ABSTRACT

BROWN, JESSICA MEI. Improving Selection Methods for Development of Freeze-Tolerant Zoysiagrass Cultivars. (Under the direction of Dr. Susana Milla-Lewis).

Zoysiagrass (*Zoysia japonica* Steud.) is a warm-season perennial turfgrass native to the Pacific Rim region and commonly grown in the southeastern United States for commercial turf and home lawns. Zoysiagrass has a wide general tolerance to abiotic stressors such as drought, shade and salinity, and relatively low input requirements compared to cool-season grasses. Although the most freeze tolerant of the warm-season grasses, its area of adaptation is mostly limited to the warm-humid and transition zones of the US. The leading freeze tolerant cultivar, Meyer, was released in 1951, and although some progress has been made in breeding for freeze tolerance in the species, the pool of cultivars with high levels of freeze tolerance is still limited. In light of the increasing desire for tolerance to extreme temperature stress, breeding efforts stand to benefit considerably from a greater knowledge base behind the physiology of winter hardiness and freeze tolerance.

Controlled environment cold acclimation and freezing tests were utilized to evaluate a 'Meyer x Victoria' mapping population for post-freeze Surviving Green Tissue (SGT) and Regrowth (RG). LSmeans were used to identify putative QTL with MQM mapping. Forty regions of interest were identified for SGT, and forty-one regions were identified for RG across multiple environments, representing between 6.4 and 12.2% of the phenotypic variation (R^2). Thirteen regions of interest overlapped with putative winter injury QTL identified in a field study. The overlapping QTL sequences of interest associated with abiotic stress response and functioned in roles such as plant growth and development, and transcriptional regulation. Overlapping QTL between the winter injury and controlled freezing studies were also compared to putative proteins associated with cold acclimation, where five expressed protein families were observed to be

present in the annotated genes, including ATPase family proteins, NADP superfamily proteins, protein kinase superfamily proteins, and auxin-responsive family proteins. These overlapping QTL of interest and their associated markers may be utilized in future breeding efforts to incorporate freezing tolerance into elite zoysia germplasm for the development of a broader pool of cultivars capable of surviving north of the transition zone.

A transcriptomic approach was utilized to identify genes involved in cold acclimation, a critical step in the development of freezing tolerance. Meyer and Victoria (a freeze susceptible cultivar) were used to assess transcriptomic response to cold acclimation. Cultivars were subjected to either a cold acclimation or non-cold acclimation treatment, then leaf samples were collected for RNA extraction. The Illumina sequencing platform was utilized to obtain high quality pair end reads, which were analyzed in correspondence with the zoysiagrass reference genome to determine differential gene expression. Differential gene expression pathways of interest were determined, after which genes associated with cold acclimation could be identified. Low molecular mass early light-inducible proteins, late embryogenesis abundant proteins, auxin-responsive proteins, and photosystem II proteins all demonstrated significant fold change differences between treatments. Expression of auxin-responsive proteins was found to be associated with cold acclimation and freezing tolerance across multiple studies in transcriptome, proteome, and genome analysis.

These findings strengthen the connection of these genomic regions and genes with increased tolerance to freeze stress, and upon further study, may be utilized to implement a marker assisted selection program with the ultimate goal of facilitating future breeding efforts to increase freeze tolerance in zoysiagrass.

Improving Selection Methods for Development of Freeze-Tolerant Zoysiagrass Cultivars

by
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DEDICATION

To my mom, for teaching me that knowledge is the one thing that can never be taken from you.

BIOGRAPHY

Jessica Mei Brown grew up in Johnstown, Pennsylvania. After graduating high school in 2013, she moved all the way from Pennsylvania to Georgia and worked full time as a machine operator in a factory for a year. In 2014, Jessica entered the University of Georgia and in 2017, she earned a BS in Genetics and a BS in Plant Biology. Following graduation, Jessica began her Masters work on zoysiagrass stress tolerance under Dr. Susana Milla-Lewis at NC State in the Department of Crop and Soil Sciences. In her free time, Jessica took art classes at the NC State Crafts Center, and volunteered with SAFE Haven for Cats and Habitat for Humanity.

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CHAPTER I

LITERATURE REVIEW

HISTORY OF ZOYSIAGRASS

Zoysiagrasses (*Zoysia* spp. Willd.), the different species commonly grouped together and referred to simply as zoysiagrass, are a warm-season turfgrass with a low growth habitat, dense texture, weed resistance, and a general tolerance to several abiotic stresses, including drought, shade, and salinity (Li et al., 2009). Because of these high levels of stress tolerance, less maintenance and chemical additives are needed to maintain zoysiagrass compared to other turfgrasses making it a lower input turf (Duble, 1996). Zoysiagrass has been used as turfgrass since the 1700s and the species are native to the Pacific Rim (Yaneshita et al., 1999). In the United States, the grass is grown in the Southern and transitional climatic zones as both ornamental and recreational turfgrasses. It is environmentally sustainable, and is well-suited for sports turf or golf courses (Patton and Reicher, 2007a) as well as being an attractive option for home and commercial lawn use within its geographic range of adaptation. The North Carolina Sod Producers Association (2016) reported that there are 21 sod producers growing 13 varieties of zoysiagrass in North Carolina.

Zoysia species are allotetraploids with a chromosome number of 40 (2n=4x=40) and a relatively small genome size of 421 Mb for *Z. japonica* Steud. (Forbes 1947; Arumuganathan et al., 1999; Tanaka et al., 2016). Eleven different species are currently recognized in the *Zoysia* genus. Their speciation is attributed to their geographic distribution throughout the Pacific Rim (Guo et al., 2014). Species can be differentiated based on leaf width, texture, color and inflorescence characteristics (Ma et al., 2007). *Zoysia* species are highly variable in terms of their adaptations. *Z. japonica* demonstrates a moderate level of freeze tolerance, while *Z. matrella* (L.) Merr. and *Z. pacifica* (Goudswaard) M. Hotta & Kuroki have relatively poor freeze tolerance (Forbes, 1952; Patton and Reicher, 2007). *Z. matrella* however demonstrates a higher level of tolerance to salinity and insect pests (Patton, 2009), and a moderately higher level of shade tolerance in comparison to *Z. japonica* (Trappe et al., 2011; Wherley et al., 2011). Due to the ability of zoysiagrass species to readily hybridize with one another (Forbes, 1952), and the continuum of genetic variability observed within and among species, there is some supporting evidence that *Z. japonica* and *Z. matrella* are actually ecotypes of the same species rather than separate species (Kimball et al., 2013). *Zoysia japonica* was first introduced to the United States from Korea in 1894 by John M.B. Sill, the consul-general at Seoul. Since its introduction, Z. *japonica*, Z. *matrella*, and *Z. pacifica*, have become the most popular zoysiagrass species in the United States, and have been widely grown since the 1930s (MacDonald and Copeland, 1997; Engelke and Anderson, 2003).

BREEDING FOR FREEZE TOLERANCE IN ZOYSIAGRASS

Freeze tolerance differs among cultivars, with *Z. japonica* cultivars being more widely used within the transition zone due to higher tolerance levels. Meanwhile, Z. *matrella* cultivars have less freeze tolerance, but are desirable for their tough, wear resistant texture. While zoysiagrass species are at the forefront of freezing tolerance for warm-season grasses, their reach does not extend far beyond the transition zone. The introduction of ‘Meyer’, a *Z. japonica* cultivar with the highest levels of winter hardiness of any zoysiagrass, increased the popularity of zoysiagrass during the 1950s (Grau and Radko, 1951). Other winter hardy turfgrasses in the transition zone typically lacked the additional forms of abiotic stress tolerance, such as heat and drought tolerance, which Meyer contained (Grau, 1952). Since Meyer’s introduction, other cultivars, such as ‘Cavalier’ (Engelke et al. 2002a), ‘Diamond’ (Engelke et al., 2002b), and ‘Zorro’ (Engelke and Reinert, 2003) have been released that are superior to Meyer in color, texture, and turf quality, but

Meyer still remains the most reliably freeze tolerant cultivar available on the market (Okeyo et al., 2011). This cultivar is a staple golf course turfgrass in the transition zone; however, its relative lack of winter hardiness in comparison to cool season grasses is a limiting factor in the commercial spread of zoysiagrass north of the transition zone. Therefore, more zoysia varieties with improved freeze tolerance should be developed to extend its' area of adaptation farther north (Fry et al., 2008).

Freeze tolerance in zoysiagrass is a complex trait, and thus far, limited progress has been made in elucidating the genetic components responsible for it. Previous studies in other crops (Bohnert et al., 1995; Lyons et al., 1979) support the notion that a plant's response to cold stress is complex, controlled by multiple genes, and subject to environmental variation. Therefore, separating genetic from non-genetic factors is difficult via traditional breeding methods. A greater understanding of the physiological and metabolic responses of zoysiagrass species to cold acclimation and freezing temperatures is needed to more efficiently identify and select materials with improved freeze tolerance.

Cold acclimation is an important aspect of freeze tolerance in zoysiagrass. Cold acclimation, a natural process that occurs during the transition from fall into winter, refers to the gradual increase in freezing tolerance as a result of the complex multigenic physiological processes that take place when a plant is exposed to low but not freezing temperatures (Guy, 1990). Winter field studies and controlled freeze experiments in zoysiagrass (Patton and Reicher, 2007a; Hinton et al., 2012) indicate that better freeze tolerance is achieved when plants are cold acclimated before being exposed to freezing temperatures. Similar types of studies in other warm-season grasses, including bermudagrass (Anderson et al., 1988; Anderson et al., 1993; Anderson et al., 2003), buffalograss (Qian et al., 2001), St. Augustinegrass (Kimball et al. 2017a, b), and saltgrass (Shahba et al., 2003), support these findings. Of the nine zoysiagrass cultivars with varying LT_{50}s studied by Hinton et al. (2012), all cultivars displayed more consistent freeze tolerance during freezing tests when collected in the winter, after cold acclimation, than samples collected in the spring, which were not cold acclimated.

Although field studies on freeze tolerance provide the most realistic data on winter survivability, field tests take longer to obtain results, due to long wait times for each winter freeze and tend to be more unreliable because of variable weather conditions. Freeze chambers can provide greater consistency and reproducibility of environmental conditions, while still corresponding well with field observations (Anderson and Taliaferro, 2002). In addition, the use of a controlled environment for freeze tests may prove to be a more efficient and cost effective method for assessing cold acclimation and freeze tolerance in warm-season turfgrass species (Qian et al., 2001; Kimball et al., 2017a, b). The consistency noted between freeze chamber tests and field evaluations confirms that similar physiological changes occur during cold acclimation, freezing, and de-acclimation in both types of environments. For zoysiagrass, freezing chambers have successfully determined the low temperature tolerance of a variety of genotypes (Dunn et al., 1999; Patton and Reicher, 2007b; Hinton et al., 2012, Pruitt, 2016). Freeze chambers have also been successfully used to evaluate the roles of various proteins, carbohydrates, and proline via cold acclimation in thirteen zoysiagrass genotypes (Patton et al., 2007a; 2007b).

LINKAGE AND QTL MAPPING IN ZOYSIAGRASS

Molecular markers are useful tools for examining genotypic variation between individuals in a population, as well as constructing linkage maps and mapping quantitative trait loci (QTL). Molecular markers that are closely linked to desirable traits can be used for selection by identifying plant material with useful alleles and thereby reducing the need for phenotypic evaluations (Stuber, 1992). These markers are most commonly single nucleotide polymorphisms (SNPs), segmental insertions/deletions (InDels), or simple sequence repeats (SSRs), also known as microsatellites (Mammadov et al., 2012). Several types of DNA-based molecular marker systems have been used in zoysiagrass in the past, including restriction fragment length polymorphism (RFLP) (Yaneshita et al., 1999), amplified fragment length polymorphism (AFLP) (Cai et al., 2004; Kimball et al., 2012), SSRs (Tsuruta et al., 2005; Cai et al., 2005; Ma et al., 2007; Li et al., 2009; Kimball et al., 2012; Holloway et al., 2018), and SNPs (Holloway et al., 2018; Huang et al., 2016; Tanaka et al., 2016; Wang et al., 2015). The high variability of SSRs make them desirable as genetic markers, in addition to their high rates of polymorphism, codominance, and abundance in the genome. They are PCR based, easily detectable, reproducible, and well suited for high throughput sampling (Kalia et al., 2011). However, their popularity has faded with the advent of the much more affordable SNPs. These markers have become the standard for molecular research, due to their even higher frequency in the genome compared to SSRs, resulting in much higher resolution genetic maps and faster discovery of marker-trait associations (Singh et al., 2013). Using these tools, the genetic diversity of zoysiagrass has been evaluated (Kimball et al., 2012; Kimball et al., 2013), linkage maps have been constructed (Yaneshita et al., 1999; Cai et al., 2004; Cai et al., 2005; Li et al., 2009, Holloway et al., 2018), and QTL have been identified (Jessup et al., 2011; Guo et al., 2014; Holloway et al., 2018).

Linkage maps work by examining recombination patterns of markers in a segregating population in order to link them to QTL of interest (Young, 2000). When a mapping population is exposed to freeze stress, those molecular markers linked to a QTL of interest will display lower recombination rates compared to those of unlinked markers. This difference in recombination rate is due to the close proximity of the marker to the associated gene on the chromosome (Mohan et al., 1996). Marker assisted selection (MAS) can be useful to improve the efficiency of selection by using DNA technology to find desirable traits, without having to wait for them to be expressed in the plant, or disentangling environmental from genetic factors. The selection of plant materials containing markers which co-segregate with a trait of interest allows those traits to be crossed into a desired genetic background. This method allows selection of superior genotypes associated with freeze tolerance with more speed and precision than was previously possible in the field (Collard et al., 2005). However, for MAS to work, molecular markers that are located near the gene of interest need to be identified. Holloway et al. (2018) developed a pseudo-F_2 mapping population of 175 individuals by crossing freeze tolerant cultivar Meyer with freeze susceptible ‘Victoria’. A high-density linkage map which contained over 2400 single nucleotide polymorphism (SNP) and SSR markers was generated. This map was subsequently used in association with phenotypic data on winter injury taken in West Lafayette, IN and Laurel Springs, NC from 2015 to 2016 with the goal of identifying genomic regions controlling freeze tolerance. Seven regions of interest were identified on chromosomes 8, 11, and 13. Sequencing analysis within these regions identified proteins related to abiotic stress tolerance; a good indicator that they may be true genic locations. These QTL must be further investigated with additional comprehensive freeze tolerance tests under controlled environmental conditions and validated in other populations before a marker assisted selection program can be implemented.

The first *Zoysia japonica* reference genome was developed in 2016 by Tanaka et al. (2016). Based on the cultivar ‘Nagirizaki’, the 334 Mb sequence predicts 59,271 protein coding genes. The reference genome was compared to the genome of three other species in the Poaceae family, those of *Brachypodium distachyon,* rice (*Oryza sativa*), and sorghum (*Sorghum bicolor*) in order to determine similarities across the family and aid in the identification of shared genetic material. An additional accession of *Zoysia japonica*, the cultivar 'Kyoto', as well as two accessions each of *Z. matrella* (L.) Merrill, and *Z. pacifica* (Goudswaard) M. Hotta & Kuroki [syn. Z. matrella (L.) Merr. var. pacifica Goudswaard] were also sequenced to investigate the genetic diversity of the species (Tanaka et al., 2016). The 'Nagirizaki' genome has an estimated 86% coverage with 3,660,698 common SNPs shared between two or more sequenced zoysiagrass accessions, out of the 7,424,163 total SNPs (Tanaka et al., 2016). Information on this reference genome will greatly aid in the identification of specific genes, such as those relevant to freezing tolerance that might be valuable in breeding.

PROTEIN EXPRESSION IN ZOYSIAGRASS

Our understanding of the physiological changes that zoysiagrass undergoes during cold acclimation is limited (Hinton et al., 2012). Meyer and Diamond (Zoysia matrella (L.) Merr.), were analyzed using electrolyte leakage under cold treatment to confirm the differential response to a freeze stimulus. After 28 days of cold stress treatment, freezing tests observed a sharp increase in relative electrolyte leakage (REL) for Diamond, but a much smoother curve for Meyer, confirming the greater freeze tolerance of Meyer. However, both cold acclimated cultivars displayed increased freeze tolerance in comparison to their non-acclimated counterparts (Xuan et al., 2013). In addition to REL, certain proteins are believed to be activated or upregulated during cold acclimation, which in turn act to enhance freeze tolerance. To gain a better understanding of the physiological and metabolic nature of freeze tolerance in zoysiagrass, Patton et al (2007a; 2007b) compared levels of compounds suspected to be associated with freeze tolerance, such as carbohydrates, organic acids, and soluble proteins at various times in the cold acclimation process. They found that carbohydrates such as glucose and sucrose increase in zoysiagrass stolons during seasonal temperature decreases, similar to what is known to occur in other warm-season turfgrasses such as saltgrass (*Distichlis spicata* (L.) Greene) (Shahba et al., 2003), centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) (Cai et al., 2004) and buffalograss (*Bouteloua dactyloides* (Nutt.) Columbus 1999) (Ball et al., 2002). However, a correlation between LT_{50} and sucrose concentration in several cold acclimated grasses showed no relationship between sucrose concentration and freezing tolerance. Despite this result, there is evidence to suggest that genotypes with poor freezing tolerance produce more soluble sugars during cold acclimation than genotypes with excellent freeze tolerance. However, total reducing sugars likely play a minute role in total freezing tolerance due to their low concentrations (Patton et al., 2007b). On the other hand, proline concentration is known to increase dramatically during cold acclimation due to an increase in synthesis. Linear and logarithmic regressions suggest an improvement in freeze tolerance in correlation with increasing proline concentration (Patton et al. 2007a). Santarius (1992) found that proline protects membranes using hydrophobic interactions, osmotic adjustment, and decreased water potential, but it is less efficient as temperatures decline. More in depth and comprehensive studies of the proteins involved in cold acclimation as well as their functions are needed before accurate and efficient selection of associated genes can occur in zoysiagrass.

One such study, by Xuan et al. (2013) performed quantitative analysis of protein accumulation in cultivars Meyer and Diamond during cold acclimation. Seventy proteins spots with a fold change of 2.0 or more were identified. Forty-five of those were functionally identified as playing a role in key functional protein classes such as redox homeostasis, signal transduction, photosynthesis, and energy metabolism among others. A close examination of the proteins involved led to several hypotheses to explain Meyer’s superior freeze tolerance. Upregulation of proteins involved in antioxidant defense, including antioxidant enzymes and resistance related proteins, during cold stress in Meyer indicated that this cultivar has a greater ability to acquire hydrogen peroxide than Diamond. Under cold acclimation, enhanced activity in the calcium and ethylene signaling pathways was observed in stolons of Meyer, while signaling pathways in Diamond were typically inhibited. Rubisco accumulation increased in Meyer, but decreased in Diamond, suggesting that an increase in photosynthesis during cold acclimation may play a role in freeze tolerance. Cold acclimation also led to the inhibition of protein folding and the enhancement of protein biosynthesis and proteolysis in Meyer stolons, suggesting that an increased protein turnover rate may be necessary for proteolysis to remove misfolded polypeptides and survive freezing stress. Overall, the upregulation of proteins in Meyer compared to Diamond suggests that a greater energy supply is needed to respond to cold stress in zoysiagrass stolons. Meyer's ability to respond to freezing stress most likely is related to its increased ROS scavenging ability, photosynthesis, protein synthesis, proteolysis, and its greater energy reserves (Xuan et al., 2013). Further research is needed on these findings, but they provide a compelling case for which proteins and pathways play a role in freeze tolerance.

Pruitt et al. (2016) began investigating the relationship between cold acclimation, protein expression, and freeze tolerance in zoysiagrass. Crown tissues of freeze tolerant 'Meyer' and freeze susceptible 'Victoria' (*Z. japonica* Steud.) were harvested for proteomic analysis after a four week acclimation period and a full freeze cycle according to the protocol of Patton et al (2007). Proteomic analysis of these tissues at the UMass Turfgrass Physiology Laboratory (Amherst, MA) identified 21 and 15 protein spots with increased abundance (up-regulated) for Meyer and decreased abundance (down-regulated) for Victoria, respectively, during cold acclimation (Brown et al., 2020). In addition, nine protein spots were found to be differentially expressed between the two cultivars in response to cold acclimation. Of the proteins identified, 31% were involved in transcription, 21% in metabolism, 10% in energy, 10% in storage, and the rest scattered among other functions. An increase in expression of proteins associated with photosynthesis, protein biosynthesis and proteolysis, energy metabolism and antioxidant defense are of particular interest for future analysis (Xuan et al., 2013). More work needs to be conducted to identify the roles of these proteins in freeze tolerance. Further investigation of these physiological changes, particularly changes in protein expression during cold acclimation, may shed light on which phenotypic traits may be useful targets for selection of more freeze tolerant varieties (Patton et al., 2007a; 2007b).

TRANSCRIPTOMIC STUDIES IN ZOYSIAGRASS

Transcriptomic analysis can be a useful tool in identifying differential genome-wide gene expression profiles following exposure to an environmental stressor, potentially drastically increasing the breadth of knowledge surrounding plant/environment interactions. Transcriptomics was developed in the late 1990's as a tool to study the expression of RNA transcripts at a particular snapshot in time. As a transient intermediate in gene expression, studying the RNA transcriptome can allow greater insight into which cellular processes are active and dormant under specific conditions. While microarrays originally dominated the field, the use of higher throughput RNA-sequencing, headed by Illumina technology, has revolutionized transcriptome sequencing (McGettigan, 2013). RNA-based markers have been utilized to examine global transcriptome profiles in response to cold stress in zoysiagrass (Wei et al., 2015) and bermudagrass (Xu et al., 2015) using Illumina sequencing technology.

Wei et al. (2015) examined the effects of cold stress (4°C) on zoysiagrass cultivar Meyer for 0 h, 2 h, and 72 h. The total RNA was extracted and the processed reads were aligned to several protein databases in BLAST. The species' with the greatest similarity in sequence identity were foxtail millet (*Setaria italica*) at 52.1% similarity, and sorghum (*Sorghum bicolor*) at 21.9% similarity. Gene ontology classification allowed identification of the possible function for the 16,206 unigenes from Meyer found in the study, which were primarily related to biological processes, cellular components, and molecular functions. Alignment to the EuKaryotic Orthologous Groups (KOG) database identified the top three functional categories as signal transduction mechanisms, general function prediction, and post-translational modification/protein turnover/chaperon related.

Transcription factors play a key role in abiotic stress response due to their role as upstream regulatory proteins. A study in zoysiagrass identified approximately 2,000 transcription factors in the unigenes, the most notable of which are bHLH, MYB-related family, ERF, NAC, and WRKY families, all of which contribute up to 37.2% of the identified transcription factors in Meyer (Wei et al., 2015). A similar study in bermudagrass identified 34 upregulated genes that had nine times higher expression in cold acclimated compared to non-acclimated plants. Of these highly expressed genes, the largest fold changes were AP2, NAC, and WRKY family related genes. All three are transcription factors that have been previously studied in Arabidopsis for their role in response to cold acclimation (Zhu et al., 2015).

Wei et al (2015) refined the ability to distinguish physiological changes of important antioxidant enzymes induced by cold acclimation. These included a 14.7% increase in peroxidase (POD) activity after 2 h, and a 10.8% increase in superoxide dismutase (SOD) activity after 72 h. Notably, the activity of catalase (CAT) and POD decreased significantly, 36% and 13.8%

respectively, after 72 h of cold (4^{o}C) exposure. They concluded that cold acclimation can induce cell desiccation as well as oxidative stress, and plays a role in the inhibition of photosynthesis and cell-to-cell substance transport in cultivar Meyer. Gene expression quantification and differential expression analysis revealed that genes related to starch hydrolysis, proline synthesis, SOD activity, and DREB response pathways among others were upregulated in response to cold treatment. A recent study by Zuo et al. (2019), which investigated the effects of the cold and freeze tolerance in zoysiagrass through overexpression of the ICE1 gene, observed a significantly higher increase in SOD and POD in the transgenic samples when exposed to cold stress at 4^{o}C, followed by freezing stress at -6^{o}C. Though the study made no conclusions on freeze tolerance, it concluded that the overexpression of ICE1 suggested an increase in cold tolerance, and also adds strength to the importance of SOD and POD in conferring tolerance when exposed to cold stress (Zuo et al., 2019), and likely plays an important role in freeze tolerance as well. These identified genes will be important focal points for further research and may lead to strong targets for breeding through marker-assisted selection.

Transcriptomic analysis allows for transcript identification and may reveal genes responsible for biological processes such as cold acclimation. Therefore the transcriptomic, and by extension, genomic diversity of zoysiagrass can be evaluated. This tool is particularly useful in combination with genomic analysis (Xu et al., 2015, Santos et al., 2018) to identify candidate genes responsible for physiological processes of interest. Transcriptomics excels in the identification of synthesized transcripts, mRNA, in response to a stimulus. RNA-sequencing has the potential to discover and identify genes involved in abiotic stress response, such as cold acclimation, by comparing the gene expression profiles of control and cold acclimated samples. Identification and mapping of differentially expressed transcripts of cold regulated genes would provide a valuable resource for breeding efforts of freeze tolerant cultivars in zoysiagrass.

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CHAPTER II

IDENTIFICATION OF QTL ASSOCIATED WITH COLD ACCLIMATION AND FREEZING TOLERANCE IN Z. JAPONICA

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Identification of QTL associated with cold acclimation and freezing tolerance in *Z. japonica*

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ABSTRACT

Zoysiagrasses (*Zoysia* spp.), a relatively low input warm-season turfgrass; have grown in popularity in the United States since their introduction in the 1890's. Over thirty improved zoysiagrass cultivars were released in the past three decades, but many lack freeze tolerance and their use is limited to warm-humid climates. In light of the desire to increase tolerance to freezing stress, breeding efforts stand to benefit considerably from a greater knowledge base underlying the physiology of winter hardiness and freeze tolerance. In the present study, controlled environment acclimation and freezing tests were utilized to evaluate a 'Meyer x Victoria' mapping population for post-freeze Surviving Green Tissue (SGT) and Regrowth (RG). LSmeans were used to identify putative quantitative trait loci (QTL) with multiple QTL model (MQM) mapping. Forty regions of interest were identified for SGT, and forty-one regions were identified for RG, accounting for between 6.4 and 12.2% of the phenotypic variation (R^2). Thirteen regions of interest overlapped with putative winter injury QTL identified in a previous field study. Overlapping QTL between the winter injury and controlled freezing studies were compared to putative proteins associated with cold acclimation, where five expressed protein families were observed to be present in the annotated genes, including ATPase family proteins, NADP superfamily proteins, protein kinase superfamily proteins, and auxin-responsive family proteins. Following further validation, these QTL and their associated markers may be utilized in future breeding efforts for the development of a broader pool of zoysiagrass cultivars capable of surviving in cool-humid climates.

Keywords Zoysiagrass, Freeze tolerance, Cold acclimation, Genetic map, QTL, SNP

INTRODUCTION

Zoysiagrasses (*Zoysia* spp. Willd.) are warm-season, allotetraploid (2n = 4x = 40) turfgrasses desirable for their low growth habit, density, fine texture, and general tolerance to drought, shade and salinity and suitable for golf and sports turf, home and commercial lawn use (Li et al., 2009). Zoysia species, particularly *Z. japonica*, are known to have the highest levels of freeze tolerance of the warm-season grasses, but their lack of winter hardiness and freezing tolerance in comparison to cool-season grasses limits its use north of the transition zone (Patton and Reicher, 2007). As evidenced by the current most freeze tolerant cultivar 'Meyer', which was released in 1951 (Grau and Radko, 1951), limited progress has been made through traditional breeding methods to increase the level of freezing tolerance in the species. Only two cultivars, 'Chinese Common' and 'Zenith' have been determined to have comparable winter hardiness to Meyer (Patton and Reicher, 2007), so there is ample room for improvement to broaden the pool of winter hardy and freeze tolerant cultivars in order to expand the commercial range of zoysiagrass north of the transition zone.

Plant response to cold stress is highly complex, controlled by multiple genes and highly influenced by variations in the environment from year to year (Lyons et al., 1979, Bohnert et al., 1995), though cold acclimation, defined as exposure to low, non-freezing temperatures plays a crucial role in increasing freezing tolerance (Guy, 1990). Due to the quantitative nature of freezing tolerance, there is a need for greater knowledge on the genetic control of the trait in order to improve selection criteria beyond phenotypic evaluations. Molecular breeding strategies enable the improvement of such quantitative traits through the use of marker assisted selection (MAS) to identify and select quantitative trait loci (QTL), and is already being used to improve zoysia cultivars (Chandra et al., 2017).

Molecular markers are small sections of DNA such as single nucleotide polymorphisms (SNP's) and simple sequence repeats (SSR's) in the genome associated with a specific location. The markers which correlate to an observed trait are QTLs. In zoysiagrass, QTLs have been identified for salt tolerance (Guo et al. 2014) and fall armyworm resistance (Jessup et al. 2011; Huang et al. 2016). The physiological components of winter hardiness in zoysiagrass have also been examined (Rogers et al. 1975; Dunn et al. 1999; Hinton et al. 2012; Patton and Reicher 2007, Holloway et al., 2018), but little progress has been achieved in identifying the genetic components of the trait. A genetic linkage map could greatly enhance the ability to identify QTLs for a trait of interest. The first linkage map for zoysia utilized RFLPs (Yaneshita et al., 1999), and were refined as technology advanced by using AFLP's (Cai et al., 2004) and SSR's (Cai et al., 2005, Li et al., 2009) for more detailed genetic analysis (Tsurata et al., 2005, Ma et al., 2007). Recently, a high density SNP-based linkage map of zoysiagrass was developed by Holloway et al. (2018). The map contains 2306 SNP markers, and 112 SSR markers resulting in 77% coverage across the genome. SSRs were used for their ease of detection and reproducibility, as well as for their reliability in high-throughput sampling (Kalia et al., 2011), while SNPs were utilized for their abundance in the genetic sequence and their efficiency as a next generation sequencing technique (Poland et al., 2012).

Few studies have identified genes related to freezing tolerance in grass species (Alm et al., 2011, Kimball et al., 2018, Poudel et al., 2019), including in zoysiagrass, St. Augustinegrass (Stenotaphrum secundatum (Walt.) Kuntze) and switchgrass (Panicum virgatum L.). Holloway et al. (2018) utilized a high density linkage map in conjunction with phenotypic data taken from winter field trials across six environments to identify putative QTL associated with winter injury in zoysiagrass. Of the identified QTL, fifty-six of them were responsible for between 7.2-21.6%

of the phenotypic variance for the four traits of interest: winter injury, establishment, pre-freeze turf quality, and post-freeze turf quality (Holloway et al., 2018). These regions were investigated for their role using the 'Nagirizaki' reference genome (Tanaka et al., 2016). Regions of particular interest on chromosome 8 were related to leucine-rich repeat receptor-like protein kinases, Rho quinine proteins, mitogen-activated kinases, G-type lectin S-receptor-like serine/threonine kinases, and DREB response element (DRE) family related proteins. On chromosomes 11, regions of interest were related to L-type lectin domain containing receptor kinases, SH3 domain containing proteins, DRE family proteins, and WD-repeat proteins. On chromosome 13, a L-type lectin domain containing receptor kinase was also detected, as well as auxin response factors (Holloway et al., 2018). In switchgrass, Poudel et al. (2019) identified several freeze tolerance genes and proteins through the use of comparative genomics, including COR47, DREB2B, zinc finger-CCCH, WRKY, GIGANTEA, HSP70, and NRT2. C-repeat binding factors (CB) related proteins are of particular interest as they play a key role in regulating cold responsive genes (COR) via dehydration-responsive elements (DRE) (Stockinger et al., 1997).

Controlled environmental evaluation in greenhouses and laboratories provides an important supplement to field studies by identifying QTL associated specifically with the traits of interest without needing to account for confounding environmental factors. QTL identified across field and controlled studies may provide greater insight into the basis of response to stress. The goal of this study was to further investigate the findings of Holloway et al (2018) by evaluating the same Meyer x Victoria mapping population for freeze tolerance under controlled environmental conditions in the greenhouse and laboratory in order to avoid escapes, as well as to include acclimation treatments for a better assessment of true freeze response.

MATERIALS AND METHODS

Plant Material

A pseudo F_2 mapping population of 175 individuals was generated from a cross between 'Meyer' (Z. *japonica*), the leading zoysiagrass freeze tolerant cultivar, and 'Victoria' (Z. *japonica*), a freeze susceptible cultivar (Holloway et al., 2018). The population was maintained at North Carolina State University (Raleigh, NC) under greenhouse conditions in pots filled with Fafard 4P potting mix (ConradFafard Inc., Agawam, MA). The plants were vegetatively propagated in 2.5-cm-diameter, 12-cm-deep cone-tainers (Ray Leach Cone-Tainers, Stuewe & Sons, Inc., Corvallis, OR) filled with USGA grade sand by taking a single stolon or rhizome containing root, crown, and shoot material. Plants were maintained at greenhouse temperature, 27 ± 2 ^{o}C and fertilized every two weeks with a 24:8:16 NPK solution of Miracle-Gro Water Soluble All Purpose Plant Food (The Scotts Miracle-Gro® Company, Marysville, OH).

Cold Acclimation and Freezing Tests

The 175 individuals of the mapping population, as well as nine commercial checks differing in their levels of freezing tolerance Meyer, Victoria, Diamond, Jamur, Empire, Zeon, Zenith, L1F, and Chinese Common were evaluated under controlled environmental conditions according to the protocol of Patton and Reicher (2007a). The evaluation included two acclimation treatments, cold acclimated (CA) and non acclimated (NA), and two freezing temperature treatments, -8^{o}C and -11^{o}C, based on the LT_{50} of the parent cultivars. The experiment was replicated a total of six times. The 184 total samples were randomized within plastic trays, fitting up to 200 cone-tainers each, leaving gaps in the center to promote air flow during freezing. Plants were allowed to establish for six weeks in the greenhouse at 27±2^{o}C. Fertilization ceased two weeks before cold acclimation began. Following greenhouse establishment, two trays of each replication were placed in a temperature controlled growth chamber for four weeks at 8/2°C day night cycles, the CA treatment. During this four week time period, the other two trays of each replication, comprising the NA treatment, were kept in an identical temperature controlled growth chamber at a consistent 22°C with the same day night cycles, in order to minimize environmental differences between the acclimation treatments. The growth chambers contained a consistent light emitting diode (LED) which have maintained a 10-h photoperiod of 300 µmol $m^{-2}s^{-1}$ active radiation (Anderson et al., 1988).

Following a four week period of an acclimation treatment, CA and NA plants were placed in freezers, one tray per freezer per treatment. Trays were randomized among the freezers for each run. Due to the capacity of the freezer and the size of the experimental populations, the full six replicate experiment was conducted over several runs. Watering was withheld for five days before freezing, in order to reach standardized water saturation levels in each cone-tainer, and in turn, maintain the experimentally determined time necessary to remove latent heat from the sand. Freezing temperatures were -8°C, and -11°C, based on the reported LT_{50}s of the parents (Patton and Reicher, 2007a; Hinton et al., 2012; Pruitt 2016). In order to minimize desiccation during the freezing period, each tray was covered in a plastic bag (Kimball et al., 2017a) before being placed into the modified commercial freezers.

The temperature was initially lowered to 0°C. Ice shavings were then sprinkled evenly over all the samples, in order to promote freezing and avoid supercooling (Kimball et al., 2017a). Then, the plants were held at -3°C for 21 hours to remove any latent heat (Anderson et al., 1993). Thermocouple sensors were placed in randomly chosen cone-tainers in each freezer in order to verify that the target temperatures were reached and maintained for the duration of the experiment. Then, the temperature was gradually decreased at a rate of -1°C hr^{-1} until reaching the target temperature, which was maintained for 3 hours. Afterward, the temperature was increased at a rate of 2°C hr^{-1} until reaching 10ºC. All trays were left to thaw at this temperature overnight before being returned to the greenhouse to monitor recovery.

Surviving green tissue (SGT) and regrowth (RG) ratings were collected once a week for six weeks post-freezing. SGT represents the severity of freezing injury that a plant sustains during freezing tests, and was rated based on percent of green leaves and nodes, on a scale of 0-5, with 0=all tissue and nodes were brown, 1=0-25%, 2=25-50%, 3=50-75%, 4=75-99%, and 5=100% green leaves and nodes. Regrowth, which is based on the percent of new green leaves and nodes, was rated on the same 0-5 scale, with a recovery rating of 0=0% RG and 5=100% RG of the turfgrass. All ratings were then converted into percentages for the sake of data reporting and interpretation (Kimball et al., 2017).

QTL Analysis

The SGT and RG data were analyzed using a mixed model (PROC MIXED) in SAS 9.4 (SAS Institute; Cary, NC). Least squares means were calculated for the mapping population without commercial checks, treating identity as a random effect. The restricted maximum likelihood approach was utilized and Kenward-Rogers was used as the denominator degrees of freedom (Kenward and Roger 1997). Check cultivars were not included in any further data analysis.

The least squares means (LS means) of SGT and RG were used in combination with the published high density linkage map of Holloway et al. (2018) to perform QTL analysis using MapQTL 6 according to their guidelines (Kyazma, 2009). Interval mapping was first used to identify putative QTL for each acclimation × temperature treatment. A 10000 permutation test was run to determine the significance of putative QTL, and Logarithm-of-odds (LOD) scores with a significance level of P=0.05 were determined to represent the cutoff of significance. A LOD threshold of 2.5 was chosen based on the average relative cumulative counts for each linkage group at the 0.95 upper boundary of significance.

Using the putative QTL identified through interval mapping, correlated markers were analyzed as cofactors under Multiple QTL Model (MQM) mapping to account for the effects of potential ‘ghost QTLs’ (Martinez and Curnow, 1992), which may reduce the residual variance in the case that a single QTL accounts for a large proportion of the total variance increasing the power of the test. Following MQM analysis, QTL where LOD ≥ 2.5 were determined within each of the treatment groups and lined up to the appropriate regions of the genome on the linkage map.

Sequence annotation

Overlapping regions were identified between the lab-based freeze QTL identified here and the field winter survival QTL identified in Holloway et al. (2018). The *Z. japonica* ‘Nagirizaki’ reference genome (Tanaka et al., 2016), as well as the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST), were utilized to assist in the determination of gene function at the approximate location of these overlapping QTL. All sequences were run using the BLASTx tool at the default parameters. Only the aligned sequences with an E value < 0.0001 were considered.

RESULTS

Freezing Tests

In controlled laboratory freezing tests, the effects of acclimation treatment, temperature, and the acclimation treatment × temperature interaction were found to be significant ($p < 0.0001$) for both SGT and RG (Table 1). The effect of genotype was significant ($p < 0.01$) when partitioned by acclimation treatment, in addition to the significant effects of acclimation treatment, temperature, and their interaction for both SGT and RG (Table 2).

Among the parents, Meyer performed better under the CA-8ºC environment for both SGT and RG, however, Victoria had higher values for both SGT and RG under the CA-11ºC and NA-8ºC environments. For both SGT and RG, transgressive segregation was observed under all environmental conditions. The progeny averages were on par with those of Victoria and higher than Meyer at the CA-11ºC environment, slightly higher than Victoria and lower than Meyer for the CA-8ºC environment, and slightly lower than Victoria and higher than Meyer for the NA-8ºC environment (Table 3).

QTL Identification

Using the 'Meyer x Victoria' high-density linkage map of Holloway et al. (2018), putative QTL for SGT and RG were identified using LS means from six experimental runs of the controlled freezing tests. Based on the significance of effects in the ANOVA (Table 2), QTL analysis was performed by acclimation treatment × temperature for both SGT and RG. A total of fifty-two QTL were identified that explained between 6.4 and 12.2% of the phenotypic variance (R^2), under a LOD threshold of 2.5 in the model (Table 4). Five of these, on chromosomes 5, 8, 12, 13, and 20, explained 10% of more of the phenotypic variance.

Forty QTL, located on all chromosomes with the exception of 16 and 17, were detected for SGT (Table 4). Nine QTL were identified at CA-11ºC, explaining between 6.4 to 9.8% of the variance. Nineteen QTL were associated at CA-8ºC, explaining 6.5 to 12.2 % of the variance, and fifteen QTL at NA-8ºC, explaining 6.4 to 11.2% of the variance (Figure 1).

For RG, forty-one QTL were identified on all chromosomes except 14 and 15 (Table 4). Twelve QTL were associated at CA-11ºC explaining 6.4 to 9.8% of the variance, fifteen at CA-

8°C explaining 6.8 to 12.2% of the variance, and fourteen at NA-8°C explaining 6.7 to 11.2% of the variance (Figure 2). The region on chromosome 5 between 80.60 cM and 116.68 cM was of particular interest as it accounted for 10.6% of the variance for regrowth.

Colocation of traits

There were twenty-nine regions of interest where putative QTL were identified for both SGT and RG: QFT1.1, QFT1.2, QFT2.1, QFT2.2, QFT3.1, QFT4.2, QFT5.1, QFT5.2, QFT6.1, QFT6.2, QFT6.3, QFT7.1, 7QFT.3, QFT8.1, QFT8.2, QFT9.1, QFT10.1, QFT11.1, QFT11.2, QFT11.3, QFT12.1, QFT12.4, QFT13.1, QFT14.1, QFT18.2, QFT19.1, QFT19.2, QFT19.4, QFT20.1, and QFT20.2. On chromosome 8, the region between 22.10 cM and 23.59 cM, explained 11.2% of the variance for surviving green tissue and regrowth, under the NA-8°C treatment. On chromosome 12, the region between 18.33 cM and 23.09 cM explained 10.4% of the variance for surviving green tissue and regrowth under the CA-11°C treatment. The region on chromosome 13 between 0.68 cM and 2.36 cM explained 12.2% of the variance for regrowth and surviving green tissue under the CA-8°C treatment. The region on chromosome 20 between 65.55 cM and 87.05 cM explained an average of 11.4% of the variance for regrowth and surviving green tissue under the CA-8°C treatment (Table 4).

The putative QTL identified as associated with winter injury in Holloway et al. (2018) were compared to putative SGT and RG QTL identified here. Thirteen regions of overlap on chromosomes 1, 3, 5, 7, 8, 12, 13, and 19 were detected (Table 5, Figure 3). These overlapping regions were detected in multiple environments. While R^2 values for freeze associated QTL ranged from 6.5 to 10.6%, those for winter injury ranged from 6.38 to 17.41%. Chromosomes 7, 12, 13 and 19 contained overlap between SGT and winter injury QTL, while chromosomes 5, 7 and 19 contained overlapping regions between RG and winter injury QTL. Furthermore, regions of overlap between all three traits were observed on chromosomes 1, 3, 7, and 8. Of particular interest is the region on chromosome 1, at 60.66 to 60.94 cM, with similar R^2 values for the winter injury study at 9.07% as well as the freeze tolerance study at 9.4%. In addition, at regions overlapping for all three traits, the area between 115.29 and 118.43 cM on chromosome 7 explains 13.67% of the variance for winter injury and 7.3% of the variance for SGT and RG, and the region on chromosome 8 between 103.38 and 104.21 cM explains 15.23 - 17.41% of the variance for winter injury and 6.7% of the variance for SGT and RG.

Sequence annotation in regions of interest

Protein coding sequences were identified from the genome sequence at each of the fifteen overlapping regions of interest (Table 5) using BLASTx. The sequences were aligned to all species with available information on BLAST, of which the most notable were *Dichanthelium oligosanthes*, rice (*Oryza sativa* L.), *Panicum miliaceum*, *Panicum hallii*, foxtail millet (*Seteria italica* L.), sorghum (*Sorghum bicolor* (L.) Moench), and maize (*Zea mays* L.). Annotated sequences within the regions of interest were identified and the top identifiable hit with an E-value of < 0.001 were compiled to investigate homology to related species (Table 6). A total of 30 homologous sequences were able to be identified, not including hypothetical, predicted, putative, probable, or unnamed proteins. On chromosome 1 was Glycerol-3-phosphate 2-O-acyltransferase 6 (GPAT) around 60.84 cM. On chromosome 3, homology was found around 99.05 cM to pectinesterase. A zinc finger CCCH domain-containing protein 22-like was also identified on chromosome 3, around 101.01 cM. On chromosome 5, cytochrome P450 was identified at the region around 83.39 cM, a truncated NADPH-dependent reductase was identified at 84.43 cM, and around 92.84 cM, D-glycerate 3-kinase, chloroplastic isoform X2 was found. On chromosome 7, around the region of 115.29 cM, homology to heat stress transcription factor A-3-like was identified. Also in chromosome 7, ATPase 2 plasma membrane-type was identified around 170.19 cM. For chromosome 8, homology to peroxidase 65 was identified around 102.38 cM. Around 122.33 cM on chromosome 12, 1-aminocyclopropane-1-carboxylate (ACC) oxidase homolog 4-like was identified. Also on chromosome 12, phenylalanine ammonia-lyase (PAL), was expressed between 123.17 cM and 123.62 cM on chromosome 12, and at 123.62 cM, auxin response factor 8-like was identified. Lastly, on chromosome 19, aspartyl protease family proteins were identified around 29.93 cM.

DISCUSSION

Significant progress has been made in the development of genomics tools for zoysiagrass. The development of RFLP, AFLP, SSR, and high density SNP linkage maps (Yaneshita et al., 1999, Cai et al., 2004, Cai et al, 2005, Tsurata et al., 2005, Ma et al., 2007, Li et al., 2009, Holloway et al., 2018) as well as the development of a reference genome for *Zoysia japonica* (Tanaka et al., 2016) has paved the way for researchers. However, identification of QTL –and their underlying genes- associated with traits of economic importance is still in the early stages. Thus far, QTL have been identified for fall armyworm resistance (Jessup et al., 2011 and Huang et al., 2016) and salinity tolerance (Guo et al., 2014). The multigenic makeup of freezing stress response increases the difficulty of partitioning its genetic components. QTL associated with winter hardiness have been identified (Holloway et al., 2018, Guo et al., 2012). However, in zoysiagrass, cold acclimation and freezing tolerance can greatly affect the winter hardiness of cultivars from year to year (Patton and Reicher, 2007, Hinton et al., 2012). Further environmental pressure, such as deep snow cover, frequent freeze events, and prolonged low temperatures (Blum, 1988) across multiple locations complicate the analysis of freezing experiments. Controlled freezing tests benefit from the ability to minimize environmental factors, in order to determine a more focused assessment of true freeze response (Patton and Reicher, 2007). This study aimed at identifying QTL associated with cold acclimation and freezing tolerance under a controlled environment, in order to reduce sources of environmental variation present in field studies and investigate genes of interest across multiple studies, as well as to determine genes of interest for cold acclimation, which is a critical process in the development of freezing tolerance.

A total of fifty-two putative QTL explaining between 6.4 and 12.2% of the phenotypic variance were identified for SGT and RG. Of those identified QTL, five explained over 10% of the variance on chromosomes 5, 8, 12, 13, and 20 and were of particular interest for their higher effect. Forty QTL were identified for SGT, and forty-one QTL were identified for RG, with significant overlap between the two traits (Table 4). Thirteen QTL regions for the two controlled freeze test traits, SGT and RG, overlapped with winter injury associated QTL (Holloway et al., 2018), on chromosomes 1, 3, 5, 7, 8, 12, 13, and 19 across multiple environments. Four regions, on chromosomes 1, 3, 7, and 8 were observed between all three traits (Table 5). Overlap between controlled freezing and winter survival has been observed in other species, including St. Augustinegrass (Kimball et al. 2018), winter wheat (Gusta et al., 2001), and rapeseed (Teutonico et al., 2003). The presence of these regions identified across multiple studies and environments strengthens their associations with freezing tolerance.

In this study, SGT and RG QTL which were detected under a cold acclimated vs non-acclimated treatment were considered to be associated with the ability to cold acclimate. Similar studies have used this same criterion to identify cold acclimation associated QTL including St. Augustinegrass (Kimball et al., 2018) and peas (Dumont et al., 2009). Thirty-eight chromosomal regions detected for SGT and/or RG traits fit under this description (Table 4). Regions associated with cold acclimation occurred on chromosomes 1, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20. The region at QFT13.1 from 0.682 cM to 2.357 cM accounted for a peak of 12.2% of the total variation, the highest of all identified QTL. The regions at QFT20.2 (65.55 to 87.07 cM) and QFT12.1 (18.33 and 23.09 cM) accounted for peaks of 11.4% and 10.4% of the variance, respectively. These regions may regulate aspects of cold acclimation, such as CBF/DREB1, ABA, or signal transduction related proteins, that contribute to the development of greater freeze tolerance (Thomashow, 1999).

Sequence annotation identified homology to the controlled freeze test QTL which overlapped with the winter field study (Table 5, Figure 3). Homologous proteins to the sequences of interest (Table 6) were investigated for functional relation to abiotic stress tolerance, particularly freezing tolerance. On chromosome 1, glucan endo-1,3-beta-glucosidase 3 is an enzyme which may be involved in carbohydrate binding and cell wall components (Li et al., 2001), and Glycerol-3-phosphate 2-O-acyltransferase 6 (GPAT) has been found to confer greater chilling tolerance through unsaturation of fatty acids (Murata et al., 1992). Pectinesterase, found on chromosome 3, is an enzyme found to support thermostability under drought as well as cold stress by modifying cell walls (Deslattes Mays et al., 2018). An overexpression of zinc finger proteins, like the zinc finger CCCH domain-containing protein 22-like found on chromosome 3, has been found to enhance cold, freeze, and other abiotic stress tolerances as part of post transcriptional regulation (Kim et al., 2005, Mukhopadhyay et al., 2004). Cytochrome P450, found on chromosome 5, is part of a class of oxidative enzymes that has been shown in *Arabidopsis thaliana* to be induced by low temperature treatment and plays a role in plant metabolism and response to stress (Bilodeau et al., 1999, Bolwell et al., 1994). NADPH-dependent reductase, also found on chromosome 5, may be related to oxidative stress, such as that which occurs under drought and freezing stimuli (Serrato et al., 2004), while D-glycerate 3-kinase, chloroplastic isoform X2, a photosynthesis related gene, plays a role in photorespiration as part of the oxidative photosynthetic carbon (C2) cycle and has been shown to be highly induced under cold stress (Boldt et al., 2005, Khan et al., 2013). On chromosome 7, heat stress transcription factor A-3-like is part of a numerous family of transcription factors that function in the dehydration response element binding (DREB) network to increase thermotolerance (Koskull-Döring et al., 2007, Li et al., 2017), and the ATPase 2 plasma membrane-type also identified on chromosome 7 functions as a proton pump and plays a role in cellular growth (Serrano, 1989). On chromosome 8, peroxidase may participate in plant cell response to osmotic stress, such as occurs in freezing, by modifying cell walls (Llorente et al., 2002). Peroxidase is also similar in function to superoxide dismutases (SOD), which scavenges reactive oxygen species (ROS) to improve abiotic stress tolerance (Lee et al., 2007). Chromosome 12 identified several homologous sequences associated with the putative winter injury and freeze tolerance regions, including 1-aminocyclopropane-1-carboxylate (ACC) oxidase homolog 4-like, an enzyme shown to be involved in conferring tolerance to abiotic and biotic stressors through ethylene biosynthesis (Gontia-Mishra et al 2014, Chen et al., 2014). Phenylalanine ammonia-lyase (PAL), also on chromosome 12, has been observed to accumulate when under low temperature conditions and exposure to light (Levya et al., 1995). When PAL was inhibited in a study in pine, reduced levels of phenols leading to reduced freezing tolerance was observed (Teklemariam and Blake, 2004). Auxin, a key component of abiotic and biotic stress response, contributes to plant growth and development on chromosome 12 (Rahman et al., 2012). Lastly, on chromosome 19, aspartyl protease family proteins were identified, which play an important role in protein degradation as a response under both environmental and developmental stimuli and in senescence, a stage of development induced by changes in environment such as abiotic stress which causes reallocation of nutrients (García-Lorenzo et al., 2006, Roberts et al., 2012).

These gene sequences were compared to results from a protein expression profile study conducted on the two parent cultivars, Meyer and Victoria, under cold acclimated conditions (Brown et al., 2020). Some of the significant proteins which were shared among the two studies were: ATPase family proteins, NADP superfamily proteins, protein kinase superfamily proteins, and auxin-responsive family proteins. The presence of these proteins in these two studies as well as across other cold stress studies in zoysiagrass (Xuan et al., 2013, Wei et al., 2015) strengthens the association between the identified QTL regions and response to freeze stress.

Further studies will need to be conducted to match the putative expressed gene transcripts of interest with the genes and proteins responsible for their expression as well as to determine the overlap between Holloway et al. (2018) to further validate regions of interest, so that candidate genes may be identified and targeted for marker assisted breeding.

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Table 1: Type 3 analysis of variance test for surviving green tissue and regrowth after freezing evaluated over six replications in 175 progeny of Meyer × Victoria.

			Surviving Green Tissue		**Regrowth**	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**	**F Value**	**Pr > F**
Genotype	174	2796	0.83	0.9463	0.83	0.9463
Acclimation (A)	1	2796	1178.09	<0.0001	824.92	<0.0001
Temperature (T)	1	2796	3121.18	<0.0001	2631.13	<0.0001
A × T	1	2796	801.03	<0.0001	571.52	<0.0001

Table 2: Type 3 analysis of variance test of fixed effects for surviving green tissue and regrowth after freezing evaluated over six replications in 175 progeny plus the 2 parents, Meyer and Victoria, partitioned by treatment.

			SGT		RG	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**	**F Value**	**Pr > F**
Genotype	176	3520	1.25	0.0174	1.25	0.0159
Acclimation (A)	1	20.1	22.09	0.0001	16.75	0.0006
Temperature (T)	1	20.1	32.52	<0.0001	28.39	<0.0001
A × T	1	20.1	8.76	0.0077	6.75	0.0172

Table 3: Adjusted least squares means for the parents, Meyer and Victoria, and progeny minimum, maximum, and average values for surviving green tissue (SGT) and regrowth (RG) for acclimation (CA, NA) x temperature (-8^{o}C, -11^{o}C) treatments. Both traits were visually evaluated on a 1-5 scale and converted into a percentage from 0-1.

	SGT			RG		
Environment[a]	**NA-8**	**CA-8**	**CA-11**	**NA-8**	**CA-8**	**CA-11**
Meyer	0.067	0.967	0.067	0.067	0.900	0.067
Victoria	0.333	0.767	0.133	0.300	0.667	0.100
Progeny Min	0.000	0.433	0.000	0.000	0.367	0.000
Progeny Max	0.500	1.000	0.400	0.500	0.933	0.333
Progeny Avg	0.210	0.796	0.134	0.202	0.697	0.111

[a] NA-8, non-acclimated at -8^{o}C, CA-8, cold-acclimated at -8^{o}C, CA-11, cold-acclimated at -11^{o}C.

Table 4: Significant quantitative trait loci (QTL) for surviving green tissue (SGT) and regrowth (RG) evaluated after cold acclimation (CA) and no-acclimation (NA) at -8ºC and -11ºC and the interaction of these traits with each other, identified using Multiple QTL Model (MQM) mapping in a Meyer x Victoria mapping population. LS means for the traits come from evaluation across six controlled freezing experiments.

QTL#	Chr.	Position (cM)	Flanking Markers	Trait	Treatment	Peak LOD > 2.5	Peak R^2
QFT1.1	1	7.94	SNP_01_34 - SNP_01_36	SGT, RG	CA-11	3.56	8.9
QFT1.2	1	13.82	SNP_01_59 - SNP_01_60	SGT, RG	NA-8, CA-11	3.23	8.1
QFT1.3	1	50.19 - 50.79	SNP_01_113 -SNP_01_114	SGT	CA-8	2.93	7.4
QFT1.4	1	59.58	SNP_01_142 - SNP_01_143	SGT	CA-8	2.75	7.0
QFT1.5	1	60.66 - 60.98	SNP_01_145 - SNP_01_152	RG	CA-11	3.75	9.4
QFT1.6	1	62.73	SNP_01_156 - SNP_01_157	SGT	CA-11	2.53	6.4
QFT2.1	2	24.73 - 27.47	SNP_02_30 - SSR_02_8	SGT, RG	NA-8	2.80	7.1
QFT2.2	2	75.59	SNP_02_62 - SNP_02_64	SGT, RG	NA-8	2.66	6.8
QFT3.1	3	97.80 - 99.05	SNP_03_74 -SNP_03_79	SGT, RG	CA-8	3.37	8.5
QFT4.1	4	49.71	SNP_04_33 - SNP_04_36	RG	CA-11	2.52	6.4
QFT4.2	4	57.36 - 57.64	SNP_04_43 - SNP_04_46	SGT, RG	CA-11	3.62	9.1
QFT5.1	5	18.14 - 32.18	SNP_05_39 - SNP_05_68	SGT, RG	CA-11, CA-8	3.58	9.0
QFT5.2	5	35.05 - 40.04	SNP_05_70 - SNP_05_74	SGT, RG	NA-8	3.25	8.2
QFT5.3	5	64.86	SNP_05_85 - SNP_05_87	RG	CA-11	2.56	6.5
QFT5.4	5	78.26	SNP_05_96 - SNP_05_99	RG	CA-11	3.63	9.1
QFT5.5	5	80.60 - 116.68	SNP_05_104 - SNP_05_181	RG	NA-8	4.26	10.6
QFT6.1	6	5.73	SSR_06_1 - SNP_06_13	SGT, RG	CA-11	2.85	7.2
QFT6.2	6	26.73 - 27.73	SNP_06_67 - SNP_06_68	SGT, RG	NA-8	3.21	8.1
QFT6.3	6	69.62 - 71.98	SNP_06_112 - SNP_06_115	SGT, RG	NA-8	3.25	8.2
QFT6.4	6	74.74	SNP_06_121 - SNP_06_122	SGT	CA-11	2.79	7.1
QFT6.5	6	80.62	SNP_06_149 - SNP_06_151	SGT	NA-8	2.50	6.4
QFT7.1	7	113.84 - 118.43	SNP_07_111 - SNP_07_119	SGT, RG	NA-8	2.88	7.3
QFT7.2	7	120.124	SNP_07_121 - SNP_07_122	SGT	CA-8	2.57	6.5
QFT7.3	7	138.31 - 143.66	SNP_07_158 - SNP_07_162	SGT, RG	NA-8	3.33	8.4
QFT7.4	7	161.65 - 172.05	SNP_07_176 - SSR_07_7	RG	CA-8	3.35	8.4
QFT8.1	8	22.10 - 23.59	SNP_08_28 - SNP_08_30	SGT, RG	NA-8	4.51	11.2
QFT8.2	8	103.38 - 104.21	SNP_08_154 - SNP_08_160	SGT, RG	NA-8	2.63	6.7
QFT9.1	9	53.24 - 54.10	SNP_09_35 - SNP_09_42	SGT, RG	NA-8	2.95	7.5
QFT10.1	10	47.83	SNP_10_46 - SSR_10_1	SGT, RG	CA-8	2.89	7.3
QFT11.1	11	12.72 - 12.73	SNP_11_1 - SNP_11_2	SGT, RG	CA-11	2.76	7.0
QFT11.2	11	35.48 - 39.67	SNP_11_17 - SSR_11_2	SGT, RG	CA-11	3.91	9.8
QFT11.3	11	129.52 - 129.77	SNP_11_121 - SNP_11_123	SGT, RG	NA-8	3.30	8.3
QFT12.1	12	18.33 - 23.09	SNP_12_8 -SNP_12_10	SGT, RG	CA-8	4.16	10.4
QFT12.2	12	41.21 - 45.96	SNP_12-20 - SNP_12_27	RG	CA-8	3.01	7.6
QFT12.3	12	62.14 - 64.15	SNP_12_28 - SNP_12_32	RG	CA-8	2.91	7.4
QFT12.4	12	94.33 - 100.31	SNP_12_38 - SNP_12_45	SGT, RG	CA-8, NA-8	3.97	9.9
QFT12.5	12	122.33 - 124.45	SNP_12_69 - SNP_12_77	SGT	CA-8	2.62	6.7
QFT13.1	13	0.68 - 2.36	SNP_13_1 - SNP_13_8	SGT, RG	CA-8	4.94	12.2

Table 4 continued: Significant quantitative trait loci (QTL) for surviving green tissue (SGT) and regrowth (RG) evaluated after cold acclimation (CA) and no-acclimation (NA) at -8°C and -11°C and the interaction of these traits with each other, identified using Multiple QTL Model (MQM) mapping in a Meyer x Victoria mapping population. LS means for the traits come from evaluation across six controlled freezing experiments.

QFT13.2	13	14.24 - 15.24	SNP_13_32 - SSR_13_4	SGT	CA-11	3.41	8.6
QFT14.1	14	0.79	SSR_14_1 -SNP_14_1	SGT	CA-8, NA-8	2.64	6.7
QFT15.1	15	70.77	SNP_15_48 - SNP_15_49	SGT	CA-8	2.73	6.9
QFT16.1	16	20.16 - 42.92	SSR_16_1 - SNP_16_10	RG	CA-11	3.41	8.6
QFT17.1	17	36.72	SNP_17_11 - SNP_17_13	RG	CA-8	2.66	6.8
QFT18.1	18	11.91	SNP_18_22 - SNP_18_24	RG	CA-8	2.82	7.2
QFT18.2	18	14.05 - 14.12	SNP_18_32 -SNP_18_35	SGT, RG	CA-8	3.66	9.2
QFT19.1	19	1.05 - 8.85	SSR_19_1 - SNP_19_18	SGT, RG	CA-8, NA-8	3.75	9.4
QFT19.2	19	27.04 - 29.93	SNP_19_57 - SNP_19_61	SGT, RG	CA-8	3.31	8.4
QFT19.3	19	59.42	SNP_19_89 - SNP_19_91	RG	CA-8	2.83	7.2
QFT19.4	19	66.31	SNP_19_108 - SNP_19_109	SGT, RG	CA-8	2.69	6.8
QFT19.5	19	76.84	SNP_19_137 - SNP_19_139	SGT	NA-8	2.55	6.5
QFT20.1	20	21.49 - 23.71	SNP_20_28 - SNP_20_32	SGT, RG	CA-8	3.18	8.0
QFT20.2	20	65.55 - 87.05	SNP_20_67 - SSR_20_6	SGT, RG	CA-8	4.61	11.4

Table 5: Overlap between winter injury QTL (Holloway et al., 2018) and surviving green tissue and regrowth QTL identified under controlled freeze trials in this study.

	Winter Injury QTL				Freeze Trial QTL				
Chr.	**Envir.**	**Flanking Markers**	**Position (cM)**	**Peak R^2**	**Traits**	**Treatmt.**	**Flanking Markers**	**Position (cM)**	**Peak R^2**
1	NCA16	Snp_01_144 - Snp_01_152	60.20 - 60.94	9.1	SGT, RG	CA-11	SNP_01_145 - SNP_01_152	60.66 - 60.94	9.4
3	INA15	Snp_03_69 - Snp_03_83	92.51 - 103.38	7.5	SGT, RG	CA-8	SNP_03_74 - SNP_03_79	97.80 - 99.05	8.5
5	INB16	SSR_05_8 - Snp_05_109	82.95 - 85.00	7.1	RG	NA-8	SNP_05_104 - SNP_05_181	80.60 - 116.68	10.6
5	INB16	Snp_05_120 - SSR_05_9	91.75 - 93.00	7.6					
7	NCA16	Snp_07_114 - Snp_07_126	115.29 - 122.65	13.7	SGT, RG	NA-8	SNP_07_111 - SNP_07_119	113.84 - 118.43	7.3
					SGT	CA-8	SNP_07_121 - SNP_07_122	120.12	6.5
7	NCA15	SSR_07_6	170.19	6.6	RG	CA-8	SNP_07_176 - SSR_07_7	161.65 - 172.05	8.4
8	NCA15	Snp_08_66 - Snp_08_176	62.98 - 113.11	17.4	SGT, RG	NA-8	SNP_08_154 - SNP_08_160	103.38 - 104.21	6.7
8	NCA16	Snp_08_139 - Snp_08_176	96.35 - 113.11	15.2					
12	NCB16	Snp_12_69	122.33	6.4	SGT	CA-8	SNP_12_69 - SNP_12_77	122.33 - 124.5	6.7
12	INA15	Snp_12_65 - Snp_12_102	118.76 - 131.59	11.2					
12	INA16	Snp_12_73 - Snp_12_82	123.41 - 124.60	10.0					
13	INA16	Snp_13_22 - Snp_13_37	10.93 - 16.73	10.3	SGT	CA-11	SNP_13_32 - SSR_13_4	14.24 - 15.24	8.6
19	INA15	Snp_19_49 - Snp_19_69	21.41 - 40.63	10.0	SGT	CA-8	SNP_19_57 - SNP_19_61	27.04 - 29.93	8.4
19	INA15	Snp_19_70 - Snp_19_108	45.85 - 65.31	12.4	RG	CA-8	SNP_19_89 - SNP_19_91	59.42	7.2
19	INA15	Snp_19_113 - Snp_19_138	69.63 - 76.84	10.0	SGT	NA-8	SNP_19_137 - SNP_19_139	76.84	6.5

Table 6. Annotated *Z. japonica* sequences identified via the NCBI BLASTx program on the significant QTL associated with both freezing tolerance and winter injury (Table 5). The protein coding sequence from the top BLAST hit was identified, where the E values <.001.

Associated Marker	**Chr**	**Location (cM)**	**Top BLAST Hit**			
			Aligned Sequence	**Species**	**E value**	**Accession #**
SNP_01_146	1	60.66	embryonic protein DC-8	*Zea mays*	2.00E-05	ACG37499.1
SNP_01_148	1	60.84	Glycerol-3-phosphate 2-O-acyltransferase 6	*Dichanthelium oligosanthes*	1.00E-08	OEL23325.1
SNP_01_149	1	60.84	glycerol-3-phosphate 2-O-acyltransferase 6	*Sorghum bicolor*	3.00E-23	XP_002465803.1
SNP_03_78	3	99.05	Pectinesterase	*Triticum urartu*	7.00E-34	EMS45437.1
SNP_03_82	3	101.01	zinc finger CCCH domain-containing protein 22-like	*Panicum miliaceum*	3.00E-06	RLN39239.1
SNP_05_105	5	83.39	cytochrome P450 72A11	*Sorghum bicolor*	3.00E-35	XP_021311069.1
SNP_05_107	5	84.43	truncated NADPH-dependent reductase	*Zea mays*	4.00E-14	AAS48099.1
SNP_05_123	5	92.84	D-glycerate 3-kinase, chloroplastic isoform X2	*Setaria italica*	8.00E-21	XP_004966356.1
SNP_07_114	7	115.29	heat stress transcription factor A-3-like	*Panicum miliaceum*	2.00E-31	RLM80050.1
SSR_07_6	7	170.19	ATPase 2, plasma membrane-type	*Sorghum bicolor*	3.00E-11	XP_002452983.1
SNP_08_154	8	102.38	Peroxidase 65	*Dichanthelium oligosanthes*	3.00E-34	OEL20961.1
SNP_12_69	12	122.33	1-aminocyclopropane-1-carboxylate oxidase homolog 4-like	*Panicum hallii*	2.00E-21	XP_025814998.1
SNP_12_71	12	123.17	Phenylalanine ammonia-lyase	*Zea mays*	7.00E-35	PWZ41109.1
SNP_12_72	12	123.17	phenylalanine ammonia-lyase-like	*Oryza sativa* Japonica Group	3.00E-25	XP_015635507.1
SNP_12_74	12	123.62	auxin response factor 8-like	*Aegilops tauschii* subsp. tauschii	4.00E-26	XP_020164753.1
SNP_19_138	19	76.84	tuliposide A-converting enzyme b2, amyloplastic-like	*Sorghum bicolor*	1.00E-16	XP_021307888.1
SNP_19_57	19	27.04	Cysteine-rich receptor-like protein kinase 10	*Zea mays*	1.00E-32	PWZ07528.1
SNP_19_58	19	29.93	aspartyl protease family protein At5g10770	*Sorghum bicolor*	1.00E-17	XP_002438291.1
SNP_19_59	19	29.93	aspartyl protease family protein At5g10770-like	*Oryza sativa* Japonica Group	3.00E-16	XP_025879436.1
SNP_19_60	19	29.93	aspartyl protease family protein At5g10770	*Sorghum bicolor*	5.00E-14	XP_002438291.1
SNP_19_61	19	29.93				
SNP_19_90	19	59.42	Homeobox-leucine zipper protein HOX6	*Dichanthelium oligosanthes*	5.00E-28	OEL19765.1

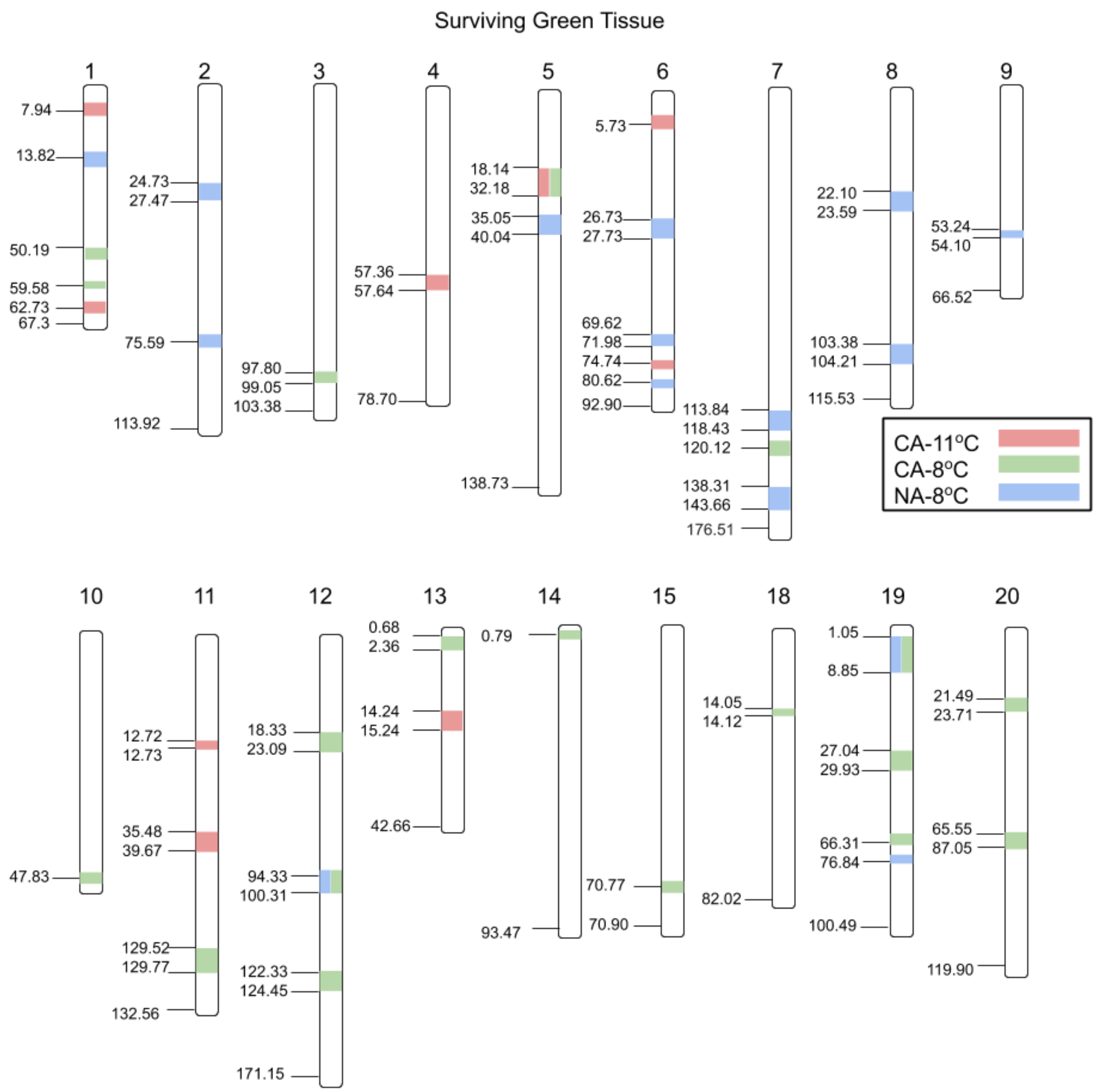

Figure 1: The chromosomes with significant QTL regions associated with surviving green tissue under an environment of cold acclimation at temperature -11°C (pink fill), cold acclimation at temperature -8°C (green fill), and non-cold acclimation at temperature -8°C (blue fill). The map positions of the closest markers are indicated to the left of the chromosomes in cM.

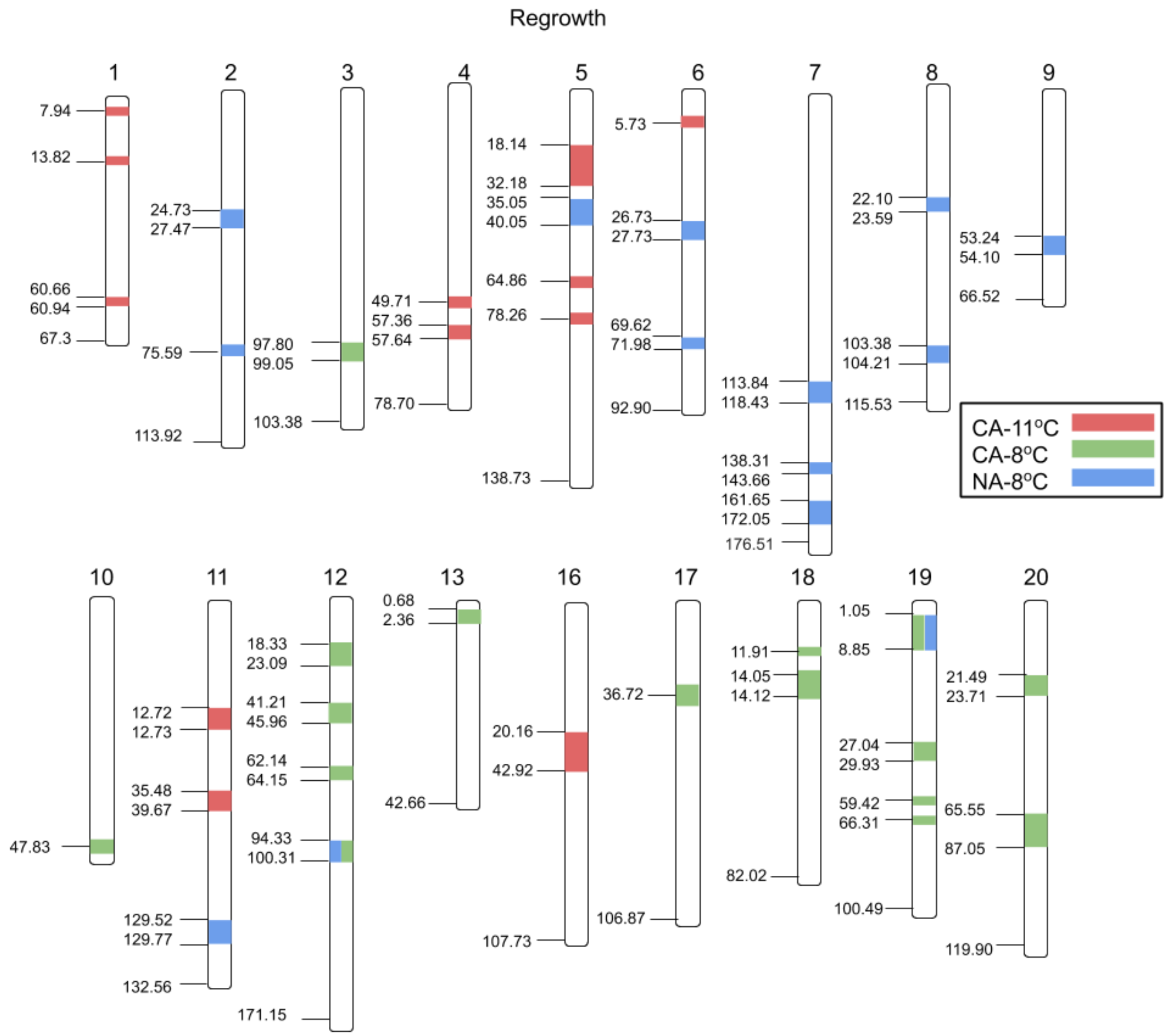

Figure 2: The chromosomes with significant QTL regions associated with regrowth under an environment of cold acclimation at temperature -11°C (pink fill), cold acclimation at temperature -8°C (green fill), and non-cold acclimation at temperature -8°C (blue fill). The map positions of the closest markers are indicated to the left of the chromosomes in cM.

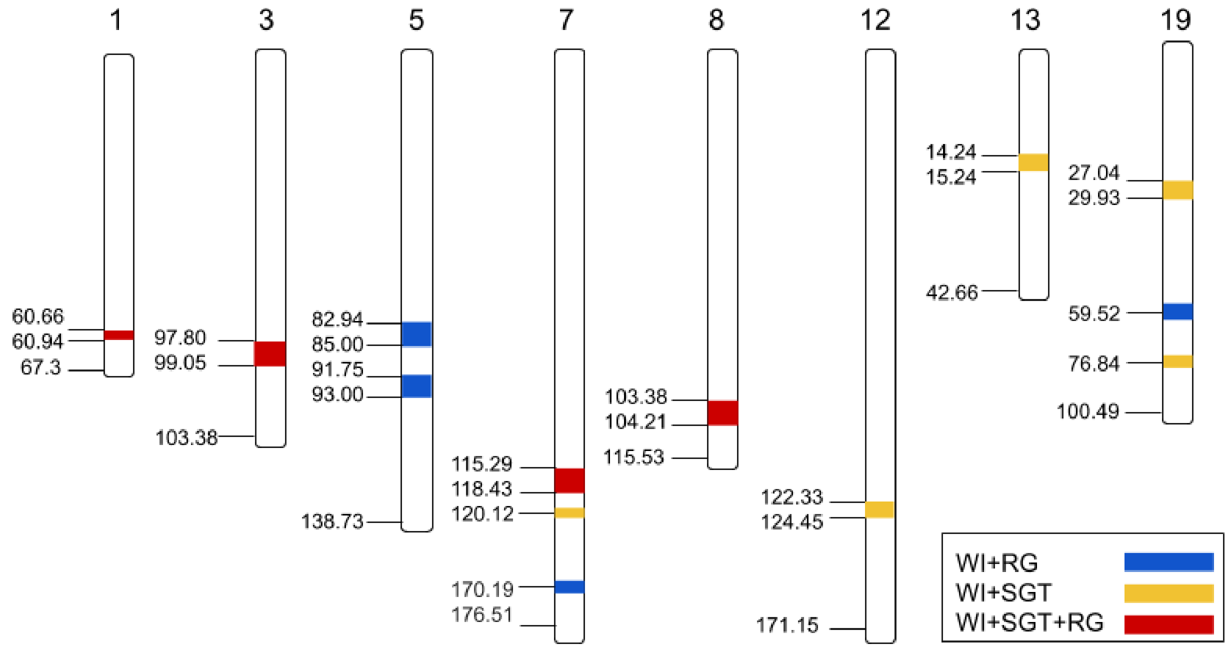

Figure 3: The overlap of winter injury associated QTL from winter field studies (Holloway et al., 2018) with surviving green tissue and regrowth associated QTL from controlled freezing studies. The map positions of the closest markers are indicated to the left of the chromosomes in cM.

CHAPTER III

TRANSCRIPTOMIC ANALYSIS OF FREEZE RESPONSE IN ZOYSIAGRASS

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Transcriptomic Analysis of Cold Acclimation Response in Zoysiagrass

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ABSTRACT

Zoysiagrass (*Zoysia japonica* Steud.) is a warm-season perennial turfgrass commonly grown in the southeastern United States for their relatively low input requirements and general tolerance to drought, shade and salinity. While zoysiagrass has the best freeze tolerance among warm-season grasses, its commercial spread is mostly limited to the transition zone and the southern US. Although some progress has been made in breeding for freeze tolerance in zoysiagrass, the pool of freeze tolerant cultivars is still limited. To deepen our understanding of the physiologic and metabolic components of freezing tolerance in zoysiagrass, a transcriptomic approach was utilized to identify genes involved in cold acclimation, a critical step in the development of freezing tolerance. Meyer, a freeze tolerant cultivar, and Victoria, a freeze susceptible cultivar were used to identify changes in expression in freeze tolerance associated transcripts. Cultivars were subjected to either a cold acclimation or non-cold acclimation treatment, then leaf samples were collected for RNA extraction. The Illumina sequencing platform was utilized to obtain high quality pair end reads, which were analyzed in correspondence with the zoysiagrass reference genome to determine differential gene expression. Differential gene expression pathways of interest were determined, after which genes associated with cold acclimation could be identified. Low molecular mass early light-inducible proteins, late embryogenesis abundant proteins, auxin-responsive proteins, and photosystem II proteins all demonstrated significant fold change differences between treatments. Expression of auxin-responsive proteins was found to be associated with cold acclimation and freezing tolerance across multiple studies in transcriptome, proteome, and genome analysis, and may be a target for future selection efforts for freeze tolerant zoysiagrass.

Keywords Zoysiagrass, Freeze tolerance, Cold acclimation, Transcriptome, RNA-sequencing

INTRODUCTION

Zoysiagrass (*Zoysia* spp.Willd.) is a warm-season turfgrass desirable for both residential and commercial turf use due to its fine leaf texture, low growth habit, low fertility requirements, and wide general tolerance to abiotic stressors such as drought, shade and salinity (Li et al., 2009; Patton et al., 2017). Freezing tolerance is a critical trait for warm-season turf, as it determines the northern boundaries a species is able to be grown in. While zoysiagrass is the most freeze tolerant of the warm-season grasses, it is still limited in range primarily to the southern and transitional climatic zones of the US (Patton et al., 2017).

The genetic complexity of abiotic stress tolerance, including freezing tolerance, slows progress in increasing freezing tolerance in the species through traditional breeding methodologies. Therefore, the identification of major-effect, genomic regions through methods like transcriptome expression via RNA-sequencing may increase the success rate of selection for freeze tolerant genotypes. RNA-sequencing allows for the precise measurement of DNA expression, in order to determine which genes are active and how much they are transcribed (Wang et al., 2009). By utilizing this tool, candidate regions associated with cold acclimation and freezing tolerance in zoysiagrass can be identified in terms of actual gene transcript expression.

Transcriptome analysis has previously been used in various *Z. japonica* cultivars to identify RNA transcripts associated with anthocyanin biosynthesis genes in leaf tissue (Ahn et al., 2015), salinity stress in roots (Xie et al., 2015), and pathogenicity of *Rhizoctonia solani* in roots (Zhu et al., 2016). Wei et al. (2015) investigated the global transcriptome profile of zoysiagrass cultivar 'Meyer' under cold acclimation conditions using gene ontology classification. They identified over 46,000 unigenes falling under twenty-five functional categories, the largest of which were signal transduction, general function, and post-translational modification/protein turnover/chaperones.

Further investigation into the unigenes induced during cold stress identified expression of genes involved in osmotic adjustment, antioxidants, nitrogen assimilation, phytohormone metabolism and signaling, and the DREB response pathway. Significant downregulation of genes involved in electron carrier activity, chlorophyll biosynthesis and carbon-fixation activity, water transport, organic substance transport, and antioxidant enzyme activity were also observed (Wei et al., 2015). These highly varied results emphasize that cold and freeze stress responses are controlled by coordinated, complex metabolic and signaling pathways.

Cold acclimation is a critical process in the development of freezing tolerance, and is defined as exposure to low but above freezing temperatures prior to a freeze stress (Patton and Reicher 2007). Areas of interest associated with cold acclimation in plant tissues include growth and water balance, compatible solute accumulation, membrane and cell wall composition changes, increases in antioxidant production, changes in gene expression, as well as changes in protein levels (Xin and Browse, 2000; Thomashow, 1999). Regulatory components that may play a prominent role in cold acclimation, as demonstrated by studies in *Arabidopsis thaliana*, include c-repeat binding factors (CBF) 1, 2, and 3 (Gilmour et al., 1998), which are also known as dehydration responsive element binding1 (DREB1) b, c and a (Liu et al., 1998, Feng et al., 2019). In Arabidopsis, these CBF related changes have been estimated to be responsible for between 12-20% of cold induced transcriptional changes (Hannah et al., 2005).

The relatively small genome size of zoysiagrass (~421 Mb) lends to the greater effectiveness of next generation sequencing techniques. Though few studies have focused on transcriptomic analysis in zoysiagrass, the availability of the assembled reference genome sequences for three zoysiagrass cultivars, one each for the three most relevant species in the *Zoysia* genus: *Z. japonica* Steud. ‘Nagirizaki’, *Z. matrella* (L.) Merr. ‘Wakaba’, and *Z. pacifica* Goudswaard ‘Zanpa’ (Tanaka et al., 2016) enhances the potential benefit of this analysis.

Research on transcriptome profiles in common bermudagrass (*Cynodon dactylon* (L.) Pers.) (Chen et al., 2015), zoysiagrass (Wei et al., 2015), and perennial ryegrass (*Lolium perenne* (L.)) (Zhang et al., 2009) under low temperature conditions have previously elucidated some of the molecular mechanisms underlying cold and freeze stress response by highlighting some of the key pathways, including the importance of nitrogen metabolism and carbon fixation in creeping bentgrass, and the DREB1 gene in zoysiagrass. This study seeks to increase the knowledge base and understanding of the molecular mechanisms involved in freeze response in zoysiagrass by investigating the transcriptomic response following cold acclimation of cultivars Meyer (freeze tolerant) and Victoria (freeze susceptible), using the Illumina RNA-Seq platform. The goal of this study was to identify candidate genes involved in the response to cold acclimation and freezing conditions through transcriptomic analysis, which may strengthen results of previous studies (Holloway et al., 2018; Brown et al., 2020). Our hope is to gain insight into the molecular mechanisms of freezing tolerance and cold acclimation, and deepen understanding of the molecular basis underlying response to freeze stress.

MATERIALS AND METHODS

Plant Materials

Zoysiagrass cultivars ‘Meyer’ and ‘Victoria’, which have been extensively reported in the literature as freeze tolerant and freeze susceptible, respectively (Dunn et al., 1999; Patton and Reicher, 2007; Patton, 2009), were used in this study. Nodes of each genetically uniform cultivar were propagated in 2x2 inch pots with Fafard 4P potting mix and grown under greenhouse conditions at 27±2C for six weeks until fully established. Plants were mowed and fertilized biweekly with 24:8:16 NPK solution of Miracle-Gro Water Soluble All Purpose Plant Food (The Scotts Miracle-Gro® Company, Marysville, OH).

Treatments and experimental design

The protocol of Chen et al. (2015) with modifications to the acclimation and freezing temperatures to suit the target cultivars was utilized. Following establishment in the greenhouse, pots were moved into temperature controlled Percival acclimation chambers (model I-36LL) for four weeks. Forty-eight pots, twenty-four Meyer and twenty-four Victoria, were held under non-acclimation (NA) conditions at a consistent 22^{o}C with 12h day and night light cycles. The other forty-eight pots were held under cold acclimation (CA) conditions at 8/2^{o}C day and night temperature and light cycles. The growth chambers contained a consistent light emitting diode (LED) which have maintained a 10-h photoperiod of 300 µmol $m^{-2}s^{-1}$ active radiation (Anderson et al., 1988). After forty-eight hours of acclimation, twelve leaf samples were collected in 1.5 mL microcentrifuge tubes in liquid nitrogen for three biological replicates each of Meyer and Victoria. The leaf samples taken from the NA chamber represented the time 0 treatment (NAT0). The leaf samples taken from the CA chamber represented time 1 treatment (CAT1). The samples were stored at -80^{o}C until RNA extraction.

RNA Extraction

Total RNA was extracted from the frozen leaf samples using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD), in accordance with the manufacturer’s protocol. Between additions of RLC buffer, the RNase-Free DNase kit was used according to manufacturer's protocol (Qiagen, Germantown, MD) to purify RNA by removing DNA. Sufficient RNA quality was measured with a RIN threshold of ≥ 5.8 by the Genomic Science Laboratory (NC State University, Raleigh, NC).

Transcriptome library preparation and sequencing

Sequencing, mapping, and primary analysis were completed by Novogene (Novogene Co., Ltd, Sacramento, CA) according to the following protocols: Total RNA was qualified via Nanodrop (OD260/OD280) for RNA purity, agarose gel electrophoresis to test RNA degradation and contamination, and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA) to check for RNA integrity. Following this quality control, mRNA was enriched via oligo(dT) beads, fragmented in fragmentation buffer, and cDNA was synthesized using random hexamers and reverse transcriptase. Second strand synthesis buffer (Illumina, San Diego, CA) was added along with dNTPs, RNase H and *Escherichia coli* polymerase I for second strand synthesis by nick-translation. cDNA library was completed following a purification round, terminal repair and poly-A tailing, ligation of sequencing adaptors, size selection and lastly PCR enrichment. The PCR library was assessed for quality before Illumina sequencing first by quantifying with a Qubit 2.0 fluorometer (Life Technologies (Carlsbad, CA)) and diluted to 1 ng/ul. Insert size was checked on an Agilent 2100 and quantified via Q-PCR where library activity must be >2 nM. RNA samples were sequenced using HiSeq Illumina sequencing platform (Illumina, San Diego, CA) by the Novogene Corporation (Sacramento, CA). The raw reads were processed for quality control by evaluating the A/T/G/C content distribution and data filtering, where reads with base quality less than 20, and uncertain nucleotides (N >10%) were discarded.

Mapping to reference genome

The transcriptome was aligned to the *Zoysia japonica* ‘Nagirizaki’ reference genome, (Tanaka et al., 2016) using the HISAT2 algorithm (v2.1.0-beta), where total mapped reads (TMR) were larger than 70% and multiple mapped reads (MMR) were no more than 10%. Mapped regions were classified as exons, introns or intergenic regions. Distribution of mapped reads in chromosomes was determined under a window size of 1K (Marquez et al., 2012).

Expression Quantification and RNA-seq Advanced QC

Gene expression level was estimated by counting reads which mapped to genes or exons, where read count was proportional to gene expression level, gene length, and sequencing depth. To compare expression levels estimated from different genes and treatments, the Reads/Fragments Per Kilo bases per Million reads (R/FPKM) method (Mortazavi et al., 2008) was utilized under a threshold of 0.1 for determining whether a gene was expressed or not. The structure and expression levels were analyzed via HTSeq v0.6.1 (Anders et al., 2015), using union mode. Co-expression Venn diagrams were used to identify similar gene expression patterns under experimental conditions. The RNA-seq correlation was determined between replicate samples. A Pearson correlation coefficient of $r \geq 0.92$, and an R2 value of ≥ 0.8 was the cutoff for reliability under ideal experimental conditions.

Differential Gene Expression Analysis

Differential gene expression levels were estimated through readcount normalization, model dependent p-value estimation, and estimation of FDR value based on multiple hypothesis testing. Two methods were utilized for the analysis. First, DESeq v1.10.1 with biological duplicates (Anders et al., 2010), which utilizes a negative binomial distribution and a screening standard of padj <0.05. Secondly, DEGseq v1.12.0 without biological duplicates (Wang et al., 2010), which utilizes a Poisson distribution and a screening standard of log2 (ratio) ≥ 2 and q-value ≤ 0.001. Volcano plots were used to infer the overall distribution of differentially expressed genes (data not shown). FPKM cluster analysis identified genes with similar expression patterns under variable experimental conditions. Venn diagrams were used to present the number of uniquely expressed genes within each cell group.

GO Enrichment Analysis

Differential expressed genes (DEGs) underwent gene ontology (GO, http://www.geneontology.org/) enrichment analysis via GOseq v2.12 (Young et al., 2010) with a corrected p-value < 0.1, to identify significantly enriched GO terms by estimating the preference of gene length. A GO enrichment bar chart was used to identify differentially expressed gene enriched GO terms and the counts of genes for each GO term, where the 30 most enriched terms were displayed. A Directed Acyclic Graph (DAG) was used to display the top ten GO enrichment results in terms of their containment relationship and extent of enrichment, separated into biological processes, molecular functions, and cellular components.

KEGG Pathway Enrichment Analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG, KOBAS v3.0 software) enrichment analysis identified significantly enriched metabolic or signal transduction pathways associated with the DEGs, where the adjusted p-value <0.05 was used. Significant homology exists between the *Zoysia japonica* genome and the *Setaria italica* genome, commonly known as foxtail millet (Bennetzen et al., 2012), due to its position as the closest relevant crop species to *Zoysia japonica*, containing approximately 52.1% similarity (Wei et al, 2015). The *Setaria italica* genome was used as the reference point for identified KEGG pathways and gene transcripts. The top twenty most significant results were plotted on a KEGG enrichment scatter plot according to Rich factor and qvalue. Enrichment pathways were generated to show significantly up and down regulated genes.

Protein-Protein Interaction Network Analysis

The STRING database (http://string-db.org/) and BLAST v2.2.28 were utilized for protein-protein interaction analysis. Results were visualized through the Cytoscape (Shannon et al., 2003) v3.7.2 software (The Cytoscape Consortium, http://manual.cytoscape.org/en/stable/).

RESULTS

Transcriptome sequencing

Following Illumina sequencing, the PCR library represented a total of 685,007,604 raw reads, which resulted in 670,006,060 clean reads, for an average of 55,833,838.33 clean reads generated per sample. The average total number of clean nucleotides generated for each sample was 8.375G. Of the clean reads, Q30 (average sequencing error rate <1%) was 93.20%, and the GC content was over 50% (Table 1).

Differential expression genes (DEGs) analysis under acclimation treatments

DEGs were defined as those genes which were significantly ($p_{adj} < 0.05$) up or down regulated in a particular sample relative to the other samples. Expression levels of genes were quantified as Fragments per Kilobase Million (FPKM) to determine consistency across biological replicates, as well as general trends for treatments and cultivars (Table 2). The treatment comparisons were cold acclimated Meyer vs cold acclimated Victoria (CM vs CV), cold acclimated Meyer vs non-cold acclimated Meyer (CM vs NM), cold acclimated Victoria vs non-cold acclimated Victoria (CV vs NV), and non-cold acclimated Meyer vs non-cold acclimated Victoria (NM vs NV). For the comparison between CM and CV, 4,143 DEGs were significant, 2,232 of which were upregulated and 1,911 downregulated. For CM vs NM, 6,675 DEGs were upregulated, and 5,895 DEGs were downregulated, out of the 12,570 significant DEGs. For CV vs NV, 11,404 DEGs were significant, of which 5,725 were upregulated and 5,679 were downregulated. Finally, for NM vs NV, 5,076 DEGs were upregulated, and 4,730 were downregulated, for 9806 significant DEGs (Figure 1).

To further observe DEG patterns, hierarchical clustering of DEGs was used to identify all genes which were significant ($p \geq 0.05$ and log2 fold change ≥ 1 or ≤ -1) in at least one of the four pairwise comparisons (Figure 2). The Venn diagram shows the unique and overlapping genes expressed among comparison groups (Figure 3). Particular attention was paid to the 2,577 unique genes expressed in group A (CVvsNV), and the 2,391 unique genes expressed in group B (CMvsNM), attributable to differences in the temperature treatment.

Gene Ontology (GO) classification of differentially transcribed genes

For GO enrichment analysis, genes were identified for each of the four comparison groups within three ontology categories: biological process, cellular component, and molecular function. For the comparison groups CM vs NM, CV vs NV, CM vs CV, and NM vs NV, there were 2,418, 2,404, 1,868, and 2,269 genes respectively involved in biological processes, 530, 528, 413, and 503 in cellular components, and 1,264, 1,231, 973, and 1,213 in molecular functions (Figure 4). Within those categories, the most enriched DEGs were further partitioned into significant sub-categories based on the number of genes involved, and whether they were up or down regulated.

Within cultivar expression differences, CM vs NM and CV vs NV, were the primary comparisons of interest (Figure 4). For CM vs NM, the major categories within molecular function were molecular function, and catalytic activity. Within cellular components, the most genes were involved in intracellular, intracellular part, and membrane, while within biological processes, single organism processes, metabolic processes, and cellular processes were the largest subcategories of genes. For CV vs NV, most genes within molecular function were involved in molecular function, transferase activity, small molecule binding, nucleotide binding, nucleoside phosphate binding, and anion binding. The largest subcategories of genes within cellular components were cellular component, membrane, intracellular, cell, and cell part, and for biological processes, most genes were involved in single organism process and single-organism cellular process.

Identification of acclimation response genes from DEG analysis

From the differential expression analysis, significant up and down regulated DEGs were investigated for homology to known sequences on BLAST swiss plot. Those which were associated with a gene in a related species were selected for further investigation. The CM vs NM and CV vs NV comparisons were of principal interest for the insights they may provide on differential response to treatment due to genetic differences between cultivars.

For the CM vs NM comparison, of the 6,675 significant DEGs that were upregulated, 4,379 were identified to be homologous (supplementary table 1). The top five genes with the greatest fold change were identified to be associated with low molecular mass early light-inducible protein HV90, chloroplastic in I (+11.968 log_2FC); late embryogenesis abundant (LEA) protein 14 in *Oryza sativa* (+11.646 log_2FC); dehydrin DHN1 in I (+11.225 log_2FC); dehydrin COR410 in I (+10.775 log_2FC); and low molecular mass early light-inducible protein HV60, chloroplastic in *Hordeum volgare* (+10.288 log_2FC). Of the 5,895 DEGs that were downregulated, 3,553 were homologous (supplementary table 2). The top five genes with the greatest fold change were associated with flowering-promoting factor 1-like protein 4 in *Oryza sativa* (-9.8437 log_2FC), amino acid transporter AVT1J in *Arabidopsis thaliana* (-9.7904 log_2FC, dirigent protein 1 in *Arabidopsis thaliana* (-9.5667 log_2FC), protein iron-related transcription factor 2 in *Oryza sativa* (-9.2231 log_2FC), and transcription factor JAMYB in *Oryza sativa* (-9.1646 log_2FC).

For the CV vs NV comparison, of the 5,725 were upregulated, 5,057 had identified homology (supplementary table 3). The top five with the greatest fold change were associated with low molecular mass early light-inducible protein HV90, chloroplastic in *Hordeum vulgare* (+10.623 log2FC), LEA protein 18 in *Arabidopsis thaliana* (+9.7227 log2FC), agmatine hydroxycinnamoyltransferase 1 in *Oryza sativa* (+9.6897 log2FC), LEA protein 14 in *Oryza sativa* (+9.6458 log2FC) and low molecular mass early light-inducible protein HV90, chloroplastic in *Hordeum vulgare* (+9.4785 log2FC). Of the 5679 genes that were downregulated, 3450 had identified homology (Supplementary Table 4). The top five with the greatest fold change were associated with auxin-responsive protein SAUR76 in *Arabidopsis thaliana* (-8.2565 log2FC), protein iron related transcription factor 2 in *Oryza sativa* (-7.6847 log2FC), probable polyol transporter 4 in *Arabidopsis thaliana* (-7.2062 log2FC), caffeoyl shikimate esterase in *Arabidopsis thaliana* (-7.08 log2FC), and ethylene-responsive transcription factor ERF073 in *Arabidopsis thaliana* (-6.9984 log2FC).

KEGG pathway enrichment analysis for DEGs

KEGG annotation of genes was performed for DEGs identified from the GO analysis. In the NM vs NV, CM vs NM, and the CV vs NV comparisons, the DEGs assigned to the KEGG database all involved 121 pathways. For CM vs CV, 122 pathways were involved (Supplementary Tables 5 and 6).

The top twenty most significant enriched pathways for each of the four comparisons were compiled for each of the four comparisons (Tables 3-6). Only pathways with a p_{adj} -value ≤ 0.05 were considered significant. When comparing transcript expression between CM vs NM (Table 4) and CV vs NV (t=Table 5), the highest input number of genes were involved in metabolism (1,258 and 1,136 genes, respectively) and biosynthesis of secondary metabolites (707 and 654 genes, respectively). Specifically, in CM vs NM, the pathways with the next largest number of genes involved were carbon metabolism, plant hormone signal transduction, and biosynthesis of amino acids. Next to metabolism and biosynthesis of secondary metabolites, which expressed large amounts of both up and down regulation of genes, carbon metabolism and biosynthesis of amino acids contained the next highest numbers of downregulated genes, while plant hormone signal transduction and starch and sucrose metabolism contained the next highest numbers of upregulated genes (Table 7). In the CV vs NV comparison, the pathways with the next highest number of genes were involved in plant hormone signal transduction, glycerophospholipid metabolism, and carotenoid biosynthesis. Next to metabolic pathways, only genes in the N-Glycan biosynthesis were significantly downregulated. Significantly upregulated pathways included biosynthesis of secondary metabolism, plant hormone signal transduction, glycerophospholipid metabolism, glycerolipid metabolism, and carotenoid biosynthesis (Table 8).

Genes involved in the KEGG pathway response to acclimation

Investigation of the DEGs with pathway annotation between the CM vs NM comparison group shows that ‘metabolic pathways’ and ‘biosynthesis of secondary metabolites’ have the largest number of genes involved, but the pathway annotation for these groups could not be differentiated. Of the significant downregulated categories (corrected p-value < 0.05), the carbon fixation in photosynthetic organisms category contained 61 downregulated DEGs with pathway annotation available, in which the most significant, with a -4.52 fold change (denoted 4.1.1.49), was phosphoenolpyruvate carboxykinase, a lyase involved in the generation of ATP (supplementary figure 1). Thirty-eight photosynthesis related DEGs were downregulated, the two most significant of which were photosystem II 13kDa protein (-3.56 fold change, denoted Psb 28), and photosystem I subunit III (-4.20 fold change, denoted PsaF) (supplementary figure 2). In addition, there were 30 downregulated genes involved in pyruvate metabolism, in which phosphoenolpyruvate carboxykinase was downregulated to the largest extent, with a -4.52 fold change (denoted 4.1.1.49), notably also involved in carbon fixation of photosynthetic organisms, as it is a precursor of pyruvate as well as oxaloacetate (supplementary figure 3). The upregulated categories of significance with pathway annotation available were plant hormone signal transduction with 75 upregulated genes, starch and sucrose metabolism with 71 upregulated genes, and endocytosis, with 56 upregulated genes. The highest fold change genes under the plant hormone signal transduction pathway were probable indole-3-acetic acid-amido synthetase GH3.1 (+5.88 fold change, denoted GH3) involved in cell enlargement and plant growth, and probable protein phosphatase 2C 68 (+6.46 fold change, denoted PP2C) involved in stomatal closure and seed dormancy (supplementary figure 4). The highest expressed upregulated genes in the starch and sucrose metabolism pathways are beta-amylase 8 isoform X2 (+6.92 fold change, denoted 3.2.1.2), soluble starch synthase 1, chloroplastic/amyloplastic (+5.41 fold change, denoted 2.4.1.21), and beta-glucosidase 26 (+5.15 fold change, denoted 3.2.1.21), a precursor to Beta-D-glucose (supplementary figure 5). The vacuolar protein sorting-associated protein 2 homolog 2 (denoted CHMP2) was the highest upregulated gene under endocytosis, with a 6.02 positive fold change (supplementary figure 6).

Then, the CV vs NV treatment was investigated for differentially expressed genes of significance. The major downregulated category of significance (corrected p-value .05) containing DEGs with pathway annotation was N-Glycan biosynthesis (33 genes), where the most downregulated gene was alpha-1,3-glucosyltransferase (-2.13 fold change, denoted ALG6) (supplementary figure 7). For upregulated DEGs with pathway annotation available in the CV vs NV comparison, plant hormone signal transduction contained the highest number (103 genes), followed by glycerophospholipid metabolism (47 genes), and glycerolipid metabolism (38 genes). In the plant hormone signal transduction pathway, probable protein phosphatase 2C 37 (denoted PP2C) exhibited a +7.65 fold change, and auxin-responsive protein SAUR71 (denoted SAUR) exhibited a +4.00 fold change (supplementary figure 8). In the glycerophospholipid metabolism pathway, phospholipase D alpha 1 (denoted 3.1.4.4) exhibited the highest fold change (+6.03), as well as choline kinase 2 (+4.70, denoted 2.7.1.32 and 2.7.1.82) (supplementary figure 9), Alpha-galactosidase (+6.05, denoted 3.2.1.22) was the highest upregulated gene in the glycerolipid metabolism pathway, followed by diacylglycerol O-acyltransferase 1-1 (+4.53, denoted 2.3.1.20) and diacylglycerol kinase 1 (+4.35, denoted 2.7.1.107)(supplementary figure 10).

DISCUSSION

Cold acclimation, a low but above freezing temperature treatment, is a critical process to the development of freezing tolerance, directly inhibiting metabolic reactions, and indirectly inhibiting cold induced osmotic stress, oxidative stress as well as numerous other genes and pathways by affecting lipid composition of membranes, sugars and proline molecules, and increasing the total soluble proteins (Guy, 1990; Thomashow, 1999). To further disentangle the differences in metabolic response to freeze, the present study compared the transcriptomes of Meyer, a freeze tolerant cultivar, and Victoria, a freeze susceptible cultivar under cold acclimated and non-cold acclimated conditions, where non-acclimation is the baseline comparison for up and down regulation.

The comparison groups CM vs NM and CV vs NV were of particular interest since they show the difference in cultivar response under the same treatment conditions, where non-acclimation is the baseline and cold acclimation is the treatment. Theoretically, any changes observed in the gene expression would be due to the treatment (Blumenberg, 2019). By observing the unique expression among these two comparison groups, we can look at how each cultivar is responding differently that may be causing Meyer to be more freezing tolerant than Victoria. In addition, differences in fold change for similarly expressed genes may indicate that up or downregulation of a particular pathway may play an important role in the development of freezing tolerance (Kim et al., 2005). The identified transcripts from the NM vs NV comparison may assist in the determination of which regions are utilized under non-acclimation conditions among both cultivars, while the CM vs CV comparison may help determine which regions are critical for cold acclimation processes. However, both of these comparison groups are confounded by the genetic differences between the cultivars, so are not of primary focus.

A similar study in bermudagrass examined a small population of genotypes identified as 'cold resistant' or 'cold susceptible', grouping resistant and susceptible accessions together for treatment purposes, where gene transcripts were associated with treatment and shared genes among a group (Chen et al., 2015). Conversely, a study on gene transcript expression in zoysiagrass specifically concentrated on the freeze tolerant cultivar Meyer to determine changes in gene expression under cold stress (Wei et al., 2015). This present study goes a step further to identify changes in gene expression under cold and freeze stress in the tolerant and susceptible cultivars, Meyer and Victoria, in order to narrow down genes in these cultivars that are responsible for their differential response, starting with an in depth examination of all the pathways and gene transcripts expressed in each of the four treatments: CM, NM, CV, and NV.

Differential gene expression among GO accessions

The gene ontology classification system divides accessions into three major categories: biological process, cellular component, and molecular function. In the comparison of the CM vs

NM compared to the CV vs NV treatment groups, significant findings ($p \geq 0.05$) as well as differential expression (fold change ≥ 2) were identified. Under the category of biological processes, photosynthesis related genes were significantly downregulated during cold acclimation in the cultivar Meyer, while biosynthetic processes, cellular homeostasis, ion transport, localization, and metabolic processes were significantly upregulated. In comparison, transmembrane transport genes were significantly downregulated in Victoria, and biosynthetic processes, localization, and metabolic regulation were the most significant upregulated processes.

Functional identification of genes

To identify key expression differences between Meyer and Victoria, the genes with the highest fold changes from differential expression analysis, as well as the most significant KEGG pathways and the highest expressed genes within those pathways, were investigated for associations with freezing stress tolerance in the CM vs NM and CV vs NV comparison groups.

Downregulated transcripts associated with low and freezing temperature abiotic stress

Of the significant identifiable genes in the CM vs NM comparison which were downregulated during cold acclimation, phosphoenolpyruvate carboxykinase (PCK) was downregulated (-4.52 fold change) as part of both the carbon fixation of photosynthetic organisms and the pyruvate metabolism pathways. This lyase was found to significantly decline in *Z. japonica* when exposed to a 10/7^{o}C chilling treatment, but showed little change in the cool season cordgrass, *Spartina anglica*, suggesting that the steep downregulation during cold acclimation of PCK may be a limiting factor in the development of greater cold tolerance in *Z. japonica* (Matsuba et al., 2008). The photosystem I subunit III (-3.56 log_2FC) and photosystem II 13kDa protein (-4.20 log_2FC) are part of the highly regulated photosynthesis processes which maintain a balance of energy absorbed vs energy consumed to optimize plant growth and development (Ensiminger, Busch and Huner, 2006). Cold stress greatly inhibits the photosynthetic system, necessitating adjustments along the pathways. Therefore, the significant change in regulation observed in the cold tolerant cultivar Meyer but not the sensitive cultivar Victoria may suggest that these transcripts are important for optimizing energy use under low temperature conditions, a hypothesis supported by similar findings in *Secale cereale* (L.) (Oquist and Huner, 1993), common bermudagrass (Chen et al., 2015), and zoysiagrass (Wei et al., 2015).

The largest downregulated change during cold acclimation in CM vs NM was flowering-promoting factor 1-like protein 4 with a -9.8437 log_2FC. A transcription factor, this gene has been shown to play a role in the gibberellin acid dependent signaling pathway in *Arabidopsis thaliana*, which in turn may modulate early plant response to reverse some inhibitory effects of abiotic stresses (Kania et al., 1997, Alonzo-Ramirez et al., 2009). This same gene was also downregulated in the CV vs NV comparison, but only at a log2FC of -2.833. The amino acid transporter AVT1J, with the next largest downregulated change in Meyer (-9.7904 log2FC), is an integral membrane protein essential for plant growth and development (Ortiz-Lopez, Chang, and Bush, 2000), but in the Victoria comparison, this same gene significantly downregulated (-3.6249 log2FC). These trends may suggest that these two significantly downregulated genes in Meyer may play a role in its increased freeze tolerance compared to Victoria. The next most downregulated genes in CM vs NM were dirigent protein 1 (-9.5667 log_2FC), and protein iron-related transcription factor 2 (-9.2231 log2FC), which were also strongly downregulated in the CV vs NV comparison (-6.4572 and -7.6847 respectively) and neither were upregulated to any significant extent. These genes may affect response to abiotic stress by modulating cell wall metabolism (Paniagua et al., 2017) and their strong change in expression in both cultivars suggests it is a key metabolic component during exposure to low temperatures. Finally, a strong downregulation of the transcription factor JAmyb was observed (-9.1646 log_2FC). However, while JAmyb was strongly downregulated in the CM vs NM comparison, it was also strongly upregulated in that same comparison (+7.813 and +6.0599 log_2FC), and upregulated to a lesser extent in Victoria (+4.1347, +4.1238, and +3.5561), but not down regulated at any significant level. When overexpressed, this gene may activate defense-associated genes involved in osmotic adjustment, ROS removal, and ion homeostasis associated with biotic and abiotic stress resistance (Yokotani et al., 2013), so these conflicting results may suggest that while expression of JAmyb is necessary for some low temperature tolerance, there may be a threshold in Meyer signaling negative regulation for stomatal closure as protection from further water loss (Yokotani et al., 2013).

In the CV vs NV comparison, the probable dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase (-2.13 fold change) in the N-Glycan biosynthesis pathway was the most downregulated gene during cold acclimation when looking at the top most significant categories. This gene is involved in protein modifications within the cell and may play a role in membrane function (von Schaewen, Frank, and Koiwa 2008). Auxin-responsive gene SAUR76 exhibited the greatest negative fold change between treatments (-8.2565 log_2FC) but when upregulated, may increase drought stress tolerance (Guo et al., 2018), a key component of both drought and freeze stress because of the water loss that occurs. Auxin responsive genes are involved in plant growth and development, as well as in the upregulation of abiotic stress responsive genes (Guo et al., 2018). By positively affecting this gene, response to abiotic stress may also be positively affected in cold sensitive cultivars. The next largest downregulated change is associated with the probable polyol transporter 4, a sugar alcohol with a fold change of -7.2062 log_2FC, which may be associated with response to water-deficit stress when expressed (Conde et al., 2015). And finally, ethylene-responsive transcription factor ERF073 (-6.9984 log_2FC) is involved in multiple stress tolerance functions, by encoding a CRT/DRE element binding factor which plays a role in multiple stress signal transduction pathways, including drought, salinity and low-temperature stress (Xu et al., 2007). These ethylene-responsive transcription factor genes showed significant upregulation in Meyer, but not in Victoria. The lack of upregulation of these genes in Victoria when exposed to low temperature stress may be a contributing factor to its cold sensitivity compared to Meyer.

Upregulated transcripts associated with low and freezing temperature stress

Gene transcripts which are highly upregulated during cold acclimation of the freeze tolerant cultivar ‘Meyer’ may signify that those genes are critical for the development of freeze tolerance, and lack of a similarly high fold change between treatments of the cold sensitive cultivar Victoria may indicate that those genes are a contributing factor to the difference in sensitivity between cultivars. In the CM vs NM comparison, the plant hormone signal transduction pathway gene, probable protein phosphatase 2C 68, with a log2 fold change of +6.56, was highly upregulated during cold acclimation and is believed to play an important role in many ABA signal transduction pathways. A similar protein phosphatase 2C (PP2C) 37 was upregulated in the CV vs NV comparison, exhibiting a +7.65 fold change as part of the carotenoid biosynthesis pathway, a component of the larger plant hormone signal transduction pathway. A study in Arabidopsis observed that downregulation of some PP2C genes during cold acclimation led to increased freezing tolerance, but also noted that PP2Cs are one of the largest families in plants involved in a variety of signaling functions (Schweighofer et al., 2004). More investigation into the specific role of the highly upregulated PP2Cs identified in this study may be needed, as other genes within the family were expressed with varying levels of up and down regulation. Within the same carotenoid biosynthesis pathway, 9-cis-epoxycarotenoid dioxygenase NCED5, chloroplastic exhibited a +8.64 fold change and phytoene synthase 2, chloroplastic exhibited a +7.71 fold change in the

Victoria comparison. 9-cis-epoxycarotenoid dioxygenase functions as a key enzyme in abscisic acid biosynthesis, where overexpression of the gene results in improved drought tolerance through reduced transpiration from leaves (Iuchi et al., 2001), potentially working simultaneously with phytoene synthase to induce response to water restriction in the leaf stomata as well as the roots (Welsch et al., 2008).

Several genes in the starch and sucrose metabolism pathways were significantly upregulated, but although they are an important component of response to low temperature stress for cold acclimation (Hannah, Hayer, and Hincha, 2005), alteration of sucrose concentrations may not be able to improve basic freezing tolerance (Klotke et al., 2004), however alterations of glucose metabolism may be important to zoysiagrass freezing tolerance (Patton et al., 2007). The largest change in expression for the Meyer comparison was also the highest change for the Victoria comparison, occurring in the low molecular mass early light-inducible protein (ELIP) HV90 chloroplastic, with a +11.968 and +10.623 log2FC respectively. The HV60 chloroplastic protein was also highly upregulated in the Meyer comparison at +10.288 log2FC. These ELIPs function as an adaptive response to high light, low temperature conditions such as those occurring during the process of cold acclimation, and may contribute to protecting photosynthetic elements from these stressors (Peng et al., 2007). A late embryogenesis abundant (LEA) protein 14 was highly expressed with a log2 fold change of +11.646 in CM vs NM, and +9.6458 in CV vs NV. LEA protein 18 was also highly expressed in the Victoria comparison at +9.7227 log2FC. High accumulations of LEA proteins are frequently linked to desiccation tolerance, such as that occurring under cold, freeze, drought, and salt stress (Ingram and Bartels, 1996, Thomashow, 1999), and has even been shown to increase tolerance to salt stress when overexpressed (Jia et al., 2014). In the Meyer comparison, dehydrin DHN1 (+11.225 log2FC) and dehydrin COR410 were highly expressed (+10.775 log2FC). These dehydrins are commonly expressed hydrophilic proteins that are induced by water related stressors, such as drought, salinity, and low temperatures and induced overexpression of dehydrins has shown a positive effect on root development, which may improve reactive oxygen species scavenging ability (Zhang et al., 2018), relate to faster recovery following water stress (Beck et al., 2007) and play an important role in cold acclimation and freezing tolerance (Patton et al., 2007).

In the CV vs NV comparison, phospholipase D alpha 1 was significantly upregulated (+6.03 log2FC) in the glycerophospholipid metabolism pathway, but though this increase occurs during cold acclimation, it is those plants which are deficient in this gene that demonstrate greater freezing tolerance in terms of membrane leakage and survival. Therefore, the upregulation of phospholipase D alpha 1 in the cold sensitive cultivar may be contributing to the sensitivity, or the changes in expression of this gene may not play a major role in affecting lipid response to low temperature stress (Welti et al., 2002). Finally, in the Victoria comparison, alpha-galactosidase was the highest upregulated gene in the glycerolipid metabolism pathway (+6.05 log2FC), causing a decrease in raffinose metabolism, though an increase in raffinose is associated with increased freezing tolerance (Shahba et al., 2003). Pennycooke et al. (2004) suggest that targeted downregulation of alpha-galactosidase may result in increased freezing stress tolerance by way of increasing raffinose concentrations.

Validation against Winter Survival and Freeze Tolerance Associated QTL

The identified gene regions from the transcriptomic analysis were compared to the QTL regions that have been previously associated with winter survival (Holloway et al., 2018) and freeze tolerance (chapter 2), as well as with the results of the protein expression analysis (Brown et al., 2020). Although direct comparisons could not be made between the gene transcripts and proteins, changes in expression during cold acclimation, low molecular mass early light-inducible proteins, late embryogenesis abundant proteins, and photosystem II proteins were all observed to have undergone significant changes in expression. Functionally, genes and proteins associated with transcription, membranes, photosynthesis, and energy were critical for the response to cold acclimation and freezing tolerance in all these studies. Across all approaches, auxin-responsive proteins were particularly emphasized as significant for freeze response. The downregulation of auxin-responsive GH3 family protein in the freeze sensitive cultivar Victoria, and the downregulation of the auxin-responsive protein SAUR76 in the CV vs NV treatment comparison strengthens its role as a key regulator of cold stress response (Rahman, 2012), and a potential area of interest to target for marker assisted selection of freeze tolerant cultivars.

Future Directions

Although above-zero cold acclimation plays a large role in the development of freezing tolerance, it was also demonstrated that subzero acclimation conditions can result in additional freezing tolerance, arising from a different set of complex biological process, as evidenced by the levels of gene expression (Herman et al., 2006). Freezing injury is typically caused by the formation of ice in intracellular plant tissues and extracellular cell walls under freezing temperatures. This extracellular ice formation results in a drop in water potential outside of the cell, leading to cellular dehydration, in a way very similar to drought and salinity stress (Guy, 1990). Therefore, the plasma membrane is considered the primary site for freeze injury to occur (Levitt 1980), as well as the chloroplast thylakoid (Hincha and Schmitt, 1992), and envelope membranes (Hincha et al., 1987). Additional leaf tissue samples were taken at the lowest freezing point, -8^{o}C, and -11^{o}C, and may be utilized for future analysis to investigate the differences in subzero temperatures vs above zero cold acclimation, and the role each play in post freeze survival and regrowth of zoysiagrass leaf tissues.

Overlapping regions among these studies strengthens associations behind true genic regions of interest. The next step will be to validate the expression of candidate genes by way of real-time quantitative PCR (RT-PCR). This will be done following the method described by Chen et al. (2012, 2013). Results will be categorized according to the strength of their correlation generated from the RNA-seq analysis.

CONCLUSIONS

This study examined the differential expression of gene transcripts between freeze susceptible 'Victoria' and freeze tolerant 'Meyer' under cold acclimated and non-cold acclimated conditions. Of the assembled genes which were identified as being differentially expressed in acclimated versus non-acclimated conditions, Meyer demonstrated significant downregulation during cold acclimation of thylakoid components, and significant upregulation during cold acclimation of genes related to transmembrane transporters and binding. In Victoria, significant downregulation during acclimation occurred in transmembrane genes, while ubiquitin ligase components and metal binding genes were upregulated. Across cultivars, cold acclimation was associated with downregulation of photosystem cellular components. Looking into specific genes in particular, the expression of low molecular mass early light-inducible proteins, LEA proteins, auxin-responsive proteins, and photosystem II proteins showed a large fold change difference between treatments, and each were associated with aspects of freezing tolerance. Auxin-responsive proteins in particular were found across transcriptome, proteome, and genome analysis to be associated with cold acclimation and freezing tolerance. These findings strengthen the connection of these genes with resistance to cold and freeze stress, and upon further study, may be utilized to facilitate future breeding efforts to increase freeze tolerance in zoysiagrass.

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Table 1: Summary of sequence assembly following Illumina sequencing.

Sample name	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20(%)	Q30(%)	GC content(%)
NM1	49,475,254	48,077,382	7.2G	0.03	97.92	94.15	57.25
NM2	51,183,836	50,010,472	7.5G	0.03	97.84	93.99	57.49
NM3	50,168,628	49,079,396	7.4G	0.03	97.88	94.04	57.49
CM1	59,804,056	58,665,446	8.8G	0.03	97.37	92.95	52.47
CM2	67,204,258	65,599,380	9.8G	0.03	97.35	92.94	51.48
CM3	53,728,046	52,608,814	7.9G	0.03	97.11	92.48	52.52
NV1	71,039,902	69,474,296	10.4G	0.03	97.47	93.19	53.68
NV2	46,536,824	45,397,712	6.8G	0.03	97.39	93.03	52.99
NV3	55,073,384	53,918,022	8.1G	0.03	97.08	92.41	53.03
CV1	61,931,458	6,0855,104	9.1G	0.03	97.43	93.12	52.9
CV2	51,018,634	49,819,784	7.5G	0.03	97.28	92.84	52.43
CV3	67,843,324	66,500,252	10.0G	0.03	97.49	93.23	52.78

NM1, NM2, NM3: Non-cold acclimated conditions, ‘Meyer’ cultivar, biological replicate 1-3.

CM1, CM2, CM3: Cold acclimated conditions, ‘Meyer’ cultivar, biological replicate 1-3.

NV1, NV2, NV3: Non-cold acclimated conditions, ‘Victoria’ cultivar, biological replicate 1-3.

CV1, CV2, CV3: Cold acclimated conditions, ‘Victoria’ cultivar, biological replicate 1-3.

Table 2: The number of genes with different expression levels determined through FPKM.

FPKM Interval	NM1 (%)	NM2 (%)	NM3 (%)	CM1 (%)	CM2 (%)	CM3 (%)	NV1 (%)	NV2 (%)	NV3 (%)	CV1 (%)	CV2 (%)	CV3 (%)
0~1	35297 (59.55)	35120 (59.25)	34999 (59.05)	34694 (58.53)	35971 (60.69)	35077 (59.18)	33754 (56.95)	34694 (58.53)	34744 (58.62)	35907 (60.58)	36262 (61.18)	36166 (61.02)
1~3	5323 (8.98)	5427 (9.16)	5482 (9.25)	5988 (10.10)	5511 (9.30)	5633 (9.50)	5243 (8.85)	5263 (8.88)	5172 (8.73)	5466 (9.22)	5468 (9.23)	5476 (9.24)
3~15	10670 (18.00)	10773 (18.18)	10783 (18.19)	9300 (15.69)	8692 (14.66)	9166 (15.46)	10194 (17.20)	9814 (16.56)	9810 (16.55)	8968 (15.13)	8803 (14.85)	8817 (14.88)
15~60	6023 (10.16)	5988 (10.10)	6081 (10.26)	6615 (11.16)	6355 (10.72)	6699 (11.30)	7675 (12.95)	7110 (12.00)	7182 12.12)	6324 (10.67)	6113 (10.31)	6176 (10.42)
>60	1958 (3.30)	1963 (3.31)	1926 (3.25)	2674 (4.51)	2742 (4.63)	2696 (4.55)	2405 (4.06)	2390 (4.03)	2363 (3.99)	2606 (4.40)	2625 (4.43)	2636 (4.45)

Table 3: The top KEGG pathways, where significance is defined as padj ≤ 0.05, in the Cold-acclimated Meyer (CM) vs Cold-acclimated Victoria (CV) comparison.

#Term	ID	Input number	Background number	P-Value	Corrected P-Value
Metabolic pathways	sita01100	494	2062	6.52E-10	7.95E-08
Biosynthesis of secondary metabolites	sita01110	301	1195	4.04E-08	2.46E-06
Carbon metabolism	sita01200	76	271	0.000407	0.012403105
Plant hormone signal transduction	sita04075	68	247	0.00118	0.028787763
Pyruvate metabolism	sita00620	31	89	0.001466	0.029811133

Table 4: The top twenty KEGG pathways, where significance is defined as padj ≤ 0.05, in the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

#Term	ID	Input number	Background number	P-Value	Padj-Value
Metabolic pathways	sita01100	1258	2062	3.16E-14	3.82E-12
Carbon metabolism	sita01200	221	271	1.10E-09	6.68E-08
Biosynthesis of secondary metabolites	sita01110	707	1195	5.05E-07	2.04E-05
Pyruvate metabolism	sita00620	80	89	2.19E-05	0.000662223
Glycolysis / Gluconeogenesis	sita00010	113	144	3.59E-05	0.000868016
Plant hormone signal transduction	sita04075	170	247	9.02E-05	0.001818593
Biosynthesis of amino acids	sita01230	165	242	0.000157	0.002716027
Pentose phosphate pathway	sita00030	53	59	0.000498	0.007531548
Starch and sucrose metabolism	sita00500	141	210	0.000704	0.009459212
RNA transport	sita03013	117	169	0.000877	0.010617429
Fructose and mannose metabolism	sita00051	57	68	0.000983	0.010815066
Ribosome biogenesis in eukaryotes	sita03008	69	89	0.001331	0.013424722
Porphyrin and chlorophyll metabolism	sita00860	41	47	0.002922	0.025692835
Glycerolipid metabolism	sita00561	57	73	0.002973	0.025692835
Nitrogen metabolism	sita00910	32	35	0.005046	0.040705178
Arginine and proline metabolism	sita00330	41	50	0.00608	0.044467065
Protein processing in endoplasmic reticulum	sita04141	127	202	0.006309	0.044467065
Citrate cycle (TCA cycle)	sita00020	42	52	0.006615	0.044467065
Phagosome	sita04145	56	76	0.007395	0.04709402
Glycerophospholipid metabolism	sita00564	73	106	0.007955	0.048126325

Table 5: The top KEGG pathways, where significance is defined as padj ≤ 0.05, in the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

#Term	ID	Input number	Background number	P-Value	Padj-Value
Metabolic pathways	sita01100	1136	2062	5.53E-09	6.69E-07
Biosynthesis of secondary metabolites	sita01110	654	1195	1.93E-05	0.000954938
Plant hormone signal transduction	sita04075	168	247	2.37E-05	0.000954938
Glycolysis / Gluconeogenesis	sita00010	101	144	0.000425	0.012845719
RNA transport	sita03013	114	169	0.000544	0.01316611
Pyruvate metabolism	sita00620	68	89	0.000733	0.013992826
Carbon metabolism	sita01200	167	271	0.00081	0.013992826
Glycerophospholipid metabolism	sita00564	77	106	0.00096	0.014520886
Protein processing in endoplasmic reticulum	sita04141	129	202	0.001158	0.015571074
Glycerolipid metabolism	sita00561	56	73	0.001918	0.021830101
N-Glycan biosynthesis	sita00510	36	40	0.001985	0.021830101
Citrate cycle (TCA cycle)	sita00020	43	52	0.002358	0.023775454
Biosynthesis of amino acids	sita01230	146	242	0.002951	0.027465925
Arginine and proline metabolism	sita00330	40	50	0.004853	0.041947462
Spliceosome	sita03040	115	188	0.005582	0.045031636

Table 6: The top twenty KEGG pathways, where significance is defined as padj ≤ 0.05, in the Non-acclimated Meyer (NM) vs Non-acclimated Victoria (NV) comparison.

#Term	ID	Input number	Background number	P-Value	Corrected P-Value
Metabolic pathways	sita01100	1106	2062	1.38E-18	1.67E-16
Carbon metabolism	sita01200	208	271	3.91E-13	2.37E-11
Ribosome	sita03010	215	314	2.81E-10	1.13E-08
Biosynthesis of secondary metabolites	sita01110	627	1195	9.23E-10	2.79E-08
Glycolysis / Gluconeogenesis	sita00010	109	144	2.20E-07	4.67E-06
Pyruvate metabolism	sita00620	78	89	2.32E-07	4.67E-06
RNA degradation	sita03018	80	112	3.56E-05	0.000537802
Citrate cycle (TCA cycle)	sita00020	47	52	3.20E-05	0.000537802
Biosynthesis of amino acids	sita01230	143	242	6.74E-05	0.000906106
Starch and sucrose metabolism	sita00500	125	210	0.000148	0.001793665
Plant hormone signal transduction	sita04075	142	247	0.000179	0.001801372
Porphyrin and chlorophyll metabolism	sita00860	41	47	0.000167	0.001801372
Pentose phosphate pathway	sita00030	46	59	0.000405	0.003766694
Arginine and proline metabolism	sita00330	40	50	0.000661	0.005711107
Protein processing in endoplasmic reticulum	sita04141	114	202	0.001174	0.009466339
Fructose and mannose metabolism	sita00051	48	68	0.00141	0.010660998
Alanine, aspartate and glutamate metabolism	sita00250	38	50	0.001704	0.012128746
Ribosome biogenesis in eukaryotes	sita03008	58	89	0.00189	0.01270577
N-Glycan biosynthesis	sita00510	32	40	0.002184	0.013909845
Phagosome	sita04145	49	76	0.004696	0.028411737

Table 7: The upregulated, downregulated, and overall significant (corrected p-value ≤ 0.05) KEGG pathways for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

Directional Change	#Term	ID	Input number	Background number	Corrected P-Value
down	Metabolic pathways	sita01100	459	2062	7.97E-06
down	Biosynthesis of secondary metabolites	sita01110	254	1195	0.022295812
down	Carbon metabolism	sita01200	87	271	3.14E-05
down	Biosynthesis of amino acids	sita01230	63	242	0.043066046
down	Carbon fixation in photosynthetic organisms	sita00710	50	79	1.71E-09
down	Photosynthesis	sita00195	34	74	0.000199304
down	Pyruvate metabolism	sita00620	30	89	0.032066737
up	Metabolic pathways	sita01100	522	2062	0.000873558
up	Biosynthesis of secondary metabolites	sita01110	310	1195	0.006999811
up	Plant hormone signal transduction	sita04075	75	247	0.034347927
up	Starch and sucrose metabolism	sita00500	71	210	0.008664151
up	Endocytosis	sita04144	56	163	0.018026553
up	Glycolysis / Gluconeogenesis	sita00010	50	144	0.020988827
up	RNA degradation	sita03018	45	112	0.006999811
up	Ribosome biogenesis in eukaryotes	sita03008	43	89	0.000873558
up	Arginine and proline metabolism	sita00330	25	50	0.010056044
up	Circadian rhythm - plant	sita04712	23	48	0.019329869
up	Fatty acid degradation	sita00071	21	47	0.042767309
all	Metabolic pathways	sita01100	981	2062	7.58E-08
all	Biosynthesis of secondary metabolites	sita01110	564	1195	0.000196786
all	Carbon metabolism	sita01200	162	271	0.000144521
all	Plant hormone signal transduction	sita04075	131	247	0.014518151
all	Biosynthesis of amino acids	sita01230	130	242	0.013171221
all	Starch and sucrose metabolism	sita00500	123	210	0.002286917
all	Glycolysis / Gluconeogenesis	sita00010	88	144	0.006457551
all	Carbon fixation in photosynthetic organisms	sita00710	69	79	2.91E-05
all	Pyruvate metabolism	sita00620	60	89	0.008184022
all	Fructose and mannose metabolism	sita00051	44	68	0.043627256
all	Pentose phosphate pathway	sita00030	41	59	0.02574561
all	Arginine and proline metabolism	sita00330	38	50	0.014518151
all	Nitrogen metabolism	sita00910	27	35	0.043627256

Table 8: The upregulated, downregulated, and overall significant (corrected p-value ≤ 0.05) KEGG pathways for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

Directional Change	#Term	ID	Input number	Background number	Corrected P-Value
down	Metabolic pathways	sita01100	420	2062	0.012283687
down	N-Glycan biosynthesis	sita00510	22	40	0.00364942
up	Biosynthesis of secondary metabolites	sita01110	268	1195	0.001579
up	Plant hormone signal transduction	sita04075	84	247	1.73E-05
up	Glycerophospholipid metabolism	sita00564	40	106	0.001579
up	Glycerolipid metabolism	sita00561	30	73	0.003153203
up	Carotenoid biosynthesis	sita00906	17	33	0.010866746
all	Metabolic pathways	sita01100	827	2062	0.000952633
all	Biosynthesis of secondary metabolites	sita01110	491	1195	0.006900843
all	Plant hormone signal transduction	sita04075	134	247	0.000952633
all	Glycerophospholipid metabolism	sita00564	62	106	0.017753494
all	Carotenoid biosynthesis	sita00906	26	33	0.032454529

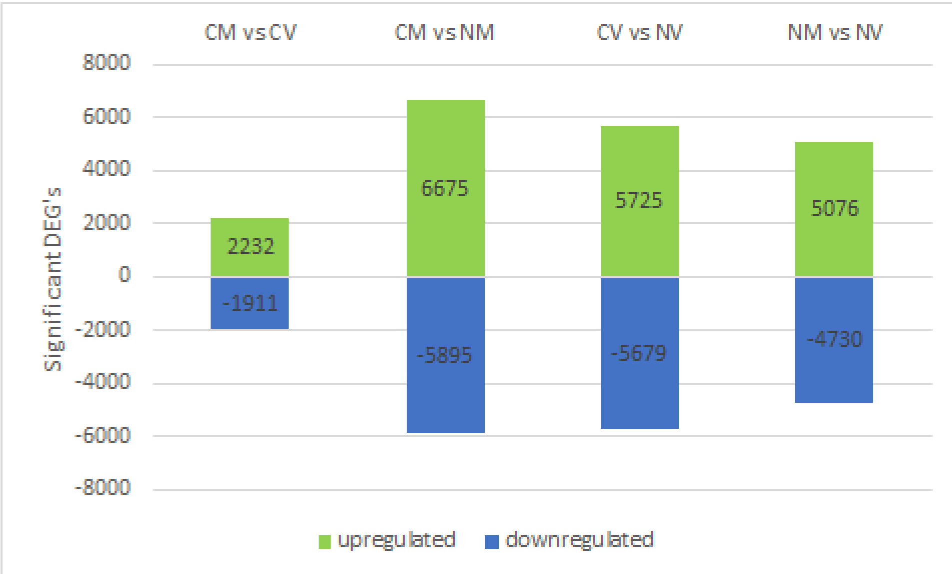


Figure 1: Histogram of significant (padj < 0.05) upregulated and downregulated Differentially Expressed Genes (DEG's), where negative values indicate how many were downregulated, and positive values indicate how many were upregulated, for the four comparison groups: cold acclimated 'Meyer' (CM) vs cold acclimated 'Victoria' (CV), CM vs non-cold acclimated 'Meyer' (NM), CV vs non-cold acclimated 'Victoria' (NV), and NM vs NV.

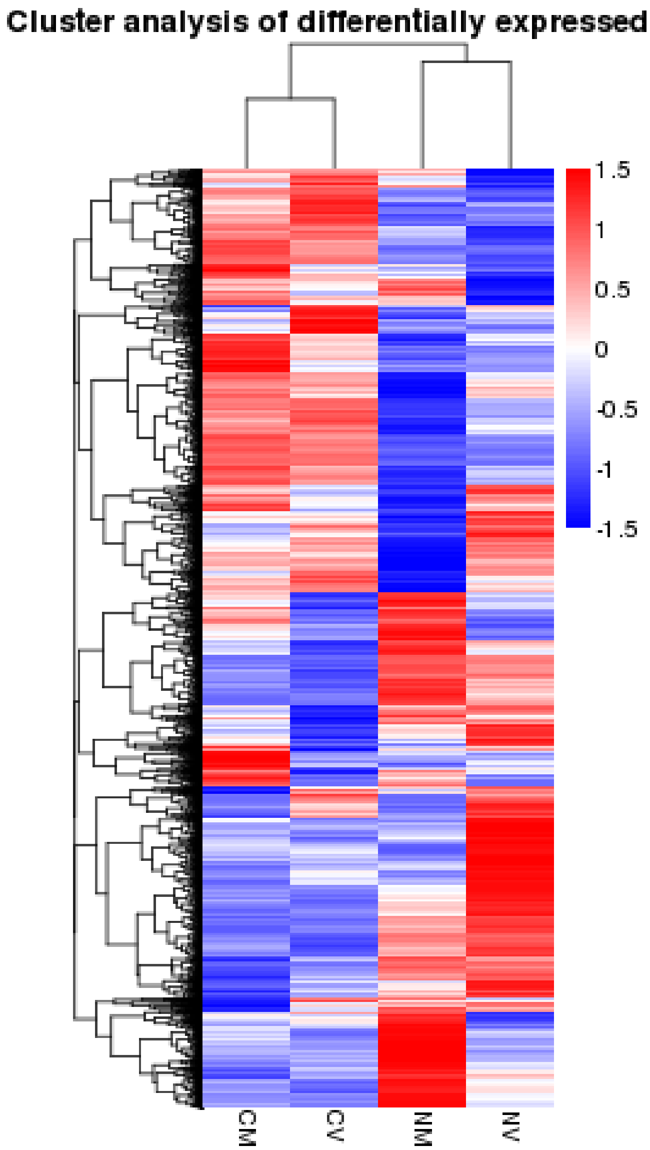


Figure 2: Hierarchical clustering of differentially expressed genes between four samples: cold acclimated 'Meyer' (CM), cold acclimated 'Victoria' (CV), non-cold acclimated 'Meyer' (NM) and non-cold acclimated 'Victoria' (NV), where blue color indicates low gene expression quantity and red color indicates high gene expression quantity. Color intensity indicates level of significance, where lighter shades indicate not significant, and deeper shades indicate significance at Fragments Per Kilobase of transcript per Million mapped reads (FPKM) >1 and log^2 fold change value >1 or < -1.

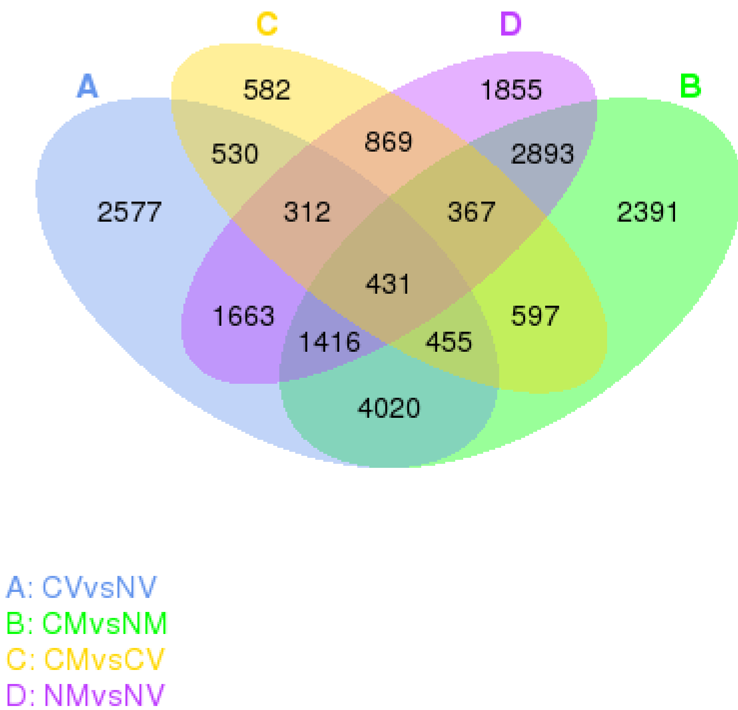
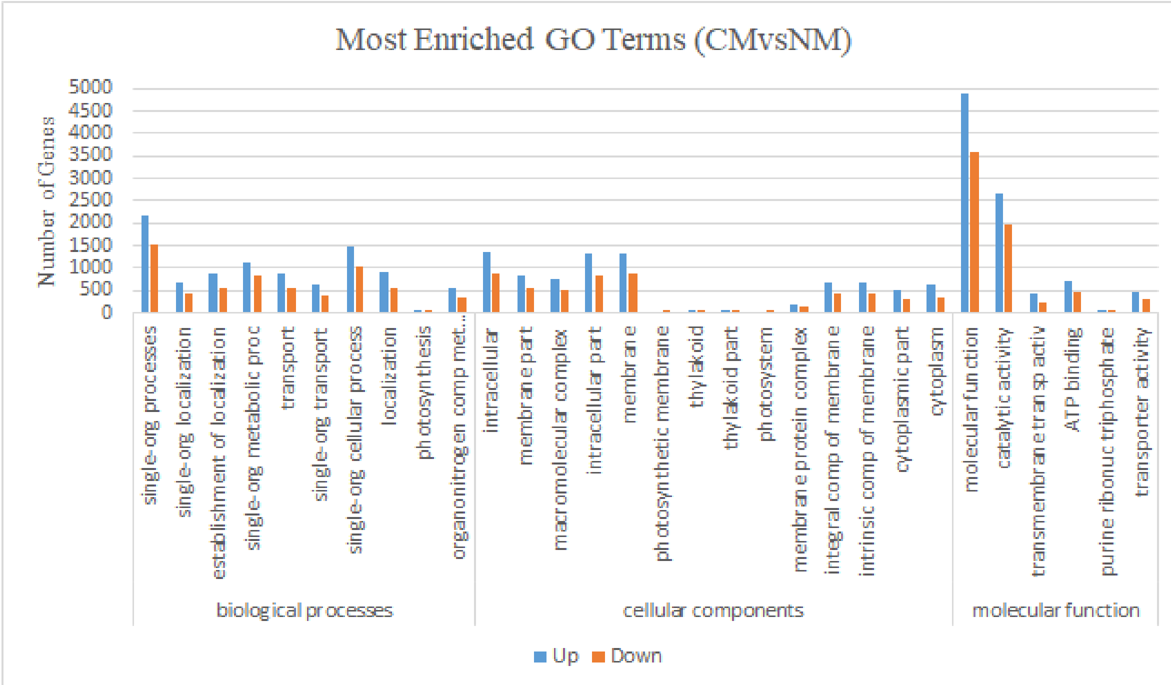
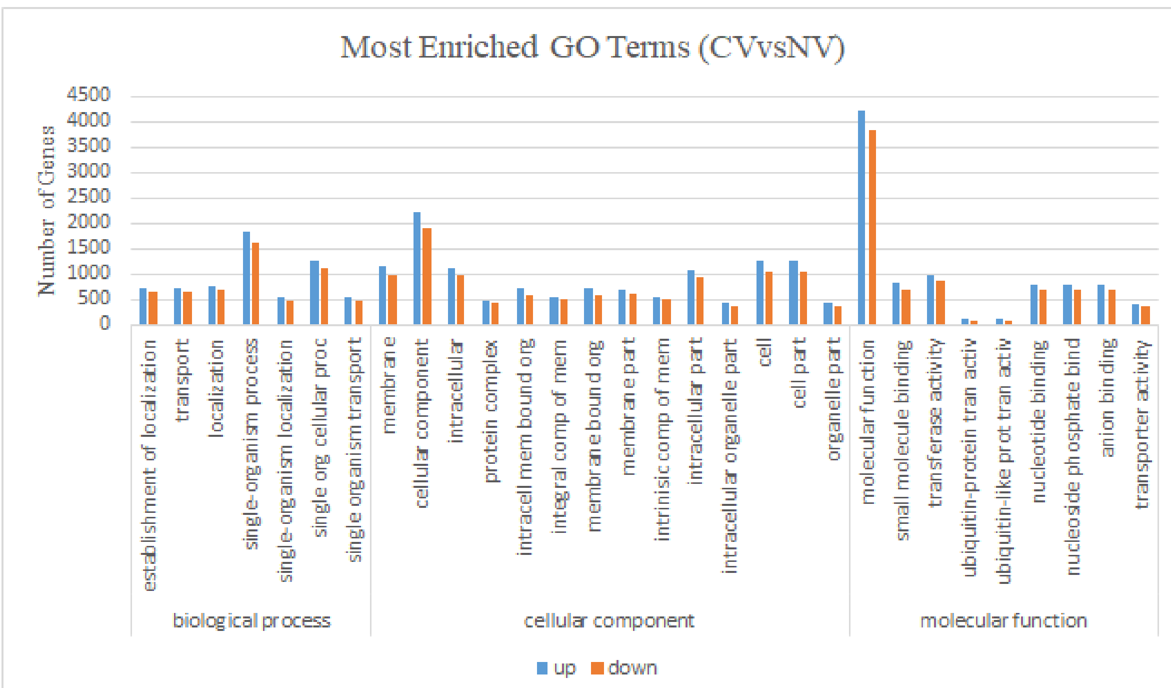


Figure 3: Venn diagram of significant (Fragments Per Kilobase of transcript per Million mapped reads (FPKM) >1) co-expression between comparisons where overlapping segments represent shared differential expressions among groups, and non-overlapping segments represent unique differential gene expressions. A: cold acclimated 'Meyer' (CM) vs cold acclimated 'Victoria' (CV), B: CM vs non-cold acclimated 'Meyer' (NM), C: CV vs non-cold acclimated 'Victoria' (NV), and D: NM vs NV.



a.



b.

Figure 4: Gene Ontology (GO) classification of the DEGs identified in the comparison between pairs of libraries: a. CM vs NM, and b. CV vs NV, annotated into three categories: biological process, cellular component and molecular function. The Y-axis is the enriched GO term, the X-axis is the number of DEGs enriched in this term for up and downregulated terms.

Supplementary Table 1: List of all differentially expressed genes with significant upregulation (log^2FoldChange ≤ -1 and padj ≤ 0.05) for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

Gene_id	readcount_CM	readcount_NM	log^2Fold Change	p_{val}	p_{adj}	Blast swiss prot
Zjn_sc00030.1.g02670.1.am.mk	44919.05	11.21185	11.968	2.72E-50	7.94E-48	sp|P14897|ELI9_HORVU Low molecular mass early light-inducible protein HV90, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//7.6445e-21
Zjn_sc00011.1.g01130.1.sm.mk	1099.753	0.343249	11.646	9.00E-46	1.88E-43	sp|Q94JF2|LEA14_ORYSJ Late embryogenesis abundant protein 14 OS=Oryza sativa subsp. japonica OX=39947 GN=LEA14 PE=2 SV=1//1.17262e-42
Zjn_sc00061.1.g01140.1.sm.mk	45264.18	18.91035	11.225	5.13E-16	1.21E-14	sp|P12950|DHN1_MAIZE Dehydrin DHN1 OS=Zea mays OX=4577 GN=DHN1 PE=1 SV=2//9.79158e-12
Zjn_sc00007.1.g09670.1.sm.mkhc	62605.96	35.74904	10.774	2.35E-114	1.24E-110	sp|P46524|CO410_WHEAT Dehydrin COR410 OS=Triticum aestivum OX=4565 GN=COR410 PE=2 SV=1//1.01795e-15
Zjn_sc00030.1.g02630.1.sm.mk	14748.18	11.79734	10.288	4.59E-69	3.47E-66	sp|P14896|ELI6_HORVU Low molecular mass early light-inducible protein HV60, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//3.08571e-32
Zjn_sc00002.1.g04260.1.am.mk	3278.178	2.745995	10.221	2.77E-19	8.70E-18	sp|P27898|MYBP_MAIZE Myb-related protein P OS=Zea mays OX=4577 GN=P PE=2 SV=1//4.51867e-28
Zjn_sc00086.1.g02840.1.sm.mk	3928.898	3.80028	10.014	1.83E-38	2.43E-36	sp|Q5UNY4|YL728_MIMIV Uncharacterized protein L728 OS=Acanthamoeba polyphaga mimivirus OX=212035 GN=MIMI_L728 PE=4 SV=1//2.52702e-54
Zjn_sc00030.1.g02690.1.am.mk	14239.09	14.73998	9.9159	5.50E-198	2.33E-193	sp|P14896|ELI6_HORVU Low molecular mass early light-inducible protein HV60, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//3.39602e-32
Zjn_sc00017.1.g00030.1.sm.mk	645.3497	0.689566	9.8702	5.16E-12	7.78E-11	sp|Q0DZE0|PAL2_ORYSJ Phenylalanine ammonia-lyase OS=Oryza sativa subsp. japonica OX=39947 GN=ZB8 PE=2 SV=1//1.28716e-63
Zjn_sc00088.1.g02310.1.sm.mk	628.9563	0.686499	9.8395	7.01E-10	8.10E-09	sp|O48716|JGB_ARATH Protein JINGUBANG OS=Arabidopsis thaliana OX=3702 GN=JGB PE=1 SV=1//8.92993e-83

Supplementary Table 2: List of all differentially expressed genes with significant downregulation (log^2FoldChange ≤ -1 and padj ≤ 0.05) for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

Gene_id	readcount_CM	readcount_NM	log^2Fold Change	p_{val}	p_{adj}	Blast swiss prot
Zjn_sc00006.1.g07620.1.sm.mk	0.32339	297.1567	-9.8437	8.31E-13	1.37E-11	sp|Q0JEF5|FLP4_ORYSJ Flowering-promoting factor 1-like protein 4 OS=Oryza sativa subsp. japonica OX=39947 GN=Os04g0282400 PE=3 SV=1//8.54541e-62
Zjn_sc00037.1.g01080.1.am.mk	0.30752	272.3233	-9.7904	6.32E-26	3.55E-24	sp|Q9LXF8|AVT1J_ARATH Amino acid transporter AVT1J OS=Arabidopsis thaliana OX=3702 GN=AVT1J PE=2 SV=1//2.04437e-73
Zjn_sc00079.1.g00420.1.sm.mk	4.144014	3142.507	-9.5667	5.62E-07	4.20E-06	sp|Q9FIG6|DIR1_ARATH Dirigent protein 1 OS=Arabidopsis thaliana OX=3702 GN=DIR1 PE=2 SV=1//3.13463e-32
Zjn_sc00009.1.g00860.1.sm.mkhc	8.402928	5021.721	-9.2231	6.77E-08	5.88E-07	sp|A2WZ60|IRO2_ORYSI Protein IRON-RELATED TRANSCRIPTION FACTOR 2 OS=Oryza sativa subsp. indica OX=39946 GN=IRO2 PE=3 SV=1//5.50516e-76
Zjn_sc00011.1.g08380.1.am.mk	1.24595	715.044	-9.1646	5.42E-26	3.06E-24	sp|Q2QZJ8|JAMYB_ORYSJ Transcription factor JAMYB OS=Oryza sativa subsp. japonica OX=39947 GN=JAMYB PE=2 SV=1//1.63056e-72
Zjn_sc00043.1.g00860.1.am.mk	1.544446	657.4895	-8.7337	1.10E-39	1.56E-37	sp|F4JIJ7|BH162_ARATH Transcription factor bHLH162 OS=Arabidopsis thaliana OX=3702 GN=BHLH162 PE=1 SV=1//2.8488e-13
Zjn_sc00001.1.g06820.1.am.mk	9.928081	3775.588	-8.571	9.97E-37	1.19E-34	sp|O64515|OCT2_ARATH Organic cation/carnitine transporter 2 OS=Arabidopsis thaliana OX=3702 GN=OCT2 PE=2 SV=1//3.21644e-160
Zjn_sc00005.1.g08060.1.sm.mkhc	3.053728	943.6183	-8.2715	5.35E-93	1.41E-89	sp|F4I4E1|BH167_ARATH Transcription factor bHLH167 OS=Arabidopsis thaliana OX=3702 GN=BHLH167 PE=2 SV=1//3.57745e-14
Zjn_sc00008.1.g11130.1.sm.mk	4.42664	1232.686	-8.1214	1.53E-86	2.58E-83	sp|Q9SA38|OCT3_ARATH Organic cation/carnitine transporter 3 OS=Arabidopsis thaliana OX=3702 GN=OCT3 PE=2 SV=1//5.82347e-167
Zjn_sc00027.1.g00770.1.am.mk	0.590145	158.2505	-8.0669	1.27E-08	1.23E-07	sp|Q58FS3|RAD_ANTMA Transcription factor RADIALIS OS=Antirrhinum majus OX=4151 GN=RAD PE=1 SV=1//9.33083e-23

Supplementary Table 3: List of all differentially expressed genes with significant upregulation (log^2FoldChange ≤ -1 and padj ≤ 0.05) for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

Gene_id	readcount_CV	readcount_NV	log^2Fold Change	p_{val}	p_{adj}	Blast swiss prot
Zjn_sc00030.1.g02670.1.am.mk	38352.71	24.3229	10.623	2.84E-109	4.24E-106	sp|P14897|ELI9_HORVU Low molecular mass early light-inducible protein HV90, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//7.6445e-21
Zjn_sc00004.1.g08640.1.sm.mk	184.7603	0.218663	9.7227	3.17E-12	4.84E-11	sp|Q96273|LEA18_ARATH Late embryogenesis abundant protein 18 OS=Arabidopsis thaliana OX=3702 GN=LEA18 PE=2 SV=1//2.99572e-13
Zjn_sc00020.1.g03270.1.am.mk	361.1525	0.437326	9.6897	1.75E-45	2.14E-43	sp|Q7XPK7|AHT1_ORYSJ Agmatine hydroxycinnamoyltransferase 1 OS=Oryza sativa subsp. japonica OX=39947 GN=AHT1 PE=1 SV=1//1.13843e-61
Zjn_sc00011.1.g01130.1.sm.mk	1383.472	1.727043	9.6458	2.61E-133	9.91E-130	sp|Q94JF2|LEA14_ORYSJ Late embryogenesis abundant protein 14 OS=Oryza sativa subsp. japonica OX=39947 GN=LEA14 PE=2 SV=1//1.17262e-42
Zjn_sc00030.1.g02680.1.am.mk	12897.87	18.08046	9.4785	2.93E-40	2.81E-38	sp|P14897|ELI9_HORVU Low molecular mass early light-inducible protein HV90, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//3.82799e-29
Zjn_sc00030.1.g02690.1.am.mk	7628.367	11.06584	9.4291	8.61E-50	1.26E-47	sp|P14896|ELI6_HORVU Low molecular mass early light-inducible protein HV60, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//3.39602e-32
Zjn_sc00063.1.g00520.1.am.mk	1172.538	1.727043	9.4071	7.37E-46	9.27E-44	sp|Q9SZ42|FAD4_ARATH Fatty acid desaturase 4, chloroplastic OS=Arabidopsis thaliana OX=3702 GN=FAD4 PE=1 SV=1//1.64688e-39
Zjn_sc00051.1.g01170.1.am.mk	823.08	1.267457	9.343	5.39E-37	4.51E-35	sp|P14897|ELI9_HORVU Low molecular mass early light-inducible protein HV90, chloroplastic OS=Hordeum vulgare OX=4513 PE=2 SV=1//1.77463e-35
Zjn_sc00002.1.g14700.1.sm.mk	82424.2	131.1514	9.2957	3.64E-235	1.52E-230	sp|Q10RZ1|BAMY2_ORYSJ Beta-amylase 2, chloroplastic OS=Oryza sativa subsp. japonica OX=39947 GN=BAMY2 PE=1 SV=1//0
Zjn_sc00012.1.g02810.1.sm.mkhc	906.2518	1.847505	8.9382	3.56E-06	2.33E-05	sp|Q42376|LEA3_MAIZE Late embryogenesis abundant protein, group 3 OS=Zea mays OX=4577 GN=MGL3 PE=2 SV=1//3.44859e-58

Supplementary Table 4: List of all differentially expressed genes with significant downregulation (log^2FoldChange ≤ -1 and padj ≤ 0.05) for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

Gene_id	readcount_CV	readcount_NV	log^2Fold Change	p_{val}	p_{adj}	Blast swiss prot
Zjn_sc00015.1.g02560.1.am.mk	0.338398	103.4895	-8.2565	2.18E-25	9.87E-24	sp|Q29PU2|SAU76_ARATH Auxin-responsive protein SAUR76 OS=Arabidopsis thaliana OX=3702 GN=SAUR76 PE=1 SV=1//2.90063e-14
Zjn_sc00034.1.g01110.1.am.mkhc	1.463276	301.0644	-7.6847	2.84E-09	2.98E-08	sp|A2WZ60|IRO2_ORYSI Protein IRON-RELATED TRANSCRIPTION FACTOR 2 OS=Oryza sativa subsp. indica OX=39946 GN=IRO2 PE=3 SV=1//1.85343e-91
Zjn_sc00023.1.g06660.1.am.mk	0.676796	99.94206	-7.2062	1.04E-23	4.26E-22	sp|Q0WUU6|PLT4_ARATH Probable polyol transporter 4 OS=Arabidopsis thaliana OX=3702 GN=PLT4 PE=2 SV=1//2.1131e-171
Zjn_sc00005.1.g01900.1.am.mk	0.448082	60.62533	-7.08	1.13E-15	2.40E-14	sp|Q9C942|CSE_ARATH Caffeoylshikimate esterase OS=Arabidopsis thaliana OX=3702 GN=CSE PE=1 SV=1//1.30625e-21
Zjn_sc00085.1.g00930.1.sm.mkhc	56.0754	7169.67	-6.9984	7.44E-82	3.66E-79	sp|Q8H0T5|ERF73_ARATH Ethylene-responsive transcription factor ERF073 OS=Arabidopsis thaliana OX=3702 GN=ERF073 PE=2 SV=1//1.3107e-12
Zjn_sc00012.1.g03460.1.am.mk	0.676796	80.27127	-6.89	3.73E-09	3.86E-08	sp|Q65X92|C3H37_ORYSJ Zinc finger CCCH domain-containing protein 37 OS=Oryza sativa subsp. japonica OX=39947 GN=Os05g0525900 PE=2 SV=1//1.01708e-106
Zjn_sc00018.1.g02480.1.am.mk	1.124878	110.9744	-6.6243	4.62E-25	2.05E-23	sp|Q9SU30|CPR1_ARATH F-box protein CPR1 OS=Arabidopsis thaliana OX=3702 GN=CPR1 PE=1 SV=2//5.41234e-09
Zjn_sc00007.1.g07620.1.am.mkhc	0.338398	30.96149	-6.5156	2.35E-08	2.17E-07	sp|F4KD68|WTR43_ARATH WAT1-related protein At5g45370 OS=Arabidopsis thaliana OX=3702 GN=At5g45370 PE=2 SV=1//1.41253e-32
Zjn_sc00092.1.g01950.1.am.mk	41.48606	3789.34	-6.5132	6.44E-129	1.79E-125	sp|Q9SI74|PMI10_ARATH Pectinesterase inhibitor 10 OS=Arabidopsis thaliana OX=3702 GN=PMEI10 PE=2 SV=1//4.05279e-16
Zjn_sc00207.1.g00210.1.am.mk	0.338398	29.73396	-6.4572	1.50E-07	1.23E-06	sp|Q9FIG6|DIR1_ARATH Dirigent protein 1 OS=Arabidopsis thaliana OX=3702 GN=DIR1 PE=2 SV=1//1.43915e-27

Supplementary Table 5: List of all KEGG pathways for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

#Term	Input number	Background number	Corrected P-Value
Metabolic pathways	1258	2062	3.82E-12
Carbon metabolism	221	271	6.68E-08
Biosynthesis of secondary metabolites	707	1195	2.04E-05
Pyruvate metabolism	80	89	0.000662223
Glycolysis / Gluconeogenesis	113	144	0.000868016
Plant hormone signal transduction	170	247	0.001818593
Biosynthesis of amino acids	165	242	0.002716027
Pentose phosphate pathway	53	59	0.007531548
Starch and sucrose metabolism	141	210	0.009459212
RNA transport	117	169	0.010617429
Fructose and mannose metabolism	57	68	0.010815066
Ribosome biogenesis in eukaryotes	69	89	0.013424722
Porphyrin and chlorophyll metabolism	41	47	0.025692835
Glycerolipid metabolism	57	73	0.025692835
Nitrogen metabolism	32	35	0.040705178
Arginine and proline metabolism	41	50	0.044467065
Protein processing in endoplasmic reticulum	127	202	0.044467065
Citrate cycle (TCA cycle)	42	52	0.044467065
Phagosome	56	76	0.04709402
Glycerophospholipid metabolism	73	106	0.048126325
Carotenoid biosynthesis	29	33	0.060509178
Circadian rhythm - plant	38	48	0.064294391
Spliceosome	116	188	0.067531423
Galactose metabolism	50	69	0.067611355
Ribosome	182	314	0.067771111
mRNA surveillance pathway	74	112	0.068336891
Oxidative phosphorylation	83	129	0.071883489
Endocytosis	101	163	0.076354618
Plant-pathogen interaction	104	171	0.095372429
Alanine, aspartate and glutamate metabolism	37	50	0.098908695
Purine metabolism	104	172	0.098908695
Sphingolipid metabolism	25	31	0.119963592
Base excision repair	32	43	0.122373078
Proteasome	41	59	0.126571511
Nicotinate and nicotinamide metabolism	17	19	0.138841002
RNA degradation	68	112	0.189175637
beta-Alanine metabolism	28	39	0.189175637
Ether lipid metabolism	22	29	0.192339301
Phenylalanine, tyrosine and tryptophan biosynthesis	34	50	0.192339301
Photosynthesis	47	74	0.193717461
Arginine biosynthesis	23	31	0.193717461
Terpenoid backbone biosynthesis	42	66	0.221007512
Riboflavin metabolism	9	9	0.241120761
Fatty acid degradation	31	47	0.247573525
alpha-Linolenic acid metabolism	33	51	0.254864116

Supplementary Table 5 continued: List of all KEGG pathways for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

Phenylalanine metabolism	30	46	0.267631227
Amino sugar and nucleotide sugar metabolism	86	153	0.268334039
Brassinosteroid biosynthesis	11	13	0.277044151
Glycine, serine and threonine metabolism	48	81	0.294945387
Valine, leucine and isoleucine biosynthesis	13	17	0.305606218
Pyrimidine metabolism	78	140	0.305606218
Sulfur metabolism	24	37	0.318351788
Ubiquitin mediated proteolysis	70	126	0.335743474
C5-Branched dibasic acid metabolism	6	6	0.335743474
Phosphatidylinositol signaling system	40	68	0.335743474
Selenocompound metabolism	13	18	0.341567062
Inositol phosphate metabolism	34	57	0.341567062
Ascorbate and aldarate metabolism	26	42	0.34206651
Glyoxylate and dicarboxylate metabolism	43	75	0.354109222
Steroid biosynthesis	23	37	0.360205983
Nucleotide excision repair	35	61	0.399351121
N-Glycan biosynthesis	24	40	0.401251682
Monobactam biosynthesis	10	14	0.401251682
Tyrosine metabolism	29	50	0.401251682
2-Oxocarboxylic acid metabolism	29	50	0.401251682
Regulation of autophagy	20	33	0.402848376
Taurine and hypotaurine metabolism	12	18	0.402848376
SNARE interactions in vesicular transport	21	35	0.402848376
Glutathione metabolism	60	113	0.426196305
Cysteine and methionine metabolism	56	105	0.426196305
Mismatch repair	22	39	0.476299864
Ubiquinone and other terpenoid-quinone biosynthesis	25	45	0.476299864
Valine, leucine and isoleucine degradation	28	51	0.476299864
Cyanoamino acid metabolism	33	61	0.476299864
One carbon pool by folate	11	18	0.49442235
Other glycan degradation	13	22	0.496619302
ABC transporters	18	32	0.500202779
Non-homologous end-joining	7	11	0.526367795
Monoterpenoid biosynthesis	8	13	0.526367795
Thiamine metabolism	8	13	0.526367795
Butanoate metabolism	14	25	0.53082511
DNA replication	29	56	0.552851195
Propanoate metabolism	15	28	0.577465835
Linoleic acid metabolism	9	16	0.579810999
Fatty acid biosynthesis	25	49	0.579810999
Peroxisome	50	101	0.579810999
Pantothenate and CoA biosynthesis	15	29	0.608099173
Flavonoid biosynthesis	21	42	0.623757579
Arachidonic acid metabolism	9	17	0.623757579
Fatty acid metabolism	41	85	0.638961443
Sesquiterpenoid and triterpenoid biosynthesis	5	9	0.638961443
Lipoic acid metabolism	3	5	0.653207003

Supplementary Table 5 continued: List of all KEGG pathways for the Cold-acclimated Meyer (CM) vs Non-acclimated Meyer (NM) comparison.

RNA polymerase	26	54	0.653207003
Histidine metabolism	8	16	0.659373279
Biosynthesis of unsaturated fatty acids	21	44	0.659373279
Isoquinoline alkaloid biosynthesis	14	29	0.659373279
Sulfur relay system	7	14	0.659373279
Basal transcription factors	24	51	0.666835717
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	11	23	0.666835717
AGE-RAGE signaling pathway in diabetic complications	5	10	0.667365385
Folate biosynthesis	9	19	0.668823304
Homologous recombination	24	52	0.668823304
Pentose and glucuronate interconversions	28	61	0.668823304
Zeatin biosynthesis	12	26	0.668823304
Lysine degradation	17	37	0.668823304
Glycosaminoglycan degradation	7	15	0.668823304
Lysine biosynthesis	8	18	0.703940773
Tryptophan metabolism	20	45	0.703940773
Protein export	25	59	0.781970784
Other types of O-glycan biosynthesis	2	5	0.781970784
Tropane, piperidine and pyridine alkaloid biosynthesis	9	24	0.845023808
Stilbenoid, diarylheptanoid and gingerol biosynthesis	8	22	0.854904868
Glycosphingolipid biosynthesis - globo series	4	12	0.856546527
Biotin metabolism	9	25	0.856546527
Glycosphingolipid biosynthesis - ganglio series	2	7	0.880591012
Synthesis and degradation of ketone bodies	3	11	0.911976726
Diterpenoid biosynthesis	8	27	0.940604714
Cutin, suberine and wax biosynthesis	9	30	0.940604714
Aminoacyl-tRNA biosynthesis	31	92	0.974865077
Phenylpropanoid biosynthesis	92	250	0.990553306
Fatty acid elongation	10	45	0.992856349

Supplementary Table 6: List of all KEGG pathways for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

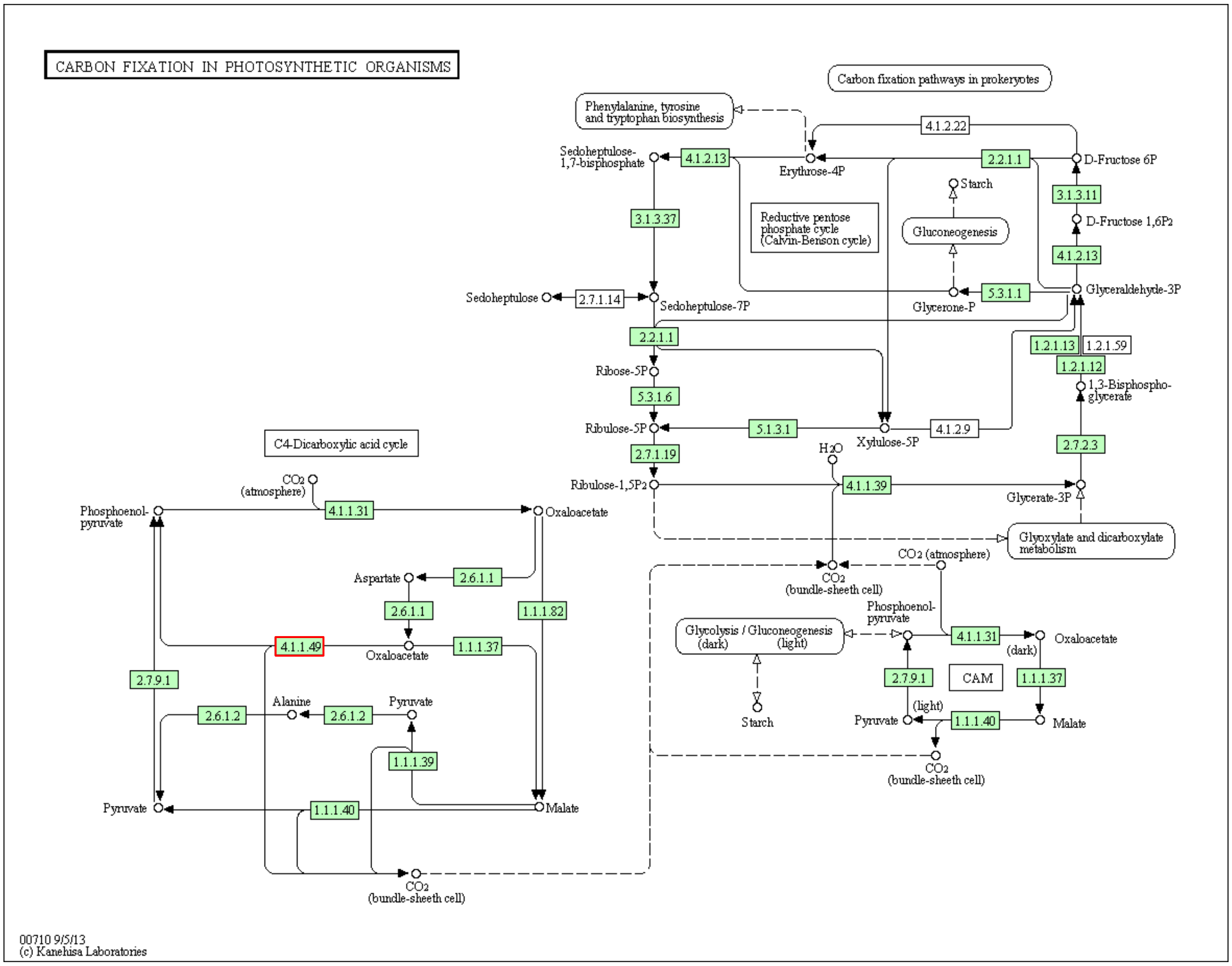
#Term	Input number	Background number	Corrected P-Value
Metabolic pathways	1136	2062	6.69E-07
Biosynthesis of secondary metabolites	654	1195	0.000954938
Plant hormone signal transduction	168	247	0.000954938
Glycolysis / Gluconeogenesis	101	144	0.012845719
RNA transport	114	169	0.01316611
Pyruvate metabolism	68	89	0.013992826
Carbon metabolism	167	271	0.013992826
Glycerophospholipid metabolism	77	106	0.014520886
Protein processing in endoplasmic reticulum	129	202	0.015571074
Glycerolipid metabolism	56	73	0.021830101
N-Glycan biosynthesis	36	40	0.021830101
Citrate cycle (TCA cycle)	43	52	0.023775454
Biosynthesis of amino acids	146	242	0.027465925
Arginine and proline metabolism	40	50	0.041947462
Spliceosome	115	188	0.045031636
Starch and sucrose metabolism	125	210	0.055699275
Porphyrin and chlorophyll metabolism	37	47	0.055699275
Regulation of autophagy	28	33	0.067729849
Phenylalanine metabolism	35	46	0.08526666
mRNA surveillance pathway	71	112	0.088305457
Carotenoid biosynthesis	27	33	0.088742325
Sulfur metabolism	28	37	0.137034148
Carbon fixation in photosynthetic organisms	51	79	0.137034148
beta-Alanine metabolism	29	39	0.137034148
Fructose and mannose metabolism	45	68	0.137034148
Endocytosis	94	163	0.145652884
Phagosome	48	76	0.180299903
Cysteine and methionine metabolism	63	105	0.180299903
Galactose metabolism	44	69	0.181987556
Folate biosynthesis	16	19	0.182515356
RNA degradation	66	112	0.182515356
alpha-Linolenic acid metabolism	34	51	0.182515356
Inositol phosphate metabolism	37	57	0.189372705
Circadian rhythm - plant	32	48	0.192618668
AGE-RAGE signaling pathway in diabetic complications	10	10	0.201559792
Ubiquitin mediated proteolysis	72	126	0.201559792
Sphingolipid metabolism	22	31	0.221827035
Terpenoid backbone biosynthesis	40	66	0.261105865
Nucleotide excision repair	37	61	0.282133916
Plant-pathogen interaction	91	171	0.320027619
Ubiquinone and other terpenoid-quinone biosynthesis	28	45	0.321690934
Riboflavin metabolism	8	9	0.351879242

Supplementary Table 6 continued: List of all KEGG pathways for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

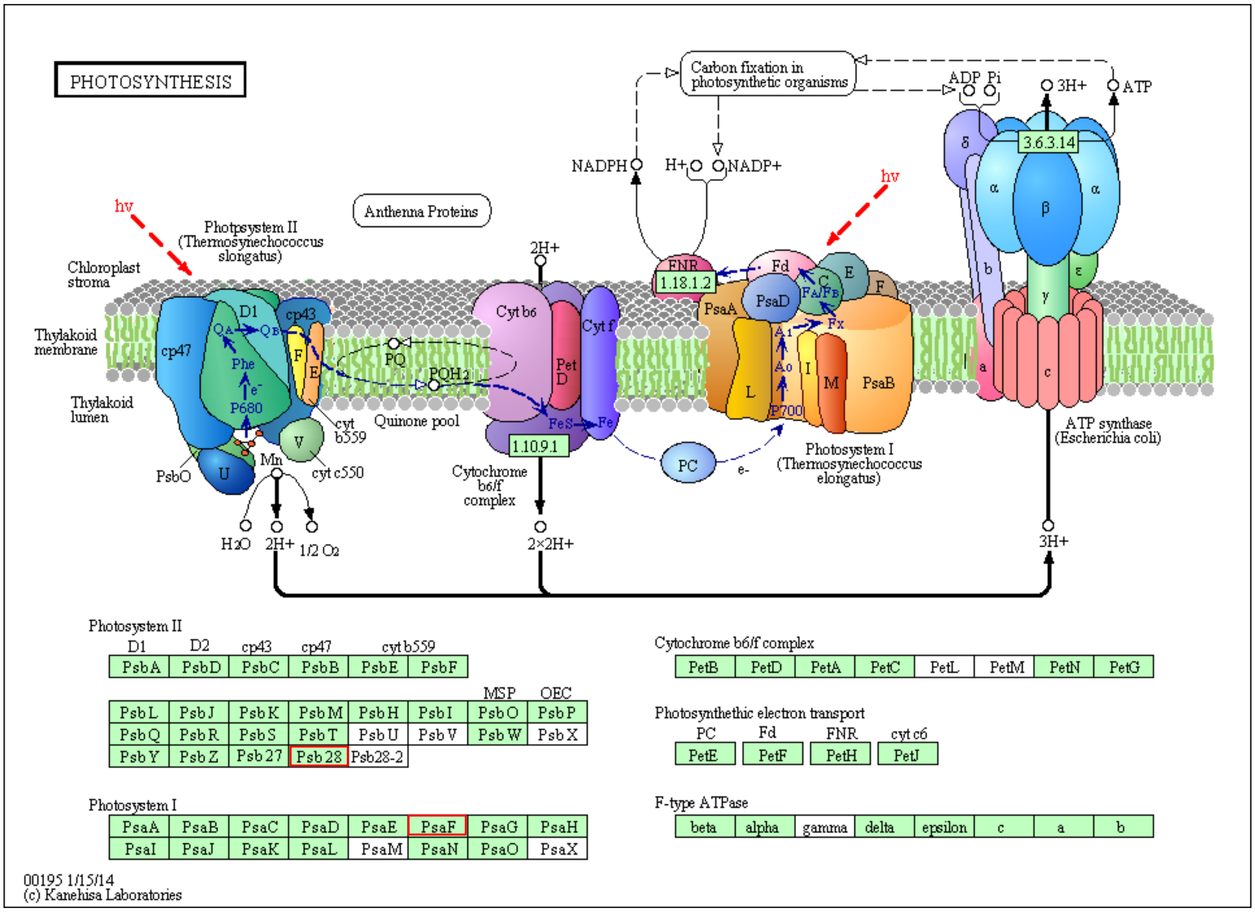
Phenylalanine, tyrosine and tryptophan biosynthesis	30	50	0.355095776
Ether lipid metabolism	19	29	0.355095776
Other glycan degradation	15	22	0.377264368
Ascorbate and aldarate metabolism	25	42	0.41495365
Alanine, aspartate and glutamate metabolism	29	50	0.41495365
Glutathione metabolism	60	113	0.41495365
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	15	23	0.415739523
Arginine biosynthesis	19	31	0.417909903
Nitrogen metabolism	21	35	0.417909903
Proteasome	33	59	0.417909903
Basal transcription factors	29	51	0.417909903
Taurine and hypotaurine metabolism	12	18	0.425497291
Brassinosteroid biosynthesis	9	13	0.473324048
Glycosaminoglycan degradation	10	15	0.475540321
Purine metabolism	86	172	0.475540321
Mismatch repair	22	39	0.483898556
Photosynthesis	39	74	0.483898556
Phosphatidylinositol signaling system	36	68	0.484854027
Sulfur relay system	9	14	0.511166418
Monobactam biosynthesis	9	14	0.511166418
Pentose phosphate pathway	31	59	0.521668172
Fatty acid degradation	25	47	0.533286739
Amino sugar and nucleotide sugar metabolism	75	153	0.533286739
Steroid biosynthesis	20	37	0.539055175
Tyrosine metabolism	26	50	0.545824744
Nicotinate and nicotinamide metabolism	11	19	0.545824744
Thiamine metabolism	8	13	0.545824744
Flavonoid biosynthesis	22	42	0.553556338
Valine, leucine and isoleucine degradation	26	51	0.574744357
Oxidative phosphorylation	62	129	0.593051725
Histidine metabolism	9	16	0.593051725
Linoleic acid metabolism	9	16	0.593051725
Butanoate metabolism	13	25	0.623576086
Glycine, serine and threonine metabolism	39	81	0.623576086
Propanoate metabolism	14	28	0.667463518
Homologous recombination	25	52	0.667463518
Non-homologous end-joining	6	11	0.668736465
SNARE interactions in vesicular transport	17	35	0.668736465
Sesquiterpenoid and triterpenoid biosynthesis	5	9	0.668736465
Biosynthesis of unsaturated fatty acids	21	44	0.669948913
One carbon pool by folate	9	18	0.673156993
Other types of O-glycan biosynthesis	3	5	0.673156993
Lipoic acid metabolism	3	5	0.673156993
2-Oxocarboxylic acid metabolism	23	50	0.710068483
Zeatin biosynthesis	12	26	0.729739123

Supplementary Table 6 continued: List of all KEGG pathways for the Cold-acclimated Victoria (CV) vs Non-acclimated Victoria (NV) comparison.

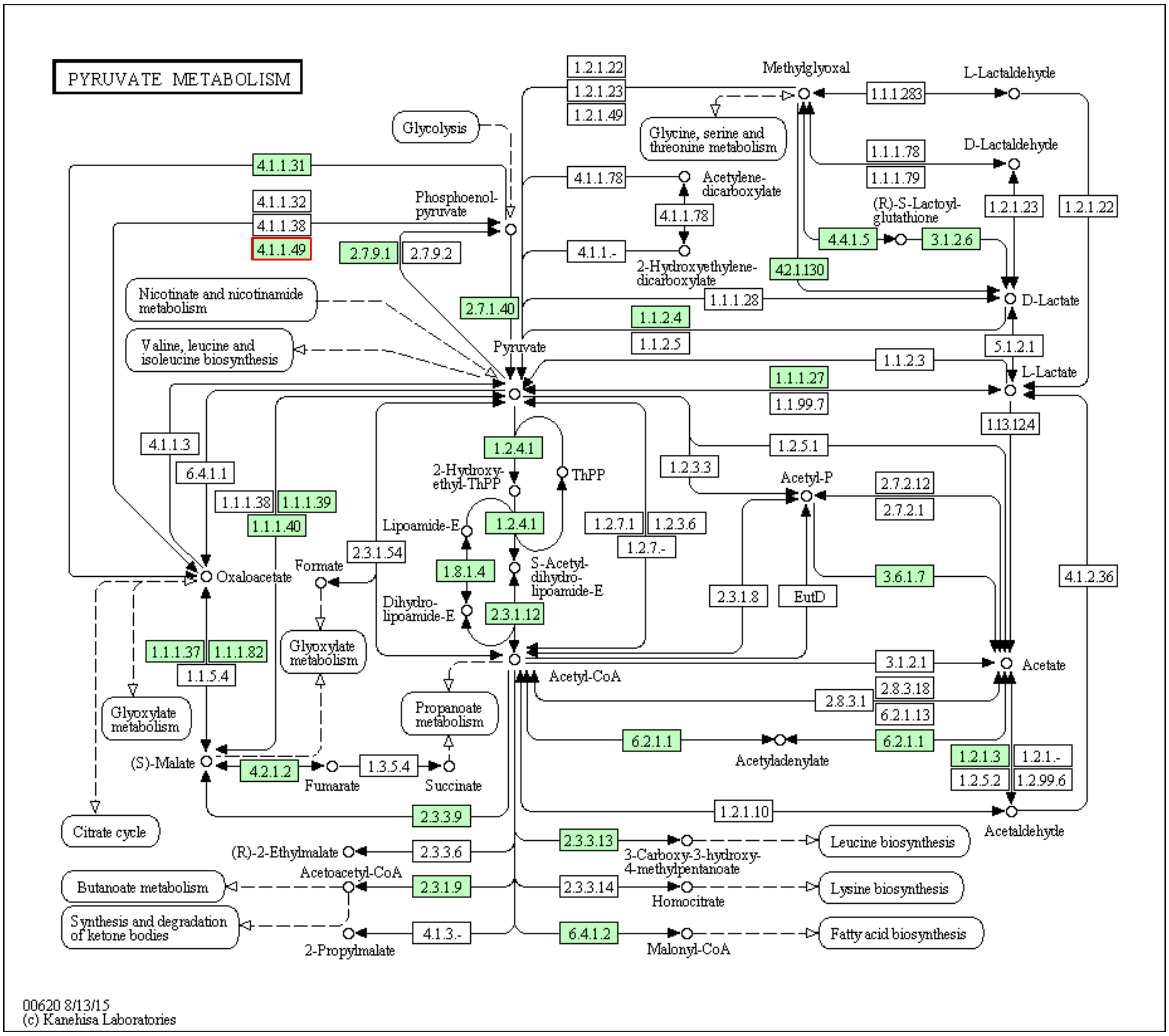
Arachidonic acid metabolism	8	17	0.729739123
DNA replication	25	56	0.743215663
Stilbenoid, diarylheptanoid and gingerol biosynthesis	10	22	0.743215663
Pantothenate and CoA biosynthesis	13	29	0.743360476
Base excision repair	19	43	0.749508002
ABC transporters	14	32	0.759157592
Peroxisome	44	101	0.759157592
Cutin, suberine and wax biosynthesis	13	30	0.759157592
Pyrimidine metabolism	61	140	0.759157592
Protein export	25	59	0.779050946
Tropane, piperidine and pyridine alkaloid biosynthesis	10	24	0.779050946
Glycosphingolipid biosynthesis - ganglio series	3	7	0.779050946
Glycosphingolipid biosynthesis - globo series	5	12	0.779050946
Isoquinoline alkaloid biosynthesis	12	29	0.779050946
Biotin metabolism	10	25	0.796511328
Fatty acid biosynthesis	20	49	0.796511328
Cyanoamino acid metabolism	25	61	0.796511328
Lysine biosynthesis	7	18	0.796511328
Fatty acid metabolism	35	85	0.796511328
Synthesis and degradation of ketone bodies	4	11	0.818764939
Ribosome biogenesis in eukaryotes	36	89	0.818764939
Valine, leucine and isoleucine biosynthesis	6	17	0.837983217
C5-Branched dibasic acid metabolism	2	6	0.837983217
Tryptophan metabolism	17	45	0.837983217
Lysine degradation	13	37	0.876503891
Monoterpenoid biosynthesis	4	13	0.876503891
Glyoxylate and dicarboxylate metabolism	28	75	0.876503891
Pentose and glucuronate interconversions	22	61	0.885255492
RNA polymerase	19	54	0.888264876
Diterpenoid biosynthesis	8	27	0.92235746
Fatty acid elongation	12	45	0.989688729
Ribosome	115	314	0.990674975
Phenylpropanoid biosynthesis	87	250	0.992176942
Aminoacyl-tRNA biosynthesis	24	92	0.995121802



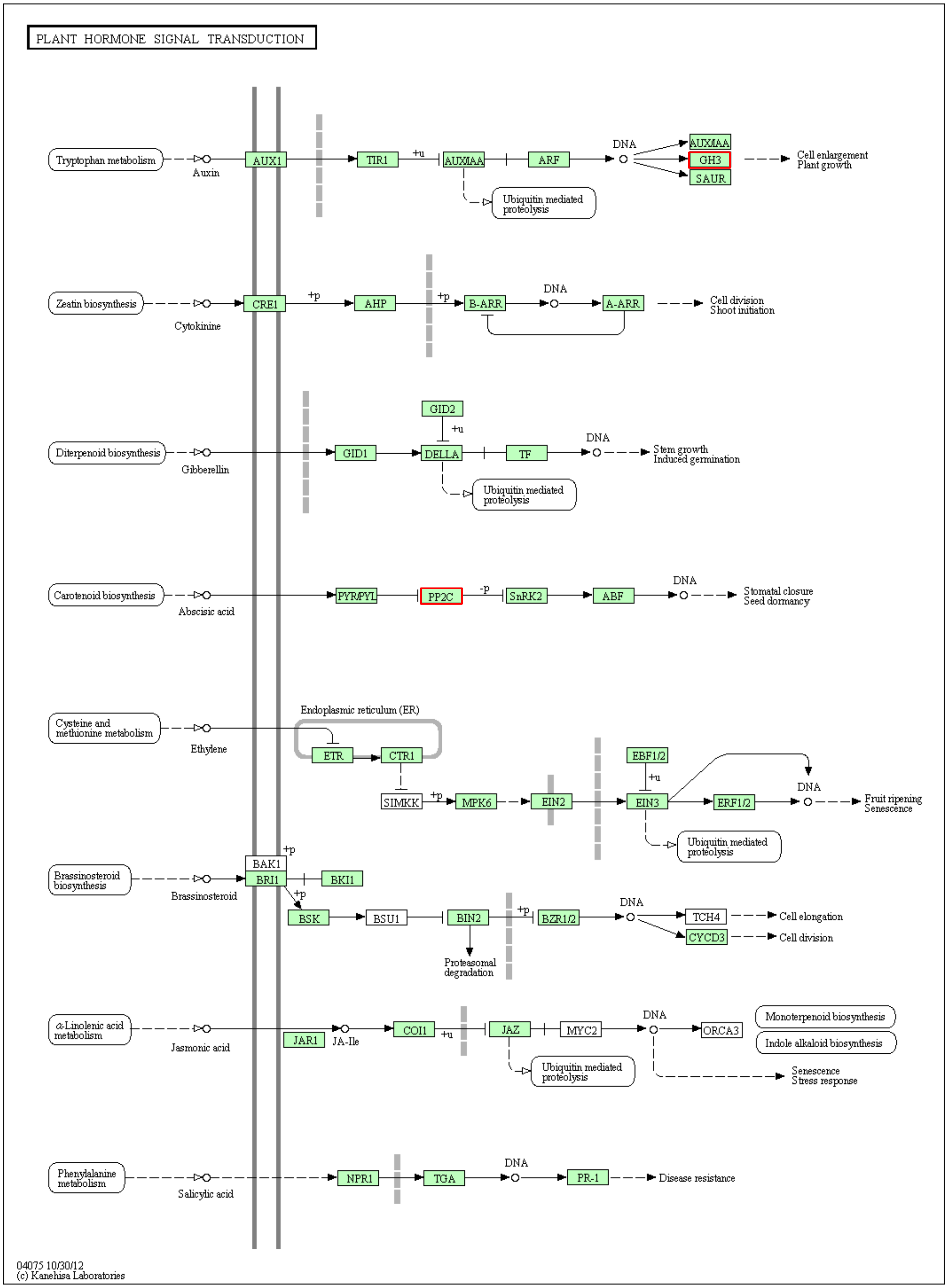
Supplementary Figure 1: Visual representation of the carbon fixation in photosynthetic organisms' pathway, where green boxes indicate downregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant downregulated genes of interest.



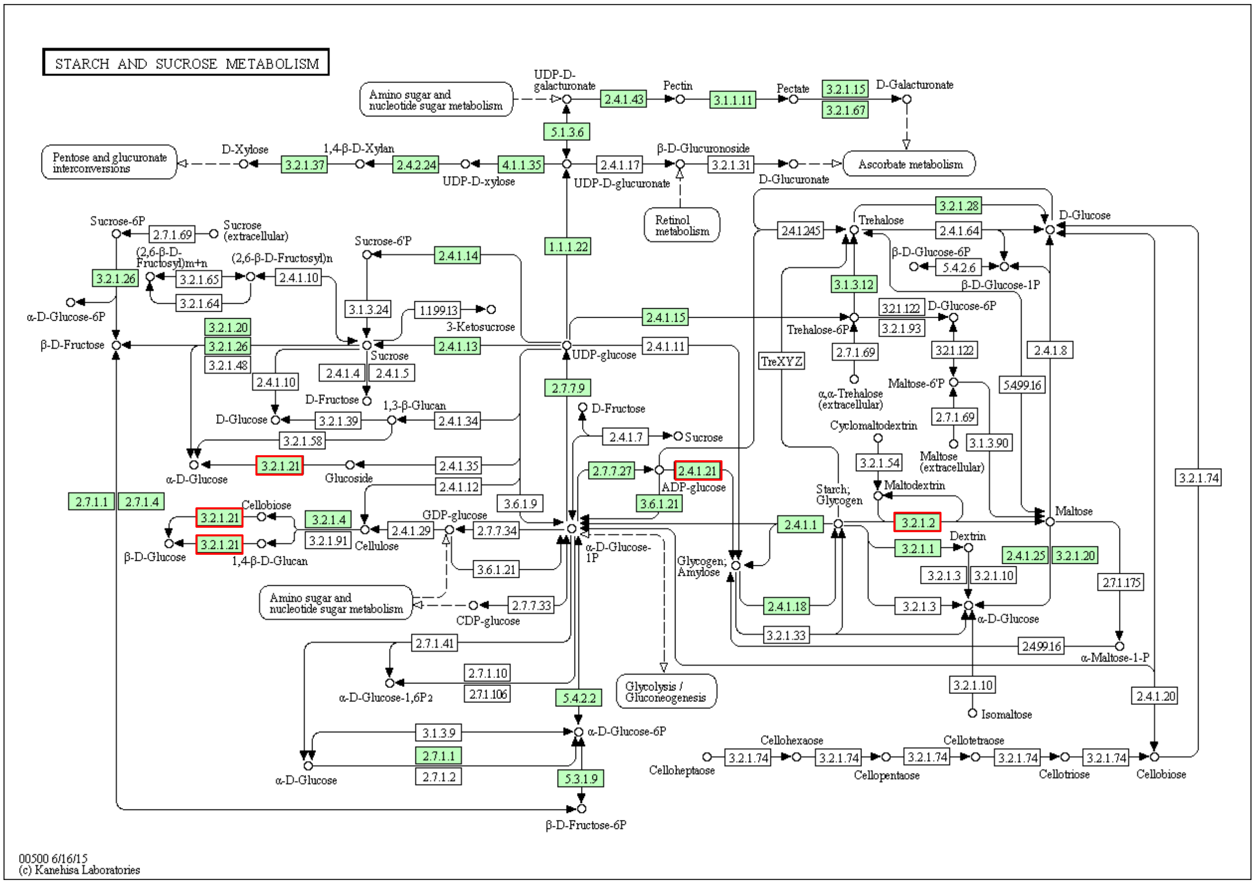
Supplementary Figure 2: Visual representation of the photosynthesis pathway, where green boxes indicate downregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant downregulated genes of interest.



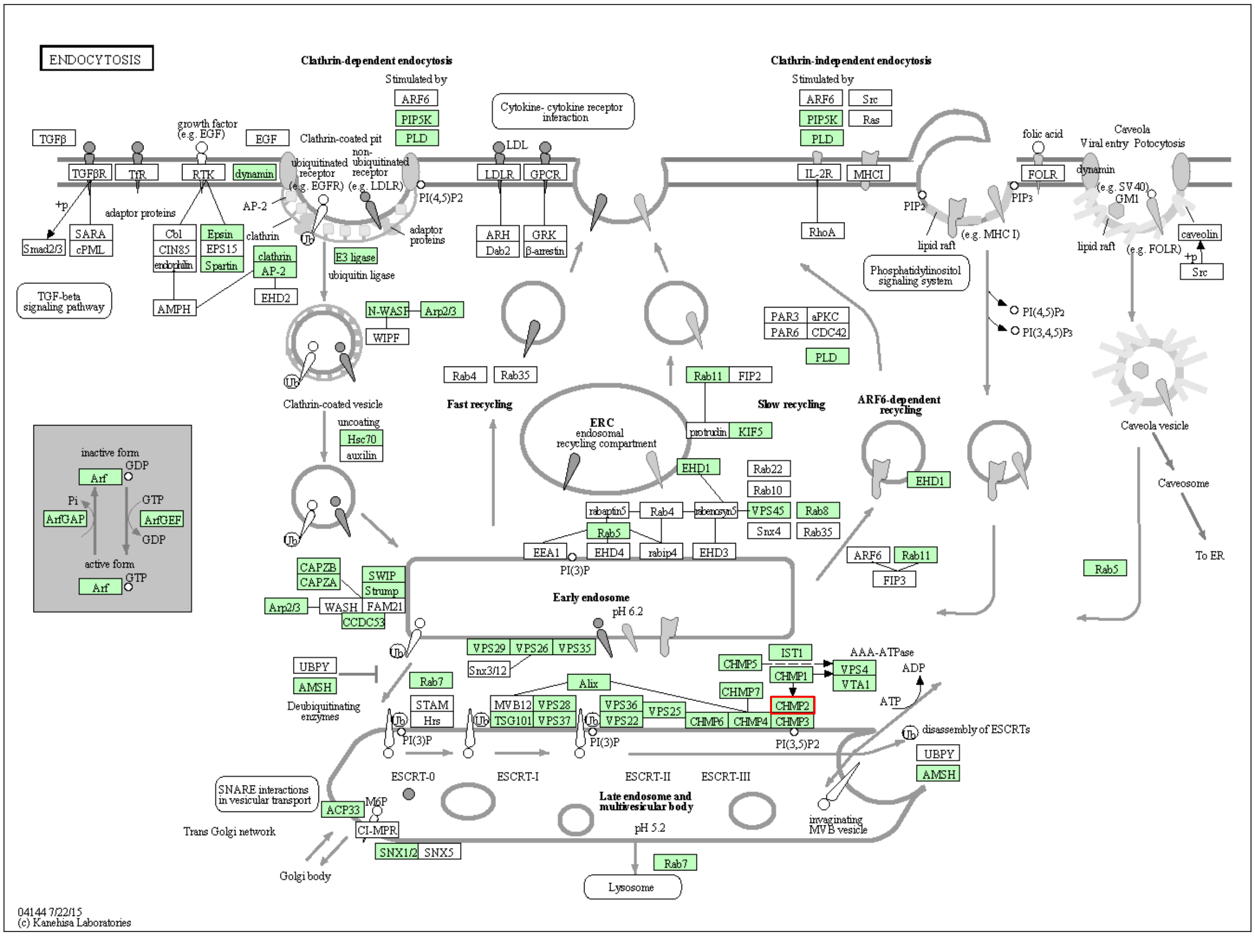
Supplementary Figure 3: Visual representation of the pyruvate metabolism pathway, where green boxes indicate downregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant downregulated genes of interest.



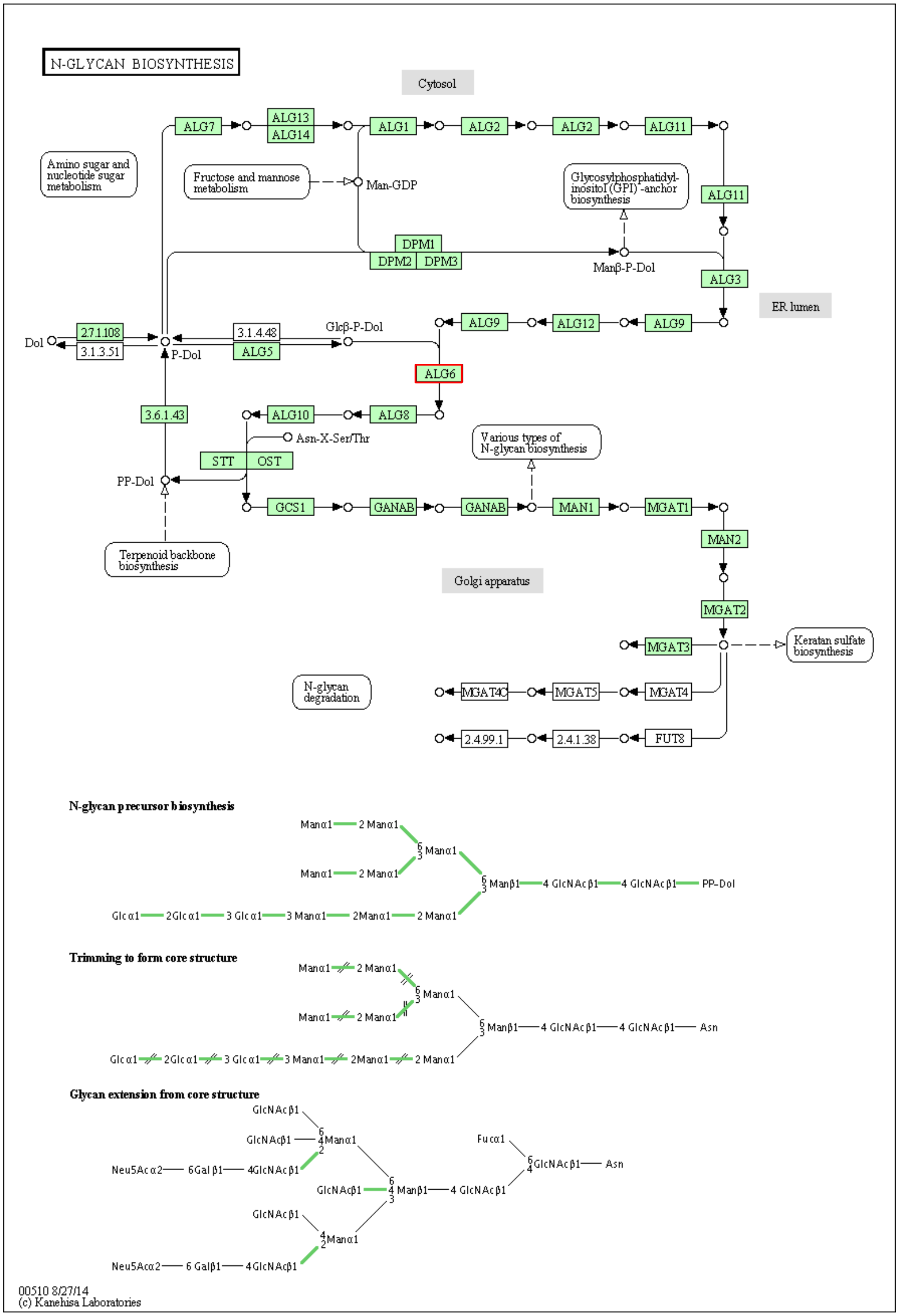
Supplementary Figure 4: Visual representation of the plant hormone signal transduction pathway, where green boxes indicate upregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant upregulated genes of interest.



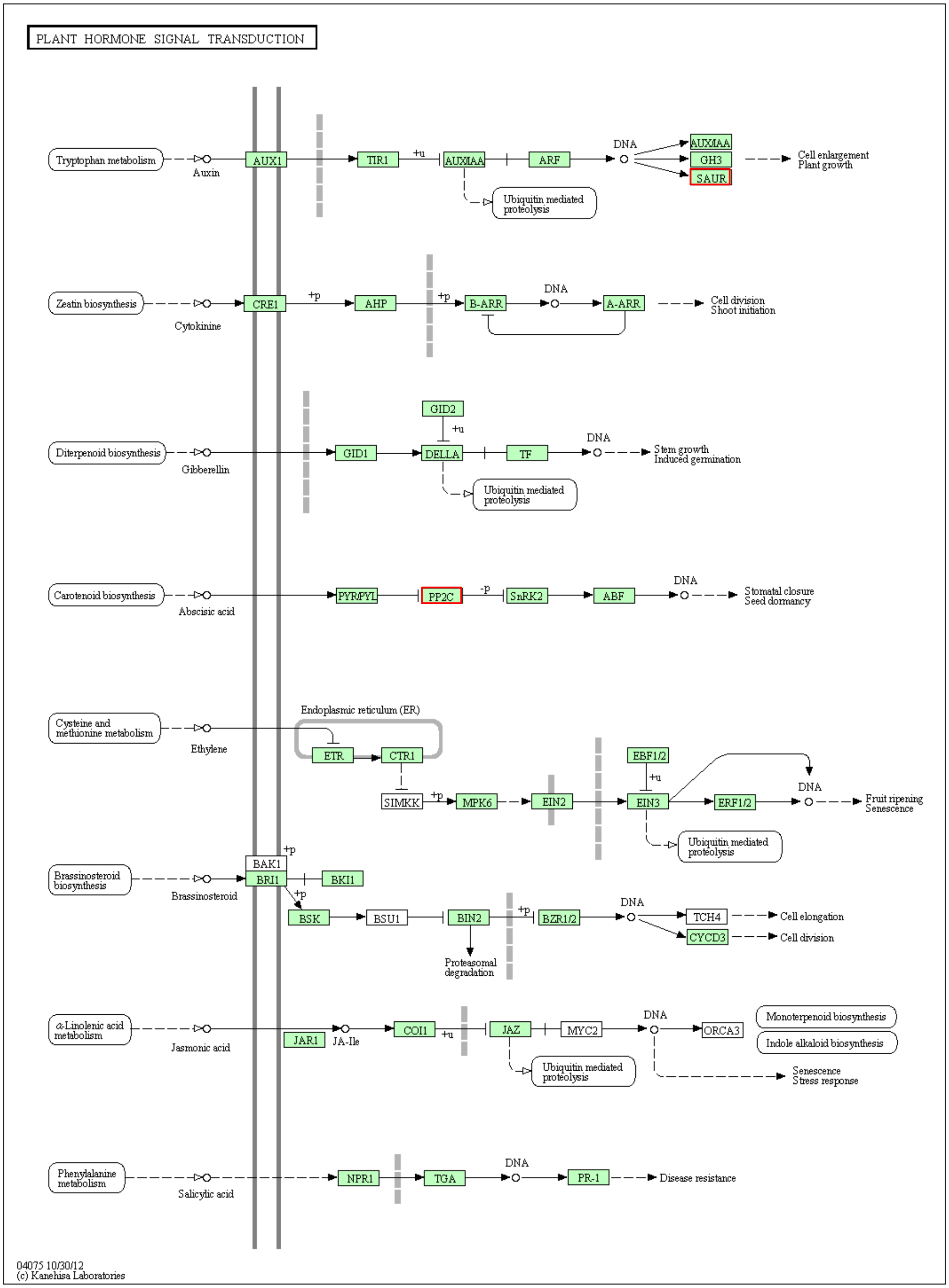
Supplementary Figure 5: Visual representation of the starch and sucrose metabolism pathway, where green boxes indicate upregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant upregulated genes of interest.



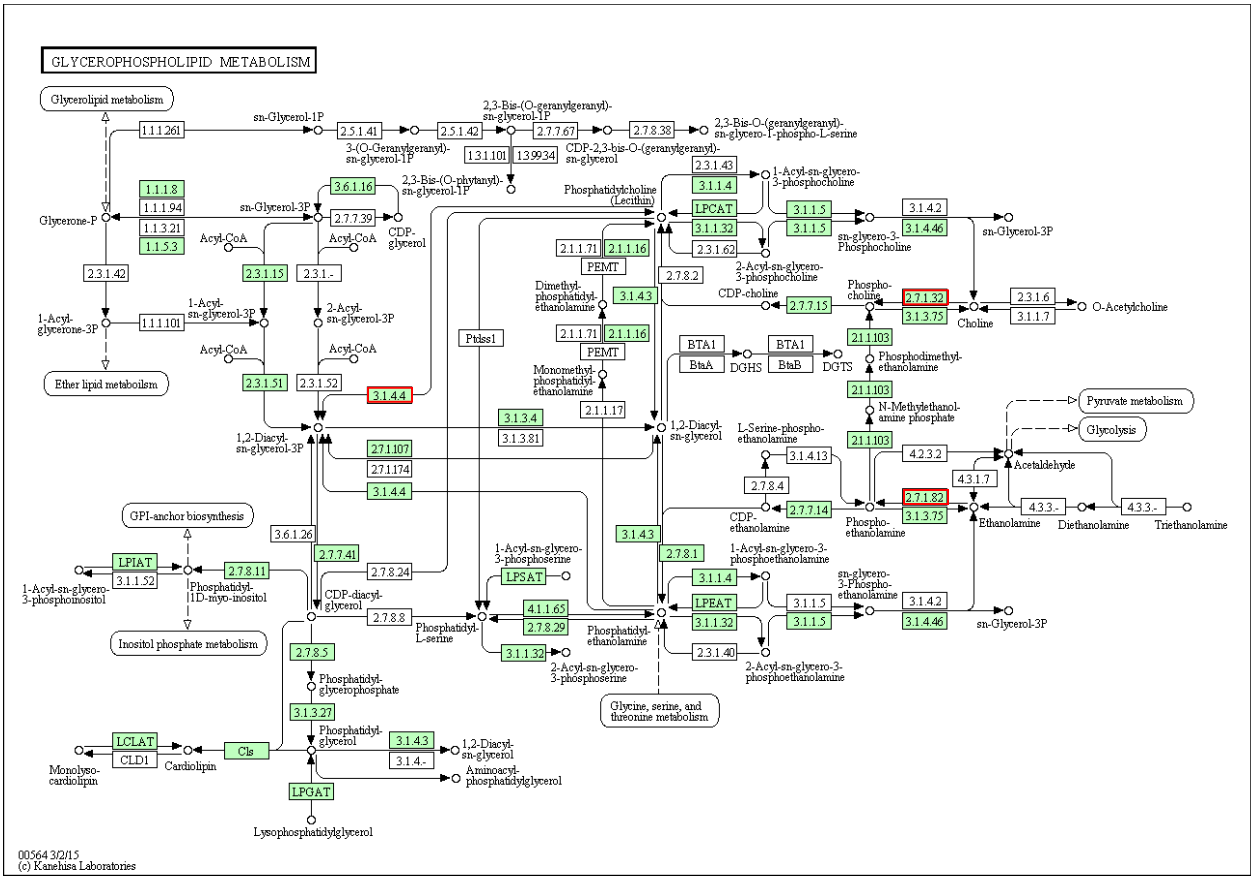
Supplementary Figure 6: Visual representation of the endocytosis pathway, where green boxes indicate upregulated changes in gene expression between the CM and NM comparison, and red boxes indicate significant upregulated genes of interest.



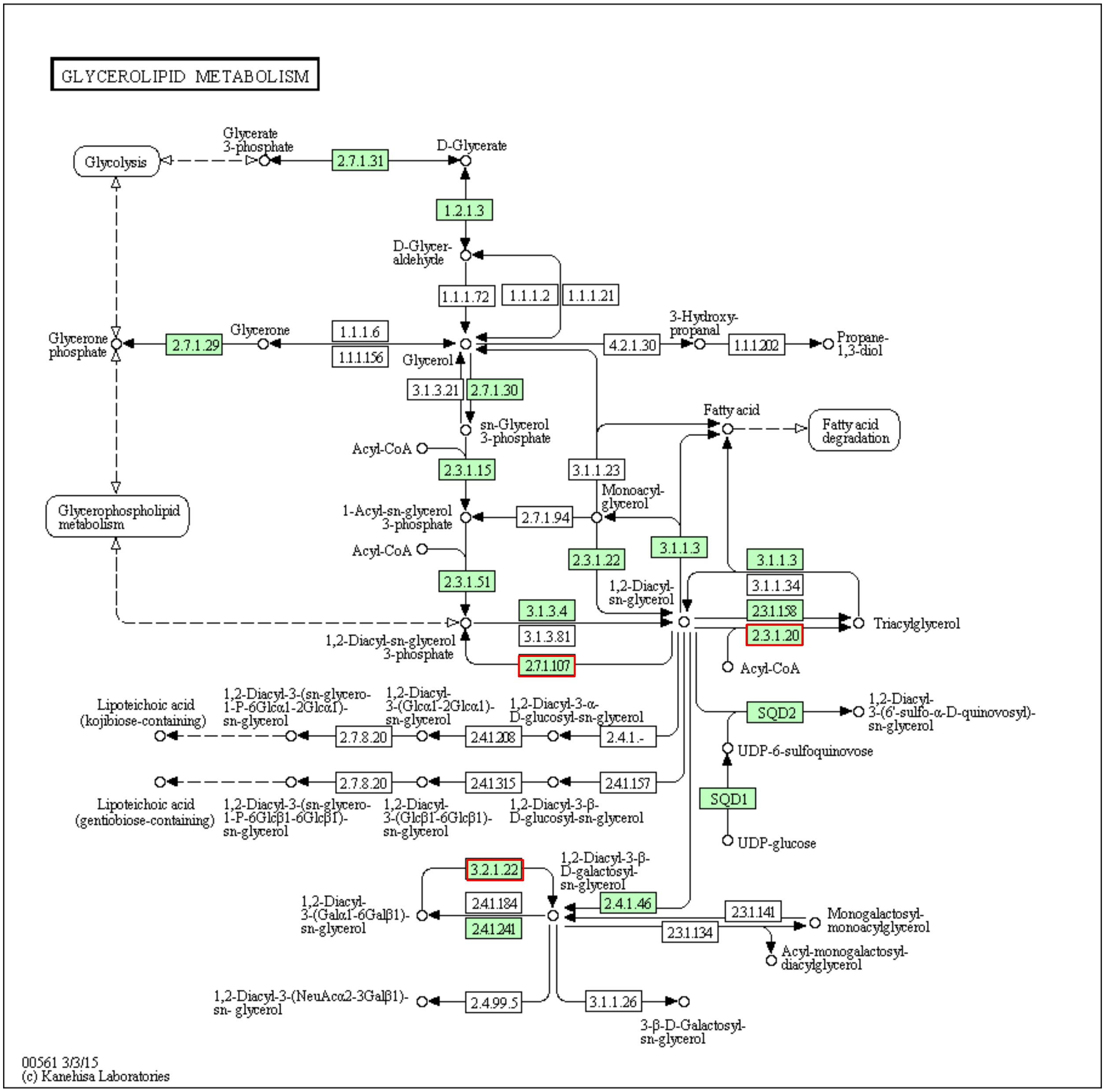
Supplementary Table 7: Visual representation of the N-Glycan Biosynthesis pathway, where green boxes indicate down regulated changes in gene expression between the CV and NV comparison, and red boxes indicate significant downregulated genes of interest.



Supplementary Figure 8: Visual representation of the Plant Hormone Signal Transduction pathway, where green boxes indicate upregulated changes in gene expression between the CV and NV comparison, and red boxes indicate significant downregulated genes of interest.



Supplementary Figure 9: Visual representation of the Glycerophospholipid Metabolism pathway, where green boxes indicate upregulated changes in gene expression between the CV and NV comparison, and red boxes indicate significant downregulated genes of interest.



Supplementary Figure 10: Visual representation of the Glycerolipid Metabolism pathway, where green boxes indicate upregulated changes in gene expression between the CV and NV comparison, and red boxes indicate significant downregulated genes of interest.

APPENDICES

Appendix A

SAS code for analysis of SGT and RG traits from controlled freeze tests on parent and progeny data without commercial checks, for Chapter II (Identification of QTL associated with cold acclimation and freezing tolerance in *Z. japonica*)

```
PROC MIXED DATA = freeze_NOCHECKS_WK6 plots= STUDENTPANEL;
CLASS run identity acclimation temperature;
MODEL SGT = acclimation|temperature|IDENTITY / DDFM=KR2;
random int treatment*temperature / subject= run ;
LSMEANS acclimation temperature  /adj=tukey;
LSMEANS acclimation *temperature/slice=( acclimation temperature);
LSMEANS identity   /cl;
LSMEANS identity* acclimation    /cl slice= ACCLIMATION;
LSMEANS identity* acclimation  /cl slice= TEMPERATURE;
LSMEANS acclimation *temperature*identity /cl slice= ACCLIMATION
*TEMPERATURE;
ods exclude lsmeans;
ODS OUTPUT LSMEANS = LSMEAN_DS_SGT_wk6;
RUN;

PROC MIXED DATA = freeze_NOCHECKS_WK6 plots= STUDENTPANEL;
CLASS run identity acclimation temperature;
MODEL RG = acclimation|temperature|IDENTITY / DDFM=KR2;
RANDOM int treatment*temperature / subject= run ;
LSMEANS acclimation temperature /adj=tukey;
LSMEANS acclimation*temperature/slice=( acclimation temperature);
LSMEANS identity   /cl;
LSMEANS identity*acclimation    /cl slice= ACCLIMATION;
LSMEANS identity*temperature /cl slice= TEMPERATURE;
LSMEANS acclimation *temperature*identity /cl slice= ACCLIMATION
*TEMPERATURE;
ODS EXCLUDE lsmeans;
ODS OUTPUT LSMEANS = LSMEAN_DS_RG_wk6;
RUN;
```

Appendix B

LS means of the 175 pseudo-F2 progeny and parent cultivars Meyer and Victoria, for two traits (RG and SGT) and 3 treatment combinations (CA-11ºC, CA-8ºC, NA-8ºC) in Chapter II (Identification of QTL associated with cold acclimation and freezing tolerance in *Z. japonica*).

Identity	RG CA-11ºC	RG CA-8 ºC	RG NA-8 ºC	SGT CA-11ºC	SGT CA-8 ºC	SGT NA-8ºC
11-TZ-4705	0.1042	0.6458	0.1667	0.1333	0.7333	0.1667
11-TZ-4706	0.1042	0.6458	0.1458	0.1333	0.8	0.1667
11-TZ-4707	0.125	0.8542	0.1458	0.1333	0.9333	0.1333
11-TZ-4708	0.0625	0.8125	0.3125	0.06667	0.9	0.3
11-TZ-4709	0.1042	0.6667	0.1667	0.1333	0.8	0.1667
11-TZ-4710	0.0625	0.5417	0.1667	0.06667	0.6667	0.1667
11-TZ-4711	0.1042	0.5	0.1458	0.1	0.5667	0.1667
11-TZ-4712	0.1042	0.75	0.0625	0.1333	0.8	0.06667
11-TZ-4713	0.125	0.625	0.1458	0.1667	0.8	0.1333
11-TZ-4714	0.1458	0.8542	0.1042	0.1333	0.9667	0.1
11-TZ-4715	0.1042	0.875	0.1042	0.1333	0.9667	0.1
11-TZ-4716	0.0625	0.8333	0.2083	0.06667	0.8667	0.2333
11-TZ-4717	0.08333	0.8542	0.1458	0.2	0.9333	0.1333
11-TZ-4718	-1.29E-15	0.7292	0.1667	7.51E-14	0.7667	0.1667
11-TZ-4719	0.0625	0.7292	0.1042	0.06667	0.8333	0.1
11-TZ-4720	0.02083	0.8125	0.1042	0.1	0.8667	0.1
11-TZ-4721	0.1042	0.5625	0.3125	0.1	0.6333	0.3333
11-TZ-4722	0.04167	0.75	0.2083	0.1667	0.8	0.2
11-TZ-4723	0.02083	0.625	0.1042	0.03333	0.7333	0.1
11-TZ-4724	0.0625	0.3958	0.1042	0.06667	0.5	0.1333
11-TZ-4725	0.125	0.7708	2.03E-14	0.1667	0.9	7.04E-14
11-TZ-4726	0.0625	0.75	0.1667	0.1	0.8333	0.1667
11-TZ-4727	0.1042	0.9167	0.1458	0.1	1	0.1667
11-TZ-4728	0.1458	0.8542	0.2708	0.1667	0.9667	0.2667
11-TZ-4729	0.1458	0.7083	0.3125	0.1667	0.8	0.3
11-TZ-4730	0.02083	0.8542	0.1667	0.06667	0.9333	0.1667
11-TZ-4731	0.125	0.8125	0.3125	0.2	0.9	0.3
11-TZ-4732	0.125	0.7083	0.0625	0.2	0.8667	0.06667
11-TZ-4733	0.1667	0.6458	0.1458	0.1667	0.7333	0.1667
11-TZ-4734	0.1042	0.6458	0.1667	0.1	0.7333	0.1667
11-TZ-4735	-1.43E-15	0.7708	0.1667	7.53E-14	0.9	0.1667
11-TZ-4736	0.02083	0.75	0.1667	0.06667	0.8667	0.1667

11-TZ-4737	0.02083	0.6042	0.1667	0.06667	0.7333	0.1667
11-TZ-4738	0.2708	0.5833	0.3125	0.3333	0.6333	0.3
11-TZ-4739	0.1875	0.8333	0.1667	0.2333	0.9	0.1667
11-TZ-4740	0.08333	0.7708	0.1667	0.1333	0.9333	0.1667
11-TZ-4741	0.02083	0.6667	0.2292	0.06667	0.7667	0.2333
11-TZ-4742	0.08333	0.3958	0.2083	0.1333	0.4667	0.2333
11-TZ-4743	0.0625	0.75	0.1458	0.1	0.8	0.1333
11-TZ-4744	0.25	0.7083	0.3333	0.2333	0.8667	0.3333
11-TZ-4745	0.02083	0.7083	0.375	0.06667	0.8333	0.3667
11-TZ-4746	0.0625	0.6875	0.2292	0.06667	0.7667	0.2333
11-TZ-4747	0.2292	0.5625	0.3125	0.2333	0.6667	0.3
11-TZ-4748	0.1042	0.7083	0.1667	0.1333	0.8	0.1667
11-TZ-4749	0.0625	0.6458	0.3333	0.1	0.7333	0.3333
11-TZ-4750	0.08333	0.625	0.0625	0.1667	0.6333	0.06667
11-TZ-4751	0.08333	0.7708	0.3125	0.1333	0.8333	0.3333
11-TZ-4752	0.125	0.5625	0.0625	0.2	0.5667	0.06667
11-TZ-4753	0.2708	0.6458	0.1667	0.3667	0.7667	0.1667
11-TZ-4754	0.2083	0.5417	0.02083	0.2333	0.6	0.06667
11-TZ-4755	0.1042	0.625	0.2083	0.1	0.6667	0.2333
11-TZ-4756	0.1042	0.6042	0.4167	0.1	0.6667	0.4
11-TZ-4757	0.3333	0.9167	0.3333	0.4	0.9667	0.3333
11-TZ-4758	0.2292	0.8542	0.4583	0.2667	0.9	0.4667
11-TZ-4759	0.0625	0.7917	0.1667	0.06667	0.8	0.1667
11-TZ-4760	0.125	0.5208	2.02E-14	0.2	0.6	7.04E-14
11-TZ-4761	0.0625	0.5417	0.1667	0.06667	0.6333	0.1667
11-TZ-4762	0.08333	0.625	0.1042	0.1667	0.7667	0.1333
11-TZ-4763	0.2292	0.7292	0.2292	0.2667	0.8333	0.2333
11-TZ-4764	0.1042	0.5208	0.1667	0.1	0.6333	0.1667
11-TZ-4765	0.0625	0.6667	0.2708	0.06667	0.8	0.3333
11-TZ-4766	0.1042	0.6042	0.2083	0.1	0.6667	0.2
11-TZ-4767	0.3125	0.7708	0.3958	0.3333	0.8667	0.4333
11-TZ-4768	0.1458	0.7292	0.2708	0.2	0.8333	0.3
11-TZ-4769	0.1042	0.7292	0.3125	0.1	0.8333	0.3333
11-TZ-4770	0.125	0.7708	0.2917	0.1333	0.8667	0.2667
11-TZ-4771	0.1042	0.6875	0.1667	0.1	0.7333	0.1667
11-TZ-4772	0.1042	0.7917	0.2292	0.1	0.9333	0.2333
11-TZ-4773	0.0625	0.625	0.1875	0.1	0.7333	0.2
11-TZ-4774	0.02083	0.75	0.3125	0.06667	0.8333	0.3333
11-TZ-4775	0.0625	0.75	0.375	0.06667	0.8333	0.4
11-TZ-4776	0.08333	0.6667	0.2292	0.1	0.8	0.2333
11-TZ-4777	0.0625	0.6667	0.3958	0.06667	0.7667	0.4

11-TZ-4778	0.1667	0.7083	0.2083	0.2	0.8	0.2
11-TZ-4779	0.2292	0.8125	0.0625	0.3333	0.8667	0.06667
11-TZ-4780	0.125	0.7708	2.00E-14	0.1667	0.8667	7.04E-14
11-TZ-4781	0.1458	0.625	2.05E-14	0.1333	0.7667	7.07E-14
11-TZ-4782	0.0625	0.7292	0.3125	0.06667	0.8	0.3
11-TZ-4783	0.0625	0.75	0.2708	0.1	0.8333	0.2667
11-TZ-4784	0.1667	0.75	0.1458	0.2	0.8333	0.1333
11-TZ-4785	0.08333	0.5625	0.3125	0.1333	0.6667	0.3
11-TZ-4786	0.3333	0.5625	0.3125	0.3333	0.6667	0.3
11-TZ-4787	0.1667	0.5625	0.3125	0.2	0.7	0.3333
11-TZ-4788	0.125	0.7708	0.1667	0.2	0.8667	0.1667
11-TZ-4789	0.1042	0.6042	0.1458	0.1333	0.7	0.1333
11-TZ-4790	0.0625	0.7083	0.0625	0.1	0.8333	0.06667
11-TZ-4791	0.2708	0.8542	0.2917	0.3667	1	0.3
11-TZ-4792	0.1042	0.6875	0.1458	0.1	0.8	0.1667
11-TZ-4793	0.1042	0.8125	0.1042	0.1667	0.9	0.1
11-TZ-4794	0.08333	0.6667	0.1667	0.2	0.7667	0.1667
11-TZ-4795	0.1667	0.7917	0.3125	0.1667	0.8667	0.3
11-TZ-4796	0.1667	0.6042	0.2917	0.2	0.7	0.3
11-TZ-4797	0.1042	0.7083	1.99E-14	0.1	0.8	7.08E-14
11-TZ-4798	0.1042	0.6458	0.1042	0.1	0.7	0.1
11-TZ-4799	0.1667	0.6875	0.25	0.1667	0.7667	0.2667
11-TZ-4800	0.25	0.75	0.3125	0.2333	0.9	0.3
11-TZ-4802	0.02083	0.6667	0.3333	0.03333	0.8	0.3333
11-TZ-4803	0.0625	0.8333	0.25	0.1	0.8667	0.2667
11-TZ-4804	0.3125	0.9375	0.2083	0.3333	1	0.2
11-TZ-4805	0.2083	0.625	0.375	0.2333	0.7	0.3667
11-TZ-4806	0.08333	0.6042	0.0625	0.1333	0.7333	0.06667
11-TZ-4807	0.04167	0.3542	0.2083	0.1	0.4333	0.2
11-TZ-4808	-1.25E-15	0.6042	0.3125	7.49E-14	0.7	0.3
11-TZ-4809	0.02083	0.5417	0.2083	0.03333	0.6667	0.2
11-TZ-4810	0.125	0.8333	0.1875	0.2	0.9	0.2
11-TZ-4811	0.08333	0.7708	0.3125	0.1333	0.8333	0.3333
11-TZ-4812	0.1458	0.625	0.1667	0.1667	0.7333	0.1667
11-TZ-4813	0.1042	0.6875	0.2292	0.1	0.7667	0.2333
11-TZ-4815	0.1042	0.6875	0.1667	0.1	0.7667	0.2
11-TZ-4816	0.0625	0.75	0.0625	0.06667	0.9	0.06667
11-TZ-4817	0.1667	0.7708	2.04E-14	0.2	0.8	7.11E-14
11-TZ-4818	0.04167	0.7708	0.1042	0.1333	0.8333	0.1
11-TZ-4819	0.0625	0.8542	0.2292	0.1	0.9	0.2333
11-TZ-4821	0.08333	0.6667	0.1667	0.1667	0.8	0.1667

11-TZ-4823	0.1042	0.7083	0.3333	0.1333	0.8333	0.3333
11-TZ-4824	0.02083	0.6875	0.2917	0.03333	0.7333	0.3667
11-TZ-4825	0.1042	0.7917	0.2708	0.1333	0.8667	0.2667
11-TZ-4826	0.1042	0.7083	0.1667	0.1333	0.8333	0.1667
11-TZ-4827	0.1042	0.8958	0.1458	0.1	0.9333	0.1667
11-TZ-4829	-1.26E-15	0.7083	0.25	7.47E-14	0.8	0.2333
11-TZ-4830	0.25	0.7917	0.3125	0.2667	0.8667	0.3
11-TZ-4832	0.2292	0.6875	0.1458	0.2333	0.7667	0.1667
11-TZ-4833	0.2083	0.7917	0.1667	0.2333	0.9333	0.1667
11-TZ-4834	0.0625	0.875	0.3125	0.1	0.9667	0.3333
11-TZ-4835	0.1667	0.9375	0.3333	0.1667	1	0.3333
11-TZ-4836	0.2708	0.9167	0.1667	0.2667	0.9667	0.1667
11-TZ-4837	0.125	0.625	0.3125	0.1667	0.8	0.3333
11-TZ-4838	0.1042	0.75	0.2083	0.1333	0.8	0.2
11-TZ-4839	0.0625	0.75	0.1458	0.06667	0.8	0.1667
11-TZ-4840	0.1042	0.6875	0.2292	0.1	0.8	0.2333
11-TZ-4841	0.1042	0.5	0.1458	0.1	0.6	0.1333
11-TZ-4842	0.2708	0.5833	0.3125	0.3	0.7	0.3
11-TZ-4843	0.2917	0.6458	0.3125	0.3	0.7667	0.3333
11-TZ-4844	0.1042	0.8958	0.1667	0.1	0.9667	0.1667
11-TZ-4845	0.125	0.6458	0.2292	0.2	0.7	0.2333
11-TZ-4846	0.0625	0.7083	2.00E-14	0.06667	0.8	7.04E-14
11-TZ-4847	0.02083	0.5208	0.1458	0.03333	0.6333	0.1333
11-TZ-4848	0.25	0.7083	0.3125	0.3	0.7667	0.3333
11-TZ-4849	0.1042	0.7708	0.1667	0.1333	0.8667	0.2
11-TZ-4850	0.0625	0.5	0.0625	0.06667	0.6333	0.1
11-TZ-4851	0.0625	0.75	0.5	0.1	0.8667	0.5
11-TZ-4852	0.0625	0.8125	0.3125	0.1	0.9333	0.3
11-TZ-4853	0.1042	0.7292	0.2708	0.1	0.9	0.2667
11-TZ-4854	0.1042	0.8958	0.1458	0.1	0.9333	0.1667
11-TZ-4857	0.25	0.8333	0.0625	0.2333	0.9333	0.06667
11-TZ-4858	0.1042	0.625	0.1458	0.1	0.7333	0.1667
11-TZ-4860	0.1042	0.7292	0.2083	0.1	0.7667	0.2333
11-TZ-4861	-1.05E-15	0.3958	0.1458	7.48E-14	0.5	0.1667
11-TZ-4863	0.0625	0.6667	0.1667	0.1333	0.7	0.1667
11-TZ-4866	0.1042	0.8125	0.1667	0.1	0.9	0.1667
11-TZ-4867	-9.58E-16	0.7917	0.3125	7.46E-14	0.9	0.3
11-TZ-4868	0.2708	0.875	0.1667	0.3	0.9333	0.1667
11-TZ-4869	0.2083	0.8958	0.3125	0.2	0.9667	0.3
11-TZ-4870	0.1042	0.6875	0.3125	0.1	0.7667	0.3333
11-TZ-4872	0.1042	0.75	0.2708	0.1333	0.8667	0.2667

11-TZ-4873	0.1042	0.8125	0.1667	0.1333	0.9	0.1667
11-TZ-4874	-1.15E-15	0.5625	2.06E-14	7.42E-14	0.6333	7.03E-14
11-TZ-4876	0.0625	0.6875	0.1458	0.06667	0.7333	0.1333
11-TZ-4877	-9.44E-16	0.8958	0.1667	7.44E-14	0.9667	0.1667
11-TZ-4880	-1.08E-15	0.6042	0.1667	0.03333	0.6667	0.1667
11-TZ-4881	0.2292	0.7083	0.3125	0.2333	0.8	0.3333
11-TZ-4882	0.1042	0.625	0.1667	0.1	0.7	0.1667
11-TZ-4883	0.0625	0.75	0.2708	0.06667	0.8667	0.2667
11-TZ-4884	0.08333	0.8333	0.1458	0.1333	0.8667	0.1333
11-TZ-4885	-9.02E-16	0.4583	0.1667	7.46E-14	0.6333	0.1667
11-TZ-4886	-1.21E-15	0.8958	0.1042	0.03333	0.9333	0.1333
11-TZ-4888	-9.99E-16	0.8125	0.4583	7.47E-14	0.8667	0.4333
11-TZ-4889	0.08333	0.7708	0.3333	0.1333	0.8667	0.3333
11-TZ-4890	0.02083	0.6875	0.3125	0.06667	0.7333	0.3
11-TZ-4891	0.0625	0.6042	0.3333	0.1	0.6667	0.3333
11-TZ-4893	0.0625	0.7708	0.1667	0.06667	0.8	0.1667
11-TZ-4894	0.0625	0.7708	0.375	0.06667	0.8333	0.3667
11-TZ-4895	0.0625	0.7083	0.2083	0.06667	0.8	0.2
11-TZ-4898	-9.99E-16	0.5625	0.1667	7.46E-14	0.6333	0.1667
11-TZ-4899	0.0625	0.5208	2.08E-14	0.1	0.5667	6.98E-14
Meyer	0.0625	0.9167	0.25	0.06667	0.9667	0.1667
Victoria	0.08333	0.6875	0.3125	0.1333	0.7667	0.3333