FUNCTIONAL GENOMICS OF COLD TOLERANCE IN BERMUDAGRASS *CYNODON DACTYLON L.*

By

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CHAPTER I

REVIEW OF LITERATURE

Bermudagrass:

Bermudagrass *Cynodon dactylon (*L.) Pers. is extensively grown in many parts of the world for different purposes. It is used as livestock herbage, ground cover to reduce soil erosion, base for biofuel production, and as turf on golf courses, sports fields and lawns (Taliaferro, 2003). Bermudagrass is the most widely used warm-season species for intensively managed turf sites in the Southern United States, and in the northern transition zone of its adaptation (Richardson, 2002). Low freezing temperatures are one of the major environmental factors that limit the geographical distribution of bermudagrass in the United States and throughout the world. Bermudagrass, like most of the warm season plants of tropical and sub-tropical origin, is very much susceptible to low freezing temperatures.

Cold temperatures in the transition zone pose problems for turf managers related to bermudagrass stress tolerance and survival. Although cool season grasses such as tall fescue (*Festuca arundinacea* Schreb.), creeping bentgrass (*Agrostis palustris* Huds), and Kentucky bluegrass (*Poa pratensis* L.) are planted in this transition zone, warm season grasses, such as: bermudagrass and zoysiagrass (*Zoysia japonica* Steud.) predominate. Zoysiagrass is more cold tolerant than bermudagrass, but because of bermudagrass's aggressive nature of growth and fast recovery from injury, it is preferred by the turf industry (Carrow, 1994).

Low temperature injury in plants:

Low temperature injury is caused by ice crystallization with resultant dehydration. Water is distributed in both the symplast and apoplast: symplastic water is found within the plasma membrane while apoplastic water is found outside the plasma membrane including, the xylem-lumen apoplast, the cell wall apoplast, and the intercellular-space apoplast (Atici and Nalbantoglu, 2003). As temperatures drop below 0°C, ice formation is generally initiated in the extracellular spaces as these fluids have a higher freezing point (lower solute concentration) than the intracellular fluid. The chemical potential of ice is less than that of liquid water at a given temperature (Thomashow, M. F. 1999). As a result, unfrozen water above the chemical potential gradient moves from inside the cell to the intercellular spaces causing cellular dehydration. Freeze induced cellular dehydration can cause different forms of membrane damages such as expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions and fracture jump lesions (Thomashow, 1999; Uemura and Steponkus, 1997; Steponkus et al. 1993). Membrane damage leads to ion leakage and cell death. Cold temperatures can also cause increased damage due to cellular ion leakage and alterations in physiological and respiratory processes (Provart, 2003; Huner, 1988, and Lyons, 1973).

Acclimation to cold temperatures:

Previous research has shown that perennial plants acquire cold tolerance by a process called cold acclimation. During cold acclimation, plants accumulate antifreeze proteins and cryoprotectants, and undergo changes in membrane lipid composition, photosynthetic carbon metabolism, and detoxification of reactive oxygen species

(Provart, 2003; Silt and Hurry, 2002). According to Thomashow (1999), non-acclimated rye was killed by freezing temperature at about -5°C, but after a period of exposure to low non-freezing temperature can survive freezing to temperatures lower than -30°C. Under cool conditions, bermudagrass growth slows down and anthocyanin production increases resulting in a purpling of foliage. If temperatures fall frequently below 0°C, the above ground foliage will be killed and regenerative parts (crown buds, rhizomes, and stolons) will go dormant (Taliaferro, 2003). The bermudagrass plant is able to sense the environmental changes in the fall that signal the coming winter and responds by expressing genes that induce acclimation to cold temperatures, enhancing freeze tolerance. The process of acclimation and dormancy reduces the damaging effects of low temperature stress.

Understanding of the nature of genes, and mechanisms responsible for freezing tolerance and cold acclimation and the genes that control them will provide for new strategies to improve the freezing tolerance in agronomically important plants. Such strategies may have potential value in traditional plant breeding programs to develop cultivars with improved freeze tolerance (Thomashow, 1999; Sarhan and Danyluk, 1998).

Genes associated with low temperatures:

Prominent genes associated with low temperature acclimation are involved in antifreezing, ion channels, osmoprotection, desaturation of fatty acids, chaperones, antioxidant and radical scavenging enzymes and molecules, pathogen defense related functions, transcription factors and protein kinases.

Cold responsive signal transduction components:

Signal transduction is initiated upon perception of signals by the receptors. Abiotic stress signals are very complex in nature. For example, low temperature stress may induce both osmotic and dehydration stress. Similarly, drought stress may also induce dehydration, osmotic and ionic stress. Hence, plants are expected to induce several signal transduction pathways to initiate a particular abiotic stress response. Abscisic acid (ABA) mediated, Ca^{+} mediated, mitogen activated protein (MAP) kinases and other kinases mediated signal transductions are well documented in the literature as stress responsive elements (Thomashow, 1999).

Cold mediated transcription regulation:

The major pathway studied during cold acclimation in Arabidopsis is CBF/ DREB1 regulatory pathway which influences the regulation of COR genes. Myb4 over expressing plants showed a significant increase in cold and freezing tolerance, as measured as membrane or Photosystem II (PSII) stability and as whole plant tolerance. Transgenic rice containing the Osmyb4 gene showed changes in expression of genes participating in different cold-induced pathways, suggesting that Myb4 represents a master switch in cold tolerance (Patzlaff et al., 2003; Vannini et al., 2004; Mattana et al., 2005). In several plant species, exogenous application of ABA increased freezing tolerance, suggesting a role in cold and freeze tolerance (Thomashow, 1999). In Arabidopsis, mutants in ABA genes *aba* and *abi* (which lack the ABA- mediated induction of COR gene expression) did not affect the COR gene expression at low temperatures suggesting the existence of ABA independent COR gene expression (Gilmour and Thomashow, 1991). A cold acclimated microarray study in Arabidopsis

using 8000 genes revealed that 30% of the cold responsive genes were CBF independent genes and fifteen of them encode transcription factors suggesting that several independent pathways were involved in the cold acclimation process (Fowler and Thomashow, 2002).

Cold responsive genes and their roles in cold acclimation:

Numerous genes induced during cold acclimation in plants probably contribute to freezing tolerance. The Arabidopsis *FAD8* gene (Gibson et al., 1994) and barley *blt4* gene (Hughes and Dunn, 1996), which encode a fatty acid desaturase and a putative lipid transfer protein, respectively, are induced in response to low temperatures. These genes might contribute to freezing tolerance by altering lipid membrane composition. Cold-responsive genes encoding molecular chaperones including a spinach *hsp70* gene and a *B. napus hsp90* gene (Krishna et al., 1995) might contribute to freezing tolerance by stabilizing proteins against freeze-induced denaturation.

Various genes encoding signal transduction and regulatory proteins, including mitogen-activated protein kinases, calcium-dependent protein kinases, and 14-3-3 proteins, have been shown to be up-regulated in response to low temperatures (Hughes and Dunn, 1996). These might contribute to freezing tolerance by controlling the expression of cold-responsive genes or by regulating the activity of proteins involved in freezing tolerance. Whether the proteins encoded by these cold-responsive genes contribute significantly to freezing tolerance remains to be determined. However, one group of proteins that accumulates during low temperature and almost certainly contributes to freezing tolerance are the recently described plant AFPs which function by inhibiting ice crystal growth and re-crystallization. Several Late Embryogenesis

Associated (LEA) genes involved in osmotic regulation like Dehydrins (Dhns) are over-expressed during low temperatures (Zee et al., 1995). Genes involved in carbohydrate metabolism (Xin and Browse, 2000) like sucrose synthase (Stitt and Hurry, 2002) were up-regulated during cold acclimation in plants and sugars are thought to have multiple roles such as cryoprotection, osmoregulation and gene regulation (Xin and Browse, 2000; Stitt and Hurry, 2002). Up-regulation of antioxidants (ascorbate peroxidase, glutathione reductase, superoxide dismutase) which scavenges the harmful reactive species were reported in the cold stressed plant cells (Tao et al., 1998). Cold acclimated bermudagrass showed increased accumulation of chitinases and unsaturated fatty acids in crown tissues (Gatschet et al., 1996; Samala et al., 1998). Reports suggest that chitinases exhibited antifreeze properties by retarding extracellular ice formation during the freezing of plant tissues (Hon et al., 1995).

Gene expression profiling tools:

Suppression subtractive hybridization:

A variety of research tools are available to investigate global gene expression levels between two mRNA populations, and identify differentially expressed transcripts (Munir et al., 2004). Examples of these tools are representational difference analysis (RDA) (Lisitsyn et al., 1993), differential display (*Rt* DD) (Liang et al., 1992) differential analysis of library expression (DAZE) (Li et al., 2004), serial analysis of gene expression (SAGE) (Velcusescu et al., 1995), techniques involving physical separation of common sequences (Akopian et al., 1995; Deleersnijder et al, 1996) and suppression subtractive hybridization (SSH) (Diatchenko et al., 1996). Despite the fact that all these techniques can result in the identification of differentially expressed genes, suppression subtractive

hybridization has been the most commonly used technique in recent years to isolate differentially expressed genes in both animals and plants.

As the name indicates, SSH utilizes two different techniques in the isolation of uniquely expressed genes. One is the subtraction step, which equalizes or normalizes the sequence concentrations (Diatchenko et al., 1996). In this step, ends of Rsa1 digested cDNA population were tagged with special adapters for each sample followed by hybridization of samples to be compared. During this hybridization, commonly present cDNA molecules within and between samples will hybridize together forming double strands. Uniquely expressed molecules will remain as single strands. A PCR based second step is applied, which exponentially amplifies the single strands and suppresses the amplification of double strands by forming hairpin-like loop structures at the ends (Diatchenko et al. 1996). SSH was used to study differentially expressed genes in Arabidopsis with response to ozone, bacterial and oomycete pathogens and the signaling compounds such as salicylic acid (SA) and Jasmonic acid (Mahalingam et al., 2003).

Microarrays:

Prior to the development of array systems, gene expression studies were performed for one gene at a time. Such techniques include northern blotting, dot blotting and quantitative RT-PCR. Other techniques such as differential display (Diatchenko et al., 2000), serial analysis of gene expression (SAGE) and total gene expression analysis (TOGA) offer multiplex technologies. These techniques have several advantages and disadvantages (Alba et al., 2004). DNA microarray technology allows researchers to investigate thousands of genes simultaneously (Park et al., 2004; Brown and Botstein, 1999) and for those organisms that have full genome sequence information available,

cDNA microarrays permits the study of whole transcriptome expression pattern in a single experiment.

Microarrays are useful in answering a number of biological questions. Microarray experiments can be carried out to find specific marker genes (Richmond and Somerville, 2000). Here, very highly induced or repressed genes can be identified for a particular situation or environment. Microarrays are used for exploratory purposes to provide clues to the function of a particular set of genes under study (Reymond, 2001). Altered gene expression of unknown genes can be studied to obtain information about response to a particular environmental stimulus (Richmond and Somerville, 2000). Gene regulatory networks can also be inferred for a particular organism in a given environment (Alba et al., 2004; Hashimoto et al., 2004).

Studies utilizing microarrays have been used in plant research to better understand processes ranging from seed filling to cold tolerance (Alba et al., 2004; Fowler and Thomashow, 2002). In recent years, cDNA microarrays were used to study global gene expression in response to abiotic stresses, such as: drought, salt and low temperatures (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002; Kathiresan et al., 2006; Manavella et al., 2006; Ueda et al., 2006). Several studies document that cold acclimation in plants involves rapid accumulation of CBF transcription factor, followed by activation of targeted COR genes, which enhances freeze tolerance. Transcription profiling experiments using microarrays in Arabidopsis against cold acclimation revealed multiple low temperature regulatory pathways in addition to the CBF cold response pathway (Fowler and Thomashow, 2002).

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CHAPTER I I

INTRODUCTION

Abstract:

Bermudagrass (*Cynodon dactylon* L pers.) is one of the most widely adapted warm-season grasses with its usage and geographic distribution limited by cold temperatures. The goal of this research was to identify genes that are differentially expressed during cold acclimation in two bermudagrass genotypes that differ in tolerance to low temperature stress. Cold tolerant genotype (MSU) and cold sensitive experimental line (Zebra) were used. Plants were cold acclimated at 8°C/2°C day/night temperatures. Crown tissue was sampled at 2 and 28 days after cold acclimation from both acclimated and non-acclimated plants. Total RNA was extracted from crown tissues and suppression subtraction hybridization (SSH) was performed to create eight normalized cDNA libraries enriched for expressed sequence tags (ESTs) representing up or down-regulated genes. A total of 3,853 clones were isolated from the eight cDNA libraries and sequenced. Sequenced ESTs were assigned putative functions and deposited in the GenBank database. ESTs were printed on the microarray slide and hybridization analysis was done for identification of differential gene expression profiles. A total of 566 differentially expressed genes were identified. Among them, genes involved in kinase signal cascade (MAPKKK, MK5 , SERK2 protein precursor) and in metabolism (sucrose synthase2, ACBP, aspartate aminotransferase) were identified as being associated with cold tolerance because as they were up-regulated only in the cold tolerant genotype MSU during cold acclimation. Further characterization of these genes will help us better understand cold tolerance in bermudagrass.

Introduction:

Bermudagrass, *Cynodon dactylon* L Pers., is a warm-season perennial that grows well in tropical, subtropical and transition regions of the world ranging from 45° North to 45° South latitudes (Harlan et al., 1970). Bermudagrass is extensively grown in lawns, golf courses, sports fields, and pastures and as ground cover for controlling soil erosion. In the United States, bermudagrass is grown mainly in the southern region of the country (Sticklen and Kenna, 1998) due to its susceptibility to freeze damage. Bermudagrass is grown in Oklahoma more extensively than any other warm-season grass because of its high level of adaptability and wearability. In Oklahoma alone it is grown on 4 million acres of pasture land as forage (http://forage.okstate.edu/). Bermudagrass is used extensively by the golfing industry (preferably in fairways and tee areas). Despite its good characters, bermudagrass usage is limited by low temperature stress (Gatschet et al., 1996).

The turfgrass breeding program at Oklahoma State University has evaluated an extensive bermudagrass germplasm collection in both field and growth chambers for tolerance to low temperatures. In this research, we characterize differences between cold tolerant and susceptible genotypes at the molecular level by determining gene expression patterns during cold acclimation. The long-range goal of this research is to understand cold tolerance and acclimation in this economically important species, and to facilitate the development of improved genotypes.

Both low non-freezing and freezing temperatures can cause direct injury to the plant cellular processes by lowering enzymatic activity and fatty acid fluidity in membranes. In addition to the direct damage, freezing temperatures can induce formation

of ice - crystals and their expansion in the extracellular spaces, which causes outward movement of water from the cell into the extracellular spaces resulting in dehydration inside the cell and shrinkage of plasma membrane away from the cell wall.

Plants can better withstand freezing if they have prior exposure to low non-freezing temperatures for a period of time: a phenomenon called cold acclimation (Thomashow, 1999; Fowler and Thomashow, 2002; Svensson et al., 2006). Freeze tolerant plants exhibit different mechanisms such as promotion of ice formation in the extracellular spaces thereby reducing the speed of ice crystal formation in the cytoplasm, or accumulation of antifreeze proteins in the apoplast which also retards the growth of ice crystals. Adaptation to low temperatures results in the expression of pathogenesis related proteins (PR proteins) and other physiological changes, such as changes in fatty acid composition of the plasma membrane. One of the major physiological changes in plants during low temperature exposure is reduced enzymatic activity (Stitt and Hurry, 2002). The most diminished physiological process is photosynthesis (Svensson et al., 2006). An initial response to low temperature is reduction in rate of carbon fixation, which in turn lowers the accumulation of sucrose in the cytosol. Optimal rates of photosynthesis require a balance between carbon fixation and sucrose synthesis (Stitt and Hurry, 2002). When this balance is disrupted, cellular damage through free radical reactions can take place. Studies in Arabidopsis showed that inhibition of sucrose synthesis and photosynthesis is reversed as cold acclimation progresses (Hurry et al., 1997). Sugars that accumulate during cold acclimation are thought to have multiple roles such as: modulating gene expression (COR genes), fatty acid and proline accumulation, cryoprotection and osmoregulation (Stitt and Hurry, 2002). Increased levels of sucrose

during cold acclimation may be due to the lower demand for photosynthate (Pollock, 1986) and plant metabolism as plants undergo the acclimation process (Levitt, 1980).

Cold acclimation involves the coordination of up-or-down-regulation of hundreds of cold regulated genes (Shinozaki and Yamaguchi-Shinozaki, 2000; Svensson et al., 2006). Cold acclimation in bermudagrass reduces damage to membranes as a result of reduction in the electrolyte leakage (Anderson et al., 1988), and the lethal low temperature killing point (Gatschet et al., 1994). Accumulation of COR proteins such as chitinases (Gatschet et al., 1996) and increased accumulation of unsaturated fatty acids (Samala et al., 1998) are also observed during cold acclimation. All these responses result from changes in gene expression.

In bermudagrass the main responses to low temperature are thought to be dormancy and senescence. Leaves lose their photosynthetic pigments and begin accumulating anthocyanins that results browning of the leaves and stems as the tissue progresses towards senescence. In the advanced stages of accumulation, regenerative tissue (Crown buds, rhizomes, and stolons) go dormant surviving the cold period to establish new growth in the coming season (Taliaferro, 2003).

Until now, cold acclimation research in bermudagrass has focused mainly on the study one or a few genes at a time to address the cold tolerance mechanism (Anderson et al., 1998; Samala et al., 1998; de los Reyes et al., 2001; Zhang et al., 2006;) This research is the first to examine global gene expression during cold acclimation in two divergent bermudagrass genotypes by utilizing microarray technology. MSU, a cold tolerant genotype with an LT_{50}value of -10.5°C and Zebra, a cold susceptible line with an

LT_{50}value of >-6.0°C (Anderson and Taliaferro, 2002) were used in this study. Our study was carried out with the following objectives.

Research Objectives:

1. Develop and sequence clones derived from subtracted cDNA libraries associated with cold acclimation in tolerant (MSU) and sensitive (Zebra) bermudagrass genotypes.
2. Identification of genes associated with cold tolerance using cDNA microarrays.
3. Identification of genes associated with cold acclimation using cDNA microarrays.

CHAPTER III

METHODOLOGY

Bermudagrass genotypes:

Bermudagrass germplasm collections at Oklahoma State University have been extensively evaluated for freezing tolerance (Anderson and Taliaferro, 2002). Among the lines evaluated, MSU (A-12195) and Zebra (Appendix fig 1) were selected for creating SSH libraries based on the wide difference between their LT_{50} (the temperature at which only 50% of the plants survive) values. MSU exhibited an LT_{50} value of −10.5°C and Zebra had an LT_{50}value of approximately −6.0°C (Anderson and Taliaferro, 2002). Both MSU and Zebra genotypes are tetraploid (2n=36), maintained by vegetative means, though they are seed bearing types, and are representative of the most tolerant and sensitive genotypes available, respectively. MSU was collected from the campus of Michigan State University, East Lansing, MI and Zebra was derived from a F_1 mutant plant with variegated chlorotic stripes on the leaves (Johnson and Taliaferro, 1975). Zebra will not over winter when grown in field plots in Stillwater, Oklahoma.

Cold acclimation and tissue collection:

The procedure for cold acclimation as described by de los Reyes et al. (2001) was followed. Mature phytomers from each genotype were transplanted into sixty 14 cm diameter pots in the greenhouse and maintained for a period of two months to obtain enough plant growth. Pots were then transferred to controlled environment chambers and grown at 28^{o} C day/24^{o} C night and 10 hour photoperiod for 2 months. Thirty pots of each genotype were transferred to a low temperature growth chamber for cold acclimation. During cold acclimation (8^{o} C day/2^{o} C night), a 10 hour photoperiod treatment was imposed. Fifteen pots for each genotype were sampled after 2- and 28-days of cold acclimation. Samples were processed separately; washed thoroughly with

cold water (~ 4^{o}C), root and shoot tissues excised and crown tissues immersed directly in RNAlater (Ambion, Austin, TX) to inhibit ribonucleases. Crown tissues at each time point were soaked overnight at 4^{o}C, blotted dry and divided approximately to 1.5 to 2.0 grams wrapped in an aluminum foil, flash- frozen in liquid nitrogen and stored at –80^{o}C for RNA isolation. The remaining 30 pots from each genotype were maintained at 28^{o}C day/24^{o}C night non-acclimating conditions under a 10 hour photoperiod. Crown tissues from these materials were harvested as described above at room temperature for the same time points (2- and 28- days) and stored at –80^{o}C for RNA isolation.

cDNA library creation:

RNA isolation:

Total RNA was extracted from crown tissues with Fenozol reagent (Activemotif, Carlsbad, CA), which contain monophasic solution of phenol and guanidine. Messenger RNA was isolated from the total RNA using a mTRAP Total (Activemotif) mRNA isolation kit. This protocol yields high quality mRNA, largely free of ribosomal RNA, proteins and genomic DNA. This procedure utilizes negatively charged high affinity hetero-oligomers consisting of thymine (T) pPNA (Phosphono-Peptide Nucleic Acids) and thymine (T) HyPNA (Hydroxy-L-proline PNA) monomers alternatively. As this poly (T) PNA probe has high affinity towards poly (A) of mRNA, it allows for high stringency washes with low salt buffers. Lower salt concentrations eliminate the formation of secondary structures of mRNA, which makes more polyA tails available for probes, thus increasing the mRNA yields. These probes have biotin molecules at their N-terminus, which in turn binds to the magnetite (Fe_3O_4) coated streptavidin beads to facilitate removal of mRNA from the solution after magnetic capture.

Differentially expressed cDNA library:

Subtracted libraries were constructed by following the method developed by (Diatchenko et al., 1996) using Clontech PCR-Select cDNA Subtraction kit (BD Biosciences, Palo Alto, CA). Both forward and reverse subtractions were performed for each genotype at each time point (2- and 28- days) to develop libraries representing both up and down-regulated genes during cold acclimation. In our study, a forward library was created from the mRNA of cold-acclimated samples after subtracting mRNA of non-acclimated samples and a reverse library was constructed from mRNA of non- acclimated samples after subtracting mRNA of cold acclimated samples. Eight libraries were created from both genotypes (Appendix Fig 2).

Subtractive hybridizations were carried out according to the manufacturer's protocol except for a few changes in the initial amount of messenger RNA and number of PCR cycles as discussed below. Three micrograms of mRNA were used for reverse transcription in each sample. The protocol was based on two populations of cDNAs, i.e. the tester and the driver. By using these two populations, two different subtractions were performed in this study. In the forward subtraction, cDNAs derived from cold acclimated tissue were used as tester and cDNAs derived from non-acclimated tissue were used as driver. The driver population was subtracted from the tester population, which resulted in the isolation of up-regulated genes due to cold acclimation. In the reverse subtractions, cDNAs derived from non-acclimated tissue were used as tester and the driver population (cDNA of acclimated sample) was subtracted from tester population. cDNAs derived from reverse subtraction were said to be down-regulated during cold acclimation.

Fifteen units of Rsa1 were used to digest 2.6 µg of cDNAs in order to get short blunt ended fragments for maximum subtraction efficiency. Each tester population was divided into two samples by ligating two different adaptors provided by the kit. In the first hybridization, a high concentration (473 ng/µl) of driver was added to each tester sample and allowed to hybridize separately at 68°C for 8 hours. More abundant transcripts within the sample will be subtracted out by forming double stranded hybrids. In the second hybridization, the two samples having adaptors were hybridized together in the presence of excess driver (which does not have adaptors to its ends) at 68°C overnight producing a variety of hybrids (in the second hybridization, abundant transcripts between samples will be subtracted out). The transcripts that had two different adaptors were exponentially amplified in the first PCR step while the one with one adaptor were amplified linearly (suppression PCR). These amplified transcripts were equalized or normalized and differentially expressed during cold acclimation. PCR1 products were run on gel along with the control checks provided with the kit to determine the subtraction efficiency. A second PCR was performed using nested primers following the protocol but the number of cycles was increased to 32. The second PCR amplification further reduces the background levels and enriches for differentially expressed transcripts which are subsequently ready to be cloned into a vector.

Cloning and transformation:

Once the populations of differentially expressed sequences were obtained by SSH, the PCR2 products were adjusted to 11ng/µl (concentration was adjusted to get optimum amount of cDNA based on size, in order to have maximum transformation efficiency). The concentrations were measured using Picogreen DNA binding fluorescent

dye (Invitrogen, Carlsbad, CA). Forty-four ng of this product was used for insertion into a plasmid vector and the plasmid was transformed into a non-pathogenic bacteria using QIAGEN PCR Cloning^{Plus}kit (Qiagen, Valencia, CA). This kit utilizes linearized plasmid vector (pDrive Cloning Vector) with 'U' overhangs at each end. These overhangs hybridize specifically to 'A' overhangs of the PCR products. This vector also allows ampicillin and kanamycin selection and blue/white colony screening.

Once the PCR products were ligated to the vector, the vectors were then transformed into QIAGEN EZ Competent cells. Transformation was done according to the Qiagen PCR Cloning^{plus} Kit protocol using heat shock with 50 ng of vector. One fifth of the ligated vector mixture was added to a tube (50µl) of competent cells. After transformation, SOC was added to the cells and plated on LB (Luria Bertani) agar/ampicillin / kanamycin media. Plates were incubated overnight at 37°C.

White colonies on X-Gal media were selected and transferred into individual wells of 96-well blocks containing 1.2 mL of 1X LB broth. Blocks were sealed with breathable tape and incubated at 37°C on a shaker @ 300 rpm for 18-20 hours. Wells were examined for bacterial growth. Freezer stocks of bacterial colonies were made by adding an aliquot of 90 µl bacterial cultures to 90 µl of 30% glycerol on 96 well assay plates. The plates were sealed and stored at -20°C for regular use, and at -80°C for long-term archiving.

Confirmation of plasmid insert:

Clones were analyzed for plasmid inserts and insert size by PCR and gel electrophoresis. The PCR reaction mix contained 10 mM Tris; pH8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.3 µM forward primer, 0.3 µM reverse primer, 50 µM dNTPs each, 0.4 units of Taq polymerase and sterile reagent grade water to make up the volume to 19µl. One µl of fresh bacterial culture was added to each well consisting of PCR reaction mixture. PCR was performed as follows: 94°C cell lysis for 30 sec, followed by 95°C denaturation for 30 sec, 68°C annealing and extension for 2 minutes for 25 cycles. During the last cycle, the extension step was extended up to 5 minutes in order to insure the synthesis of full-length copies. PCR products were analyzed by gel electrophoresis using 2% agarose gel in 1X TAE buffer. Each colony was checked for the number of inserts and approximate insert size. Of the clones recovered, 72.5% had a single insert and a size ≥200 bp (Appendix Figure no.3).

DNA sequencing:

After eliminating clones that had inserts less than approximately 200 bp, 3,930 clones were shipped in freezing medium to Dr. Andrew Patterson's laboratory, University of Georgia, Athens for sequencing. The freezing media contained LB broth with Ampicillin and the following ingredients: 0.036 M K_2HPO_4 (anhydrous), 13.2 µM KH_2PO_4, 1.7 µM Sodium Citrate, 0.4 µM $MgSO_4.7H_2O$, 6.8 µM $(NH_4)_2SO_4$, 22 ml autoclaved 100% glycerol. Clones were sequenced from the 3' prime end with a Qiagen 5'-TGTAAAACGACGGCAGT-3' primer. Vector sequences were trimmed by passing through Pipeonline 3.0 : an annotated EST processing and functional data sorting software from Oklahoma State University Bioinformatics Laboratory, and 3845 high

quality sequences (Appendix Table no.1) were deposited in EST GenBank database (dbEST). http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

Contig assembly and analysis:

EST sequences from this study and a bermudagrass fungal disease (Spring Dead Spot, SDS) resistance study (Zhang et al., 2006) were assembled using the TGICL and CAP3 programs. TGICL is a software program for fast clustering of large EST datasets against the modified version of NCBI's megablast (Zhang et al., 2000). The resulting clusters were assembled using CAP3 sequence assembly program (Huang and Madan, 1999).

Fabrication of microarray slides:

Plasmid isolation:

Plasmid isolation and purification was performed at the S.R.Noble Foundation, Ardmore, Oklahoma. A Biomek 2000 was used with an in-house protocol http://www.noble.org/plantbio/genomics/printlayout_1_32465_32465.html.

Along with the clones obtained from the cold acclimation experiment, 749 ESTs from the SDS library (Zhang et al., 2006) were also included for plasmid preparation. Purified plasmid pellets were resuspended in 100 µl of TE buffer and stored at -20°C. Two µl of purified plasmid was diluted 1000 fold in 0.22 mm filtered autoclaved double distilled water. PCR reactions were set up to amplify inserts using

M13F (5'-TGTAAAACGACGGCCAGT-3') and

M13R (5'-TCACAGAGGAAACAGCTATGAC-3')

primers and one unit of MangoTaq DNA polymerase (Bioline, Boston, MA). PCR parameters were as follows: 94°C for 2 min initial denaturation, 95°C for 30 s

denaturation, 51°C for 30 s annealing, 72°C for 1.5 min extension for 34 cycles with a 5 min 72^{o} C extension step at the end in order to get full length sequences.

Purification of PCR product:

An ethanol precipitation method was used for purification of PCR products prior to printing on microarray slides. Three volumes of 95% ethyl alcohol and one-tenth volume of 3M sodium acetate was added and mixed in each well followed by storage at -80°C for one hour precipitating the DNA. The precipitate was centrifuged at 12,000 rpm for 30 min, the aliquot was drained and the pellet was washed with 80 µl of 70% ethyl alcohol. The pellet was air dried at room temperature for 10-15 min and dissolved in 12 µl of 3X SSC. Samples were checked randomly for concentration by NanoDrop spectrophotometer and single band purity was checked with 1% agarose gel electrophoresis. Final concentrations were adjusted to 200 ng/µl. Purified DNA samples were stored at -20°C until further use.

Preparation of spiking and control DNA:

AFGC microarray control set was obtained from Nottingham Arabidopsis Stock Center (http://arabidopsis.org.uk), which contained 8 transgene controls and 10 spiking controls. DNA was prepared according to the AFGC consortium protocol. PCR products were purified using the ethanol precipitation method as described before. Final concentration was adjusted to 200 ng/µl and stored in 3X SSC for printing. Bermudagrass actin, 18S ribosome, chitinase, and wheat Actin genes were used as internal controls. Bermudagrass actin and 18 S ribosome clones were provided by Dr. Zhang, OSU, and chitinase gene clones were provided by Dr. Taliaferro's laboratory, Oklahoma State

University. Clones were amplified using M13 forward and reverse primers. PCR products were purified as described above.

Microarray printing:

The purified PCR products present in 96 well plates (48 plates) were transferred to 384 well plates. A single 384 well plate, which was prepared in duplicate, was test printed and stained with Syto61 (Molecular Probes, Eugene, OR) to assess the quality of printing. Prepared probes along with controls and spikes from 12 plus 384 well plates were printed on Arrayit Superamine2 slides (Telechem International, Sunnyvale, CA). Fifty slides were printed in 17X17 formats with three replications consisting of 48 subarrays. Controls were spotted twice in each replication. A total of 4,624 genes replicated three times consisting of 13,872 spots were printed utilizing the Omnigrid-100 Microarrayer (Gene Machines, San Carlos, CA). After printing, the slides were left in the machine for one hour to allow maximum binding, then baked at 80°C overnight for cross linking and stored desiccated in vacuum at room temperature until used. A single slide was stained with Syto61 to assess the quality of printing (Appendix Figure no.4).

Array hybridization:

Target and spike RNA preparation:

Target RNA was prepared as mentioned previously. SP1, SP5, SP9 spike RNAs were prepared from purified PCR products using Riboprobe System3 *in vitro* transcription kit (Promega, Madison, WI). DNAse treatment was performed as mentioned in the above protocol and heat inactivated at 95°C for 5 min. *In vitro* synthesized RNA was purified using RNeasy minikit (Qiagen, Valencia, CA). Purified spike RNA was

stored in RNA storage solution (Ambion Inc, Austin, TX) at -80°C after quantification. The working solution was adjusted to one ng/µl for hybridization.

Microarray slide treatment and pre-hybridization:

Array slides to be hybridized were rinsed in 0.1% SDS for 2-3 min to remove excess DNA, and then rinsed in autoclaved nanopure water for few seconds. Slides were placed into a beaker of boiling dd H_2O (95°C) for 8 min to denature DNA. Slides were quickly spun dry for 20 s, incubated in a pre-warmed BlockIt high throughput blocking buffer (Telechem Int., Sunnyvale, CA) to block the amine groups that did not bind to DNA during printing and, to eliminate fluorescent background followed by incubation at 50°C until ready for cDNA hybridization.

cDNA synthesis and hybridization:

Target cDNAs were prepared using 1.5 µg of mRNA from cold acclimated and non-acclimated samples for both the genotypes. In general, control RNA was labeled with Alexafluor 546™ and cold acclimated RNA with Alexafluor 647™ using Array 900 kit from Genisphere (Hatfield, PA). cDNA synthesis was scaled up to 20 µl, in each reaction, and 1 ng of SP1, SP5, SP9 spike mRNAs were added along with the experimental mRNA. Dye specific RNA-RT primer mix containing Superscript II enzyme was used to reverse transcribe (Invitrogen, Carlsbad, CA) mRNA to cDNA. The reaction was stopped by adding 0.1 M NaOH/50 mM EDTA, and heated for 10 min at 65°C.

cDNA hybridization mix was prepared by combining the control and cold acclimated cDNA in a tube along with formamide-based hybridization buffer and LNA™ dT Blocker. The reaction mix was scaled up to a total volume of 81 µl, and applied to the

prepared slide. A 24x60 mm LifterSlip (Erie Scientific Company, Portsmouth, NH) glass cover slip was placed on top of the hybridized array and the slides were kept in individual hybridization chambers. Approximately 15µl of 3xSSC solution was added to both wells on each side of the hybridization chamber and carefully clamped shut and incubated at 50°C overnight.

cDNA hybridization washes:

After hybridization, slides were washed in pre-warmed (65°C) 2X SSC, 0.2% SDS solution for 5 min, followed by 2X SSC 0.2% SDS, 2X SSC and 0.2X SSC washes at room temperature for 15 min each. Finally, slides were rinsed in ddH_2O at room temperature for a few minutes, spun dry and stored at 50°C until ready for 3DNA (3-dimensional nucleic acid) dendrimer hybridization.

3DNA dendrimer hybridization and washes:

In the dark room, 3DNA capture reagents, AF546 and AF647 were used to make hybridization mix according to the Array 900 kit protocol (Genisphere, Hatfield, PA) making the volume up to 81 ul. Hybridizations were performed as mentioned earlier for four hours in a hybridization chamber shielded from light. Post 3DNA dendrimer hybridization washes were performed as mentioned above and slides were stored in air tight slide tubes until ready for scanning.

The entire experiment was replicated three times for each hybridization (control and cold acclimated) and each time point and each genotype separately. A dye swapping (Appendix Figure no.5) experiment was done for Zebra 2 day time point in order to investigate dye bias. Self on self was performed for MSU 28 day samples as an extra experiment to see whether any genes are biased to a particular dye.

Data Collection and analysis:

Data Collection:

Hybridization signal intensities of the slides were captured using ScanArray Express (Perkin Elmer, Wellesley, MA). Pixel resolution was set to 10 microns, laser settings were kept constant (90 for Alexa 647, and 80 for Alexa 546) and photo multiplier tube settings were adjusted to optimize the data. Spike control spots and internal control spots were considered while adjusting the settings. Alexafluor 647 was read through red channel and Alexafluor 546 through green channel. Images obtained from both channels were analyzed utilizing GenePix Pro 4.0 (Axon Instruments, Union City, CA). Spots were aligned with gal file (A text file with specific information about the location, size, and name of each spot on the slide). This file includes information that GenePix uses to create an array list. In addition, this file is necessary for creating the microarray grid http://www.stanford.edu/~btomooka/gal/howto_create_a_gal_file.htm). Spot intensities less than 200 or greater than 65,000 relative fluorescence units (RFU) in both the channels were discarded. Spots having too much background and mechanically damaged spots were flagged and removed from further analysis. Experimental gene expression values were converted to log-transformed ratios and compared with control values. A 2-fold change was represented by a log 2 ratio of 1.0 for up-regulation and -1.0 for the down-regulation.

Analysis:

Linear Models for Microarray Data (Limma package, Smyth G K, 2004) were used through Bioconductor software (www.bioconductor.org) for microarray data

analysis. The following statistical parameters were applied during analysis. (Appendix figure numbers 5 to 7 will show the quality of normalization):

- Background correction: Robust regression background correction method
- Normalization within array: Loess normalization pin-by-pin intensity dependant normalization
- Normalization between arrays: Quantile normalization
- P values: False discovery rate control
- B-statistics: Emperical Bayes approach (ebayes)

Those ESTs with a p value < 0.001 and 2 fold changes in expression at any time point, and in any one genotype were considered as differentially expressed. Based on the above criteria, there were1470 differentially expressed ESTs from this study.

Assembly of differentially expressed ESTs into unigene set with CAP3 software (http://genome.cs.mtu.edu/sas.html) resulted in 94 contigs and 472 singlets giving 566 unigenes. FASTA formatted sequences of these unigenes were annotated by searches of the Universal Protein Resource (UniProt, www.pir.uniprot.org) database using BLASTx algorithm with an e-value of 0.1, and for those that did not match any known protein in the UniProt record were searched against NCBI's unigene database using tBlastx algorithm with an e-value of 0.1. Following the annotation of sequences with the similarities found in UniProt database, Gene Ontology (GO) terms (GoID) were linked to the sequences using GO stat (Beißbarth and Speed, 2004; Khatri and Draghici, 2005) and only those having GO term (Gene Ontology function) were selected with a P-value cutoff of 0.1. A gene ontology map was created and used in Genesis software for further visualization of gene functions

(http://genome.tugraz.at/genesisclient/genesisclient_faq.shtml). For ease of analysis and interpretation, genes having annotations were clustered based on their expression pattern for 2- and 28- day samples separately. Histograms were developed using Microsoft Excel, or expression images were prepared using Genesis.

CHAPTER IV

RESULTS

cDNA library construction:

Because very little EST information was available in the public databases for bermudagrass (663 ESTs from GenBank) during the initial periods of the study, we constructed our own cDNA library specifically targeting genes induced by cold temperatures. In order to enrich cold acclimation response genes from house keeping and commonly abundant genes, Suppression Subtractive Hybridization (SSH) was applied between cold acclimated and non-acclimated mRNA populations after 2 and 28 days of acclimation for MSU and Zebra yielding 3,845 ESTs. All Sequenced ESTs were deposited in GenBank EST database. ESTs were assembled into contigs using CAP3 assembly program (Table1), which yielded 2,440 unigenes consisting 2,219 singlets.

Microarray analysis:

Microarray analysis was performed to establish differences in gene expression profiles in crown tissue at 2 and 28 days of cold acclimation in cold tolerant (MSU) and sensitive (Zebra) bermudagrass genotypes. Differentially expressed ESTs were selected if the $\log_2$ ratio was >1 or <1 (2 fold differences in expression) and the p-value was less than 0.001. Microarray analysis yielded 1,473 differentially expressed ESTs representing 32.2% of the 4,570 total ESTs, including 3,850 ESTs from cold acclimation library and 720 ESTs from SDS library (Zhang et al., 2006). Alignment of these differentially expressed genes using CAP3 resulted in 94 contigs and 472 singlets giving 566 unigenes. From here onwards contigs were referred to as genes. All the genes that showed two-fold differential expression were grouped as up and down-regulated at each time point and genotype. Figure 1A shows 116 genes in MSU and 83 genes in Zebra up-regulated at 2 days after initiation of cold acclimation (DCA), sharing 19% or 37 common genes

between MSU and Zebra. Fig 1C indicates that 108 genes in MSU and 109 genes in Zebra were up-regulated at 28 days after initiation of cold acclimation (DCA), sharing 35 genes or 16% of the 217 up-regulated genes. In fig 1B, 192 genes in MSU and 113 genes in Zebra were down-regulated at 2 DCA sharing 77 genes or 25% of 305 total down-regulated genes. In fig 1D, 172 genes in MSU and 146 genes in Zebra were down-regulated at 28 DCA, sharing 56 genes or 17% of 318 total down-regulated genes. Percentage of differentially expressed genes common to both the genotypes was higher at 2 DCA (56%) than at 28 DCA (33%). On average, only 20% of the up-regulated and 27% of the down-regulated genes were common between the cold resistant and susceptible genotypes.

. Seventy-three contigs out of 566 unigenes had GO functions from the genomic databases and functions were visualized using Genesis software. In the microarray analysis, where two or more ESTs from the same gene but with different lengths the expression value of lengthiest EST was used. Fig 2 shows the functional category of these annotated genes. Nearly 53% of genes were involved in metabolism followed by binding (14.9%), catalytic activity (14.2%), physiological process (9.9%) and protective and regulatory mechanisms (7.8%).

Table2 shows the above contigs along with putative functions and expression values. Based on this table in MSU there were 46 differentially expressed genes; 18 up and 28 down-regulated genes at two day acclimation and 49 differentially expressed genes; 23 up and 26 down-regulated genes at 28 day cold acclimation. In Zebra, 31 differentially expressed genes; 22 up-regulated and 9 down-regulated genes at 2 day acclimation and 37 differentially expressed genes; 23 up and 14 down-regulated genes at

28 day cold acclimation in their acclimated crown tissue compared to their non-acclimated tissue. The gene UBA/THIF-type NAD/FAD binding showed highest up – regulation among the annotated genes in both genotypes and at both time points. The expression value of UBA/THIF-type NAD/FAD binding was up-regulated 123 and 23 fold in MSU and 64 and 14.6 fold in Zebra after 2 and 28 days respectively in cold acclimated crown tissue compared to their non-acclimated control. The gene Dehydrin 4 showed very high down-regulation in both genotypes at both the time points. Dehydrin 4 was down-regulated 17.8 and 32 fold in MSU and 5.5 and 6.5 fold in Zebra after 2 and 28 DCA, respectively, in cold acclimated tissues when compared to non-acclimated crown tissues. Detailed expression pattern of genes in both genotypes are discussed below using histograms.

Divergent gene expression profiles between MSU and Zebra after 2 days:

We selected two genotypes, MSU and Zebra, based on the divergent response to cold temperatures: MSU being the most tolerant and Zebra the most susceptible bermudagrasses available. Figure 3 illustrates the most divergent expression pattern of genes significantly up-regulated between these two genotypes after 2 days cold acclimation. Xylanase inhibitor XIP-III, chloroplast 30S ribosomal protein S15, phosphoribosyltranferase, and sucrose synthase-2 were up-regulated more than 3 fold, while protein TAR1 was up-regulated greater than two fold in MSU cold acclimated crown tissue when compared to non-acclimated crown tissue at 2 days. Neither of the above-mentioned genes were significantly up-regulated in Zebra. Chloroplast 30S ribosomal protein S8 and S3, DNA-directed RNA polymerase beta chain were up-regulated greater than three-fold and DNAJ-related protein ZMDJ1 and another isoform

of chloroplast 30S ribosomal protein S8 were up-regulated greater than two-fold in Zebra cold acclimated crown tissue compared to non-acclimated crown tissue in 2 day sample (Fig 3). Again, neither of the genes up-regulated in Zebra were significantly up-regulated in MSU.

Figure 4 illustrates the expression pattern for 18 genes that were down-regulated in MSU but not in Zebra after 2 days of cold acclimation when compared to non-acclimated crown tissue. The dehydrin DHN1gene was the most highly down-regulated gene in MSU followed by Legumine-like protease 8 precursors, membrane protein precursor, adenine nucleotide translocase, and initiation factor elf-4. The remaining genes shown in figure 4 were down-regulated at least two-fold in acclimated tissues compared to non-acclimated tissues.

Figure 5 represents down-regulated genes only in one genotype but not in the other after 2 DCA. Catalase was down-regulated more than 6-fold in MSU cold acclimated crown tissues compared to non-acclimated tissues at 2 day time point. Polyprotein was more than four-fold, protein translation factor SUI1 homolog more than three-fold, serine/threonine kinase 2-fold down-regulated in Zebra cold acclimated crown tissue at 2 days.

Divergent gene expression profiles between MSU and Zebra after 28 days:

There were nine genes up-regulated in cold acclimated MSU crown tissue at 28 days, but not in Zebra cold acclimated crown tissue (Fig 6). Acyl CoA-Binding Protein (ACBP) was up-regulated more than five-fold followed by chloroplast 30S ribosomal protein S15, MAP cascade associated MK5 protein, and sucrose synthase-2 were up-regulated more than three-fold. Mitogen-activated protein (MAP) kinase kinase kinase

(MAPKKK or MAP3K), SERK2 protein precursor, ribosomal protein L37, tetrameric ubiquitin and aspartate aminotransferase were up-regulated more than two fold. These are all directly or indirectly involved in low temperature signal transductions in a variety of organisms.

Figure 7 shows down-regulated genes only in MSU cold acclimated crown tissues, but not in Zebra. There were 16 genes significantly down-regulated only in MSU after 28 days cold acclimation. Catalase and putative membrane protein precursor were down-regulated more than 5-fold and legumain-like protease precursor, ribonuclease EG, protein translation factor SuI1 homolog, protein ORF106b, 64c and aspartic proteinase were all down-regulated greater than three-fold.

Figure 8 illustrates the genes that are either up or down-regulated in Zebra while being statistically neutral in MSU after 28 days of cold acclimation. Polyprotein was down-regulated more than 5-fold, Xylanase inhibitor XIP-III and GATA-binding transcription factor were down-regulated more than two-fold in zebra cold acclimated crown tissue compared to its non-acclimated crown tissue at 28 days. Elongation factor 1-alpha, LIM transcription homolog, phenylalanine ammonia-lyase and ATP synthase A chain were up-regulated more than two-fold in 28 day acclimated crown tissue.

Differences in gene expression profiles between 2 and 28 days cold acclimation in MSU:

There were six genes at 2 days (fig 3) and nine genes at 28 days (fig 6) that were up-regulated in cold acclimated crown tissue compared to non-acclimated crown tissue. Only two genes, chloroplast 30S ribosomal protein S15 and sucrose synthase-2 were up-regulated at both 2 and 28 day time points in acclimated tissue. There were 18 genes at 2

days (figure 4) and 16 genes at 28 days (figure 7) that were down-regulated in cold acclimated crown tissue when compared to the non-acclimated tissue. There were only five common genes including legumain-like protease precursor, putative membrane protein precursor, putative ribonuclease EG, phytepsin precursor, hypothetical protein orf 106b that were down-regulated at both the time points when compared to the non-acclimated crown tissue.

Similarities in gene expression profiles of MSU and Zebra at 2 days after cold acclimation:

Genes up- or down-regulated early in the cold acclimation process may be involved in a cold shock response. There were 18 genes that were up-regulated in both MSU and Zebra cold acclimated tissue after 2-DCA when compared to their non-acclimated counter parts (Fig 9). There were 13 genes up-regulated in both MSU and Zebra, at 2 DCA including: UBA/THIF-type NAD/FAD binding protein 123- and 28-fold, putative cystatin 12- and 5-fold, predicted seven transmembrane receptor more than 10- and 2-fold, sucrose synthase 2 more than 8- and 4-fold, aspartate aminotransferase more than 7.5- and 3.5-fold and actin more than 7.5- and 2-fold up-regulated in MSU and Zebra, respectively. Only five genes were down-regulated upon exposure to low temperatures in both the genotypes crown tissues compared to non-acclimated tissues. Dehydrin 4 18- and 5-fold, Delta 1-pyrroline-5-carboxylate synthetase 4- and 3-fold, ORF64c 4- and 2-fold were down-regulated in MSU and Zebra, respectively. RNA-binding region RNP-1 and NADP-dependent malic enzyme were more than 2.5- and 2-fold down-regulated respectively in crown tissues of both the genotypes at 2- DCA.

Similarities in gene expression profiles of MSU and Zebra at 28 days after cold acclimation:

There were 18 genes that showed up-regulation in both the genotypes at 28-DCA (Fig 10). UBA/THIF-type NAD/FAD binding gene was up-regulated 63- and 14-fold, sucrose synthase 2 gene 7- and 2-fold, Cysteine protease-14 7- and 3-fold, putative cystatin 6- and 6-fold, another Putative cystatin 5- and 2-fold, predicted seven transmembrane receptor 5- and 3-fold, actin 5- and 3-fold, ADP-ribosylation factor 5- and 2-fold up-regulated in MSU and Zebra, respectively. On contrary, the gene Phosphoribosyltransferase was up-regulated more in Zebra than in MSU (6- and 4- fold in Zebra and MSU respectively).

There were 11 genes that showed significant two-fold down-regulation in both genotypes (Fig 11). Dehydrin 4; 34- and 6-fold, Dehydrin DHN1; 18- and 2-fold, Glyoxalase I; 6- and 3-fold, ER6 protein 5- and 3-fold, DNA-directed RNA polymerase beta chain 4- and 2-fold down-regulated in MSU and Zebra, respectively. On contrary, the gene Delta 1-pyrroline-5-carboxylate synthetase was down-regulated 2- fold in MSU and 3- fold in Zebra in contrast to the above expression pattern.

Of the 13 genes that showed up-regulation after 2-DCA, all but aspartate aminotransferase were up-regulated at 28 days as well in both genotypes (figs 9 and 10). Elongation factor eEF1 gamma chain, putative membrane protein, hypothetical protein , phenylalanine ammonia-lyase, putative glucan endo-1,3-beta-glucosidase GVI precursor, phosphoribosyltransferase were up-regulated only at 28 days in acclimated crown samples in both the genotypes. Three genes including Dehydrin 4, Delta 1 pyrroline-5-

carboxylate synthetase, and NADP-dependent malic enzyme showed continuous down-regulation in both genotypes at both time points (figs 9 and 11).

The above results were based on the genes with functional annotations. There were 493 genes that were differentially expressed but without functional annotations from the available databases, indicating an unknown function. Table 3 shows genes up-regulated more than 4-fold in MSU after 2 days acclimation and table 4 shows genes up-regulated at least 3-fold at 28 DCA. These genes did not show any significant response in Zebra except accession number DN985563 and DN986562, which was 2- fold up- and down-regulated, respectively, in Zebra 28 days acclimated crown tissue.

CHAPTER V

DISCUSSION

Significant differences were observed in gene expression profiles of MSU and Zebra during cold acclimation. The relationship of these profiles to possible cold tolerance mechanisms operating in bermudagrass are discussed below.

Genes associated with cold acclimation:

Cold acclimation associated genes are genes that showed continuous up-regulation at both the time points during cold acclimation as well as genes up-regulated at 28-DCA. Of the 13 genes that showed up-regulation at 2-DCA, all but aspartate aminotransferase were up-regulated at 28-DCA as well in both genotypes (figs 9 and 10). There are 15 genes that were associated with cold acclimation including UBA/THIF-type NAD/FAD binding protein, sucrose synthase2, cysteine protease14, putative cystatin (2), predicted seven transmembrane receptor, actin, ADP-ribosylation factor, ribosylation factor, DELLA protein GAI1, ribosomal protein(2), Cytochrome P450 like_TBP, Elongation factor eEF1 gamma chain and putative membrane protein in their order of magnitude of up-regulation in MSU (figs 9 and 10).

Ubiquitin associated enzyme was very highly up-regulated in both genotypes (132-fold in MSU and 28-fold Zebra) at 2-DCA as well as at 28- DCA (63-fold in MSU and 14-fold in Zebra). The expression was very high at 2-DCA and nearly reduced to half in 28-DCA crown tissues in both the genotypes compared to non-acclimated tissue (figs 9 and 10). Until recently, ubiquitinization genes were thought to function in marking proteins for degradation in the proteosomes, but it is now known that ubiquitin genes may have several roles in eukaryotes, including: vesicular trafficking, kinase activation, regulating gene expression response to stress, DNA repair, growth factor signaling and

gene silencing (Conaway et al., 2002; Hurley et al., 2006). Probably this may be the first report showing up-regulation of UBA genes involved in cold acclimation in plants.

Elongation factor eEF1 gamma chain, putative membrane protein, hypothetical protein , phenylalanine ammonia-lyase, putative glucan endo-1,3-beta-glucosidase GVI precursor, phosphoribosyltransferase were up-regulated only at 28- days in acclimated crown samples in both the genotypes. Three genes including Dehydrin 4, Delta 1 pyrroline-5- carboxylate synthetase, NADP-dependent malic enzyme showed continuous down-regulation in both the genotypes at both time points (fig 9 and 11).

Alterations in carbohydrate metabolism, during cold acclimation has been well documented in plants (Crespi et al., 1991; Stitt and Hurry, 2002). Sucrose accumulation was observed in cold acclimated bermudagrass (Zhang , Ervin and Labrnage., 2006). Significant up-regulation of sucrose synthase was found for both MSU and Zebra during cold acclimation. However, as was observed for actin, a two-to three- fold increase was observed in MSU when compared to Zebra (figs 9 and 10).These findings agree with the above reports (Zhang , Ervin and Labrnage., 2006), where they observed higher amounts of sucrose accumulation in stolons of tolerant bermudagrass genotype than the sensitive ones during low temperature acclimation. Increased levels of sucrose synthase under cold conditions may associate with the transport of sucrose to vacuole where it is used for fructan synthesis, or for direct accumulation as sucrose (Calderón and Pontis, 1985; Crespi et al., 1991).

In our studies actin was up-regulated in cold acclimated samples throughout the cold acclimation period, suggesting that actin gene expression is also associated with cold acclimation. Expression levels of actin in MSU was approximately 7.5- fold at 2

DCA and 5.5- fold at 28 DCA, while in Zebra, expression levels were 2.5- and 3.5- fold, respectively, in acclimated samples compared to their non-acclimated counterparts. Cold tolerant MSU showed a higher level of expression than sensitive Zebra. Actin is known to be involved in calcium induced signal transductions via changes in the cell cytoskeleton (Buchanan et al., 2000; Örvar et al., 2000; Drobak et al., 2004).

Cysteine proteases are a group of plant proteases having a common catalytic mechanism that involves a cystine amino acid in the active site. Both cysteine proteases and cysteins were up-regulated in both genotypes at both time points during cold acclimation. Plant cysteins are involved in storage protein turnover, programmed cell death, tolerance to biotic and abiotic stress, senescence, differentiation of mesophyll cells into tracheary elements and xylogenesis (Arai and Abe, 2000; Belenghi et al., 2003; Kiggundu et al., 2006). Plant cystein proteases are also involved in scavenging reactive oxygen species (ROS) as 1-cysteine peroxiredoxin and 2-cysteine peroxiredoxin during unfavorable conditions (Rouhier and Jacquot, 2002).

Genes associated with cold tolerance:

Genes those were up-regulated only in crown tissues of cold tolerant genotype MSU at 2 and 28 DCA, but not in Zebra compared to their non-acclimated crown tissues. These genes are therefore of great interest to researchers interested in cold tolerance. During 2- day acclimation, Phosphoribosomal transferase, protein TAR1 (Transcript Antisense to Ribosomal RNA), sucrose synthase 2, chloroplast 30s ribosomal protein S15 and xylanase inhibitor XIP-III were up-regulated at least greater than two fold (fig 3). After 28- days acclimation, acyl-CoA- Binding protein, chloroplast 30s ribosomal protein S15, sucrose synthase 2, protein kinase MK5, MAPKKK, SERK2 protein precursor,

ribosomal protein L37, aspartate aminotrasferase were up-regulated in MSU crown tissue at least greater than two fold (fig 6).

Acyl-CoA-Binding Protein (ACBP) binds medium and long chain acyl coA esters with very high affinity, and participates in acyl coA transportation, metabolism and cell signaling (Knudsen 1991; Takle et al., 2005). In the present study, ACBP was nearly seven-fold up-regulated in fully acclimated MSU crown tissue, however no expression was observed in Zebra. Over- expression of ACBP was observed in *catfish* in response to both high- and low-temperature stresses (Ju et al., 2002; Takle et al., 2005). This is the first report of ACBP being over-expressed in plant tissues during cold acclimation.

A splice variant of sucrose synthase 2 from clone JN-133 derived from bermudagrass cDNA library constructed against Spring Dead Spot fungus (*Ophiosphaerella herpotricha)* infection (Zhang et al., 2006) was up-regulated approximately three-fold in 2-day and 28-day acclimated samples of MSU but not in cold susceptible Zebra. This clone has 38 percent sequence identity with the other sucrose synthase 2 reported above that was up-regulated in both the genotypes following cold acclimation. This sucrose synthase 2 transcript may be genotype specific and may contribute positively to cold tolerance in MSU.

Mitogen Activated Protein (MAP) kinases may enhance freezing tolerance in plants by controlling expression of freeze tolerance genes, or by regulating proteins involved in freezing tolerance (Thomashow, 1999). In our study, a MAP kinase kinase kinase (MAPKKK) was induced more than two-fold and protein kinase MK5 was induced three-fold in fully acclimated MSU crown tissues. In Zebra crown tissues, these genes were nearly neutral. MAP kinase signal transduction is one of the major signaling

pathways in plant cells (Buchanan et al., 2000). MAP KKK is involved in localization of signals to the nucleous, whereas protein MK5 is involved in activation of transcription factors and export of signals to cytoplasm as a result of phosphorylation (Levy et al., 1998; Shi et al., 2003). These kinases may be involved in low temperature induced MAP kinase signal transduction cascades, which may function in increasing cold tolerance in MSU.

Negative expression of genes in MSU:

Dehydrins are the most commonly reported genes or proteins induced by low temperature stress (Pearce, 1999; Zhu et al., 2000) or other biotic stresses. Low temperature stress studies (Zhu et al., 2000) reported that chilling induced Dehydrins help to prime plant cells for acclimation to more intense cold, which in turn helps adaptation to dehydration during freeze thaw cycles. The same study showed that 11 different Dehydrin genes were present in barley. DHN5 and DHN8 were highly induced during cold acclimation (4˚C) whereas DHN1 and DHN4 showed no expression at 4˚C but were induced only at freezing temperatures (-4˚C) and under dehydrated conditions.

Interestingly in our study, DHN1 was highly down-regulated (16 fold) after two days acclimation in MSU but with no changes in expression in Zebra. DHN4 was down-regulated at 2-days (18-and 5-fold in MSU and Zebra respectively) and in fully acclimated condition, 34 and 6 fold in MSU and Zebra, respectively. We do not know the exact reason why the dehydrins were down-regulated in bermudagrass crowns while in other plant species they are up-regulated in response to stress. However, most reports on dehydrins concern above ground plant parts such as leaves and stems or even roots rather than below ground storage organs such as crown tissues and tubers. Because

bermudagrass crowns undergo dormancy at low temperatures and the crown tissues are the only surviving part during winter, the tolerant species may exhibit some anti-senescent behavior that reduces expression of what would normally be an induced gene. In our study, many genes that were typically shown to be induced during senescence or programmed cell death treatments were down-regulated, including: autophagy related genes and phytepsin precursor in MSU only. In contrast in the cold susceptible genotype (Zebra) there was no change in expression of these genes indicating that they are most likely specific to the tolerant phenotype.

Studies on expression of antioxidant enzymes in dormant potato (Rojas-Beltran et al., 2000) showed that catalase, ascorbate peroxidase, glutathione peroxidase were down-regulated in dormant potato tubers, and in another study by Zhang et al., (2006) catalase and ascorbate were down-regulated in bermudagrass stolons with cold acclimation treatment. Our findings with MSU agree with those previous studies where catalase was nearly 8-fold down-regulated at 28-days and ascorbate peroxidase 2-fold down-regulated after 2-days cold acclimation in MSU, in contrast with most other cold acclimation reports (Prasad, 1996). This specific relationship may be further explained by studying organ specific expression pattern or metabolic condition of the organ under low temperature stress.

Vacuole autophagy is a major pathway by which eukaryotic cells degrade macromolecules (Xiong and Fei, 2006). Protein 8 precursor has been shown to be involved in autophagy and in this study was down-regulated in MSU only. Macromolecules such as carbohydrates, lipids, proteins, and nucleic acids degradation is

one of the major physiological processes involved in cold acclimation (Buchanan et al., 2000).

Adomet synthase analogs are inhibitors of the enzymes involved in plant senescence (Miura and Chiang, 1985). Adomet synthase 4, S- adenosyl homocystein hydrolase 2 were up-regulated with senescence promoting treatment (Buchanan-Wollaston et al., 2005). In this study all these genes were at least two fold down-regulated in MSU upon cold treatment, indicating some kind of mechanism may be inhibiting senescence in this tolerant genotype.

CHAPTER VI

CONCLUSIONS

In this study, we were able to identify 566 differentially expressed genes from crown tissues of bermudagrass genotypes, MSU and Zebra. These genes were associated with metabolism, signal transduction, dormancy regulation, and senescence related functions. UBA/THIF-type NAD/FAD binding protein, sucrose synthase2, cysteine protease14, putative cystatin , predicted seven transmembrane receptor, actin, ADP-ribosylation factor, DELLA protein GAI1, ribosomal protein, Cytochrome P450 like _TBP were identified as genes associated with cold acclimation. Genes involved in kinase signal cascade (MAPKKK, MK5, SERK2 protein precursor) and in metabolism (sucrose synthase2, ACBP, aspartate aminotransferase) were identified as associated with cold tolerance as they were up-regulated only in cold tolerant MSU during cold acclimation period. Because cold tolerance involves cross talk between multiple pathways, characterizing these genes along with highly induced genes of unknown function may help us better understand their role in cold tolerance. This study constitutes the first large scale analysis of gene expression pathways during cold acclimation in bermudagrass. Results from this study will serve as a basis for further investigations into the cold acclimation and tolerance mechanisms associated with bermudagrass stress response.

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Table 1. SSH library constructed from cold acclimated and non-acclimated crown tissues of MSU and Zebra genotypes.

Number of ESTs per Contig	Number of Contigs present in the Library
>100	2
51-100	2
41-50	3
31-40	4
21-30	9
11-20	17
6-10	38
4	19
3	31
2	96
1	2219

Table 2. Differentially expressed genes along with putative functions. Expression values of cold-acclimated (2- and 28- DCA) MSU and Zebra samples compared to non-acclimated crown tissue. Expression values are presented in $\log_2$ ratio of acclimated versus non-acclimated. Values highlighted in red are significantly up-regulated and values highlighted in green are significantly down-regulated to the cold treatment.

NCBI_ACCNO	Gene Annotation	MSU2day	ZEBRA2day	MSU28day	ZEBRA28day
BQ826386	UBA/THIF-type NAD/FAD binding	6.9447295	4.8254417	5.9871737	3.8711759
DN985561	Putative cystatin	3.6474832	2.4084302	2.7786933	2.6650519
BQ826351	Predicted seven transmembrane receptor	3.3510839	1.3272689	2.4781262	1.7333794
BQ826306	Sucrose synthase 2	3.1080527	2.1387591	2.9312584	1.3375572
DN987085	Aspartate aminotransferase	2.9993902	1.8749051	1.0012607	-0.318024
BQ826366	Actin	2.9198938	1.2876016	2.4683434	1.8208593
BQ826269	Cysteine protease 14	2.2044694	1.5401132	2.8805816	1.572175
BG322336	DELLA protein GAI1	2.15375	1.6457149	2.2150441	1.3674872
BQ826352	Phosphoribosyltransferase	1.7831031	0.8023872	2.0997029	2.6529291
DN989054	Cytochrome P450 like_TBP	1.7463639	1.3006583	1.7085863	1.6305076
DN988264	Ribosomal protein	1.7059346	1.1372447	2.0721384	2.1499535
DN985563	Putative cystatin	1.6773851	2.5108163	1.7685945	1.2547607
BQ826279	Sucrose synthase 2	1.5936691	0.5157641	1.5869716	-0.445505
DN986166	Protein TAR1	1.4141322	-0.419781	0.5137259	-0.195842
BQ826101	ADP-ribosylation factor	1.3438014	1.0448144	2.3901072	1.2272336
BQ826337	Chloroplast 30S ribosomal protein S15	1.2931257	0.3443007	1.6868091	-0.078718
BQ825943	Ribosomal protein L11	1.1178125	1.0638047	1.9172044	1.3115296
DN989000	Xylanase inhibitor XIP-III	1.0769322	0.2236291	0.1506169	-1.463526

NCBI_ACCNO	Gene Annotation	MSU2day	ZEBRA2day	MSU28day	ZEBRA28day
DN986874	MAP kinase kinase kinase	0.9494656	-0.648677	1.3045142	-1.032075
DN988922	DNA-directed RNA polymerase beta'' chain	0.9310986	1.677305	-1.030116	-0.097639
BQ826267	Ribosomal protein L37	0.895426	0.2813094	1.0408914	-0.079334
BQ825936	Hypothetical protein	0.8389976	1.4029806	0.7015051	1.3659786
BQ825934	Acyl-CoA-binding protein	0.8238421	2.7833579	0.4797992	0.5690703
DN987534	Tetrameric ubiquitin	0.8143426	0.5923101	1.0344824	0.6674843
DN985397	Chloroplast 30S ribosomal protein S3	0.7322523	1.9054078	-0.19917	0.4613791
BQ826357	Phenylalanine ammonia-lyase	0.7093678	0.7099068	1.2776332	1.4076171
DN989089	SERK2 protein precursor	0.7035203	0.1025863	1.085501	0.4611631
BQ826239	Putative glucan endo-1,3-beta-glucosidase GVI precursor	0.7006804	0.608736	1.4701337	1.5555698
BG322354	Elongation factor eEF1 gamma chain	0.460155	0.3802183	1.092744	1.0618969
DN988485	Chloroplast 30S ribosomal protein S8	0.42891	1.0576978	0.2973967	0.4617689
DN986445	Polyprotein	0.4021353	-2.264951	0.235396	-2.578101
BQ826356	Protein kinase MK5	0.3867817	-0.53122	1.5624252	0.5071654
DN987969	Chloroplast 30S ribosomal protein S8	0.3859523	1.6272466	-1.374519	0.6755227
BQ826329	ATP synthase a chain	0.2817623	0.8588129	-0.102874	1.6757313
BG322318	Phenylalanine ammonia-lyase	0.2231359	0.6019954	0.8821825	1.311273
BQ826412	DnaJ-related protein ZMDJ1	0.1860836	1.1441431	0.0960737	-0.000211
DN987448	Putative membrane protein	0.1607325	0.0718237	1.0261998	1.0012858
DN987040	Protein kinase c inhibitor-like protein	0.1001278	0.3245168	-1.473112	-1.393204
DN987058	Serine/threonine kinase	-0.098808	-1.031583	0.3924512	0.1916487

NCBI_ACCNO	Gene Annotation	MSU2day	ZEBRA2day	MSU28day	ZEBRA28day
DN987118	DNA-directed RNA polymerase beta'' chain	-0.208808	1.5480224	-2.18473	-1.063778
BQ826445	LIM transcription factor homolog	-0.231747	0.2334924	0.2913201	1.2206721
DN985584	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	-0.443989	0.2388722	-1.278644	-0.27214
DN987179	Putative GATA-binding transcription factor	-0.4563	-0.888166	-0.653945	-1.281797
BQ826365	Aspartic proteinase	-0.464385	-0.384204	-1.625974	-0.873579
BQ826400	Putative RUB1 conjugating enzyme	-0.615621	0.2860379	-1.260727	-0.364452
DN988817	Hypothetical protein orf111-b	-0.651609	-0.625483	-1.040541	-0.585593
BQ826431	Protein translation factor SUI1 homolog	-0.916078	-1.60146	-1.733557	-0.005653
DN986977	Arginyl-tRNA synthetase	-0.949252	0.028945	-1.02272	-1.194494
BQ826355	Eukaryotic translation initiation factor 5	-0.966854	-0.665219	-1.318704	-0.666843
DN987314	Putative cystatin	-1.003089	-1.251802	-0.629079	-1.206351
DN988572	Phytepsin precursor	-1.093194	-0.103642	-1.185582	-0.937016
DN988920	Autophagy-related protein 8 precursor	-1.142014	-0.349193	-0.751946	-0.756892
DN987474	Putative S-adenosylhomocystein hydrolase 2	-1.29195	-0.376958	-0.227774	-0.277637
DN987494	NADP-dependent malic enzyme	-1.323357	-1.236406	-0.343895	-0.745344
BG322311	Ascorbate peroxidase	-1.388983	-0.364043	-0.129119	-0.054714
DN985426	RNA-binding region RNP-1	-1.409733	-1.469386	-1.750765	-1.44255
DN987247	Possible ribonuclease EG	-1.481065	-0.182577	-1.900672	-0.496481
DN987399	Elongation factor 1-alpha	-1.493166	0.6293251	-0.345418	1.0890687

NCBI_ACCNO	Gene Annotation	MSU2day	ZEBRA2day	MSU28day	ZEBRA28day
DN987352	MGC81508 protein	-1.530031	0.0832188	-0.071033	0.5676296
DN988913	Putative AdoMet synthase 4	-1.591066	0.4968381	-0.424664	1.1045384
DN986971	Cysteine proteinase 1 precursor	-1.634687	-0.399215	-0.903714	-0.269862
BQ826426	ER6 protein	-1.681853	-0.259698	-2.3698	-1.657233
DN989106	Hypothetical protein orf106b	-1.827677	-0.624111	-1.611801	-0.708578
DN987028	Glyoxalase I	-1.921043	-0.770999	-2.76018	-1.856976
BG322346	Initiation factor eIF-4 gamma, middle;	-2.015314	-0.662967	-1.205918	-0.103234
DN985684	Putative adenine nucleotide translocase	-2.026924	-0.954285	-1.226331	-0.996865
DN985524	Delta 1-pyrroline-5-carboxylate synthetase	-2.042782	-1.707423	-1.030663	-1.756721
DN987440	ORF64c	-2.130656	-1.083357	-1.63693	-0.614486
DN987047	Putative membrane protein precursor	-2.275362	-0.103975	-2.646495	-0.482407
DN987297	Catalase	-2.652563	1.2559149	-2.926355	-0.785543
DN987302	Legumain-like protease precursor	-3.393221	-0.229817	-1.960843	-0.220915
DN987480	Dehydrin DHN1	-4.013999	-0.985375	-4.177508	-1.342307
DN985603	Dehydrin 4	-4.237808	-2.471023	-5.103497	-2.714651

Table 3. Genes with out matches to gene databases (no annotations) significantly ($p \leq 0.001$) up-regulated in crown tissues of MSU, not in Zebra, at 2- days after initiation of cold acclimation.

NCBI_ACCNO	MSU_Log2 _Ratio	P-Value	ZEBRA_Log2_Ratio	P-Value
BQ826214	3.28	1.64E-07	0.9	0.0084
BQ826339	3.01	8.11E-06	0.96	0.001
DN988506	2.44	0.00013	0.2	0.5872
DN986845	2.4	7.59E-07	-0.18	0.6282
DN986562	2.34	8.16E-07	-0.47	0.1801
DN986579	2.32	9.90E-07	-0.39	0.5314
DN986723	2.25	1.28E-05	-0.29	0.3691
DN986836	2.23	1.28E-05	-0.19	0.8357
DN986700	2.22	2.41E-05	-0.19	0.7987
DN987367	2.21	8.27E-05	-0.03	0.9777
DN986555	2.19	5.57E-06	-0.09	0.9334
DN986688	2.14	1.18E-05	0.97	0.0001
DN986596	2.14	0.00021	0.06	0.9552
DN985640	2.11	0.00398	-0.54	0.0426
DN986321	2.11	2.88E-06	0	0.995

Table 4. Genes with out matches to gene databases (no annotations) significantly ($p \leq 0.001$) up-regulated in crown tissues of MSU, not in Zebra at 28- days after initiation of cold acclimation.

NCBI_ACCNO	MSU_Log2 _Ratio	P-Value	ZEBRA_Log2_Ratio	P-Value
BQ826032	4.24	1.37E-07	0.13	0.608
BQ825934	2.78	7.9E-05	0.57	0.00198
DN985563	2.51	1.4E-05	1.25	0.09268
BQ826261	2.49	6.12E-07	0.65	0.00197
DN987367	2.39	7.11E-07	-0.15	0.82133
BQ826017	2.37	3.7E-06	-0.33	0.1969
DN986836	2.29	1.25E-07	-0.13	0.37946
DN988509	1.88	4.7E-05	0.95	0.00059
DN986555	1.88	3.14E-07	-0.32	0.07264
BQ826209	1.8	7E-06	0.45	0.04149
DN986903	1.77	1.2E-06	0.41	0.3664
BQ826214	1.75	0.00019	0.69	0.0176
DN986699	1.75	1.6E-06	-0.14	0.83547
DN986846	1.73	1.1E-06	0.03	0.92618
DN986562	1.72	4.61E-07	-1.06	0.02997

Figure 1. Venn diagrams showing distribution of differentially expressed genes in crown tissues of MSU and Zebra. A and C showing up-regulated genes in MSU and Zebra cold acclimated for 2- and 28- days compared to non-acclimated sample at 2- and 28- days respectively and B &D are showing down-regulated genes in MSU and Zebra cold acclimated for 2- and 28- days compared to non-acclimated sample at 2- days and 28- days respectively.

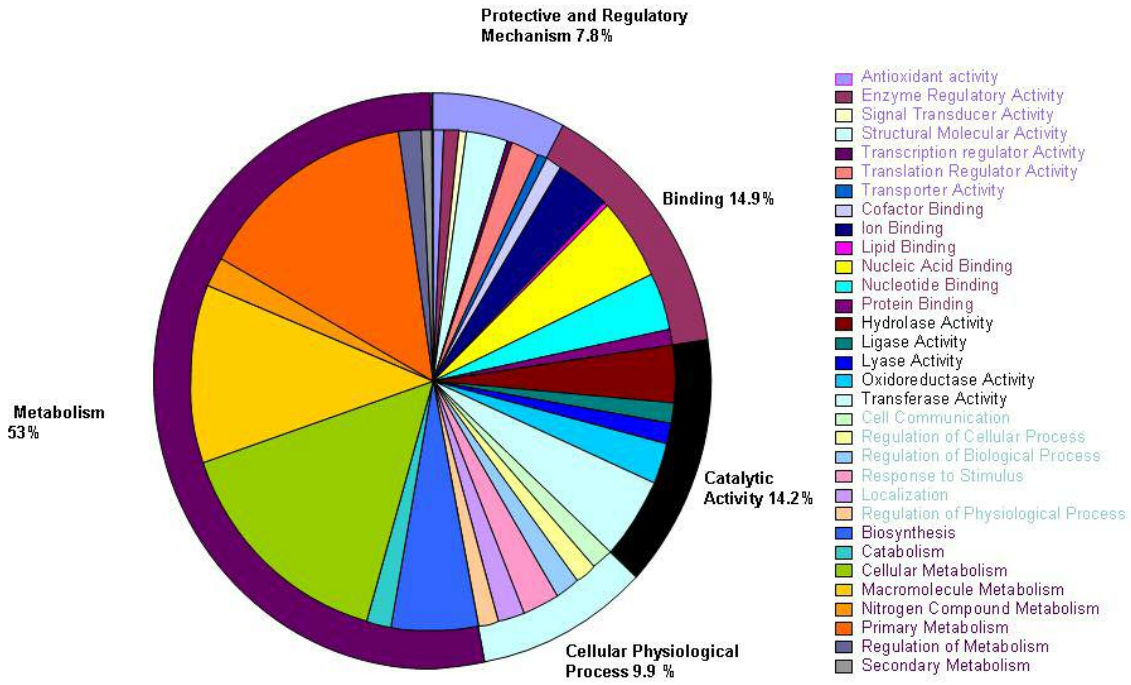


Figure 2. Functional categories of differentially expressed genes based on Genesis software. Outer pie has the broader categories and the inner pie has more sub-categories.

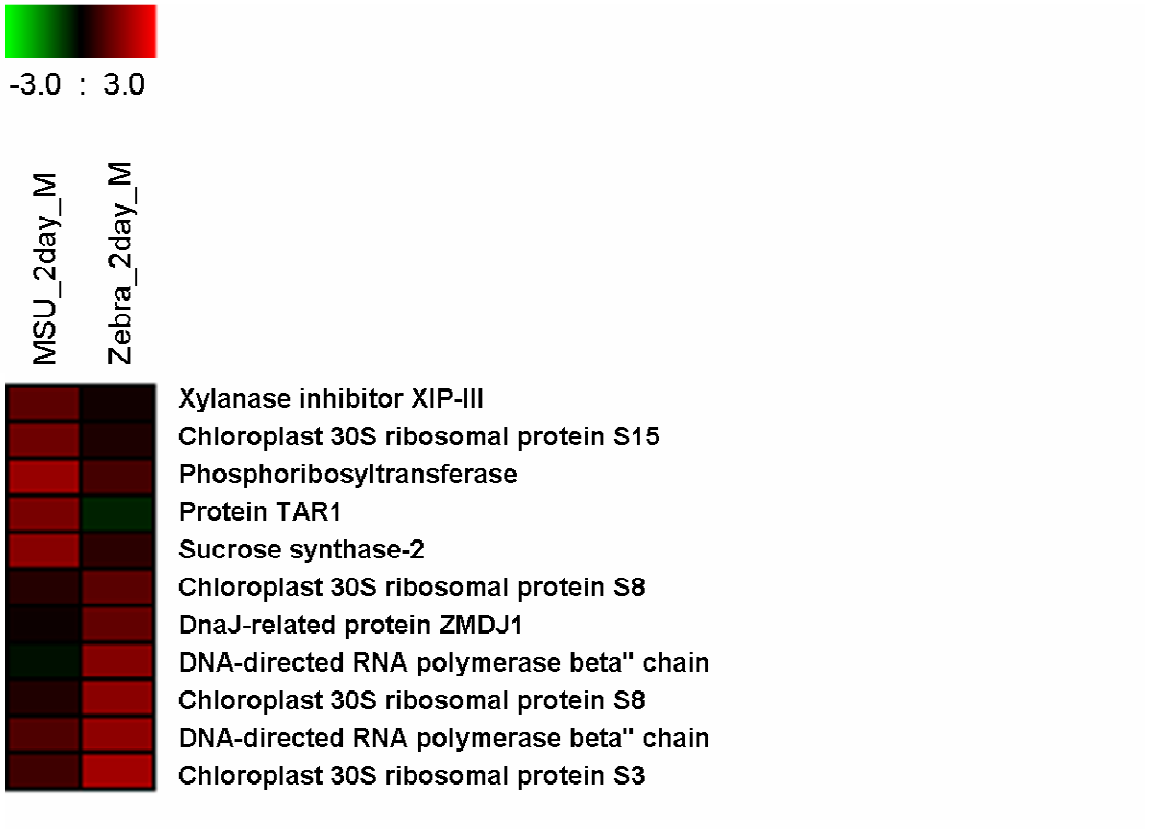


Figure 3. Up-regulated genes (at least 2-fold) in crown tissues of MSU or Zebra but not in both, at 2- days after initiation of cold acclimation.

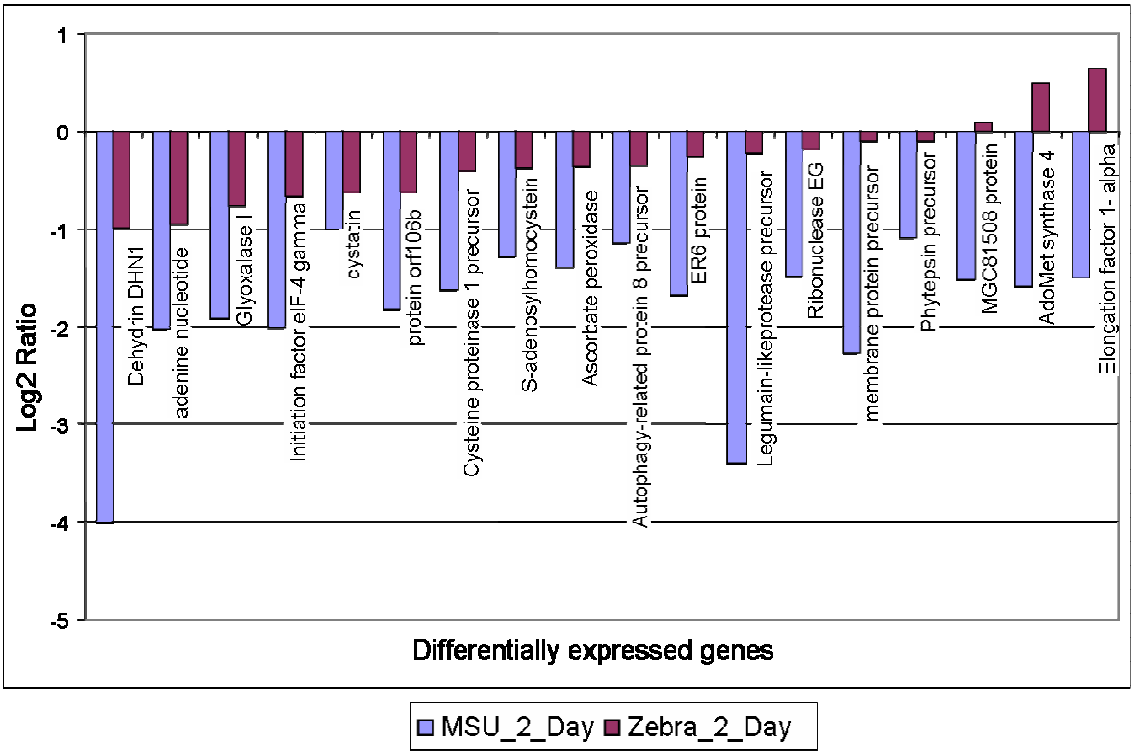


Figure 4. Down-regulated genes (at least 2- fold) in crown tissues of MSU at 2- days after initiation of cold acclimation.

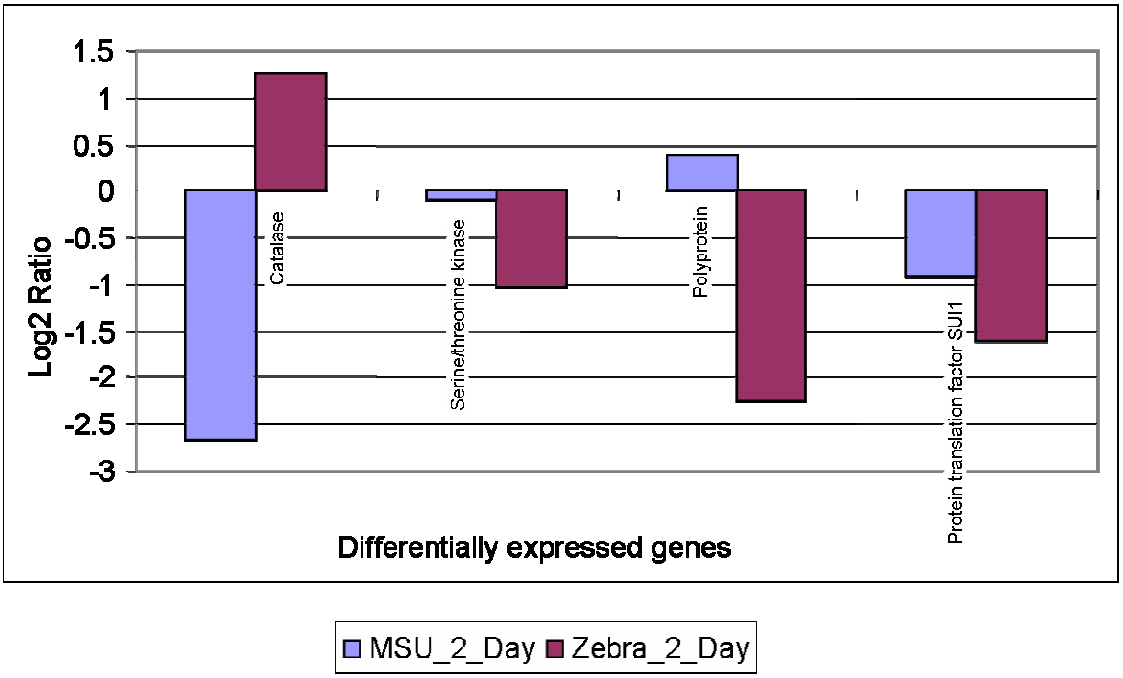


Figure 5**.** Down-regulated genes (at least 2- fold) in crown tissues of MSU or Zebra but not in both, at 2- days after initiation of cold acclimation.

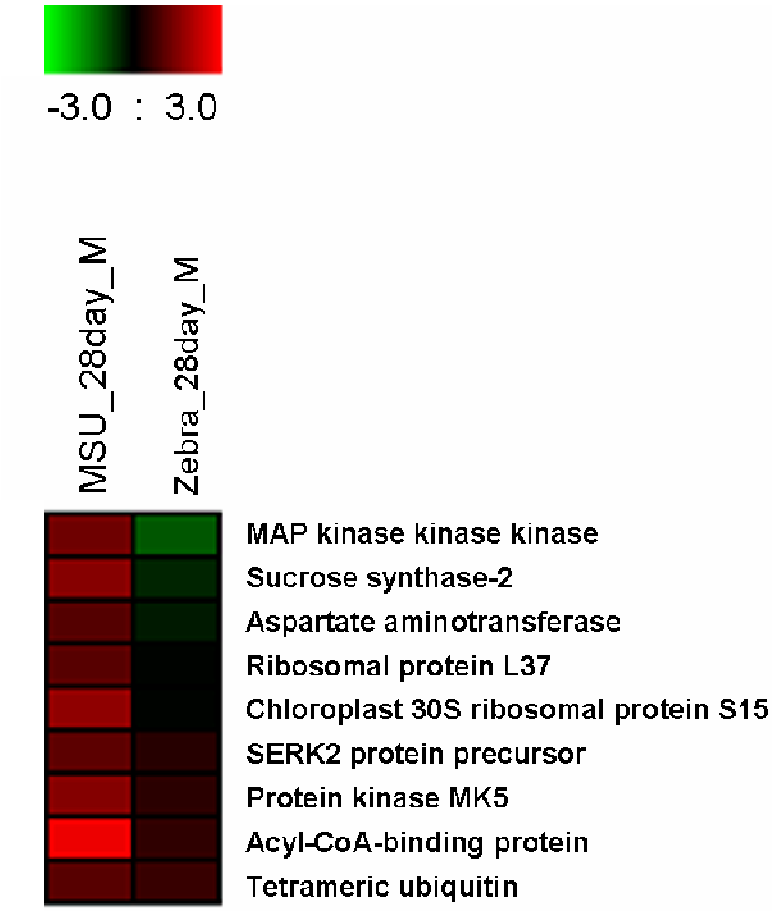


Figure 6. Up-regulated genes (at least 2- fold) in crown tissues of MSU at 28- days after initiation of cold acclimation.

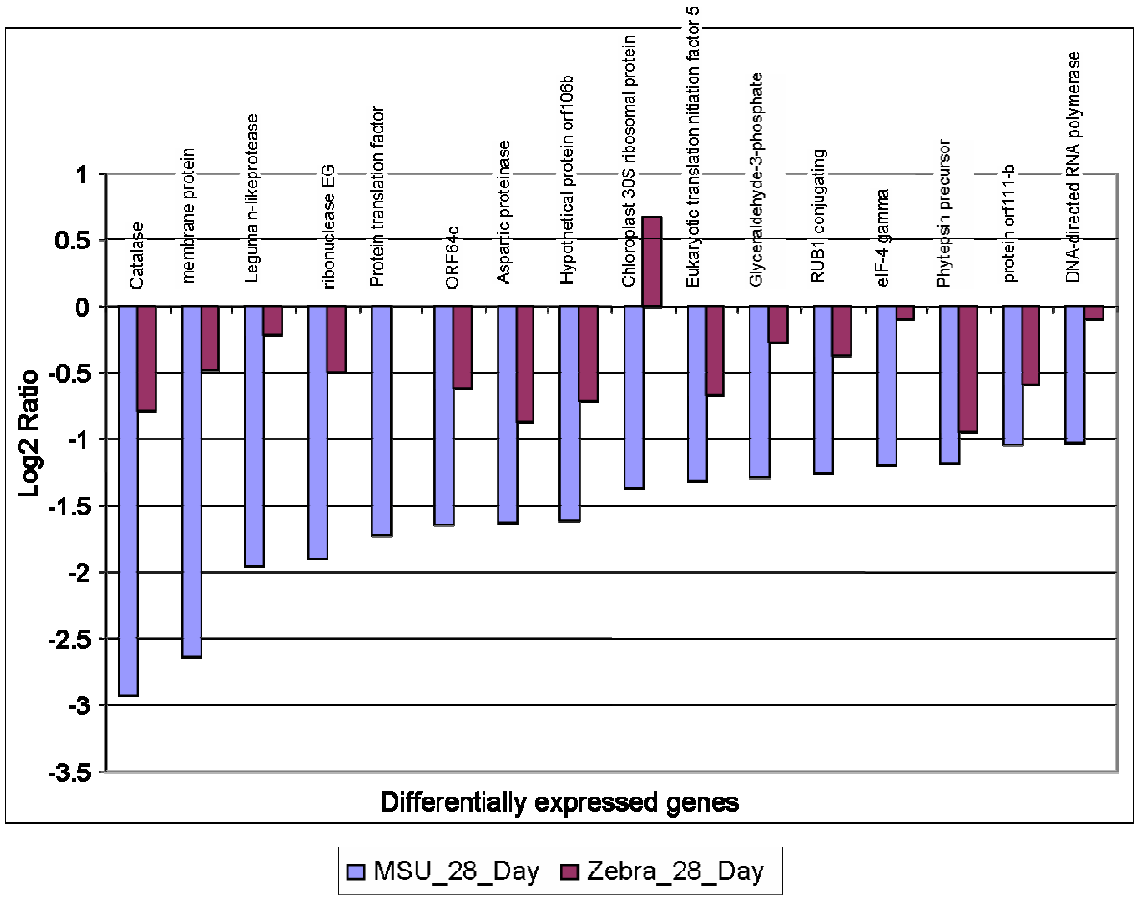


Figure 7. Down-regulated genes (at least 2- fold) in crown tissues of MSU at 28- days after initiation of cold acclimation.

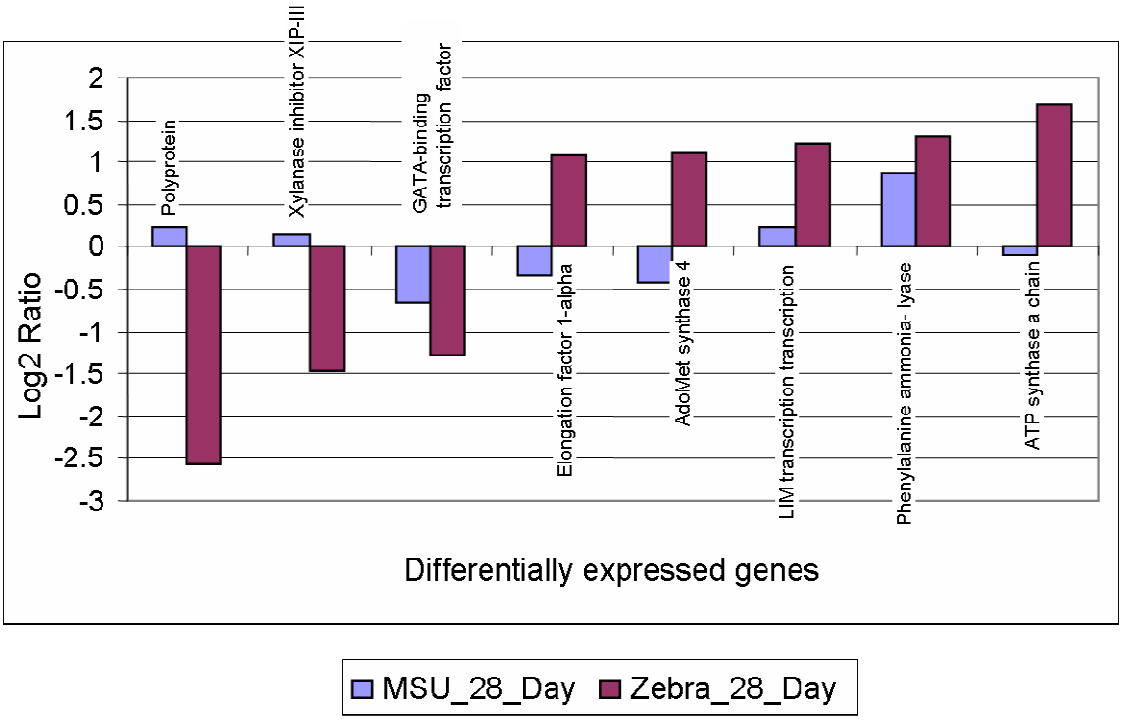


Figure 8. Up or down-regulated genes (at least 2- fold) in crown tissues of Zebra at 28-days after initiation of cold acclimation.

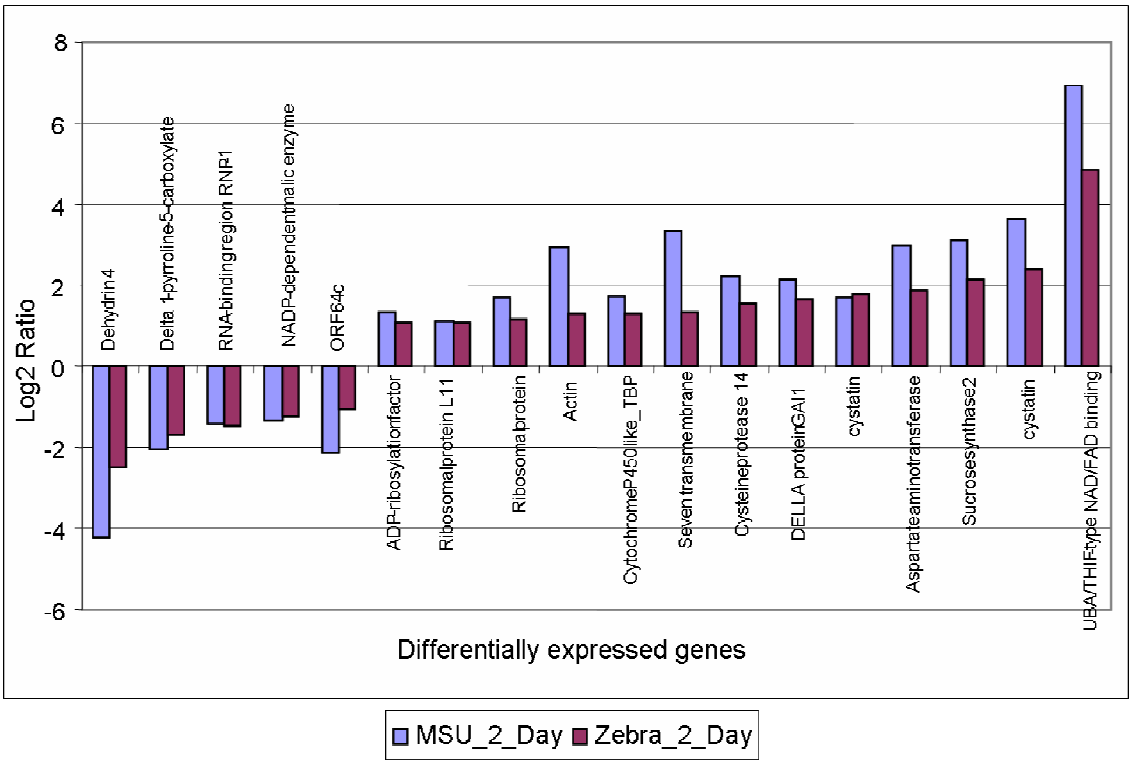


Figure 9. Up or down-regulated genes (at least 2- fold) in crown tissues of both MSU and Zebra at 2- days after initiation of cold acclimation.

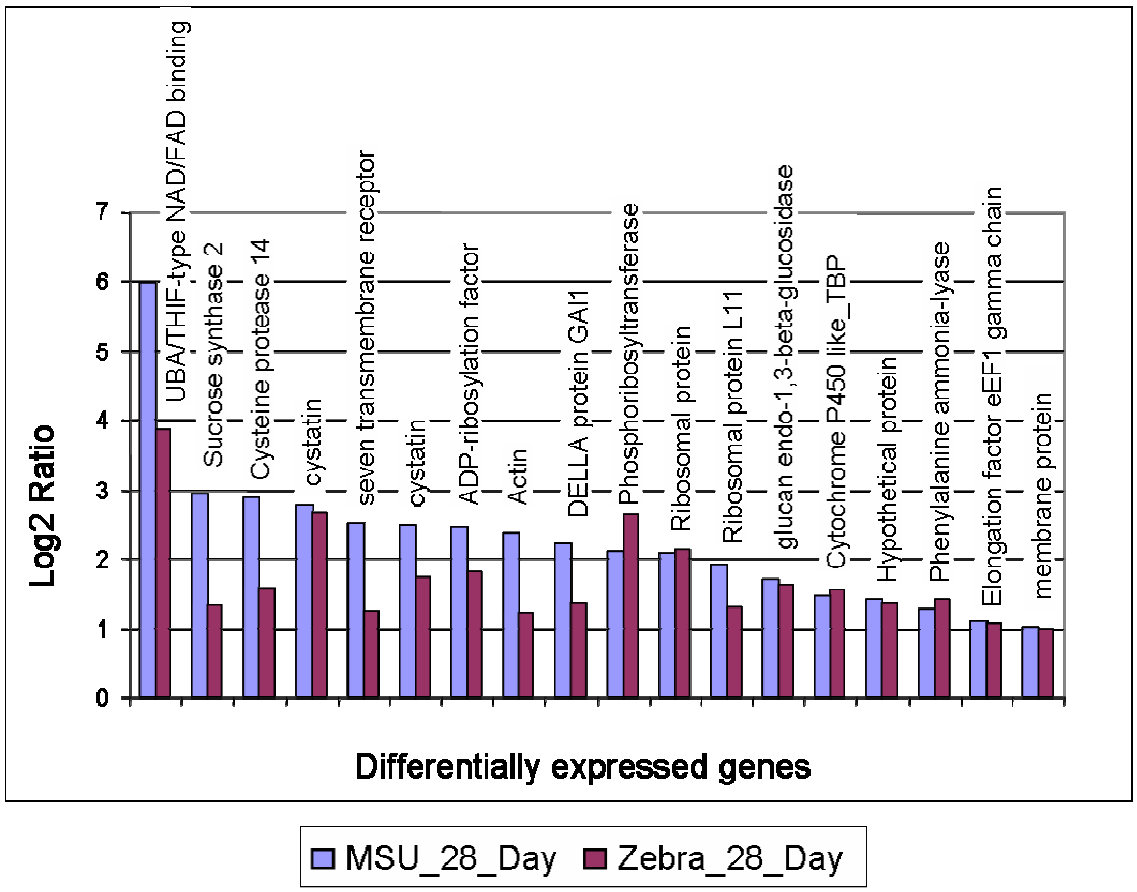


Figure 10. Up-regulated genes (at least 2- fold) in crown tissues of both MSU and Zebra at 28- days after initiation of cold acclimation.

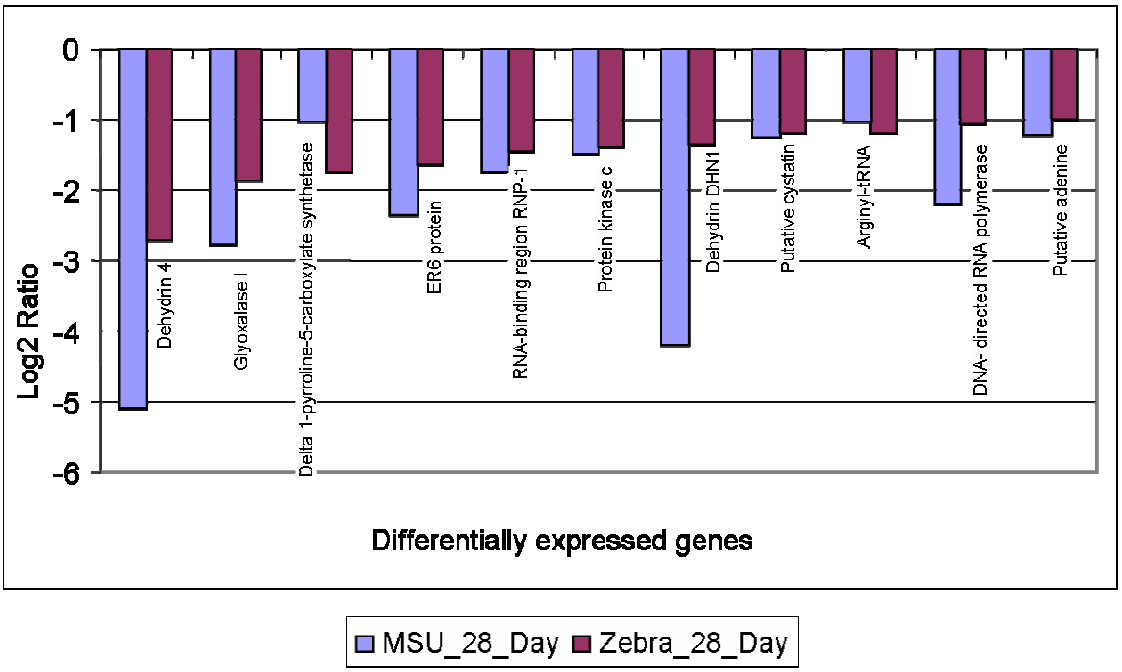


Figure 11. Down-regulated genes (at least 2- fold) in crown tissues of both MSU and Zebra at 28- days after initiation of cold acclimation.

APPENDIX

Genotype	Up Regulated transcripts		Down Regulated transcripts	
	2 days	28 days	2 days	28 days
MSU	480	480	429	621
Zebra	576	768	299	192

Table 5. Number of clones sequenced from the cDNA library

Figure12. Bermudagrass varieties MSU and Zebra

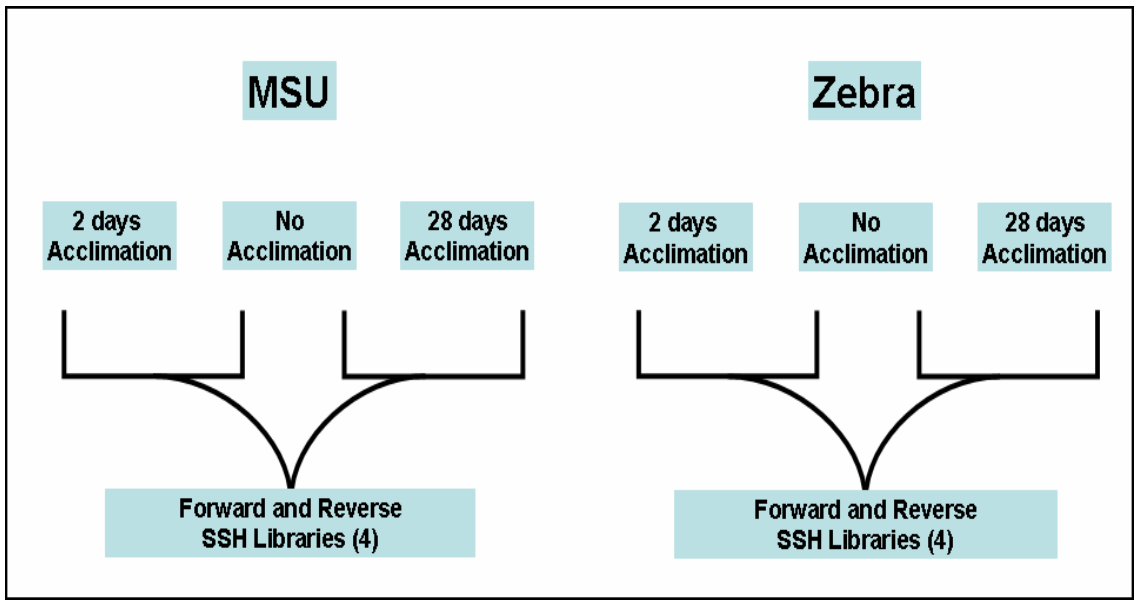


Figure 13. Layout of cDNA construction

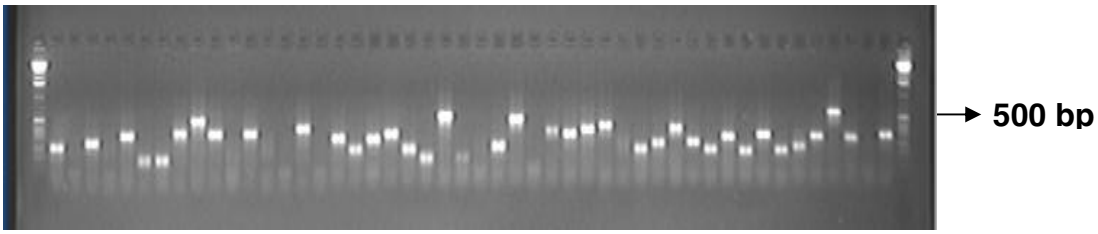


Figure 14. Representative gel of cDNA library screening.

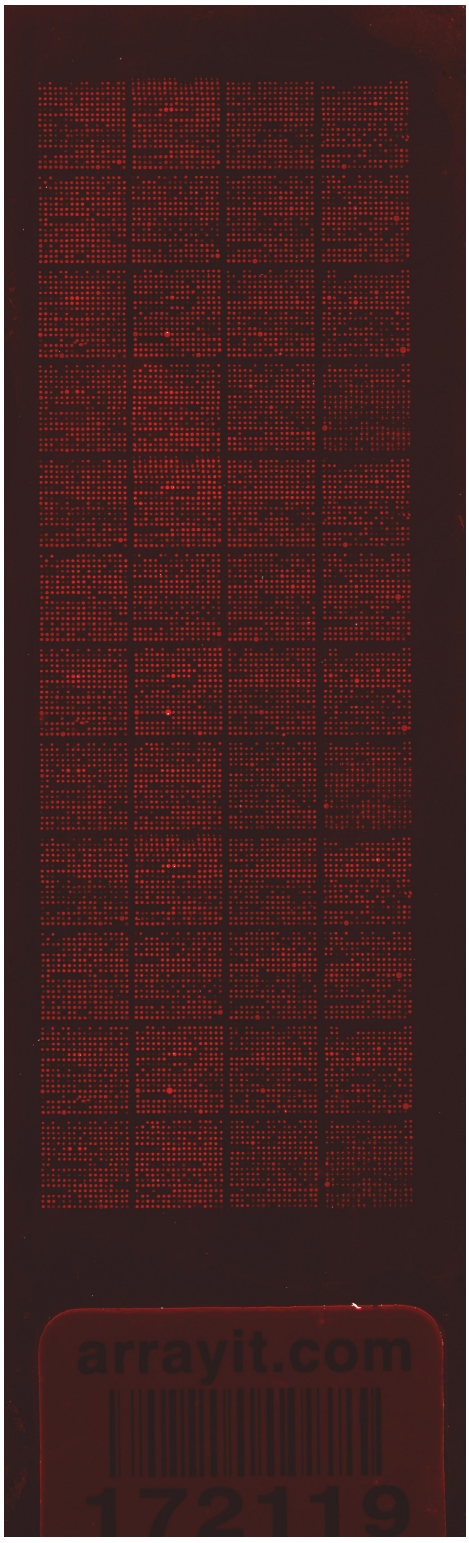


Figure 15. Representative image of Syto 61 stained microarray

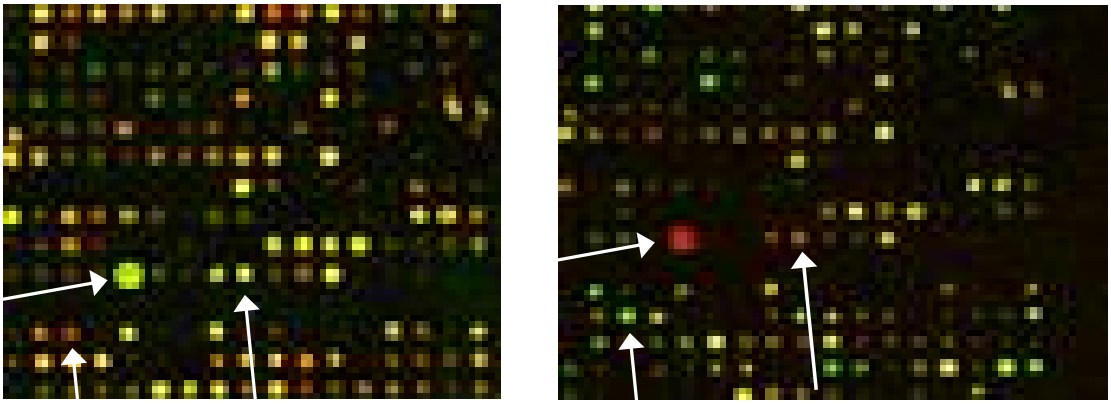
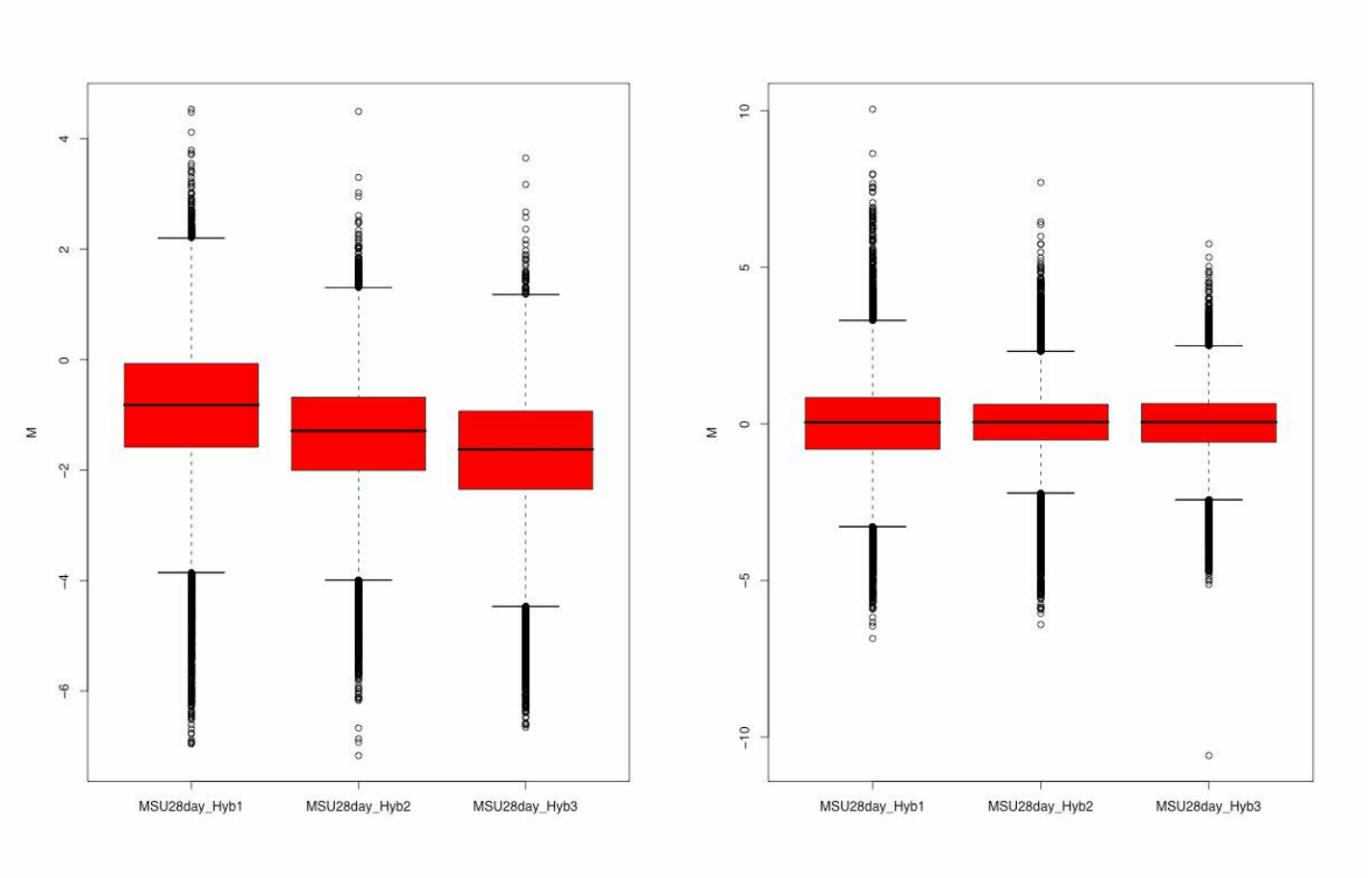
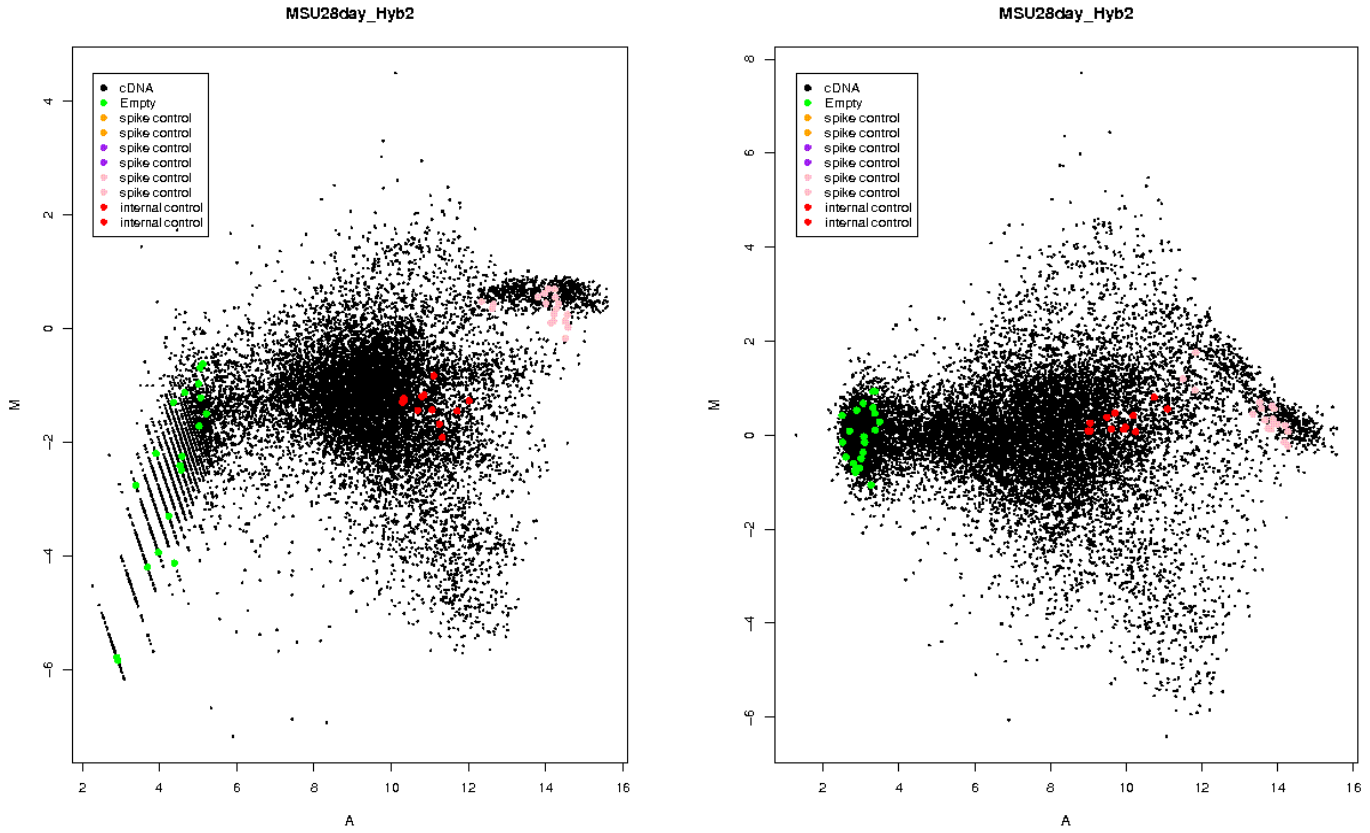


Figure 16. Representative portion of the slides showing dye swap experiment



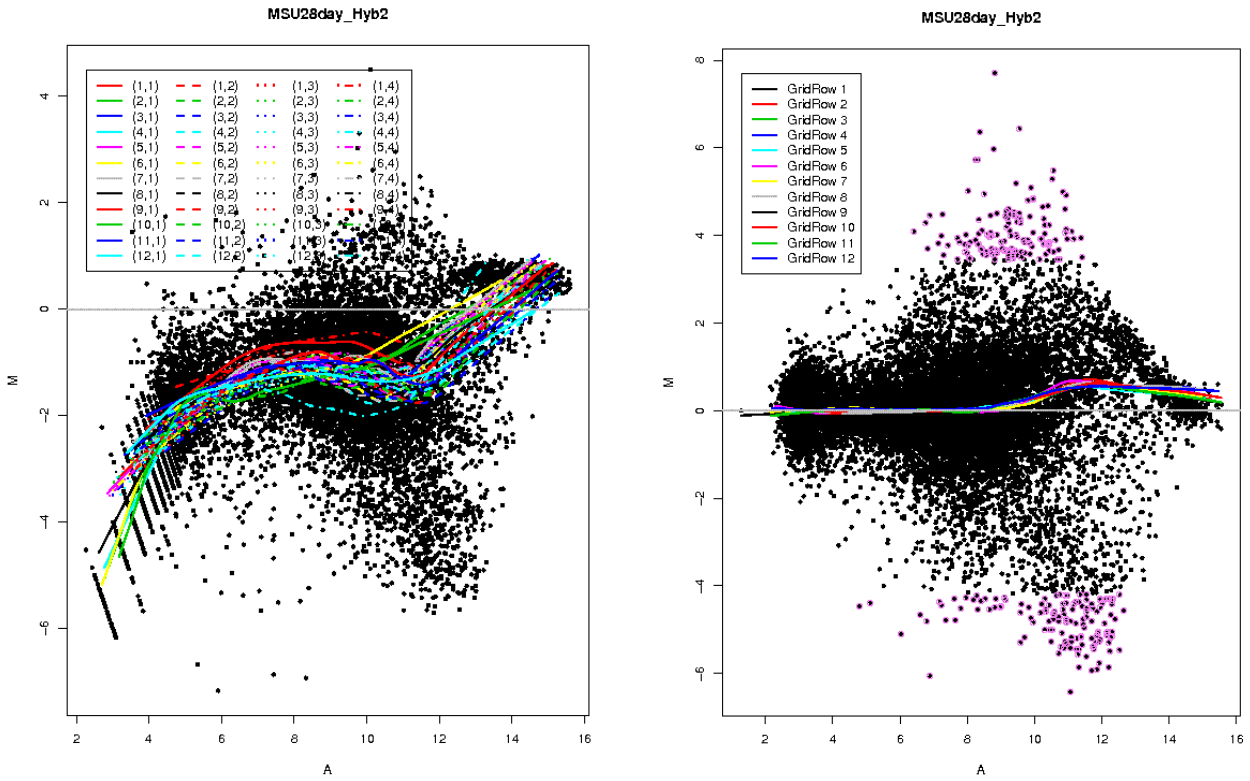
A B

Figure 17. Representative box plot. Figure 6a is before normalization and 6b is after normalization for MSU 28 sample for the three biological replications.



A B

Figure 18. Representative scatter-plot showing control spots. Figure 7a is before normalization and 7b is after normalization for MSU 28 sample for second biological replication. After normalization alignment of empty spots, internal control and spike control ratios near zero can be seen.



A B

Figure 19. Representative scatter-plot showing grids. Figure 8a is before normalization and 8b is after normalization for MSU 28 sample for second biological replication. After normalization alignment of grid rows near zero can be seen.

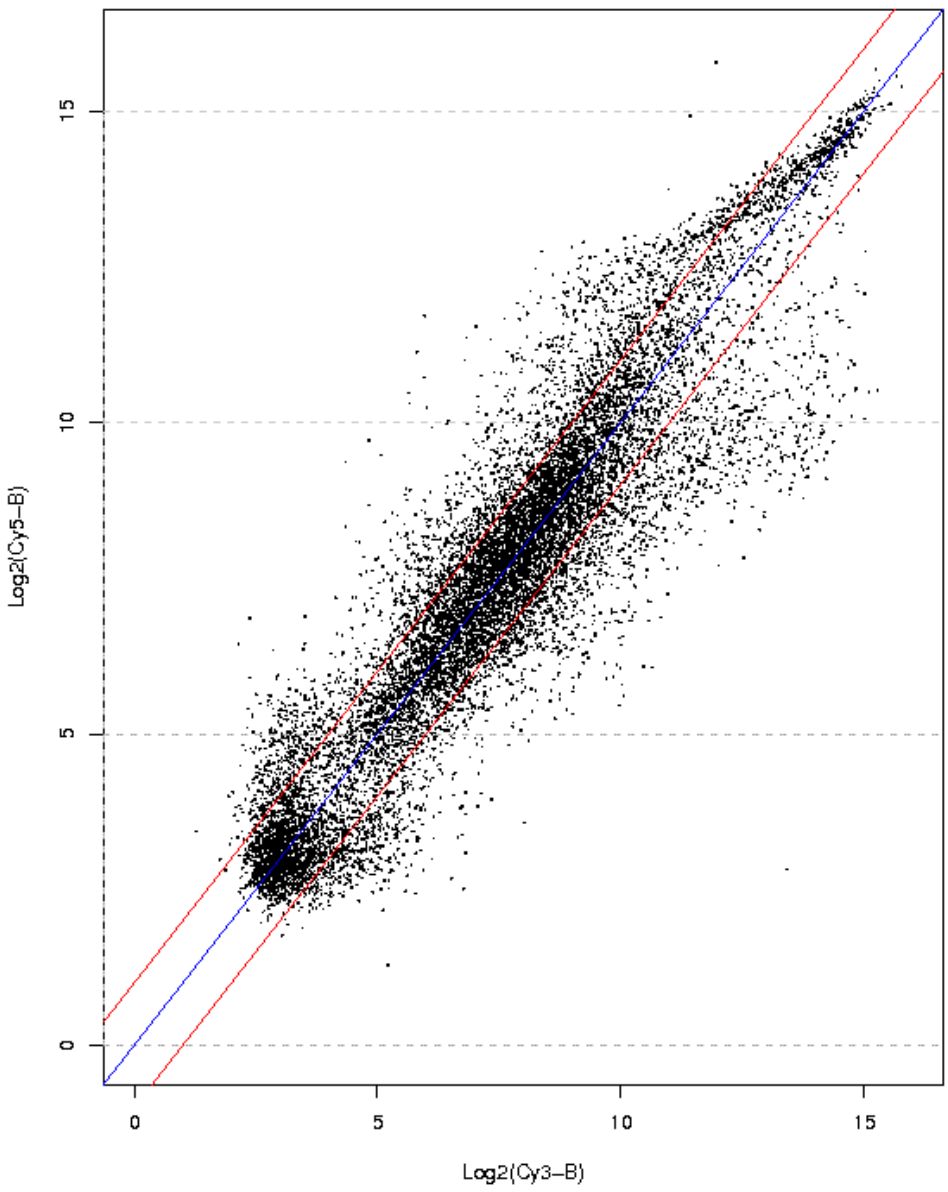


Figure 20. Intensity plot showing genes are not biased to any dye used in the microarray hybridization

VITA

Kalpalatha Melmaiee

Candidate for the Degree of

Doctor of Philosophy

Dissertation: FUNCTIONAL GENOMICS OF COLD TOLERANCE IN BERMUDAGRASS CYNODON DACTYLON L.

Major Field: Plant Science

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Experience: Graduate Research Associate, Department of Plant and Soil Sciences, Oklahoma State University, August 2002 to December 2006. Agricultural Officer, Department of Agriculture, Andhra Pradesh, India, October1999 to March 2001. Research Associate, International Crops Research Institute for the Semi–Arid Tropics (ICRISAT) Asia center, Patancheru, India, September 1996-April 1999.

Professional Memberships: American Society of Plant Biologists, American Association for Advancement of Science, Crop Science Society of America.

Name: Kalpalatha Melmaiee Date of Degree: May, 2007

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: FUNCTIONAL GENOMICS OF COLD TOLERANCE IN BERMUDAGRASS CYNODON DACTYLON L.

Pages in Study: 88 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Science

Scope and Method of Study: Bermudagrass (*Cynodon dactylon* L pers.) is one of the most widely adapted warm-season grasses with its usage and geographic distribution limited by cold temperature. The goal of this research was to identify genes that are differentially expressed during cold acclimation in two bermudagrass genotypes (MSU and Zebra) that differ in tolerance to low temperature stress. Microarray analysis was performed to study gene expression at global level.

Findings and Conclusions: In this study, we were able to identify 566 differentially expressed genes from tissues of MSU and Zebra bermudagrasses. These genes associated with metabolism, signal transduction, dormancy regulation, and senescence related functions. UBA/THIF-type NAD/FAD binding protein, sucrose synthase2, cysteine protease14, putative cystatin , predicted seven transmembrane receptor, actin, ADP-ribosylation factor, DELLA protein GAI1, ribosomal protein, Cytochrome P450 like _TBP were identified as genes associated with cold acclimation. Genes involved in kinase signal cascade (MAPKKK, MK5, SERK2 protein precursor) and in metabolism (sucrose synthase2, ACBP, aspartate aminotransferase) were identified as associated with cold tolerance as they were up-regulated only in cold tolerant MSU during cold acclimation period. Because cold tolerance involves cross talk between multiple pathways, characterizing these genes along with highly induced genes of unknown function may help us better understand their role in cold tolerance. This study constitutes the first large-scale analysis of gene expression pathways during cold acclimation in bermudagrass. Results from this study will serve as a basis for further investigations into the cold acclimation and tolerance mechanisms associated with bermudagrass stress response.

ADVISER'S APPROVAL: Michael P. Anderson