PLASTIDIC MEMBRANE LIPID RESPONSE TO ABIOTIC TEMPERATURE STRESS IN CEREALS

by

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To my parents, Walter and Sharon, who have shown me insurmountable love and support.

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ABSTRACT

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Current crop and climate modeling studies predict temperature extremes that may add future challenges to global food and agriculture production systems due to yield decreases in our staple cereal crops. Although there have been some temperature stress adaptation traits and a few associated genes discovered in plants for enhanced thermotolerance, very little is known about these traits in our major cereal crops, particularly in maize. Furthermore, the limited availability of appropriate selection environments and accurate phenotyping, including functional traits for selection, have been major hurdles to overcome in making meaningful gains towards improved thermotolerance in breeding programs. Previous reports have established that dynamic changes in leaf membrane lipids occur when exposed to temperature stress and many have tried to identify specific lipid classes, individual species, or levels of unsaturation as indicators of tolerance or susceptibility. In this study, several types of cereals, with special emphasis on maize, are studied with the goal to expand the understanding of the leaf lipid membrane characteristics and responses when exposed to temperature stress and to find evidence of heritable lipid biomarker(s) that could be used in breeding for enhanced thermotolerance. Leaf lipids for maize inbred lines and twelve hybrids exhibiting differential tolerance to high temperature stress were analyzed after growing in a controlled environment at optimal and high temperature stress conditions. It was hypothesized that the newly introduced ratios of monogalactosyldiacylglycerol (MGDG)

and digalactosyldiacylglycerol (DGDG) containing acyl chains with total 36 carbons and 6 double bonds (36:6) compared to those with 36 carbons and 5 double bonds (36:5), here termed the MGDG and DGDG "Unsaturation Ratios" can be used to differentiate the changes in the plastidic lipid membrane unsaturation levels and to aid in identifying heat tolerant genotypes. An analysis of the MGDG and DGDG Unsaturation Ratios was performed on twenty-five diverse parents of the nested-association mapping (NAM) population, Mo17, and one hundred and ninety-one B73 x B97 recombinant inbred lines (RILs) grown in field conditions. The selected lipid phenotypes were found to be as diverse as the inbred lines in which they were measured and showed a large differential between the temperate inbred lines B73 and B97. Allelic variation controlling the differences in MGDG and DGDG Unsaturation Ratios was identified in the B73 x B97 RIL subpopulation through linkage mapping analysis. Finally, an analysis of the MGDG and DGDG Unsaturation Ratios was performed across eight of the world's most important cereal crops. The results of these studies provide preliminary evidence that the MGDG and DGDG Unsaturation Ratios may be beneficial lipid biomarkers that can be used to screen germplasm in breeding programs for improved thermotolerance for our most important cereal crops with the potential to differentiate tolerance in germplasm even without the presence of the ideal selection environment.

CHAPTER 1. ABIOTIC TEMPERATURE STRESS AND PLANT LEAF LIPIDS

1.1 Introduction

Global demand for cereal crops is predicted to significantly increase due to multiple market influences. The world's population is predicted to approach 10 billion people in 2050 according to the United Nations (UN) Department of Economic and Social Affairs (United Nations, 2015). The rising affluence and urbanization of developing nations is driving social transformation and shifting dietary preferences towards increased meat and refined sugar consumption (Tilman and Clark, 2014). Biofuel production is projected to steadily grow, co-opting corn, soybean, and sugarcane as common feedstocks (Trumbo and Tonn, 2016). Climate change is predicted to negatively impact crop yields due to many environmental challenges such as elevated average temperatures, severe droughts, and increased flooding (Cheeseman, 2016). Land limitations and diminishing availability of water resources are two major constraints limiting sustainable options of expanding global croplands (Ramankutty et al., 2018). There is also a changing social acceptance of industrial agriculture and an increasing demand for sustainable intensification of agriculture, which will require new and innovative approaches to meet production demand (Davis et al., 2016). Public and private sector stakeholders in the global agricultural complex, large and small scale, will be facing these and many additional challenges.

Yield gains in maize and other crops during the 20th and early 21st centuries are mostly attributed to improvements in plant breeding, such as single-cross maize hybrids, increased plant density, biotechnology, and effective management practices (Mansfield

and Mumm, 2014). These efforts have thus far met and sustained the productivity needs of our food, feed, fiber, and fuel producers and consumers. However, to meet future demand, there is a need to sustainably continue maximizing genetic gain and yield potential, while improving yield stability through enhanced stress tolerance.

1.2 Abiotic Stress

Abiotic stress is caused by any non-living environmental factor that is harmful to the plant and negatively affects its cellular homeostasis, growth, development, fitness, and/or production (Cramer et al., 2011; Mickelbart et al., 2015). Globally, yield, production, and economic losses occur due to various abiotic stressors, translating into realized annual economic and societal setbacks. Although there are many different abiotic stresses that negatively impact plants, there are common responses such as initial stress detection, signal transduction, differential gene expression, and ultimately changes in metabolism or physiology to better cope with the underlying stresses (Knight and Knight, 2001; Chinnusamy et al., 2004; Wang et al., 2016; Zhu, 2016). Common abiotic stresses associated with crop production include heat, chilling, freezing, drought, flooding, salinity, metal toxicity, and ion deficiency (Campos et al., 2004; Bailey-Serres and Voesenek, 2008; Lobell, et al., 2013; Farooq et al., 2015; Mickelbart et al., 2015). The effect of these stresses may be dependent on the time, magnitude, and duration of the exposure and whether the occurrence is sudden or gradual (Yeh, et al., 2012). For example, moderate drought stress may only lead to stomatal closure and restricted gas exchange, whereas chronic drought stress may critically perturb cellular homeostasis leading to metabolic or photosynthetic failure and ultimately plant death (Shao et al., 2008). Abiotic stresses occurring during vegetative stages may delay or inhibit plant

development, whereas stress during the reproductive stages can negatively affect grain yield or prevent reproductive success (Wilhelm, et al., 1999).

Frequently, more than one abiotic stress can occur during a growing season or even at the same time. For example, heat and drought stress often coincide where high temperature acts to exacerbate the drought (Mittler, 2006). As a defense mechanism, plants often respond to drought stress by closing stomata to conserve water. The combination of drought and heat stress can lead to closed stomata, increased leaf temperature, and high respiration with decreased photosynthetic efficiency, ultimately leading to poor yield and decreased viability (Mittler, 2006). The effects of abiotic stresses can either be temporary or permanent. As an example, short-term drought stress during vegetative growth stages may result in leaf rolling, which will stop once the stress is alleviated; however, chronic drought stress may lead to permanent cellular death within the leaf tissue and decreased photosynthetic efficiency (Hsiao et al., 1984).

Plants have evolved complex stress tolerance mechanisms through natural selection involving many cellular, physiological, and biochemical processes (Kotak et al, 2007). A plants' ability to adapt or acclimate to a stressful environment and maintain cellular homeostasis through its reproductive cycle while other plants succumb to the stress indicates an underlying genetic diversity that can be targeted in breeding programs for enhanced stress tolerance.

1.3 Breeding for Abiotic Stress Tolerance

Efforts to breed and develop crops for enhanced abiotic stress tolerance have had limited success (Richards, 1996; Tester and Bacic, 2005; Nuccio et al., 2018). There are

many likely reasons why minimal improvements have been achieved. Most industrial breeding programs are working with a relatively narrow pool of elite germplasm that may not contain useful traits for selection and are unlikely to invest the resources in a pre-breeding program to introduce variation from less adapted sources such as landraces. However, the development of new breeding tools such as genomic selection (GS) may soon change this paradigm. Gorjanc et al. (2016) proposed guidelines on how to initiate a pre-breeding program using genomic selection to efficiently and effectively introduce polygenic variation from landraces. Additionally, genomic selection for yield under drought stress has been shown to be more effective than conventional phenotypic selection in tropical maize (Beyene et al., 2015). Recently, high-throughput phenotyping (HTP), remote sensing, and machine learning have been deployed to collect spatial and temporal field data that can be quickly analyzed to detect useful variation in stress tolerance (Honsdorf et al., 2014; Singh et al., 2016). The introduction of useful genetic diversity combined with the scale and efficiency of high-throughput phenotyping and genomic selection will provide future breeders unparalleled opportunities to identify and develop new useful genetic variation and achieve meaningful gains and success in breeding for abiotic stress tolerance.

Useful genetic variation for abiotic stress tolerance is not very useful if it cannot be identified. Appropriate target environments for phenotyping heat, drought, or chilling stress are very difficult to naturally come by and lack control or repeatability for multi-year analyses. Managed stress environments (MSE) representative of the target phenotypic environments are essential for high quality phenotyping data and reducing experimental error (Masuka et al., 2012). Having a managed environment suitable for

repeatable evaluation of germplasm for stress tolerance will also allow for the identification and development of precise phenotyping traits or tools for improved assessment in field conditions (Bita and Gerats, 2013).

Although many genes have been identified in plants that contribute to enhanced tolerance, the development of molecular markers to use in marker-assisted selection (MAS) for abiotic stress improvement have been difficult (Bita and Gerats, 2013). The difficulty is likely due to the polygenic nature, complex mechanisms, and genetic background interactions involved. Therefore, it has been suggested that developing biomarkers based on morphological, physiological, or biochemical characteristics associated with the stress tolerance may be an effective strategy to identify genetic variation for breeding (Wahid et al., 2007; Bita and Gerats, 2013).

1.4 Heat Stress

Heat stress in plants occurs, in general, when environmental temperatures climb above the normal temperature range that supports optimal growth and reproductive fitness (Kotak et al., 2007; Edreira et al., 2014; Rezaei et al., 2015; Mesihovic et al., 2016). When temperatures occur outside the optimal range, it is thought to disrupt cellular homeostasis and impede normal growth, development, and reproduction in plants (Kotak et al., 2007). In cases of severe heat stress, plant death may result. Common indicators used to rate heat stress, for example in maize, are leaf firing, tassel blast, increased anthesis-silking interval (ASI), premature senescence, and chlorophyll fluorescence (Chaele et al., 2007; Chen et al., 2010; Edreira et al., 2011; Zaidi et al., 2016).

Heat stress can occur during episodes of elevated temperature that are short and transient or long and chronic (Wahid et al., 2007). It can cause morphological, physiological, and biochemical changes that negatively affect plants at all stages of plant growth and development, including seeds, seedling, vegetative or reproductive growth (Barnabás et al., 2008; Ashraf and Harris, 2013). Leaves, flowers, pollen, seed or kernel development, cells and their processes can all be affected (Barnabás et al., 2008; Chen et al., 2010; Chen et al., 2012). Negative impacts to grain yield and quality can result if heat stresses occur during the reproductive stages such as pollen shed and silking in maize (Cantarero et al., 1999; Chen et al., 2010). A multi-year field study by Chen et al. (2010) described maize as being particularly sensitive to heat stresses after the eighth leaf stage (V8) when the stalk is rapidly elongating, and the tassel is developing, also developing leaves during the V10 to V14 stages have increased susceptibility to leaf firing, thus decreased photosynthetic capacity when damaged. The photosynthetic apparatus is accepted by many to be one of the most temperature sensitive component of plants with photosystem II (PSII) likely as the most heat-sensitive component (Berry and Björkman, 1980; Sinsawat et al., 2004). In many crops, exposure to 35-40°C for less than one hour is enough to disrupt or inhibit photosynthesis and temperatures around 45°C render it permanently non-functional (Berry and Björkman, 1980; Salvucci et al., 2004).

Reactive oxygen species (ROS) are produced in low levels as byproducts of normal cellular metabolic and physiological processes typically associated with the mitochondria, chloroplasts, and peroxisomes (Suzuki and Mittler, 2005; Gill and Tuteja, 2010). They are formed when high-energy electrons react with molecular oxygen (O_2) yielding peroxides (H_2O_2), superoxide (O_2^-), singlet oxygen ($^1O_2$) or hydroxyl radicals ($HO^\bullet$), which are

damaging to the plant cell DNA, proteins, lipids, or other macromolecules (Suzuki and Mittler, 2005). Under normal cellular homoeostasis, the production of ROS molecules is balanced out by ROS scavenging enzymatic and non-enzymatic mechanisms (Gill and Tuteja, 2010). Superoxide dismutases (SOD), primarily found in chloroplasts, catalyze the conversion of superoxide into molecular oxygen or hydrogen peroxide and are often referred to as the first line of defense against ROS (Alscher et al., 2002; Gill et al., 2015). Catalase (CAT) is a peroxide scavenging enzyme, mostly found in the peroxisomes and mitochondria, which catalyze the conversion of peroxide into molecular oxygen and water (Willekens, et al., 1997; Anjum et al., 2016). Additional plant antioxidant enzymes include ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaiacol peroxidase, and glutathione S-transferase, all working to maintain optimal ROS levels in both optimal and stress environments (Leterrier et al., 2005; Gill and Tuteja, 2010; Gill et al., 2013; Das and Roychoudhury, 2014; Maruta et al., 2016). Non-enzymatic antioxidants include ascorbic acid, reduced glutathione, carotenoids, flavonoids, and α-tocopherol (Gill and Tuteja, 2010; Das and Roychoudhury, 2015). During exposure to stress, there can be a rapid increase in the production of ROS, referred to as the oxidative burst, causing damage and disruption of normal cellular structures and processes, however studies have indicated they may also act to initiate signal transduction pathways aimed at mediating downstream pathways for protecting the plant against stress (Miller et al., 2008).

Heat-shock proteins (HSPs) play a major role in the maintenance of cellular homeostasis when plants are under heat stress, as well as other abiotic stress responses (Iba, 2002). Conserved families of HSPs include the HSP60, HSP70, HSP90, HSP100, and the

small HSPs (sHSPs) (Wang et al., 2004). Most are found in the cellular cytoplasm, nucleus, chloroplasts, mitochondria, or endoplasmic reticulum (ER) (Vierling, 1991). In general, these proteins can act as molecular chaperones, preventing denatured protein aggregation, assisting in protein refolding, and stabilizing proteins and membranes during heat stress conditions (Wang et al., 2004). HSP genes are, in general, transcriptionally regulated through the binding of activated heat-shock factors (HSFs) often to upstream promoter regions, known as heat-shock elements (HSEs) (Iba, 2002). When heat stress is perceived by the plant, a signal is triggered that activates the heat-shock response, in part, defined by the transient expression of HSP activated through HSFs, providing protection and enhanced thermotolerance (Iba, 2002; Kotak et al., 2007). The discovery and increased understanding of the heat-shock response during exposure to high temperature has facilitated a large body of research into transgenic approaches for improving heat stress tolerance in plants.

A major area of inquiry is how lipid membranes in plants adapt and change to maintain cellular homeostasis during temperature extremes. A significant body of literature indicates that lipids play a key role in both normal cellular processes and stress responses due, in part, to being major components of cellular membranes. Lipid membranes provide the compartmentalization required for normal cellular processes (Ohlrogge and Browse, 1995). Thus, their disruption can lead to major perturbations in plant cell homeostasis, the results of which can be detrimental to growth and development (Bonaventure et al. 2003; Walls and Browse, 2002; Kobayashi et al. 2007; Aronsson et al. 2008). The plasma membrane is primarily comprised of phospholipids while the membranes of plastids (e.g., chloroplasts, amyloplasts) are principally composed of galactolipids (Rochester et al. 1987;

Waters and Langdale, 2009). The galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) constitute the bulk of the lipids present in the chloroplast membranes with DGDG being almost exclusive to the thylakoid membranes (Härtel et al. 1997). Lipid membranes have been proposed as one of the primary heat sensors in plants leading to downstream signal transduction and the activation of the heat shock response (HSR) (Saidi et al., 2011; Mittler et al., 2012). Previous studies have shown that a calcium (Ca^{2+}) influx across the plasma membrane occurs in response to high temperatures, leading to a HSR and the development of enhanced heat stress tolerance (Knight, 1999; Saidi et al., 2011). The increased levels of cytosolic calcium may communicate the detection of heat stresses to downstream processes through Ca^{2+}-dependent protein kinases (CDPK) or calmodulin (CaM)-dependent protein kinases (Knight, 2000; Cheng et al., 2002; Liu et al., 2008). It has been shown that an increase in membrane fluidity may also act as a heat sensor that mediates a mitogen-activated protein kinase (MAPK) called heat shock-activated MAPK (HAMK) and is thought to communicate the signal through the cytoskeleton, Ca^{2+} levels, and CDPKs (Sangwan et al., 2002).

As environmental temperatures increase, the cellular lipid membranes in plants become increasingly fluid and destabilized (Wahid et al., 2007). Membrane bound proteins may become denatured or the membrane itself may become increasingly porous and no longer able to function as the boundary of ions or enzymes (Wahid et al., 2007). To counteract these affects, the cells decrease the levels of unsaturation in certain membrane lipid species (Wahid et al., 2007). Currently it is not clear how this decrease in unsaturation is controlled by high temperatures; however, it is not believed to be controlled at the level

of transcription of known lipid genes (Falcone et al., 2004). For example, in *Arabidopsis thaliana*, the fatty acid desaturase (FAD) 8 gene was discovered to actively transcribe during cold temperatures, which alleviates the negative effects of the chilling stress by increasing the levels of unsaturated membrane lipids compared to lines with a non-functional FAD8 gene (Gibson et al., 1994). A similar gene or transcriptional process has not been described during high temperature stresses. Although not fully understood how, previous researchers have described results where plants exposed to high temperature stresses preferentially decrease the levels of lipid membrane unsaturation to a more thermodynamically favorable level of less unsaturation (Murata and Los, 1997; Iba, 2002; Larkindale and Huang, 2004). In a study of transgenic tobacco plants with an altered ability to synthesize trienoic fatty acids, the transgenic plants did not show common symptoms of heat stress when compared to the wild-type plants from the same genetic background (Murakami et al., 2000). The decreased levels of unsaturation may increase the temperature at which the typically highly unsaturated galactolipids phase-separate into nonbilayer structures that are thought to disrupt membrane organization leading to some of the common heat stress phenotypes (Murakami et al., 2000). The composition and structure of thylakoid membranes within chloroplasts are thought to play a role in a plant's susceptibility to damage in heat stress environments where the lipid composition of these membranes may be a significant factor contributing to differential levels of thermotolerance (Süss and Yordanov, 1986; Kim and Portis, 2005). Evidence was shown that the ability of wild-type *A. thaliana* to acclimate to heat stresses was due to an increase in the relative amount of DGDG, an increase in the ratio of DGDG-to-MGDG, and a general decrease of fatty acid levels of unsaturation (Chen et al., 2006). Increased levels of

DGDG relative to MGDG is thought to increase stability of the thylakoid membrane and promote the bilayer structure and dynamics required for normal membrane and protein function at higher temperatures (Süss and Yordanov, 1986; Hölzl et al., 2006). Possible reasoning may be due to DGDG being a lipid with a large polar head group that forms what is described as a lamellar phase (Lα) in aqueous solution and thought to promote the formation of a bilayer in the cytoplasm environment, whereas MGDG organizes into a hexagonal phase (HexII) structure that does not promote bilayer formation in this environment (Webb and Green, 1991; Härtel et al., 2000). In maize, Chen et al. (2010) suggested that phosphatidic acid (PA) was the only lipid that showed a significant difference between the heat tolerant inbred (B76) and a heat susceptible inbred (B106) and proposed that it may be important in maintaining membrane stability during high temperature stress (Chen et al., 2010). Besides membrane stability, PA has been identified as a signaling molecule triggered during stress (Munnik, 2001; Testerink et al., 2005; Wang, 2005). In wheat, it was observed that levels of triacylglycerols (TAGs) containing fully unsaturated (18:3) acyl chains increased by threefold when exposed to high temperature stress which may be due to free fatty acids from lipid membrane remodeling (Narayanan et al., 2016).

As the emergent properties of the lipidome that lead to improved heat stress tolerance in model plants are being studied, limited information is available in our most important cereal crops, including maize. It is necessary to identify the rules that govern the complexity of high temperature tolerance to enable new tools and understanding to make necessary gains in breeding for improved high temperature tolerance.

1.5 Cold Stress

Low temperatures, either in periods of short or prolonged duration, are a major environmental limitation to the global cultivation of important cereal crops such as rice, maize, sorghum, and millet (Graham and Patterson, 1982; Mula et al., 2009; Rodríguez et al., 2014; Shakiba et al., 2017; Parra-Londono et al., 2018). Cold stress is typically categorized as either chilling stress, which generally involves temperatures below 20°C or above 0°C, and freezing stress, involving temperatures below 0°C (Chinnusamy et al., 2007). Enhanced tolerance or adaptability to suboptimal temperatures, particularly during the early growing season and reproductive stages could expand production possibilities and provide improved yield protection (Parra-Londono et al., 2018). Chilling stress can be a major issue in temperate, high elevation, and subtropical rice production, where temperatures below 17°C have been shown to prompt decreased germination rates, delayed maturity, and/or reduced yield (Andaya and Mackill, 2003; Shakiba et al., 2017). Maize is a chilling sensitive crop in which low temperature stress primarily affects the early growth stages during germination and photosynthetic development leading to poor germination, viability, and ultimately decreased yields (Rodríguez et al., 2014). Sorghum is a warm season crop with major production in arid and semi-arid regions in which chilling stress during germination and early growth stages at temperatures below 15°C, dependent on the time or duration, can lead to decreases in seed germination, reduced post-emergence development rates, and reduced yield (Smith and Frederiksen, 2000; Parra-Londono et al., 2018). Most of pearl millet production occurs in the arid and semi-arid areas within Africa and Asia where it is well adapted to elevated temperatures (Serba et al., 2017). Although there are minimal studies in chilling stress responses of pearl millet, it has been reported that low temperatures during early seed germination can result in major decreases in yield

at harvest, however testing during cool seasons have shown promising results for adaptation to cooler temperatures (Mula et al., 2009; Yeshvekar et al., 2017).

An active area of research is understanding how plants sense suboptimal environmental temperatures before triggering the appropriate responses. Cellular membranes are thought to be at least one of the cold sensing mechanisms plants possess (Chinnusamy et al., 2007; Takahashi et al., 2013; Barrero-Sicilia et al., 2017). At lower temperatures, the lipid membranes in plant cells undergo dynamic changes (Örvar et al., 2000; Barrero-Sicilia et al., 2017). In response to chilling stress, there is a general trend of increasing lipid content and levels of unsaturation that is commonly ascribed to as an attempt to maintain membrane fluidity and homeostasis at cold temperatures (Örvar et al., 2000). The decrease in membrane fluidity has been shown to lead to an increase in cytosolic Ca^{2+} concentration due to negatively affecting normal Ca^{2+} channel functioning, which may also be an initial sensor leading to signaling of transduction pathways (Beck at al., 2007).

Chilling stresses occur at temperatures above freezing but below optimal for normal physiological or cellular functioning. For example, in maize, chilling stress can occur in general between 0°C and 20°C (Chinnusamy et al., 2007; Hu et al., 2017). Plants that are chilling sensitive, exhibit chlorosis, decreased leaf expansion, impairment in growth, decrease in viability, fitness, or death, where the magnitude is highly dependent on the time, duration, and temperature in which the stress is presented (Thakur et al., 2010; Hu et al., 2017). Chilling stress can lead to metabolic disruption of the photosynthetic process or structures such as perturbation to photosystem I (PSI) and PSII, inhibitions of electron transport, decreased CO_2 assimilation, development of defective chloroplasts, and

decreased stomatal conductance (Kratsch and Wise, 2000; Allen and Ort, 2001). Another major issue during cold stress is the occurrence of an imbalance between water uptake and transpiration that can lead to leaf dehydration (Beck et al., 2007). Increased production of ROS can cause oxidative damage to lipid membranes; however, there is an important balance in which ROS may act as a secondary messenger in the perception and signal transduction response to cold stress (Mahajan and Tuteja, 2005).

Chilling tolerance mechanisms in plants are adaptive characteristics that mitigate or deter the deleterious effects of suboptimal temperature during the life cycle and the effectiveness of their response are often associated with certain low temperature adapted species or as genetic variants of otherwise low temperature sensitive species (Fowler et al., 1999). Plants have evolved many strategies to endure and survive suboptimal temperature stresses, including many morphologic, physiologic, metabolic, or molecular adaptations that are modulated through a multitude of stress signaling and gene expression networks (Fowler et al., 1999; Janmohammadi et al., 2015). Dynamic alterations in lipid membranes occur in response to low temperature, such as an increase in fatty acid unsaturation and membrane stabilizing head-groups (Mahajan and Tuteja, 2005). For example, in *Arabidopsis thaliana*, FAD8 gene transcription is increased in plants at low temperatures, suggesting its role in increasing unsaturation in response to cold stress (Gibson et al., 1994). Cold-responsive genes (COR) that are regulated by low temperatures have been identified with dehydration-responsive elements (DRE/C-repeat) in their promoter regions in which the transcription factors DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR 1 (DREB1)/C-REPEAT BINDING FACTOR (CBF) (Shinozaki et al., 2003). Another transcription factor, INDUCER OF CBF EXPRESSION 1 (ICE1) has been

identified in *A. thaliana* that is constitutively expressed and able to bind to and activate CBF expression, thus playing an important role in cold tolerance (Chinnusamy, et al., 2003).

Cold acclimation in plants occurs following exposure to low temperature (e.g. 2°C – 10°C). Improved cold acclimation in plants increases resilience and expands the temperature range of tolerance (Graham and Patterson, 1982; Fowler et al., 1999; Xin and Browse, 2000). The magnitude of acclimation has been shown to be dependent on rate, stage, duration, or temperature and can be interrupted, reversed, or resumed (Fowler et al., 1999). Plants that have been exposed to low temperatures and able to be cold acclimatized, not only show improved chilling stress tolerance upon subsequent stress, but also have increased freezing tolerance at temperatures below 0°C (Thomashow, 1999). A plant's ability to cold acclimate after exposure to nonlethal suboptimal temperatures and increase freezing tolerance, greatly improves its survival and reproductive success when faced with unpredictable future weather patterns.

Cold stress tolerance has been studied extensively in both model and crop species; however, practical breeding application of the various genes associated with control of cold tolerance mechanisms is still in development. Much of the research effort focused on breeding for cold tolerance, for example in maize, prioritizes the improvement of germination and establishment (Rodríguez et al., 2007). Due to the ability to control the environment, many of the cold tolerance studies in plants, such as for germination or establishment, occur in the growth chamber. However, it has been observed that cold tolerance phenotypes identified in controlled environments do not necessarily correlate well with those observed in field settings, therefore it has been recommended to leverage

both, with field trials used to provide performance confirmation (Menkir and Larter, 1985; Revilla et al., 2000; Rodríguez et al., 2007). There have been many studies which show that cold tolerance is a multigenic trait with complex environmental interactions and quantitative inheritance (Sutka, 2001; Revilla et al., 2016; Shakiba, et al. 2017). Many of these studies have identified QTL associated with traits that are highly correlated or associated with cold tolerance, such as germination rate, chlorophyll fluorescence, or seedling growth; however, the impact of these QTLs has been minimal for improving low temperature tolerance in our staple food crops (Revilla et al., 2016). For these reasons, it is necessary to have a clear definition and understanding of the target selection criteria and how it can be effectively integrated into the breeding program. For example, the use of chlorophyll fluorescence as a tool to select for cold tolerance has shown some promise in selecting for improved photosynthetic capacity in maize (Fracheboud et al., 1999). Selection on physiological characteristics or biomarkers that integrate various mechanisms that confer cold tolerance, may be beneficial and help improve the efficiency of breeding for this trait (Noble and Rogers, 1992). Due to their importance and overall physiological interconnectedness to cold stress, lipid membranes may provide an ideal target for identification of biomarkers highly associated with cold tolerance in plants (Chen et al., 2013).

CHAPTER 2. ASSOCIATION OF MAIZE LEAF GALACTOLIPID UNSATURATION LEVELS WITH FIELD-BASED HIGH TEMPERATURE TOLERANCE

2.1 Abstract

Crop and climate modeling studies suggest that elevated daytime and nighttime temperatures will present future challenges to global food production due to yield decreases in our staple crops from the accompanying stresses. Although some high temperature adaptation traits and their associated genes have been discovered in plants, almost nothing is known about the contributions of these traits to the adaptation of the major cereal crop, maize (*Zea mays* L.). The identification of useful genetic variation associated with sustained productivity during increased temperature stress will be key to safeguarding yield potential during an uncertain future climate.

Previous studies have shown leaf membrane lipids undergo dynamic changes when exposed to high temperature stresses, one of which is a relative decrease in the fully unsaturated species of the plastidic lipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), suggesting a physiological process that may be a key trait for enhanced tolerance.

This study aims to expand our understanding of the leaf membrane lipid response linked to high temperature stresses. It was hypothesized that ratios of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) containing acyl chains with total 36 carbons and 6 double bonds (36:6) compared to those with 36 carbons and 5 double bonds (36:5), here termed the MGDG and DGDG "Unsaturation Ratios", may be used to differentiate changes in the plastidic lipid

membrane unsaturation levels with potential to classify heat tolerant maize genotypes.

The maize inbred lines, B73 (heat susceptible), LPS-F32 (heat tolerant), and 12 CIMMYT hybrid lines with differential heat tolerance phenotypes were grown in a controlled environment at near-optimal temperature (30^0C daytime/20^0C nighttime) and under heat stress conditions (42^0C daytime/35^0C nighttime) and the leaf lipid content was then analyzed using electrospray ionization with tandem mass spectrometry (ESI-MS/MS). A comparison of B73 and LPS-F32 leaf lipid content showed increased levels of MGDG(36:5) and DGDG(36:5) lipid species in the heat tolerant line compared to the heat susceptible line when grown in both near-optimal temperature and heat stress environments.

An analysis of the MGDG and DGDG Unsaturation Ratios from the 12 hybrid lines revealed a high correlation with the in-field measured heat stress phenotypes. Together, these findings suggest that the level of unsaturation in plastidic membrane lipids is genetically controlled and may be associated with heat stress adaptation in maize. The preliminary analysis indicates that the MGDG and DGDG Unsaturation Ratios may have potential to be used as lipid biomarkers to screen maize genotypes with useful genetic variation for increased thermotolerance in a temperature-controlled environment, which may reduce the need for preliminary heat stress trait screening in unpredictable field environments.

2.2 Introduction

Farmers have long been challenged with producing crops in unpredictable and stressful environments. Despite these challenges, the adoption of improved management

practices and germplasm development have resulted in steady increases in crop yields (Cober and Morrison, 2015; Ruffo et al., 2015). However, recent climate modeling studies suggest that high daytime and nighttime temperatures may present a hurdle to future increases in global crop productivity (Lobell et al., 2011; Challinor et al. 2014; Ray et al., 2015).

Most plant species have developed mechanisms that enable them to survive and reproduce in the presence of biotic and/or abiotic stresses. To cope with high temperature stresses, plants have developed basal and acquired thermotolerance mechanisms (Larkindale et al., 2005). Basal thermotolerance is the inherent ability of a plant to tolerate high temperature stresses. Acquired thermotolerance describes how exposure to a brief period of sub-lethal heat stress enables a plant to better cope with subsequent exposure to high temperature stresses (Chen et al. 2006). Much research in diverse plant species (e.g., *Arabidopsis thaliana, Phaseolus vulgaris, Solanum lycopersicum*) has been devoted to understanding these key thermotolerance mechanisms (Wahid et al., 2007). It is critical that the findings of these studies be leveraged in major field crops, such as maize, when developing strategies for improving heat stress tolerance and, by extension, protecting yield potential.

An increasing body of literature indicates that lipids play a key role in both normal cellular processes and stress responses in a large part due to being major components of cellular membranes. Lipid membranes provide the compartmentalization required for normal cellular processes (Ohlrogge and Browse, 1995), thus, their disruption can lead to major perturbations in plant cell homeostasis, the results of which can be detrimental to growth and development (Bonaventure et al., 2003; Walls and

Browse, 2002; Kobayashi et al., 2007; Aronsson et al., 2008). The plasma membrane is primarily comprised of phospholipids while the plastid membranes (e.g., chloroplasts, amyloplasts) are principally composed of galactolipids (Rochester et al., 1987; Waters and Langdale, 2009). The galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) make up most lipids present in the chloroplast with DGDG being almost exclusive to the thylakoid membranes (Härtel et al., 1997).

Many studies have shown that saturation levels and composition of lipid species in the plastids vary in response to changes in temperature (Chen et al., 2006; Theocharis et al., 2012). Lipids that have fatty acyl chains with higher levels of double bonds are less viscous than lipids with higher levels of single bonds. This is, in part, due to the unsaturated bonds making it harder for the lipids to pack together, therefore requiring less thermal energy to increase membrane fluidity. Alternatively, membranes with higher levels of saturated fatty acyl groups are able to pack together more tightly, requiring higher levels of thermal energy to increase membrane fluidity. An example of this is illustrated in *A. thaliana* by the second plastid specific omega-3 fatty acid desaturase 8 (AtFAD8) gene, which is actively transcribed and compensates for the lowered activity of AtFAD7 when the plant is exposed to cold temperatures (Gibson et al., 1994; Román et al., 2015). The contribution of this gene increases the levels of (18:3) fatty acids within the chloroplast envelope membrane, which is thought to alleviate higher membrane viscosity caused by sub-optimal environmental temperatures. However, for high temperature environments, a similar gene or transcriptional process directly leading to changes in lipid membrane saturation levels has not been discovered (Gibson et al., 1994).

Researchers have shown that plants preferentially decrease the levels of lipid membrane unsaturation towards a more thermodynamically favorable saturated state when exposed to high temperature, which may relieve the effects of the stress (Murata and Los, 1997; Iba, 2002; Larkindale and Huang, 2004). Murakami et al. (2000) used transgenic tobacco plants that are unable to synthesize the fully unsaturated trienoic fatty acids to show that plants with decreased levels of lipid membrane unsaturation had enhanced thermotolerance as compared to the wild-type plants. This study of transgenic tobacco plants supports the idea that thermotolerance in plants is strongly influenced by the lipid composition of the thylakoid membrane of the chloroplast (Süss and Yordanov, 1986; Kim and Portis, 2005). The lipid species MGDG and DGDG are thought to contribute to the functional intactness of the photosystem (PS) I and II complexes, with evidence that DGDG provides functional stability for the light harvesting complex (LHC) II and the oxygen evolving complex (Kelly and Dörmann, 2002; Hölzl et al., 2006). Chen et al. (2006) has shown that heat tolerance in *A. thaliana* may be associated with a relatively increased DGDG level as well as a higher ratio of DGDG-to-MGDG (DGDG:MGDG ratio). A higher DGDG:MGDG ratio is thought to enhance thylakoid stability by maintaining the bilayer characteristics required for normal membrane and protein function when exposed to elevated temperatures (Süss and Yordanov, 1986; Hölzl et al., 2006).

While associations between lipids and high temperature stress are being investigated in model plants, limited research in this area has been focused in maize. One study in maize suggested that phosphatidic acid (PA) levels were significantly higher in a heat tolerant inbred as compared to a heat susceptible inbred line and thus

proposed that it may be important in maintaining membrane stability during high temperature stress (Chen et al., 2010). Identifying the simple and complex relationships between membrane lipids and high temperature tolerance is a possible avenue for identifying useful genetic variation that can be directly applied in maize breeding programs for improving heat stress tolerance. The objective of this study is to add to the knowledge of maize leaf lipid characteristics during heat stress and to identify potential lipid biomarker(s) associated with increased thermotolerance which may be used in future genetic studies of high temperature stress or applied within a maize breeding program aimed to improve heat stress tolerance.

2.3 Materials and Methods

2.3.1 Genetic Material

The maize inbred lines, B73 and La Posta Seq-F32 (LPS-F32), and hybrid genotypes B73xLPS-F32, B73xDTPWC9-F115, B73xDTPYC9-F46-1, B73xLa Posta Seq C7-F103, B73xDTPWC9-F2, B73xLa Posta Seq C7-F71, B73xDTPYC9-F74, B73xLa Posta Seq C7-F86, B73xDTPYC9-F46-3, B73xLa Posta Seq C7-F153, B73xLa Posta Seq C7-F64, B73xLa Posta Seq C7-F32, B73xLa Posta Seq C7-F78 were chosen for their contrasting levels of previously observed thermotolerance. B73 was chosen based on being the major reference genome which was developed through the efforts of the Maize Genome Sequencing Consortium (MGSC) (Wei et al., 2009; Schnable et al., 2009), and because it is an historically important Iowa Stiff Stalk Synthetic (BSSS) breeding line, which is susceptible to heat stress. LPS-F32 is an International Maize and Wheat Improvement Center (CIMMYT) tropical inbred line developed through the Drought Tolerance for Asia breeding program and shows higher levels of heat stress

tolerance. The hybrid lines are crosses between CIMMYT inbred lines from the Drought Tolerance for Asia and B73 which were chosen because they have shown differential levels of tolerance to heat stress.

2.3.2 Growth Chamber Conditions

For each growth chamber experiment, 3 seeds of each maize line used in lipid analyses were planted in 10 cm diameter plastic pots containing Metro-Mix® 510 (The Scotts Company, Marysville, OH) and were thinned to a single plant shortly after emergence. Five replicates of each line were grown in individual pots arranged in a randomized complete block design. Plants were watered as needed to avoid water stress. Plants were fertilized weekly with Miracle-Gro® All Purpose Plant Food using 0.38 mL L^{-1} of water (The Scotts Company, Marysville, OH).

For B73 leaf lipid characterization and B73, LPS-F32, and B73xLPS-F32 comparison for biomarker discovery experiments, the near-optimal temperature settings were 30°C daytime and 20°C nighttime (hereafter referenced as Near-optimal) with a 12 hour (h) light and 12 h dark photoperiod. The high temperature settings were 42°C daytime and 35°C nighttime (hereafter referenced as Heat) with a 12 h light and 12 h dark photoperiod. The near-optimal temperature samples for lipids were taken at the 6- to 8-leaf stage. Six leaf punch samples were collected from the topmost leaf with a visible collar from four-five biological replicates of each genotype. After four days of growth at the high temperature treatment, six leaf punches were collected from the topmost leaf with a visible collar from the same plants.

For the 12 CIMMYT hybrids, temperature and photoperiod conditions were the same as previously described. However, once the plants reached the 6- to 8 leaf stage, the

treatment samples taken were (1) near-optimal temperature (Near-optimal), (2) after 4 hours of high temperature (Heat + 4 Hours), (3) after 4 days of high temperature (Heat + 4 Days), and 10 days after resetting near-optimal temperature (Near-optimal + 10 Days).

For the field-based experiment, the 12 CIMMYT hybrids were grown in the high temperature environment of Patancheru, India in 2011.

2.3.3 Lipid Analysis

Six leaf punches from single plants were immediately placed in individual 20 mL glass vials with PTFE-lined screw caps (National Scientific, Rockwood, TN, USA) containing 3 mL of 75°C isopropanol with 0.01% butylated hydroxytoluene (BHT) and heated in a water bath for fifteen minutes to deactivate phospholipase D enzymes. Samples were stored at -80°C until processed for lipid extraction. Total leaf lipids were extracted according to the procedure described by Welti et al. (2002). Final lipid extracts were dried using a Savant Concentrator (Thermo Fisher Scientific Inc, Waltham, MA, USA). The lipid extracts were shipped overnight on dry ice in 2 mL glass vials with polytetrafluoroethylene (PTFE)-lined screw caps (National Scientific, Rockwood, TN, USA) to the Kansas Lipidomics Research Center (KLRC) at Kansas State University. Total leaf lipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to KLRC developed protocol (Xiao et al., 2010).

2.3.4 Phenotypic Analysis

Field-based phenotyping was performed by CIMMYT-Asia at the ICRISAT campus in Patancheru, India, a location offering a more predictable, high-temperature summer growing environment. In 2011, maize lines were planted in 2 replicates in an alpha-lattice design with 3 checks for phenotypic observations. Normal field management

practices were performed to ensure healthy plant growth and pest management. Furrow irrigation was performed as needed to prevent water stress.

To examine the impacts of high temperature stress, leaf firing (LF), tassel blast (TB), and leaf senescence (LS) phenotypes were scored at flowering time. Final grain yield was measured at the end of the growing season. The LF was defined as the severity of necrosis of the leaves at the top of the plant. The trait was evaluated using a 1-10 scale with 1 and 10 indicating no leaf firing and severe leaf firing, respectively. TB was defined as the extent of tassel desiccation and evaluated using a 1-10 scale, with 1 indicating no tassel blast and 10 indicating complete tassel blast with no viable pollen. LS was defined as necrotic leaves at the bottom of the plants and was evaluated on a 1-10 scale, with 1 being little to no leaf senescence and 10 representing major leaf senescence. Grain yield was measured at the end of the growing season as tons per hectare (t ha^{-1}).

2.3.5 Statistical Analysis

SAS Software version 9.2 (Cary, NC, USA) was used for all statistical analyses. Best linear unbiased estimates (BLUEs) were calculated using SAS PROC MIXED to obtain the LSMEANS for field-based phenotypes of the 12 CIMMYT hybrid lines. PROC CORR was used to calculate the correlations between the field-based phenotypes and leaf lipids. JMP Pro version 11 (Cary, NC, USA) was used to calculate All Pairs Tukey HSD tests to group the hybrid lines with leaf firing, tassel blast, and senescence field-based phenotypes.

2.4 Results

2.4.1 B73 Leaf Lipid Analysis

An analysis of eleven lipid classes encompassing 156 species was performed to characterize the constitution of the leaf lipid content of B73 grown in a controlled environment at near-optimal temperatures (30°C daytime/20°C nighttime) and after four days exposed to high temperature stress (42°C daytime/35°C nighttime) (Table 2.1; Figure. 2.1). The highest concentrations of individual lipids observed were the galactolipids MGDG and DGDG (Table 2.1). These lipids collectively constitute more than 90% of B73 total leaf lipids when grown in a controlled environment at either near-optimal or high temperature. At the near-optimal temperature, the major acyl species identified for galactolipids are DGDG(34:3), DGDG(36:5), and DGDG(36:6) and MGDG(36:5) and MGDG(36:6) (Figure 2.1). The major acyl species identified for phospholipids are phosphatidylglycerol (PG)(32:0), PG(32:1), PG(34:2), PG(34:3), and PG(34:4); phosphatidic acid (PA)(36:4); phosphatidylcholine (PC)(34:2), PC(34:3), PC(36:4), and PC(36:5); phosphatidylethanolamine (PE)(34:2), PE(34:3), PE(36:4), and PE(36:5); phosphatidylinositol (PI)(34:2) and PI(34:3); and phosphatidylserine (PS)(34:2), PS(34:3), PS(40:2), and PS(42:2) (Figure 2.1).

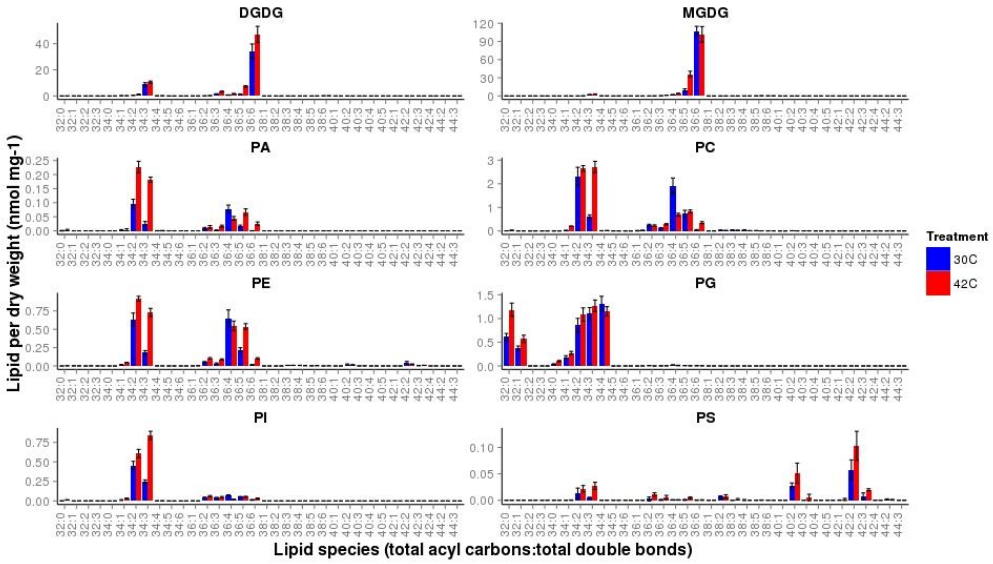

Figure 2.1 B73 leaf lipid content per dry weight (nmol mg^{-1} dry wt) comparison when grown at near-optimal (30°C daytime/20^{0}C nighttime) (blue) and high temperature (42°C daytime/35^{0}C nighttime) (red) controlled environment conditions.
DGDG digalactosyldiacylglycerol, MGDG monogalactosyldiacylglycerol, PA phosphatidic acid, PC phosphatidylcholine, PE phosphatidylehanolamine, PG phosphatidylglycerol, PI phosphatidylinositol, PS phosphatidylserine.

The comparison of B73 grown at near-optimal and high temperature revealed significant differences in membrane lipid content and saturation levels. Most lipid species had significant changes when exposed to heat stress, with a general trend towards both greater total and individual species lipid content. The major chloroplast associated membrane lipids, MGDG, DGDG, and PG, showed significant increases when exposed to high temperature stress compared to near-optimal growing temperatures (Table 2.1). MGDG increased from 122.6 to 147.0 nmol lipid mg^{-1} dry leaf weight (hereafter referenced as nmol mg^{-1} dry wt), DGDG increased from 48.0 to 72.1 nmol mg^{-1} dry wt, and PG increased from 4.5 to 5.7 nmol mg^{-1} dry wt. The major plasma membrane associated lipids, PC, PE, PI, PS, and PA, also showed significant increases when exposed to high temperature stress (Table 2.1). Lipid content increased from 6.2 to 8.1

nmol mg^{-1} dry wt for PC, from 1.9 to 3.1 nmol mg^{-1} dry wt for PE, from 0.94 to 1.7 nmol mg^{-1} dry wt for PI, from 0.12 to 0.26 nmol mg^{-1} dry wt for PS, and from 0.23 to 0.58 nmol mg^{-1} dry wt for PA. The DGDG:MGDG ratio significantly increased and the MGDG and DGDG Unsaturation Ratios significantly decreased when exposed to high temperature stress (Table 2.1).

Table 2.1 B73 leaf lipid total content (nmol mg^{-1} dry wt) of each head group class, DGDG:MGDG, MGDG and DGDG Unsaturation Ratios show significant differences when grown at near-optimal temperature (30°C daytime/20^{0}C nighttime) compared to high temperature (42°C daytime/35^{0}C nighttime) for 4 days.

Name	Heat stress experiment		P-value
	30°C/20°C	42°C/35°C	
	nmol lipid mg^{-1} dry weight		
DGDG	48.0 ± 7.0	72.1 ± 7.9	0.004**
MGDG	122.6 ± 9.8	147.0 ± 18.1	0.068
PG	4.5 ± 0.5	5.7 ± 0.6	0.031*
LysoPG	0.12 ± 0.002	0.20 ± 0.03	0.007**
LysoPC	0.02 ± 0.00	0.03 ± 0.01	0.036*
LysoPE	0.01 ± 0.00	0.03 ± 0.00	0.000***
PC	6.2 ± 0.96	8.1 ± 0.51	0.020*
PE	1.9 ± 0.30	3.1 ± 0.14	0.001***
PI	0.94 ± 0.09	1.7 ± 0.09	0.000***
PS	0.12 ± 0.04	0.26 ± 0.06	0.012*
PA	0.23 ± 0.04	0.58 ± 0.05	0.000***
DGDG:MGDG	0.39	0.49	0.003**
DGDG Unsaturation Ratio	24.17	6.51	0.005**
MGDG Unsaturation Ratio	10.86	2.82	0.003**

Statistical significance between environments indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$

DGDG digalactosyldiacylglycerol, MGDG monogalactosyldiacylglycerol, PG phosphatidylglycerol, PC phosphatidylcholine, PE phosphatidylethanolamine, PI phosphatidylinositol, PS phosphatidylserine, PA phosphatidic acid

Relative changes in lipid content were further analyzed to elucidate any important changes in either saturation levels or composition that may be involved in high temperature stress adaptation. In this study, total levels of MGDG and DGDG decreased 74.0 % and 73.1%, respectively, when exposed to high temperature stress. The DGDG:MGDG ratio increased 25.6% from 0.39 for the optimal temperature treatment to 0.49 for the high temperature treatment (Table 2.1). MGDG and DGDG both increase the less unsaturated (36:5) species in the presence of high temperature stress, however the increase does not necessarily coincide with a similar decrease in the (36:6) species. The MGDG(36:5) and DGDG(36:5) lipid species increased 264.7% and 408.8%, respectively, after four days of high temperature stress. The MGDG(36:6) species decreased by 5.5% while the DGDG(36:6) species increased by 37.0%. The effect of the large increase in the (36:5) species and the comparatively small decrease or increase of the (36:6) species resulted in a net increase of the saturation levels, thus lowering the trienoic fatty acid composition of the MGDG and DGDG lipids within the chloroplastic membranes. This increase in saturation level during exposure to high temperature stress is consistent with previous studies (Murakami et al., 2000; Chen et al., 2006).

Because the chloroplast is the site of the heat labile photosynthetic machinery, it is important for the membranes to maintain proper composition and viscosity to sustain physiological homeostasis. The galactolipids, MGDG and DGDG, are the major plastidic membrane lipids essential to maintaining photosynthetic efficiency (Kalisch et al., 2016). The B73 MGDG and DGDG Unsaturation Ratios both decreased ($p < 0.01$) in response to exposure to high temperature stress (Table 2.1). The MGDG Unsaturation Ratio decreased 74.1% from 10.8 grown at 30°C to 2.8 when grown at 42°C and the DGDG

Unsaturation Ratio decreased 73.1% from 24.2 grown at 30°C to 6.5 when grown at 42°C. These results are consistent with the assumption that maize plastidic lipid unsaturation levels decrease when exposed to elevated temperatures. Overall, these results provide clear evidence that the maize inbred line B73 actively regulates membrane lipid composition and saturation levels in response to high temperature and suggests that MGDG and DGDG Unsaturation Ratios may provide potential lipid biomarkers for screening maize germplasm for high temperature tolerance.

2.4.2 B73 and LPS-F32 Leaf Lipid Comparison

To gain further insight into maize lipid responses to heat stress, a comparison of the effect of high temperature stress on the leaf membrane lipids of the heat tolerant LPS-F32 tropical inbred line with that of heat susceptible B73 temperate inbred line was made. The B73xLPS-F32 hybrid was also included in the comparative analysis. Field observations in a high temperature environment provided evidence that B73 is susceptible to heat stress while LPS-F32 has comparably enhanced thermotolerance (Figure. 2.2).

Figure 2.1 2011 summer field observation of maize inbred lines B73 (left) and LPS-F32 (right) grown in the high temperature environment of Patancheru, India.

Exposure to high temperature stress caused a decrease in the DGDG Unsaturation Ratios of LPS-F32, B73, and B73xLPS-F32 by 39.6%, 57.9%, and 46.4%, respectively, compared to the near-optimal temperature environment. The MGDG Unsaturation Ratio decreased 44.0%, 60.1%, and 54.7% in LPS-F32, B73, and B73xLPS-F32, respectively (Table 2.2, Figure 2.3). Similar to other studies, these results indicate that the overall unsaturation levels of the chloroplast membranes are decreasing at high temperatures. Interestingly, these ratios appear to follow a trend in which the susceptible B73 inbred has the highest baseline MGDG and DGDG Unsaturation Ratios at optimal temperature while the heat tolerant LPS-F32 has the lowest levels. When LPS-F32 is combined with B73 as a hybrid, these levels appear to be intermediate between B73 and LPS-F32 in both the optimal and high temperature environments (Figure 2.3).

Table 2.2 Total DGDG:MGDG, MGDG and DGDG Unsaturation Ratios of the maize lines B73, LPS-F32, and B73xLPS-F32 show significant differences when grown at high temperature (42°C daytime/35^{0}C nighttime) compared to near-optimal temperature (30°C daytime/20^{0}C nighttime).

Name	B73		P-value	LPS-F32		P-value	B73xLPS-F32		P-value
	30°C/20°C	42°C/35°C		30°C/20°C	42°C/35°C		30°C/20°C	42°C/35°C	
	nmol mg^{-1}dry wt			*nmol mg^{-1}dry wt*			*nmol mg^{-1}dry wt*		
DGDG:MGDG	0.27	0.40	0.003**	0.30	0.36	0.011*	0.30	0.35	0.030*
DGDG Unsaturation Ratio	14.49	6.10	0.000***	4.78	2.89	0.025*	7.33	3.92	0.025*
MGDG Unsaturation Ratio	8.74	3.48	0.028*	3.80	2.13	0.064	5.13	2.32	0.010**

Statistical significance between environments indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$
DGDG digalactosyldiacylglycerol, MGDG monogalactosyldiacylglycerol

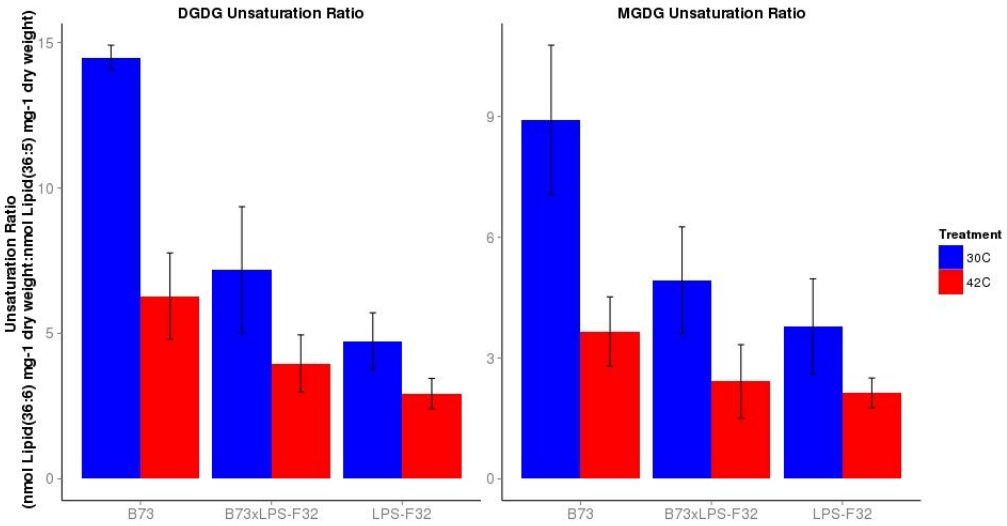

Figure 2.2 MGDG and DGDG Unsaturation ratios of the maize inbred lines, B73, LPS-F32, and B73xLPS-F32 at near-optimal temperature (30^0C daytime/20C^{0}C nighttime) (blue) and after 4 days of high temperature (42^0C daytime/35^0C nighttime) (red) growing conditions. MGDG monogalactosyldiacylglycerol and DGDG digalactosyldiacylglycerol.

2.4.3 Heat Stress Field Observations

To characterize the performance of maize hybrid lines used in this study, field-based heat stress phenotypic data were obtained for twelve CIMMYT hybrids with diverse thermotolerance in the high temperature environment of Patancheru, India in 2011. As shown in Table 2.3, grain yield and leaf firing were the only heat stress-associated phenotypes to exhibit significantly different responses between the hybrids. B73xLPS-F78 and B73xLPS-F32 had significantly lower levels of leaf firing while B73xDTPWC9-F115 had significantly higher levels of leaf firing. B73xLPS-F32 was the highest yielding hybrid with the least amount of tassel blast and next to lowest leaf firing score. The hybrid lines, B73xLPS-F64 and B73xLPS-F32, had the lowest levels of leaf senescence, respectively. B73xDTPWC9-F115 and B73xLPS-C7-F71 had the lowest grain yields with the highest levels of leaf firing and senescence (Table 2.3). The 2011 summer field evaluation identified the B73xLPS-F32 hybrid as the most heat stress tolerant among those evaluated.

Table 2.3 2011 summer field evaluation of CIMMYT hybrids in the high temperature environment of Patancheru, India. Grain yield was measured in tons per hectare (t ha^{-1}), Tassel blast, Senescence, and Leaf firing were measured using a 1 – 10 rating, with 1 indicating little to no presence and 10 indicating complete presence of the phenotype.

Hybrid	Grain Yield (t ha^{-1})	Tassel Blast (1-10)	Senescence (1-10)	Leaf Firing (1-10)
B73xLPS-C7-F32	3.03a	2.8a	2.8a	1.7b
B73xLPS-C7-F153	2.21a	3.2a	3.2a	2.8ab
B73xLPS-C7-F64	1.84ab	3.5a	2.3a	3.0ab
B73xLPS-C7-F78	1.24b	3.1a	4.5a	1.5b
B73xDTPYC9-F46-3	2.22a	6.3a	3.4a	3.7ab
B73xLPS-C7-F103	2.04a	3.6a	3.1a	8.9ab
B73xDTPWC9-F2	2.57a	8.3a	4.0a	6.1ab
B73xLPS-C7-F86	1.34b	4.9a	4.6a	4.6ab
B73xDTPYC9-F46-1	-	3.0a	4.3a	8.4ab
B73xDTPYC9-F74	1.63b	9.8a	4.1a	4.9ab
B73xLPS-C7-F7	1.09b	5.4a	5.1a	7.1ab
B73xDTPWC9-F115	1.05b	4.9a	5.2a	9.2a

Within a column, values followed by different lowercase letters indicate statistically significant differences at $p<0.05$.

2.4.4 Field and Growth Chamber Comparison

To understand whether the role of membrane unsaturation in heat stress adaptation of maize can be correlated with field observations, the twelve maize hybrid lines that were demonstrated to have differential phenotypic response to heat stress under field conditions were grown in a growth chamber and total leaf lipids were analyzed. The MGDG and DGDG Unsaturation Ratios were calculated to indicate lipid unsaturation in each of the genotypes. There was a significant correlation between the plastidic membrane DGDG Unsaturation Ratio for hybrids grown in high temperature conditions in the growth chamber for 4 hours and 4 days and heat-induced tassel blast for those same hybrids grown in the field (Table 2.4). DGDG Unsaturation Ratio values from hybrids grown under the near-optimal temperature, at the high temperature for 4 hours, and at a return to near-optimal temperature for 10 days were significantly correlated with field-based leaf senescence (Table 2.4). The MGDG Unsaturation Ratio from hybrids grown at high temperature for 4 days and a return to near-optimal temperature for 10 days was significantly correlated with field-based tassel blast while the MGDG Unsaturation Ratio from hybrids grown at high temperature for 4 hours, 4 days, and a return to near-optimal temperature for 10 days was significantly correlated with field-based leaf senescence (Table 2.4).

Table 2.4 Correlations of MGDG and DGDG Unsaturation Ratios from developing hybrid leaves at near-optimal growing conditions (near-optimal), high temperature for 4 hours (heat + 4 hours), high temperature for 4 days (heat + 4 days), and a return to near-optimal temperature for 10 days (near-optimal + 10 days) with the heat stress phenotypes tassel blast, leaf firing, and leaf senescence.

		DGDG Unsaturation ratio		MGDG Unsaturation ratio	
		Correlation	P-value	Correlation	P-value
Near-optimal (30°C daytime/20^{0}C nighttime)	Tassel Blast	0.562	0.057	0.166	0.605
	Leaf Firing	0.364	0.245	0.235	0.463
	Leaf Senescence	0.685	0.014*	0.222	0.489
Heat + 4 Hours (42°C daytime/35^{0}C nighttime)	Tassel Blast	0.608	0.036*	0.466	0.127
	Leaf Firing	0.292	0.357	0.380	0.223
	Leaf Senescence	0.723	0.008**	0.681	0.015*
Heat + 4 Days (42°C daytime/35^{0}C nighttime)	Tassel Blast	0.679	0.015*	0.651	0.022*
	Leaf Firing	0.499	0.098	0.451	0.141
	Leaf Senescence	0.463	0.130	0.335	0.287
Near-optimal + 10 Days (30°C daytime/20^{0}C nighttime)	Tassel Blast	0.416	0.179	0.583	0.047*
	Leaf Firing	-0.081	0.802	-0.219	0.495
	Leaf Senescence	0.893	<0.000***	0.732	0.007**

Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$

2.5 Discussion

The analysis of B73 leaf lipids when grown at near-optimal temperature revealed that plastidic MGDG was the major galactolipid, accounting for 66% of the total leaf lipids measured. More than 85% of this MGDG pool was composed of fully unsaturated MGDG(36:6) (18:3/18:3) species. DGDG accounted for 25% of the total lipids analyzed, with 71.5% of the DGDG pool composed of the fully unsaturated DGDG(36:6) (18:3/18:3) species. It is well known that the thylakoid membrane of plants has a high degree of unsaturation, and the maize inbred line B73 is no different (Chapman et al., 1983; Wang and Benning, 2012). One proposed reason is that the α-linolenic fatty acid (18:3) may allow for the unique membrane folding characteristics of the thykaloid grana which is required for proper functioning (Wang and Benning, 2012). Once fatty acid biosynthesis is initiated in the plastid stroma, they can be incorporated into either the prokaryotic or eukaryotic glycerolipid synthesis pathway.

Out of the six phospholipid classes analyzed in B73 at near-optimal temperature treatment, total PC levels were the highest among all phospholipid classes at 6.2 nmol mg^{-1} dry wt, constituting 44.4% of all phospholipids measured or 3.3% of total lipids (Figure 2.1). Total PG and PE content were the next most abundant with 4.5 and 1.9 nmol mg^{-1} dry wt, respectively. Finally, trace amounts of PI, PA, and PS were identified with 0.94, 0.23, and 0.13 nmol mg^{-1} dry wt, respectively. These results are consistent with previous studies revealing the major constituents of the plasma membrane to be from the PC and PE classes (Rochester et al., 1987; Welti et al., 2002). PGs are primarily found in chloroplasts and are the only phospholipids present in thykaloid membranes, where they have been suggested to play a role in the structural integrity of the photosynthetic apparatus (Sato et al., 2000; Hagio et al., 2002). Although trace amounts of PI are

identified in this study, this class of lipid has been shown to play many structural, signaling, and regulatory roles in the cell (Ghosh and Bankaitis, 2011). Recent research has suggested that increased levels of PI from the overexpression of the phosphatidylinositol synthase gene in maize can contribute to increased drought tolerance through favorable changes in membrane composition (Liu et al., 2013). PA is the simplest phospholipid analyzed, reflecting its role as a precursor lipid for the synthesis of many other lipids. The low levels of PA found in B73 leaf lipids do not reflect its importance in membrane biosynthesis, structure, and signal transduction. PA has been shown to be involved in altering the local curvature of membranes which may allow for their adaptive functioning (Kooijman et al., 2003; Kooijman et al., 2005). PA has also been established as a second messenger that is involved in signaling due to environmental stressors such as cold, heat, drought, and wounding (Testerink and Munnik, 2011). PS is a low abundant plant lipid, which has been suggested to play a role in plant cell death signaling (O'Brien et al., 1998; Xiao-Yong et al., 2003; Devaiah et al., 2006). A characterizing attribute of signaling lipids, such as PI, PA and, PS, is that they are in low abundance and have rapid turnover such as observed in this study from B73 leaves.

Previous studies in *A. thaliana* have shown that an increase in total DGDG, a rise in the DGDG:MGDG ratio, and a decrease in lipid unsaturation are all associated with increased tolerance to high temperature stress in plants (Chen et al., 2006). However, these associations have not been well studied in maize. Previous research has shown high correlations between the maize leaf lipidome and important agronomic traits associated with hybrid breeding, indicating a potential use in predicting phenotypic performance of certain traits (Riedelsheimer et al., 2013). Chen et al. (2010) suggest that increased levels

of PA during heat stress may serve as an important signaling mechanism for high temperature tolerance in maize. Results from this study revealed significant increases in PA levels during the high temperature treatment, particularly for the PA(34:2) and PA(34:3) species. In the current study, we focused on the plastidic lipids, MGDG and DGDG, as major effectors involved in maintaining cellular homeostasis during high temperature stress.

The ability of sessile organisms such as maize to survive temperature stresses depends on their capacity to maintain physiological and cellular homeostasis. Developmental and morphological traits that render plants tolerant to heat stress have been identified in cultivated and wild relatives of crops (Wahid et al. 2007). Although these characteristics are generally under the control of polygenic inheritance, efforts to move these traits into elite genetic backgrounds are gaining interest as the challenge of high temperature stress continues to grow. The protective role played by membrane lipids and their unsaturation properties is only one of multiple plant mechanisms that may impart heat tolerance. There are many cellular and physiological processes that are activated when plants are exposed to high temperature stress environments. Most notable is the transcriptional activation of heat shock proteins, which act as molecular chaperones to refold denatured proteins or remove damaged proteins (Wang et al., 2004).

Since there is increasing evidence that a decrease in trienoic fatty acyl groups in the chloroplastic lipid membranes is associated with enhanced thermotolerance (Murakami et al., 2000), the use of the novel MGDG and DGDG Unsaturation Ratios was proposed to reflect the relationship of lipid unsaturation composition of the plastidic membranes with enhanced thermotolerance in maize. Because there does not appear to be

specific gene(s) that regulates changes in lipid saturation levels directly in response to high temperatures, the MGDG and DGDG Unsaturation Ratios may be used to characterize the complex membrane lipid unsaturation changes that occur during high temperature stress.

This study provides evidence that correlates field based high temperature stress phenotypes in maize with changes in levels of galactolipids and unsaturation. Although these results suggest a relationship between the MGDG and DGDG Unsaturation Ratio and heat stress associated phenotypes in maize, further analysis must be made before any conclusions are final. Additional years of field observation as well as direct sampling of leaf tissue from field-grown plants are required. The MGDG and DGDG Unsaturation ratios have the potential to be important biomarkers that can be used to screen germplasm for enhanced thermotolerance in environments with or without high temperature stress. This has practical use for plant breeding, particularly in regions where proper high temperature environments are transient and unpredictable.

Future multi-year population-based experiments should be undertaken to further examine the impact of high temperature stress on leaf lipids and the utility of the MGDG and DGDG Unsaturation Ratios to predict high temperature tolerance in maize. These experiments are needed to allow for a more comprehensive examination of the impact of high temperature stress on the maize leaf lipids, the associated genetics, genes associated with beneficial lipid phenotypes, and their practical use in breeding maize for improved tolerance to high temperature environments.

2.6 Conclusion

The MGDG and DGDG lipids make up the majority of lipids analyzed in maize leaves. They were found to be highly unsaturated and dynamically change in both amount and level of unsaturation when exposed to high temperature stress in a controlled environment.

Preliminary analysis indicates the MGDG and DGDG Unsaturation Ratios may be useful lipid biomarkers to aid in the selection of high temperature stress tolerant germplasm. They were shown to significantly decrease in maize lines exposed to high temperature stress compared to near-optimal growing environment. High temperature tolerant lines, such as LPS-F32, had significantly lower levels than heat susceptible B73 in both near-optimal and high temperature environments. This provides an indication that the MGDG and DGDG Unsaturation Ratios may be useful tools for identifying high temperature tolerant germplasm even when the necessary environmental conditions are not present.

CHAPTER 3. LEAF LIPID ANALYSIS IN THE NESTED ASSOCIATION MAPPING (NAM) POPULATION FOUNDERS AND B73 X B97 RILS SUBPOPULATION

3.1 Abstract

The study of maize leaf lipids has been limited and historically focused on limited germplasm. Maize is a very diverse crop and goes with expectation that this diversity would be reflected in the lipid profiles across germplasm representative of this diversity. The 26 parental inbred lines of the nested association mapping (NAM) population were profiled for total leaf lipid content by electrospray ionization tandem mass spectrometry (ESI-MS/MS) reported as nanomoles of lipid per milligram dry weight (nmol mg^{-1} dry wt). The lipid profiles exhibited variation in lipid composition, especially in ratios of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) containing acyl chains with total 36 carbons and 6 double bonds (36:6) compared to those with 36 carbons and 5 double bonds (36:5), here termed the MGDG and DGDG "Unsaturation Ratios". One of the NAM populations derived from B73 x B97, was further analyzed for total leaf lipid content by ESI-MS/MS due to the large phenotypic difference of the parental components for the MGDG and DGDG Unsaturation Ratios. Linkage mapping identified two major quantitative trait loci (QTL) associated with known fatty acid desaturase (FAD) 7 and FAD8 genes in maize. These results provide preliminary work indicating the diverse NAM population may be an underutilized resource for identifying novel or functional alleles that may be beneficial in the improvement of maize through lipid characterization and mapping.

3.2 Introduction

Maize is one of the world's most important crops and used for food, feed, fiber, and fuel production (Wheals et al., 1999; Shiferaw et al., 2011; Saravana Bavan and Mohan Kumar, 2012; Ranum et al., 2014; West et al., 2014). Tremendous efforts are being made to continuously improve and adapt elite maize germplasm for changing climates and an ever-growing population (Gazal et al., 2017; Myers et al., 2017; Steduto et al., 2018). The genetic diversity of maize will provide the foundation of future efforts to identify beneficial alleles through breeding with the goal to promote the fitness and production of maize in diverse and dynamic environments (Abberton et al., 2016; Dwivedi et al., 2016).

The eye of the breeder has traditionally been the principal phenotyping tool for identifying potentially useful genetic diversity and has successfully shaped maize into the production powerhouse it is today. Today, new technologies such as high-throughput phenotyping and genomic selection are maturing as routine tools for crop improvement. Although the effectiveness of these tools continues to progress, there is a great need to continue bringing innovation to breeders that will help in addressing current and future challenges (Schrag et al., 2018). In the case of identifying heat stress tolerant maize germplasm for breeding programs, an appropriate environment is required to accurately and reproducibly phenotype for these traits. The transient and unpredictable nature of environmental temperature in field phenotyping, both within and across years, provides a challenge to the maize improvement community.

There are abundant studies in model organisms such as the flowering plant *Arabidopsis thaliana* characterizing total leaf lipids and suggesting certain leaf lipid content and unsaturation levels are associated with improved environmental tolerance or

acclimation to both heat and cold. Mutant and wild-type studies have indicated that membrane fatty acid composition and polyunsaturation are important determinants that influence photosynthetic and plant growth stability at high temperatures (Hugly et al., 1989; Falcone et al., 2004; Higashi et al., 2015). Gibson et al. (1994) discovered the temperature dependent role of fatty acid desaturase 8 (FAD8) was to increase or maintain omega-3 (ω-3) desaturase activity to maintain growth at low growth temperatures, whereas, trienoic fatty acid content was indicated in a study of a triple *fad3-2 fad7-2, fad8 Arabidopsis* mutant to be important for maintenance of the chloroplasts during plant growth at low temperatures (Routaboul et al., 2000). Limited information is available regarding maize lipids or their association with improved thermotolerance. Evidence that lipid membrane thermostability is important for maize high temperature tolerance was provided through the comparison of the inbred lines B76 (more tolerant) and B106 (less tolerant) in which phosphatidic acid (PA) was suggested as playing an important role (Chen et al., 2010). A low temperature dependent ω-3 desaturase was also discovered in maize (ZmFAD8), which showed increased expression at 5°C and minimal expression at normal growing temperatures when compared to ZmFAD7 (Berberich et al., 1998).

The maize NAM population was developed as a public resource to enable the study of the genetic basis of complex quantitative traits simultaneously through both linkage analysis and association mapping techniques (Yu et al., 2008). Twenty-five diverse founder inbred lines, representing the diversity of maize from a global collection, were crossed to B73 to develop 25 bi-parental families of 200 recombinant inbred lines (RILs) in each subpopulation (Yu et al., 2009; McMullen et al., 2009). There are many advantages to conducting genetic and phenotypic studies using the NAM population. It is an immortal

population with high density genotyping readily available as a public resource. It has been extensively phenotyped, characterized, and used to study the genetic architecture or map QTL in many different environments for traits such as flowering time (Buckler et al., 2009), leaf architecture (Tian et al., 2011), Northern Corn Blight (NCB) resistance (Poland et al., 2011), Southern Leaf Blight (SLB) resistance (Kump et al., 2011), kernel composition (Cook et al., 2012), and kernel oil biosynthesis (Li et al., 2013).

The aim of the present study is to build preliminary work in discovering potential lipid biomarker(s) that are genetically controlled, heritable, and can be used to classify thermotolerance within a diverse set of maize inbred lines with or without the dependence of the appropriate environment. The goal is to investigate the hypothesis that there is great diversity within maize leaf lipids, specifically the MGDG and DGDG Unsaturation Ratios, when analyzed within the diverse NAM founder inbred lines. This diversity will be used to interrogate the genetic bases for these traits.

3.3 Materials and Methods

3.3.1 Genetic Material

The twenty-five diverse founder lines of the maize Nested-Association Mapping (NAM) Population and Mo17 (Table 3.1) were characterized for total leaf lipids and analyzed for multiple lipid phenotype traits. The experiment was planted in 2011 at the Purdue University Agronomy Center for Research and Education (ACRE) farm in West Lafayette, IN. The field experimental design was a randomized complete block with five replicates and standard field management practices in place.

The NAM founder lines were observed in a single environment during each summer of 2013-2015 in the high temperature environments of either Patancheru or Daulatabad, India and characterized for agronomic traits associated with plant health, ear number and grain weight, and the associated heat stress traits, leaf firing and tassel blast. Ear number was calculated as number of ears per plot divided by plot plant number. Grain weight was measured in grams (g). The leaf firing and tassel blast traits were observed one to two weeks after flowering and calculated as a percent (%) incidence per plot (Zaidi et al., 2016). The field experimental design consisted of single row plots in a lattice design with 2-3 local checks and standard management practices to minimize non-experimental stresses such as drought.

The B73 x B97 NAM RIL subpopulation was evaluated in a single environment during the summers of 2014 and 2015 in the high temperature environment of Daulatabad, India and characterized for agronomic traits associated with plant health, ear number and grain weight. The field experiment design consisted of single row plots in a lattice design with 2-3 local checks and standard management practices to minimize non-experimental stresses.

Table 3.1 Maize Nested-Association Mapping (NAM) founder and Mo17 inbred lines studied at Purdue ACRE in 2011.

Inbred	Developed	Class	Heterotic Group	Heterotic Subgroup
B73	Iowa, USA	Temperate	SS	B73
B97	Iowa, USA	Temperate	NSS	NSS-Mixed
CML52	Mexico	Tropical	TS	TZI
CML69	Mexico	Tropical	TS	Suwan
CML103	Mexico	Tropical	TS	CML-late
CML228	Mexico	Tropical	TS	Suwan
CML247	Mexico	Tropical	TS	CML-early
CML277	Mexico	Tropical	TS	CML-P
CML322	Mexico	Tropical	TS	CML-early
CML333	Mexico	Tropical	TS	CML-P
HP301	Indiana, USA	Popcorn	Popcorn	NA
IL14H	Illinois, USA	Sweet corn	Sweet corn	NA
Ki3	Thailand	Tropical	TS	Suwan
Ki11	Thailand	Tropical	TS	Suwan
Ky21	Kentucky, USA	Temperate	NSS	K64W
M37W	South Africa	Temperate	Mixed	NA
M162W	South Africa	NA	NSS	K64W
Mo18W	Missouri, USA	Temperate	Mixed	NA
Mo17	Missouri, USA	Temperate	NSS	CO109:Mo17
MS71	Michigan, USA	Temperate	NSS	NSS-X
NC350	North Carolina, USA	Tropical	TS	NC
NC358	North Carolina, USA	Temperate	TS	TZI
Oh7B	Ohio, USA	Temperate	Mixed	NA
Oh43	Ohio, USA	Temperate	NSS	M14:Oh43
P39	Indiana, USA	Sweet corn	Sweet corn	NA
Tx303	Texas, USA	Temperate	Mixed	NA
Tzi8	Nigeria	NA	TS	TZI

One hundred and ninety-one of the NAM B73 x B97 subpopulation recombinant inbred lines (RILs) were planted in 2013 at the Purdue University ACRE farm in West Lafayette, IN. Total leaf lipids were characterized and analyzed for various lipid phenotypes. The field experiment design was a randomized complete block design with four replicates with standard management practices used.

3.3.2 Total Leaf Lipid Analysis

Six leaf punches from single plants were immediately placed in individual 20 mL glass vials with PTFE-lined screw caps (National Scientific, Rockwood, TN, USA) containing 3 mL of 75°C isopropanol with 0.01% butylated hydroxytoluene (BHT) and heated in a water bath for fifteen minutes to deactivate phospholipase D enzymes. Samples were stored at -80°C until processed for lipid extraction. Total leaf lipids were extracted according to the procedure described by Welti et al. (2002). Final lipid extracts were dried using a Savant Concentrator (Thermo Fisher Scientific Inc, Waltham, MA, USA). The lipid extracts were shipped overnight on dry ice in 2 mL glass vials with polytetrafluoroethylene (PTFE)-lined screw caps (National Scientific, Rockwood, TN, USA) to the Kansas Lipidomics Research Center (KLRC) at Kansas State University. Total leaf lipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to KLRC developed protocol (Xiao et al., 2010).

3.3.3 Genotypic Data

Genotypic data for the B73 x B97 NAM RIL subpopulation were generated using a panel of 1536 SNPs by the Illumina GoldenGate Assay (Illumina, San Diego, CA) with SNP calls calculated using Illumina BeadStudio software (Ilumina, San Diego, CA) as previously described by McMullen et al. (2009). A quality score of 1 to 4 was assigned to each SNP with quality 1 representing high quality and quality 4 representing a failed assay (McMullen et al., 2009). In summary, there were 956 quality 1 SNPs, 255 quality 2 or 3 SNPs, and 325 quality 4 SNPs. A total of 1,211 SNPs were available for genetic analysis (McMullen et al., 2009). SNPs were chosen where the B73 allele is rare within the population with 974 chosen from random genes, 329 chosen from candidate genes, and 233 chosen from inbred line sequence alignments provided by Pioneer Hi-bred International (McMullen et al., 2009; www.panzea.org).

3.3.4 Statistical Analysis

QTL mapping was performed with R statistical software using a modified version of interval mapping (Lander and Botstein, 1989; R core Team, 2017). The log of odds (LOD) threshold was calculated using permutation test with 1000 repetitions (Doerge and Churchill, 1996).

Pearson correlation was calculated between average MGDG and DGDG Unsaturation Ratios in the NAM B73 x B97 RIL subpopulation using R statistical software (R core Team, 2017).

Inferential statistics for phenotypic means comparison such as Tukey honestly significant difference (HSD) and t-tests were calculated using the R package agricolae: statistical procedures for agricultural research (de Mendiburu, 2017).

A mixed-model was run with ASReml Release 4.1.0.2080mw-64bit using replication, block, and year as fixed effects and genotype as a random effect to create best linear unbiased predictions (BLUPs) to evaluate the combined 2014-2015 B73 x B97 NAM RIL subpopulation trial data in the high temperature environment of Daulatabad, India.

3.4 Results

3.4.1 NAM Founder Inbred Line Experiment

3.4.1.1 Lipid Sampling Environmental Conditions

The environmental conditions for the NAM founder experiment during the 2011 growing season at the Purdue University ACRE farm in West Lafayette were comparable to the 50-year historical average (Table 3.2). May and June of 2011 were modestly warmer than the 50-year average, 0.4 and 1.1 degrees Celsius (°C), respectively during emergence and much of the vegetative growth. The leaf sampling occurred at the V12 to R1 growth stages during the month of July which was 2.8 °C warmer on average than the 50-year historic average.

Table 3.2 Weather data for the 2011 growing seasons with a 50 year (1962-2011) historical average for comparison.

	Average Temperature (°C)		Maximum Temperature (°C)		Minimum Temperature (°C)	
	2011	50 year	2011	50 year	2011	50 year
May	16.6	16.2	22.3	22.3	10.9	10.1
June	22.4	21.3	28.0	27.4	16.8	15.3
July	25.8	23.0	31.8	29.0	19.8	17.0
August	22.2	21.8	29.0	28.0	15.3	15.6
September	17.5	18.2	23.6	25.0	11.3	11.4

* Weather data downloaded from National Oceanic and Atmospheric Administration (NOAA) www.ncdc.noaa.gov

3.4.1.2 Lipid Phenotypic Results

Average total monogalactosyldiacylglycerol (MGDG) measured in the NAM founder inbred lines from 33.60 to 79.88 nanomoles per milligram of dry weight (nmol mg^{-1} dry wt) (Table 3.3). Ki3 had the lowest levels of average total MGDG, whereas CML322 had the highest levels of average total MGDG. B73 and B97, had similar levels of average total MGDG at 72.73 and 72.47, respectively (Figure 3.1).

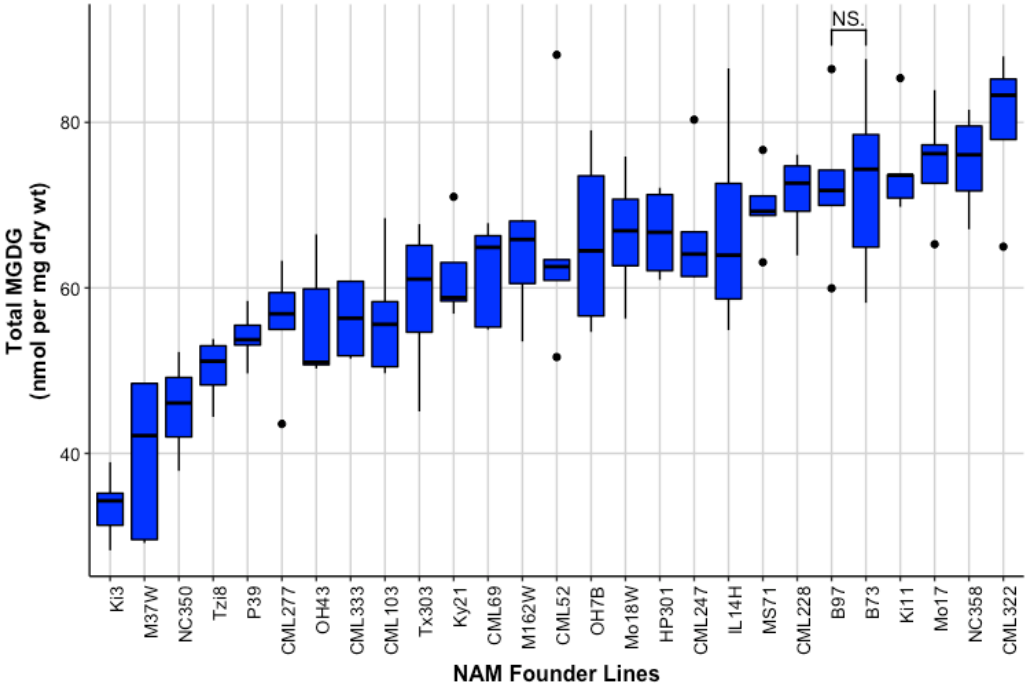

Figure 3.1 Average total MGDG lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Average MGDG(36:6) lipid content measured in the NAM founder inbred lines ranged between 22.64 to 68.26 nmol mg^{-1} dry wt (Table 3.3). Ki3 had the lowest level of average MGDG(36:6), whereas the temperate inbred NC358, developed in North Carolina, USA and part of the TZI subheterotic group, had the highest level of average MGDG(36:6). B73 levels of average MGDG(36:6) were 65.29 nmol mg^{-1} dry wt, which is significantly higher ($p<0.05$) than B97 levels, 50.18 nmol mg^{-1} dry wt (Figure 3.2).

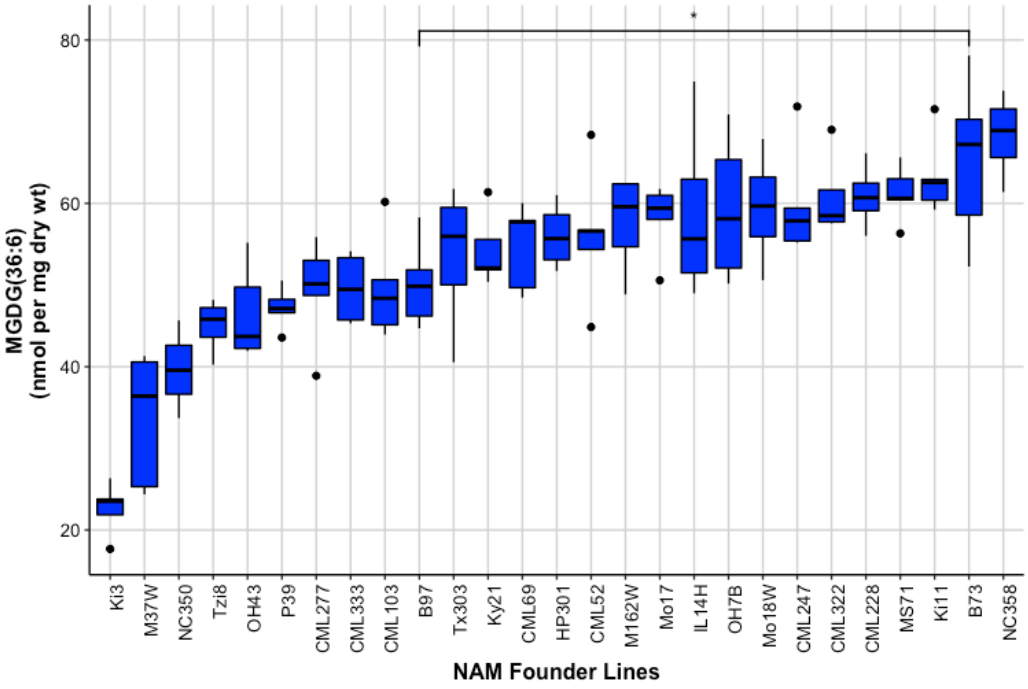

Figure 3.2 Average MGDG(36:6) lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Average MGDG(36:5) lipid species measured in the NAM founder inbred lines ranged between 2.54 to 17.42 nmol mg^{-1} dry wt (Table 3.3). The temperate inbred M162W, developed in South Africa and part of the NSS heterotic group, had the lowest level of average MGDG(36:5), whereas B97 had the highest levels of average MGDG(36:5) nmol mg^{-1} dry wt. B73 levels of average MGDG(36:5) at 5.14 nmol mg^{-1} dry wt were significantly lower ($p<0.01$) than B97 (Figure 3.3).

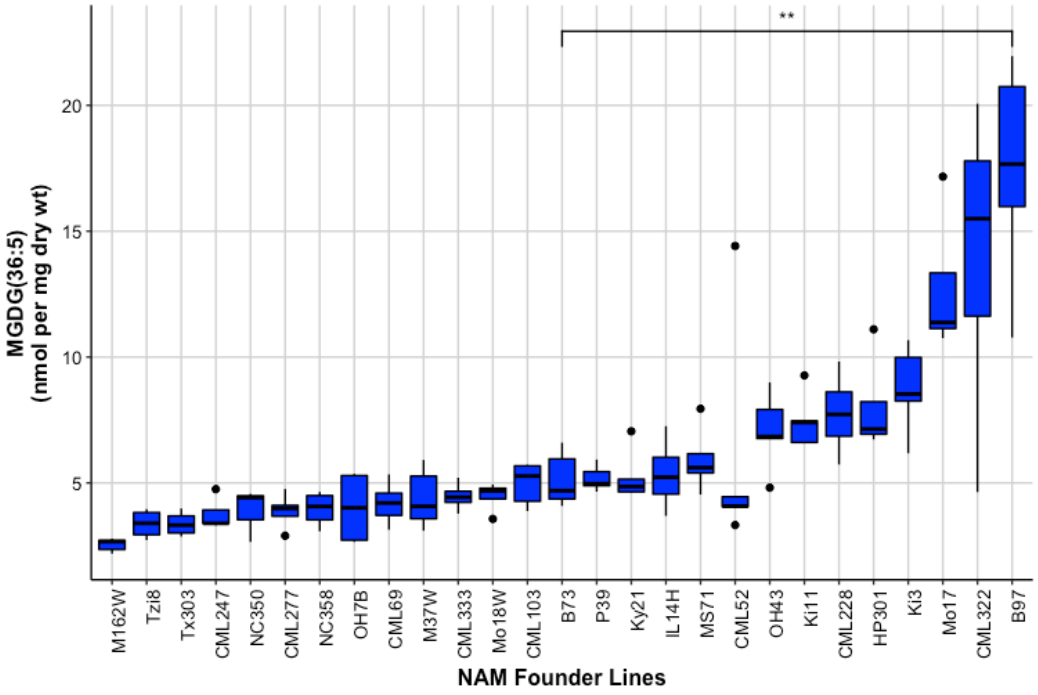

Figure 3.3 Average MGDG(36:5) lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Table 3.3 Selected MGDG lipid data analyzed in the maize NAM founder inbred lines grown at Purdue University ACRE in 2011. Inbred lines followed by a different lowercase letter are significantly different ($p<0.05$) according to Tukey HSD.

Inbred	Rep No.	Total MGDG		MGDG (36:6)		MGDG (36:5)	
		nmol $mg^{-1} \pm SD$	Tukey HSD	*nmol* $mg^{-1} \pm SD$	Tukey HSD	*nmol* $mg^{-1} \pm SD$	Tukey HSD
B73	5	72.73±11.53	abc	65.29±10.09	ab	5.14±1.09	de
B97	5	72.47±9.51	abc	50.18±5.35	bcde	17.42±4.41	a
CML52	5	65.34±13.60	abcde	56.19±8.38	abcde	6.07±4.68	de
CML69	5	61.85±6.25	abcde	54.76±5.30	abcde	4.20±0.84	de
CML103	5	56.51±7.58	bcdef	49.65±6.46	cde	4.97±0.84	de
CML228	4	71.33±5.37	abcd	60.89±4.16	abcd	7.75±1.72	cde
CML247	5	66.77±7.91	abcde	59.95±6.89	abcd	3.76±0.61	de
CML277	5	55.63±7.43	bcdef	49.33±6.45	cdef	3.89±0.68	de
CML322	4	79.88±10.21	a	60.89±5.46	abcd	13.92±6.67	ab
CML333	4	56.25±5.28	bcdef	49.60±4.66	cdef	4.46±0.58	de
HP301	4	66.63±5.73	abcde	56.02±4.20	abcde	8.03±2.06	cd
IL14H	4	67.33±13.88	abcde	58.80±11.52	abcd	5.35±1.50	de
Ki3	5	33.60±4.03	g	22.64±3.20	g	8.73±1.74	bcd
Ki11	5	74.65±6.22	ab	63.33±4.83	abc	7.47±1.09	de
Ky21	5	61.63±5.72	abcde	54.26±4.41	abcde	5.27±1.02	de
M162W	5	63.23±6.25	abcde	57.58±5.81	abcd	2.54±0.26	e
M37W	5	39.57±9.66	fg	33.59±8.21	fg	4.39±1.17	de
Mo17	5	75.07±6.82	ab	58.16±4.48	abcd	12.75±2.66	abc
Mo18W	4	66.49±8.19	abcde	59.46±7.24	abcd	4.47±0.61	de
MS71	5	69.78±4.88	abcd	61.20±3.45	abcd	5.93±1.27	de
NC350	3	45.41±7.21	efg	39.65±5.99	efg	3.88±1.06	de
NC358	4	75.19±6.41	ab	68.26±5.35	a	3.96±0.72	de
Oh43	5	55.67±7.25	bcdef	46.56±5.76	def	7.07±1.55	de
Oh7B	4	65.67±11.65	abcde	59.33±9.64	abcd	4.01±1.51	de
P39	5	54.08±3.23	cdef	47.22±2.54	def	5.18±0.51	de
Tx303	4	58.73±9.97	abcdef	53.56±9.37	abcde	3.38±0.51	de
Tzi8	4	50.13±4.22	defg	45.02±3.49	def	3.37±0.59	de

Average total digalactosyldiacylglycerol (DGDG) measured in the NAM founder inbred lines ranged from 12.89 to 32.45 nmol mg^{-1} dry wt (Table 3.4). Ki3 had the lowest level of average total DGDG, whereas B97 had the highest levels of average total DGDG. B73 had relatively similar levels of average total DGDG as B97 at 31.60 nmol mg^{-1} dry wt (Figure 3.4).

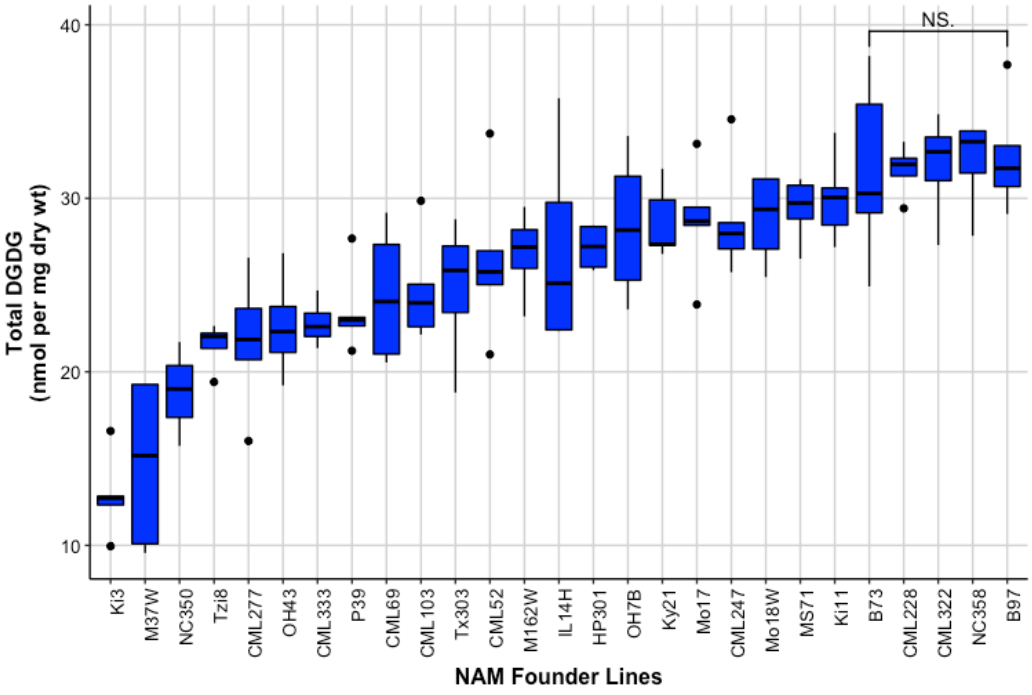

Figure 3.4 Average total DGDG lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Average DGDG(36:6) lipid species measured in the NAM founder inbred lines had a range between 8.01 to 22.75 nmol mg^{-1} dry wt (Table 3.4). Ki3 had the lowest level of average DGDG(36:6), whereas the tropical inbred CML228, developed in Mexico and part of the Suwan subheterotic group, had the highest level of DGDG(36:6). B73 and B97 had relatively similar levels of average DGDG(36:6) at 22.26 and 17.80 nmol mg^{-1} dry wt, respectively (Figure 3.5).

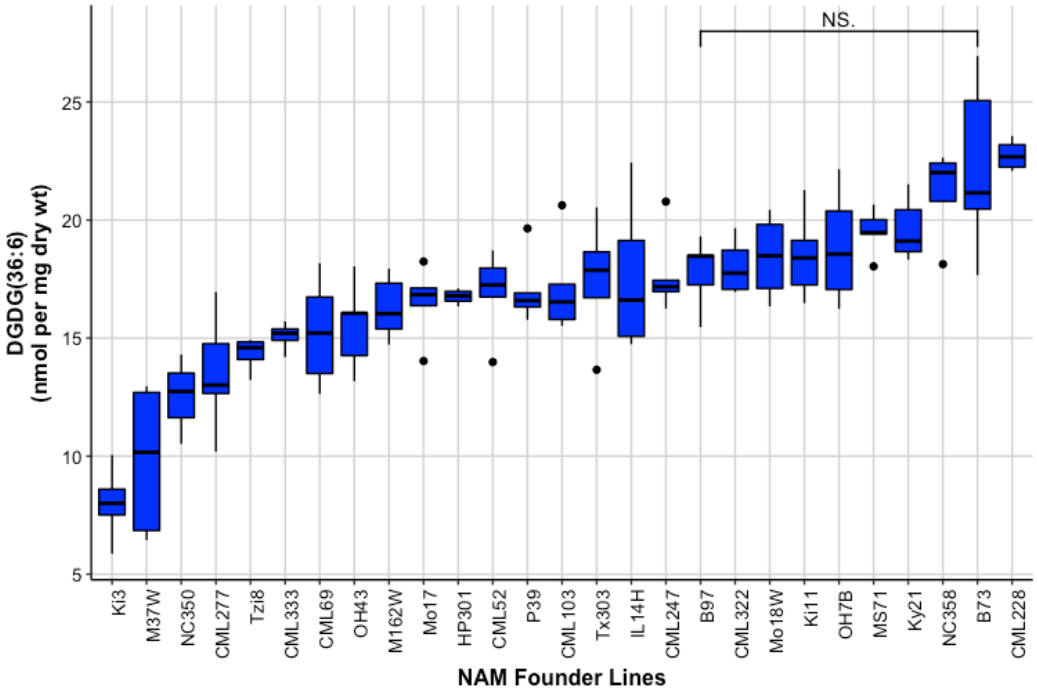

Figure 3.5 Average DGDG(36:6) lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Average DGDG(36:5) lipid species measured in the NAM founder inbred lines ranged between 0.66 to 5.83 nmol mg^{-1} dry wt (Table 3.4). M162W had the lowest level of average DGDG(36:5), whereas B97 had the highest level of average DGDG(36:5). B73 had significantly lower ($p<0.001$) levels of DGDG(36:5) at 1.23 nmol mg^{-1} dry wt than B97 (Figure 3.6).

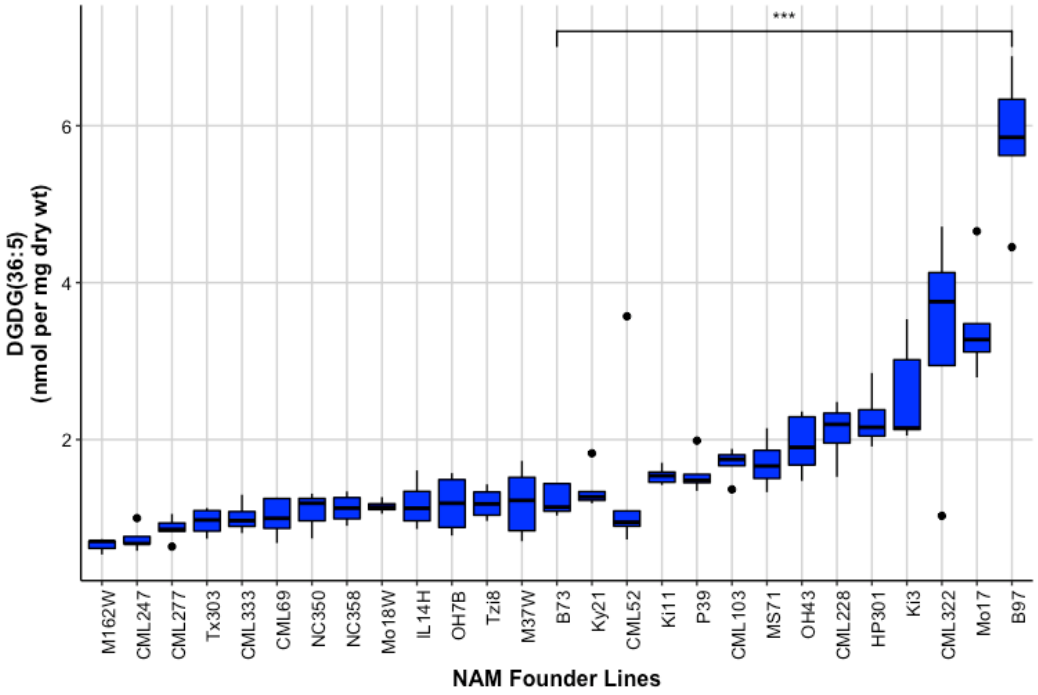

Figure 3.6 Average DGDG(36:5) lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Table 3.4 Selected DGDG lipid data analyzed in the maize NAM founder inbred lines grown at Purdue University ACRE in 2011. Inbred lines followed by a different lowercase letter are significantly different ($p<0.05$) according to Tukey HSD.

Inbred	Rep No.	Total DGDG		DGDG (36:6)		DGDG (36:5)	
		nmol mg^{-1} ± SD	Tukey HSD	*nmol mg^{-1} ± SD*	Tukey HSD	*nmol mg^{-1} ± SD*	Tukey HSD
B73	5	31.60±5.25	abc	22.26±3.72	a	1.23±0.20	de
B97	5	32.45±3.27	a	17.80±1.50	abcdef	5.83±0.91	a
CML52	5	26.50±4.62	abcdef	16.93±1.81	bcdef	1.45±1.19	de
CML69	5	24.43±3.80	abcdef	15.25±2.27	cdef	1.01±0.25	de
CML103	5	24.72±3.09	abcdef	17.15±2.06	bcdef	1.69±0.20	de
CML228	4	31.65±1.61	abc	22.75±0.69	a	2.10±0.41	cde
CML247	5	28.79±3.40	abcde	17.73±1.77	abcdef	0.74±0.16	de
CML277	5	21.76±3.90	efg	13.51±2.53	efg	0.86±0.15	de
CML322	4	31.88±3.23	abc	18.03±1.27	abcdef	3.31±1.60	ab
CML333	4	22.82±1.41	cdefg	15.08±0.64	cdef	1.01±0.21	de
HP301	4	27.18±1.40	abcdef	16.76±0.33	bcdef	2.27±0.41	cd
IL14H	4	27.08±6.32	abcdef	17.60±3.54	abcdef	1.18±0.33	de
Ki3	5	12.89±2.38	h	8.01±1.53	h	2.58±0.67	bcd
Ki11	5	30.01±2.50	abcd	18.51±1.85	abcd	1.54±0.11	de
Ky21	5	28.61±2.11	abcde	19.61±1.34	abc	1.37±0.26	de
M162W	5	26.81±2.41	abcdef	16.28±1.34	bcdef	0.66±0.08	e
M37W	5	14.68±4.74	gh	9.82±3.10	gh	1.20±0.44	de
Mo17	5	28.73±3.30	abcde	16.52±1.55	bcdef	3.46±0.71	abc
Mo18W	4	28.83±2.79	abcde	18.44±1.91	abcde	1.15±0.09	de
MS71	5	29.38±1.83	abcde	19.52±0.97	abc	1.70±0.32	de
NC350	3	18.82±3.00	fgh	12.52±1.90	fgh	1.08±0.30	de
NC358	4	32.07±2.88	ab	21.20±2.09	ab	1.12±0.20	de
Oh43	5	22.65±2.88	defg	15.52±1.87	cdef	1.94±0.38	de
Oh7B	4	28.38±4.51	abcdef	18.88±2.64	abcd	1.18±0.39	de
P39	5	23.54±2.44	bcdef	17.05±1.51	bcdef	1.56±0.25	de
Tx303	4	24.82±4.31	abcdef	17.49±2.85	abcdef	0.95±0.18	de
Tzi8	4	21.54±1.44	efg	14.33±0.77	defg	1.19±0.21	de

Average total leaf lipid measured in the NAM founder inbred lines ranged from 53.95 to 123.87 nmol mg^{-1} dry wt (Table 3.5). The Ki3 tropical inbred line, developed in Thailand and part of the Suwan subheterotic group, had the lowest levels of average total leaf lipid, whereas the tropical inbred CML322, developed in Mexico and part of the CML-early subheterotic group, had the highest levels of average total leaf lipid. The temperate inbred lines, B73 and B97, had similar levels of average total leaf lipids at 117.45 and 116.49, respectively (Figure 3.7).

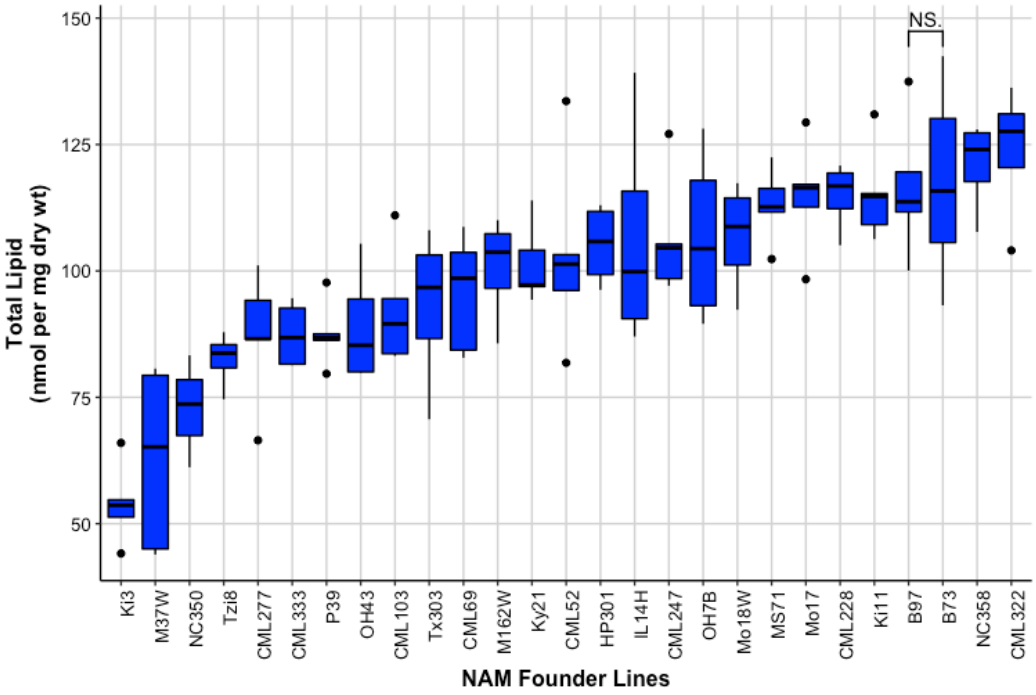

Figure 3.7 Average total leaf lipid content (nmol mg^{-1} dry wt) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

MGDG and DGDG Unsaturation Ratios for the NAM founder inbred lines were calculated as average nmol lipid(36:6) mg^{-1} dry wt/nmol lipid(36:5) mg^{-1} dry wt. The MGDG Unsaturation Ratio ranged from 2.69 to 22.73. Ki3 had the lowest MGDG Unsaturation Ratio, whereas M162W had the highest MGDG Unsaturation Ratio (Table 3.5) The B73 MGDG Unsaturation Ratio was 12.83, which is significantly different ($p<0.001$) from B97, which had the second lowest MGDG Unsaturation Ratio at 3.03 (Figure 3.8).

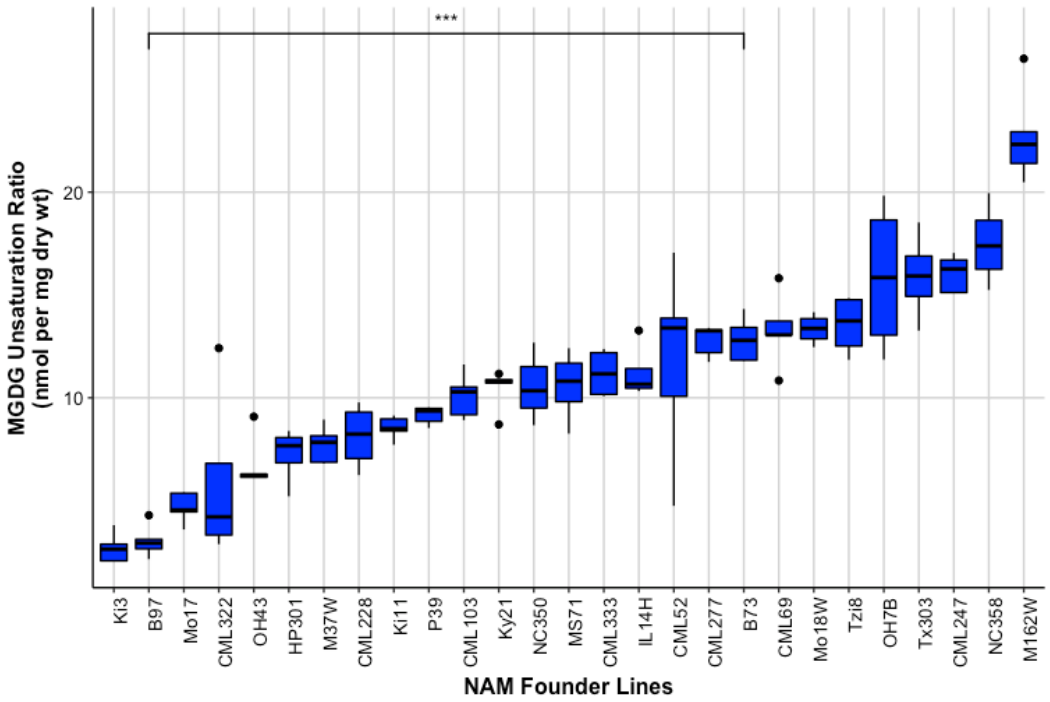

Figure 3.8 MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

The DGDG Unsaturation Ratio ranged from 3.11 to 24.98 (Table 3.5). B97 had the lowest DGDG Unsaturation Ratio, whereas M162W had the highest DGDG Unsaturation Ratio. The B73 DGDG Unsaturation Ratio was 18.13, which is significantly different ($p<0.001$) from B97 (Figure 3.9).

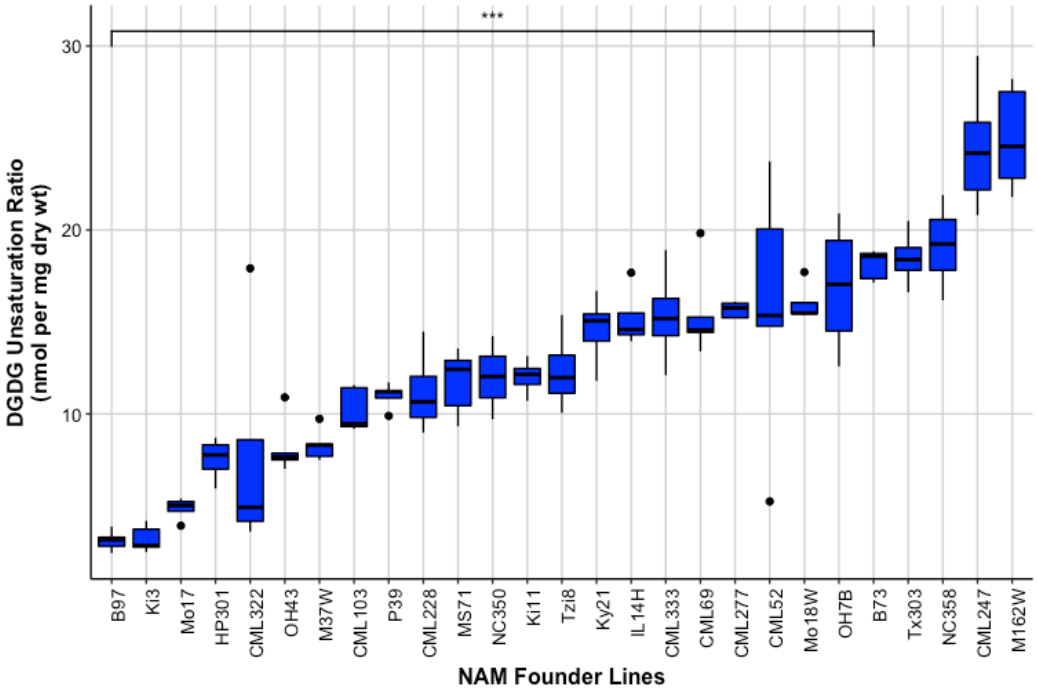

Figure 3.9 DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) measured in the NAM founder lines at Purdue ACRE in 2011. Statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Table 3. 5 MGDG and DGDG Unsaturation Ratios (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) measured in the maize NAM founder inbred lines grown at Purdue University ACRE in 2011. Inbred lines followed by a different lowercase letter are significantly different ($p<0.05$) according to Tukey HSD.

Inbred	Rep No.	Total Lipid		MGDG Unsaturation Ratio		DGDG Unsaturation Ratio	
		nmol $mg^{-1} \pm SD$	Tukey HSD	nmol (36:6) mg^{-1} / nmol (36:5) mg^{-1}	Tukey HSD	nmol (36:6) mg^{-1} / nmol (36:5) mg^{-1}	Tukey HSD
B73	5	117.45±19.48	abc	12.83	bcdef	18.13	bc
B97	5	116.49±13.69	abcd	3.03	k	3.11	h
CML52	5	103.22±18.93	abcdef	11.83	cdefg	15.83	bcde
CML69	5	95.62±11.58	abcdef	13.30	bcde	15.50	bcde
CML103	5	92.37±11.40	abcdefg	10.10	efghi	10.19	efg
CML228	4	114.87±7.01	abcde	8.12	fghij	11.19	defg
CML247	5	106.49±12.07	abcdef	16.05	bc	24.50	a
CML277	5	86.96±12.95	defg	12.78	bcdef	15.66	bcde
CML322	4	123.87±13.90	a	5.93	ijk	7.84	fgh
CML333	4	87.40±6.90	cdefg	11.20	defgh	15.35	bcde
HP301	4	105.22±8.23	abcdef	7.23	ghijk	7.55	fgh
IL14H	4	106.47±23.59	abcdef	11.23	defgh	15.20	bcde
Ki3	5	53.95±7.90	h	2.69	k	3.20	h
Ki11	5	115.29±9.55	abcd	8.54	fghij	12.01	cdef
Ky21	5	101.30±7.95	abcdef	10.45	efghi	14.59	bcde
M162W	5	100.66±9.80	abcdef	22.73	a	24.98	a
M37W	5	62.82±17.84	gh	7.72	ghij	8.31	fgh
Mo17	5	114.78±11.13	abcde	4.67	jk	4.86	gh
Mo18W	4	106.81±11.15	abcdef	13.34	bcde	16.02	bcde
MS71	5	113.08±7.35	abcde	10.59	efghi	11.73	defg
NC350	3	72.71±11.10	fgh	10.56	efghi	11.99	cdefg
NC358	4	120.94±9.36	ab	17.49	b	19.14	ab
Oh43	5	89.01±10.89	bcdefg	6.77	hijk	8.18	fgh
Oh7B	4	106.63±17.97	abcdef	15.85	bcd	16.90	bcd
P39	5	87.59±6.47	cdefg	9.15	efghij	10.99	defg
Tx303	4	93.04±16.33	abcdefg	15.91	bcd	18.47	abc
Tzi8	4	82.50±5.65	efgh	13.54	bcde	12.35	bcdef

3.4.1.3 High Temperature Field Experiments

High temperature field observations during the summers of 2013-2015 in Patancheru and Daulatabad, India show significant heat stress differentiation between the NAM founder inbred lines, B73 and B97 (Table 3.6). The three years of data add support to the increased high temperature tolerance of B97 compared to B73. In 2013, B73 produced zero ears, had zero grain field weight, showed an average of 88.20% leaf firing and an average of 35.83% tassel blast per plot. B97 had an average of 0.96 ears per plant, an average of 350.00 g of grain per plot, with zero leaf firing or tassel blast. The local checks, CL02450 and CML451, had an average of 0.85 ears per plant, an average of 200.00

g of grain per plot, no leaf firing or tassel blast and an average of 1.16 ears per plant, an average of 112.50 g of grain per plot, with zero leaf firing or tassel blast, respectively. In 2014, B73 produced an average 0.14 ears per plant, an average of 27.50 g of grain per plot, had an average of 12.50% leaf firing with zero tassel blast. B97 produced an average of 0.79 ears per plant, an average of 135.00 g of grain per plot with zero leaf firing or tassel blast. Three local checks were observed. CL02450 produced an average of 0.59 ears per plant, an average of 105.00 g of grain per plot with zero leaf firing or tassel blast. CML451 produced an average of 0.69 ears per plant, an average of 100.00 g of grain per plot, and zero leaf firing or tassel blast. CML472 produced an average of 0.42 ears per plant, an average of 40.00 g of grain per plot with an average of 47.40% leaf firing and zero tassel blast. In 2015, B73 showed an average of 89.74% tassel blast, but did not have average ear number, grain weight, or leaf firing data. B97 produced an average of 0.28 ears per plant, an average of 73.33 g of grain per plot, and showed zero leaf firing or tassel blast. Three local checks were observed with CML470 producing an average of 0.61 ears per plant, an average of 73.33 g of grain per plot with an average of 58.94% leaf firing and an average of 3.70% tassel blast. CML472 produced an average of 0.22 ears per plant, an average of 20.00 g of grain per plot, and had an average of 46.31% leaf firing and an average of 73.74% tassel blast. CML474 produced an average of 0.62 ears per plant, an average of 173.33 g of grain per plot with zero leaf firing or tassel blast.

Table 3. 6 Maize Nested-Association Mapping (NAM) founder inbred lines, B73 and B97, high-temperature field data observed during summer 2013-2015 in India. T-test statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Inbred	Type	Year	Rep No.	Ear Number (ears/row)	Field Weight (g)	Leaf Firing (%)	Tassel Blast (%)
B73	NAM Inbred	2013	4	0.00±0.00**	0.00±0.00 NS	88.20±9.12***	35.83±21.51*
B97	NAM Inbred	2013	4	0.96±0.35**	350.00±404.15 NS	0.00±0.00***	0.00±0.00*
CL02450	Local Check	2013	4	0.85±0.21	200.00±400.00	0.00±0.00	0.00±0.00
CML451	Local Check	2013	8	1.16±0.49	112.5±318.20	0.00±0.00	0.00±0.00
B73	NAM Inbred	2014	4	0.14±0.17*	27.50±48.56 NS	12.50±25.00NS	0.00±0.00 NS
B97	NAM Inbred	2014	4	0.79±0.36*	135.00±86.99 NS	0.00±0.00 NS	0.00±0.00 NS
CL02450	Local Check	2014	4	0.59±0.13	105.00±30.00	0.00±0.00	0.00±0.00
CML451	Local Check	2014	4	0.69±0.0.39	100.00±99.33	0.00±0.00	0.00±0.00
CML472	Local Check	2014	4	0.42±0.09	40.00±67.33	47.40±20.89	0.00±0.00
B73	NAM Inbred	2015	3	NA	NA	NA	89.74±17.77***
B97	NAM Inbred	2015	3	0.28±0.12	73.33±11.55	0.00±0.00	0.00±0.00***
CML470	Local Check	2015	3	0.61±0.27	73.33±23.09	58.94±20.27	3.70±6.41
CML472	Local Check	2015	3	0.22±NA	20.00±NA	46.31±7.43	73.74±23.54
CML474	Local Check	2015	3	0.62±0.27	173.33±102.63	0.00±0.00	0.00±0.00

3.4.2 NAM B73 x B97 RILs Experiment

3.4.2.1 Lipid Sampling Environmental Conditions

The NAM B73 x B97 RIL subpopulation was evaluated during the 2013 growing season at the Purdue University ACRE farm in West Lafayette, which had similar, to above average, monthly temperatures compared to the 50-year historical average (Table 3.7). May and June of 2013, during emergence and much of the vegetative growth, were modestly warmer, an average of 2.1 and 0.4 °C, respectively, than the 50-year average. The leaf sampling occurred at the V8 to V12 growth stages during the month of July which was 1.0 °C cooler on average than the 50-year historic average.

Table 3.7 Weather data for the 2013 and 2014 Purdue University ACRE growing seasons in West Lafayette, IN with a 50 year (1962-2013) historical average for comparison.

	Average Temperature (°C)			Maximum Temperature (°C)			Minimum Temperature (°C)		
	2013	2014	50 year	2013	2014	50 year	2013	2014	50 year
May	18.4	17.0	16.3	24.8	23.5	22.5	12.1	10.5	10.2
June	21.8	22.7	21.4	27.4	29.1	27.4	16.1	16.3	15.4
July	22.1	20.0	23.1	27.7	26.6	29.1	16.5	13.5	17.1
August	21.4	22.0	21.8	28.1	28.2	28.0	14.7	15.7	15.6
September	19.0	16.8	18.2	26.9	24.5	25.0	11.1	9.2	11.4

* Weather data downloaded from National Oceanic and Atmospheric Administration (NOAA) www.ncdc.noaa.gov

3.4.2.2 Lipid Phenotypic Results

Selected MGDG and DGDG lipid phenotype distributions of the maize NAM B73 x B97 RIL subpopulation grown at Purdue University ACRE farm in 2013 are found in Figures 3.10 and 3.11, respectively. The parental values, mean, maximum, and minimum are summarized in Table 3.8. Correlation of the MGDG and DGDG Unsaturation Ratio traits had an r^2 of 0.482 (Figure 3.12).

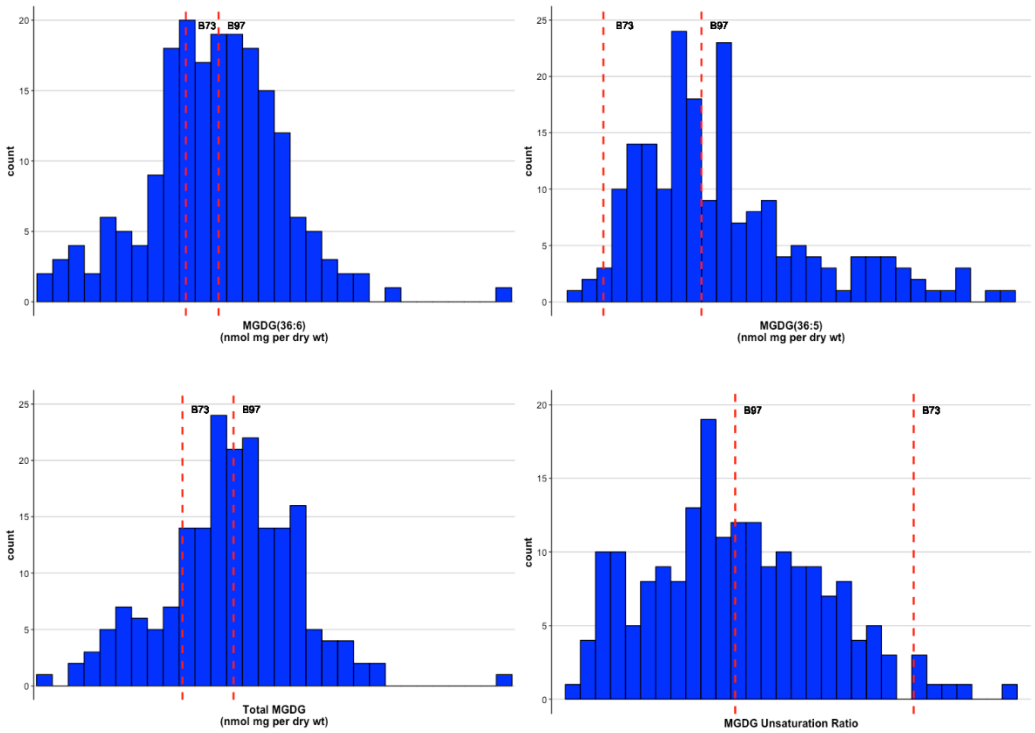

Figure 3. 10 Selected MGDG lipid phenotypic distributions in maize NAM B73 x B97 RIL subpopulation grown at Purdue ACRE in 2013. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

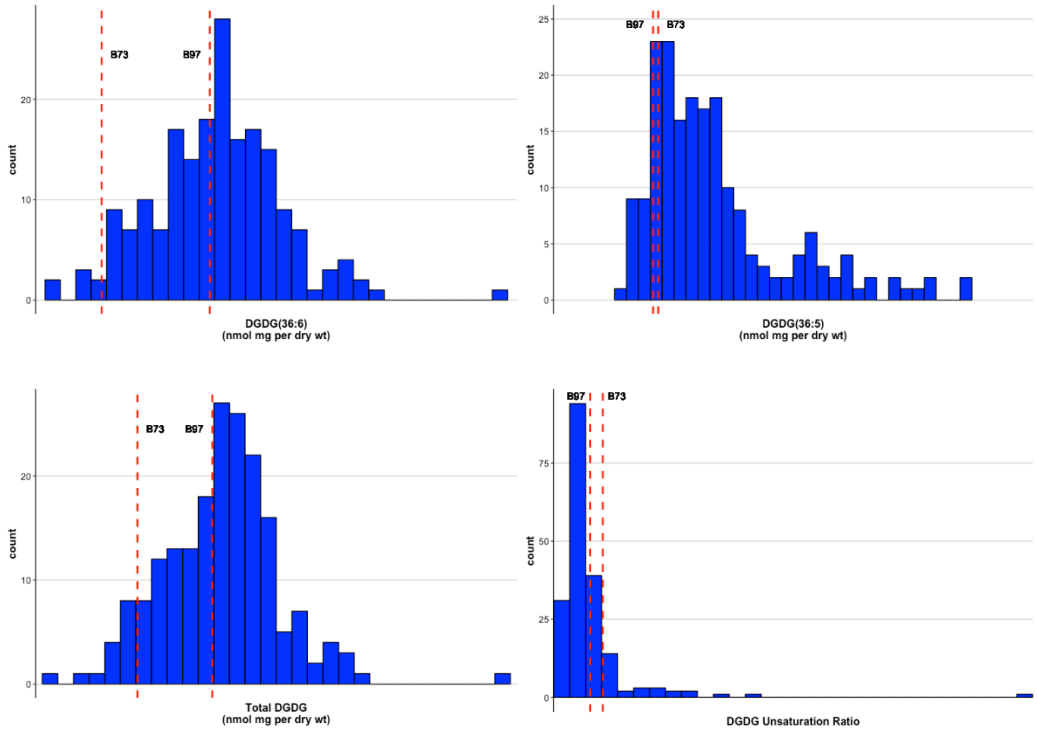

Figure 3. 11 Selected DGDG lipid phenotypic distributions in maize NAM B73 x B97 RIL subpopulation grown at Purdue ACRE in 2013. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

Table 3. 8 Summary of selected MGDG and DGDG lipid phenotypes for parental and NAM B73 x B97 RILs grown at Purdue University ACRE in 2013.

Phenotype	Parental Inbreds		Recombinant Inbreds		
	B73	B97	Mean	Maximum	Minimum
	nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight				
MGDG Unsaturation Ratio	18.63	10.92	10.92	22.75	3.88
DGDG Unsaturation Ratio	68.41	98.77	68.14	1122.29	9.81
	nmol mg^{-1} dry wt				
MGDG(36:6)	84.59±8.47	90.65±8.55	89.79±16.98	142.23	57.37
MGDG(36:5)	4.54±1.07	8.30±2.24	9.23±4.01	20.29	3.68
Total MGDG	94.71±7.81	105.34±11.50	104.78±19.62	162.63	66.94
DGDG(36:6)	25.00±2.58	31.96±5.85	32.44±5.63	50.28	21.49
DGDG(36:5)	0.37±0.21	0.32±0.03	0.93±0.73	3.43	0.03
Total DGDG	37.50±4.54	44.26±7.56	45.05±7.26	69.79	28.91

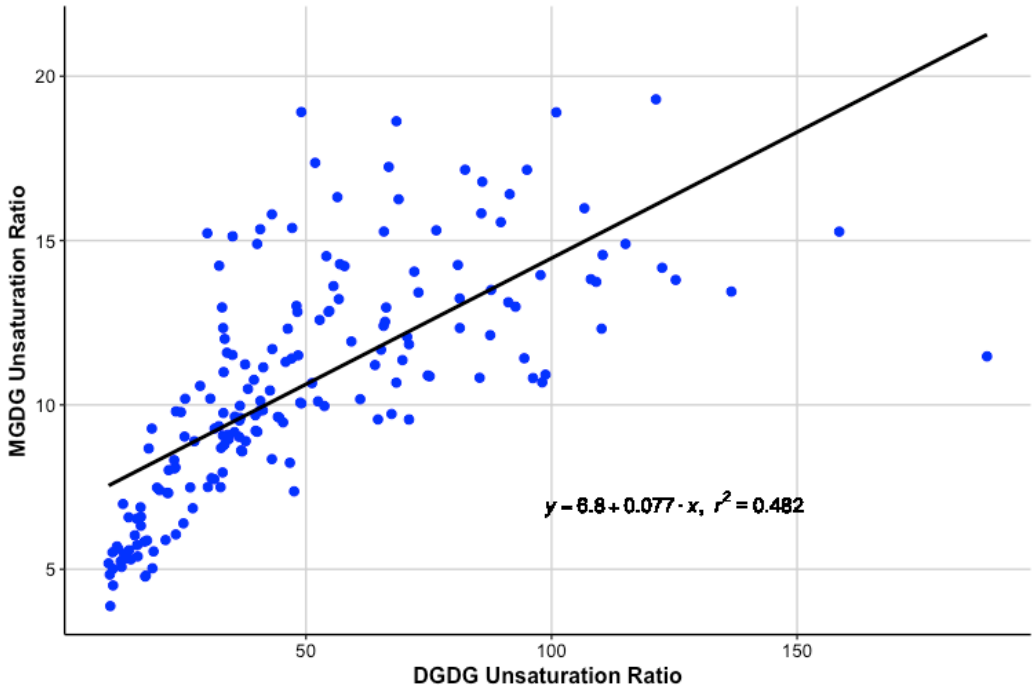

Figure 3.12 Pearson correlation of MGDG Unsaturation Ratio vs DGDG Unsaturation Ratio phenotypes for NAM B73 x B97 RILs grown at Purdue University ACRE in 2013. Unsaturation Ratios calculated as nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight.

3.4.2.3 B73 x B97 NAM RIL Subpopulation Genotyping

The B73 x B97 NAM RIL subpopulation QTL study for the MGDG and DGDG Unsaturation Ratio phenotypes was performed using 692 single nucleotide polymorphism (SNP) genetic markers assayed across the parental lines, B73 and B97, and 191 individual RILs. The genetic markers were dispersed across the genome with an average spacing of 3,663,360 base pairs between SNPs, with an average of 69 genetic markers analyzed per chromosome, resulting in an average coverage of 202,791,633 base pairs (Table 3.9).

Table 3.9 Summary of NAM B73 x B97 RIL physical map used for interval mapping QTL analysis.

Chr	Marker Number	Marker Coverage (bp)	Min Distance (bp)	Max Distance (bp)	Ave Distance (bp)
1	109	297,570,924	160	29,506,379	2,914,288
2	87	233,876,337	263	40,690,976	2,721,010
3	80	228,614,270	79	30,519,501	2,908,723
4	69	245,123,514	48	29,105,477	3,450,905
5	86	216,431,558	179	63,637,821	4,053,298
6	38	167,148,576	264	33,134,289	4,281,145
7	47	169,955,290	191	56,563,656	6,094,707
8	69	172,057,947	57	25,160,717	4,501,757
9	59	150,138,200	26	26,661,240	2,591,292
10	48	146,999,711	217	19,427,785	3,116,470
Average	69	202,791,633	148	35,440,784	3,663,360
Total	692	2,027,916,327			

3.4.2.4 B73 x B97 NAM RIL Subpopulation QTL Analysis

A total of 191 B73 x B97 RILs were used to evaluate the genetic architecture of the MGDG and DGDG Unsaturation Ratio phenotypic traits at Purdue University ACRE in 2013. Interval Mapping (IM) was used for QTL analysis with a total of 3 major QTL identified for both the MGDG and DGDG Unsaturation Ratio traits. One major QTL on chromosome 1 and two QTL, one minor and one major, on chromosome 9 were identified for these traits (Figures 3.13 and 3.14).

MGDG Unsaturation Ratio QTL analyses are summarized in Table 3.10. The major QTL identified on chromosome 1 has a peak marker (PZA00240.6), with a significance LOD score of 12.92, and physical position at base pair 41,231,748. This QTL explains 46.4% of the phenotypic variation with an additive effect of 1.96 from the B97 allele. The first QTL identified on chromosome 9 has a peak marker (PZA01861.1) with a significance LOD score of 6.81 and physical position at base pair 85,740,054. This QTL explains 28.0% of the phenotypic variation with an additive effect of 1.50 from the B97 allele. The second major QTL on chromosome 9 has a peak marker (PZA01096.1) with a significance LOD score of 11.76 and physical position at base pair 133,450,713. This QTL explains 41.2% of the phenotypic variation with an additive effect of 1.82 from the B97 allele.

DGDG Unsaturation Ratio QTL analyses are summarized in Table 3.10. The major QTL identified on chromosome 1 has a peak marker (PZA03742.1), with a significance LOD score of 4.36 and physical position at base pair 44,535,423. This QTL explains 19.0% of the phenotypic variation with an additive effect of 0.32 from the B97 allele. The first QTL identified on chromosome 9 has a peak marker (PZA03596.1) with a significance LOD score of 2.68 and located at base pair 90,436,248. This QTL explains

12.1% of the phenotypic variation with an additive effect of 0.25 from the B97 allele. The second major QTL on chromosome 9 has a peak marker (PHM1766.1), with a significance LOD score of 3.64 and physical position at base pair 136,401,627. This QTL explains 16.1% of the phenotypic variation with an additive effect of 0.29 from the B97 allele.

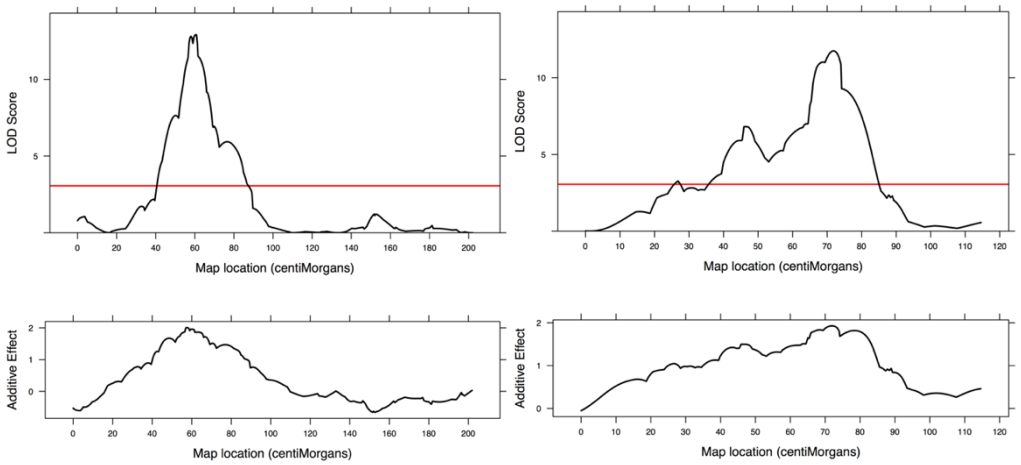

Figure 3.13 Graphical interval mapping results for MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) in the maize NAM B73 x B97 RIL subpopulation at Purdue University ACRE in 2013. Global LOD threshold is 3.06. Significant QTL identified on chromosome 1 (left) and chromosome 9 (right).

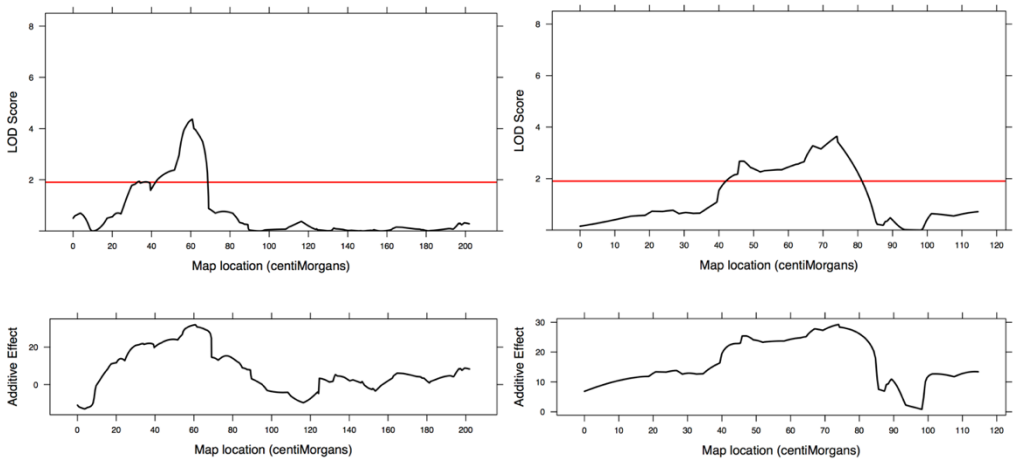

Figure 3.14 Graphical interval mapping results for DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) in the maize NAM B73 x B97 RIL subpopulation at Purdue University ACRE in 2013. Global LOD threshold is 1.90. Significant QTL identified on chromosome 1 (left) and chromosome 9 (right).

Table 3.10 Summary of interval mapping QTL results for MGDG and DGDG Unsaturation Ratios (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) in NAM B73 x B97 RILs grown at Purdue University ACRE in 2013.
†Additive effect of B97 allele

Chromosome	Peak Marker	Physical Position (bp)	LOD	Additive Effect†	R^2
MGDG Unsaturation Ratio (Global LOD Significance Threshold = 3.06)					
1	PZA00240.6	41,231,748	12.92	1.96	0.464
9	PZA01861.1	85,740,054	6.81	1.50	0.280
9	PZA01096.1	133,450,713	11.76	1.82	0.412
DGDG Unsaturation Ratio (Global LOD Significance Threshold = 1.90)					
1	PZA03742.1	44,535,423	4.36	31.91	0.190
9	PZA03596.1	90,436,248	2.68	25.41	0.121
9	PHM1766.1	136,401,627	3.64	29.20	0.161

3.4.2.5High Temperature Field Experiments

High temperature field observations during the summers of 2014-2015 in Daulatabad, India show heat stress differentiation between the B73 x B97 NAM RILs subpopulation (Table 3.11). The two years data indicates B97 has increased high temperature tolerance compared to B73 when comparing the agronomic traits, grain weight and ear number, to assess general plant health; however, a statistical significance was not observed. In summary, B73 produced an average of 0.57 ears per plant and yielded 178.72 g of grain per plot, whereas B97 produced an average of 0.61 ears per plant and yielded 191.03 g of grain per plot. The grain per plot average for the population was 154.94 g, with a maximum of 271.31 g and a minimum of 111.46 g. On average, the population had 0.56 ears/plant, with a maximum of 0.72 and minimum of 0.47.

There is a weak negative linear relationship between the MGDG and DGDG Unsaturation ratios analyzed in 2013 at Purdue ACRE with grain weight and ear number observed in the high temperature environments in 2014-2015 (Figure 3.15). The MGDG Unsaturation Ratio has a Pearson correlation with both grain weight and ear number of -

0.05. The DGDG Unsaturation Ratio has a correlation with grain weight of -0.03 and with ear number of -0.06.

Table 3. 11 Summary of multi-year analysis of selected agronomic phenotypes for parental and NAM B73 x B97 RILs grown at Daulatabad, India in 2014-2015. Prediction±SE

Phenotype	Parental Inbreds		Recombinant Inbreds		
	B73	B97	Mean	Maximum	Minimum
Grain Weight (g)	178.72±43.71	191.03±43.70	154.94±61.56	271.31	111.46
Ear Number (ears/plot)	0.57±0.08	0.61±0.08	0.56±0.11	0.72	0.47

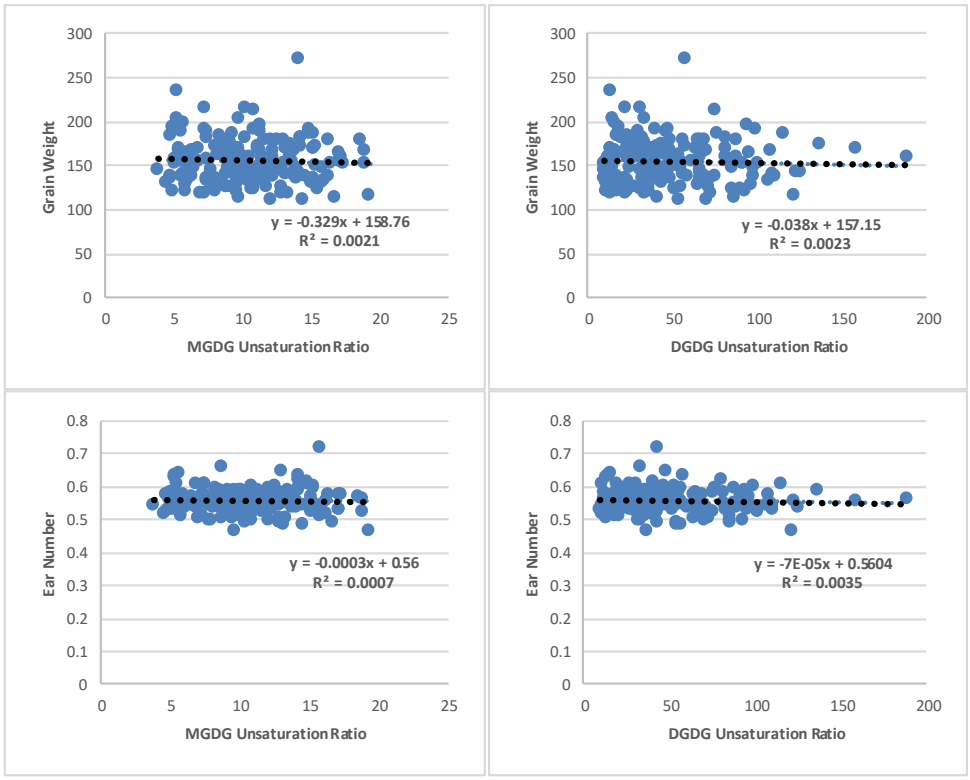

Figure 3. 15 Comparison of multi-year analysis of selected agronomic phenotypes for NAM B73 x B97 RILs grown at Daulatabad, India in 2014-2015 with MGDG and DGDG Unsaturation Ratios analyzed at Purdue University ACRE in 2013. Unsaturation Ratios calculated as nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight. Grain weight units are g/plot and ear number units are ears/plant.

3.4.3 Phenotypic Evaluation Over Time and Growth Stage

3.4.3.1 Diurnal Experiment

To acquire preliminary data and assess potential variability concerning time of day and temperature fluctuations, a study of selected MGDG and DGDG lipid traits in maize inbred B73 was performed over a 24-hour time period with samples taken every 3 hours. This analysis was performed at the Purdue University ACRE in 2013 and was intended to provide a snapshot of the diurnal response to guide future experimental design and study.

The MGDG(36:6), MGDG(36:5), Total MGDG, and MGDG Unsaturation Ratio traits follow similar trends over the 24 hour period (Figure 3.16). When temperatures are cooler during the early morning or later evening, trait values tend to increase. As daytime progresses and temperatures increase, trait values tend to decrease. Although these observed and predictable trends occur, only a few of the 3-hour time intervals analyzed are statistically different for each of the selected MGDG lipid phenotypes (Table 3.12). Only MGDG(36:5) was significantly different between the 4pm and 7pm samplings ($p<0.05$). MGDG(36:5) was also significantly different between the 7am – 1pm ($p<0.05$) and 7am – 4pm ($p<0.01$) sampling intervals.

The selected DGDG lipid traits exhibited more variability over the 24-hour period. DGDG(36:6) and Total DGDG showed the familiar trend of increased trait values during cooler early morning and later evening (Figure 3.17). The DGDG(36:5) trait values decreased as temperatures increased in the morning between 7am and 10am, increased as temperatures increased in the afternoon between 1pm and 4pm, decreased in the evening at 7pm as temperatures were increased, increased as temperatures decreased during the night at 10pm, decreased at late night between 1am and 4am as temperatures continued to decrease (Figure 3.17). The DGDG Unsaturation Ratio followed the trend of increased trait values during cooler early morning and later evening with the exception of an increase at 7pm, a time of increased temperature (Figure 3.17). The observed and predictable trends observed for the selected DGDG lipid phenotypes were statistically different for only a few of the 3-hour sampling intervals (Table 3.13). DGDG(36:6) was statistically different between the 7am – 10am ($p<0.01$) and 4pm – 7pm ($p<0.05$) time intervals. DGDG(36:5) was significantly different between the 7am – 10am and 7pm – 10pm ($p<0.05$) time intervals. Total DGDG was significantly different between the 7am – 10am ($p<0.01$) time interval. The DGDG Unsaturation Ratio was significantly different between the 1am – 4am and 4am – 7am ($p<0.05$) time intervals.

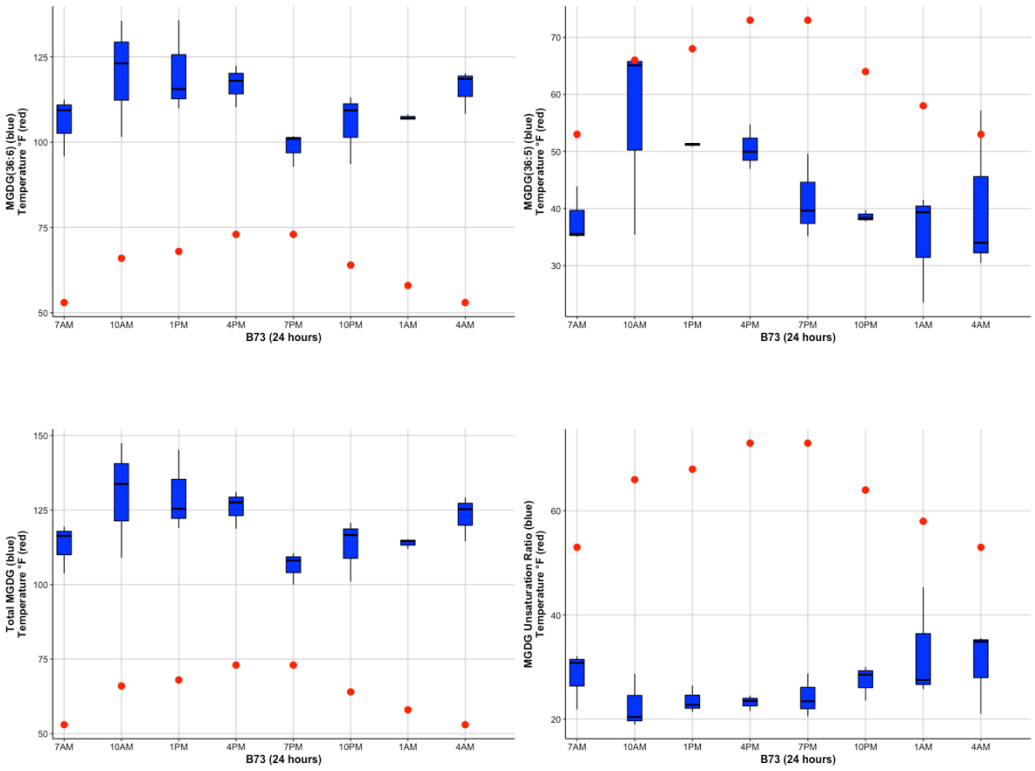

Figure 3. 16 Diurnal (24-hour) analysis of selected MGDG lipid phenotypes in maize inbred line B73 at Purdue University ACRE in 2013. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Boxplots: Lipids (nmol mg^{-1} dry wt). Dot plots: Temperature (°F).

Table 3. 12 Time interval comparisons of selected MGDG lipid phenotypes analyzed in maize inbred line B73 over a 24-hour period at Purdue University ACRE in 2013. Lipids measured in nmol mg^{-1} dry wt.
T-test statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Trait	Time Interval	Mean Difference	p-value
MGDG(36:6)	7am – 10am	14.17	0.1059
	10am – 1pm	-0.34	0.9698
	1pm – 4pm	3.53	0.7855
	4pm – 7pm	18.36	0.0919
	7pm – 10pm	6.83	0.4973
	10pm – 1am	-1.87	0.7841
	1am – 4am	-8.47	0.1805
	4am – 7am	9.79	0.3574
	7am – 1pm	14.51	0.1763
	7am – 4pm	10.98	0.2627
	7am – 7pm	7.38	0.3926
MGDG(36:5)	7am – 10am	1.75	0.3107
	10am – 1pm	0.45	0.6979
	1pm – 4pm	0.06	0.8161
	4pm – 7pm*	0.91	0.0464
	7pm – 10pm	-0.29	0.5306
	10pm – 1am	0.38	0.5405
	1am – 4am	-0.58	0.5217
	4am – 7am	0.24	0.7055
	7am – 1pm*	1.30	0.0505
	7am – 4pm**	1.24	0.0072
	7am – 7pm	-0.33	0.1869
Total MGDG	7am – 10am	16.90	0.1237
	10am – 1pm	0.16	0.9872
	1pm – 4pm	4.07	0.7585
	4pm – 7pm	19.61	0.0838
	7pm – 10pm	6.57	0.5339
	10pm – 1am	-1.12	0.8807
	1am – 4am	-9.14	0.2045
	4am – 7am	9.88	0.3785
	7am – 1pm	16.74	0.1435
	7am – 4pm	12.66	0.2190
	7am – 7pm	6.95	0.4286
MGDG Unsaturation Ratio	7am – 10am	-5.58	0.4638
	10am – 1pm	-0.86	0.8322
	1pm – 4pm	0.34	0.8678
	4pm – 7pm	-1.05	0.6695
	7pm – 10pm	3.13	0.2541
	10pm – 1am	-5.50	0.3851
	1am – 4am	2.37	0.7070
	4am – 7am	2.24	0.2822
	7am – 1pm	-4.73	0.2572
	7am – 4pm	-5.07	0.1783
	7am – 7pm	4.02	0.1531

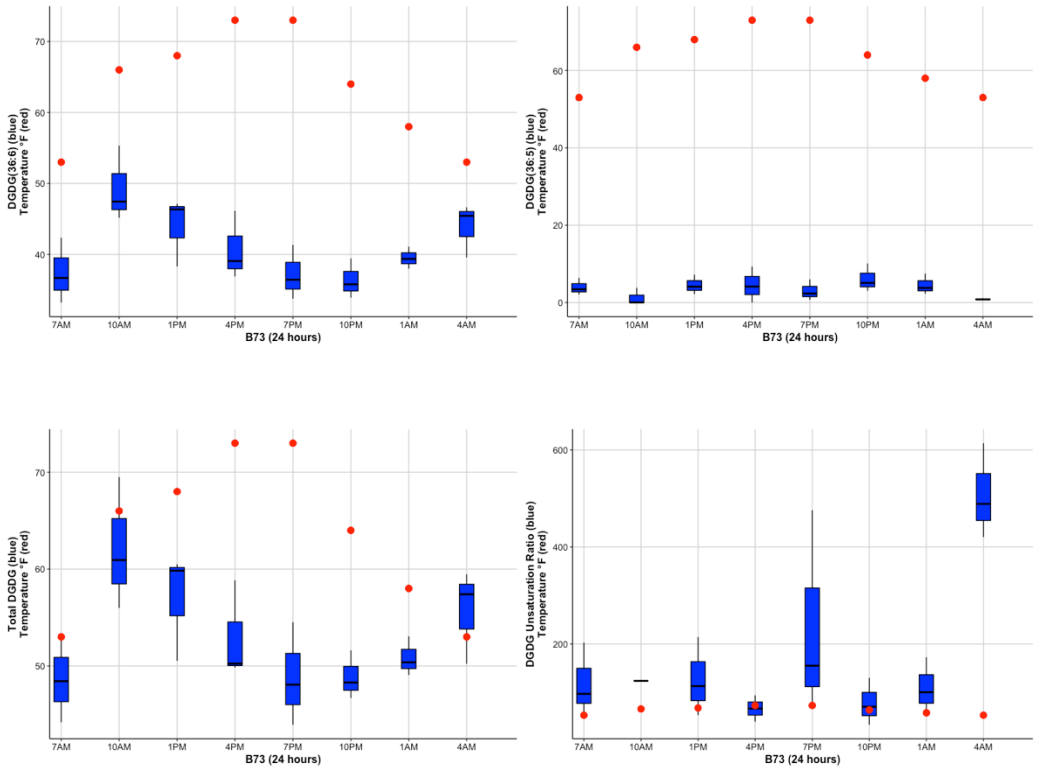

Figure 3. 17 Diurnal (24-hour) analysis of selected DGDG lipid phenotypes in maize inbred line B73 at Purdue University ACRE in 2013. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Boxplots: Lipids (nmol mg^{-1} dry wt). Dot plots: Temperature (°F).

Table 3.13 Time interval comparisons of selected DGDG lipid phenotypes analyzed in maize inbred line B73 over a 24-hour period at Purdue University ACRE in 2013. Lipids measured in nmol mg^{-1} dry wt.
T-test statistical significance between B73 and B97 inbred lines indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

Trait	Time Interval	Mean Difference	p-value
DGDG(36:6)	7am – 10am **	11.89	0.0029
	10am – 1pm	5.38	0.2810
	1pm – 4pm	3.22	0.2521
	4pm – 7pm*	3.51	0.0327
	7pm – 10pm	-0.81	0.8506
	10pm – 1am	-3.11	0.3397
	1am – 4am	-4.40	0.1786
	4am – 7am	6.47	0.3115
	7am – 1pm	6.51	0.2264
	7am – 4pm	3.28	0.5741
	7am – 7pm	0.24	0.9608
DGDG(36:5)	7am – 10am*	-0.27	0.0216
	10am – 1pm	-0.32	0.2762
	1pm – 4pm	0.00	0.9962
	4pm – 7pm	0.15	0.7615
	7pm – 10pm*	0.31	0.0298
	10pm – 1am	0.16	0.3099
	1am – 4am	0.36	0.1293
	4am – 7am	-0.31	0.1437
	7am – 1pm	0.05	0.6213
	7am – 4pm	0.05	0.8123
	7am – 7pm	0.09	0.7412
Total DGDG	7am – 10am**	13.49	0.0097
	10am – 1pm	5.19	0.3706
	1pm – 4pm	3.98	0.3329
	4pm – 7pm	4.13	0.0622
	7pm – 10pm	0.02	0.9964
	10pm – 1am	-1.97	0.5248
	1am – 4am	-4.86	0.1990
	4am – 7am	7.04	0.3224
	7am – 1pm	8.30	0.1707
	7am – 4pm	4.32	0.5027
	7am – 7pm	-0.19	0.9738
DGDG Unsaturation Ratio	7am – 10am	4.72	0.9231
	10am – 1pm	-2.92	0.9561
	1pm – 4pm	-59.99	0.3537
	4pm – 7pm	166.31	0.3103
	7pm – 10pm	-155.10	0.2485
	10pm – 1am	-31.28	0.5006
	1am – 4am*	-398.23	0.0215
	4am – 7am*	388.43	0.0443
	7am – 1pm	7.65	0.9103
	7am – 4pm	-52.34	0.3824
	7am – 7pm	-113.97	0.5319

3.4.3.2 Growth and Developmental Stages

A two-part study investigated MGDG and DGDG Unsaturation Ratios at a range of developmental stages in B73 and B97. Phenotypic data was collected and analyzed at the 4-leaf (4L), 8-leaf (8L), 12-leaf (12L), and reproductive (R) stages to provide insight into the effects of growth and developmental stages. The first analysis evaluated how the MGDG and DGDG Unsaturation Ratios compare between B73 and B97 at each developmental stage. The second analysis compared each developmental stage within B73 and B97.

For the first analysis, the MGDG Unsaturation Ratio trait was significantly different between B73 and B97 during the 4L, 8L, R ($p<0.01$) and 12L stages ($p<0.001$) (Figure 3.18). The DGDG Unsaturation Ratio was significantly different between B73 and B97 at the 4L, 8L, and 12L stages ($p<0.05$), whereas it was not found to be significantly different at the R stage (Figure 3.19).

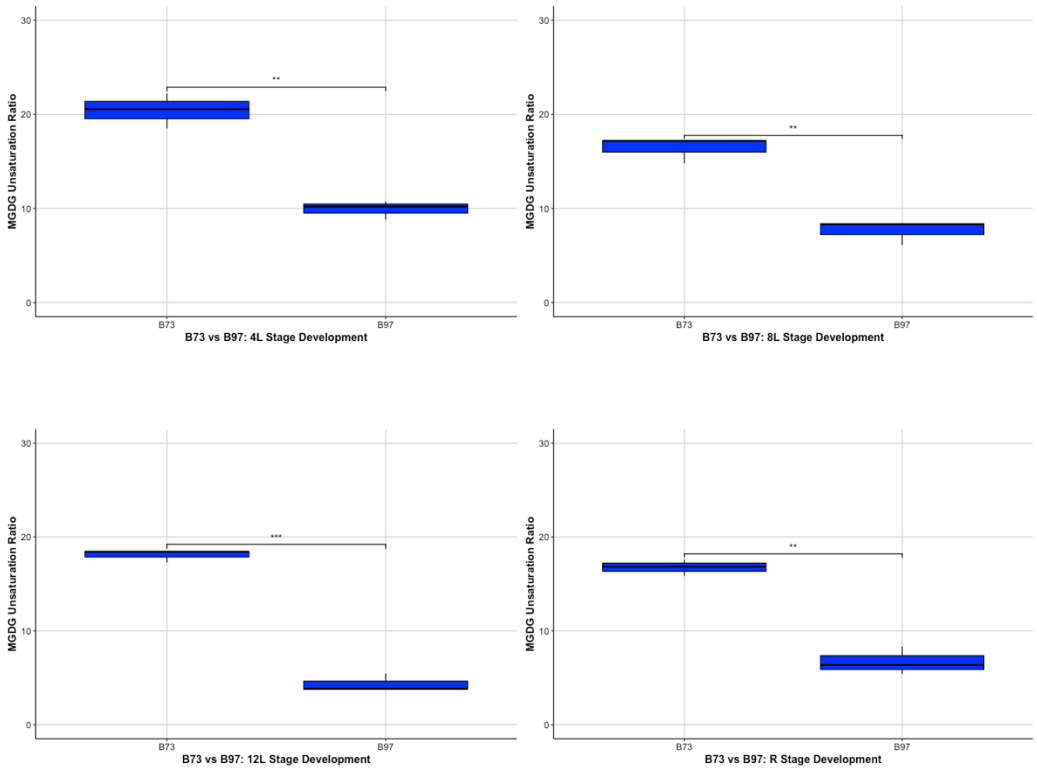

Figure 3.18 Leaf stage comparison between maize inbred lines B73 and B97 MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L Stage Development (top left), 8L Stage Development (top right), 12L Stage Development (bottom left), and R Stage Development (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

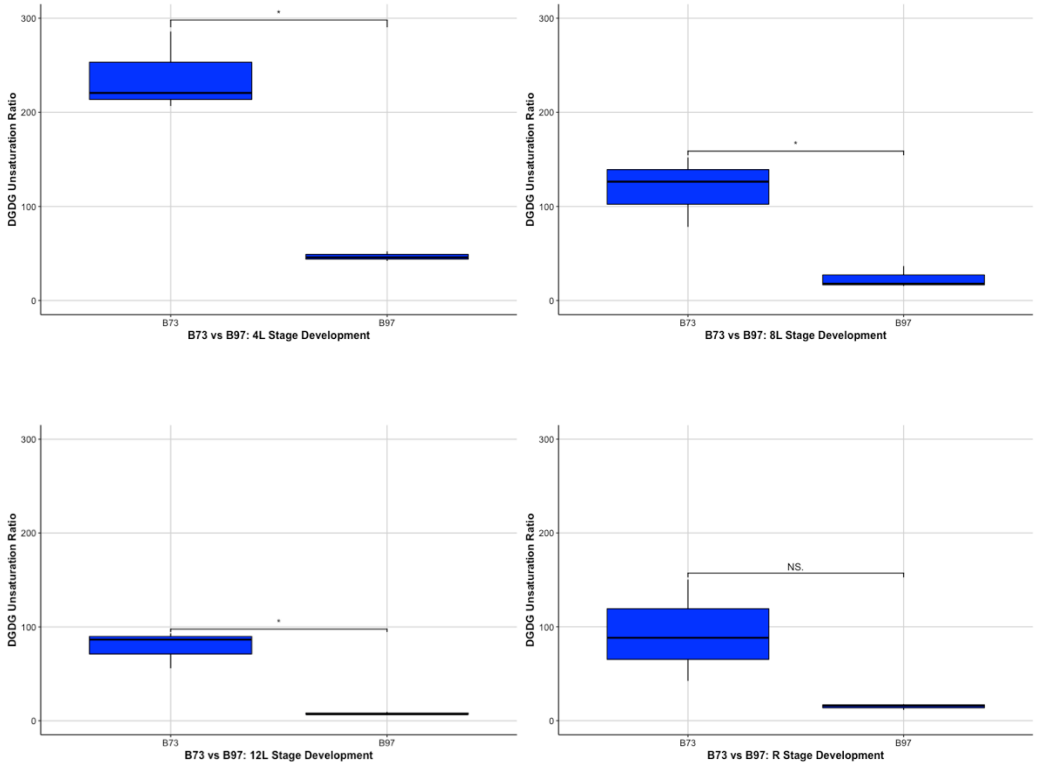

Figure 3.19 Leaf stage comparison between maize inbred lines B73 and B97 DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L Stage Development (top left), 8L Stage Development (top right), 12L Stage Development (bottom left), and R Stage Development (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

For the second analysis, the MGDG and DGDG Unsaturation Ratios were compared between each developmental stage in B73 and B97. The B73 MGDG Unsaturation Ratio was significantly different ($p<0.05$) only between 4L and 8L developmental stages, whereas there was no significant difference between other developmental stage comparisons (Figure 3.20). The B97 MGDG Unsaturation Ratio was significantly different ($p<0.01$) between 4L stage vs 12L stage and significantly different ($p<0.05$) between 4L stage vs R stage and 8L stage and 12L stage (Figure 3.21). No other developmental stage comparison resulted in a significant difference.

The B73 DGDG Unsaturation Ratio was significantly different ($p<0.05$) between 4L stage vs 8L stage, 4L stage vs 12L stage, and 4L stage vs R stage, whereas there was no significant difference between the later developmental stage comparisons (Figure 3.22). The B97 DGDG Unsaturation Ratio was significantly different ($p<0.01$) between 4L stage vs 12L stage and 4L stage vs R stage and significantly different ($p<0.05$) between 12L stage vs R stage (Figure 3.23). No other developmental stage comparison resulted in a significant difference.

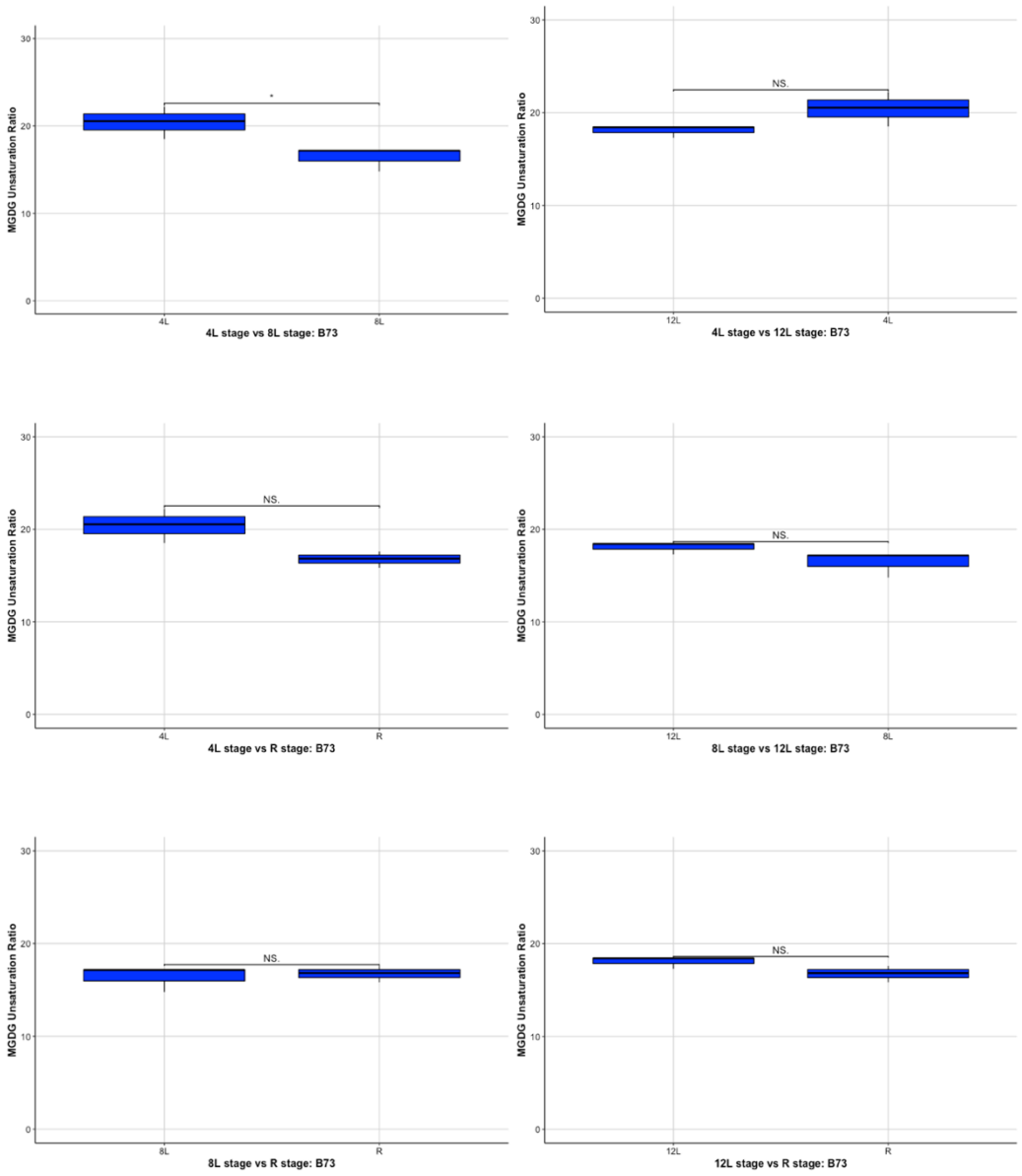

Figure 3.20 Leaf stage comparison of maize inbred line B73 MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L stage vs 8L stage (top left), 4L stage vs 8L stage (top right), 4L stage vs R stage (middle left), 8L stage vs 12L stage (middle right), 8L stage vs R stage (bottom left), and 12L stage vs R stage (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

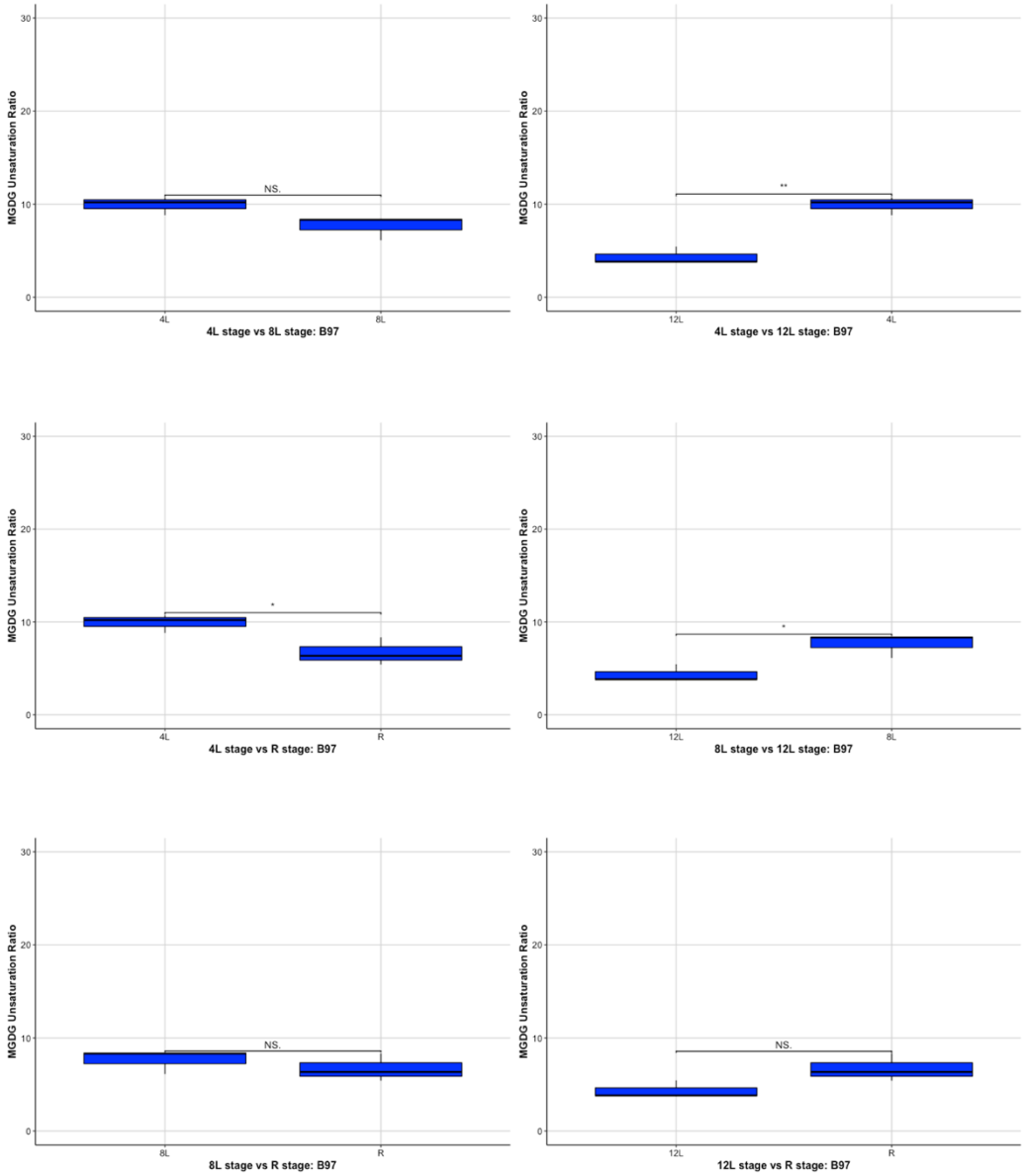

Figure 3.21 Leaf stage comparison of maize inbred line B97 MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L stage vs 8L stage (top left), 4L stage vs 8L stage (top right), 4L stage vs R stage (middle left), 8L stage vs 12L stage (middle right), 8L stage vs R stage (bottom left), and 12L stage vs R stage (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

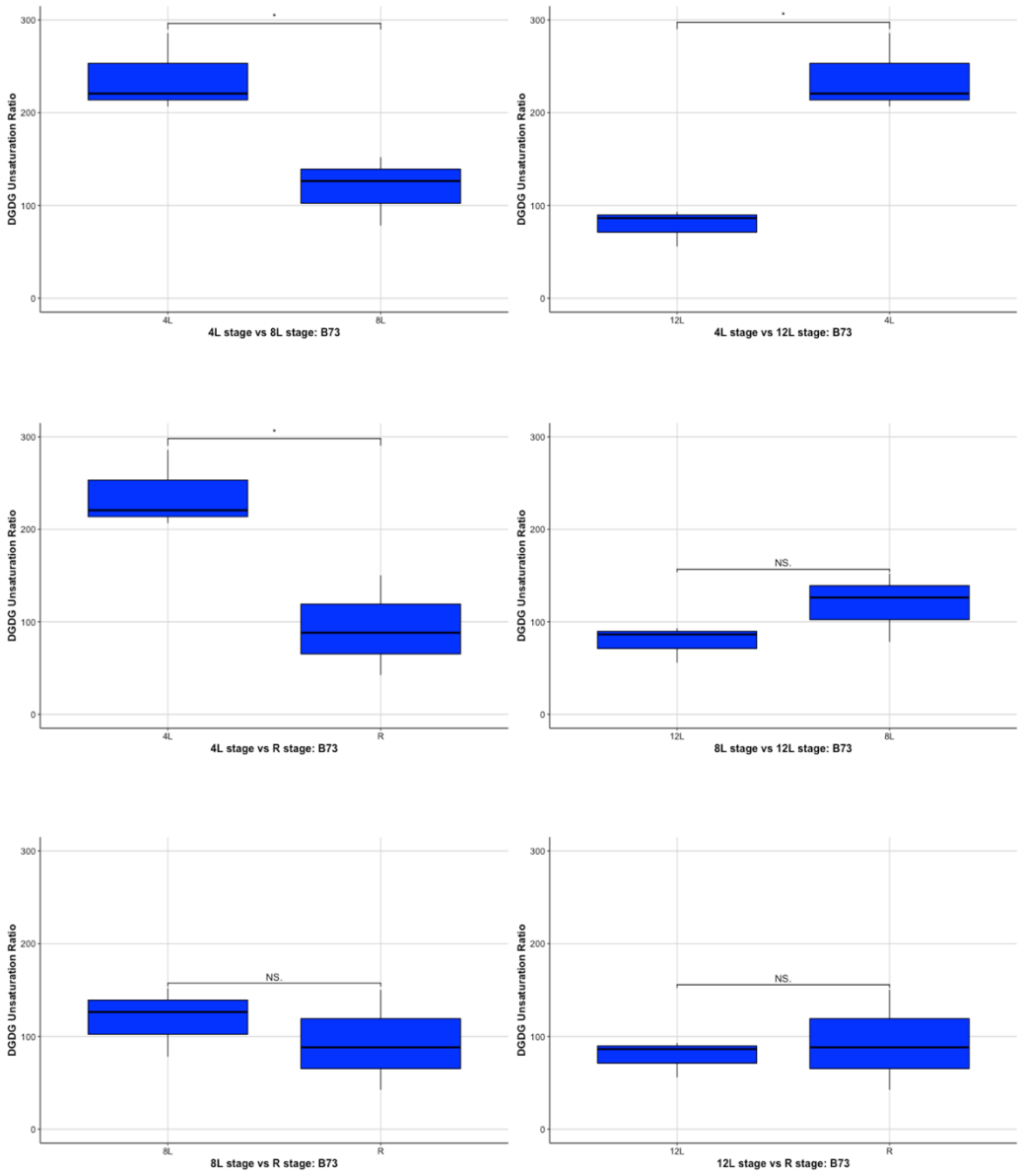

Figure 3.22 Leaf stage comparison of maize inbred line B73 DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L stage vs 8L stage (top left), 4L stage vs 8L stage (top right), 4L stage vs R stage (middle left), 8L stage vs 12L stage (middle right), 8L stage vs R stage (bottom left), and 12L stage vs R stage (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

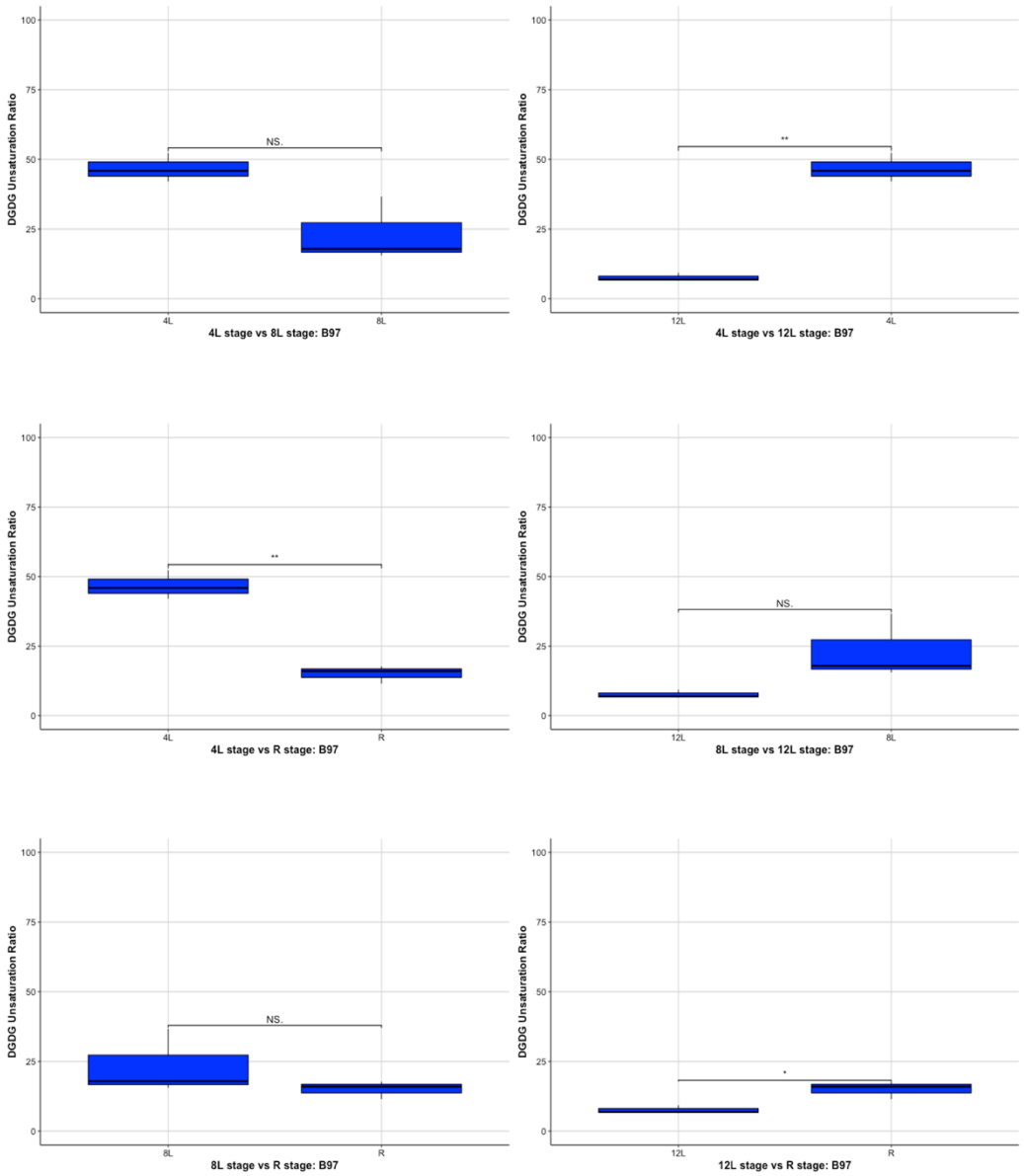

Figure 3.23 Leaf stage comparison of maize inbred line B97 DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) at Purdue University ACRE in 2013. 4L stage vs 8L stage (top left), 4L stage vs 8L stage (top right), 4L stage vs R stage (middle left), 8L stage vs 12L stage (middle right), 8L stage vs R stage (bottom left), and 12L stage vs R stage (bottom right). Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

3.5 Discussion

The 2011 and 2013 growing seasons at the Purdue University ACRE in West Lafayette, IN experienced average monthly temperatures comparable to the past 50-year monthly averages. Specifically, the sampling of the NAM founder lines for total leaf lipid analysis occurred during the month of July 2011, which was on average 2.8°C warmer than the 50-year average. The sampling of the B73 x B97 NAM RIL subpopulation for total leaf lipid analysis occurred during the month of July 2013, which saw an average of 1.0°C cooler than the 50-year average. These conditions were advantageous for the preliminary in field total leaf lipid analysis of the NAM founder lines and NAM RIL subpopulation where a baseline level in a non-stress environment was desired. Previous studies have shown the optimal temperature from planting to tassel emergence for most maize is between 29.2-32.4°C with a lethal maximum temperature between 43.1-48.9°C (Sánchez et al., 2014). Because the diverse set of NAM founder lines are evenly split between temperate and tropical adaptation, the modest increase in average monthly temperature may have been close to an optimal growing environment. Both B73 and B97 were developed in the US corn belt, where a 1°C cooler average temperature in July would still fall within the optimal or normal range for the growing environment.

Few, if any studies have explored the total leaf lipid content within a broad and diverse set of maize germplasm. For example, Chen et al. (2010) evaluated only two temperate inbred lines, B76 and B106, for leaf lipids associated with high temperature tolerance, concluding phosphatidic acid (PA) plays a role in tolerance. The diversity of the NAM founder inbred lines was reflected in the analysis of selected lipid phenotypic traits studied. The tropical inbred, CML322, which was developed in Mexico, had over

2x higher average total leaf lipid than the tropical Thailand inbred, Ki3. This same trend occurred when analyzing the major plastidic lipids between these lines, total MGDG and total DGDG, in which Ki3 had on average levels greater than 2x less than the inbreds with the highest levels. Future study should be undertaken to understand why a large difference in total leaf lipid and major plastidic lipids occurs and whether any physiological implications or environmental adaptation exists because of the difference.

The MGDG and DGDG Unsaturation Ratios had a greater than 8x difference between the inbred lines with the lowest and highest values for these traits. The MGDG and DGDG Unsaturation Ratios of B73, the common female inbred across all NAM RIL subpopulations, are among the highest of all NAM founder inbred lines. This provides multiple bi-parental subpopulations to evaluate the genetics involved in these traits. B73 and B97, the temperate parental lines of the B73 x B97 RIL subpopulation, were significantly different ($p<0.001$) for both MGDG and DGDG Unsaturation Ratios.

High temperature field environment observations during the summers of 2013-2015 in India provided evidence that B73 and B97 have differential tolerance to high temperature stress with B73 more susceptible and B97 more tolerant. The agronomic traits, ear number and grain weight, provide information on the relative health of the plants. In each year, B73 produced minimal to no ears or grain, whereas B97 consistently produced ears and grain weight, often within the range of the local checks. These results indicate that B97 can achieve reproductive success reliably in a high temperature environment, whereas B73 appears more often succumb to the stress and fail to reproduce. Leaf firing and tassel blast are associated as high temperature stress traits and depending on the severity, lead to developmental and/or reproductive issues. In each

year, B73 reliably showed significantly higher percentages of leaf firing and tassel blast than B97. In all three years, B97 showed zero leaf firing or tassel blast, which was on par with or better than the local checks. In general, these results provide a good indication of the overall increased high temperature stress tolerance of B97 over B73.

The B73 x B97 NAM RIL subpopulation evaluated during the 2014-2015 summers in the high temperature environment of Daulatabad, India provided additional evidence of the differential heat stress tolerance between B73 and B97 in both the inbred parental lines and RIL subpopulation. Using the agronomic traits, ear number and grain weight, to assess and compare the relative plant health, there was clear segregation within the subpopulation for differential heat stress tolerance. There was greater than 2x difference between the minimum and maximum grain weight measured and a 1.5x difference between the minimum and maximum ear number per plant. A comparison of the MGDG and DGDG Unsaturation Ratios analyzed at Purdue ACRE in 2013 with the agronomic traits observed in the high temperature environment in 2014-2015 showed a weak negative linear relationship. For the hypothesis that a smaller MGDG or DGDG Unsaturation Ratio is associated with increased heat stress tolerance to be true, a negative linear relationship with relative plant health traits such as ear number and grain weight would be expected. For example, RILs with the smallest MGDG and DGDG Unsaturation Ratios would be expected to have the highest heat stress tolerance, thus maintain plant health through reproduction, yielding the largest number of ears and grain. Based on the available data and weak relationships observed, no conclusion from this analysis can be made regarding the relationship of the MGDG and DGDG Unsaturation Ratios with heat stress tolerance. Additional studies are recommended in which the RIL

subpopulation is evaluated in multiple high temperature environments suitable for temperate germplasm over multiple years with the simultaneous analysis of the MGDG and DGDG Unsaturation Ratios.

QTL analysis of the MGDG and DGDG Unsaturation Ratios in the B73 x B97 NAM RIL subpopulation identified three significant QTL associated with each trait (Table 3.8). In each QTL, the B97 allele is associated with the decrease in the MGDG and DGDG Unsaturation Ratios. The major MGDG Unsaturation Ratio QTL peak marker on chromosome 1 was located just over 3.5 million base pairs upstream of the FAD8 gene (GRMZM2G074401) while the major QTL peak marker on chromosome 9 was located just over 4.3 million base pairs upstream of the FAD7 gene (GRMZM2G128971). The major DGDG Unsaturation Ratio QTL peak marker on chromosome 1 was located just over 250,000 base pairs upstream of the FAD8 gene (GRMZM2G074401) and the major QTL peak marker on chromosome 9 was located just over 1.4 million base pairs upstream of the FAD7 gene (GRMZM2G128971). In each case, the FAD8 and FAD7 genes are located within the significant QTL region of chromosomes 1 and 9, respectively, suggesting that phenotypic diversity of these lipid traits may be driven, in part, by allelic differences associated with these genes.

Evaluation of the selected MGDG and DGDG lipid phenotypes over time and growth stages, provided important insight into future experimental design strategies. The selected lipid traits analyzed followed trends in relation to the temperature and time of day. Increased levels of unsaturation were predicted during early morning and late evening as temperatures are cooler and decreased levels of unsaturation were predicted during the mid-day as temperatures are warmer. The fully unsaturated MGDG(36:6) and

DGDG(36:6) lipids decrease as temperatures reach their maximum between 4pm and 7pm, whereas the less unsaturated MGDG(36:5) and DGDG(36:5) lipids increase as temperatures approach their daily maximum. The MGDG and DGDG Unsaturation Ratios were lowest, reflecting a decrease in unsaturation, during mid-day as temperatures are elevated. Although these trends were observed, few statistical differences were found between the 3-hour sampling intervals. Because larger projects like the B73 x B97 NAM RIL subpopulation can take longer than 3 hours to sample, longer intervals were analyzed for statistical differences. MGDG(36:5) was the only lipid trait found to have significant difference between the longer sampling time intervals, 7am – 1pm ($p < 0.05$) and 7am – 4pm ($p < 0.01$). This study of selected MGDG and DGDG lipid traits reveals that the levels of unsaturation follow predictable trends over the course of a 24-hour period in relation to the environmental temperature. Although, these preliminary results indicate that few significant differences are detected as sampling takes place over the course of the day, steps in the experimental design should be taken to minimize the length of time between the beginning and end of a sampling experiment. These results will aid future studies and experimental design.

The assessment of MGDG and DGDG Unsaturation Ratio phenotypes at various growth and developmental stages of the maize inbred lines B73 and B97 provides important insight into understanding how these phenotypic traits are dependent upon developmental stage for expression. Previous experiments have shown that B73 and B97 have differential MGDG and DGDG Unsaturation Ratios, however these phenotypes were evaluated at a single growth point. This study provides evidence that comparison of MGDG and DGDG Unsaturation Ratios between B73 and B97 are statistically different

during early, 4L and 8L, as well as the later, 12L and R developmental stages (Figures 3.17 and 3.18). The exception was the DGDG Unsaturation Ratio at the R stage, however a relatively large sample variation for B73 may be causing the lack of significance. These results indicate that the MGDG and DGDG Unsaturation Ratios can differentiate between maize inbred lines at various stages during growth and development in field conditions.

The comparison of developmental stages within B73 and B97 suggests that the stage phenotypic sampling is significant and should be considered in the experiment design. The implication may be most relevant when evaluating MGDG and DGDG Unsaturation Ratio phenotypes within germplasm with diverse maturity backgrounds, as this may introduce confounding results. MGDG Unsaturation Ratio phenotypes were significantly different in B73 between the 4L and 8L stages ($p<0.05$) and in B97 between 4L and 12L ($p<0.01$), 4L and R, and 8L and R ($p<0.05$). The DGDG Unsaturation Ratio phenotypes were significantly different in B73 between 4L and 8L, 4L and 12L, and 4L and R ($p<0.05$) and in B97 between 4L and 12L, 4L and R ($p<0.01$), and 12L and R ($p<0.05$).

There are many additional developmental and environmental factors beyond temperature, time of day, and developmental stage that may influence lipid phenotypic traits like the MGDG and DGDG Unsaturation Ratios. Previous research has shown that light significantly affects lipid levels in *Arabidopsis thaliana* (Burgos et al., 2011). Future experiments should be designed taking these factors into consideration.

3.6 Conclusion

This study provides a framework in which future studies of environmental effects on leaf lipids in maize can be developed. The maize NAM founder inbred lines are shown to be as diverse in leaf lipid content as they are genotypically and geographically adapted. B73 and B97 were selected for genetic evaluation through linkage mapping analysis due to their observed differences in high temperature tolerance and differences in multiple MGDG and DGDG lipid traits of interest. Linkage mapping analysis of the MGDG and DGDG Unsaturation Ratios identified major QTL associated with ZmFAD7 and ZmFAD8 genes, providing evidence of the genetic inheritance of these unique lipid traits which could be used in developing improved germplasm.

As future research is planned to study how environmental changes effect leaf lipid content and the impact these changes have on the agronomic and physiological performance of maize, this study provides a foundation that will be influential in the planning process. The time of day sampling leaf lipids occurs was shown to have some significance when comparing B73 sampled over a 24-hour period, although this significance does not appear to be a major influence, it should be considered important when planning large projects in which sampling may take the majority of the day. The growth and developmental stages appear to have significance when comparing B73 and B97 leaf lipids across different stages. These two maize inbred lines were statistically distinguishable from each other based on the MGDG and DGDG Unsaturation Ratios across all developmental stages analyzed, apart from the DGDG Unsaturation Ratio at the R stage. However, this study indicates that sampling around the same developmental stage should be considered to best compare within inbred lines. This may be important when

experiments consist of germplasm with diverse maturity or environmental stress is changing developmental timelines. Results from this study provide preliminary justification for minimizing the time between the beginning and end of sampling and to sample at similar growth and development stages to minimize confounding results in field based total leaf lipid analysis.

CHAPTER 4. LEAF LIPID ANALYSIS OF MAJOR CEREAL CROPS AT COLD, NORMAL, AND HOT TEMPERATURES

4.1 Abstract

The continued development of cereal cultivars with improved yield potential and stress adaptation is fundamental to ensure global food supply (Reynolds et al., 2016). High temperatures and chilling temperatures are increasingly common environmental stressors, which depending on the time, duration, and intensity during the growing season, can lead to large yield losses. Many challenges exist in identifying functional genetic variability or important traits that can be translated into yield protection and improved adaptation to temperature stresses. The cereal crops that provide the majority of the world's calories, have origins of adaptation from diverse regions and environments which may provide insight into temperature adaptation mechanisms. Plastidic membrane lipids have been shown to play important roles in protecting plants during temperature stress. In this study, eight major cereal crops were investigated to measure the influence of chilling and high temperature stress on plant growth and leaf lipid content and composition. The results from this study suggest the ratios of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) containing acyl chains with total 36 carbons and 6 double bonds (36:6) compared to those with 36 carbons and 5 double bonds (36:5), here termed the MGDG and DGDG "Unsaturation Ratios" respond in predictable ways in chilling and heat stress environments and may differentiate between cool- and warm-season adapted cereal crops, providing evidence to their utility as lipid biomarkers for screening for temperature stress tolerant germplasm.

4.2 Introduction

Cereals make up a major proportion of the global crop production and demand (Rötter et al., 2015; Haberer et al., 2016; Henry et al., 2016). Improving the adaptability of society's most important cereal crops to environmental temperature fluctuations is of increasing interest as one component of a broader effort in securing the global food supply in an uncertain and changing climate. This is reflected in the breadth of research dedicated to studying future caloric needs of a growing population and how best to mitigate the negative effects or embrace the opportunities of a changing climate (Lobell et al., 2011; Lipper et al., 2014; Vervoort et al., 2014). Foundational genomic resources and technologies have been developed in the major cereal crops, benefitting many additional areas of agriculture and plant breeding (Goff et al., 2002; Yu et al., 2002; Paterson et al., 2009; Schnable et al., 2009; Kawahara et al., 2013; Mayer et al., 2012; Mayer et al., 2014; Law et al., 2015). This research is aiding the development of germplasm with improved abiotic stress tolerance (Chen et al., 2017; Muthusamy et al., 2017; Ohama et al., 2017). The protection of crop yield potential through improved tolerance to chilling and heat stress may help to alleviate some of the challenges associated with unpredictable temperature changes.

The primary cereals, maize, wheat, rice, sorghum, pearl millet, barley, oat, and rye, have origins of adaptation from diverse regions and environments (Table 4.1). They have a diverse range of temperature adaptation and can be broadly categorized into warm-season and cool-season. Wheat, barley, oat, and rye are broadly cool-season, whereas maize, sorghum, rice, and pearl millet are generally categorized as warm-season (Al-Khatib and Paulsen, 1999; May et al., 2007). This diversity of adaptation may present

insight into new mechanisms that could be leveraged to screen for functional diversity or locating genes associated with improved tolerance to chilling or heat stress. Many cereal crops have an inherent range of cold temperature tolerance that limits their geographical cultivation (Knight and Knight, 2012). However, tolerance to chilling stress has increased in importance as economically important crops are being adapted to higher latitudes, such as maize in Europe (Sobkowiak et al., 2016), or experience longer periods of cool temperatures during the early growing season (Revilla et al., 2000). Previous studies have shown mechanisms involved in chilling tolerance to include an adaptive photosynthetic apparatus and cell wall/membrane modifications (Sobkowiak et al., 2016). Specifically, proportions of certain saturated and unsaturated membrane fatty acids and their relationship to chilling tolerance have been investigated in many plant models (Miquel et al., 1993; Kodama et al., 1995; Somerville, 1995; Routaboul et al., 2000). Transgenic tobacco plants engineered with a fatty acid desaturase 7 (FAD7) gene from *Arabidopsis thaliana* resulted in increased levels of trienoic fatty acids and enhanced chilling tolerance (Kodama et al., 1994). The cloned FAD8 gene in *A. thaliana* provided the first evidence of a temperature-regulated gene which increases or maintains plastidic membrane trienoic fatty acids when plants are exposed to low temperatures (Gibson et al., 1994). Soon after, the maize FAD8 gene was cloned and showed similar activity at 5°C in response to chilling stress (Berberich et al., 1998).

Previous studies have estimated that regions where much of the world's cereal crops are produced have experienced an average annual temperature increase around 1°C over the previous 100 years, a trend that is expected to continue and possibly accelerate over the next 100 years (Zhao et al., 2017). Multiple crop-modelling studies estimate that

crop yields in the United States could drastically decrease by the end of the century due to high temperature stresses (Schlenker and Roberts, 2009; Schauberger et al., 2017). Heat stress associated yield loss is often attributed to stunted growth, developmental abnormalities, poor seed set, or plant death. Due to the sessile nature of plants, numerous adaptations have evolved to protect against high environmental temperature stress conditions, in which no single response mechanism is sufficient for adequate defense (Hong and Vierling, 2000; Iba, 2002; Kotak et al., 2007; Wahid et al., 2007; Bita and Gerats, 2013). Much research has been undertaken that investigates the role lipid membranes play in association with heat stress tolerance in plants. Various mechanisms have been proposed based on observed changes of membrane lipid composition and fatty acid saturation levels in response to heat stress which are thought to maintain membrane stability and cellular homeostasis (Welti et al, 2007). Complementary studies using transgenic tobacco plants with a silenced FAD7 gene showed a decrease in trienoic fatty acids which was associated with increased heat stress tolerance compared to wild-type plants (Murakami et al., 2000; Hiremath et al., 2017).

In this study, total leaf lipids of eight primary and economically important cereal crops with diverse geographical and environmental origins of adaptation were analyzed at normal, chilling, and heat stress conditions with the goal to identify potential lipid phenotypes that may be useful in screening for improved temperature tolerant germplasm.

4.3 Materials and Methods

4.3.1 Genetic Material

Two unique cultivars from eight different types of cereals were selected for a total of sixteen lines (Table 4.1). Seed sources were requested from the USDA Germplasm Resources Information Network (GRIN; www.ars-grin.gov).

Table 4. 1 List of cereals used in the leaf lipid analysis grown in controlled growth chamber temperature environments (1) cold – 20°C day/10°C night, (2) normal – 30°C day/20°C night, and (3) hot – 40°C day/30°C night.

Cereal	Scientific Name	PlantID	Growing Season	Photosynthetic Pathway
Barley	*Hordeum vulgare*	Morax	Cool-season	C_3
Barley	*Hordeum vulgare*	Steptoe		
Oat	*Avena sativa*	Kanota	Cool-season	C_3
Oat	*Avena sativa*	Ogle		
Rye	*Secale cereale*	Lo7	Cool-season	C_3
Rye	*Secale cereale*	Lo225		
Spring Wheat	*Triticum aestivum*	Chinese Spring	Cool-season	C_3
Spring Wheat	*Triticum aestivum*	Halberd		
Rice (Japonica)	*Oryza sativa subsp. Japonica*	Nipponbare	Warm-season	C_3
Rice (Indica)	*Oryza sativa subsp. Indica*	Kasalath		
Maize	*Zea mays*	B73	Warm-season	C_4
Maize	*Zea mays*	Mo17		
Pearl Millet	*Pennisetum glaucum*	ICMP85410	Warm-season	C_4
Pearl Millet	*Pennisetum glaucum*	Tift 454		
Sorghum	*Sorghum bicolor*	BTx623	Warm-season	C_4
Sorghum	*Sorghum bicolor*	IS3620C		

4.3.2 Experimental Design

Two lines from eight different cereal crops were grown in the Purdue University Horticulture Plant Growth Facility in 2015. Three sets of three replicates of each line were grown in three individual growth chamber experiments using random complete block design (RCBD) (Fernandez, 2007). Each 10 cm diameter plastic pot containing Metro-Mix® 510 (The Scotts Company, Marysville, OH) was planted with three seeds, which were thinned to one individual per pot after emergence. The initial growth chamber environments were set at 30°C day and 20°C night with a 12-hour light cycle. After two weeks of growth, each individual growth chamber was set to a final environmental designation: (1) Normal – 30°C day and 20°C night, (2) Cold – 20°C day and 10°C night, and (3) Hot – 40°C day and 30°C night. After three weeks, samples were taken for total leaf lipid analysis and fresh and dry biomass weights were measured in grams (g).

4.3.3 Lipid Extraction

Six leaf punches from single plants were immediately placed in individual 20 mL glass vials with PTFE-lined screw caps (National Scientific, Rockwood, TN, USA) containing 3 mL of 75°C isopropanol with 0.01% butylated hydroxytoluene (BHT) and heated in a water bath for fifteen minutes to deactivate phospholipase C enzymes. Samples were stored at -80°C until processed for lipid extraction. Total leaf lipids were extracted according to the procedure described by Welti et al. (2002). Final lipid extracts were dried using a Savant Concentrator (Thermo Fisher Scientific Inc, Waltham, MA, USA). The lipid extracts were shipped overnight on dry ice in 2 mL glass vials with polytetrafluoroethylene (PTFE)-lined screw caps (National Scientific, Rockwood, TN,

USA) to the Kansas Lipidomics Research Center (KLRC) at Kansas State University. Total leaf lipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to KLRC developed protocol (Xiao et al., 2010).

4.3.4 Statistical Analysis

Inferential statistics for phenotypic means comparison such as Tukey honestly significant difference (HSD) and t-tests were calculated using the R package agricolae: statistical procedures for agricultural research (de Mendiburu, 2017).

4.4 Results

4.4.1 Cereal Lipid Diversity at Normal Temperature

The normal temperature environment (30°C day/20°C night) was used as the baseline for evaluating changes in selected leaf lipid phenotypes in cold (20°C day/10°C night) and hot (40°C day/30°C night) environments. Average total lipid amounts evaluated at the normal temperature environment of the various cereals were highly diverse (Figure 4.1). Rice had the lowest average total lipid, with Kasalath (Indica) having a lower amount than Nipponbare (Japonica), 84.36 and 100.48 nmols mg^{-1} dry wt, respectively. In maize, average total lipids were significantly higher in B73 than Mo17 ($p < 0.05$) at 174.71 and 127.98 nmols mg^{-1} dry wt, respectively (Table 4.2). Pearl Millet had average total lipid levels of 142.19 and 191.96 nmols mg^{-1} dry wt for Tift454 and ICMP85410, respectively. Average total lipid levels in Sorghum were 185.62 and 232.03 nmols mg^{-1} dry wt for IS3620C and BTx623, respectively. In barley, average total lipid levels were 212.28 and 266.41 nmols mg^{-1} dry wt for Morex and Steptoe, respectively. Spring wheat average total lipid levels were 222.02 and 243.85 nmols mg^{-1} dry wt for Chinese Spring and Halberd, respectively. Average total lipids in Oat were 232.17 and

256.87 nmols mg^{-1} dry wt for Ogle and Kanota, respectively. In Rye, average total lipids were 258.98 and 272.54 nmols mg^{-1} dry wt for Lo225 and Lo7, respectively.

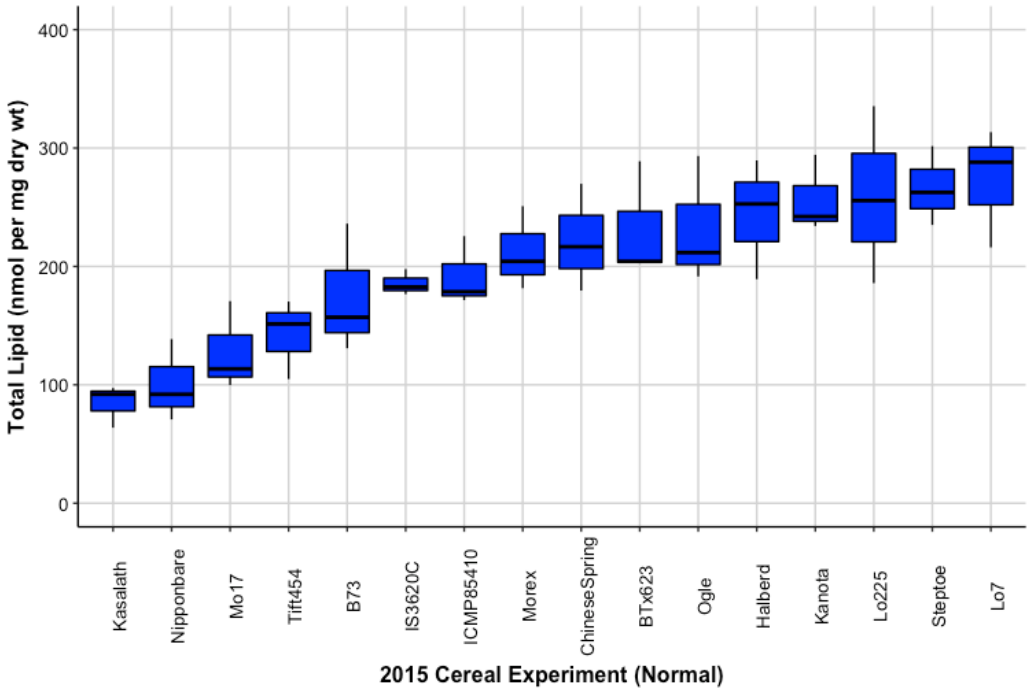

Figure 4. 1 Boxplots of the total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. Lipids measured in nmol mg^{-1} dry wt.

Table 4.2 Total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) temperature environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.
†Significantly different $p<0.05$

Cereal	PlantID	Total Lipid	p-value
Rice(Indica)	Kasalath	84.36±18.01	0.6484
Rice(Japonica)	Nipponbare	100.48±34.70	
Maize	B73	174.71±54.80	0.0431†
Maize	Mo17	127.98±37.64	
PearlMillet	Tift454	142.19±33.70	0.0872
PearlMillet	ICMP85410	191.96±29.38	
Sorghum	IS3620C	185.62±10.91	0.2972
Sorghum	BTx623	232.03±49.27	
Barley	Morex	212.28±35.37	0.2470
Barley	Steptoe	266.41±33.49	
SpringWheat	ChineseSpring	222.02±45.34	0.1063
SpringWheat	Halberd	243.85±50.78	
Oat	Ogle	232.17±53.92	0.6344
Oat	Kanota	256.87±32.65	
Rye	Lo225	258.98±74.81	0.7088
Rye	Lo7	272.54±50.54	

Significant variation was observed in selected MGDG and DGDG lipid phenotype levels at normal growing temperature (30°C day/20°C night) (Tables 4.3 and 4.4; Figures 4.2 and 4.3). The average MGDG(36:6) lipid content was less in rice than all other cereal crops. Kasalath (Indica) and Nipponbare (Japonica) levels were 39.71 and 49.62 nmol mg^{-1} dry wt, respectively. The highest levels of average MGDG(36:6) were identified in Rye, with Lo7 and Lo225 at 134.86 and 137.85 nmol mg^{-1} dry wt, respectively. The average MGDG(36:5) lipid content of rice was lower than the other cereal crops with Kasalath (Indica) and Nipponbare (Japonica) levels of 1.83 and 2.03 nmol mg^{-1} dry wt, respectively. Average Oat MGDG(36:5) levels were the highest of all other cereal crops. Kanota and Ogle average levels were 39.38 and 40.76 nmol mg^{-1} dry wt, respectively. The pearl millet lines, Tift 454 and ICMP85410, were the only pair of cereal crops with significantly different levels between them ($p<0.001$). Average levels of rice total MGDG lipid content

were lowest in rice compared to the other cereal crops analyzed. Kasalath (Indica) and Nipponbare (Japonica) levels were 43.31 and 54.23 nmol mg^{-1} dry wt, respectively. Rye, on average, had the highest levels of average total MGDG lipid content with Lo225 and Lo7 levels at 153.11 and 159.54 nmol mg^{-1} dry wt, respectively. The maize lines, B73 and Mo17, were the only pair of cereal crops with average total MGDG lipid levels significantly different ($p < 0.05$) from one another. Average levels of the MGDG Unsaturation Ratio were lowest in Oat compared to the other cereal crops analyzed. Ogle and Kanota levels were 2.06 and 2.62 nmol mg^{-1} dry wt, respectively. Alternatively, rice had the highest average level of MGDG Unsaturation Ratio. Kasalath (Indica) and Nipponbare (Japonica) levels were 34.10 and 38.11 nmol mg^{-1} dry wt, respectively. The average Unsaturation Ratios of B73 and Mo17 were significantly different ($p < 0.01$), with Mo17 lower than B73 levels at 6.64 and 17.26, respectively. The rye lines, Lo7 and Lo225, were the only other pair of cereal crops analyzed which showed significant difference ($p < 0.05$) in average MGDG Unsaturation Ratio levels.

The average levels of DGDG(36:6) were lowest in rice compared to the other cereal crops analyzed (Table 4.4). Kasalath (Indica) and Nipponbare (Japonica) levels were 9.82 and 13.04 nmol mg^{-1} dry wt, respectively. Average levels of DGDG(36:6) were highest in Rye, with Lo225 and Lo7 having 46.16 and 49.19 nmol mg^{-1} dry wt, respectively. B73 and Mo17 average levels of DGDG(36:6) were found to be significantly different ($p < 0.05$). Compared to the other cereal crops analyzed, rice had the lowest average levels of DGDG(36:5). Kasalath (Indica) and Nipponbare (Japonica) levels were 0.50 and 0.62 nmol mg^{-1} dry wt, respectively. Oat had the highest average levels of DGDG(36:5) with Ogle and Kanota having 9.39 and 10.59 nmol mg^{-1} dry wt, respectively. Pairs of cereal lines with

significant differences of DGDG(36:5) levels include B73 and Mo17 ($p < 0.05$) and the pearl millet lines, Tift 454 and ICMP85410 ($p < 0.01$). The sorghum line, BTx623, had the lowest overall average total DGDG lipid content at 14.89 nmol mg^{-1} dry wt, with rice on average having the lowest average levels. Kasalath (Indica) and Nipponbare (Japonica) had levels of 18.00 and 22.05 nmol mg^{-1} dry wt, respectively. Rye had the highest levels compared to the other cereal crops analyzed. Average total DGDG content in Lo225 and Lo7 were 66.17 and 69.67 nmol mg^{-1} dry wt, respectively. Average levels of total DGDG were significantly different between B73 and Mo17 ($p < 0.05$). The average DGDG Unsaturation Ratio levels were lowest in oat, with Ogle and Kanota levels at 3.22 and 3.28 nmol mg^{-1} dry wt, respectively. The highest overall level of average DGDG Unsaturation Ratio was found in the sorghum line, BTx623 at 54.13. The highest average levels were found in rice with Kasalath (Indica) and Nipponbare (Japonica) at 30.63 and 54.13, respectively. Average DGDG Unsaturation Ratio levels were significantly different between the barley lines, Morax and Steptoe, Tift 454 and ICMP85410, rice lines, Nipponbare and Kasalath, rye lines, Lo7 and Lo225 ($p < 0.05$), and B73 and Mo17 ($p < 0.01$).

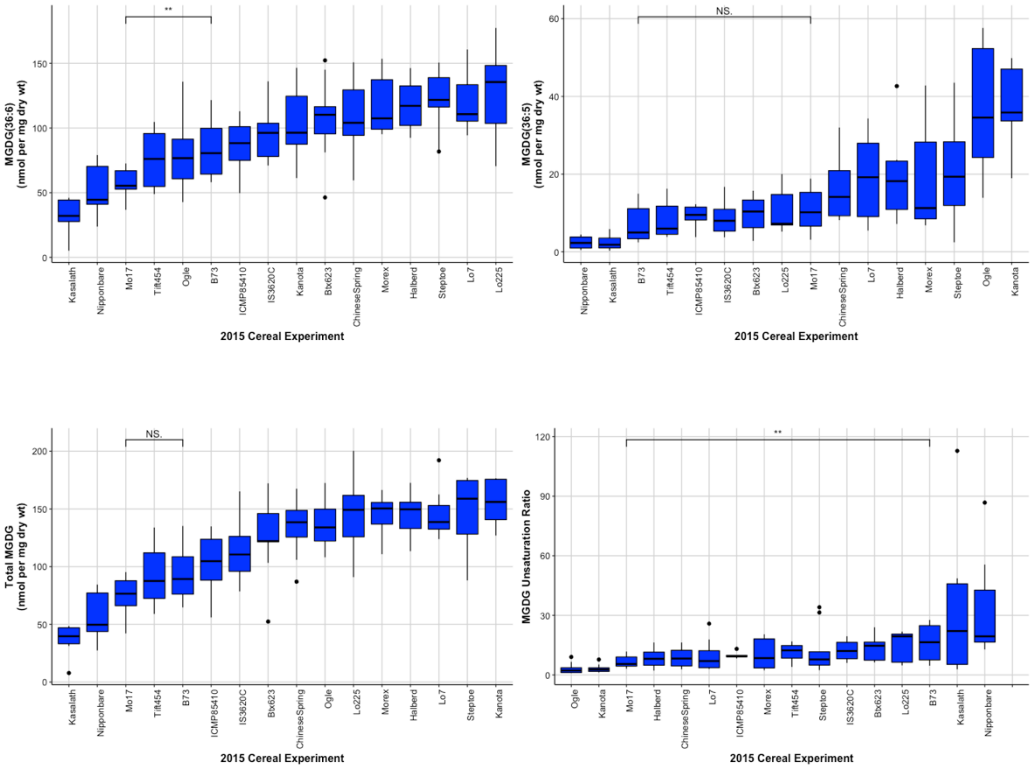

Figure 4.2 Boxplots of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

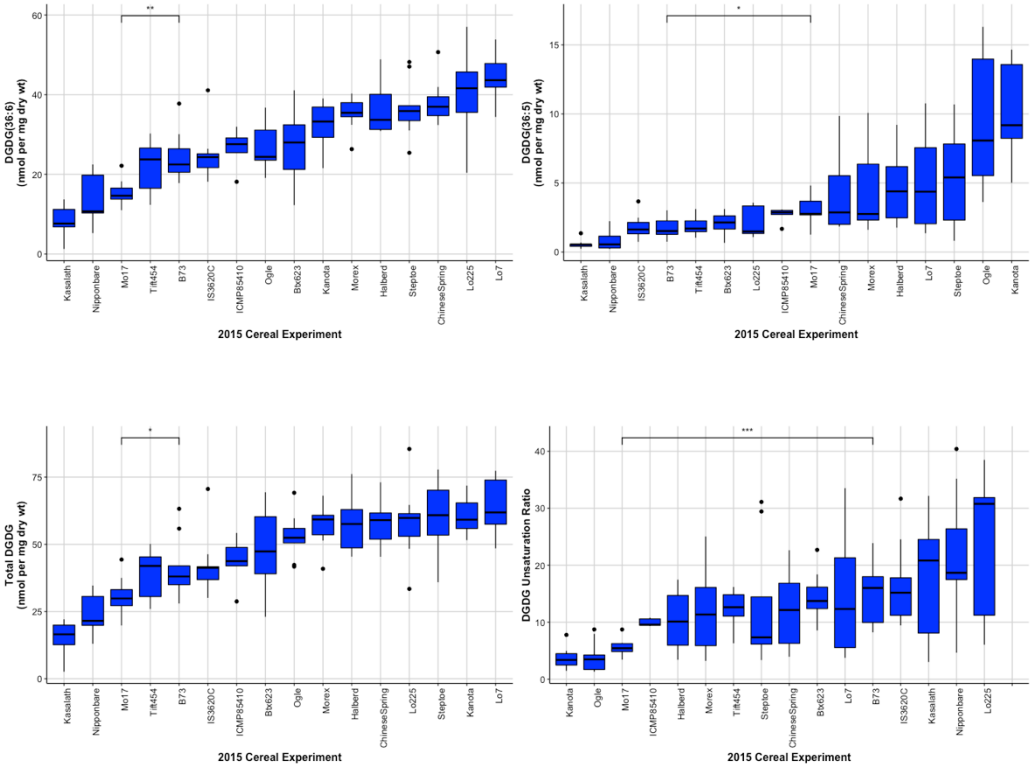

Figure 4.3 Boxplots of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

Table 4.3 Selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	PlantID	Rep	MGDG(36:6)	MGDG(36:5)	Total MGDG	MGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morax	3	108.53±13.91	12.96±4.62	127.56±20.21	8.87±2.34
Barley	Steptoe	3	134.05±14.85	18.00±4.01	159.05±17.97	7.65±1.45
Maize	B73	3	88.85±29.49	5.24±2.02	98.38±33.28†	17.26±1.61††
Maize	Mo17	3	55.74±14.66	9.71±5.91	70.77±22.58†	6.64±2.32††
Oat	Kanota	3	101.71±9.45	39.38±8.06	153.62±19.90	2.62±0.28
Oat	Ogle	3	79.71±10.50	40.76±14.77	137.66±30.75	2.06±0.41
Pearl Millet	ICMP85410	1	92.07±19.28	9.73±1.68††	109.32±23.64	9.43±0.34
Pearl Millet	Tift 454	3	67.00±17.92	4.88±1.00††	78.04±19.98	13.85±3.22
Rice (Japonica)	Nipponbare	3	49.62±19.42	2.03±2.12	54.23±22.53	38.11±20.12
Rice (Indica)	Kasalath	3	39.71±10.38	1.83±1.49	43.31±8.50	34.10±22.80
Rye	Lo7	3	134.86±25.13	17.06±7.19	159.54±34.27	8.72±3.02†
Rye	Lo225	3	137.85±38.38	7.39±2.93	153.11±45.44	19.20±2.54†
Sorghum	BTx623	3	121.53±20.41	8.86±3.89	138.03±25.40	14.70±3.44
Sorghum	IS3620C	3	99.28±8.97	8.41±1.80	113.70±11.53	12.01±1.45
Spring Wheat	Chinese Spring	3	108.60±19.00	13.04±3.37	127.85±23.06	8.52±1.33
Spring Wheat	Halberd	3	117.24±16.75	17.78±8.33	142.67±26.53	7.81±3.90

†Significantly different $p<0.05$

††Significantly different $p<0.01$

Table 4.4 Selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	PlantID	Rep	DGDG(36:6)	DGDG(36:5)	Total DGDG	DGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morax	3	32.08±5.00	2.96±0.76	50.52±9.21	11.06±1.59†
Barley	Steptoe	3	40.37±5.80	5.26±1.23	65.51±10.77	7.85±1.35†
Maize	B73	3	28.66±8.05†	1.84±0.80†	46.45±14.85†	16.22±2.21††
Maize	Mo17	3	16.58±4.83†	3.18±1.23†	32.46±10.31†	5.37±0.77††
Oat	Kanota	3	33.74±3.99	10.59±2.93	62.08±7.98	3.28±0.59
Oat	Ogle	3	27.80±3.50	9.39±0.88	54.01±13.76	3.22±0.88
Pearl Millet	ICMP85410	1	28.32±3.33	2.87±0.15††	46.67±6.63	9.84±0.66†
Pearl Millet	Tift 454	3	20.49±7.14	1.41±0.32††	37.07±9.73	14.24±2.18†
Rice (Japonica)	Nipponbare	3	13.04±6.13	0.62±0.51	22.05±8.07	26.03±9.35†
Rice (Indica)	Kasalath	3	9.82±2.62	0.50±0.09	18.00±4.06	20.31±7.73†
Rye	Lo7	3	49.19±5.16	4.11±1.94	69.67±10.46	14.04±6.59†
Rye	Lo225	3	46.16±10.74	1.52±0.42	66.17±18.62	30.63±1.33†
Sorghum	BTx623	3	31.24±8.70	2.13±0.75	14.89±1.22	54.13±13.25
Sorghum	IS3620C	3	24.86±0.47	1.57±0.13	41.48±0.21	15.96±1.59
Spring Wheat	Chinese Spring	3	38.75±10.35	2.99±1.13	56.64±14.57	13.52±2.90
Spring Wheat	Halberd	3	39.86±9.02	4.77±2.58	61.93±15.49	10.40±5.71

†Significantly different $p<0.05$

††Significantly different $p<0.01$

The selected MGDG lipid phenotype levels at normal growing temperature (30°C day/20°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.4). The average MGDG(36:6), MGDG(36:5), and Total MGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season cereal crops, whereas the MGDG Unsaturation Ratio was significantly ($p<0.05$) less in cool-season versus warm-season cereal crops.

The selected DGDG lipid phenotype levels at normal growing temperature (30°C day/20°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.5). The average DGDG(36:6), DGDG(36:5), and Total DGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season

cereal crops, whereas the DGDG Unsaturation Ratio was less in cool-season versus warm-season cereal crops, however a statistical significance was not observed.

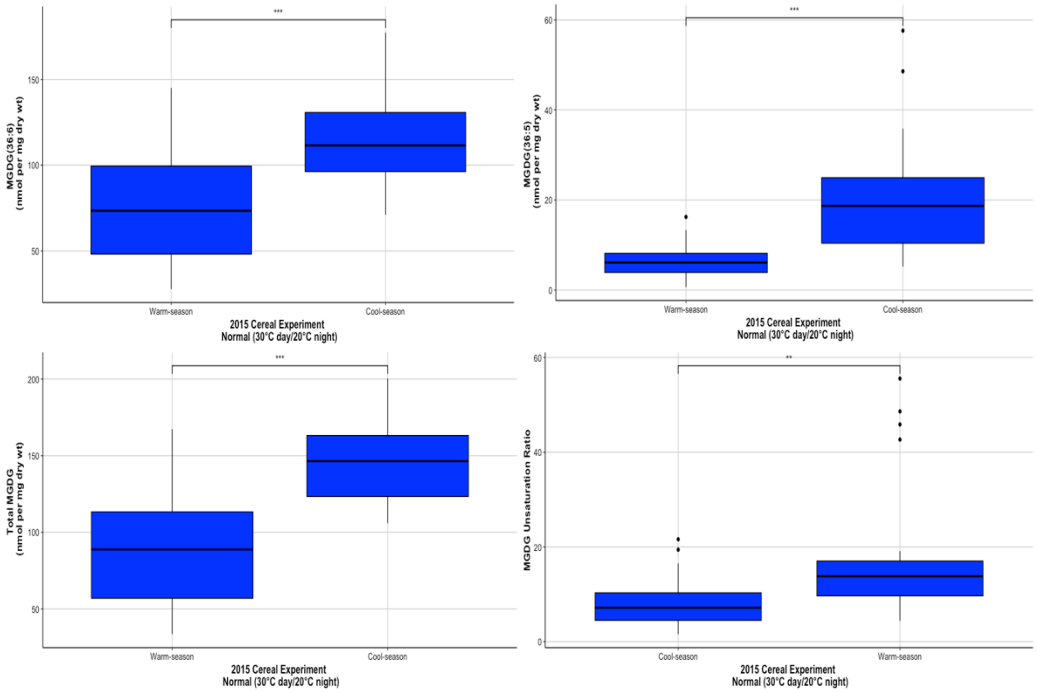

Figure 4.4 Boxplot comparison between warm-season and cool-season cereals of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

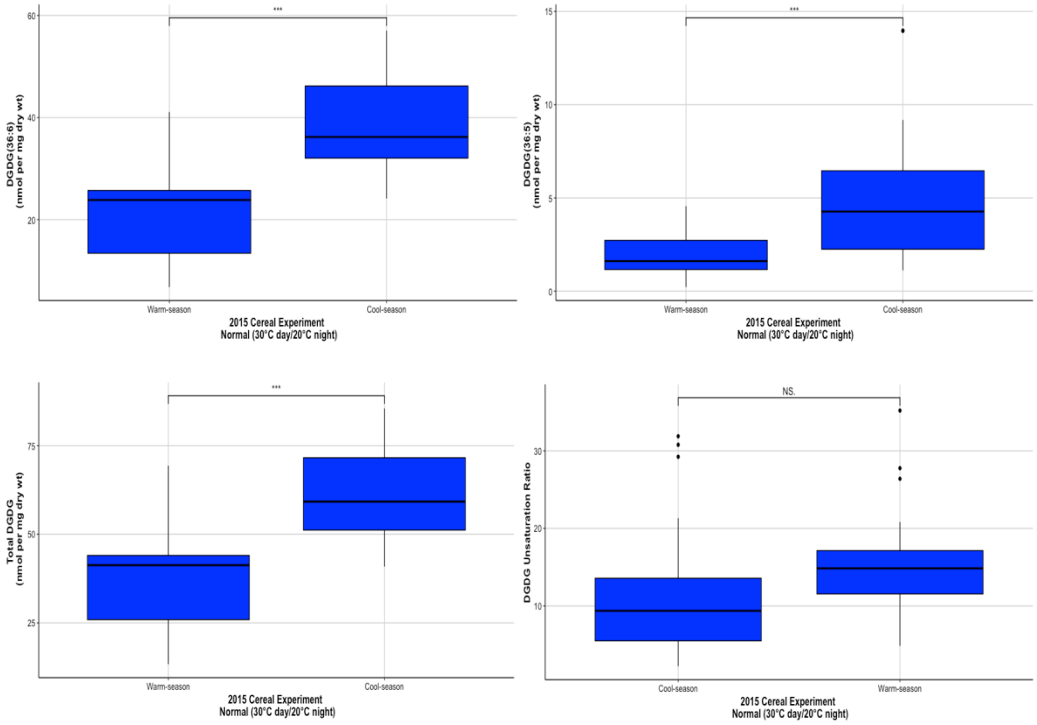

Figure 4.5 Boxplot comparison between warm-season and cool-season cereals of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 30°C day/20°C night (normal) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

4.4.2 Cereal Lipid Diversity at Cold Temperature

The cold temperature environment (20°C day/10°C night) was used to simulate chilling stress on the cereal crops and analyze its effect on selected lipid phenotypes. Total lipid amounts evaluated at the cold temperature environment of the various cereals were highly diverse (Table 4.5; Figure 4.6). Rice had the lowest average total lipid, with Kasalath (Indica) having a significantly ($p < 0.05$) lower amount than Nipponbare (Japonica), 83.67 and 127.38 nmols mg^{-1} dry wt, respectively. The oat line Kanota had the highest average total lipid at 276.69 nmols mg^{-1} dry wt.

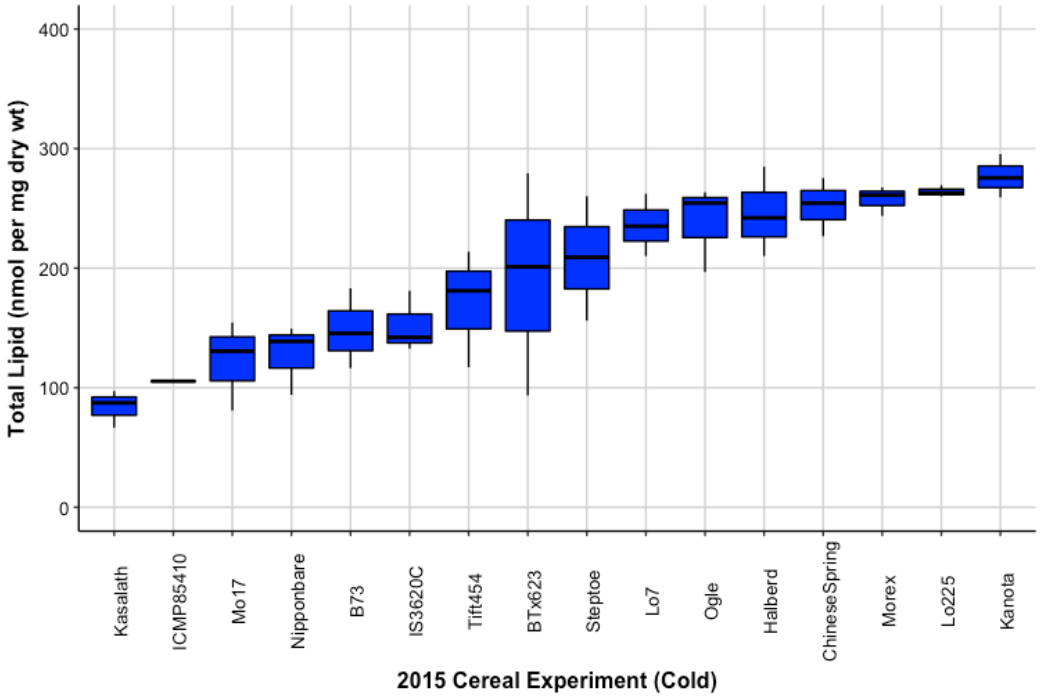

Figure 4.6 Boxplots of the total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) environment. Lipids measured in nmol mg^{-1} dry wt.

Table 4.5 Total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) temperature environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.
†Significantly different $p<0.05$

Cereal	PlantID	Total Lipid	p-value
Rice(Indica)	Kasalath	83.67±15.65	0.0325†
Rice(Japonica)	Nipponbare	127.38±29.33	
Maize	B73	148.35±33.47	0.2883
Maize	Mo17	122.00±37.56	
PearlMillet	Tift454	170.69±49.10	NA
PearlMillet	ICMP85410	105.41	
Sorghum	IS3620C	151.98±25.67	0.5272
Sorghum	BTx623	191.32±93.34	
Barley	Morex	257.43±12.37	0.2737
Barley	Steptoe	208.49±52.09	
SpringWheat	ChineseSpring	252.13±24.47	0.7657
SpringWheat	Halberd	245.65±37.50	
Oat	Ogle	238.26±36.25	0.2512
Oat	Kanota	276.69±18.16	
Rye	Lo225	264.08±4.80	0.2227
Rye	Lo7	235.81±26.13	

The selected MGDG and DGDG lipid phenotype levels at cold growing temperature (20°C day/10°C night) showed a diverse range (Tables 4.6 and 4.7; Figures 4.7 and 4.8). The average MGDG(36:6) lipid content was less in the Indica rice line, Kasalath, than all other cereal crops. Kasalath (Indica) and Nipponbare (Japonica) levels were 39.42 and 63.66 nmol mg^{-1} dry wt, respectively. The highest levels of average MGDG(36:6) were identified in the Rye line, Lo225, at 146.00 nmol mg^{-1} dry wt, respectively. The average MGDG(36:5) lipid content of rice was lower than the other cereal crops. Kasalath (Indica) and Nipponbare (Japonica) levels were 1.2 and 2.34 nmol mg^{-1} dry wt, respectively. Average total MGDG lipid content was lowest in Kasalath at 41.86 nmol mg^{-1} dry wt and highest in the oat line, Kanota, at 170.19 nmol mg^{-1} dry wt. The oat lines, Kanota and Ogle, had the lowest average MGDG Unsaturation Ratio levels

of all the cereal crops analyzed at 5.51 and 6.41 nmol mg^{-1} dry wt, respectively. The rice lines, Nipponbare (Japonica) and Kasalath (Indica), had the highest average MGDG Unsaturation Ratio levels at 44.69 and 55.44 nmol mg^{-1} dry wt, respectively. The maize lines, B73 and Mo17, were the only pair of cereal crops analyzed with significantly different ($p < 0.5$) MGDG Unsaturation Ratios from one another at 19.89 and 7.04 nmol mg^{-1} dry wt, respectively.

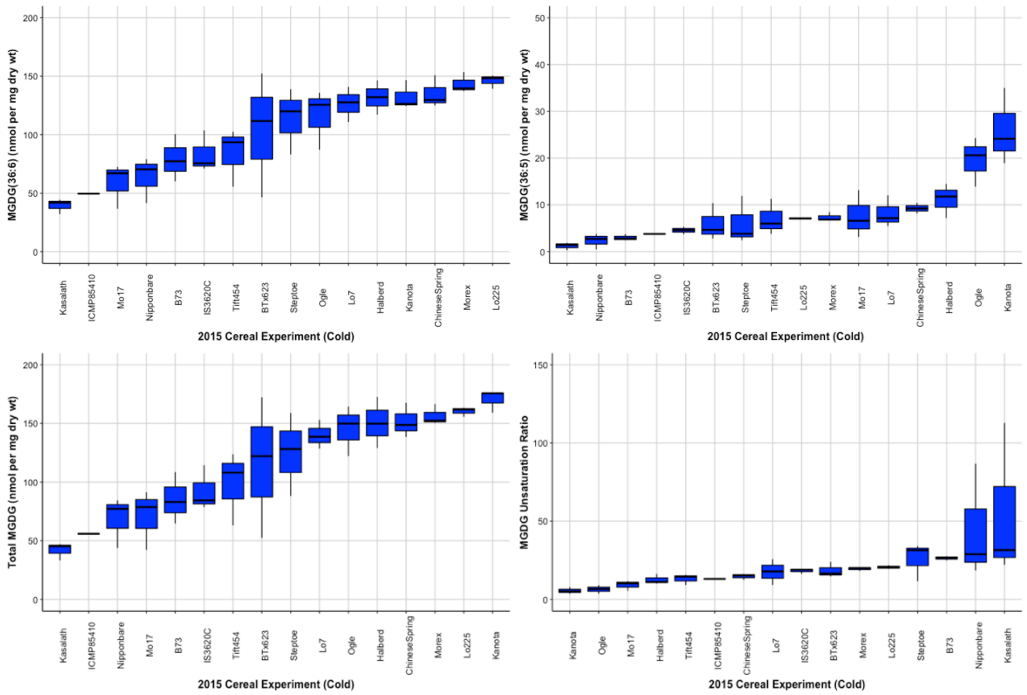

Figure 4.7 Boxplots of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

The average levels of DGDG(36:6) were lowest in Indica rice compared to the other cereal crops analyzed (Table 4.7). Kasalath levels were 10.67 nmol mg^{-1} dry wt. Average levels of DGDG(36:6) were highest in Rye, with Lo7 and Lo225 having 40.74 and 43.17 nmol mg^{-1} dry wt, respectively. Rice had the lowest average levels of DGDG(36:5). Kasalath (Indica) and Nipponbare (Japonica) levels were 0.43 and 0.79 nmol mg^{-1} dry wt, respectively. The highest average levels of DGDG(36:5) were from Oat with Ogle and Kanota having 4.59 and 6.65 nmol mg^{-1} dry wt, respectively. The lowest average Total DGDG was found in the Indica rice, Kasalath and Japonica rice Nipponbare, which were significantly different at 17.83 and 29.41 nmol mg^{-1} dry wt, respectively. The oat line, Kanota, had the highest average level at 60.63 nmol mg^{-1} dry wt. The lowest levels of average DGDG Unsaturation Ratio was found in the Oat lines, with Kanota and Ogle having levels of 5.74 and 6.98, respectively. The Rye line, Lo225, had the highest overall average DGDG Unsaturation Ratio level at 34.44. The average DGDG Unsaturation Ratio levels were significantly different ($p < 0.05$) between the maize lines, B73 and Mo17, at 19.89 and 7.04, as well as significantly different ($p < 0.05$) between the sorghum lines, BTx623 and IS3620C, at 17.83 and 24.20, respectively.

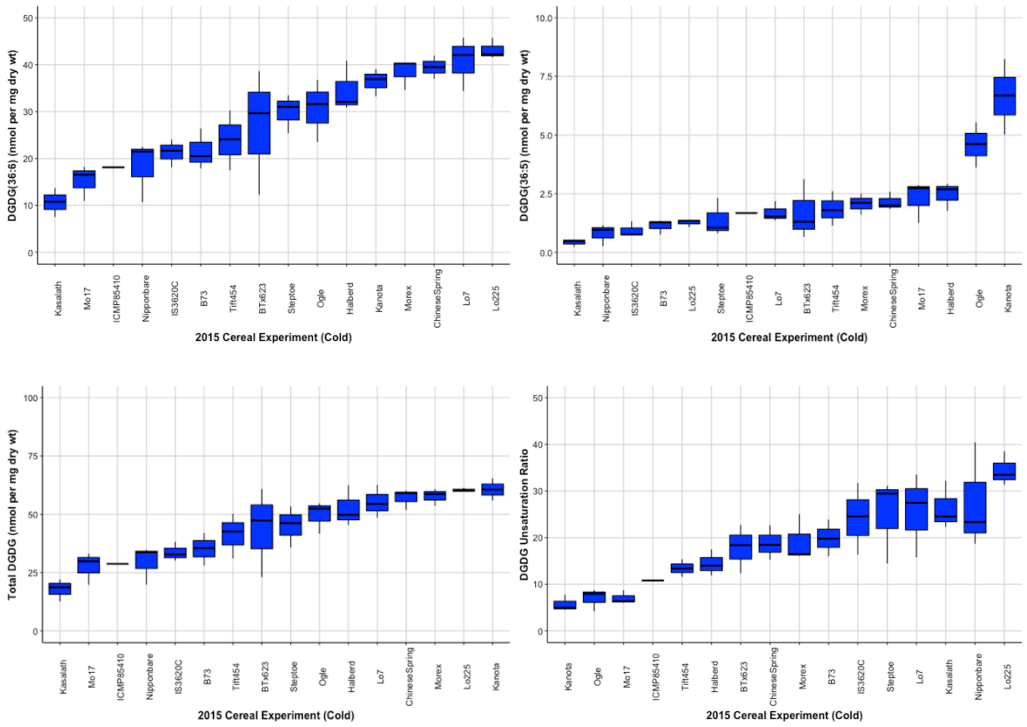

Figure 4.8 Figure 4.8 Boxplots of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (normal) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

Table 4.6 Selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	PlantID	Rep	MGDG(36:6)	MGDG(36:5)	Total MGDG	MGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morax	3	143.47±8.78	7.39±0.95	156.39±8.79	19.52±1.26
Barley	Steptoe	3	114.01±28.37	6.07±5.13	125.05±35.49	25.71±12.25
Maize	B73	3	79.28±20.23	3.00±0.70	85.44±22.07	26.36±1.43†
Maize	Mo17	3	58.76±19.29	7.62±5.09	70.75±25.60	9.14±3.24†
Oat	Kanota	3	132.50±12.22	26.01±8.20	170.19±9.60	5.51±2.11
Oat	Ogle	3	116.16±25.67	19.59±5.26	145.48±21.47	6.41±2.73
Pearl Millet	ICMP85410	1	49.68± NA	3.79± NA	55.92± NA	13.12± NA
Pearl Millet	Tift 454	3	83.85±24.94	7.04±3.84	98.32±31.45	13.06±3.50
Rice (Japonica)	Nipponbare	3	63.66±19.65	2.34±1.70	68.47±21.67	44.69±36.78
Rice (Indica)	Kasalath	3	39.42±6.50	1.20±0.83	41.86±7.56	55.44±49.90
Rye	Lo7	3	126.42±15.12	8.22±3.41	140.08±12.30	17.61±8.31
Rye	Lo225	3	146.00±5.96	7.08±0.16	160.30±4.14	20.63±1.12
Sorghum	BTx623	3	103.43±53.47	5.95±3.95	115.58±60.19	18.39±4.93
Sorghum	IS3620C	3	83.38±17.72	4.55±0.80	92.43±19.23	18.29±1.66
Spring Wheat	Chinese Spring	3	135.12±11.25	9.27±0.93	151.59±12.03	14.70±1.66
Spring Wheat	Halberd	3	131.82±14.65	11.14±3.70	150.48±21.83	12.55±3.32

†Significantly different $p<0.05$

Table 4.7 Selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/20°C night (normal) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.
†Significantly different $p<0.05$

Cereal	PlantID	Rep	DGDG(36:6)	DGDG(36:5)	Total DGDG	DGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morax	3	38.39±3.27	2.07±0.45	57.68±3.73	19.20±5.07
Barley	Steptoe	3	29.97±4.13	1.40±0.81	45.17±8.79	25.00±9.18
Maize	B73	3	21.61±4.37	1.12±0.32	35.16±7.00	19.89±3.94†
Maize	Mo17	3	15.26±3.79	2.29±0.89	27.61±6.95	7.04±1.47†
Oat	Kanota	3	36.41±2.93	6.65±1.61	60.63±4.76	5.74±1.78
Oat	Ogle	3	30.63±6.65	4.59±0.96	49.64±6.96	6.98±2.39
Pearl Millet	ICMP85410	1	18.13± NA	1.68± NA	28.75± NA	10.80± NA
Pearl Millet	Tift 454	3	23.94±6.38	1.85±0.73	41.30±9.56	13.45±1.87
Rice (Japonica)	Nipponbare	3	18.23±6.54	0.79±0.47	29.41±8.23†	27.48±11.45
Rice (Indica)	Kasalath	3	10.67±3.09	0.43±0.17	17.83±4.78†	26.33±5.19
Rye	Lo7	3	40.74±5.80	1.69±0.43	55.19±7.08	25.59±9.03
Rye	Lo225	3	43.17±2.22	1.26±0.16	60.45±0.85	34.44±3.67
Sorghum	BTx623	3	26.85±13.41	1.70±1.27	43.74±19.14	17.83±5.18†
Sorghum	IS3620C	3	21.29±2.98	0.94±0.33	33.69±4.14	24.20±7.66†
Spring Wheat	Chinese Spring	3	39.46±2.04	2.15±0.31	56.99±3.63	18.79±3.01
Spring Wheat	Halberd	3	34.58±5.45	2.46±0.61	52.58±8.84	14.46±2.82

The selected MGDG lipid phenotype levels at cold growing temperature (20°C day/10°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.9). The average MGDG(36:6), MGDG(36:5), and Total MGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season cereal crops, whereas the MGDG Unsaturation Ratio was less in cool-season versus warm-season cereal crops, however a statistical significance was not observed.

The selected DGDG lipid phenotype levels at cold growing temperature (20°C day/10°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.10). The average DGDG(36:6), DGDG(36:5), and Total DGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season cereal crops, whereas the DGDG Unsaturation Ratio was less in cool-season versus warm-season cereal crops, however a statistical significance was not observed.

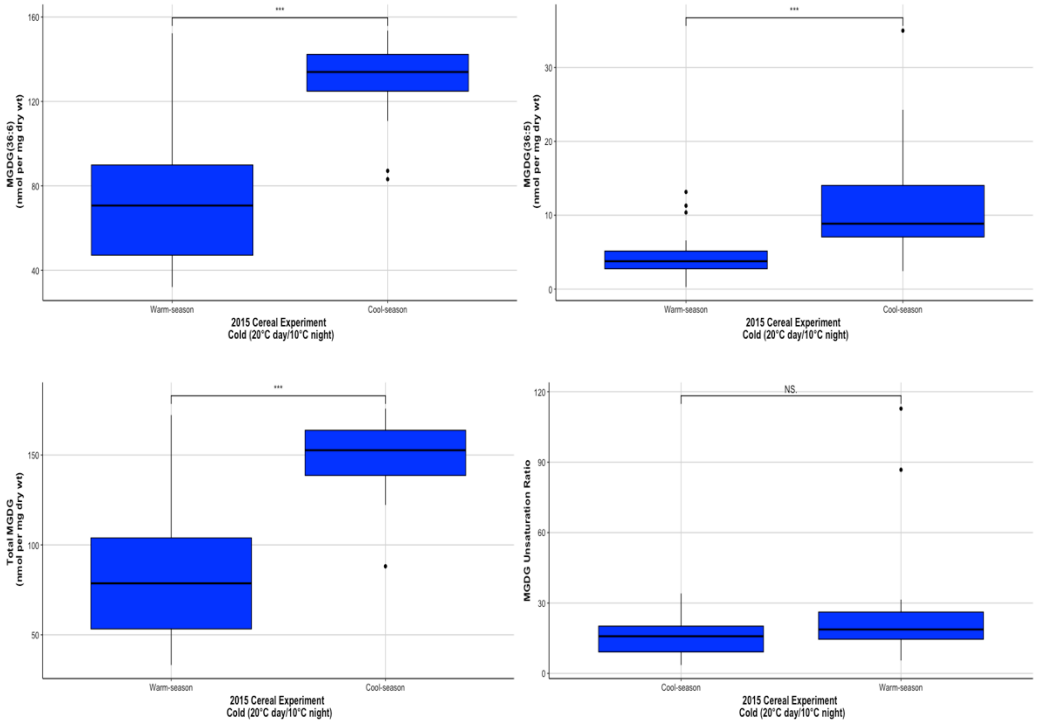

Figure 4.9 Boxplot comparison between warm-season and cool-season cereals of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

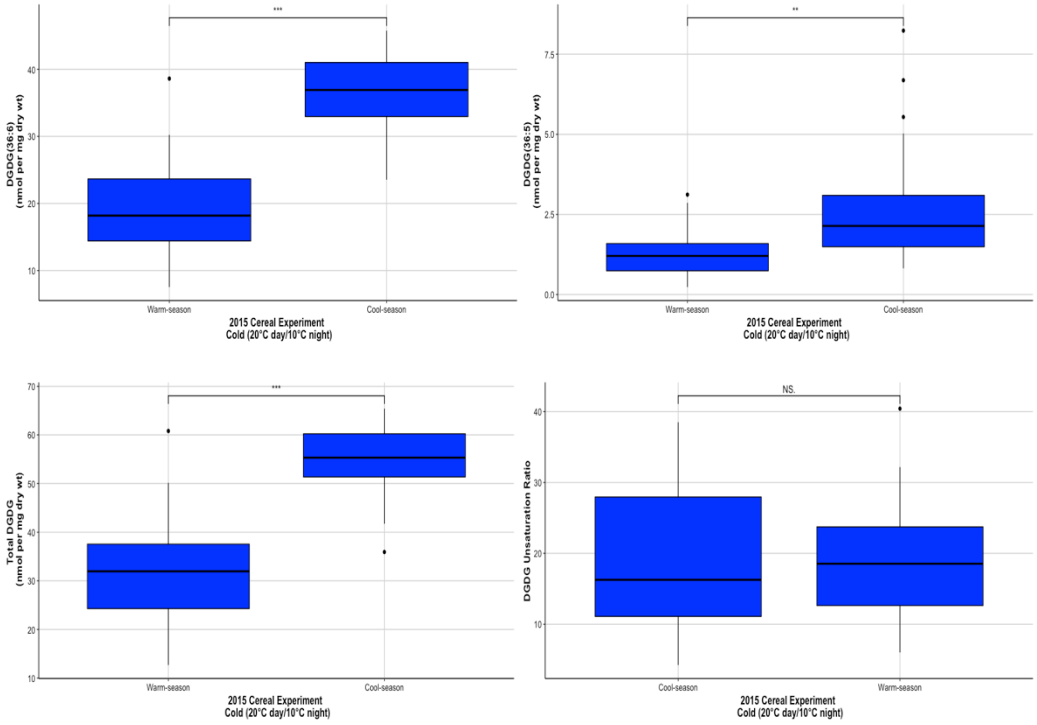

Figure 4.10 Boxplot comparison between warm-season and cool-season cereals of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 20°C day/10°C night (cold) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

4.4.3 Cereal Lipid Diversity at Hot Temperature

The hot temperature growth chamber environment (40°C day/30°C night) was used to simulate heat stress on the cereal crops and analyze its effect on selected lipid phenotypes. The total lipid content of the various cereals evaluated in the hot temperature environment were found to be highly diverse (Table 4.8; Figure 4.11). The lowest average total lipid was identified in rice, with Kasalath (Indica) and Nipponbare (Japonica) levels, 57.79 and 96.13 nmols mg^{-1} dry wt, respectively. The barley lines Morex and Steptoe had the highest average total lipid at 257.42 and 273.27 nmols mg^{-1} dry wt, respectively.

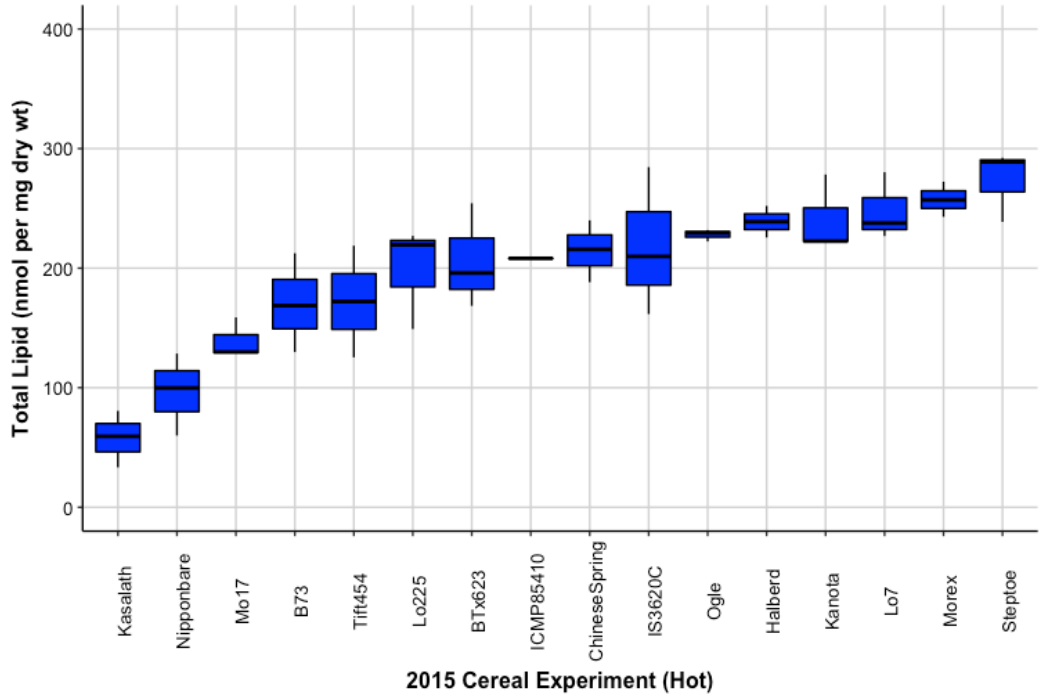

Figure 4.11 Boxplots of the total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. Lipids measured in nmol mg^{-1} dry wt.

Table 4.8 Total leaf lipid content analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) temperature environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	PlantID	Total Lipid	p-value
Rice(Indica)	Kasalath	57.79±23.73	0.1899
Rice(Japonica)	Nipponbare	96.13±34.45	
Maize	B73	170.34±41.37	0.1931
Maize	Mo17	139.21±16.99	
PearlMillet	Tift454	172.09±66.05	NA
PearlMillet	ICMP85410	208.16±NA	
Sorghum	IS3620C	218.64±61.94	0.3671
Sorghum	BTx623	206.25±43.87	
Barley	Morex	257.42±14.74	0.2980
Barley	Steptoe	273.27±30.00	
SpringWheat	ChineseSpring	214.62±25.93	0.3162
SpringWheat	Halberd	238.85±18.74	
Oat	Ogle	227.86±4.94	0.6006
Oat	Kanota	241.04±32.26	
Rye	Lo225	198.51±43.02	0.1027
Rye	Lo7	248.24±28.25	

†Significantly different $p<0.05$

The selected MGDG and DGDG lipid phenotype levels at hot growing temperature (40°C day/30°C night) showed a diverse range (Tables 4.9 and 4.10; Figures 4.12 and 4.13). The average MGDG(36:6) lipid content was lowest in the rice lines, Kasalath (Indica) and Nippobare (Japonica) than all other cereal crops with levels of 20.09 and 40.96 nmol mg^{-1} dry wt, respectively. The highest levels of average MGDG(36:6) were found in the barley line, Steptoe, at 112.44 nmol mg^{-1} dry wt. The average MGDG(36:5) lipid content was considerably lower in rice than all other cereal crops evaluated. Kasalath and Nipponbare levels were 2.66 and 4.17 nmol mg^{-1} dry wt, respectively. The highest average levels of MGDG(36:5) were found in the oat lines, Kanota and Ogle, at 47.84 and 50.44 nmol mg^{-1} dry wt, respectively. The rye lines, Lo7 and Lo225, had significantly different ($p <0.05$) levels during heat stress at 30.52 and 16.98 nmol mg^{-1} dry wt, respectively.

Average total MGDG lipid content was lowest in rice with Kasalath and Nipponbare levels at 26.23 and 49.43 nmol mg^{-1} dry wt, respectively. The highest levels were identified in the barely lines, Morex and Steptoe, at 151.30 and 159.62 nmol mg^{-1} dry wt, respectively. The oat lines, Ogle and Kanota, had the lowest average MGDG Unsaturation Ratio levels of all the cereal crops analyzed at 1.04 and 1.48, respectively. The rice line, Nipponbare, had the highest average MGDG Unsaturation Ratio level at 16.26, which was significantly different ($p < 0.05$) from Kasalath at 4.37. The rye lines, Lo7 and Lo225, were also significantly different ($p < 0.05$) from one another at 3.30 and 5.49, respectively.

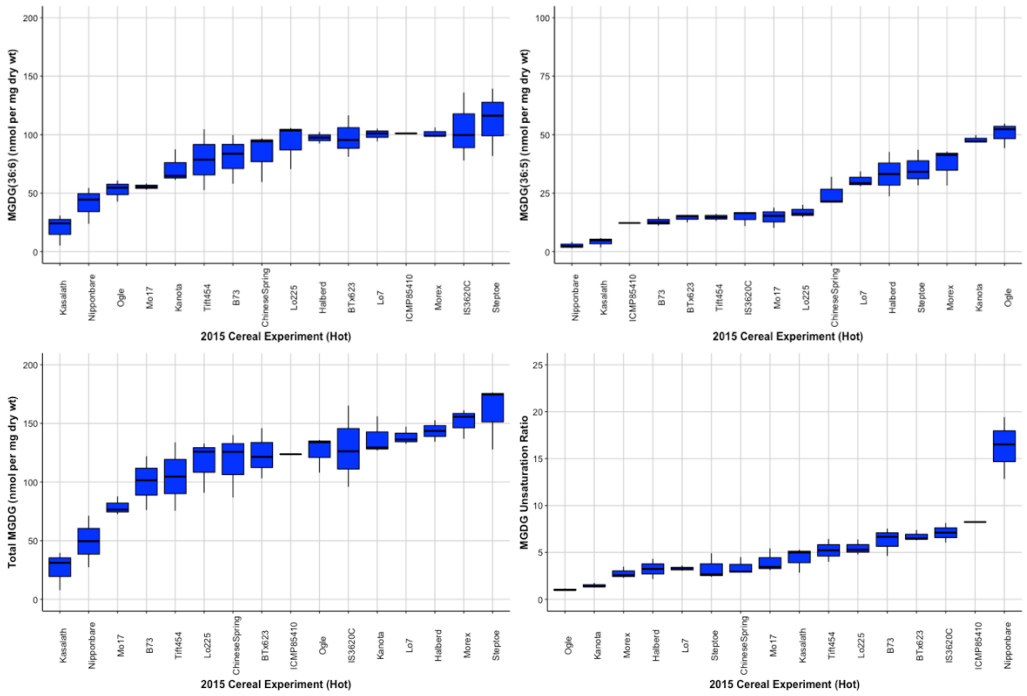

Figure 4.12 Boxplots of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

Table 4.9 Selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	Plant ID	Rep	MGDG(36:6)	MGDG(36:5)	Total MGDG	MGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morax	3	101.18±4.33	37.46±8.02	151.30±12.79	2.79±0.63
Barley	Steptoe	3	112.44±28.94	35.33±7.68	159.62±27.54	3.328±1.39
Maize	B73	3	80.54±21.01	12.87±1.94	99.89±23.00	6.28±1.49
Maize	Mo17	3	55.56±2.72	14.76±4.35	78.96±7.92	4.00±1.26
Oat	Kanota	3	71.18±14.19	47.84±1.77	137.48±16.17	1.48±0.24
Oat	Ogle	3	52.71±9.16	50.44±5.51	125.99±15.55	1.04±0.10
Pearl Millet	ICMP85410	1	101.05± NA	12.25± NA	123.74±NA	8.25± NA
Pearl Millet	Tift 454	2	78.65±36.83	14.70±2.19	104.71±41.27	5.22±1.73
Rice (Japonica)	Nipponbare	3	40.96±15.68	2.66±1.44	49.43±21.91	16.26±3.30†
Rice (Indica)	Kasalath	3	20.09±13.36	4.17±2.10	26.23±16.50	4.37±1.34†
Rye	Lo7	3	100.21±5.54	30.52±3.36†	138.66±7.67	3.30±0.28†
Rye	Lo225	3	93.23±19.78	16.98±2.72†	116.53±22.49	5.49±0.83†
Sorghum	BTx623	3	97.69±17.77	14.47±1.72	123.58±21.47	6.73±0.59
Sorghum	IS3620C	3	104.58±29.36	14.72±3.26	129.14±34.73	7.10±1.04
Spring Wheat	Chinese Spring	3	83.53±20.83	24.79±6.26	117.59±27.46	3.43±0.93
Spring Wheat	Halberd	2	97.41±7.09	33.16±12.42	143.55±12.99	3.25±1.53

†Significantly different $p<0.05$

The average levels of DGDG(36:6) were lowest in Indica rice compared to the other cereal crops analyzed (Table 4.10). Average Kasalath and Nipponbare levels were 4.82 and 8.64 nmol mg^{-1} dry wt, respectively. Average levels of DGDG(36:6) were highest in the Rye line Lo7, with levels at 43.17 nmol mg^{-1} dry wt. Rice had the lowest average levels of DGDG(36:5). Kasalath (Indica) and Nipponbare (Japonica) levels were 0.82 and 1.03 nmol mg^{-1} dry wt, respectively. The highest average levels of DGDG(36:5) were from Oat with Kanota and Ogle having 12.53 and 21.63 nmol mg^{-1} dry wt, respectively. The two rye lines analyzed, Lo7 and Lo225, were the only cereal pair that had significantly different ($p < 0.05$) levels of DGDG(36:5) at 9.22 and 3.43 nmol mg^{-1} dry wt, respectively. The lowest average Total DGDG was found in rice, with Kasalath

and Nipponbare levels at 10.20 and 20.28 nmol mg^{-1} dry wt, respectively. The barely line, Steptoe, had the highest average level at 69.29 nmol mg^{-1} dry wt. The lowest levels of average DGDG Unsaturation Ratio was found in the Oat lines, with Ogle and Kanota having levels of 1.47 and 2.12, respectively. The Japonica rice line, Nipponbare, had the highest overall average DGDG Unsaturation Ratio level at 13.60. The average DGDG Unsaturation Ratio levels were significantly different ($p < 0.05$) between the maize lines, B73 and Mo17, at 9.15 and 4.20, respectively.

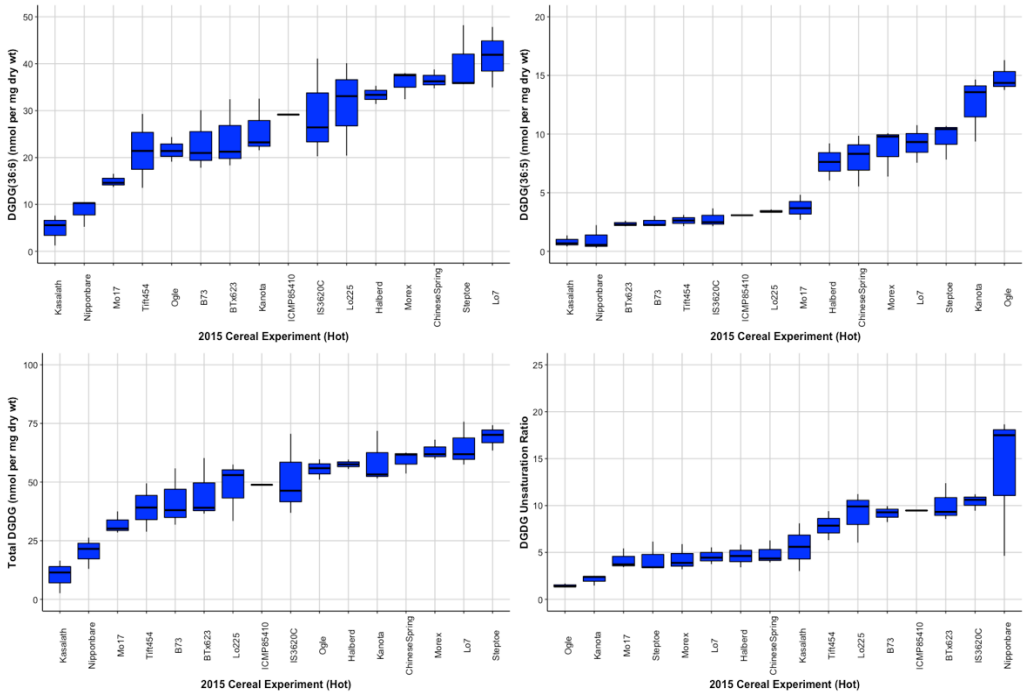

Figure 4.13 Boxplots of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt.

Table 4.10 Selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. A t-test comparison was performed for each cereal pair. Lipids measured in nmol mg^{-1} dry wt.

Cereal	PlantID	Rep	DGDG(36:6)	DGDG(36:5)	Total DGDG	DGDG Unsaturation Ratio
			nmol mg^{-1} dry wt			
Barley	Morex	3	35.99±3.09	8.75±2.06	63.25±4.32	4.33±1.39
Barley	Steptoe	3	39.94±7.17	9.65±1.58	69.29±5.46	4.31±1.60
Maize	B73	3	22.96±6.37	2.48±0.47	41.89±12.48	9.15±0.87†
Maize	Mo17	3	14.96±1.44	3.73±1.07	32.05±4.80	4.20±1.08†
Oat	Kanota	3	25.77±5.92	12.53±2.80	58.89±11.26	2.12±0.56
Oat	Ogle	3	21.63±2.65	14.81±1.33	55.55±4.33	1.47±0.20
Pearl Millet	ICMP85410	1	29.15	3.08	48.85	9.47±NA
Pearl Millet	Tift 454	3	21.42±11.13	2.63±0.68	39.15±14.57	7.86±2.19
Rice (Japonica)	Nipponbare	3	8.64±2.94	1.03±1.05	20.28±6.74	13.60±7.78
Rice (Indica)	Kasalath	3	4.82±3.26	0.82±0.49	10.20±7.04	5.57±2.54
Rye	Lo7	3	41.56±6.45	9.22±1.61†	65.03±9.54	4.58±0.91
Rye	Lo225	3	31.20±9.99	3.43±0.13†	47.97±12.79	9.06±2.69
Sorghum	BTx623	3	24.00±7.44	2.35±0.25	45.30±13.03	10.08±2.02
Sorghum	IS3620C	3	29.26±10.71	2.77±0.80	51.27±17.38	10.42±0.89
Spring Wheat	Chinese Spring	3	36.59±2.04	7.90±2.19	59.29±4.91	4.86±1.25
Spring Wheat	Halberd	2	33.35±2.73	7.63±2.24	57.57±2.84	4.63±1.72

†Significantly different $p<0.05$

The selected MGDG lipid phenotype levels at cold growing temperatures (20°C day/10°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.14). The average MGDG(36:6), MGDG(36:5), and Total MGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season cereal crops, whereas the MGDG Unsaturation Ratio was significantly ($p<0.05$) less in cool-season versus warm-season cereal crops.

The selected DGDG lipid phenotype levels at cold growing temperature (20°C day/10°C night) have differential levels when comparing between the warm-season and cool-season cereals (Figure 4.15). The average DGDG(36:6), DGDG(36:5), and Total DGDG lipid content was significantly ($p<0.05$) less in warm-season versus cool-season cereal crops, whereas the DGDG Unsaturation Ratio was significantly ($p<0.05$) less in cool-season versus warm-season cereal crops.

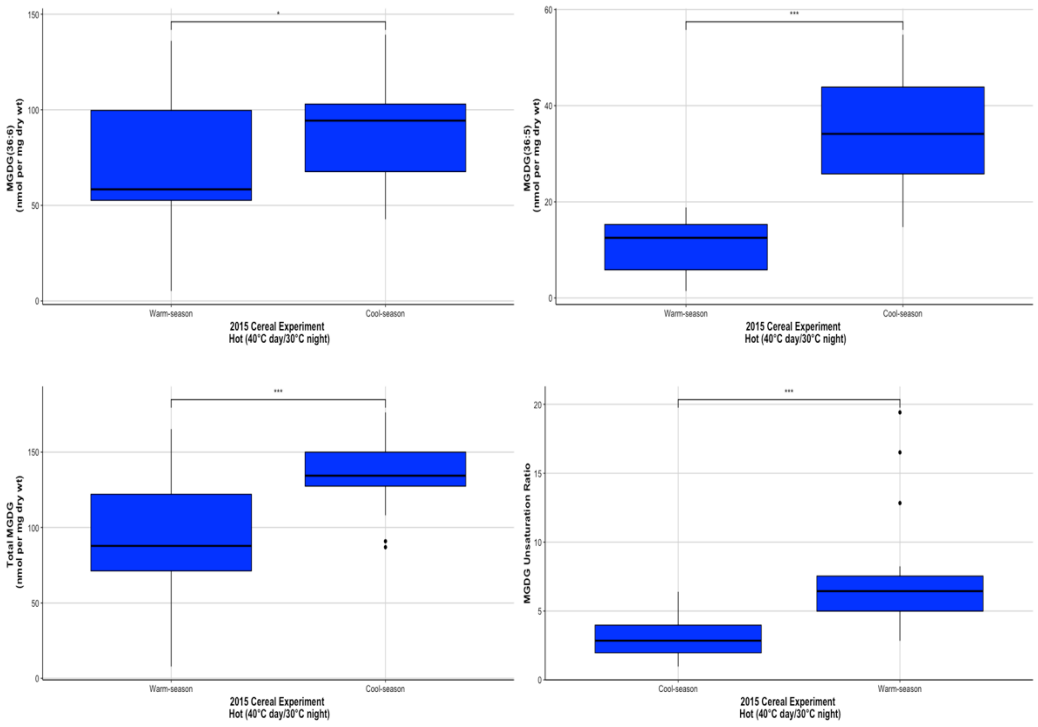

Figure 4. 14 Boxplot comparison between warm-season and cool-season cereals of the selected MGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. MGDG(36:6) (top left), MGDG(36:5) (top right), Total MGDG (bottom left), and MGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

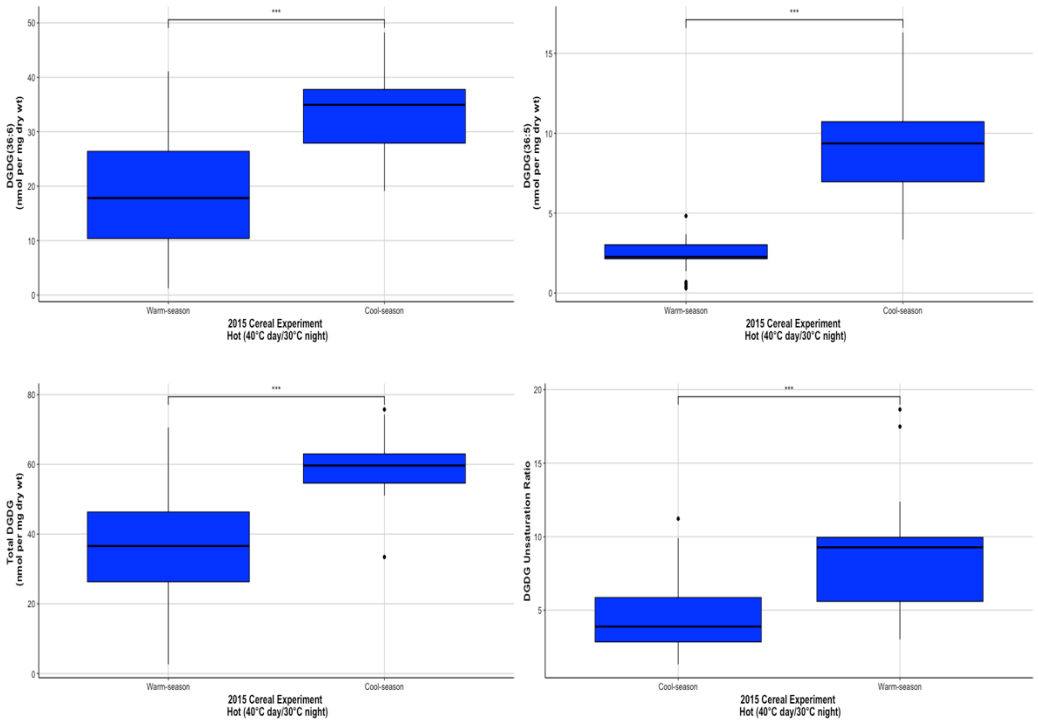

Figure 4.15 Boxplot comparison between warm-season and cool-season cereals of the selected DGDG lipid phenotypes analyzed in sixteen diverse cereal lines in a controlled 40°C day/30°C night (hot) environment. DGDG(36:6) (top left), DGDG(36:5) (top right), Total DGDG (bottom left), and DGDG Unsaturation Ratio (bottom right). Lipids measured in nmol mg^{-1} dry wt. Statistical significance indicated as (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (NS) no significance.

4.4.4 Cereal Lipid Phenotype Comparisons between Normal, Cold, and Hot Temperature Environments

To evaluate the differential response of the selected MGDG and DGDG lipid phenotypes when exposed to chilling stress and heat stress, comparisons were made for each cereal line between normal versus cold and normal versus hot growth chamber temperature environments (Tables 4.11 and 4.12). Figures 4.16 and 4.17 provide an overview comparison of the selected MGDG and DGDG lipid phenotypes for each cereal line at cold, normal, and hot environments. In general, as temperatures increase, the levels of MGDG and DGDG unsaturation increases for all cereal crops analyzed.

Despite an overall trend of an increased MGDG and DGDG Unsaturation Ratios at colder temperatures, most of the cereal lines analyzed in this study did not have significantly different levels when comparing the cold temperature and normal temperature environments. The maize line B73, barley line Morex, and spring wheat line Halberd were the only cereal lines with a significant difference ($p < 0.05$) between MGDG Unsaturation Ratios in the normal and cold temperature environments (Table 4.11), whereas none of the lines were significantly different for the DGDG Unsaturation Ratio phenotype (Table 4.12).

The overall trend at the warmer temperature environment is a decrease in MGDG and DGDG Unsaturation Ratios (Figures 4.16 and 4.17). The MGDG Unsaturation Ratio for the maize line B73, oat lines Kanota and Ogle, pearl millet line Tift 454, rye line Lo225, and sorghum line IS3620C were all significantly different ($p < 0.05$), as well as the barely line Steptoe and spring wheat line Chinese Spring were significantly different ($p < 0.01$), when comparing between the normal and hot temperature environments (Table 4.11). The DGDG Unsaturation Ratio for the maize line Mo17, Japonica rice line Nipponbare, sorghum line IS36620C, spring wheat line Chinese Spring were all significantly different ($p < 0.05$), as well as the barley line Steptoe, oat line Kanota, and rye line Lo225 were significantly different ($p < 0.01$), when comparing between the normal and hot temperature environments (Table 4.12).

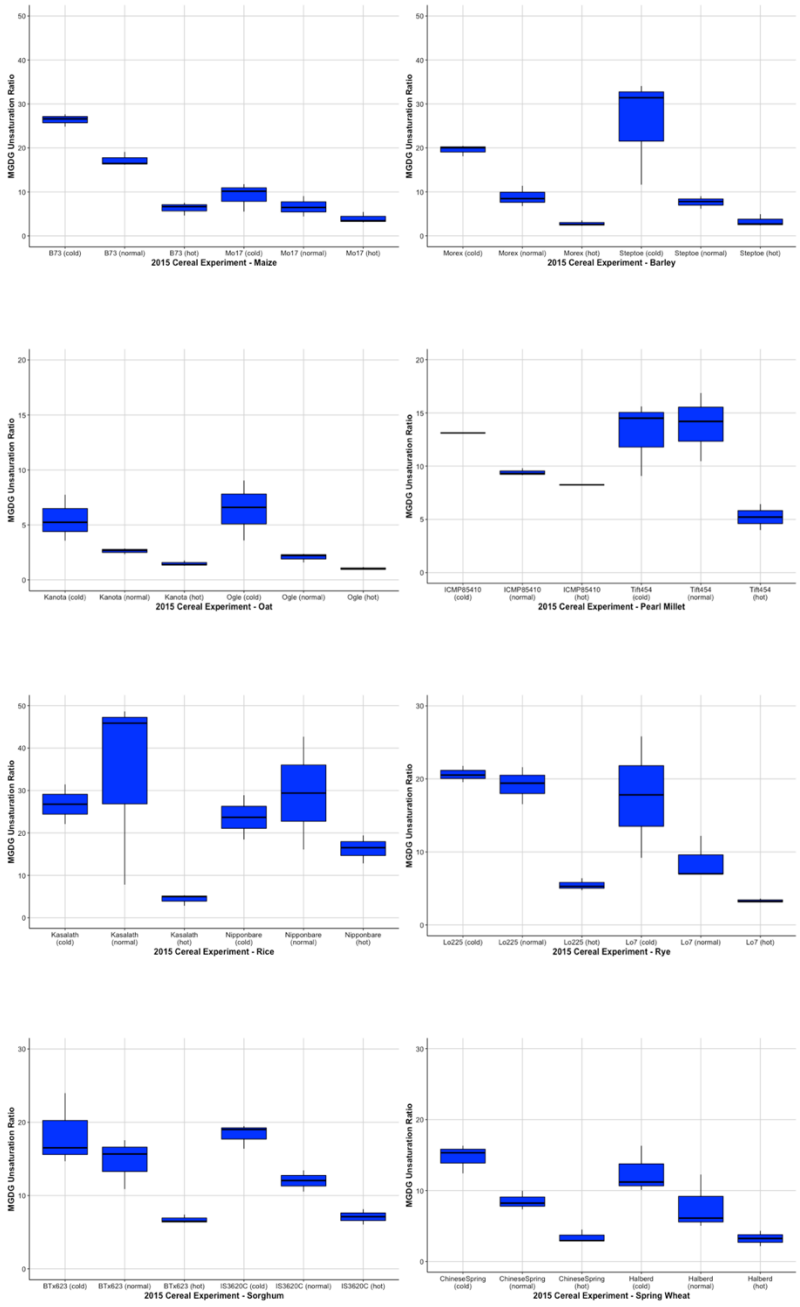

Figure 4.16 Boxplots comparing MGDG Unsaturation Ratios analyzed in sixteen diverse cereal lines in 20°C day/10°C (cold), 30°C day/20°C (normal), and 40°C day/30°C (hot) environments.

Table 4.11 MGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) comparison between Normal vs Cold and Normal vs Hot temperature environments. A t-test comparison was performed for each treatment pair.

Cereal	PlantID	Normal	Cold	P-value	Normal	Hot	P-value
Maize	B73	17.26±1.61	26.36±1.43	0.0335†	17.26±1.61	6.28±1.49	0.0252†
Maize	Mo17	6.64±2.32	9.14±3.24	0.0804	6.64±2.32	4.00±1.26	0.0614
Barley	Morex	8.87±2.34	19.52±1.26	0.0149†	8.87±2.34	2.79±0.63	0.0691
Barley	Steptoe	7.65±1.45	25.71±12.25	0.1053	7.65±1.45	3.33±1.39	0.0091††
Oat	Kanota	2.62±0.28	5.51±2.11	0.1293	2.62±0.28	1.48±0.24	0.0325†
Oat	Ogle	2.06±0.41	6.41±2.73	0.1316	2.06±0.41	1.04±0.10	0.0422†
Pearl Millet	ICMP85410	9.43±0.34	13.12±NA	NA	9.43±0.34	8.25±NA	NA
Pearl Millet	Tift 454	13.85±3.22	13.06±3.50	0.8467	13.85±3.22	5.22±1.73	0.0305†
Rice (Indica)	Kasalath	34.10±22.80	55.44±49.90	0.4761	34.10±22.80	4.37±1.34	0.1617
Rice (Japonica)	Nipponbare	38.11±20.12	44.69±36.78	0.7787	38.11±20.12	16.26±3.30	0.1539
Rye	Lo225	19.20±2.54	20.63±1.12	0.2271	19.20±2.54	5.49±0.83	0.0141†
Rye	Lo7	8.72±3.02	17.61±8.31	0.2981	8.72±3.02	3.30±0.28	0.0760
Sorghum	BTx623	14.70±3.44	18.39±4.93	0.1493	14.70±3.44	6.73±0.59	0.0753
Sorghum	IS3620C	12.01±1.45	18.29±1.66	0.0657	12.01±1.45	7.10±1.04	0.0237†
Spring Wheat	Chinese Spring	8.52±1.33	14.70±2.03	0.0502	8.52±1.33	3.43±0.93	0.0044††
Spring Wheat	Halberd	7.81±3.90	12.55±3.32	0.0218†	7.81±3.90	3.25±1.53	0.1738

†Significantly different $p<0.05$
††Significantly different $p<0.01$

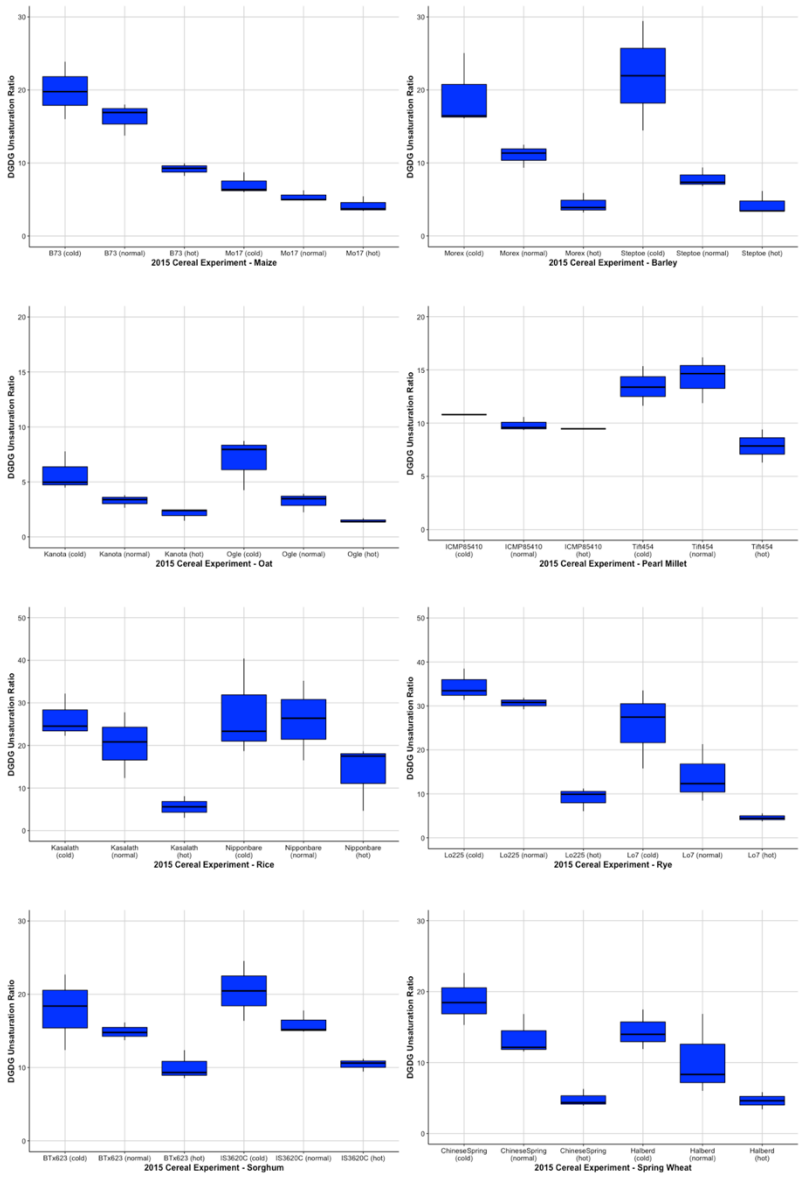

Figure 4.17 Boxplots comparing DGDG Unsaturation Ratios analyzed in sixteen diverse cereal lines in 20°C day/10°C (cold), 30°C day/20°C (normal), and 40°C day/30°C (hot) environments.

Table 4. 12 DGDG Unsaturation Ratio (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) comparison between Normal vs Cold and Normal vs Hot temperature environments. A t-test comparison was performed for each treatment pair.

Cereal	PlantID	Normal	Cold	p-value	Normal	Hot	p-value
Maize	B73	16.22±2.21	19.89±3.94	0.0824	16.22±2.21	9.15±0.87	0.0561
Maize	Mo17	5.37±0.77	7.04±1.47	0.0576	5.37±0.77	4.20±1.08	0.0231
Barley	Morex	11.06±1.59	19.20±5.07	0.1649	11.06±1.59	4.33±1.39	0.0596
Barley	Steptoe	7.85±1.35	25.00±9.18	0.0723	7.85±1.35	4.31±1.60	0.0043
Oat	Kanota	3.28±0.59	5.74±1.78	0.0981	3.28±0.59	2.12±0.56	0.0068
Oat	Ogle	3.22±0.88	6.98±2.39	0.1516	3.22±0.88	1.47±0.20	0.0657
Pearl Millet	ICMP85410	9.84±0.66	10.80±NA	NA	9.84±0.66	9.47±NA	NA
Pearl Millet	Tift 454	14.24±2.18	13.45±1.87	0.7691	14.24±2.18	7.86±2.19	0.0725
Rice (Indica)	Kasalath	20.31±7.73	26.33±5.19	0.3239	20.31±7.73	5.57±2.55	0.1085
Rice (Japonica)	Nipponbare	26.03±9.35	27.48±11.45	0.8647	26.03±9.35	13.60±7.78	0.03051
Rye	Lo225	30.63±1.33	34.44±3.67	0.2993	30.63±1.33	9.06±2.69	0.0056
Rye	Lo7	14.04±6.59	25.59±9.03	0.3099	14.04±6.59	4.58±0.91	0.1023
Sorghum	BTx623	14.89±1.22	17.83±5.18	0.3293	14.89±1.22	10.09±2.02	0.1097
Sorghum	IS3620C	15.96±1.59	24.20±7.66	0.1514	15.96±1.59	10.42±0.89	0.0316
Spring Wheat	Chinese Spring	13.52±2.90	18.79±3.69	0.1878	13.52±2.90	4.86±1.25	0.0131
Spring Wheat	Halberd	10.40±5.71	14.46±2.82	0.1427	10.40±5.71	4.63±1.72	0.2171

†Significantly different $p<0.05$
††Significantly different $p<0.01$

The average MGDG and DGDG Unsaturation Ratios showed consistent and predominantly significant ($p<0.05$) differences when individually compared between cold, normal, and hot growing environments and within warm- or cool-season adaptation groups (Figure 4.18). The average MGDG and DGDG Unsaturation Ratios consistently measured at the lowest levels in the hot temperature environment, intermediate levels in the normal temperature environment, and the highest levels at the cold temperature environment. The phenotypic results were found to be statistically ($p<0.05$) different within each adaptation group when comparing between temperature environments for all scenarios with the exception of the MGDG Unsaturation Ratio measured in warm-season adapted cereals when comparing between cold versus normal temperature and normal versus hot temperature environments and the DGDG Unsaturation Ratio measured in

warm-season adapted cereals when comparing between cold versus normal temperature environments (Table 4.13).

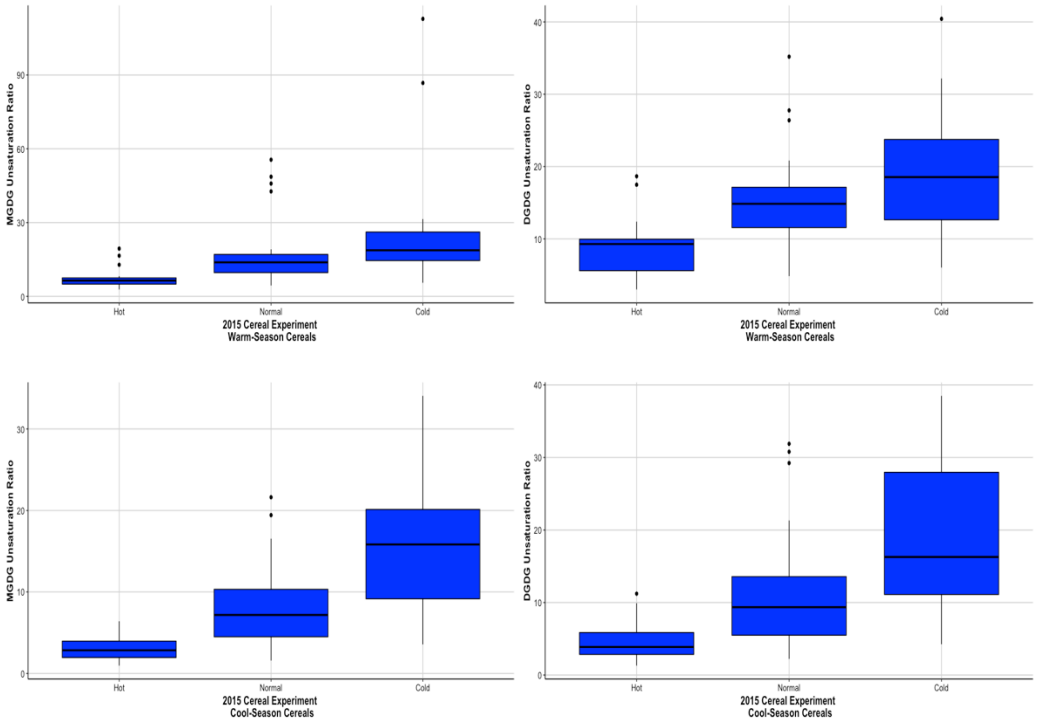

Figure 4.18 Boxplot comparison between warm-season (top) and cool-season (bottom) adapted cereals analyzed in controlled 30°C day/20°C night (normal), 20°C day/10°C night (cold), and 40°C day/30°C night (hot) environments. Warm-season MGDG Unsaturation Ratio (top left), Warm-season DGDG Unsaturation Ratio (top right), Cool-season MGDG Unsaturation Ratio (bottom left), and Cool-season DGDG Unsaturation Ratio (bottom right). Unsaturation Ratios in nmol lipid (36:6) mg^{-1} dry wt/nmol lipid (36:5) mg^{-1} dry wt.

Table 4. 13 Comparison of warm-season and cool-season adapted average MGDG and DGDG Unsaturation Ratios (nmol lipid(36:6) mg^{-1} dry weight/nmol lipid(36:5) mg^{-1} dry weight) between Cold, Normal, and Hot temperature environments. Average values followed by a different lowercase letter are significantly different ($p < 0.05$) according to Tukey HSD.

Environment	MGDG Unsaturation Ratio	DGDG Unsaturation Ratio	MGDG Unsaturation Ratio	DGDG Unsaturation Ratio
	nmol lipid (36:6) mg^{-1} dry wt/ nmol lipid (36:5) mg^{-1} dry wt			
	Warm-season		Cool-season	
Cold (20°C day/10°C night)	25.88^{a}	19.07^{a}	15.33^{a}	18.78^{a}
Normal (30°C day/20°C night)	18.26^{ab}	15.36^{a}	8.18^{b}	11.75^{b}
Hot (40°C day/30°C night)	7.28^{b}	8.77^{b}	3.00^{c}	4.41^{c}

4.4.5 Cereal Temperature Stress Evaluation

This study evaluated two unique lines from six different cereal crops that originate from diverse adaptive environments. Depending on latitude and geographical location, the environments have differing average temperatures during the growing season best suited for optimal growth of the individual crops, in general warm-season and cold-season adaptation. To evaluate the temperature stress tolerance and growth chamber environment most accommodating to vegetative growth of each cereal line, the fresh and dry aerial weights were measured after lipid sampling.

The highest average dry weight was used as a proxy for most agreeable temperature environment for vegetative growth (Table 4.14). For maize, B73 grew best at normal temperature, whereas Mo17 preferred the hot environment. Both barley and pearl millet lines preferred the normal temperature environment, whereas both rye and spring wheat lines preferred the cold temperature environment. The oat line Kanota preferred the cold temperature and Ogle grew best in the normal temperature environment. The Indica rice line, Kasalath, grew best in the cold temperature while the Japonica line, Nipponbare, preferred the normal temperature. For sorghum, BTx623 had the most growth in the hot environment and IS3620C grew best in the normal temperature environment.

Table 4.14 Cereal fresh and dry weights measured after cold (20°C day/10°C night), normal (30°C day/20°C night), and hot (40°C day/30°C night) environment experiments in grams (g). The most favorable temperature environment based on highest average dry weight is indicated in bold.

Cereal	PlantID	Fresh Weight (g)			Dry Weight (g)		
		Cold	Normal	Hot	Cold	Normal	Hot
Maize	B73‡	181.8±16.1	222.4±32.8	173.8±24.7	41.0±7.5	**41.5±3.6**	27.9±2.9
Maize	Mo17§	195.0±40.2	252.5±51.8	234.7±44.5	44.9±19.1	48.1±11.6	**53.4±25.9**
Barley	Morex‡	58.0±12.2	70.2±3.1	27.9±0.5	6.8±1.3	**8.5±0.7**	4.7±0.4
Barley	Steptoe‡	46.4±5.9	55.3±3.6	21.6±2.2	5.9±0.6	**6.6±0.3**	3.9±0.5
Oat	Kanota†	53.1±6.4	49.8±5.2	15.2±2.2	**6.2±0.7**	5.5±0.3	2.4±0.4
Oat	Ogle‡	39.4±2.7	52.4±2.5	16.1±1.9	4.8±0.5	**5.9±0.4**	2.6±0.2
Pearl Millet	ICMP85410‡	89.5±NA	102.6±5.5	65.3±NA	15.4±NA	**19.0±3.3**	6.6±NA
Pearl Millet	Tift 454‡	121.9±17.9	175.8±13.7	214.5±31.0	22.5±4.5	**34.3±4.2**	31.3±2.8
Rice (Indica)	Kasalath†	7.1±6.1	6.7±4.1	4.1±1.7	**1.4±1.1**	1.2±0.7	0.9±0.3
Rice (Japonica)	Nipponbare‡	2.5±1.4	5.9±2.9	3.3±1.8	0.6±0.3	**1.2±0.6**	0.8±0.4
Rye	Lo225†	20.9±2.5	16.5±1.7	2.9±0.3	**2.7±0.3**	2.4±0.3	1.0±0.2
Rye	Lo7†	16.2±2.3	13.3±4.5	6.3±0.5	**2.2±0.3**	1.9±0.6	1.2±0.2
Sorghum	BTx623§	80.0±15.2	137.4±5.0	198.7±10.6	17.7±5.1	28.6±4.9	**41.4±1.6**
Sorghum	IS3620C‡	79.9±14.1	123.0±28.4	128.6±15.7	16.8±3.0	**28.3±6.6**	24.8±3.4
Spring Wheat	Chinese Spring†	42.7±18.3	38.1±11.1	19.2±1.6	**4.7±2.0**	4.4±1.0	3.2±0.3
Spring Wheat	Halberd†	40.8±10.3	20.2±8.4	18.0±1.8	**4.5±1.0**	2.3±1.0	3.2±0.1

†Cold environment preference
‡Normal environment preference
§Hot environment preference

4.5 Discussion

In this study, the normal temperature environment (30°C day/20°C night) was adopted as the de facto baseline for evaluating the changes of selected lipid phenotypes in cold (20°C day/10°C night) and hot (40°C day/30°C night) environments. A comparison of selected average MGDG and DGDG lipid content of eight major cereal crops provides new insight into the composition of the plastidic membranes (Tables 4.2, 4.3, and 4.4). The normal temperature environment comparison of average total leaf lipids revealed that the cereal crops designated in the warm-season group, rice, maize, sorghum, and pearl

millet, had the lowest levels compared to the cool-season group, wheat, barley, rye, and oat. Rice, the only C_3 plant of the warm-season group, had the lowest average total lipid content. Both average MGDG(36:6) and DGDG(36:6) lipid levels were, in general, lowest in the warm-season cereal group and highest in the cool-season group. The importance of increased levels of unsaturation in cold environments is exemplified by the discovery of the cold-induced FAD8 gene in Arabidopsis and maize, which actively transcribes during cold-stress, increasing or maintaining the amount of unsaturated lipids (Gibson et al., 1994; Berberich et al., 1998). These studies suggest having higher levels of the fully unsaturated MGDG(36:6) lipid in the plastidic membranes would be beneficial to cool-season adapted plants to maintain appropriate membrane composition for homeostasis at lower temperatures (Kodama et al., 1994). In fact, the current study shows the cool-season cereal lines had generally higher average amounts of the fully unsaturated MGDG(36:6) and DGDG(36:6) lipids than the warm-season adapted lines at normal temperature. Additionally, the cool-season cereals also generally had higher average amounts of the less unsaturated MGDG(36:5) and DGDG(36:5) lipids. The MGDG Unsaturation Ratio was lowest in the cool-season and highest in the warm-season cereal crops. The higher levels of the less unsaturated lipids in the cool-season cereals compared to the warm-season cereals is likely due to the overall higher total lipid content found in the cool-season cereals.

A comparison of the percent change of lipid phenotypes in cereal lines between normal and cold and normal and hot temperature environments begins to reveal trends among the cool-season and warm-season crops. The average MGDG and DGDG Unsaturation Ratios show differentiation between the warm-season and cool-season

adapted cereal crops when comparing the normal temperature versus the cold temperature environmental treatments (Figures A.5 and A.9). The warm-season adapted lines, in general, had more modest increases of average MGDG and DGDG Unsaturation Ratios than the cool-season adapted lines, which generally had larger increases. Larger MGDG or DGDG Unsaturation ratios indicate a relative higher amount of fully unsaturated MGDG(36:6) or DGDG(36:6) lipid content compared to the less unsaturated MGDG(36:5) or DGDG(36:5) lipid content. This difference in magnitude of change in lipid unsaturation between the normal and cold temperature environments across diverse cereals, which are either warm- or cool-season adapted, provides evidence that higher levels of unsaturation within the plastidic membranes may be a cold temperature adaptation.

The comparison of the percent change in average MGDG and DGDG Unsaturation Ratios between the normal and hot temperature environmental treatments revealed a differentiation between the warm- and cool-season adapted cereal crops (Figures A.14 and A.18). The warm-season adapted lines, in general, had smaller decreases of average MGDG and DGDG Unsaturation Ratios than the cool-season adapted lines, which generally had larger decreases. The smaller MGDG and DGDG Unsaturation Ratios indicate a relative lower amount of the fully unsaturated MGDG(36:6) and DGDG(36:6) lipid content compared to the less unsaturated MGDG(36:5) and DGDG(36:5) lipid content. This relative difference in magnitude of change in lipid unsaturation between the normal and hot temperature environments is of interest, providing evidence that lower levels of unsaturation with the plastidic membranes may be a warm temperature adaptation.

The comparison of dry weights after each temperature treatment provides evidence of the most agreeable temperature environment for each cereal line. In general, the cool-season adapted lines preferred the cold and normal temperature environments and the warm-season adapted lines preferred the normal and hot temperature environments. In this study, the maize line Mo17 preferred the hot temperature whereas B73 preferred the normal temperature environment. This indicates that Mo17 may have higher heat stress tolerance when compared to B73. The oat line Kanota preferred the cold temperature environment, whereas Ogle preferred the warmer normal environment. The Japonica rice line Nipponbare preferred the normal temperature environment whereas the Indica rice line preferred the cold temperature environment. The sorghum line IS3620C preferred the normal temperature environment, whereas BTx623 preferred the warmer hot temperature environment. The differential environmental preferences of these cereal lines will be useful in future genetic studies. The cereal lines that do not show differences in environmental preference will not be as useful in future analysis, therefore more studies will be needed to identify germplasm with differential thermotolerance in barley, pearl millet, spring wheat, and rye.

4.6 Conclusion

This analysis of eight major cereal crops from different adaptive backgrounds provides additional evidence into how selected MGDG and DGDG lipid phenotypes change depending on environmental temperature stress. In a cold stress environment, the level of MGDG and DGDG unsaturation decreases, whereas in the heat stress environment, the level of MGDG and DGDG unsaturation increases. These changes are

reflected by the MGDG and DGDG Unsaturation Ratios.

This study provides additional preliminary results which indicate the MGDG and DGDG Unsaturation Ratios may function as lipid biomarkers which can be used to differentiate temperature adaptation in cereals.

CHAPTER 5. CONCLUSION

5.1 Conclusion

This research investigated total leaf lipids in cereal crops and their association with high and cool temperature stresses. The focus was prioritized on maize plastidic lipids and their levels of unsaturation in relation to high temperature stress; however, a broader study of the plastidic lipids in eight major cereal species at both high and cool temperature stresses were examined. The preliminary study of the inbred line B73 provided support to previously published research that the plastidic lipid classes, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), make up most of the lipids identified in maize leaves. These lipids were found to be highly unsaturated and to dynamically change in both amount and level of unsaturation when exposed to high temperature stress in a controlled environment. It was hypothesized that ratios of MGDG and DGDG lipids containing acyl chains with total 36 carbons and 6 double bonds (36:6) compared to those with 36 carbons and 5 double bonds (36:5), here termed the MGDG and DGDG "Unsaturation Ratios", may be used to differentiate changes in the plastidic lipid membrane unsaturation levels with potential to identify heat tolerant maize genotypes. Lower levels of MGDG and DGDG Unsaturation Ratios reflect a relative decrease in unsaturation of plastidic membrane lipids and therefore were hypothesized to be indicative of an increase in high temperature tolerance.

Field observations in a high temperature environment identified maize inbred lines with contrasting high temperature tolerance. The CIMMYT inbred line, LPS-F32, exhibited increased high temperature tolerance as compared to the temperate B73 inbred line. An analysis of B73, LPS-F32, and B73xLPS-F32 revealed significant decreases in the MGDG and DGDG Unsaturation Ratios after exposure to high temperature stresses compared to a near-optimal growing environment. The high temperature tolerant line, LPS-F32, had significantly lower levels than the heat susceptible B73 in both the near-optimal and high temperature environments. This preliminary analysis indicated that the MGDG and DGDG Unsaturation Ratios may be useful lipid biomarkers to aid in the selection of high temperature stress tolerant germplasm and provided an indication that they may be useful for identifying high temperature tolerant germplasm even when high temperature environmental conditions are not present.

The diverse set of twenty-five maize Nested Association Mapping (NAM) population founder inbred lines and B73 were used to investigate the variation of the MGDG and DGDG Unsaturation Ratios and to identify a subpopulation with significantly contrasting trait levels for analysis. The analysis of MGDG and DGDG Unsaturation Ratios in field-grown maize revealed significant levels of variation between the NAM founder inbred lines. B73 and B97 were selected for multi-year phenotypic observation in a field-based high temperature environment due to their significant differences in MGDG and DGDG Unsaturation Ratios. B97 is a temperate inbred line with the lowest levels of MGDG and DGDG Unsaturation Ratios which significantly contrast with the higher levels found in B73. As predicted based on the MGDG and DGDG Unsaturation Ratios, B97 was found to have increased high temperature tolerance

compared to the high temperature susceptible B73 after multi-year field-based high temperature environment observations. Linkage mapping analysis of the MGDG and DGDG Unsaturation Ratios in the B73 x B97 NAM RIL subpopulation identified major QTL associated with ZmFAD7 and ZmFAD8 genes, providing evidence of genetic variation of these lipid traits.

The time of day sampling leaf lipids occurs was investigated and shown to have some significance when comparing B73 sampled over a 24-hour period and it should be considered important when planning large projects in which sampling may take most of the day. The growth and developmental stages appear to have significance when comparing B73 and B97 leaf lipids across different stages. These two maize inbred lines were statistically distinguishable from each other based on the MGDG and DGDG Unsaturation Ratios across all developmental stages analyzed, apart from the DGDG Unsaturation Ratio at the R stage. However, this study indicates that sampling around the same developmental stage should be considered to best compare within inbred lines. This may be important when experiments consist of germplasm with diverse maturity or environmental stress is changing developmental timelines. Results from this study provide preliminary justification for minimizing the time between the beginning and end of sampling and to sample at similar growth and development stages to minimize confounding results in field based total leaf lipid analysis.

The analysis of eight major cereal crops from different adaptive backgrounds provides additional evidence into how the MGDG and DGDG Unsaturation Ratio traits change depending on environmental temperature stress. In a cold stress environment, the level of MGDG and DGDG unsaturation decreases, whereas in the heat stress

environment, the level of MGDG and DGDG unsaturation increases. These changes are reflected by the MGDG and DGDG Unsaturation Ratios. This study provides additional preliminary results which indicate the MGDG and DGDG Unsaturation Ratios may function as lipid biomarkers which can be used to differentiate temperature adaptation in cereals.

In conclusion, leaf lipid content and levels of unsaturation in cereal crops, specifically the plastidic lipid membranes, dynamically change when the plants are exposed to cool temperature and heat stresses. The MGDG and DGDG Unsaturation Ratios have been shown to significantly differentiate known high temperature tolerant from susceptible maize inbred lines and to identify a high temperature tolerant maize inbred line. However, additional multi-year and multi-environment research is needed to fully characterize and evaluate the efficacy of the MGDG and DGDG Unsaturation Ratio biomarkers for classifying thermotolerance of maize.

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APPENDIX

STANDARDIZED PERCENT CHANGE OF SELECTED LIPID PHENOTYPES IN CEREALS BETWEEN NORMAL, COLD, AND HOT ENVIRONMENTS

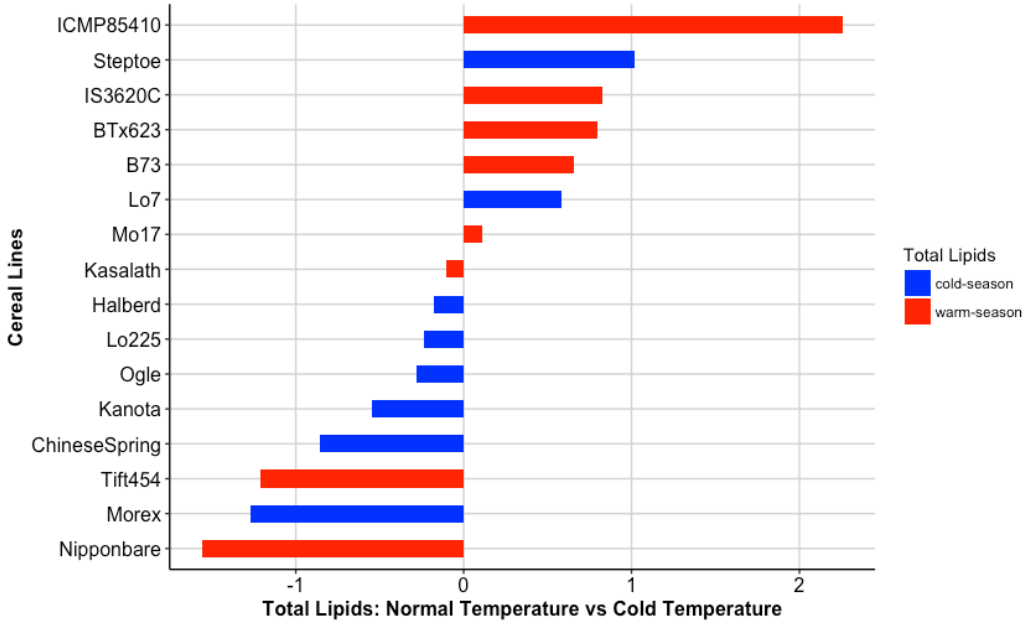

Figure A.1 Standardized percent change of the cereal lines' Total Lipid content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

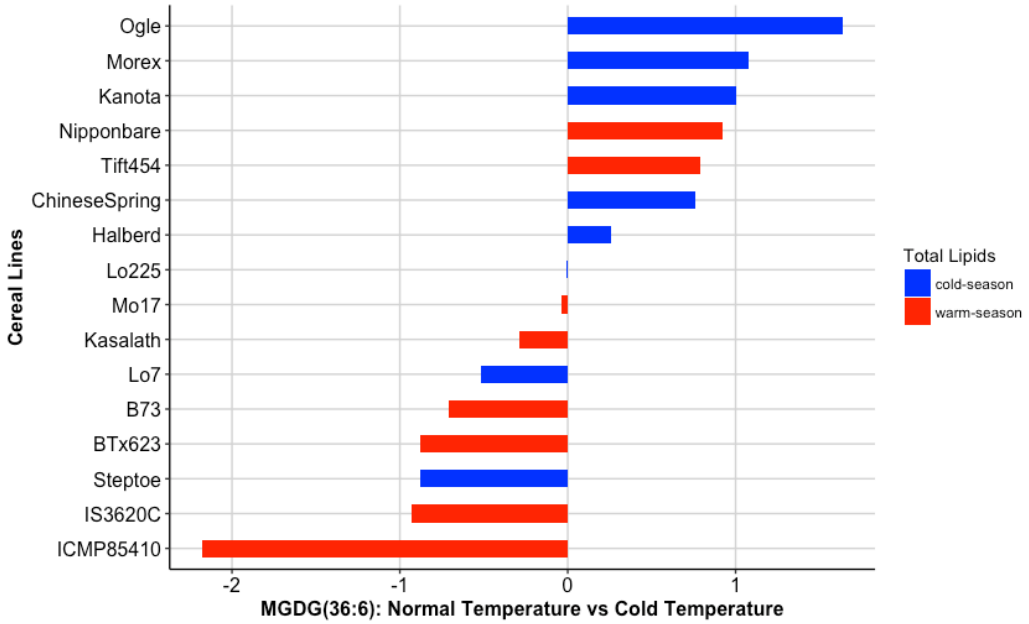

Figure A.2 Standardized percent change of the cereal lines' MGDG(36:6) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

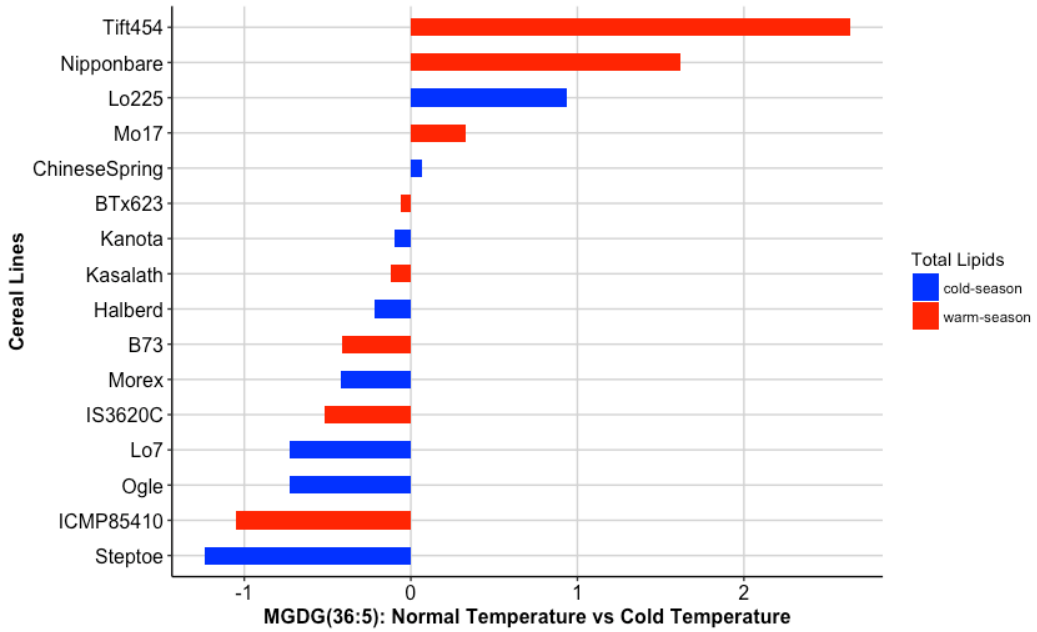

Figure A.3 Standardized percent change of the cereal lines' MGDG(36:5) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

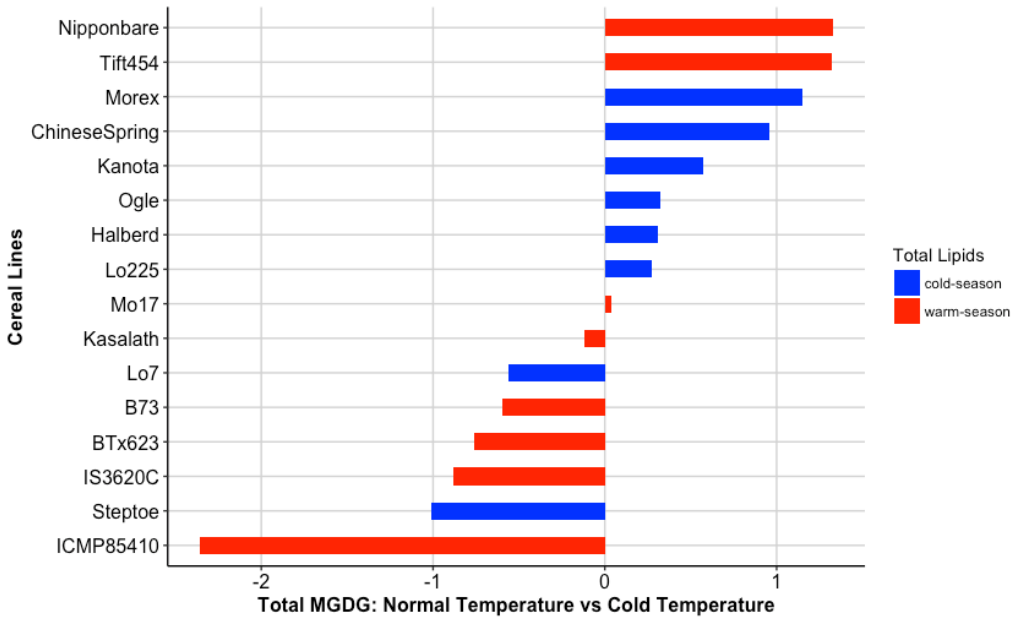

Figure A.4 Standardized percent change of the cereal lines' Total MGDG content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

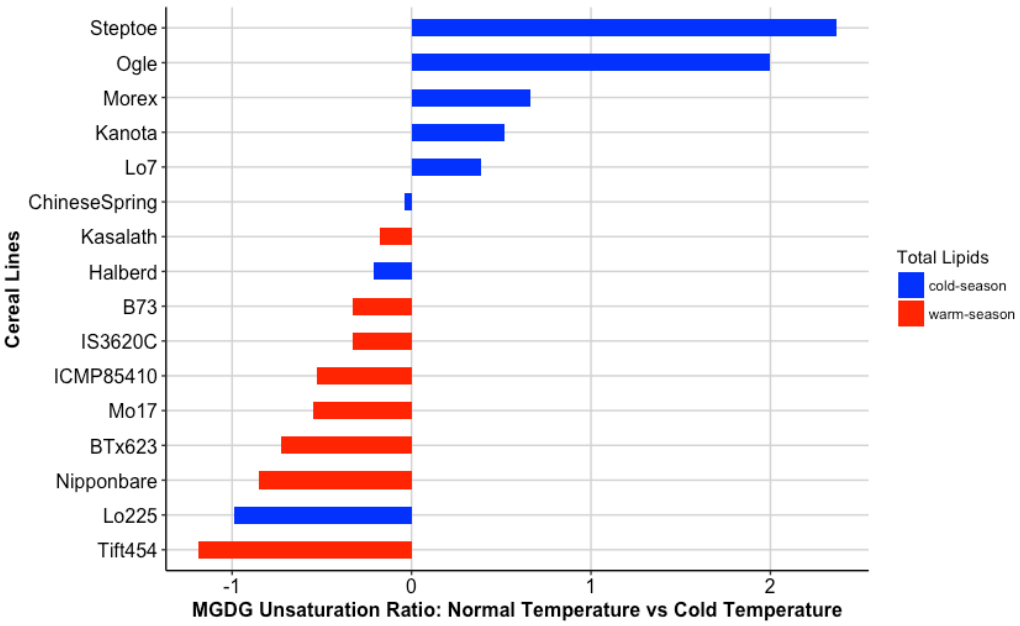

Figure A.5 Standardized percent change of the cereal lines' MGDG Unsaturation Ratio content between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

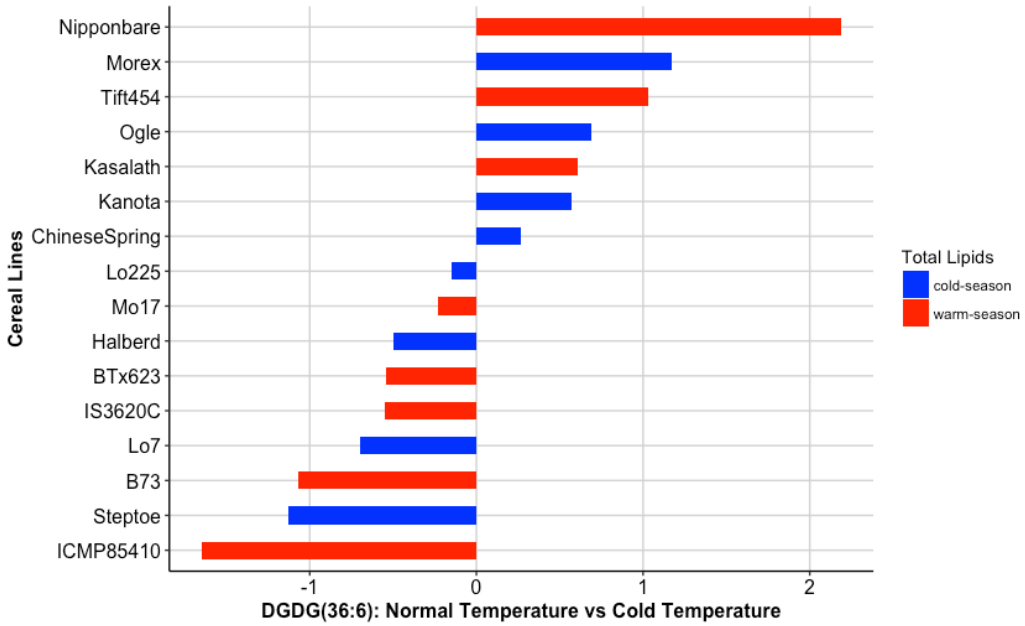

Figure A.6 Standardized percent change of the cereal lines' DGDG(36:6) (nmol mg^{-1} dry wt) content between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

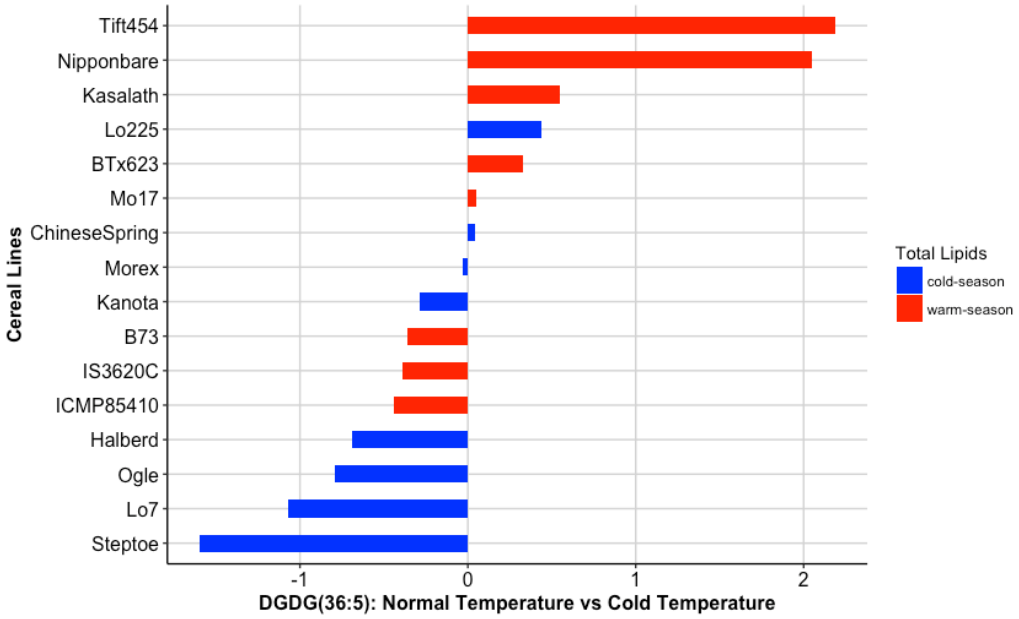

Figure A.7 Standardized percent change of the cereal lines' DGDG(36:5) (nmol mg^{-1} dry wt) content between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

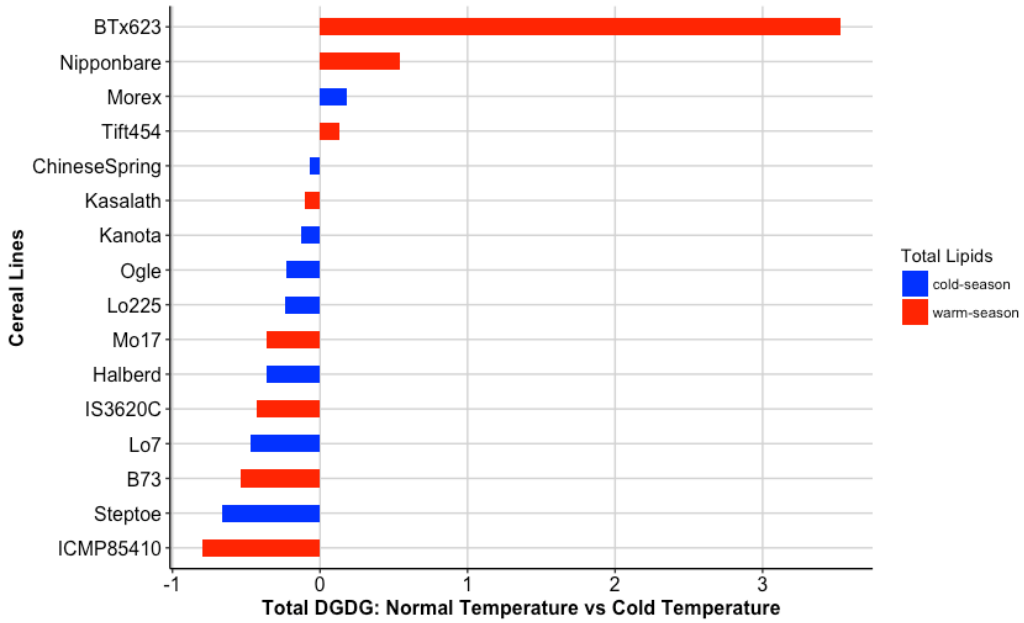

Figure A.8 Standardized percent change of the cereal lines' Total DGDG (nmol mg^{-1} dry wt) content between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

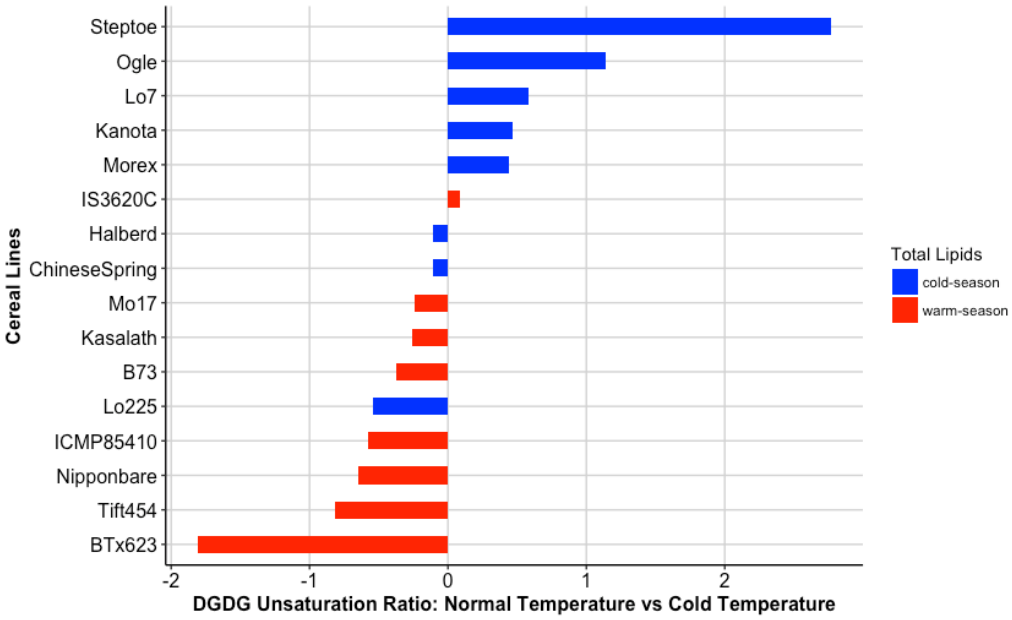

Figure A.9 Standardized percent change of the cereal lines' DGDG Unsaturation Ration content between normal (30°C day/20°C night) and cold (20°C day/10°C night) temperature treatments.

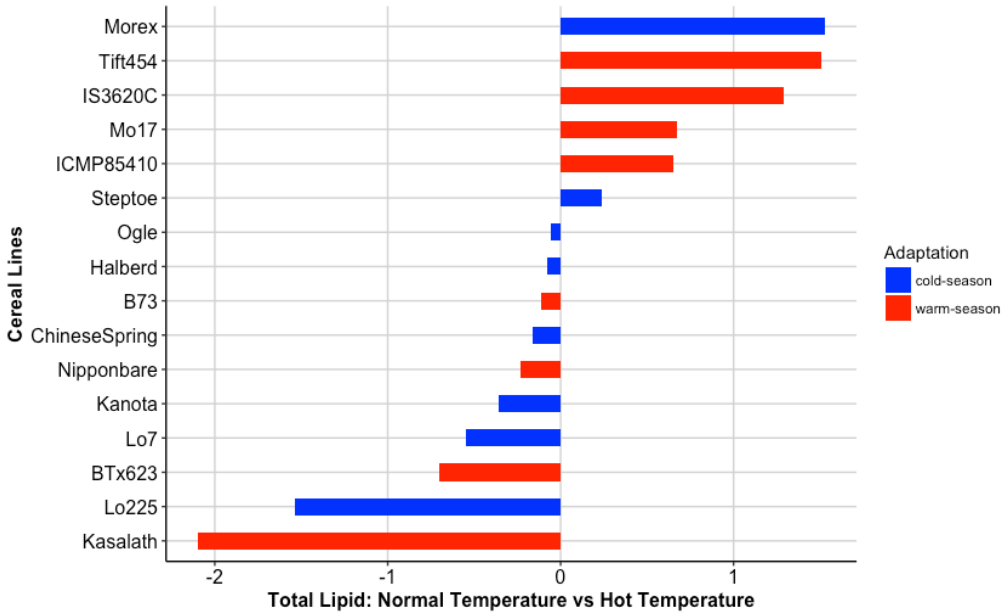

Figure A.10 Standardized percent change of the cereal lines' Total Lipid content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

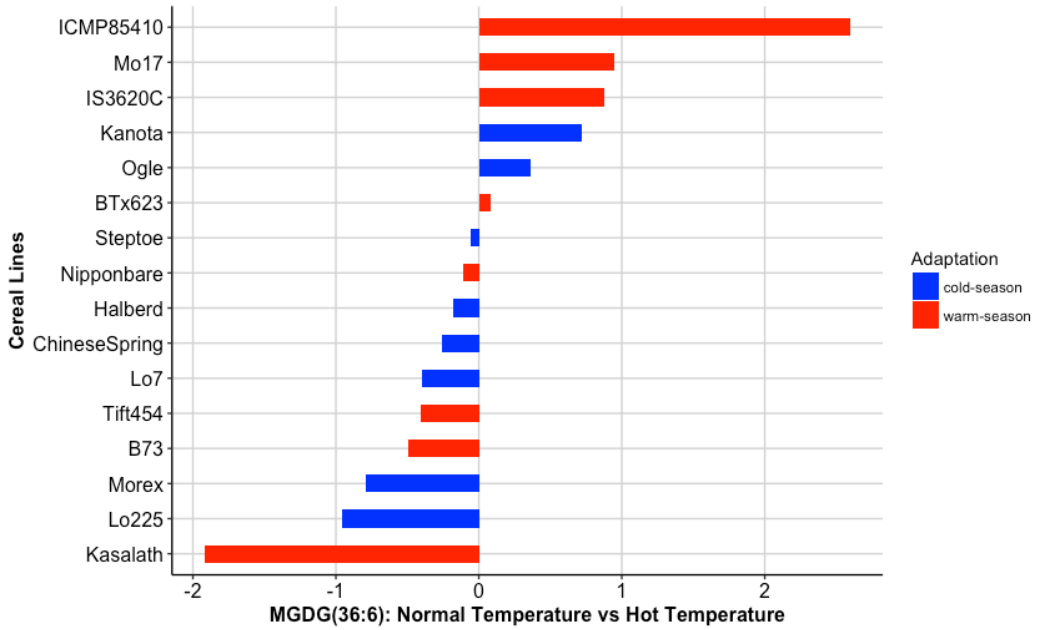

Figure A.11 Standardized percent change of the cereal lines' MGDG(36:6) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

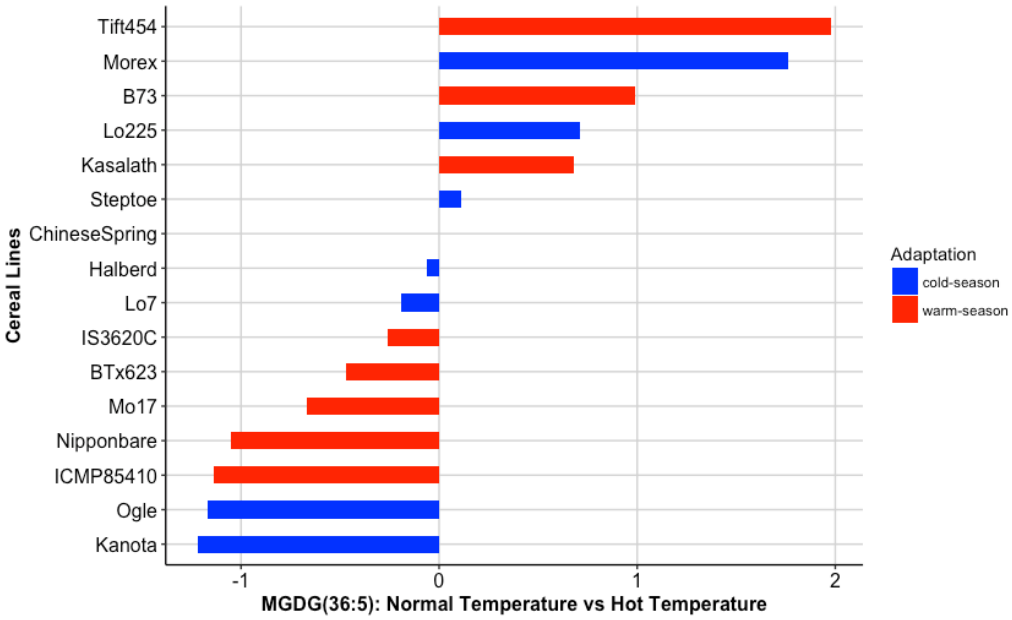

Figure A.12 Standardized percent change of the cereal lines' MGDG(36:5) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

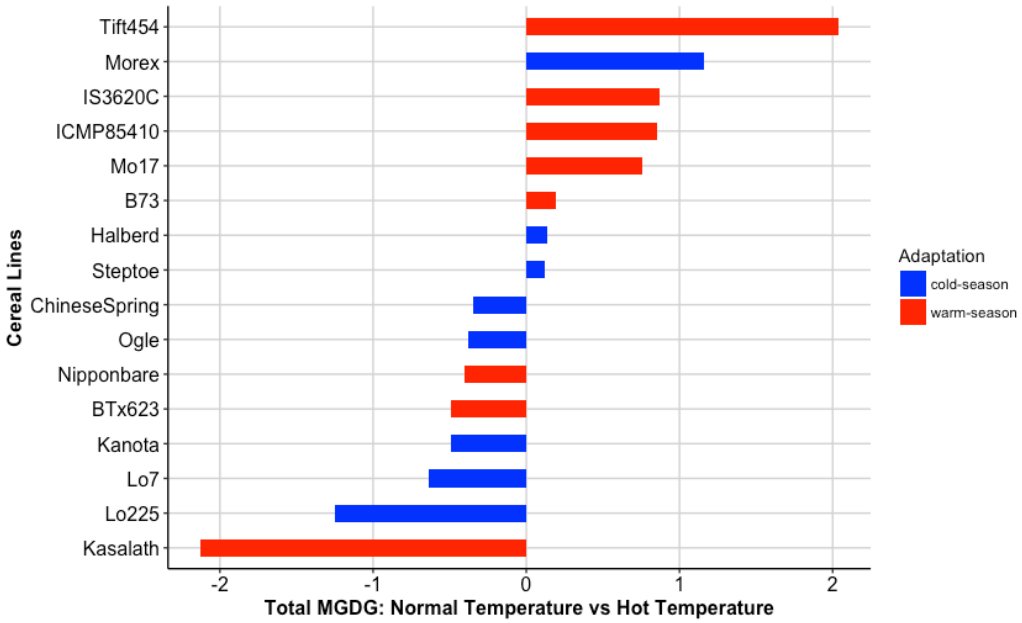

Figure A.13 Standardized percent change of the cereal lines' Total MGDG content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

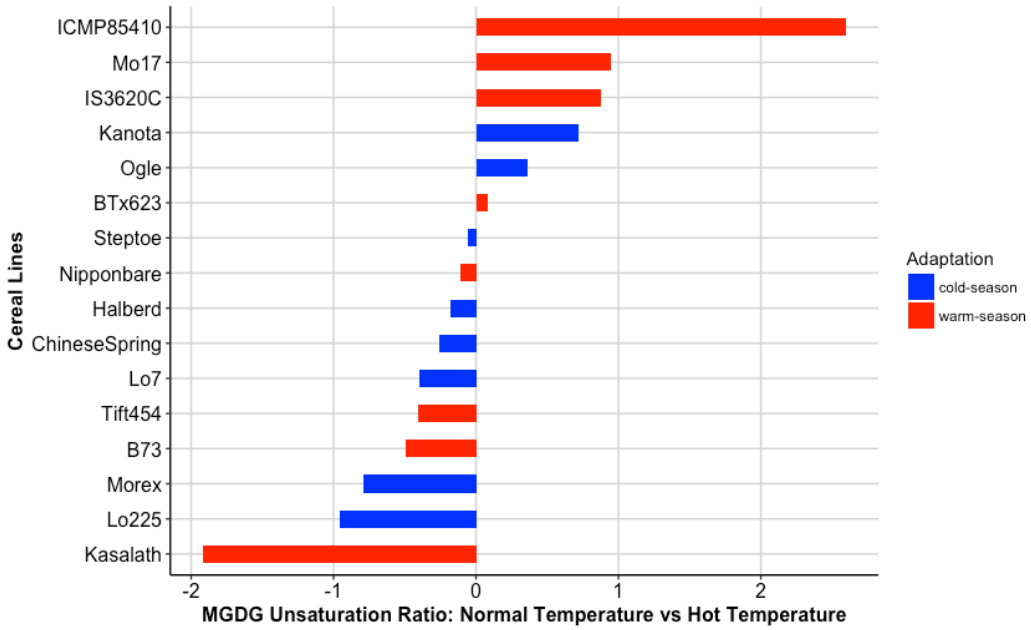

Figure A.14 Standardized percent change of the cereal lines' MGDG Unsaturation Ratio between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

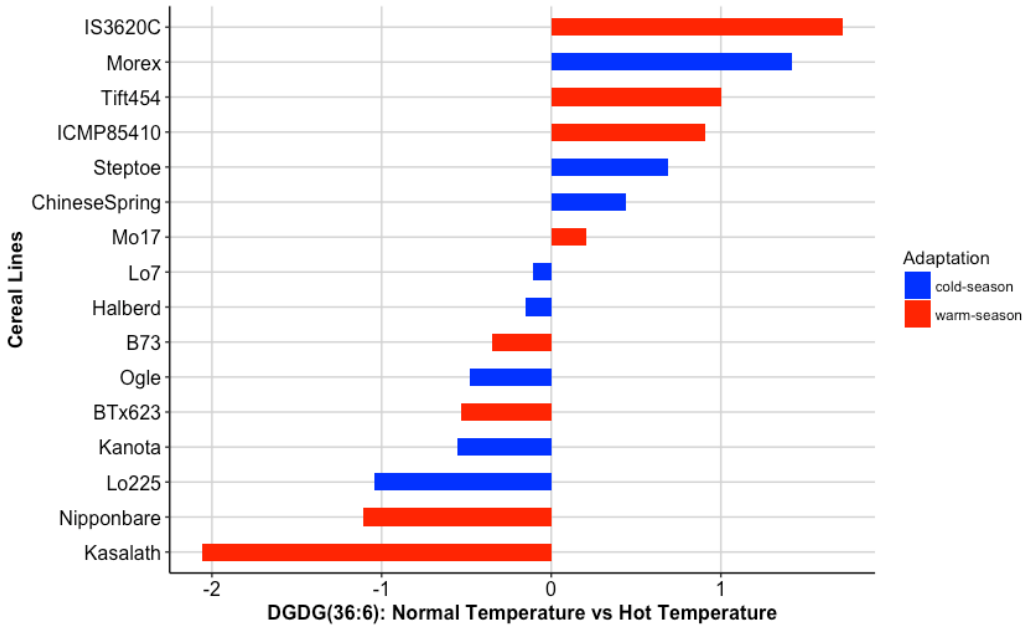

Figure A.15 Standardized percent change of the cereal lines' DGDG(36:6) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

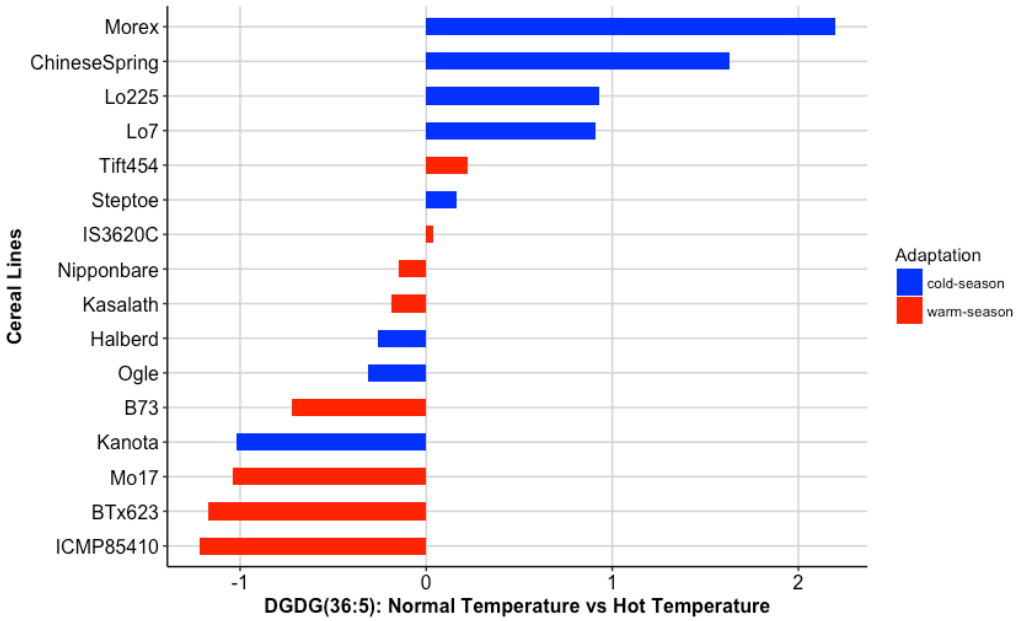

Figure A.16 Standardized percent change of the cereal lines' DGDG(36:5) content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

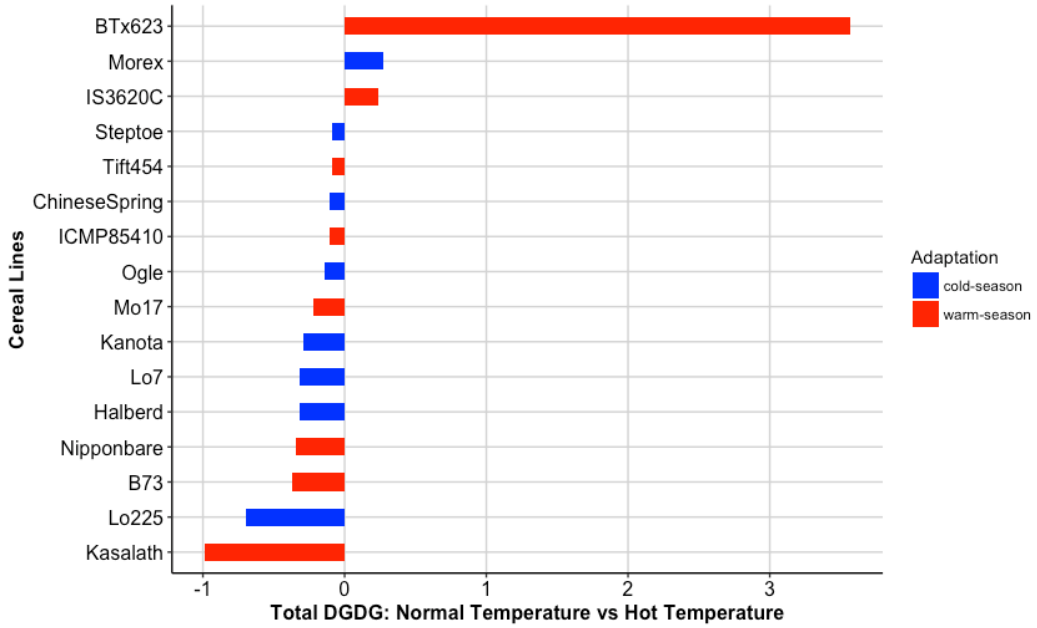

Figure A.17 Standardized percent change of the cereal lines' Total DGDG content (nmol mg^{-1} dry wt) between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

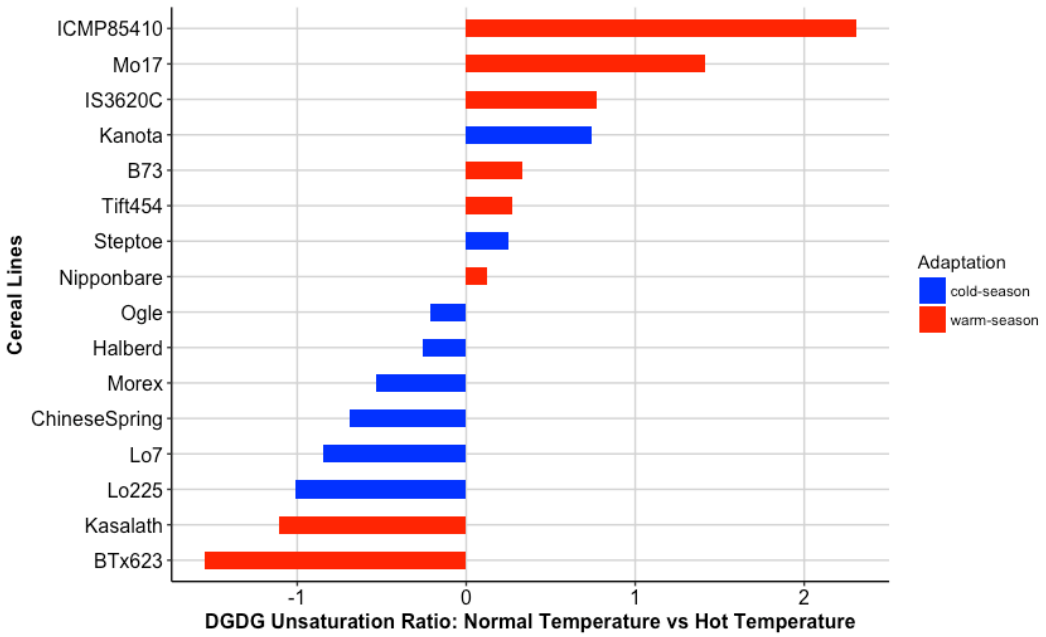

Figure A.18 Standardized percent change of the cereal lines' DGDG Unsaturation Ratio between normal (30°C day/20°C night) and hot (40°C day/30°C night) temperature treatments.

PUBLICATION

Zheng, P, Babar, M. D. A., Parthasarathy, S., Gibson, R., Parliament, K., Flook, J., Patterson, T., Friedemann, P., Kumpatla, S., and Thompson, S. (2014). A truncated FatB resulting from a single nucleotide insertion is responsible for reducing saturated fatty acids in maize seed oil. Theoretical and Applied Genetics, 127, 1537-15

PATENT

Ren, R., Nagel, B. A., Gibson, R., Gao, Y. S., Mammadov, J. (2018). Maize cytoplasmic male sterility (CMS) S-type restorer Rf3 gene, molecular markers and their use. Patent No.: US 9,883,643