures at 28 compared to 2 days after treatment.

Bermudagrass is grown throughout the southern portion of the United States for turf and forage purposes. This grass combines excellent stress and wear tolerance making it the premier turfgrass for the golf and athletic turf industries. However, the Achilles heel of this species is a limited level of cold tolerance which keeps it from being extensively used in more northerly regions. For now, bermudagrass is restricted to regions south of the revised Arborday Hardiness Zone seven (Figure 1). North of region seven, cold stresses are a common and expensive occurrences resulting in increased labor and replacement costs to golf course superintendents, home owners, and athletic field managers.

Improving tolerance to low temperatures would have the effect of not only reducing cost, but also providing an opportunity to extend the region of adaptation for this extraordinarily useful grass to more northerly areas. However, improving cold tolerance may require that we understand the cold tolerance mechanism to a greater extent than is currently available. This research seeks to extend our current understanding of cold tolerance to the global genetic level using microarray analysis of gene expression under cold acclimating conditions.

Oklahoma State University is home to one of the largest collections of bermudagrass germplasms throughout the world assembled by Dr. Charles Taliaferro (shown above) and currently directed by Dr. Yanqi Wu.

MICHAEL ANDERSON, Ph.D., Associate Professor, Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, OK; KALPALATHA MELMAIEE, Ph.D., Post Doctoral Research Scientist, Department of Agriculture and Natural Resources, Delaware State University; Dover, DE; SATHYA ELAVARTHI, Ph.D., Assistant Professor, Department of Agriculture and Natural Resources, Delaware State University; and ARRON GUENZI, Ph.D., former Associate Professor, Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, OK.

USGA Turfgrass and Environmental Research Online 11(1):1-8.
TGIF Record Number: 195711

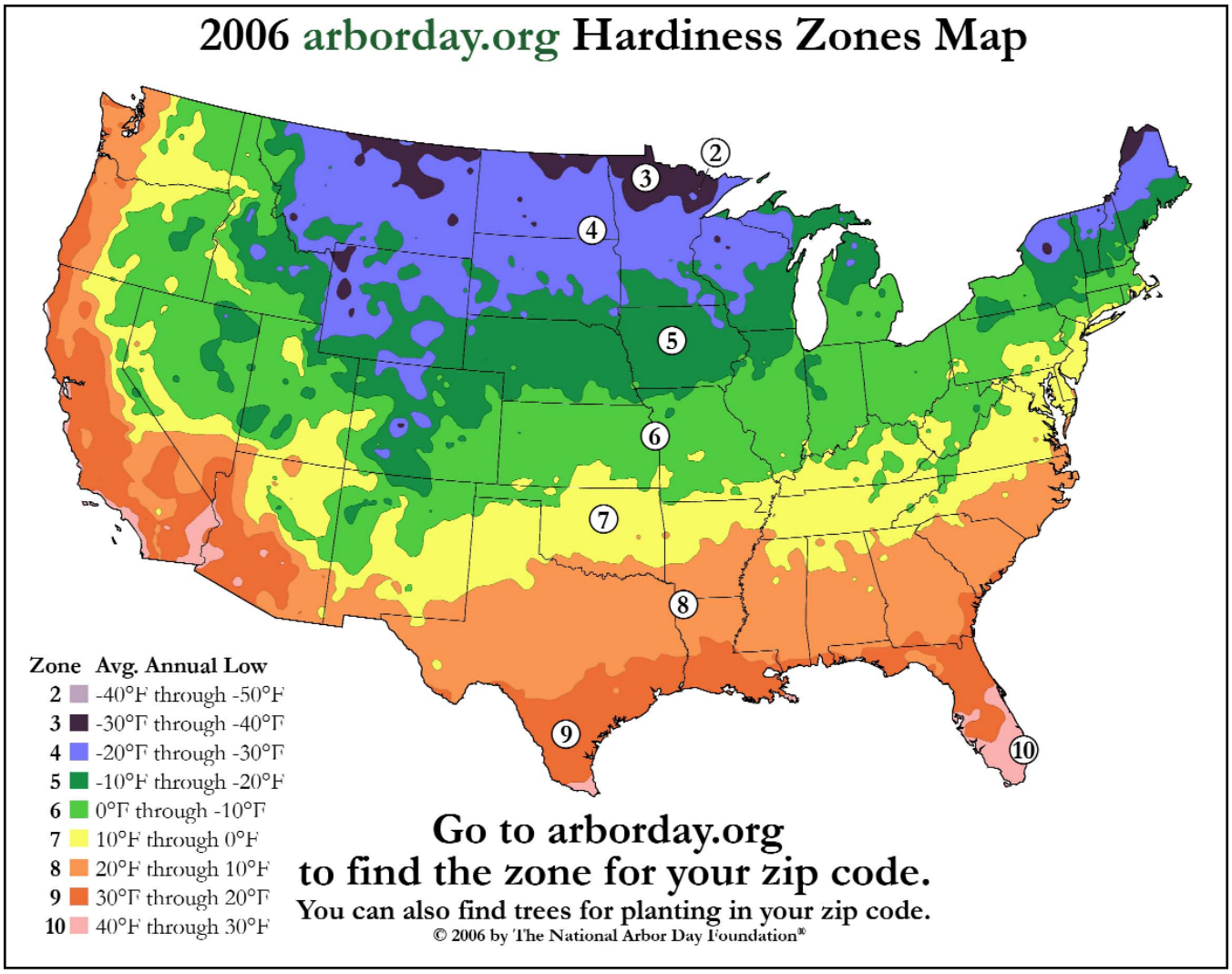

Figure 1. Arborday.org hardiness zone map adjusted to 2006 conditions. You can find this map at http://www.arborday.org/media/zones.cfm. Bermudagrass is currently restricted to regions south of the revised Arborday hardiness zone seven.

Oklahoma State University is home to one of the largest collections of bermudagrass germplasms throughout the world, assembled by Dr. Charles Taliaferro and currently administered and enlarged by Dr. Yanqi Wu. Evaluations of germplasm by Dr. Jeff Anderson in the Department of Horticulture have revealed significant differences among bermudagrass lines with respect to cold tolerance (1, 2). In order to study cold tolerance in bermudagrass, it was necessary to select contrasting cold tolerant and susceptible types: 'MSU' and' Zebra', respectively. 'MSU' was collected from the campus of Michigan State University in the late 90s, and 'Zebra' was discovered as a chance mutant that exhibited a horizontal striped pattern on the leaves (Figure 2).

This research seeks to better understand the way bermudagrass adapts to cold conditions at the biochemical level. Up until now little is known on a global scale concerning how bermudagrass adapts to cold conditions. Previous research at OSU revealed that cold temperatures stimulated the production of chitinases--proteins active in inhibiting pathogens and reducing growth of damaging ice crystals within cellular tissues (3).

Extensive work in the lab of X. Zhang at Virginia Tech University showed increased abscissic acid (ABA) and decreased cytokinin: hormones that control growth and development and stress response, in cold acclimated bermudagrass tissues (7). Further research in the same lab showed an increase in dehydrins during acclima-

tion, a protein associated with dehydration stress. Furthermore, proline and general protein levels were also shown to increase in the same study (8). Earlier research showed increases in soluble sugars in acclimating bermudagrass crown tissues (6).

While significant advances have been made, progress so far has been limited to research on one gene or physiological activity at a time, among the thousands of genes or processes that are likely to change as a result of cold treatment. What is needed to gain greater understanding of this process is an examination of many genes on a global scale. This can only be done by using advanced genomic techniques, such as microarray analysis, and high powered computer statistical analysis.

To examine gene expression on a global scale, it was necessary to use a technique that can look at gene expression of many genes at once. Here we chose to use microarray analysis to identify genes that differ in response to cold temperatures, termed differentially expressed genes. The work was conducted primarily by Kalapalatha Melmaiee, a former Ph.D student with some assistance from her husband Dr. Sathya Elavarthi, both former students at Oklahoma State University, advised by Dr. Michael Anderson and Dr. Arron Guenzi. Microarray analysis is capable of detecting changes in gene expression for thousands of genes simultaneously to a high degree of accuracy.

The technique consists of the robotic spotting of thousands of small amounts of cDNA from individual genes on a small glass microscope slide (Figure 3) and using a mixture of cDNA from treated and control plants that had been labeled with a chemical probe that gives off light at different wavelengths when stimulated. Probes were developed from treated and control tissues in order to probe the thousands of genes for their response to either cold or normal room temperature conditions.

We exposed ‘Zebra’ and ‘MSU’ plants in two growth chambers under room temperature or cold non-freezing (4º C) conditions. After the

Figure 2. ‘MSU’ and ‘Zebra’ bermudagrass lines with close-up of ‘Zebra’ striped leaf inserted in lower right hand corner.

treatments, plants were harvested for their crown tissues, and these tissues were extracted for their messenger ribonucleic acids (mRNA), which contains chemical encoded information that is used to make enzymes and enzymes are the agents that actually do the cellular work of adjusting to cold conditions. The mRNA was converted to cDNA, to make it easier to work with, and the cDNA from each treatment was subjected to a technique called subtractive hybridization that selects for only those cDNAs that are increased or decreased relative to control tissues, termed differentially expressed.

The differentially expressed cDNAs were placed in a circular plasmid and inserted singly into bacteria. The collection of bacteria were grown to amplify the cDNA separately in mass, and the cDNA in the circular plasmid were collected and spotted on a glass plate using a highly sophisticated robotic spotting device. A total of 4,589 DNA fragments each corresponding to a specific gene were spotted three times for at total of 13,767 spots per microscope slide.

Included in these thousands of genes were 744 genes from a study that examined changes in gene expression in bermudagrass exposed to the disease spring dead spot. These spring dead spot responsive genes were provided by Dr. Zhang of the Samuel Roberts Nobel Foundation (5). Spring dead spot resistance has in the past shown strong association with resistance to cold temperature stress, so it will be interesting to see how many of these genes show responses to cold temperatures. Thus, the cDNA that was spotted came from those genes that showed enhanced or suppressed expression. Each gene cDNA sequence was compared with previously researched cDNA sequences information stored in the National Center for Biotechnology Information computers in order to determine their identities in association with known genes.

The probes were constructed from cDNA isolated from either treated or control tissues of 'Zebra' and 'MSU' at 2 and 28 days of cold treatment. The slides containing nearly 4,500 genes were exposed to a mixture of treated and control probes, each probe using a different fluorescent

Kalapalatha Melmaiee sitting next to Omnigrid robotic microarray spotter and computer for microarray analysis.

marker molecule resulting in a specific hybridization to each of the corresponding genes on the slide. Those with differentially expressed genes will hybridize with more probes and give off more light at the specific wavelength for each probe. The slides were scanned using a powerful fluorescent microscope-like scanner for light emissions to determine the level of expression at the two distinct wavelengths of the treated and control fluorescent markers. The data was analyzed using powerful computer software programs producing a false colorized image and identifying those genes that were differentially expressed.

Of the 4,589 genes spotted on the slide, 586 were shown to respond to cold temperatures in bermudagrass crown tissues of 'Zebra' or 'MSU' at 2 or 28 days. This meant that under cold treatment conditions, only about 13% of the genes examined responded in some way to cold treatment. Of the 586 differentially expressed genes, only 97 showed any similarity to known genes in the National Center for Biotechnology Information (NCBI) data base. This was signifi-

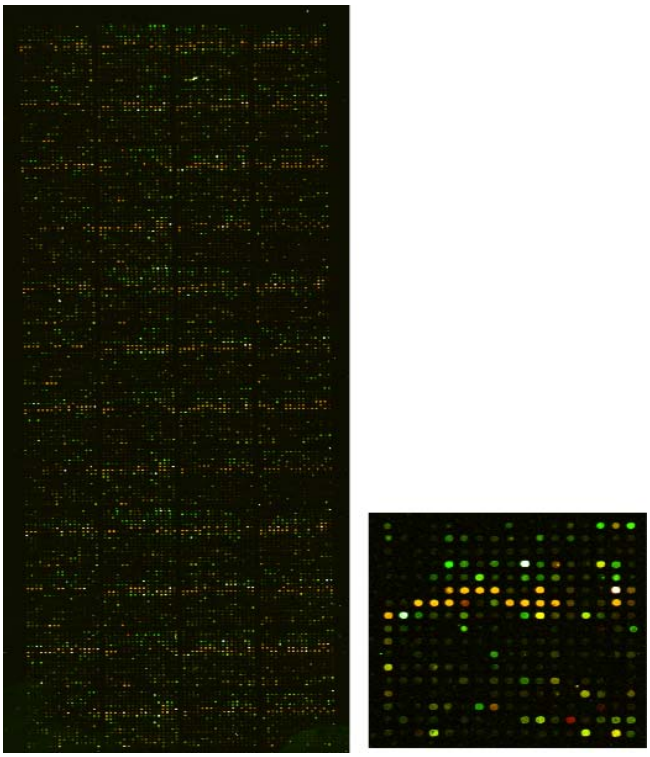

Figure 3. Total image (left) and close up (right) of microarray slide under stimulation showing fluorescent stimulation of light for red color indicating enhanced genes and green color indicating surpressed genes. This particular slide was treated with probes from MSU tissue acclimated at 2 days.

cant that only about 17% of the genes were identifiable while 83% were genes that have no known function or gene association. Most studies with plants show a much higher number of identifiable genes. This means that there is something about the gene make up of bermudagrass crown tissue that is different from other plant species studied so far.

Of the 20 genes that showed the most differential expression, only one of them (senescence associated protein gene, SAP gene) showed similarity with known genes (Figure 4). But this one showed the highest level of expression of all genes showing up to a 123-fold increase in gene activity with cold treatments. Many of these highly enhanced genes were more enhanced in resistant 'MSU' than susceptible 'Zebra' meaning that whatever gives 'MSU' its level of resistance also results in greater levels of gene expression for the most differentially expressed genes (Table 1).

Looking at timing, it appeared also that most genes were more highly expressed at the 28-day period than at the earlier 2-day treatment period. This probably indicates that as bermudagrass crowns acclimate, there is a continued enhancement or deepening in the cold tolerance mechanism that requires more changes in gene expression from acclimating genes.

Microarray analysis also provides an opportunity to examine specific genes of interest. In this study several genes stood out including sucrose synthase, a gene that is intimately connected with sugar metabolism. Previous reports showed clearly that sugars increases in bermudagrass crowns (6). Sugars are known to protect against cold treatment by stabilizing cellular membranes, reducing the freezing temperature of ice formation, and reducing stress due to highly reactive oxygen.

The SAP gene in this study showed the strongest response of all as mentioned above. Studies in *Arabidopsis* have linked this gene to dark-induced senescence and abscissic acid (ABA) treatment. Senescence is a process whereby tissues age in preparation for cellular death, and ABA is a hormone that is often associated with dormancy reactions in seeds and buds. While bermudagrass, a warm-season perennial species, is not known to undergo a classical dormancy reaction, there appears to be some elements of the cold acclimation response at the molecular level that resembles dormancy- perhaps a kind of pseudo dormancy.

Future research may focus on distinguishing between conventional dormancy reactions as occurs in buds or seeds and those that occur in warm-season grasses like bermudagrass. Another gene, the gene for the Acyl CoA binding protein, was highly enhanced in bermudagrass crown tissues. This gene codes for proteins that help transport CoA, a compound associated with energy and lipid production, to the chloroplasts and other membranes within the cell. These, in turn, have a major affect on fatty acid composition and especially the phospholipid fraction (4). Fatty acid composition is a major factor in cold acclimation in many living organisms.

One gene response that was very surprising was that of the dehydrins which showed a dra-

	Number of Genes Expressed			
	MSU	**Zebra**	**2 days**	**28 days**
Enhanced Genes	157	88	98	147
Depressed Genes	273	68	127	214
	Difference in Expression			
	MSU	**Zebra**	**2 days**	**28 days**
Enhanced Genes	3.58	1.32	1.63	1.68
Depressed Genes	2.77	1.76	1.81	2.13

Table 1. Overall changes in expression in 'MSU' and 'Zebra' at 2 and 28 days of cold acclimation. Top of table indicates the number of genes while the bottom of the table indicates fold differences in level of expression.

matic suppression in bermudagrass crown tissues. Dehydrins are known to be enhanced under stress conditions, even in bermudagrass crown tissues (8). Here the opposite occurred, showing almost a 9-fold decrease in response to cold temperatures. This indicates that not all dehydrins are stress-induced and, in fact, some may be suppressed suggesting that this gene response is not so simple or straight forward as we may think. Another gene with the greatest suppression was similar to a family of bacterial proteins called universal stress proteins that have been identified in bacteria and some plants which some think provides a measure of stress endurance. Why this protein was suppressed is not known at this time.

On the whole, there were more genes that were suppressed than enhanced, and this was especially evident in 'MSU' compared to ' Zebra'. This enhanced suppression may be important to turn off genes that are no longer needed under pseudo-dormant conditions. There were many more differentially expressed genes in 'MSU' than 'Zebra'. The reason for this is unclear, but suggests that resistant biotypes are more metabolically fluid compared to the susceptible biotypes. This ability to change expression of many genes may be one aspect of resistance mechanisms that is over-looked. Overall level of gene expression was greatest in 'MSU compared to 'Zebra' suggesting that gene expression is more powerfully expressed in resistant biotypes.

The study showed some interesting surprises as is common in many studies of an exploratory nature, especially those conducted in unknown or little researched territory. It is clear that bermudagrass contains many genes that are not very similar to those previously studied in plants or other organisms. This makes it especially difficult to construct an overall scheme that tells us how bermudagrass acclimates to cold temperatures. Previous studies have focused on one aspect at a time, but in contrast, this study looked at global-scale changes in gene expression. How this information might be used to provide better bermudagrass varieties that are more adapted to cold temperatures is an important question. This study is clearly only a beginning, but with time the identities of many of these unknown genes will be uncovered through the efforts of plant scientists throughout the world working on plants of many species and submitting their results to global databases.

As information accumulates and connections are made, patterns will emerge that will suggest new ways to enhance cold resistance. Some of these genes may become targets for biotechnology manipulations to engineer cold resistance. Some may serve as useful markers for breeding programs. Future possibilities are endless. However, before any real and sustained progress is possible, more fundamental knowledge concerning bermudagrass crown acclimation is necessary.

We need to look at some of these specific genes across a wide variety of bermudagrass genotypes differing in cold tolerance. More phys-

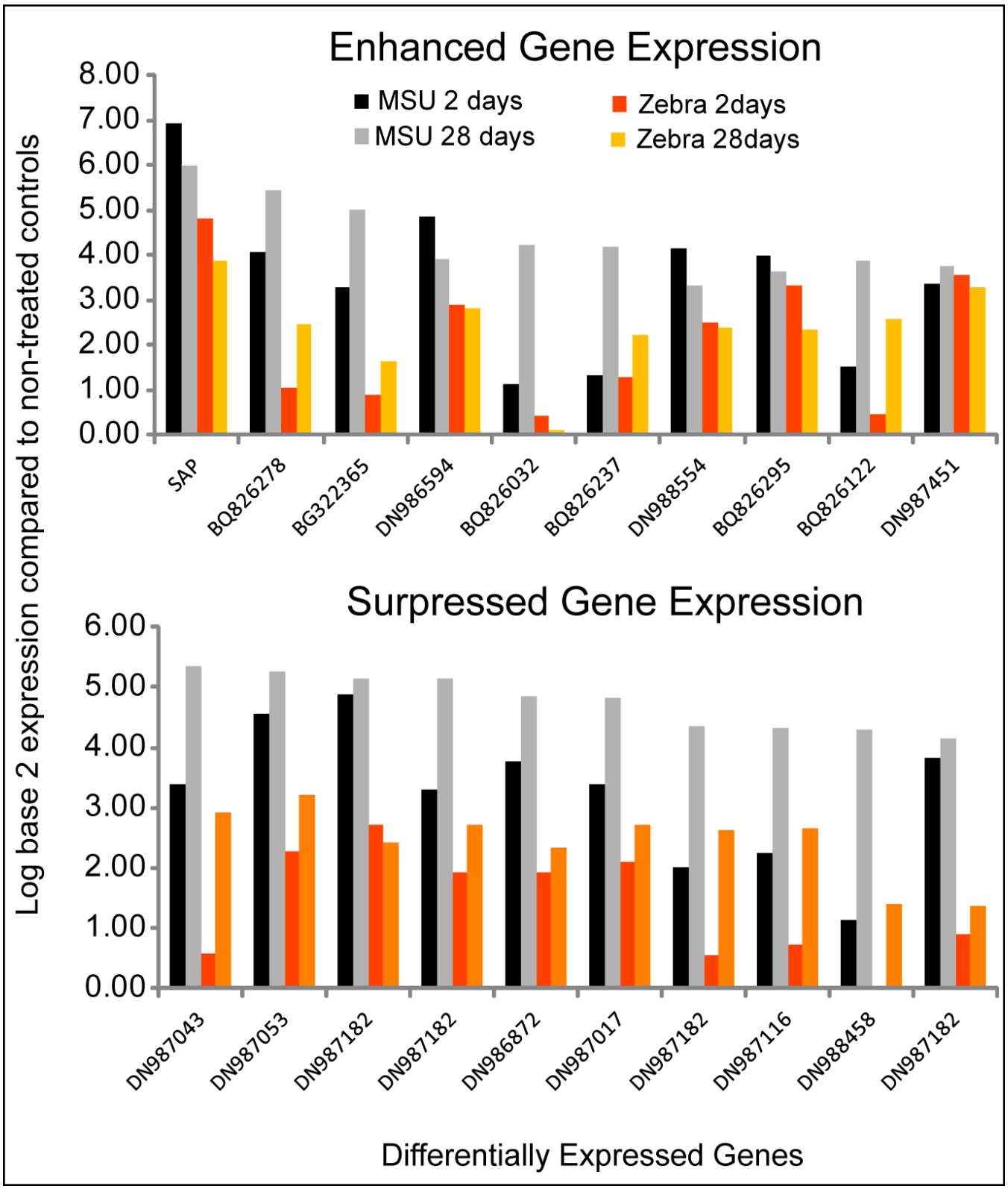

Figure 4. Charts showing the level of the most enhanced (top) and surpressed (bottom) genes in 'MSU' and 'Zebra' at 2 and 28 days cold acclimation. The values are expressed exponentially as a log base 2 number indicating the fold expression difference between treated and control crown tissues.

iological studies such as those conducted in Zhang lab need further emphasis. Unfortunately, most physiological or molecular studies on grass species are conducted in only two grass families containing familiar grasses such as wheat, corn, sorghum, or rice. Bermudagrass is a member of a grass family that is distinct and little studied. Furthermore, very little research has been conducted at this level using below-ground regenerative tissues, especially in warm-season perennial species. In fact, little is known about bud dormancy or pseudo-dormancy from non-temperate species.

These are likely the primary reason why we know so little and why few of our bermudagrass genes match those in the current databases. We now know the identities of 97 differentially expressed genes, and we have the sequences of 489 unknown genes that may prove useful in a number of studies to breed for enhanced cold tolerance. This study represents a dramatic step forward in better understanding cold acclimation in bermudagrass on a global scale and will serve as a basis to direct future studies to increase our current understanding of bermudagrass physiology and biochemistry as it relates to cold acclimation.

Acknowledgements

The authors would like to thank Dr. Patricia Canaan for her help in analyzing the microarray data, Dr. Jeff Anderson for his contribution in evaluating bermudagrass lines, Dr. Charles Taliaferro for providing the genotypes and for his encouragement and support and the Lab of Dr. Andrew Patterson for sequencing the bermudagrass cDNAs. The authors also express appreciation to USGA's Turfgrass and Environmental Research Program for its financial suupport of this work.

Literature Cited

1. Anderson, J. A., and C. M. Taliaferro. 2002. Freeze tolerance of seed-producing turf bermudagrasses. *Crop Science* 42:190-192. (TGIF Record 78169)

2. Anderson, J. A., M. P. Kenna, and C. M. Taliaferro. 1988. Cold hardiness of 'Midiron' and 'Tifgreen' bermudgrass. *HortScience* 23:748-750. (TGIF Record 13163)

3. de los Reyes, B. G., C. M. Taliaferro, M. P. Anderson, U. Melcher, and S. McMaugh. 2001. Induced expression of the class II chitinase gene during cold acclimation and dehydration of bermudagrass (*Cynodon* sp.). *Theoretical and Applied Genetics* 103:297-306. (TGIF Record 99995)

4. Kojima, M., J. Casteel, J. A. Miernyk, and J. J. Thelen. 2007. The effects of down-regulating expression of *Arabidopsis thaliana* membrane-associated acyl-CoA binding protein 2 on acyl-lipid composition. *Plant Science* 172:36-44.

5. Zhang, Y., A. C. Guenzi, M. P. Anderson, C. M. Taliaferro, and R.A. Gonzales. 2006. Enrichment of bermudagrass genes associated with tolerance to the spring dead spot fungus *Ophiosphaerella herpotricha*. *Physiological and Molecular Plant Pathology* 68:105-118. (TGIF Record 195652)

6. Zhang, X. Z., and E. H. Ervin. 2008. Metabolic defense responses of bermudagrass during acclimation to freezing stress. *Acta Horticulturae* 783:181-194. (TGIF Record 136260)

7. Zhang, X., E.H. Ervin, C. Waltz, and T. Murphy. 2011. Metabolic changes during cold acclimation and deacclimation in five bermudagrass varieties: II. cytokinin and abscisic acid metabolism. *Crop Science* 51:847-853. (TGIF Record 177722)

8. Zhang, X., K. Wang, E. H. Ervin, C. Waltz, and T. Murphy. 2011. Metabolic changes during five bermudagrass varieties. I. proline, total amino ccid, protein, and dehydrin expression. *Crop Science* 51:838-846. (TGIF Record 177721)