PHYSIOLOGICAL AND LIPIDOMIC CHARACTERIZATION OF HIGH TEMPERATURE STRESS AND TRAITS ASSOCIATED WITH TOLERANCE IN WHEAT

by

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B.S., Kerala Agricultural University, Kerala, India, 2007
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

High temperature is a major environmental factor that limits wheat productivity. Climate models predict greater increases in night temperature than in day temperature. The objectives of this research were to quantify the effects of high day and night temperatures during anthesis on physiological (chlorophyll fluorescence, chlorophyll concentration, leaf level photosynthesis, lipid peroxidation and membrane damage), biochemical (reactive oxygen species [ROS] concentration and antioxidant capacity in leaves) and yield traits and membrane lipid profile and identify the lipids that are associated with high temperature response in wheat. Winter wheat genotypes Ventnor (heat tolerant) and Karl 92 (heat susceptible) were grown at optimum temperatures (25/15°C, maximum/minimum) until the onset of anthesis. Thereafter, plants were exposed to high night (HN, 25/24°C), high day (HD, 35/15°C), high day and night (HDN, 35/24°C) or optimum temperatures. Compared with optimum temperature, HN, HD and HDN increased ROS concentration, lipid peroxidation and membrane damage and decreased antioxidant capacity, photochemical efficiency, photosynthesis, seed set, grain number and grain yield. Impact of HN and HD was similar on all traits, when stress was imposed for seven days. High day and night temperatures resulted in significant changes in the amount of plastidic and extra-plastidic lipids and lipids with oxidized acyl chains (ox-lipids) in both genotypes. The decrease in lipid unsaturation levels of complex lipids at high temperatures was predominantly due to decrease in 18:3 fatty acid and increase in 18:1 and 16:0 fatty acids. We identified novel odd-numbered long-chain fatty acid-containing phospholipids, which were highly responsive to high temperature stress. Ventnor had higher amounts of sterol glycosides (SG) and lower amounts of ox-lipids at high temperatures than Karl 92; thus SGs and ox-lipids may be potential biomarkers for heat tolerance and susceptibility, respectively, in wheat. Co-occurring lipids, which are up-or-down-regulated together through time under high day and night temperatures formed groups, which were experiencing coordinated metabolism. These results suggest that high day and night temperatures during anthesis cause damage of a similar magnitude to wheat, if stress is imposed for a short term (seven days) and compositional changes in lipid profile in response to high temperature contribute to heat tolerance.

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Table of Contents

List of Figures

List of Tables

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Dedication

To all farmers who have been sowing seeds of hope on our planet

Chapter 1 - Literature Review

Importance of Wheat

Wheat (*Triticum aestivum* L.) is the second most important cereal crop in the world after rice (*Oryza sativa* L.) (FAO, 2015). It is a widely adapted crop grown in a vast range of environments ranging from temperate, irrigated areas to tropical, high-rainfall areas and from warm, humid conditions to cold, dry conditions (Acevedo et al., 2002). Wheat is one of the earliest domesticated food crops in the world (Curtis, 2002) and is believed to have originated in south-western Asia or more specifically, in the fertile crescent about 8000 to 10000 years ago (Gill and Friebe, 2002).

Wheat is an important staple food in most part of the globe and is used to make flour for variety of foods such as breads, biscuits, cookies, cakes, breakfast cereal, pasta, noodles and couscous (Cutis, 2002). Wheat is the most important source of carbohydrate in a vast majority of countries (Curtis, 2002). Its nutritional content also includes minerals, vitamins and lipids. A predominantly wheat-based diet provides more fibers than a meat-based diet (Johnson et al., 1978). Wheat is also used as an animal feed, biofuel and as a construction material for roofing thatch and for the manufacture of adhesives, paper additives and alcohol (Curtis, 2002). Considering its increased importance in global food-economics system, the demand for wheat grain is expected to increase in the future with the increase in global population.

Classification of Wheat

Botanical classification of bread wheat is outlined in Fig. 1.1. The subtribe Triticinae in which the genus *Triticum* belongs to, includes four wild wheat genera namely, *Aegilops, Agropyron, Haynaldia* and *Secale*. Major cultivated species of wheat are bread wheat (*T. aestivum*; hexaploid) and durum wheat (*T. turgidum ssp. durum*; tetraploid). Primitive classification of wheat, based on morphological characters, classify wheat into three series: einkorn, emmer and dinkel. Based on growing season, wheat is classified into winter wheat and spring wheat. Winter wheat is planted in autumn, usually between September and December and harvested in July. It uses autumn moisture for germination, sprouts before freeze, becomes dormant until soil gets warmed up in the next spring and uses early spring sunshine, warmth and rainfall for growth (Curtis, 2002). It requires a short period of cold temperature (0-5°C) to flower

(Curtis, 2002). Development of winter wheat requires exposure of seedlings to a temperature of 3-8°C. Spring wheat is sown in April (in spring as the name indicates) and harvested in August (late summer). Spring wheat does not require low temperature for development. If winter wheat is sown in spring, it cannot head, flower and set seeds in the same growing season, however, if seeds get vernalization treatment, winter wheat will flower even if planted in spring. Winter wheat requires the ability of 'winter hardiness' to survive in the winter. Based on grain hardness, wheat is classified into hard wheat (high protein content, used for bread) and soft wheat (low protein content, used for pastries). Based on grain color, wheat is classified into red and white wheat.

Morphology of a Wheat Plant

A wheat plant produces two types of roots; seminal roots and nodal roots (Kirby, 2002). The shoot of a wheat plant shows determinate growth, i.e. it terminates with a spike (Kirby, 2002). It commonly produces 1-9 tillers per plant (Peterson, 1965). Generally, winter wheat produces more tillers than spring wheat and late-maturing varieties produce more tillers than early maturing varieties. Not all tillers produce an inflorescence in wheat (Gallagher and Biscoe, 1978).

Wheat inflorescence is called as a spike, which can be lax, dense or compact depending upon the distance between different florets within the spikelet. Each spikelet consists of a central, zig-zag, jointed axis called rachis. The two boat shaped bracts at the base of rachis is called glumes which become the chaff or hull at maturity. Florets start from nodes or joints of rachis and each floret has the potential to produce a grain at maturity. Each flower consists of lemma, palea, three stamens and a feathery stigma (Peterson, 1965). Lemma and palea encase the wheat grain. Lemma contains awns at the end; however bread wheat can be awned or awneless. In wild wheat, glume and lemma are difficult to be removed at threshing; making them 'hulled wheat'. The remaining species produce 'naked wheat'.

Wheat grain is called a caryopsis and consists of a single seed enclosed in a membranous pericarp. Wheat endosperm consists of starchy endosperm and aleurone layer. Starchy endosperm contains storage proteins, referred to as gluten consisting of four types of proteins namely, gliadins, glutenins (major components), albumins and globulins (minor components) (Wieser et al., 1998).

Growth and Development

Growth habit of wheat can be prostrate (winter), erect (spring) and intermediate (semi-winter). Haun's scale (Haun, 1973), Feeke's scale (Large, 1954) and Zadoks' scale (Zadoks et al., 1974) are used to indicate wheat growth stages.

Germination in wheat is generally not dependent upon the light conditions; it proceeds even in light or dark. Generally, the seed requires 30-40% moisture content for germination. Optimum temperature for germination is 20-25°C. Usually, emergence is noticed in about 7-14 days after sowing (Gallagher and Biscoe, 1978).

Flowering starts in the spike of main stem followed by the tillers in the order of their emergence. Anthesis starts at the middle of the spike and proceeds to upper and lower portions (Evans et al., 1972). Anthesis within a spike completes within 3-5 days (Kirby, 2002). Ripening of grain comprises of 'water -ripe (pre-milk stage), milky- ripe (milk stage), mealy- ripe (soft-dough), waxy-ripe (hard dough or yellow ripe), fully-ripe (ripe) and dead-ripe stages (Hanft and Wych, 1982).

Wheat is a long day plant. It performs the C_3 type of photosynthesis (Acevedo et al., 2002). Base temperature (T_{base}) for physiological processes in wheat is 0-4°C (Acevedo et al., 2002).

Genetics

The genus *Triticum* possesses a basic chromosome number of seven (1x=7; Gill and Friebe, 2002). Cultivated species of wheat belong to three ploidy groups; diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42; Gill and Friebe, 2002). The polyploid wheats contain both homologous (genetically identical chromosomes within the genome) and homoeologous (genetically related chromosomes in different genomes) chromosomes; homoeology is observed throughout the tribe Triticeae (Gill and Friebe, 2002).

Compared to other crops, study and manipulation of wheat genome is increasingly challenging due to its complex hexaploid nature and large size with increased number of repetitive sequence (90%; Skovmand et al., 2002). Bread wheat genome has a size of ~17 Gb (organized into 21 pairs of chromosomes, each of the seven pairs belonging to one of the three genomes A, B and D (Gill et al., 2004; Eversole et al., 2014). The unique heterochromatic banding (C-banding) patterns (Gill et al., 1991) and molecular karyotyping of wheat

chromosomes (Pederson and Langridge, 1997) make their identification efficient. The evolution of cultivated wheat species involved alloploidization among related genomes (Kilian et al., 2007). Phylogenetic evolution of bread wheat is shown in Fig. 1.2 (Gill and Friebe, 2002).

Wheat genetic resources, which serve as the gene pool for crop improvement can be categorized into modern cultivars under current use, obsolete cultivars, which were commonly cultivated in the past and identified in the pedigrees of modern cultivars, landraces, wild relatives of Triticeae tribe, genetic and cytogenetic stocks and breeding lines (Frankel, 1977; FAO, 1983). Collection of wild wheat species in their region of origin, collection of landraces in places where they have not been collected before and acquisition of improved, obsolete and new cultivars from all over the world are important for wheat germplasm collection and preservation activities (Skovmand et al., 2002; Gill et al., 2006).

Effect of High Temperature Stress on Wheat

High temperature stress is a major environmental factor that limits yield in wheat. Every 1°C increase above a mean temperature of 23°C decreases wheat yield by ~10% (Gibson and Paulsen, 1999). More than 40% of total wheat area is affected by high temperature stress (Reynolds et al., 2001). High temperature affects wheat yield either through chronic stress by prolonged, moderately high temperatures up to 32°C or through heat-shock, which is sudden, but comparatively brief exposure to 33°C and above (Paulsen, 1994; Savin et al., 1997, Yang et al., 2002). The impact of high temperature stress on crop depends up on intensity, rate of increase, duration of stress and stage of crop development (Wahid et al., 2007; Prasad et al., 2008a). High temperature stress induces several alterations in physiological, biochemical and molecular components of wheat crop production.

Effect of High Temperature Stress on Physiological Traits

Photosynthesis

Photosynthesis is one of the most sensitive processes to high temperature in wheat (A1-Khatib and Paulsen, 1984). Photosynthetic rates exhibit a sharp decline when wheat plant is exposed to high temperature stress during vegetative or reproductive phase (A1-Khatib and Paulsen, 1984; Grover et al., 1986). Optimum temperature for photosynthesis has a broad range (20 to 30°C) in wheat, however, the rate photosynthesis declines rapidly at temperatures >30°C

(Wardlaw, 1974). Net photosynthetic rate throughout the crop cycle is a major parameter controlling biomass production and grain yield of wheat under heat stress (Reynolds et al., 2000). Al-Khatib and Paulsen (1990) reported that high temperature (32/27°C day/night) at seedling stage or from anthesis to maturity decreased average leaf photosynthetic rate of wheat by 32 and 11%, respectively. Prasad et al. (2008b) reported reduction in photosynthetic rates due to high night temperatures (>14°C) in wheat. Photosynthesis happening at awns is more temperature tolerant compared to that in leaves (Blum, 1986). Mobilization of stem reserves from pre-anthesis photosynthesis to developing grains is increasingly important for grain filling when current photosynthesis is inhibited by heat stress (Yang et al., 2002). Considerable genetic variability has been reported in wheat for net photosynthetic rate under high temperature conditions (Reynolds et al., 2000).

Disruptions in the structure and function of chloroplasts, reduction in chlorophyll content and inactivation of chloroplast enzymes under high temperature are major reasons for decreased photosynthesis in wheat under heat stress (Xu et al., 1995; Farooq et al., 2011). Reduction in net photosynthetic rate under heat stress during grain filling period is closely associated with chlorophyll loss (Reynolds et al., 2000) and variation in the ratio between chlorophyll *a* and chlorophyll *b* due to premature leaf senescence (Al-Khatib and Paulsen, 1984; Harding et al., 1990). High temperature initially accelerates thylakoid membrane breakdown, which leads to electrolyte leakage and disruption of all photochemical reactions, especially, photosystem II (PS II) and cytochrome f/b6-mediated reactions, which ultimately lead to drastic reduction in rate of photosynthesis (Al-Khatib and Paulsen, 1990; Harding et al., 1990). Photosystem II has a major role in the responses of photosynthetic reactions to high temperature. Photosystem II is more sensitive to high temperature than PS I (Heckathorn et al., 1998). In wheat, high temperature causes significant damage to different sites of PS II (Sharkova, 2001). Since wheat is a cool season crop, its PS II is more sensitive to heat stress compared to warm season crops such as rice and pearl millet (*Pennisetum glaucum*; Al-Khatib and Paulsen, 1999). Heat stress also causes cessation of photophosphorylation due to damage of thylakoid membrane (Dias and Lidon, 2009).

Stomatal conductance

Increased stomatal conductance (mmol m^{-2} s^{-1}, measure of rate of CO_2 entering or water vapor exiting through the stomata) leads to transpirational cooling and canopy temperature

depression (CTD). Higher stomatal conductance and associated leaf cooling provides an avoidance type of heat resistance to high temperature (Lu et al., 1998). Reynolds et al. (1998) showed a positive correlation between stomatal conductance and yield in wheat grown under hot environments. Lu et al. (1998) identified stomatal conductance as a useful selection criterion in wheat for high yields. Positive correlation is reported between CTD and stomatal conductance and between CTD and yield in wheat grown under hot environments (Amani et al., 1996; Reynolds et al., 1998; Ayeneh et al., 2002). Prasad et al. (2008b) reported that stomatal conductance remained unaffected across night temperatures of 14, 17 and 20°C and slightly increased by 10% at night temperature of 23°C.

Respiration

Impact of heat stress on respiration is relatively less understood (Prasad et al., 2008a). With increases in temperature, respiration cost increases and reaches the point that rate of photosynthesis cannot compensate for respiratory losses, and that leads to carbon starvation (Levitt, 1980). Responses of respiration to high temperature vary with the age of organs in crop plants (Paulsen, 1994). Generally, respiration exponentially increases with increasing temperatures from 0 to 35 or 40°C, reaches the maximum around 40 to 50°C and then decreases with further increases in temperature above 50°C (Prasad et al., 2008a).

Effect of High Temperature Stress on Biochemical Traits

Enzyme activities

Response of photosynthesis to high temperature stress is closely related to temperature dependence of the major photosynthetic enzyme, Rubisco (Prasad et al., 2008a). Under high temperature conditions, rate of inactivation of Rubisco exceeds activase's ability to promote Rubisco activation (Salvucci and Crafts-Brandner, 2004), which leads to reduced activation and activity of Rubisco. In addition, under high temperature stress, solubility of CO_2 is decreased at a greater extent than O_2, which favors the oxygenation activity of Rubisco, which leads to increased photorespiration and reduced photosynthesis (Lea and Leegood, 1999). Endogenous levels of Rubisco activase plays an important role in determining wheat productivity under heat stress conditions (Ristic et al., 2009). High temperature (40°C) decreases the abundance of large and small subunits of Rubisco and Rubisco activase (Demirevska-Kepova et al., 2005). Krishnan

et al. (1989) observed genetic variability in wheat for synthesis of the small subunit of Rubisco at 34°C.

Starch synthase (SS) is one of the key enzymes responsible for endosperm starch biosynthesis and grain filling. High temperatures above 25°C decrease the activity of SS in wheat (Hawker and Jenner, 1993; Keeling et al., 1993, 1994). In addition, high temperature also regulates SS gene expression at the transcriptional level and reduces the relative levels of transcripts for that enzyme more than for other starch biosynthetic enzymes in wheat (Hurkman et al., 2003). Considering the enzymes involved in endosperm starch synthesis pathway, soluble starch synthase (SSS) is highly thermosensitive, especially at temperatures above 34°C (Keeling et al., 1993). Soluble starch synthase has a T_{opt} of 20-25°C and temperatures above 25°C adversely affect the activity of this enzyme (Keeling et al., 1993), which results in reduced grain growth and starch accumulation (Prakash et al., 2003). This effect is found to be apparently reversible in wheat after a short period of exposure to elevated temperature (Keeling et al., 1993). However, prolonged exposure to elevated temperature causes knockdown or complete loss of activity of SSS, which is much slower to reverse in wheat endosperm (Keeling et al., 1993). Even short periods of heat stress (30-40ºC) causes a decline in the rate of starch deposition due to reduction in the activity of SSS. Keeling et al. (1993) reported that several other enzymes in starch biosynthesis pathway, including alkaline pyrophosphatase, phosphoglucomutase, UDP- glucose pyrophosphorylase, hexokinase, phosphoglucoisomerase, sucrose synthase, ADP- glucose pyrophosphorylase and bound starch synthase remained unaffected under elevated temperatures (25-45°C).

Membrane stability

Membrane thermostability is highly correlated with yield in wheat under hot environments (Shanahan et al., 1990; Reynolds et al., 1994; Fokar et al., 1998a). Studies have documented high genetic variability and heritability of this trait in wheat (Fokar et al., 1998a). Reynolds et al. (1994) reported that membrane thermostability of heat-acclimated flag leaves under field conditions as well as seedlings grown in controlled conditions was associated with heat tolerance in 16 spring wheat cultivars at several hot environments. Level of unsaturation of membrane-phospholipids is linked to the extent of heat induced damage to membranes (Reviewed by Fu et al., 2011). Thylakoids harbor chlorophyll, and damage of thylakoid membrane under heat stress leads to chlorophyll loss in wheat (Ristic et al., 2007). Sairam et al.

(1997) reported increased membrane stability in heat tolerant wheat genotypes and association of that character to activity of antioxidant enzymes. Even though cell membrane thermostability has been identified as a potentially powerful selection criterion for high temperature tolerance in wheat (Reynolds et al., 2001), some studies have reported that this trait alone cannot serve for that purpose (Blum et al., 2001).

Heat shock proteins

Plants produce specific proteins termed as heat shock proteins (HSP) in response to high temperature stress. Heat shock proteins are usually undetectable at non-stressed conditions (Kimpel and Key, 1985). They can be categorized in to high molecular weight (ranges from 68 to 110 kD) and low molecular weight groups (ranges from 15 to 27 kD). High molecular weight HSPs are present in all organisms studied to date. Low molecular weight HSPs are the most abundant class of HSPs in higher plants, and the 15 to 18 kD HSPs are unique to plants (Kimpel and Key, 1985; Ho and Sachs, 1989). Most, but not all, heat shock proteins are molecular chaperones. Molecular chaperones are proteins produced in plants in response to heat stress (Fu et al., 2011). They bind with partially unfolded or denatured proteins to stabilize them, and protect them from thermal aggregation and facilitate their re-folding during recovery when stress is relieved (Vierling, 1991; Feder and Hofmann, 1999). Some HSPs are also involved in eliminating potentially harmful proteins arising from misfolding, denaturation or aggregation and play an important role in maintaining cellular homeostasis (Wang et al., 2004). Heat shock proteins consists of classical and non-classical proteins. Most of the classical HSPs exhibit chaperon activity, binding with denatured proteins to prevent their thermo-aggregation (Fu et al., 2011). Non-classical HSPs include plastid protein synthesis elongation factor EF-Tu and peptidyl-prolyl cis/trans isomerases (Fu et al., 2011).

Krishnan et al. (1989) showed a positive correlation between synthesis of specific low molecular weight HSPs and heat tolerance in wheat. Wheat exhibits genetic variability for the synthesis of HSPs in response to high temperature stress (Krishnan et al., 1989). Vierling and Nguyen (1992) reported that acquired heat tolerance in wheat is associated with the level of expression of HSP genes during the initial 2 h of heat stress. It is reported that HSPs are synthesized even before leaf temperatures reach lethal levels for growth and development (Hendershot et al., 1992). Ristic et al. (2008) reported that EF-Tu is upregulated in wheat under heat stress and genotypes with increased accumulation of this protein are tolerant to heat stress.

EF-Tu improves heat tolerance through its chaperone activity (Rao et al., 2004; Fu et al., 2008). Fu et al. (2008) reported that transgenic wheat carrying an EF-Tu gene from maize (*Zea mays* L.) exhibited decreased thermal aggregation of leaf proteins and thylakoid membrane damage and increased rate of photosynthesis under heat stress. Transgenic wheat plants with EF-Tu gene produced superior grain yield based on number of grains per plant, total grain weight per plant and individual grain weight, compared to their non-transgenic counterparts under heat stress (Fu and Ristic, 2010). Modification of the expression of plastidal EF-Tu and/or selection of genotypes with increased activity might help to improve heat tolerance of wheat (Rao et al., 2004; Fu et al., 2008).

Reactive oxygen species

Heat stress often induces accumulation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl ion (OH^-) and singlet oxygen (O^{-1}) in leaves which leads to oxidative stress (Schoffl et al., 1999; Sairam et al., 2000). Chloroplast, mitochondria and peroxisomes are major cellular organelles that generate ROS under stress (Salin, 1991; Sairam and Srivastava, 2002; Diego et al., 2003). Heat stress induced membrane and protein damage results in increased ROS content (Dat et al., 1998a, 1998b). Over-excitation of chlorophyll molecules under high temperature also results in accumulation of ROS (Djanaguiraman et al., 2010a). In general, high temperature induced imbalance between photosynthesis and respiration leads to oxidative damage in plants (Fitter and Hay, 1987). Accumulation of ROS causes disruption of DNA strands and activation of cell wall hydrolyzing enzymes such as nucleases, proteases and lipases resulting in loss of cell structure (Imlay and Linns, 1988; Paliyath and Droillard, 1992). It also causes denaturation of proteins and lipid peroxidation in membranes, which lead to membrane damage and membrane leakiness, and ultimately results in decreased photosynthetic rate and enhanced cell death (Noctor and Foyer, 1998; Liu and Huang, 2000; Djanaguiraman et al., 2009).

Antioxidants

The antioxidant defense system in plants has both enzymatic and non-enzymatic components. Major antioxidant enzymes in plants include superoxide dismutase, catalase, peroxidase, ascorbate-gluthatioine pathway enzymes such as ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase,

Halliwell-Asada pathway enzymes and dehydroascorbate reductase (Keleş and Öncel, 2002; Djanaguiraman and Prasad, 2010). Non-enzymatic antioxidants include glutathione, ascorbic acid, β-carotene and tocopherols (reviewed by Farooq et al., 2011).

Under non-stressed conditions, antioxidant enzymes scavenge ROS and protect cells from oxidative damage. Under stressed conditions, the general antioxidant status diminishes or antioxidant enzymes reduce their activity and the amount of ROS increases in the cell (Prochazkova et al., 2001; Srivalli and Khanna-Chopra, 2004; Djanaguiraman et al., 2010a,b). Superoxide dismutase, catalase and peroxidase system converts ROS to O_2 and water (Raychaudauri, 2008). As a result of ascorbate-gluthatioine pathway, H_2O_2 will be reduced to water (Fu et al., 2011). Superoxide dismutase is the only enzyme that can scavenge O_2^-, whereas catalase and peroxidase scavenge H_2O_2 (Djanaguiraman et al., 2010a,b). Increased antioxidant enzyme (superoxide dismutase, catalase, glutathione reductase and peroxidase) activities are reported in heat tolerant genotypes in wheat (Kraus et al., 1995; Sairam et al., 1997; Sairam et al., 2000). Non-enzymatic antioxidants, such as ascorbic acid is also important for protection of wheat plants from heat-induced oxidative damage (Sairam et al., 2000). Agarwal et al. (2005) found that application of abscisic acid and salicylic acid increased the activity of antioxidant enzymes and decreased the amount of ROS in heat stressed wheat plants, and this decreased oxidative damage resulted in increased chlorophyll and carotenoid contents, relative water content, membrane stability index, leaf area and total biomass compared to control plants.

High Temperature Sensitive Stages in Wheat

Vegetative Stages

High temperature, usually, decreases the duration of all growth stages in wheat. Shpiler and Blum (1986) reported that high temperature from emergence to anthesis significantly decreased duration of all the development stages, GS1 (emergence to double-ridge), GS2 (double ridge to anthesis) and GS3 (anthesis to grain maturation) in wheat; and duration of GS2 was most sensitive to high temperature. The developmental stage at which wheat plant is exposed to heat stress will determine the degree of damage (Slafer and Rawson, 1995). The double-ridge stage is highly sensitive to heat stress (Slafer and Rawson, 1995). High temperature at double-ridge stage damages the development of spikelet primordia on the apex (Johnson and Kanemasu, 1983).

Prasad et al. (2008b) reported that plant height and tiller number remained unaffected, whereas, biomass showed a curvilinear decrease under high night temperatures (>23°C) in wheat.

Reproductive Stages

Heat stress during reproductive development is a major constraint to wheat production in most parts of the world. Wheat plant suffers from greater damage when heat stress occurs during the reproductive phase than vegetative phase due to the direct effect of high temperature on grain number and grain weight (Wollenweber et al., 2003). Generally, plant reproductive tissues have less temperature tolerance compared to vegetative tissues (Fu et al., 2011). Reproductive development is accelerated when wheat is grown in hot environments (reviewed by Hall, 1992, 1993).

Pollen and ovule formation

The most heat sensitive stage of reproductive development in wheat is the period from the onset of meiosis in pollen or embryo sac mother cells to the early development of micro or megaspores (Saini and Aspinall, 1982). Pollen formation is one of the most heat sensitive processes in cereals including wheat (Saini and Aspinal, 1982). High temperature induced pollen sterility is mainly due to the irregularities during microsporogenesis (Jager et al., 2008). Inability of pollen to synthesize HSP is thought to be the reason for its increased sensitivity to high temperature (Young et al., 2001). Saini and Aspinall (1982) reported 35% reduction in pollen viability in wheat plants exposed to high temperature of 30°C for 3 d compared to normal temperature (20°C). Female fertility is also affected by high temperature in wheat (Saini and Aspinall, 1982). Heat stress that coincides with meiosis in megaspore mother cells leads to abnormal ovary development with small sized or no embryo sac and reduced nucellus (Saini et al., 1983).

High temperature stress causes many ultra-structural changes to pollen grains which lead to pollen sterility in wheat (Saini et al., 1984). Saini et al. (1984) reported that high temperature (30°C for 3 d) during meiosis caused premature degeneration of tapetal cells which resulted in pollen sterility in wheat. These sterile pollen grains contained apparently normal exine, very little or no intine or cytoplasm and no starch.

Anthesis

High temperature stress causes severe damage to plants when it occurs during or soon after anthesis (Prasad et al., 2006). Wheat plants are highly susceptible to heat stress during the period between flower initiation and anthesis, and the effect is marked by the reduction in kernel number (Acevedo et al., 2002). T_{min}, T_{opt} and T_{max} for successful anthesis are reported as 9, 18-24 and 31°C respectively in wheat (MacDowel, 1973, Russel and Wilson, 1994).

Seed set

Generally, high temperature stress just before or during anthesis is associated with reduced seed set percentage in wheat, mainly due to (a) production of nonviable pollen or ovule, (b) hindered pollen tube growth and (c) inability of fertilized embryo-sac for the transition to a seed (Saini and Aspinall., 1982; Saini et al., 1983). Seed set in wheat is most sensitive to high temperature stress when the stress coincides with the time period between the onset of meiosis in pollen mother cell to the formation of microspores and their early development (Saini and Aspinall, 1982). High temperature stress at this highly sensitive stage hinders pollen performance, which leads to drastic reduction in seed set (Saini and Aspinall, 1982). High temperature stress during meiosis in megaspore mother cell results in abnormal ovary development and reduced pollen tube growth in wheat, which leads to reduced seed set (Saini et al., 1983).

Embryo abortion

Embryo abortion has been reported in wheat due to heat stress (Saini et al., 1983). Hays et al. (2007) reported that high temperature induced increase in ethylene production presumably causes embryo abortion and decreased kernel weight in wheat.

Yield Components and Yield

Grain number

Grain number is a major component of yield in wheat (Evans, 1978). High temperature accelerates the development of the spike and reduces the number of spikelets and grains per spike (reviewed by Farooq et al., 2011). Genetic variation is reported in wheat for grain number under heat stress (Fokar et al., 1998b). Temperatures above 31°C just before anthesis cause reduction in grain number due to pollen sterility (Wheeler et al., 1996). Fischer (1985) reported 4% reduction in number of wheat grains per unit area for each degree increase (from 15-22°C) in

mean temperature during the one month period before anthesis. Ferris et al. (1998) reported that 10°C increase in maximum temperature at mid anthesis caused 40% reduction in grain number per spike. Shpiler and Blum (1991) examined 21 spring wheat cultivars and found that heat tolerance was associated with increases in grain number per spike. Prasad et al. (2008b) documented a linear decrease in grain number per spike with increases in night temperatures from 14 to 23°C.

Individual grain weight

Al-Khatib and Paulsen (1990) reported that high temperature (32/27°C) from anthesis to maturity caused 20% reduction in average grain weight of wheat. Stone and Nicolas (1998) observed that high temperature (40/21°C) during grain filling decreased individual grain weight of wheat by 14%, compared to the control (21/16°C). Gibson and Paulsen (1999) imposed high temperature stress starting from 15 d after anthesis (grain number was set by this time) until maturity, and quantified yield reduction due to decreased individual grain weight as 18%. Elevated temperatures reduce the time period between anthesis and physiological maturity, which results in a reduction in individual grain weight (Warrington et al., 1977; Shpiler and Blum, 1986). Decreased size of endosperm cells in grains and reduced starch deposition due to reduced activity of SSS are major reasons for decreased individual grain weight under heat stress (Hoshikawa, 1962; Bhullar and Jenner, 1985; Hawker and Jenner, 1993). Stone and Nicolas (1995) reported that a sudden increase in temperature (from 20 to 40ºC) caused a greater reduction in individual grain weight than a gradual increase in temperature (6ºC h^{-1}, from 20 to 40ºC) in a heat sensitive wheat variety, but this trend was absent in the heat tolerant wheat variety. Considerable genetic variability has been identified in wheat for individual grain weight under heat stress (Stone and Nicolas, 1994; Fokar et al., 1998b).

Grain filling duration

High temperature-induced decrease in grain filling duration results in a reduction of individual grain weight (Al-Khatib and Paulsen, 1984; Gibson and Paulsen, 1999; Viswanathan and Khanna-Chopra, 2001). Grain filling duration decreases by 2-8 d for every 1°C increase above 15-20°C in wheat (reviewed by Streck, 2005). Prasad et al. (2008b) reported that grain filling duration in wheat was decreased by 3 and 7 d at night temperatures of 20 and 23°C, respectively, compared to a night temperature of 14°C.

Grain filling rate

Under optimum temperature conditions, decreased grain filling duration is compensated by increased grain filling rate, but this compensation does not happen under high temperature stress, which results in a significant reduction in individual grain weight (Sofield et al., 1977). Reduction in leaf and spike photosynthesis (Blum et al., 1994) and decreased remobilization of stem reserves (Yang et al., 2002) are major reasons for decreased grain filling rate at high temperatures. Increased grain filling rate is a useful trait that can be used for the improvement of heat tolerance in wheat (Dias and Lidon, 2009).

Total grain yield

Gibson and Paulsen (1999) found that high temperature stress of 35/20°C from 10 d after anthesis until maturity decreased grain yield by 78%, grain number by 63% and individual grain weight by 29%. Reduction in number of spikes per unit area, number of fertile spikes per plant, number of grains per spike and grain weight lead to reduction in grain yield of wheat under heat stress (Warrington et al., 1977; Acevedo et al., 2002). Lobell et al. (2005) reported that grain yield in wheat is more sensitive to increases in daily T_{min} than T_{max}. The same authors observed 10% decrease in wheat yield for every 1°C increase in night temperature above optimum in Mexico.

Grain quality

High temperature stress during the post-heading period adversely affects grain quality in wheat (Blumenthal et al., 1993, Stone et al., 1997, Gibson et al., 1998). High temperature stress influences grain protein content and composition (Stone and Nicolas, 1998). It reduces starch deposition in grains (Spiertz et al., 2006) and leads to more nitrogen per unit of starch (Reviewed by Farooq et al., 2011). High temperatures (>35°C) during grain filling negatively affect dough properties in wheat since it increases the ratio between gliadin and glutenin in grains, which produces a weak dough (Blumenthal et al., 1993). Heat stress also reduces noodle swelling power of wheat flour (Stone and Nicolas, 1994).

Breeding for High Temperature Tolerance

Genetic resources available for the improvement of bread wheat consist of primary, secondary and tertiary gene pools (Skovmand et al., 2002). Conventional breeding approach

utilizes an artificially created mutagenized population also for bread wheat improvement (Fu et al., 2011). Genetic variability for heat tolerance has been identified in cultivated wheat (Wardlaw et al., 1989a,b; Al-Khatib and Paulsen, 1990; Ferrara et al., 1994; Reynolds et al., 1994; Hede et al., 1999) and its wild relatives (Damania and Tahir, 1993; Waines, 1994; Sun and Xu, 1998). Genetic variability for heat tolerance has been reported in wild *Triticum* and *Aegilops* species (Edhaie and Waines, 1992). These authors tested accessions from nine different countries of the world and found that all of the heat tolerant accessions were from eastern Israel, western Jordan and southwestern Syria. The authors also suggested that these three regions can be exploited for heat tolerant genotypes of bread and durum wheat that could be incorporated into wheat breeding programs. Landraces also exhibit significant genetic variability for heat tolerance (reviewed by Farooq et al., 2011). Heat tolerant accessions belonging to *Aegilops geniculata*, *A. speltoides*, *A. searsii* and *A. longissima* have been identified (Ehdaie and Waines, 1992; Khanna-Chopra and Viswanathan, 1999; Zaharieva et al., 2001). Pradhan et al. (2012) screened 52 accessions of *Aegilops sp.* for tolerance to an extended period (16 d) of heat stress at anthesis and identified tolerant genotypes of *A. speltoides* and *A.geniculata* based on grain number and grain size. They also reported that tremendous genetic variability exists in *Aegilops sp.* for heat tolerance that can be exploited in breeding for heat tolerance at reproductive stage. High temperature tolerance have also been reported in different genotypes of synthetic wheat (Yang et al., 2002; Trethowan and Mujeeb-Kazi, 2008; Kurahashi et al., 2009). Synthetic wheats are valuable genetic stocks and can act as bridges for introgression of alien genes into cultivated wheat varieties (Siddiqui, 1976; Jiang et al., 1994; Rajaram et al., 1997).

Traits Associated with Heat Tolerance

Canopy Temperature Depression

Canopy temperature depression is the difference between air temperature and canopy temperature. Canopy temperature depression shows high genetic correlation with yield in wheat in both warm and temperate environments (Reynolds et al., 2001). Canopy temperature depression is significantly correlated with biomass, grains m^{-2}, spikes m^{-2} and grains $spike^{-1}$ under high temperature conditions in wheat (Reynolds et al., 2001). It is a heritable trait and can serve as a useful criterion for evaluating heat tolerance in both early and advanced generations (Reynolds et al., 1998; Reynolds et al., 2001). Canopy temperature depression is a function of

many important physiological mechanisms such as metabolism, partitioning and vascular transport, and is therefore, a powerful trait for selection (Reynolds et al., 2001). However, this trait is highly sensitive to environmental factors such as radiation, evapotranspiration and wind. It shows highest potential as a selection criterion in low relative humidity environments.

Early Heading

Early heading is an important and effective trait of wheat genotypes that contribute to heat tolerance (Tewolde et al., 2006). Tewolde et al. (2006) concluded that early-heading cultivars out-yielded later-heading cultivars in environments with risks of post-heading heat stress. Compared to later heading cultivars, early heading cultivars showed many important tolerant traits including (a) longer post-heading period and grain-filling duration, (b) completion of significant fraction of grain-filling duration earlier in the season before the onset of heat stress, (c) retention of more number of green leaves at anthesis and (d) less number of leaves lost due to senescence at anthesis. However, early heading may not be a useful trait to select cultivars suitable for locations where cool temperature or frost limits the early heading trait.

Time of Day of Flowering

Anther dehiscence, pollen shed, pollen tube growth, pollination and fertilization are highly sensitive to high temperature stress, while the embryo formed after fertilization is comparatively more tolerant (Prasad et al., 2006). The time of day of flowering (TDF) is the time of the day at which anthesis commences in a crop (Sheehy et al., 2007). Air temperature increases rapidly after sunrise and exceeds the critical temperature of 35°C at around 10:00 AM in high temperature damage-prone areas (Nishiyama and Blanco, 1980; Prasad et al., 2006). Therefore, shifting of TDF to early hours of the morning is one way to escape high temperature-induced spikelet sterility in plants (Nishiyama and Blanco, 1980; Prasad et al., 2006). Genetic variability has been reported for TDF in rice, where TDF varied from 7:00 AM to 12:30 PM (Prasad et al., 2006).

Increased Partitioning

Grain filling under heat stress is largely associated with partitioning of stem reserves in wheat (Fokar et al., 1998b; Yang et al., 2002). Increased ability to utilize mobilized stem reserves contributes to increased grain number and grain weight under heat stress (Fokar et al.,

1998b). Increased partitioning of reserves from leaves, stem or other plant parts is a potential strategy to improve grain filling and yield in wheat under heat stress (Wardlaw, 1974). Considerable genetic variability is reported for assimilate partitioning under heat stress in wheat (Yang et al., 2002). Increased partitioning under heat stress is a 'true tolerance mechanism' exhibited by tolerant genotypes.

Heat Susceptibility Index

Heat susceptibility index (HSI) for grain yield is calculated using the formula, HSI = (1- Y/Yp) / D; where, Y is the average grain yield per plant of a genotype at high temperature, Yp is the average grain yield per plant of the same genotype at optimum temperature, D is the stress intensity, which is calculated as 1- X/Xp, in which X is the mean Y of all genotypes, and Xp is the mean Yp of all genotypes (Fischer and Maurer, 1978). Low HSI is a useful criterion to select heat tolerant genotypes in cultivated and wild wheat (Khanna-Chopra and Viswanathan, 1999; Yang et al, 2002; Pradhan et al., 2012). Generally, genotypes with HSI ≤ 0.5 are considered as highly heat tolerant, genotypes with HSI > 0.5 to ≤ 1.0 are considered as moderately tolerant and genotypes with HSI > 1.00 are considered as susceptible (Khanna-Chopra and Viswanathan, 1999).

Chlorophyll Content

Measurement of chlorophyll content in leaves is useful for high throughput screening for heat tolerance among wheat genotypes (Ristic et al., 2007). Chlorophyll loss is closely associated with heat-induced thylakoid membrane damage in wheat (Ristic et al., 2007). Genetic variability exists in wheat for the ability to retain chlorophyll content under heat stress (Wardlaw et al., 1980; Blum, 1986; Ristic et al., 2007).

Decreased Membrane Damage

Increased membrane stability, which is an indication of decreased membrane damage is a selection criterion used to select heat tolerant wheat genotypes under both controlled and field conditions (Blum et al., 2001; Reynolds et al., 2001). Genotypes vary in the extent of membrane damage under heat stress (Fokar et al., 1998a). Since cellular membrane stability is a highly heritable trait (Fokar et al., 1998a), it has a potential application in breeding wheat for heat tolerance. Fokar et al. (1998a) found that increased membrane stability was associated with

increased grain weight per spike in wheat. Measurement of electrical conductivity quantifies heat induced electrolyte leakage from tissues, thus, it evaluates the damage to plasma membrane (Blum and Ebercon, 1981; Ibrahim and Quick, 2001); measurement of chlorophyll *a* fluorescence indicates damage to thylakoid membranes (Krause and Weis, 1984; Maxwell and Johnson, 2000; Sayed, 2003); and TTC assay quantifies damage to mitochondrial membranes (Fokar et al., 1998a).

Decreased ROS Production and Increased Amount of Antioxidants

Protection against oxidative damage is an important component in the determination of heat tolerance in plants. Increased antioxidant activity is correlated with reduced oxidative damage and acquired thermotolerance in wheat (Sairam et al., 2000; Almeselmani et al., 2009). The beneficial effects of increased antioxidant enzyme activity and decreased oxidative damage is reflected in many physiological and biochemical parameters such as increased chlorophyll and carotenoid contents, relative water content and membrane stability and whole plant characteristics such as increased leaf area and total biomass (Agarwal et al., 2005; Almeselmani et al., 2009). To exploit the association between antioxidant activity and heat tolerance, external growth regulators are applied in wheat to increase the activity of antioxidants and to reduce heat damage. For example, abscisic acid and salicylic acid when sprayed on leaves of wheat genotypes increase the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase and decrease the contents of ROS such as hydrogen peroxide and thiobarbituric acid reactive substances (Agarwal et al., 2005).

Heat Shock Proteins

Since the synthesis of HSPs is related to acquisition of heat tolerance in wheat (Vierling and Nguyen, 1992), it offers a useful trait for selecting for heat tolerance. Manipulation of expression patterns of HSPs has the potential to improve temperature tolerance of wheat (Fu et al., 2008; Fu and Ristic, 2010). Transgenic wheat plants with increased heat tolerance have been developed using HSP (plastid protein synthesis elongation factor- EF-Tu) genes from maize (Fu et al., 2008; Fu and Ristic, 2010).

Cell Membranes

Physical state or phase of membranes is influenced by the spatial arrangement and motional freedom of lipid molecules (van Meer et al., 2008). In gel and liquid crystalline (fluid) phases, the lattice structure of the membrane is 'lamellar' or bilayer type (pairs of layers that face one another) (Fig. 1.3). In this structure, the polar head groups of the lipids face the aqueous phase on both sides of the bilayer and the nonpolar hydrocarbon tails oppose each other in the bilayer (i.e. head groups outside and hydrocarbon tails inside). In contrast, polar head groups are inside and the hydrophobic, hydrocarbon tails are outside in solution in case of hexagonal II phase (Voet et al., 2008) (Fig. 1.3). The hydrocarbon tails are packed randomly in the fluid phase, while gel phase possess a closer packing of head groups and extended lipid chains (Garvey et al., 2013). Thus, gel phase is a more orderly array of lipids than fluid phase (Voet et al., 2008). Hexagonal phase is characterized by a hexagonal symmetry with a central circular water channel (Garvey et al., 2013) that gives a tubular shape for the membranes (Cullis and DeKruijff, 1979). Membranes have a curved morphology in hexagonal II phase and a flat, planar morphology in the gel and fluid phases (Epand, 1998). Individual lipids possess a cone shape in the hexagonal phase and a cylindrical shape in the gel and fluid phases (Cullis and DeKruijff, 1979).

In gel phase, hydrocarbon chains are ordered in the 'all-trans' conformation (Seddon and Templer, 1995). Upon heating, the gel phase melts to form the fluid phase. In the fluid phase, fatty acid double bonds in the hydrocarbons mainly have the *cis* configuration (Voet et al., 2008). The presence of *cis* double bonds introduces bends in the fatty acid chains and reduces tight packing of adjacent lipid molecules (Huang, 2006).

In the fluid phase, the interfacial area per molecule expands by 15-30%, compared to gel phase (Seddon and Templer, 1995). In the gel phase, the area per lipid molecule is less, the lipid tails are almost fully extended and straight, the mobility is considerably decreased and the bilayer thickness is more, compared to fluid phase (Koynova and Caffrey, 1998). For gel and fluid phases (bilayer phases), the packing ratio (ratio of effective cross sectional area of the polar head group to the cross sectional area of the hydrocarbon chain region) is one or close to one. For hexagonal II phase, the packing ratio will be less than one, which means that there will be greater cross-sectional area available for the hydrocarbon chain tails and less area available for the head groups (Voet et al., 2008).

The lipids are more mobile when they are in liquid-crystalline (fluid) phase than in gel phase (Voet et al., 2008). In the fluid phase, the lipids can diffuse freely inside the membrane bilayer. They can even move from the membrane surface to the solution because of the hydrophobicity of the tail group. In the gel phase, lipids are packed more orderly and are comparatively immobile. Lipids can undergo lateral diffusion (movement in the plane of bilayer) and transverse diffusion (also called flip-flop movement; movement across the bilayer; relatively rare event) in the fluid phase, whereas in gel phase, lipids almost lack these movements (Voet et al., 2008). In the gel phase, lipids can only undergo a hindered long-axis rotation on a time scale of 100 nsec (Seddon and Templer, 1995). Lipids are more mobile in hexagonal II phase than in fluid or gel phase.

Temperature can cause lipid transitions from one phase to another. High temperature causes a transition from gel phase to fluid phase and then to hexagonal II phase (Quinn, 1985). The temperature at which the phase transition occurs is called the transition temperature (Voet et al., 2008). Each lipid species will have its own characteristic transition temperature. Factors such as length and unsaturation of the hydrocarbon that makes up the tail group and electrostatic properties of the head group species affect the transition temperature. When the hydrocarbon length increases, vander Waals interactions become stronger. This results in an increase of transition temperature as it requires more energy to disrupt the ordered packing. Similarly, transition temperature increases with degree of saturation (Voet et al., 2008). It is well established that plants can decrease lipid unsaturation levels at high temperatures and increase it at low temperatures to maintain optimal membrane fluidity (Cyril et al., 2002; Larkindale and Huang, 2004; Chen et al., 2006; Welti et al., 2007).

Usually, phosphatidyl choline (PC) or digalactosyldiacylglycerol (DGDG) exist either in gel or fluid phases (Seddon et al., 1983). The lipid species that forms a hexagonal II phase most often is phospatidylethanolamine (PE). The head group of PE binds fewer water molecules compared to head group of PC. This lower hydration seems to promote hexagonal II phase in PE. Monogalactosyldiacylglycerol (MGDG) and phosphatidic acid (PA; in the presence of Ca^{2+}) tend to form hexagonal phases (Cullis and DeKruijff, 1979; Verleij et al., 1982). The presence of cations in a lipid monolayer promotes a non-bilayer phase (Killian et al., 1994). van Meer et al. (2008) reported that phase behavior of lipids is determined by degree of ordered packing of hydrocarbon chains and diffusion efficiency. The heterogeneity in membrane lipid composition

broadens the temperature over which membranes undergo transitions between the phases, i.e. the transition is not as sharp as a function of temperature as it is for a single lipid. Proteins associated with lipids in a membrane affect membrane physical properties, phase behavior, thickness and curvature (Seddon and Templer, 1995; Mitra et al., 2003). Proteins can modify the packing of acyl chains in lipids, rigidify the lipids and increase the transition temperature for phase changes (Tsvetkova et al., 2002).

The lipid compositions of different plant organs can be different. For example, triacylglycerols (TAG), the main storage lipids and energy reserves, are mainly found in seeds. Around 60% of the weight of a seed is made up of TAGs. TAGs are stored in the embryo or endosperm tissues of seeds and are used to provide energy for a germinating seedling (www.lipidlibrary.aocs.org). Rochester et al. (1987) observed relatively greater amounts of sterols and less amounts of phospholipids (PC and PE) in roots compared to leaves. Sterols are important for cell elongation, vascular differentiation and hormone (auxin and ethylene) signaling in roots (Clouse, 2002). Larkindale and Huang (2004) found greater amount of unsaturated lipids and less amount of saturated lipids in leaves than in roots. Djanaguiraman et al. (2013) found greater amounts of MGDG and DGDG and less amounts of PA in leaves than in pollen.

Phospholipase A, phospholipase C, phospholipase D, desaturases and lipoxygenases are some of the major enzymes related with lipid metabolic reactions. Phospholipase A cleaves acyl side chains of phospholipids and releases lysophospholipids (Voet et al., 2008). Phospholipase A1 hydrolyzes the fatty acid at the *sn*-1 position of phospholipids and phospholipase A2 at the *sn*-2 position. Phospholipase C cleaves phospholipids in to diacylglycerol and a phosphate-containing head group (cleaves before the phosphate). Phospholipase D hydrolyzes phospholipids to produce phosphatidic acid and free head groups (Welti et al., 2002) (cleaves after the phosphate). Phospholipase C and D cleave the first and the second phosphodiesteric bond, respectively, and therefore, are called phosphodiesterases, whereas phospholipase A releases fatty acids from phospholipids (Wang et al., 2012). Desaturase removes hydrogen atoms from a fatty acid and introduces double bonds into the fatty acid. Saturated fatty acids are converted to unsaturated fatty acids through the action of desaturases (Voet et al., 2008). Lipoxygenase catalyzes the dioxygenation (incorporation of both atoms of molecular oxygen) of polyunsaturated fatty acids in lipids containing a cis,cis-1,4- pentadiene structure to produce an

unsaturated fatty acid hydroperoxide. Lipoxygenase activity leads to the synthesis if oxylipins which are the products of fatty acid oxidation (Porta and Rocha-Sosa, 2002).

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Figures

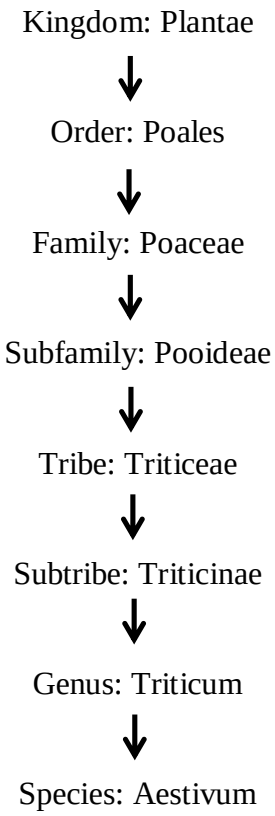

Figure 1.1. Botanical classification of bread wheat (*Triticum Aestivum* L.)

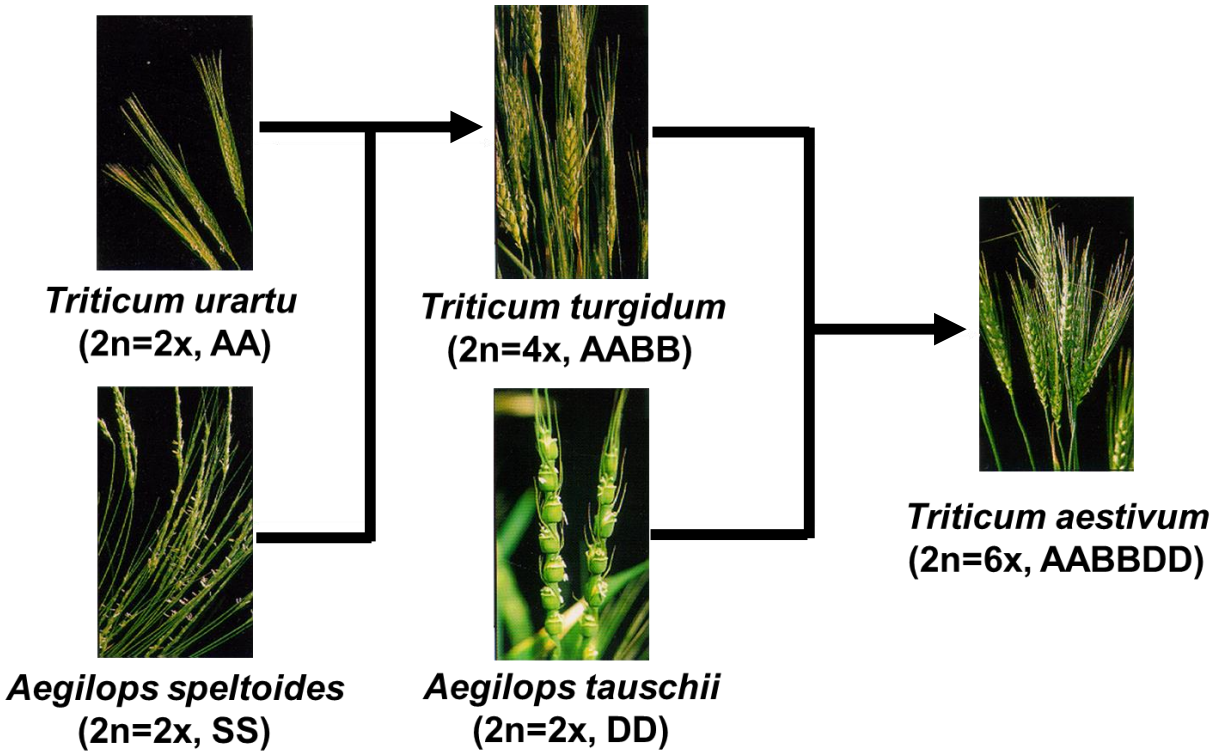

Figure 1.2. Evolution of bread wheat (*Triticum Aestivum* L.). B genome is closely related to S genome and *A. speltoides* is believed to be the donor of B genome to bread wheat (Gill and Friebe, 2002).

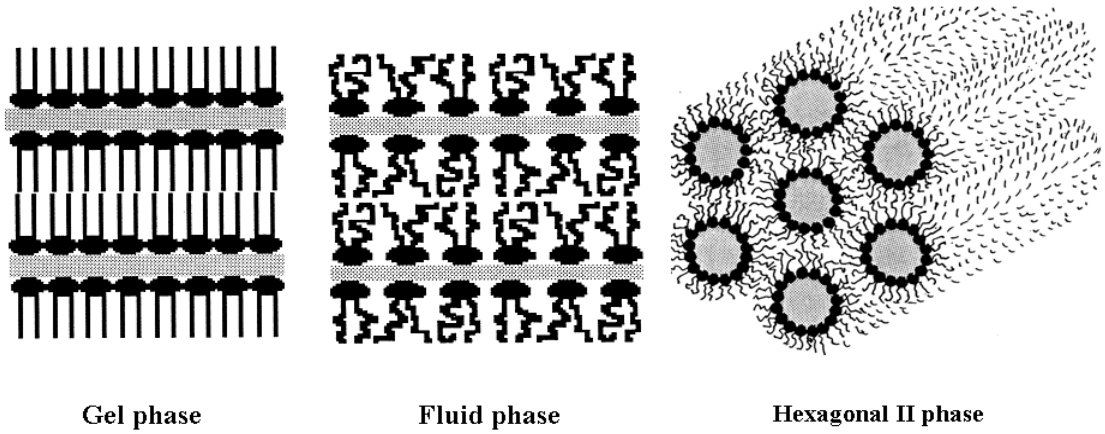

Figure 1.3. Gel, fluid and hexagonal II phases of membranes (Seddon and Templer, 1995; Koynova and Caffrey, 1998).

Chapter 2 - General Introduction

Global mean surface air temperature has increased by 0.5°C in the 20th century (Intergovernmental Panel on Climate Change 2007). This increase is brought about by increases in day maximum and night minimum temperatures. More regions on the globe were affected by increases in night minimum temperatures than increases in day maximum temperatures in the past century (Easterling et al., 1997) (Fig. 2.1). Night minimum temperature has increased more than twice that of day maximum temperature worldwide in the past century (Harvey, 1995; Easterling et al., 1997, 2000) (Fig. 2.2). This resulted in a decrease in the diurnal temperature range (difference between the maximum and minimum temperature during a day) for many parts of the world (Karl et al., 1991, 1993). Climate models predict similar trends for the future, i.e. relatively greater increase in night minimum temperatures compared with day maximum temperatures over the globe.

Most studies have investigated the combined effects of high day and night temperatures on wheat. The few reports existing on differential effects of high day and high night temperatures on wheat are highly contradictory. For example, early reports of Asana and Williams (1965) and Wardlaw et al. (1980) showed that high day and high night temperatures have similar impacts on grain growth of wheat. In contrast, Lobell and Ortiz-Monasterio (2007) reported that historical wheat yields in the Yanqui Valley of Mexico were strongly and negatively correlated with night minimum temperatures but not correlated with day maximum temperatures. Therefore, better understanding of the differential responses of wheat plants to high day and high night temperatures is needed to anticipate the impacts of climate change on wheat production and to develop appropriate management practices.

The objectives of this dissertation were to quantify and compare the effects of high day and high night temperatures on wheat and to identify the traits and mechanisms associated with high day and high night temperature stress response in wheat.

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Figures

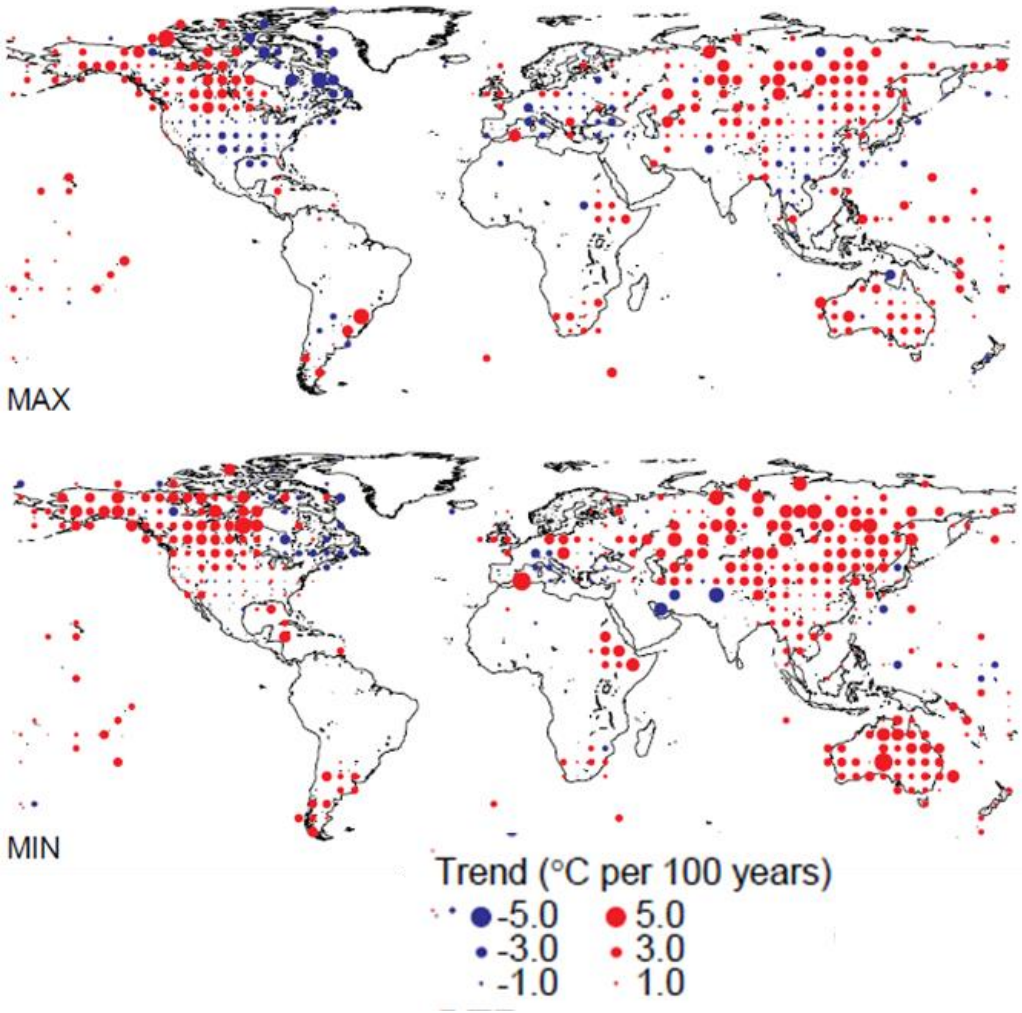

Figure 2.1. Trends in day maximum and night minimum temperatures over the globe (Easterling et al., 1997).

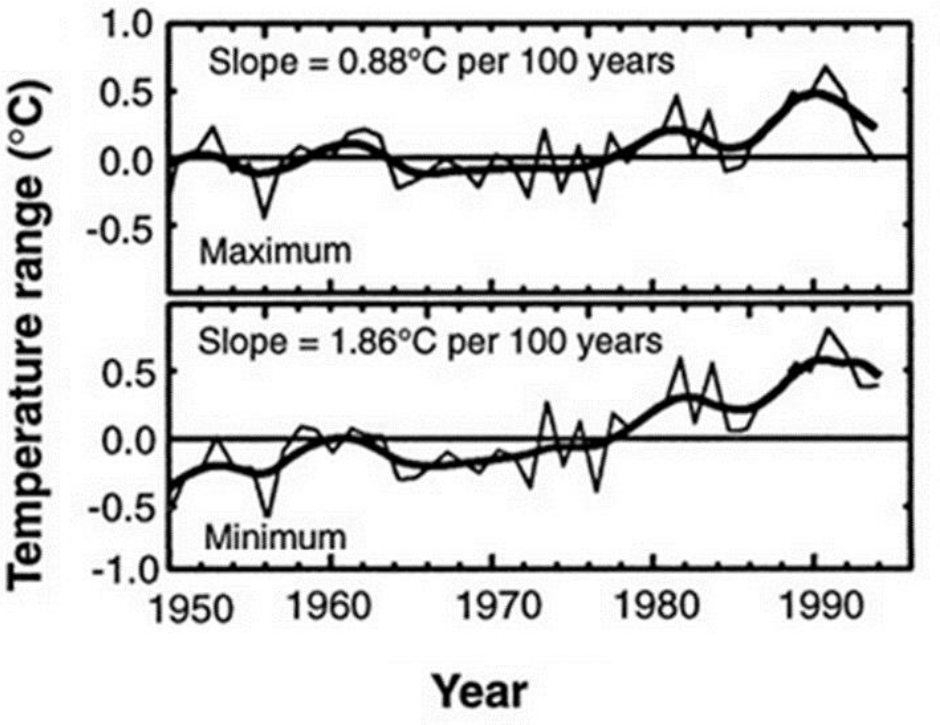

Figure 2.2. Time series data showing annual average day maximum temperature and night minimum temperature over the globe (Easterling et al., 1997).

Chapter 3 - Impact of High Night and High Day Temperature Stress on Winter Wheat

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Abstract

High temperature is a major environmental factor that limits wheat (*Triticum aestivum* L.) productivity. Climate models predict greater increases in night temperature than in day temperature. The objective of this research was to compare the effects of high day and high night temperatures during anthesis on physiological (chlorophyll fluorescence, chlorophyll concentration, leaf level photosynthesis and membrane damage), biochemical (reactive oxygen species [ROS] concentration and antioxidant capacity in leaves), growth and yield traits of wheat genotypes. Winter wheat genotypes (Ventnor and Karl 92) were grown at optimum temperatures (25/15°C, maximum/minimum) until the onset of anthesis. Thereafter, plants were exposed to high night (HN, 25/24°C), high day (HD, 35/15°C), high day and night (HDN, 35/24°C), or optimum temperatures for 7 days. Compared with optimum temperature, HN, HD and HDN increased ROS concentration and membrane damage and decreased antioxidant capacity, photochemical efficiency, leaf level photosynthesis, seed set, grain number and grain yield per spike. Impact of HN and HD was similar on all traits. Greater impact on seed set, grain number and grain yield per spike was observed at HDN, compared with HN and HD. These results suggest that HN and HD during anthesis cause damage of a similar magnitude to winter wheat.

Introduction

High temperature stress is a major environmental factor that limits yield in wheat (*Triticum aestivum* L.). High temperatures of 30 to 40°C are common in wheat growing regions during the crop life cycle and it can decrease yields by 50% or more (Chowdhury and Wardlaw, 1978; Paulsen, 1994). Wheat crops are often exposed to high temperature stress during the reproductive stages of crop development in most wheat-growing regions of the world. High temperatures during reproductive development cause serious damage to wheat when they occur just before or during anthesis (Saini and Aspinall, 1982). Global mean surface air temperature has increased by 0.5°C in the 20th century and is predicted to increase further by 1.5 to 4.5°C by the end of 21st century (Intergovernmental Panel on Climate Change, 2007). This increase is brought about by differential changes in day maximum and night minimum temperatures. In the past century, daily minimum temperature has increased more than twice that of daily maximum temperature worldwide (Easterling et al., 1997). In addition, most climate models predict relatively greater increase in night temperatures compared with day temperatures, resulting in a narrowing of the diurnal temperature range (difference between the maximum and minimum temperature during a day) (Dai et al., 2001).

The impact of high temperature stress on growth and yield of wheat is well documented (Porter and Gawith, 1999; Farooq et al., 2011). High temperature causes damage to thylakoid membranes, which leads to chlorophyll loss (Ristic et al., 2007) and decreases efficiency of photosystem II (PSII) (Yang et al., 2002a; Pradhan et al., 2012) and photosynthesis (Al-Khatib and Paulsen, 1990, 1999; Prasad et al., 2008). At the cellular level, high temperature causes decreased antioxidant activity and leads to increased production of reactive oxygen species (ROS) and oxidative damages such as lipid peroxidation and membrane damage (Sairam et al., 2000). High temperature often leads to decreased seed set, grain number, grain filling duration, grain filling rate and individual grain weight (Al-Khatib and Paulsen, 1984; Wheeler et al., 1996; Ferris et al., 1998; Gibson and Paulsen, 1999; Prasad et al., 2008). Collectively, the effects of high temperature result in decreased grain yield and harvest index (Al-Khatib and Paulsen, 1990; Stone and Nicolas, 1994; Wheeler et al., 1996; Ferris et al., 1998; Gibson and Paulsen, 1999).

Most studies have investigated the combined effects of high day and night temperatures on wheat. The few reports existing on differential effects of high day and night temperatures on wheat are highly contradictory. Early reports of Asana and Williams (1965) and Wardlaw et al.

(1980) showed that high day and night temperatures have similar impacts on grain growth of wheat. Lobell and Ortiz-Monasterio (2007) reported that historical wheat yields in the Yanqui Valley of Mexico were strongly and negatively correlated with night minimum temperatures but not correlated with day maximum temperatures. In the San Luis-Mexicali Valley of Mexico and the Imperial Valley of California, different trends were reported; for example, historical yield correlations with day maximum and night minimum temperatures were similar (Lobell and Ortiz-Monasterio, 2007). Using different crop models, studies have reported that negative effects of high temperature on wheat yields in the USA are decreased when the increments in night minimum temperatures are greater than the increments in day maximum temperatures (Rosenzweig and Tubiello, 1996; Dhakhwa and Campbell, 1998). Anthropogenic climate change is characterized by differential warming between day and night (Lobell and Ortiz-Monasterio, 2007). Therefore, better understanding of the differential responses of wheat plants to high day or night temperatures is needed to anticipate the impacts of climate change on wheat production and to develop appropriate management practices. Even though Prasad et al. (2008) quantified the impacts of night temperature on physiology and growth of spring wheat, they did not investigate the differential response to day versus night temperature increases. Comparative responses of wheat to high day or night temperature stress in terms of physiological processes and reproductive function are not well understood.

The objectives of this research were to quantify and compare the effects of high day and high night temperatures during anthesis on physiological (chlorophyll *a* fluorescence, chlorophyll concentration, leaf level photosynthesis and plasma membrane damage), biochemical (ROS concentration and total antioxidant capacity in leaves), growth (plant height, tiller number and vegetative dry weight) and yield (spike number, seed set, grain number, grain yield and individual grain weight) traits of wheat genotypes. We test the hypothesis that high day and high night temperatures during anthesis cause ROS-induced oxidative damage and decrease leaf level photosynthesis, seed set, grain number and grain yield in wheat.

Materials and Methods

This study was conducted in controlled-environment facilities at the Department of Agronomy, Kansas State University, Manhattan, KS, USA. Experiments were conducted in 2012 (Experiment 1) to determine the impact of high day and high night temperature stress on

physiological, biochemical, growth and yield traits of winter wheat genotypes. The experiment was repeated with the same treatment structure, but with new randomization in 2013 (Experiment 2).

Plant Material and Growth Conditions

Seeds of two winter wheat genotypes, Karl 92 [Crop Reg. no. CV-832, P1564245 (Sears et al., 1997), heat-susceptible (Yang et al., 2002b; Hays et al., 2007)] and Ventnor [Crop Reg. no. not yet available, heat-tolerant (Al-Khatib and Paulsen, 1990; Yang et al., 2002a,b)] were sown in 4-cm-deep trays containing commercial Sunshine Metro Mix 200 potting soil (Hummert International, Topeka, KS, USA). Both genotypes were open-flowering type. Seeds were sown at a depth of about 2 cm. The seedlings were raised in a growth chamber (Conviron Model CMP 3244, Winnipeg, MB, Canada) maintained at 25/15°C (day maximum/night minimum; Al-Khatib and Paulsen, 1984). After 10 d, seedlings were vernalized for 56 d at 4°C and 8 h photoperiod. Following vernalization, two seedlings of the same genotype were transplanted into 1.6-L pots (24 cm length and 10 cm width, MT49 Mini-Treepot, Stuewe & Sons, Inc., Tangent, OR, USA). Rooting medium in pots was commercial Sunshine Metro Mix 200 potting soil. The rooting medium was fertilized with Osmocote (Scotts, Marysville, OH, USA), a controlled-release fertilizer with19:6:12 N:P_2O_5:K_2O, respectively, at 5 g per pot before transplanting. There were 40 pots of each genotype, with a total of 80 pots in the growth chamber. Pots were watered daily and kept in trays containing about 1-cm-deep water throughout the experiment to avoid water stress. Position of pots was changed randomly at 7-d intervals to avoid positional effects. After seedling establishment, seedlings were thinned to one per pot, which was maintained until maturity. At this time, a systemic insecticide, Marathon 1% G (a.i.: Imidacloprid: 1–[(6–Chloro–3–pyridinyl)methyl]–N–nitro–2–imidazolidinimine) was applied at 1.5 g per pot to avoid infestation of sucking insect pests.

At the onset of anthesis (Feekes growth stage 10.5.1), four temperature regimes were established: optimum temperature (OT, 25/15°C), high night temperature (HN, 25/24°C), high day temperature (HD, 35/15°C) and high day and night temperature (HDN, 35/24°C). The temperature treatments were randomly assigned to growth chambers. Each growth chamber had 10 plants of each genotype. Out of these 10, five plants were used to collect leaf samples for the destructive measurements of ROS concentration, plasma membrane damage and total antioxidant

capacity. The remaining five plants were used for the measurement of chlorophyll *a* fluorescence, chlorophyll concentration, photosynthesis and growth and yield traits. Pots were arranged randomly in all growth chambers. During the stress period, position of pots was changed randomly every day in all growth chambers to avoid positional effects. The plants were maintained in the high temperature regimes for 7 d. After that, they were returned to the original growth chamber (25/15°C), where they remained until final harvest at maturity. Similar management practices were followed in both experiments.

In all temperature regimes, day maximum temperature was held for 8 h from 09:00 h to 17:00 h. Similarly, night minimum temperature was held for 8 h from 21:00 h to 05:00 h. The transition period between the day maximum and night minimum temperatures was 4 h. Relative humidity in all growth chambers was set at 85%. Air temperature and relative humidity were continuously monitored at 15-min intervals in all growth chambers throughout the experiment using a HOBO data logger (Onset Computer Corporation, Bourne, MA, USA). The photoperiod was 16 h (from 05:00 h to 21:00 h) (Prasad et al., 2011b), and photon flux density (400 to 700 nm) provided by cool fluorescent lamps was about 900 μmol m^{-2} s^{-1} at 15-cm away from the lamps and 600 μmol m^{-2} s^{-1} at the top of the plant canopy. The temperature, relative humidity and light settings were similar in both experiments.

Quality Control of Growth Chambers

The quality of temperature controls in all four growth chambers are given in Supplemental Fig. 3.S1. The average day (between 09:00 h and 17:00 h) and night (between 21:00 h and 05:00 h) temperatures at OT (25/15°C), HN (25/24°C), HD (35/15°C) and HDN (35/24°C) observed in Experiment 1 were 24.3(±0.6)/15.3(±0.3)°C, 25.2(±0.2)/24.2(±0.2)°C, 34.8(±0.1)/15.1(±0.1)°C and 34.9(±0.1)/24.2(±0.2)°C, respectively. In Experiment 2, average day and night temperatures were 24.2(±0.8)/14.9(±0.01)°C, 24.4(±0.5)/23.5(±0.4)°C, 34.4(±0.5)/15.0(±0.01)°C and 35.1(±0.1)/24.1(±0.1)°C, respectively. Relative humidity during day and night were similar across all temperature regimes and experiments and ranged from 72 to 88%.

Data Collection

At the onset of anthesis, the main stem of all plants was tagged for the measurements of various physiological, biochemical, growth and yield traits. All traits were measured on five

plants of each genotype at each of the four temperature regimes in both experiments. Physiological (chlorophyll *a* fluorescence, chlorophyll concentration, photosynthesis and plasma membrane damage) and biochemical (ROS concentration and total antioxidant capacity in leaves) traits were measured on days 1, 4 and 7 during the stress period and day 2 during the recovery period (after the stress was relieved). Growth and yield traits were measured at maturity.

Chlorophyll a Fluorescence, Photosynthesis and Chlorophyll Concentration

Chlorophyll *a* fluorescence, photosynthesis and chlorophyll concentration were measured on attached fully expanded flag leaves of the tagged stems of five plants per genotype from all temperature regimes. All traits were measured at midday (between 10:00 h and 14:00 h). Chlorophyll *a* fluorescence and photosynthesis were measured halfway between the ligule and the tip of the flag leaf. Chlorophyll *a* fluorescence parameters were measured using a modulated fluorometer (OS30p, OptiSciences, Hudson, NH, USA). The minimum fluorescence (Fo) and maximum fluorescence (Fm) were measured on 60-min dark-adapted flag leaves. A ratio of variable fluorescence (Fv, the difference between maximum and minimum fluorescence) to maximum fluorescence (Fm) represents maximum quantum yield of PSII (Maxwell and Johnson, 2000). A decrease in the Fv/Fm ratio indicates decreased photochemical efficiency of PSII, and an increase in the Fo/Fm ratio indicates thylakoid membrane damage (Krause and Weis, 1984; Maxwell and Johnson, 2000).

Leaf level photosynthesis was measured using the LI-COR 6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA). Measurements were taken at day growth temperature and ambient CO_2 conditions (390 μmol mol^{-1}). The internal light-emitting diode (LED) light source in the LI-COR 6400 was set at 1600 μmol m^{-2} s^{-1} to have a constant and uniform light across all measurements.

Chlorophyll concentration was measured using a self-calibrating chlorophyll meter (Soil Plant Analyzer Development [SPAD], Model 502, Spectrum Technologies, Plainfield, IL, USA). Measurements were taken at five different areas along the flag leaf blade, and the readings were averaged to get a single value for a plant.

Reactive Oxygen Species Concentration and Plasma Membrane Damage in Leaves

Reactive oxygen species concentration and plasma membrane damage in leaves were measured using molecular probes. Reactive oxygen species production was assessed by the Image-iT LIVE green ROS detection kit (Molecular Probes Inc., Eugene, OR, USA; Kristiansen et al., 2009; Prasad and Djanaguiraman, 2011), following the manufacturer's protocol. The assay is based on 5–(and–6)–carboxy–2′,7′–dichlorodihydrofluorescein diacetate (carboxy–H_2DCFDA), a fluorogenic marker for ROS in cells. The nonfluorescent carboxy-H_2DCFDA dye penetrates cells and is cleaved by cellular esterases, producing a reduced fluorescein compound. In the presence of ROS, this compound is oxidized and emits a bright green fluorescence. A stock solution of 10 mM carboxy-H_2DCFDA was prepared by adding 50 µL dimethylsulfoxide (DMSO) to one vial of carboxy-H_2DCFDA (275 µg, supplied by LIVE green ROS detection kit, Molecular Probes). The vial was vortexed until the powder dissolved completely. A working solution of 25 µM carboxy-H_2DCFDA was prepared by adding 5 µL of the 10 mM carboxy-H_2DCFDA stock solution to 2 mL of warm (30°C) Hanks' Balanced Salt Solution (HBSS) buffer. The leaf specimens (slices from middle portion of the leaves) were collected from tagged stems, placed in microcentrifuge tubes, immediately frozen in liquid nitrogen and transported to the laboratory. The leaf specimens were washed in warm (30°C) HBSS buffer and immediately immersed in 25 µM carboxy-H_2DCFDA working solution. The leaves were incubated for 30 min at 37°C and protected from light. Leaves stained with the Live green ROS detection stain were observed using a Zeiss LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA). The fluorescent images of stained leaves were taken with a 488-nm Argon LASER line with 505/530 nm excitation/emission bandpass filter. Only the central portion of leaf specimens was used for imaging; the edges were not used to avoid the areas damaged when cutting the leaf specimens. Images of leaf specimens were analyzed using the public domain software, ImageJ (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/; Abramoff et al., 2004) and the fluorescence intensities were recorded.

Plasma membrane damage was assessed with SYTOX green nucleic acid stain (Molecular Probes Inc., Eugene, OR, USA; Truernit and Haseloff, 2008; Prasad and Djanaguiraman, 2011). SYTOX green stain is a high-affinity nucleic acid stain that penetrates cells with damaged plasma membranes and produces bright green fluorescence. It cannot penetrate intact plasma membranes of live cells. The leaf specimens (slices from middle portion

of the leaves) were collected from tagged stems, placed in microcentrifuge tubes, immediately frozen in liquid nitrogen and transported to the laboratory. The leaf specimens were washed in warm (30°C) HBSS buffer and immediately immersed in SYTOX green stain solution (1 μM) for 15 min. Leaves stained with SYTOX green stain were observed using a Zeiss LSM 5 PASCAL laser scanning confocal microscope. The fluorescent images of stained leaves were taken with a 488-nm Argon LASER line with 505/530 nm excitation/emission bandpass filter. As described above, only the central portion of the leaf specimens was used for imaging. Images of leaf specimens were analyzed using ImageJ software and the fluorescence intensities were recorded.

Total Antioxidant Capacity in Leaves

Total antioxidant capacity in leaves was measured using OxiSelect total antioxidant capacity (TAC) assay kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's protocol. The TAC assay is based on the reduction of copper (II) to copper (I) by antioxidants. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. Absorbance values are proportional to the sample's total antioxidant capacity. Leaf samples were prepared according to the procedure of Gillespie et al. (2007). The leaf samples were collected from tagged stems, placed in microcentrifuge tubes, immediately frozen in liquid nitrogen and transported to the laboratory. Samples were stored at –80°C until processing. The leaf samples were weighed using a Model A-160 Balance (Denver Instrument Company, Bohemia, NY, USA) equipped with an Unstable Indicator and a Filter setting of 'Normal' to minimize weighing time, which was always < 1 minute per sample to avoid moisture absorption by the sample, before carrying out the TAC assay. To pulverize the tissue, three tungsten carbide beads were placed in each sample tube. Tissues were pulverized by grinding them using a Retsch Mixer Mill MM 400 (Verder Scientific Inc., Newtown, PA, USA) for 2 min at 30 Hz. Samples were inserted into pre-cooled Teflon adaptors in the Mixer Mill while pulverization. Samples were extracted with ice-cold 50% acetone (1 mL per sample tube). Samples were thoroughly shaken manually after adding acetone. Samples were centrifuged (4500 g for 30 min at 4°C) with a Labnet Prism R Refrigerated Microcentrifuge (Labnet International Inc., Edison, NJ, USA). The TAC assay was carried out immediately after extraction because antioxidant capacity changes quickly. The

supernatant was added to the 96-well microtiter plate (20 µL per well), and 180 µL of the 1X reaction buffer (supplied by TAC assay kit, Cell Biolabs) was added to each well and mixed thoroughly using an Orbital-Genie orbital shaker (Scientific Industries Inc., Bohemia, NY, USA). An initial absorbance was obtained by reading the plate at 490 nm using an ELx800 absorbance microplate reader (Biotek, Winooski, VT, USA). To initiate the reaction, 50 µL of the 1X copper ion reagent (supplied by TAC assay kit, Cell Biolabs) was added to each well, and the plate was incubated for 5 min on the orbital shaker. To terminate the reaction, 50 µL of 1X stop solution was added to each well. The final absorbance was obtained by reading the plate again at 490 nm using the microplate reader. Net absorbance was calculated by subtracting the initial absorbance from the final absorbance. Total antioxidant capacity was determined by comparison with known concentrations of uric acid standards and was expressed as mM uric acid equivalents (UAE) per g of leaf tissue (fresh weight basis).

Growth and Yield Traits

At maturity, the numbers of filled and unfilled grains were estimated from the tagged spikes of five plants per genotype from all temperature regimes. Individual spikelets were checked for grain by pressing the floret between the thumb and index finger. Seed set was determined as the ratio of spikelets with grain to the total number of spikelets and expressed as a percentage. Data on plant height and tiller number were recorded at maturity on five plants per genotype from all temperature regimes. Plant height was determined as the distance between base of the plant and flag leaf ligule. Plants were hand-harvested by cutting them at the soil level. Spike number per plant was recorded at the time of harvest. Vegetative parts (leaves + stems) and spikes (main spike and other spikes separately) were dried at 60°C and 40°C, respectively for 10 d for determination of dry weight (Smith, 1973). Vegetative dry weight was determined as the weight of leaves + stems and reported on a per-plant basis. Spikes were threshed after drying to separate grains. Grain number per main spike was counted manually. Grain yield per main spike and per plant were determined. Individual grain weight was determined by dividing grain yield per main spike by number of grains per main spike. Harvest index was estimated as the ratio of grain yield to the total aboveground biomass (vegetative dry weight + spike weight) for each plant.

Data Analyses

The experimental design was a randomized complete block with a split plot treatment structure. Temperature was the main plot factor and genotype was the split plot factor. Day of high temperature stress (DOS; indicates sampling time) was treated as the split-split plot factor for physiological and biochemical traits. The treatment factor, temperature had four levels (OT, HN, HD and HDN), genotype had two levels (genotypes Karl 92 and Ventnor) and DOS had four levels (days 1, 4, 7 and 9; day 9 indicates the recovery period because stress was relieved on day 7). There were two replications (Experiments 1 and 2) for the main plot treatment factor (temperature) and five replications (five plants) for the split plot treatment factor (genotype). Analysis of variance was performed using the GLM procedure in SAS (Version 9.2, SAS Institute) for physiological, biochemical, growth and yield traits. The Type 3 F-tests were performed using the TEST option on the RANDOM statement in the GLM procedure. Experiment (block), temperature and genotype were used as class variables. DOS was included as a class variable for physiological and biochemical traits. Experiment and experiment × temperature interaction were treated as random effects, and temperature and genotype were treated as fixed effects. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$).

Results

Analysis of variance showed that the temperature × genotype × DOS interaction was not significant for any of the traits recorded (Table 3.1). The temperature × genotype interaction was significant only for grain yield per plant and harvest index. The temperature × DOS interaction was significant only for ROS concentration.

Chlorophyll Concentration, Chlorophyll a fluorescence and Photosynthesis

Chlorophyll concentration was not significantly affected by temperature regimes (Table 3.1). In contrast, chlorophyll *a* fluorescence (Fo/Fm and Fv/Fm ratios) and photosynthesis were significantly affected by temperature regimes (Table 3.1). The effects of HN, HD and HDN were similar on Fo/Fm, Fv/Fm and photosynthesis (Fig. 3.1). Values of Fo/Fm ratio were significantly increased by HN (11%), HD (17%) and HDN (21%) (Fig. 3.1a), which indicates increased thylakoid membrane damage due to high day and high night temperatures. Values of Fv/Fm ratio were significantly decreased by HN (2%), HD (3%) and HDN (4%) (Fig. 3.1b), which indicates

decreased photochemical efficiency of PSII due to high day and high night temperatures. Photosynthesis was significantly decreased by HN (12%), HD (20%) and HDN (25%) (Fig. 3.1c).

Reactive Oxygen Species Concentration, Plasma Membrane Damage and Total Antioxidant Capacity

Effects of temperature regimes over time were significant for ROS concentration in leaves (Table 3.1; Fig. 3.2). Temperature effects were significant from day 4 during the stress period (Fig. 3.2). When averaged across all measurement times, ROS concentration was significantly greater at HN (82%), HD (89%) and HDN (118%) compared with OT (Fig. 3.2e; Fig. 3.3a, 3.3b, 3.3c and 3.3d). The effects of HN, HD and HDN on ROS concentration were similar (Fig 3.2e). The temperature treatments, HN, HD and HDN caused significant damage to plasma membranes (Percentage increases were 32, 27 and 45%, respectively) (Fig. 3.1d; Fig. 3.3e, 3.3f, 3.3g and 3.3h). The temperature treatments, HN, HD and HDN had similar impacts on plasma membrane damage. Total antioxidant capacity in leaves decreased due to HN (16%), HD (17%) and HDN (16%) compared with OT (Fig. 3.4). Impacts of HN, HD and HDN on total antioxidant capacity were similar.

Growth and Yield Traits

Plant height, tiller number and vegetative dry weight were not significantly influenced by high day and high night temperatures (Table 3.1). Similarly, spike number and individual grain weight were not affected by temperature regimes (Table 3.1; Fig. 3.5d). However, HN, HD and HDN significantly decreased seed set (15, 18 and 37%, respectively), grain number per spike (13, 17 and 41%, respectively) and grain yield per spike (13, 16 and 43%, respectively) (Table 3.1; Fig 3.5a, 3.5b and 3.5c). The effects of HN and HD were similar on the above traits, whereas HDN had a greater impact than HN and HD.

Genotypes differed for grain yield per plant and harvest index in response to temperature regimes (Table 3.1). The heat-susceptible genotype Karl 92 showed a significant decrease in grain yield per plant at HN, HD and HDN (Fig. 3.6a). The temperature treatments, HN and HD had similar impacts on grain yield per plant of Karl 92, whereas HDN had a greater impact than HN and HD. Harvest index of Karl 92 decreased only at HDN (Fig. 3.6c). Temperature regimes

had no significant effects on grain yield per plant and harvest index of the heat-tolerant genotype, Ventnor (Fig. 3.6b and 3.6d).

Discussion

High temperature treatments (HN, HD and HDN) increased ROS concentration, plasma membrane damage and Fo/Fm ratio (thylakoid membrane damage) and decreased total antioxidant capacity, Fv/Fm ratio (maximum quantum yield of PSII) and photosynthesis in leaves of winter wheat genotypes (Karl 92 and Ventnor) (Fig. 3.1 through 3.4). These effects were produced by 10 and 9°C elevations in day maximum and night minimum temperatures, respectively, compared with an optimum temperature of 25/15°C. High temperature treatments, HN and HD had similar impacts on the above traits. This indicates that these traits are temperature-driven, regardless of whether stress occurs during day or night.

Increased ROS concentration observed under high temperature may be a result of decreased antioxidant activity (Prochazkova et al., 2001; Djanaguiraman et al., 2010). Increased ROS production, in turn leads to blockage of PSII reaction center and electron flow, which results in increased Fo/Fm ratio, decreased Fv/Fm ratio and downregulation of PSII photochemistry (Djanaguiraman et al., 2010). All these changes lead to decreased photosynthesis (Djanaguiraman et al., 2010). Increased ROS concentration could also cause damage to the membranes (Allen, 1995). Increased permeability of the compromised thylakoid membranes under high temperature leads to proton leakage, which decreases synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), resulting in decreased photosynthesis (Bukhov et al., 1999; Schrader et al., 2004).

High temperature treatments (HN, HD and HDN) decreased seed set, grain number per spike and grain yield per spike (Fig. 3.5). Decreased grain number was a result of decreased seed set. Decreased seed set under high temperature stress may be due to poor functionality of pollen and/or ovule such as decreased pollen germination, pollen longevity and rate of pollen tube growth and poor fertilization (Saini and Aspinal, 1982; Saini et al., 1983; Shivanna et al., 1991; Kakani et al., 2002; Prasad et al., 2006, 2011a; Weerakoon et al., 2008; Prasad and Djanaguiraman, 2011; Djanaguiraman et al., 2013a,b). However, pollen or ovule performance was not estimated in this research to pinpoint the exact cause of decreased seed set. Individual grain weight (Fig. 3.5d) is mainly determined by grain filling duration and grain filling rate, even

though it is influenced by mobilization of stored pre-anthesis assimilates (Bidinger et al., 1977; Calderini et at., 2001). Individual grain weight was not affected by high day and high night temperatures in this study mainly because plants did not experience high temperature stress during the grain filling stage (high temperature was imposed during anthesis). Decreased grain yield per spike happens due to decreased grain number or individual grain weight. Because high day and high night temperatures did not decrease individual grain weight, decreased grain yield per spike in the present study was due to decreased grain number.

The impact of HN and HD was similar on seed set, grain number per spike and grain yield per spike (Fig. 3.5). One reason may be poor pollen and ovule performance (such as decreased pollen germination, pollen longevity and rate of pollen tube growth and poor fertilization) occurring under both HN and HD, which leads to decreased seed set resulting in decreased grain number and yield. Djanaguiraman et al. (2013b) reported that pollen germination is decreased by high temperature stress during anthesis, regardless of whether stress occurs during day or night, even though anther dehiscence takes place during the day. In addition, high temperature also hinders the development of fertilized embryo sacs (Saini and Aspinall, 1982), which could happen even if the stress occurs during day or night. Thus, HDN had greater impacts on seed set, grain number per spike and grain yield per spike compared to HN and HD.

Genotypes differed in grain yield per plant and harvest index under high day and high night temperatures (Table 3.1; Fig. 3.6). The exact reason for these differential responses cannot be substantiated with the results obtained from this research. High temperature stress was started on each plant at the onset of anthesis in the main spike. At this time, tillers did not reach the flowering stage. Thus, tillers experienced high temperature stress prior to anthesis, possibly before pollen maturation. Further research is needed to determine whether the heat-tolerant genotype Ventnor and heat-susceptible genotype Karl 92 differ in responses to high temperature stress in terms of grain yield and harvest index, when stress is imposed prior to anthesis.

The diurnal temperature range for HN, HD and HDN were 1, 20 and 11°C, respectively. The average temperatures (T_{avg}, sum of maximum and minimum temperatures/2) for HN, HD and HDN were 24.5, 25 and 29.5°C, respectively. Both HN and HD (different diurnal temperature range, but similar T_{avg}) had similar impacts on all traits measured in this study. This result is supported by Djanaguiraman et al. (2013b); HN (30/29°C) and HD (39/20°C) had different diurnal temperature range and similar T_{avg}, and had similar impacts on photosynthesis,

pollen germination and yield in soybean [*Glycine max* (L.) Merr.]. Even though HN and HD had similar impacts on yield traits (seed set, grain number per spike and grain yield per spike), HDN (greater diurnal temperature range than HN, lower diurnal temperature range than HD and greater T_{avg} than HN and HD) had greater impacts than HN and HD. These results imply that the T_{avg} rather than the diurnal temperature range was critical in determining the effects of high temperature stress on yield traits of wheat. This result is consistent with the classical reports of Fischer (1985) who suggested that the effects of high temperature on grain number in wheat are driven through the changes in T_{avg}.

The high temperature stresses used in this research were 25/24°C (HN), 35/15°C (HD) and 35/24°C (HDN). Therefore, the high temperature effects quantified by this research refer to 10 and 9°C elevations for day maximum and night minimum temperatures, respectively, compared with an optimum temperature of 25/15°C. Further research is needed to evaluate the results over a wide range of day maximum and night minimum temperatures.

Conclusions

This research quantified the effects of HN (25/24°C), HD (35/15°C) and HDN (35/24°C) during anthesis on physiological, biochemical, growth and yield traits of winter wheat genotypes (Karl 92 and Ventnor). The high temperature treatments, HN, HD and HDN increased ROS concentration and membrane damage and decreased antioxidant capacity, photochemical efficiency and leaf level photosynthesis compared with OT. The high temperature treatments, HN, HD and HDN decreased grain yield per spike through decreased grain number and seed set. Impacts of HN and HD were similar on all traits including seed set, grain number per spike and grain yield per spike. Compared with HN and HD, HDN had greater impacts on seed set, grain number and grain yield per spike. Overall, the results from this research suggest that HN (an elevation of 9°C from the optimum) and HD (an elevation of 10°C from the optimum) cause damage of a similar magnitude to winter wheat.

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Figures and Tables

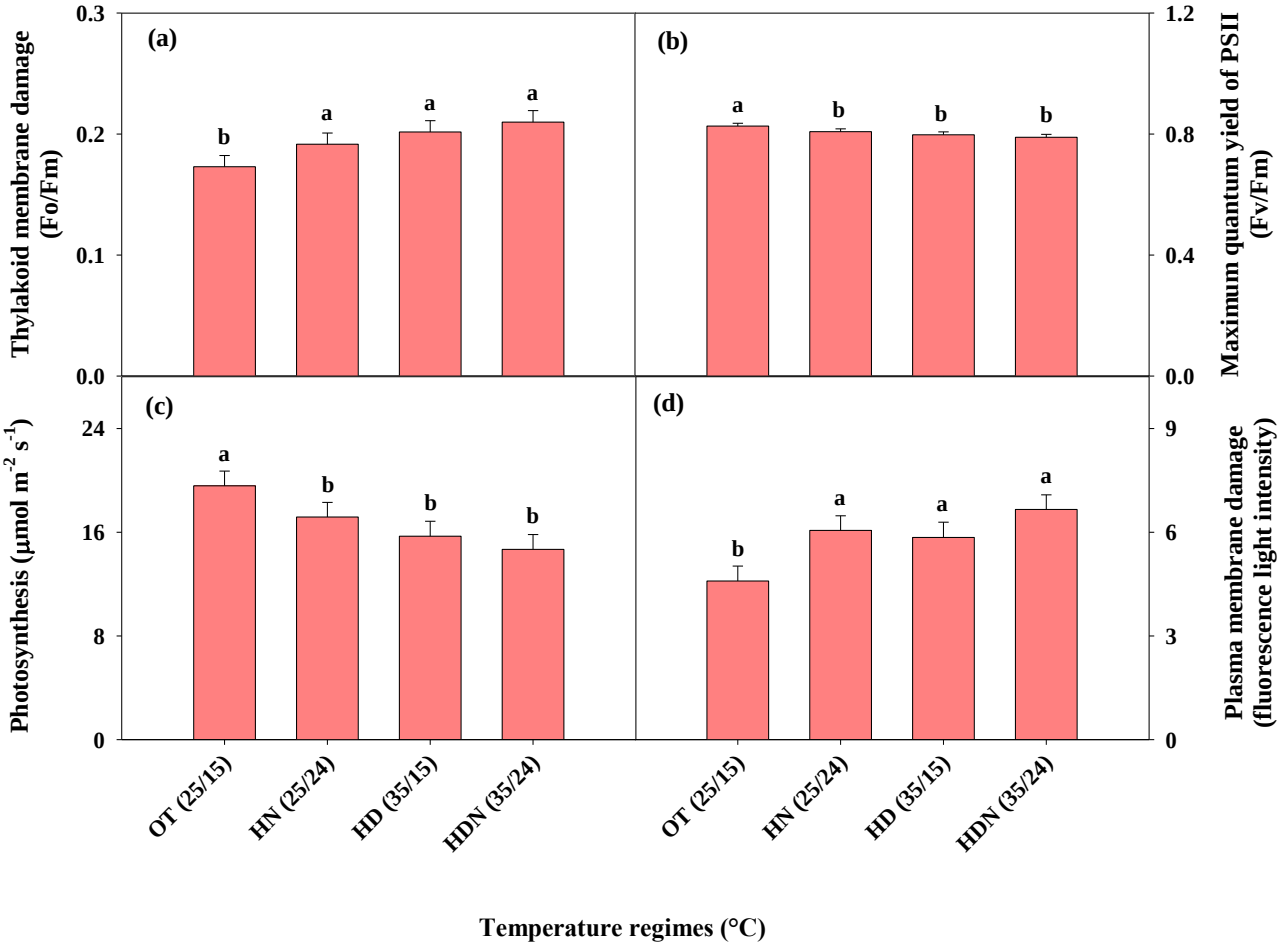


Figure 3.1. Effects of temperature regimes (optimum temperature [OT, 25/15°C], high night temperature [HN, 25/24°C], high day temperature [HD, 35/15°C] and high day and night temperature [HDN, 35/24°C]) on various physiological traits in leaves.

Data are averaged across two experiments, two genotypes, five replications (plants) of each genotype and four measurements taken on each plant on days 1, 4 and 7 during the stress period and day 2 during the recovery period (after the stress was relieved). Each datum indicates mean value and error bars denote standard errors. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$). Means with different letters are significantly different according to the LSD test at $P < 0.05$.

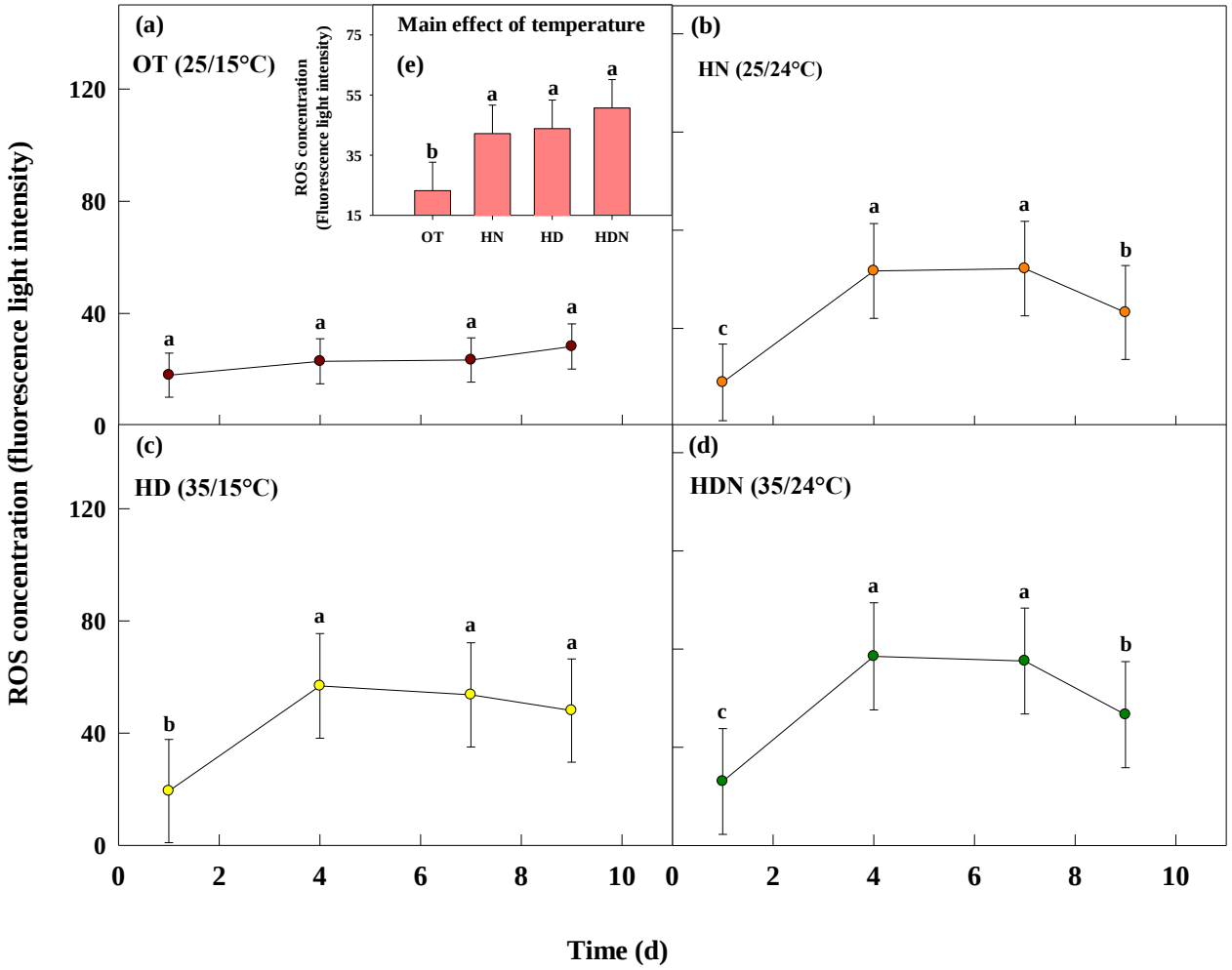


Figure 3.2. Time series data (a, b, c, d) on reactive oxygen species (ROS) concentration in the leaves measured at optimum temperature (OT, 25/15°C), high night temperature (HN, 25/24°C), high day temperature (HD, 35/15°C) and high day and night temperature (HDN, 35/24°C) and main effect of temperature on ROS concentration in the leaves (e).

High temperature stress was started on day 1 and relieved on day 7. The estimates of ROS concentration on day 9 in Fig. a, b, c and d correspond to the measurements taken during the recovery period (stress was relieved on day 7). Data are averaged across two experiments, two genotypes and five replications (plants) of each genotype in Fig, a, b, c and d. Each datum in Fig, a, b, c and d indicates mean value and error bars denote standard errors. Data are averaged across two experiments, two genotypes, five replications (plants) of each genotype and four measurements taken on each plant on days 1, 4, 7 and 9 after the start of high temperature stress, in Fig. e. Each datum in Fig. e indicates mean value and error bars denote standard errors. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$). Means with different letters are significantly different according to the LSD test at $P < 0.05$.

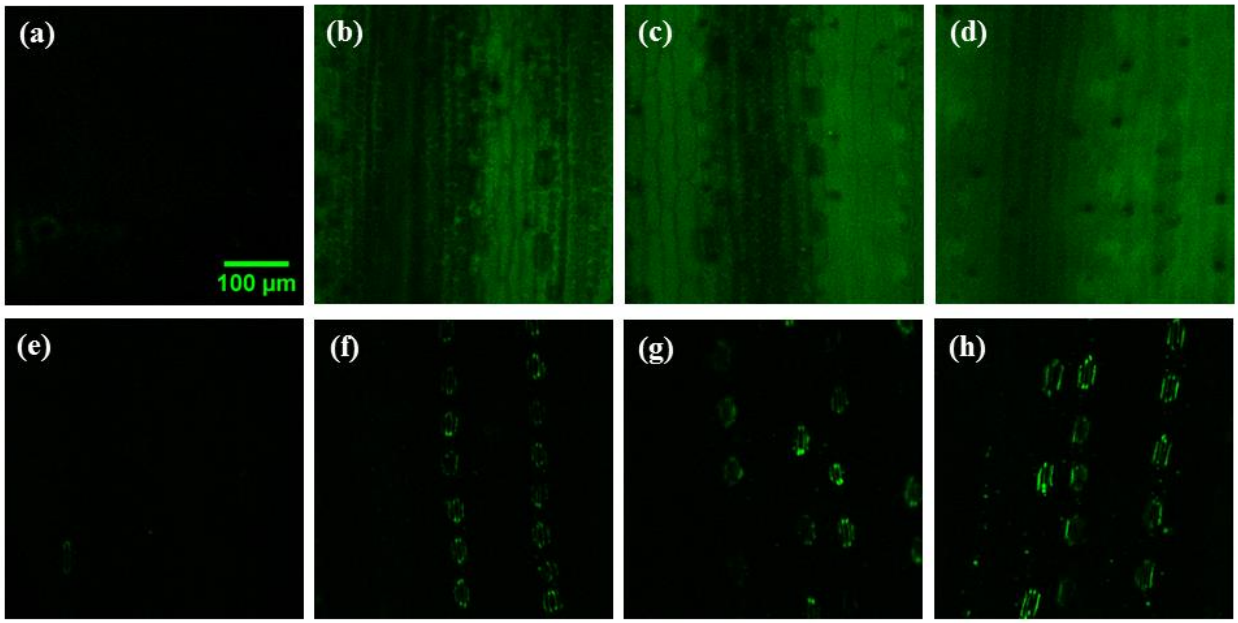


Figure 3.3. Representative images showing effects of different temperature regimes (optimum temperature [a, e], high night temperature [b, f], high day temperature [c, g] and high day and night temperature [d, h]) on reactive oxygen species (ROS) concentration (a, b, c, d) and plasma membrane damage (e, f, g, h) in wheat leaves.

Reactive oxygen species concentration and plasma membrane damage in leaves were quantified by LIVE green ROS detection stain and SYTOX green nucleic acid stain, respectively. Leaf specimens were observed using a laser scanning confocal microscope. Black unstained areas of leaves and green stained areas of leaves by LIVE green ROS detection stain indicate the absence and presence of ROS, respectively. The intensity of green color is proportional to the amount of ROS. Likewise, black unstained areas of leaves and green stained areas of leaves by SYTOX green nucleic acid stain indicate the absence and presence of plasma membrane damage, respectively. The intensity of green color is proportional to plasma membrane damage.

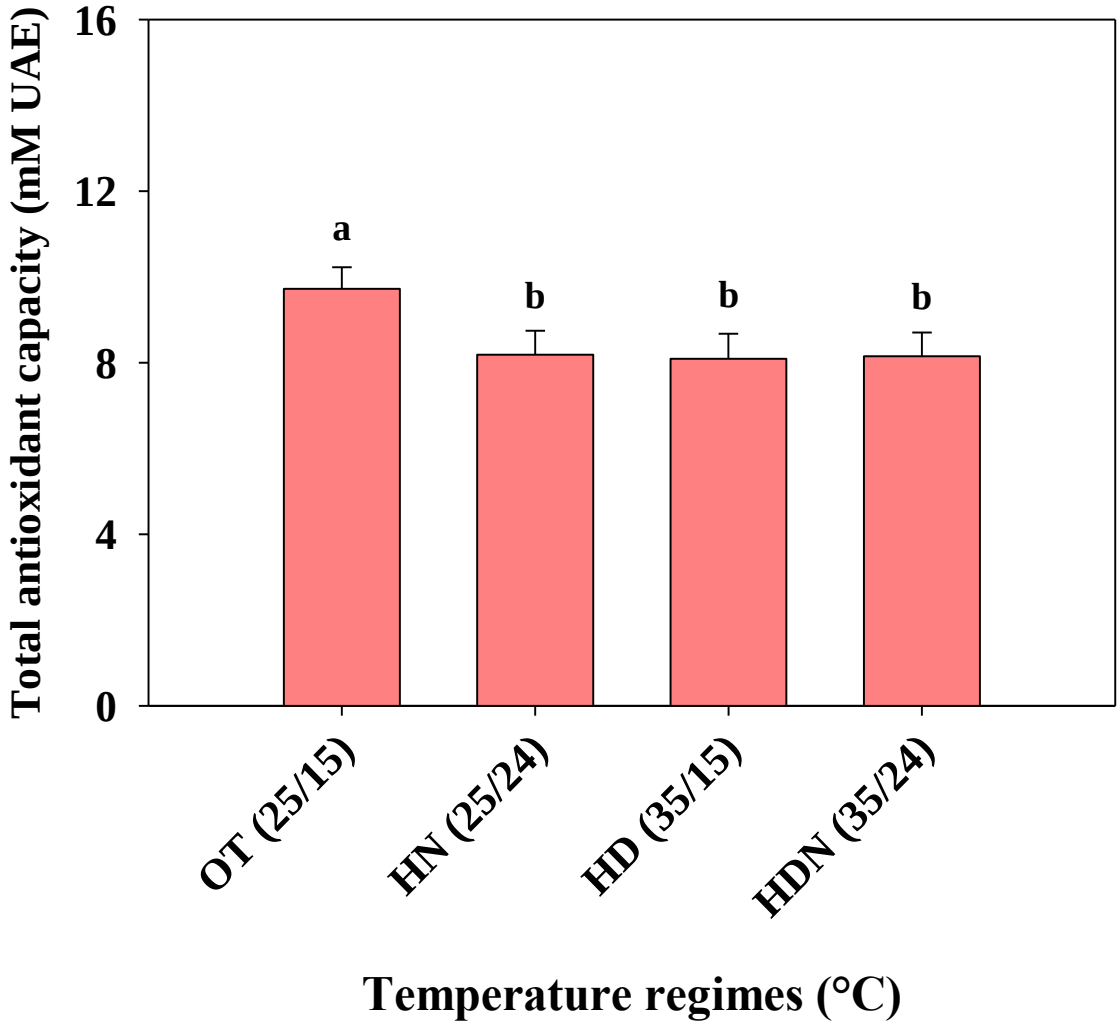


Figure 3.4. Effects of temperature regimes (optimum temperature [OT, 25/15°C], high night temperature [HN, 25/24°C], high day temperature [HD, 35/15°C] and high day and night temperature [HDN, 35/24°C]) on total antioxidant capacity (mM uric acid equivalents [UAE] per g of leaf tissue) in wheat.

Data are averaged across two experiments, two genotypes, five replications (plants) of each genotype and four measurements taken on each plant on days 1, 4 and 7 during the stress period and day 2 during the recovery period (after the stress was relieved). Each datum indicates mean value and error bars denote standard errors. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$). Means with different letters are significantly different according to the LSD test at $P < 0.05$.

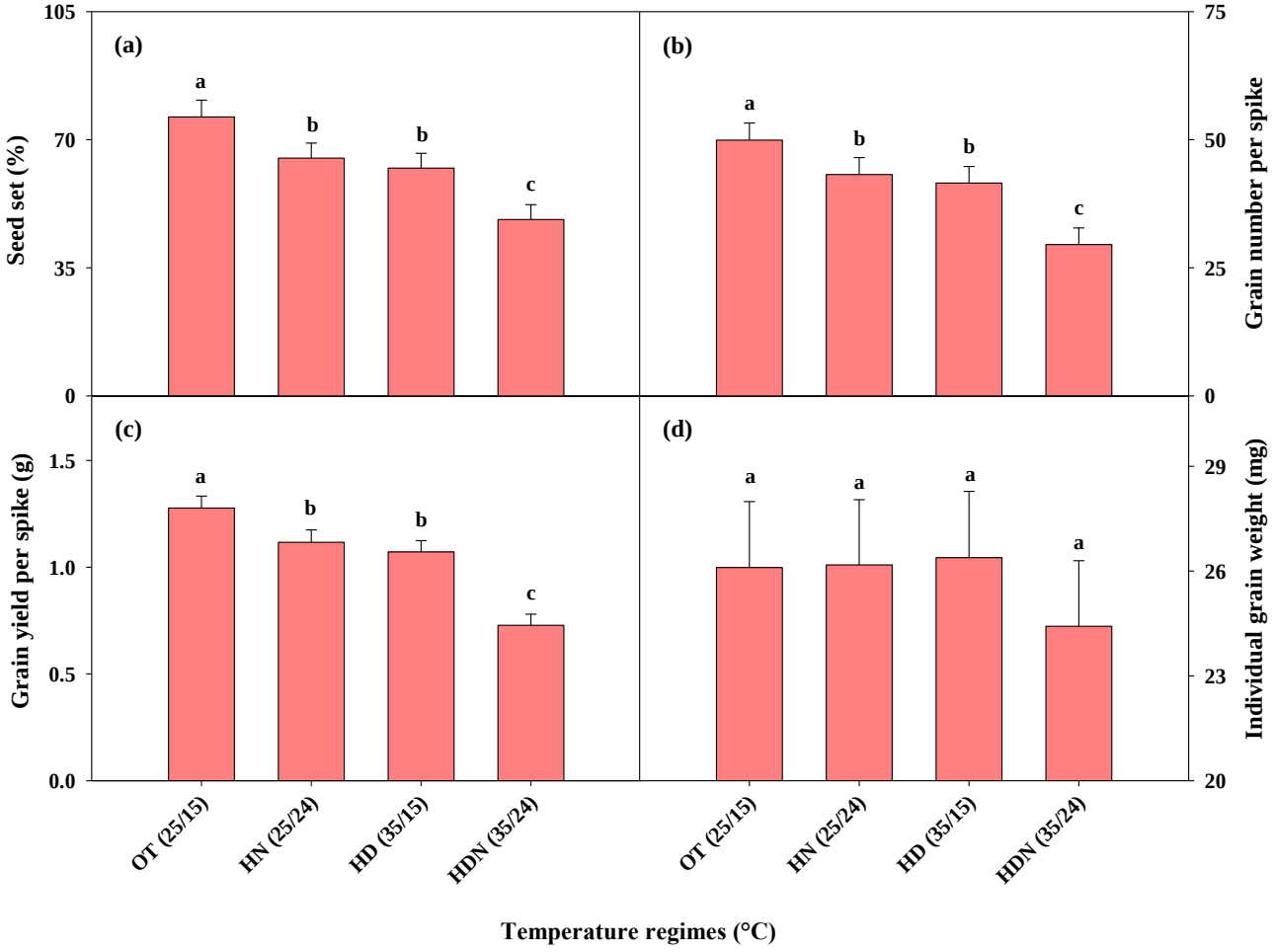


Figure 3.5. Effects of temperature regimes (optimum temperature [OT, 25/15°C], high night temperature [HN, 25/24°C], high day temperature [HD, 35/15°C] and high day and night temperature [HDN, 35/24°C]) on yield traits.

Only the main spike was sampled to determine the above traits. Data are averaged across two experiments, two genotypes and five replications (plants) of each genotype. Each datum indicates mean value and error bars denote standard errors. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$). Means with different letters are significantly different according to the LSD test at $P < 0.05$.

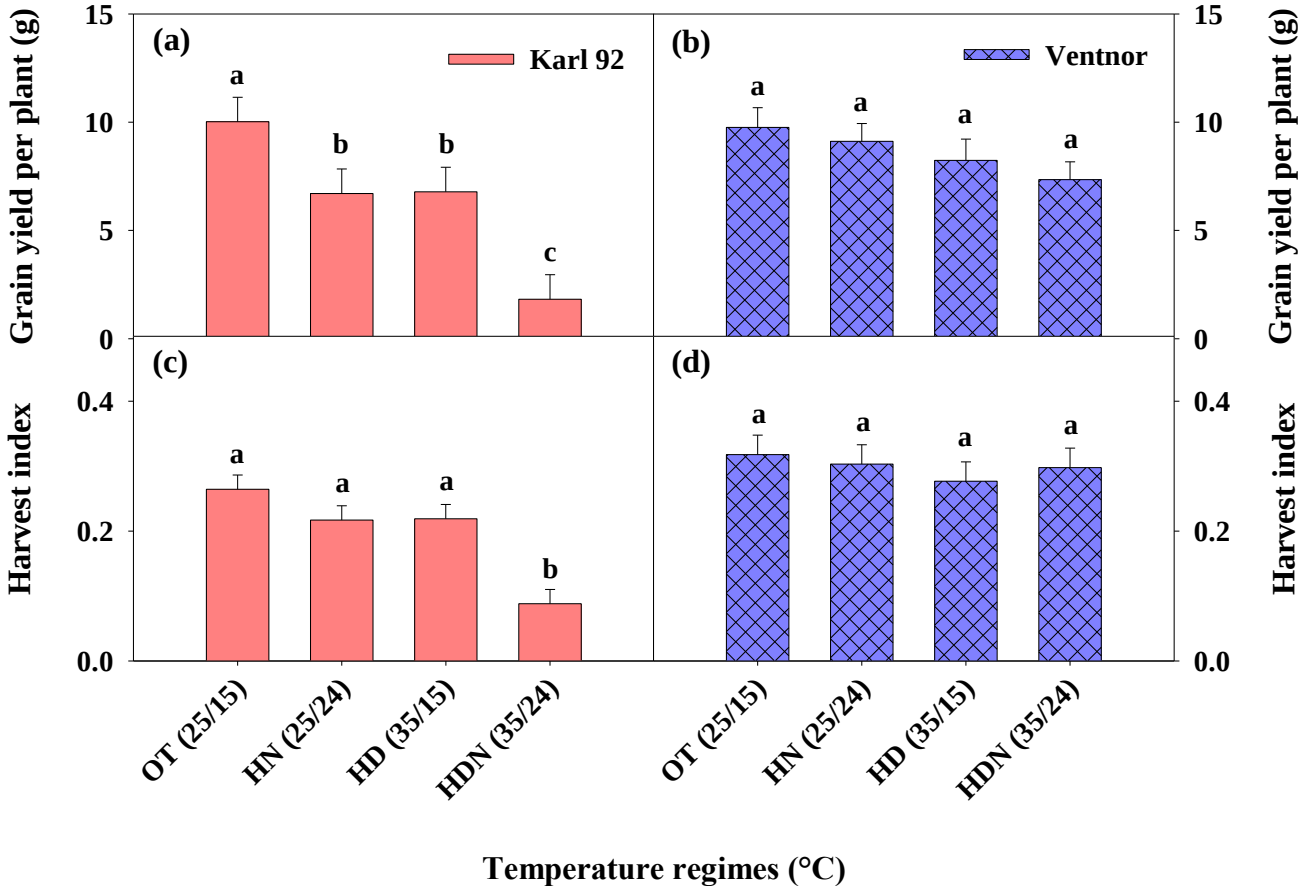
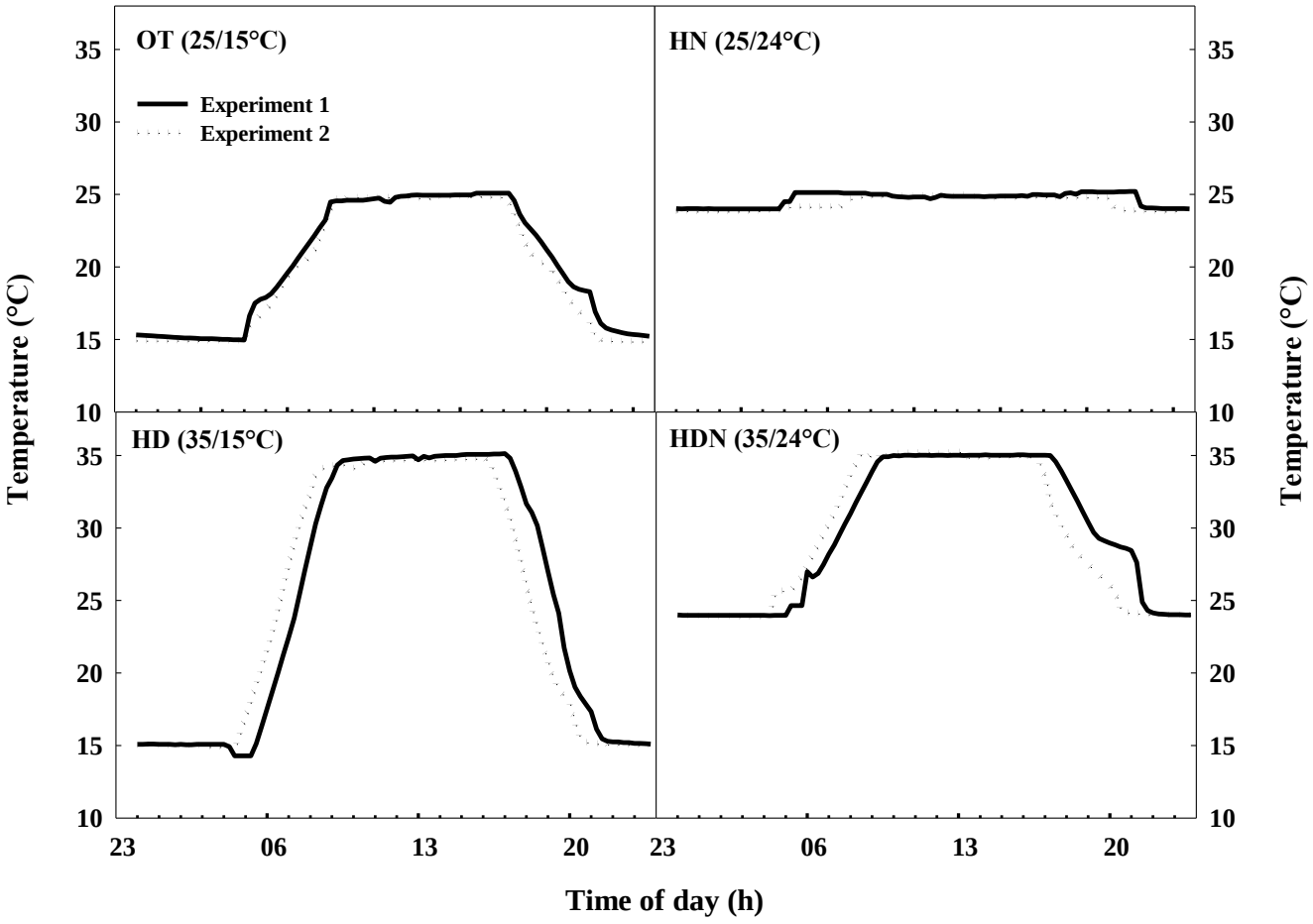


Figure 3.6. Effect of temperature regimes (optimum temperature [OT, 25/15°C], high night temperature [HN, 25/24°C], high day temperature [HD, 35/15°C] and high day and night temperature [HDN, 35/24°C]) on grain yield per plant and harvest index of genotypes Karl 92 and Ventnor. Data are averaged across two experiments and five plants. Each data point indicates mean value and error bars denote standard errors. Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$). Means with different letters are significantly different according to the LSD test at $P < 0.05$.



Supplemental Figure 3.S1. The observed air temperature inside the growth chambers at optimum temperature (OT), high night temperature (HN), high day temperature (HD), and high day and night temperature (HDN) regimes in experiment 1 and 2. In all temperature regimes, day maximum temperature was held for 8 h from 09:00 h to 17:00 h. Similarly, night minimum temperature was held for 8 h from 21:00 h to 05:00 h.

Table 3.1. Probability values of effects of temperature (T), genotype (G), T × G interaction, day of stress (DOS), and T × DOS interaction on various biochemical, physiological, growth and yield traits.

Traits	T	G	T × G	DOS	T × DOS	T × G × DOS	Main effect of temperature (mean±standard error†)			
							Optimum temperature	High night temperature	High day temperature	High day and night temperature
Total antioxidant capacity (mM uric acid equivalents)	0.0010	0.0342	0.9569	0.0366	0.4208	0.8047	9.72±0.501	8.18±0.553	8.09±0.583	8.15±0.551
Reactive oxygen species concentration (fluorescence light intensity)	0.0120	0.1416	0.0962	<.0001	0.0004	0.3293	23.2±9.45	42.2±9.47	43.8±9.51	50.7±9.49
Plasma membrane damage (fluorescence light intensity)	0.0126	0.7865	0.6511	<.0001	0.6888	0.0545	4.58±0.429	6.05±0.423	5.85±0.441	6.66±0.424
Maximum quantum yield of PSII (Fv/Fm)	0.0142	0.1342	0.9016	0.1877	0.1502	0.3054	0.827±0.009	0.807±0.009	0.797±0.009	0.789±0.009
Thylakoid membrane damage (Fo/Fm)	0.0136	0.1345	0.9022	0.1887	0.1518	0.3111	0.173±0.009	0.192±0.009	0.202±0.009	0.210±0.009
Leaf level photosynthesis ($\mu mol\ m^{-2}\ s^{-1}$)	0.0458	0.0508	0.4835	<.0001	0.1079	0.3302	19.6±1.13	17.1±1.13	15.6±1.15	14.6±1.13
Chlorophyll concentration (SPAD units)	0.6419	0.0054	0.4508	0.6679	0.4478	0.2166	58.9±1.03	58.1±1.03	58.5±1.04	58.0±1.04
Plant height (cm)	0.5011	<.0001	0.0546	–‡	–	–	29.3±6.99	28.6±6.99	24.7±6.99	25.7±6.99
Tiller number	0.8263	<.0001	0.3586	–	–	–	17.0±1.18	16.7±1.18	15.6±1.18	15.5±1.18
Vegetative dry weight per plant (g)	0.3634	<.0001	0.5749	–	–	–	30.5±3.53	26.2±3.50	22.9±3.50	20.1±3.50
Seed set (%)	0.0169	<.0001	0.2412	–	–	–	76.2±4.60	64.9±4.09	62.2±4.04	48.1±4.04
Grain number per spike	0.0005	0.0001	0.6332	–	–	–	50±3.34	43±3.30	41±3.22	29±3.22
Grain yield per spike (g)	0.0396	0.0051	0.1847	–	–	–	1.28±0.056	1.11±0.058	1.07±0.053	0.728±0.051
Individual grain weight (mg)	0.7977	0.3167	0.0540	–	–	–	26.1±1.88	26.1±1.86	26.3±1.89	24.4±1.87
Spike number per plant	0.8775	<.0001	0.2770	–	–	–	16.8±1.15	16.7±1.15	17.8±1.18	17.2±1.15
Grain yield per plant (g)	0.1858	0.9958	0.0090	–	–	–	8.89±1.28	6.91±1.27	6.11±1.30	3.58±1.27
Harvest index	0.1943	<.0001	0.0013	–	–	–	0.291±0.023	0.260±0.023	0.248±0.023	0.193±0.023

† Means and standard errors were estimated using the MIXED procedure in SAS. Separation of means was done using the LSD test ($P < 0.05$).

‡ Not available.

Chapter 4 - High Day and Night Temperature Stress Results in Major Lipid alterations in Wheat

Abstract

Climate models predict greater increases in night temperature than in day temperature. Understanding how wheat (*Triticum aestivum* L.) plants regulate membrane lipid composition under high day and night temperatures is critical to develop climate resilient wheat varieties. We hypothesized that molecular species of triacylglycerol (TAG), sterol glycosides (SG), acylated sterol glycosides (ASG) and ox-lipids (lipids with oxidized acyl chains) are associated with high day and night temperature response in wheat. We used an automated electrospray ionization-tandem mass spectrometry approach to determine the changes in lipid profile under high temperature stress in wheat leaves. Both the heat tolerant genotype Ventnor and susceptible genotype Karl 92 decreased the level of lipid unsaturation under high day and night temperatures. The decrease in lipid unsaturation levels of complex lipids was predominantly due to decrease in 18:3 fatty acid and increase in 18:1 and 16:0 fatty acids. The amounts of 18:3-acyl containing TAGs increased under high temperature stress, suggesting a possible role of TAGs in high temperature stress adaptation by sequestering the 18:3 fatty acids from membrane lipids. We identified novel odd-numbered long-chain fatty acid-containing phospholipids, which were highly responsive to high temperature stress. The heat tolerant genotype Ventnor had higher amounts of SGs and saturated species of ASG and lower amounts of ox-lipids at high temperatures, compared to the susceptible genotype Karl 92. This result demands further studies including several genotypes with varying degrees of heat tolerance to confirm SGs and ox-lipids as potential biomarkers for heat tolerance and susceptibility, respectively in wheat.

Introduction

High temperature stress is a major environmental factor that limits yield in wheat (*Triticum aestivum* L.). High temperatures of 30 to 40°C are common in wheat-growing regions of the world during the crop life cycle. Every 1°C increase in mean temperatures above 23°C during grain filling decreases wheat yield by more than 10% (Gibson and Paulsen, 1999). The risk of heat stress in wheat is expected to increase in the future with climate change as global mean surface air temperature is predicted to increase by 1.4–3.1°C by the end of 21st century (Intergovernmental Panel on Climate Change, 2013). This increase would be brought about by increases in day-maximum and night-minimum temperatures. In the past century, average daily minimum temperature has increased more than twice compared to the increase in average daily maximum temperature over the globe (Harvey, 1995; Easterling et al., 1997, 2000). Therefore, understanding the responses of wheat plants to high night, as well as high day, temperatures is critical to anticipate the impacts of climate change on wheat production and to develop climate resilient wheat varieties.

The impacts of combination of high day and night temperatures on plant physiological processes are well documented. For example, high day and night temperatures increase reactive oxygen species (ROS) content in wheat leaves (Narayanan et al., 2014). Increased ROS leads to peroxidation of lipids, which results in the production of malondialdehyde (MDA) (Bowler et al., 1992; Liu and Huang, 2000; Me`ne-Saffrane´ et al., 2009). Both ROS and MDA are recognized as toxic by-products of aerobic metabolism and, at the same time, as mediators of signaling stress response. Stress induced lipid peroxidation and other changes in membrane lipid profile can lead to membrane damage, electrolyte leakage and cell death (Liu and Huang, 2000; Welti et al., 2002). Membranes have long been proposed as major targets of high temperature stress. In plants, the thylakoid membranes are particularly sensitive to high temperature stress (Sharkey, 2005). Any damage to thylakoid membranes results in the reduction of leaf photosynthesis as these membranes are the sites of light dependent reactions of photosynthesis (Allen and Forsberg, 2001; Sharkey, 2005).

Plants regulate membrane lipid composition and fatty acid unsaturation levels, in response to changes in temperature, in order to maintain optimal fluidity and integrity of membranes (Larkindale and Huang, 2004; Chen et al., 2006; Zheng et al., 2011). For example, heat tolerant Arabidopsis (*Arabidopsis thaliana*) plants increased digalactosyldiacylglycerol

(DGDG) and the ratio of DGDG to monogalactosyldiacylglycerol (MGDG) under high temperature stress (Chen et al., 2006; Welti et al., 2007) because smaller head group of MGDG promotes a non-bilayer phase, while the larger head group of DGDG promotes a fluid phase (Webb and Green, 1991). Welti et al. (2002) reported that Arabidopsis mutants with freezing tolerance maintained higher levels of phosphatidylcholine (PC), a bilayer-promoting promoting lipid, compared to wild types. In addition, it is well established that plants can decrease lipid unsaturation levels at high temperatures and increase it at low temperatures, to maintain optimal membrane fluidity (Larkindale and Huang, 2004; Chen et al., 2006; Welti et al., 2007).

Innovative applications of mass spectrometry have revealed roles of plant lipids, particularly galactolipids and phospholipids, in stress response. However, the biological significance of other plant lipids, such as triacylglycerols (TAG), sterol derivatives (sterol glycoside [SG] and acylated sterol glycoside [ASG]) and ox-lipids (lipids with oxidized acyl chains), are just beginning to be uncovered. It has been reported that active recycling of fatty acids happen in TAGs as fatty acids are released from and reincorporated into TAGs (Hernández et al., 2012). Thus, TAGs may play a role in high temperature stress response by sequestering highly unsaturated fatty acids from the membrane lipids and/or releasing saturated fatty acids that could be incorporated into the membrane lipids in order to decrease the overall unsaturation level of membrane lipids. In plants, sterols present in rafts (lipid microdomains on membranes) have been found to improve the ordered packing of lipid molecules within the bilayer and regulate membrane dynamics that makes the membrane less sensitive to thermal shock (Beck et al., 2007; Hodzic et al., 2008). Beck et al. (2007) proposed that the presence of an ethyl group on the alkyl chain of sitosterol and stigmasterol (major plant sterols) increases van der Waals interaction within the rafts, and thereby increasing membrane cohesion. Recently, Vu et al. (2012) found that mechanical, biotic and low temperature stresses lead to different changes in the ox-lipid profiles of Arabidopsis. Ox-lipids may act as signaling molecules that initiate stress response in plants (Andersson et al., 2006), and are produced enzymatically (lipoxygenase) and nonenzymatically (through the action of ROS) (Zoeller et al., 2012). It is well established that the nonenzymatic oxidation of polyunsaturated fatty acids results in the production of MDA (Me`ne-Saffrane´ et al., 2009; Ayala et al., 2014). Thus, ox-lipids, in addition to ROS and MDA, might serve as stress indicators as well as signaling molecules. In this study, we test the hypothesis that molecular species of TAG, sterol lipids (SG and ASG) and ox-lipids are associated with high day

and/or night temperature stress response in wheat. The objectives of our study were to characterize the responses of two winter wheat genotypes (heat tolerant genotype Ventnor and heat susceptible genotype Karl 92) to high day and night temperatures based on yield and physiological traits, determine the changes in lipid composition and unsaturation levels of wheat genotypes under high temperature stress and to identify the lipid changes that are related with high temperature tolerance or susceptibility of the wheat genotypes.

Genotypes Ventnor and Karl 92 were grown at optimum temperature (OT; 25/15°C, maximum/minimum) until the onset of flowering. Thereafter, plants were exposed to high night (HN; 25/24°C), high day (HD; 35/15°C), high day and night (HDN; 35/24°C) or optimum temperatures for 12 days. For lipid extraction, leaf samples were collected from five plants per genotype from each temperature regime on the last day of stress. A direct infusion automated electrospray ionization-tandem mass spectrometry (ESI-MS/MS) approach was used to quantitatively profile the lipid molecular species in order to determine the changes in lipid composition and saturation levels under high day and night temperature stresses in wheat genotypes. We are presenting an unprecedentedly comprehensive analysis of lipid changes under high temperature stress in wheat with the inclusion of novel odd-numbered long-chain fatty acid (OLCFA)-containing lipids, which were highly responsive to high temperature stress.

Materials and Methods

Plant Material and Growth Conditions

Two experiments with the same treatment structure and measurement conditions were conducted in controlled-environment facilities at Kansas State University, USA in 2013 and 2014. In both experiments, seedlings of two winter wheat (*Triticum aestivum* L.) genotypes, Ventnor (heat tolerant [Al-Khatib and Paulsen, 1990, Yang et al., 2002a,b]) and Karl 92 (heat susceptible [Yang et al., 2002b, Hays et al., 2007]) were raised in Sunshine Metro Mix 200 potting soil at 25/15°C (day-maximum/night-minimum). Ten-day-old seedlings were vernalized at 4°C and 8 h photoperiod for 56 d. Following vernalization, seedlings were transplanted into 1.6-L pots (one seedling per pot) and maintained at 25/15°C in a growth chamber. Rooting medium in pots (Sunshine Metro Mix 200 potting soil) was fertilized with Osmocote, a controlled-release fertilizer with 19:6:12 N:P_2O_5:K_2O, respectively, at 5 g per pot before transplanting. At seven days after transplanting, a systemic insecticide, Marathon 1% G (a.i.:

Imidacloprid: 1–((6–Chloro–3–pyridinyl) methyl)–N–nitro–2–imidazolidinimine), was applied at 1.5 g per pot to avoid infestation of sucking insect pests. Plants were watered daily to avoid water stress. Position of pots was changed randomly at 7-day intervals to avoid positional effects.

At the onset of flowering (Feekes growth stage 10.5.1), plants were transferred to one of the four temperature regimes: OT (25/15°C; Al-Khatib and Paulsen, 1984), HN (25/24°C; Prasad et al., 2008), HD (35/15°C; Al-Khatib and Paulsen, 1984) and HDN (35/24°C). The temperature treatments were randomly assigned to growth chambers. The quality of temperature control in all four growth chambers is given in Supplemental Fig. 4.S1. Each growth chamber had 15 plants of each genotype. Out of these 15, five plants were used for the measurement of thylakoid membrane damage, photosynthesis and yield traits. Another five plants were used to collect leaf samples for the measurements of RS and MDA contents. The remaining five plants were used to collect leaf samples for lipid extraction. During the stress period, the position of pots was changed randomly every day in all growth chambers to avoid positional effects. The plants were maintained in the various temperature regimes for 12 d. After that, they were returned to the original growth chamber (25/15°C), where they remained until final harvest at maturity.

In all temperature regimes, day-maximum and night-minimum temperatures were held for 8 h and the transition period between maximum and minimum temperatures was 4 h (Supplemental Fig. 4.S1). Relative humidity in all growth chambers was set at 85%. Air temperature and relative humidity were continuously monitored at 15-min intervals in all growth chambers throughout the experiment using a HOBO data logger (Onset Computer Corporation). The photoperiod was 16 h, and photon flux density (400 to 700 nm) provided by cool fluorescent lamps was about 900 µmol m^{-2} s^{-1} at 15 cm away from the lamps and 700 µmol m^{-2} s^{-1} at the top of the plant canopy.

Measurement of Physiological and Yield Traits

At the onset of flowering, the main stem of five plants per genotype in each growth chamber was tagged for the measurements of chlorophyll *a* fluorescence (to estimate thylakoid membrane damage), photosynthesis and yield traits. Chlorophyll *a* fluorescence and photosynthesis were measured on attached fully expanded flag leaves of the tagged stems at day of stress (DOS) 12 (last day of temperature treatment). These traits were measured halfway between the ligule and the tip of the flag leaf at midday (between 10:00 and 14:00 h).

Chlorophyll *a* fluorescence parameters were measured using a modulated fluorometer (OS30p, OptiSciences). The minimum fluorescence (Fo) and maximum fluorescence (Fm) were measured on 60-min dark-adapted flag leaves. Thylakoid membrane damage was estimated as the ratio of Fo to Fm (Krause and Weis, 1984, Maxwell and Johnson, 2000). Leaf level photosynthesis was measured using the LI-COR 6400 portable photosynthesis system (LI-COR). Measurements were taken at daytime growth temperature and ambient CO_2 conditions (390 µmol mol^{-1}). The internal light-emitting diode (LED, the light source) in the LI-COR 6400 was set at 1600 µmol m^{-2} s^{-1} to have a constant and uniform light across all measurements.

Reactive species (ROS and reactive nitrogen species [RNS]) content was estimated using OxiSelect in vitro ROS/RNS assay kit (Cell Biolabs). This assay is based on a proprietary quenched, ROS/RNS specific fluorogenic probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ), which is first primed with a quench removal reagent, and then stabilized in the highly reactive DCFH form. Both ROS and RNS react with DCFH and oxidize it to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Fluorescence intensity is proportional to the RS content within the sample (Wang and Joseph, 1999). To estimate RS content, leaf samples (~ 50 mg) were collected from flag leaves of five plants per genotype from each temperature regime at DOS 12 and stored at -80°C until processing. Weighed leaf samples were pulverized by grinding them using a Retsch Mixer Mill MM 400 (Verder Scientific Inc.) for 2 min at 30 Hz. The pulverized leaf tissues were used for the estimation of RS content as per the manufacturer's protocol. Briefly, pulverized leaf tissues were suspended in 1 ml of 1X phosphate buffered saline and centrifuged at 10000 g for 5 min. The supernatant (50 µL) was transferred to a black colored 96-well microplate and incubated for 5 min at room temperature with a catalyst (1X). Freshly prepared DCFH solution (100 µL) was added to each well and incubated for 45 min by protecting the wells from light. After incubation, fluorescence from samples was read at 485 nm excitation/535 nm emission wavelengths using a Victor3 fluorescence plate reader (PerkinElmer). The RS contents of samples were determined by comparison with the predetermined DCF standard curve and was expressed as nmol DCF per g of leaf tissue (fresh weight basis).

Malondialdehyde content in leaf samples were measured using OxiSelect thiobarbituric acid reactive substances (TBARS) assay kit (Cell Biolabs) as an estimate of lipid peroxidation. Lipid peroxides are unstable indicators of oxidative stress in cells and they decompose to form

complex end products such as MDA (Kappus, 1985). The TBARS assay is based on the reactivity of MDA with two molecules of thiobarbituric acid (TBA) via an acid-catalyzed nucleophilic-addition reaction. The resulting pinkish-red fluorescent 1:2 MDA:TBA adduct has an absorbance maximum at 532 nm and can be measured colorimetrically (Heath and Packer, 1968; Kappus, 1985; Janero, 1990). In the present study, for the estimation of MDA content, leaf samples (~ 100 mg) were collected from flag leaves of five plants per genotype from each temperature regime at DOS 12 and stored at -80°C until processing. Weighed leaf samples were pulverized by grinding them using a Retsch Mixer Mill MM 400 (Verder Scientific Inc.) for 2 min at 30 Hz. The pulverized leaf tissues were used for the estimation of MDA content as per the manufacturer's protocol. Briefly, pulverized leaf tissues were suspended in 1 ml of 1X phosphate buffered saline and 10 µL of 100X butylated hydroxytoluene (BHT), and centrifuged at 10000 g for 5 min. The supernatant (100 µL) was incubated with 100 µL of sodium dodecyl sulfate lysis solution for 5 min at room temperature. Thiobarbituric acid (250 µL) was added into each sample and incubated at 95°C for 45 min. After cooling to room temperature on ice for 5 min, all samples were centrifuged at 3000 rpm for 15 min. The supernatant (200 µL) was transferred to a 96-well microplate and the absorbance was read at 532 nm using an Epoch spectrophotometer (BioTek). The MDA contents of samples were determined by comparison with a predetermined MDA standard curve and was expressed as µmol per g of leaf tissue (fresh weight basis).

For the estimation of seed set, the numbers of filled and unfilled grains on the tagged stems were recorded three weeks after flowering. Individual florets were checked for grain by pressing them between the thumb and the index finger. Seed set was determined as the ratio of florets with grain to the total number of florets, and expressed as a percentage. At maturity, plants were hand-harvested by cutting them at the soil level. Vegetative parts (leaves + stems) and spikes (main spike and other spikes separately) were dried at 65°C for 7 d and 40°C for 10 d, respectively for determination of dry weight. Spikes were threshed after drying to separate grains. Grain number per main spike was counted manually. Grain yield per main spike and per plant were determined. Individual grain weight was determined by dividing grain yield per main spike by number of grains per main spike. Harvest index was estimated as the ratio of grain yield to the total aboveground biomass (vegetative dry weight + spike weight) for each plant.

Lipid Extraction

For lipid extraction, leaf samples were collected from five plants per genotype from each temperature regime between 13:00 and 15:00 h at DOS 12. At sampling, the middle one third of flag leaves were cut and immediately chopped into 6 mL of isopropanol with 0.01% BHT at 75°C in a 50-mL glass tube with a Teflon-lined screw-cap (Thermo Fisher Scientific). Tubes were kept at 75°C for 15 min to deactivate lipid-hydrolyzing enzymes. After cooling the samples to room temperature, 3 mL of chloroform and 1.2 mL of water were added, and samples were stored at -80°C until analysis. The lipid extraction procedure was previously described by Vu et al. (2012). Briefly, the lipid extract in isopropanol, BHT, chloroform and water was shaken on an orbital shaker at room temperature for 1 h and transferred to a new glass tube using a Pasteur pipette, leaving the leaf pieces in the original tube. Four milliliters of chloroform:methanol (2:1) were added to the leaves, the samples were shaken on an orbital shaker at room temperature overnight, and the solvent was transferred to the first extract. The addition, shaking and transfer steps were performed four times until the leaf pieces of every sample appeared white. At this stage, the solvent was evaporated from the extract in an N-EVAP 112 nitrogen evaporator (Organomation Associates, Inc.). Finally, the lipid extract was dissolved in 1 mL of chloroform and stored at -80°C. The extracted leaf pieces were dried in an oven at 105°C overnight, cooled and weighed to express the lipid content on a dry weight basis. Dry weights were determined using a balance (Mettler Toledo AX), which had a detection limit of 2 µg. The precision and accuracy of the balance were previously described by Vu et al. (2012).

ESI-MS/MS lipid profiling

An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Xiao et al., 2010, supplemental data) with modifications and incorporation of a quality controlled approach. From the extracts that were dissolved in 1 mL of chloroform, an aliquot of 15 to 70 µL, corresponding to approximately 0.2 mg dry weight, was added to each of two vials (vial 1 and vial 2). Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added to vial 1 in the following quantities (with some small variation in amounts in different batches of internal standards): 0.6 nmol PC(di12:0), 0.6 nmol PC(di24:1), 0.6 nmol LysoPC(13:0), 0.6 nmol LysoPC(19:0), 0.3 nmol PE(di12:0), 0.3

nmol PE(di23:0), 0.3 nmol LysoPE(14:0), 0.3 nmol LysoPE(18:0), 0.3 nmol PG(di14:0), 0.3 nmol PG(di20:0(phytanoyl)), 0.3 nmol LysoPG(14:0), 0.3 nmol LysoPG(18:0), 0.23 nmol PI(16:0-18:0), 0.16 nmol PI(di18:0), 0.2 nmol PS(di14:0), 0.2 nmol PS(di20:0(phytanoyl)), 0.3 nmol PA(di14:0), 0.3 nmol PA(di20:0(phytanoyl), 0.31 nmol TAG(tri17:1), 0.36 nmol DGDG(16:0-18:0), 0.95 nmol DGDG(di18:0), 1.51 nmol MGDG(16:0-18:0), and 1.3 nmol MGDG(di18:0). To vial 2, only the last four internal standards were added, using half the amount as vial 1. The sample and internal standard mixture in each vial were combined with solvents, such that the composition of chloroform:methanol:300 mM ammonium acetate in water was 300:665:35 (v / v / v), and the final volume was 1.4 mL.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API4000, ABSciex). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 $\mu L\ min^{-1}$.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected from the sample in vial 1 with the following scans: PC and LysoPC, $[M + H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and LysoPE, $[M + H]^+$ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); PG and LysoPG, $[M + NH_4]^+$ in positive ion mode with NL 189.0; PI, $[M + NH_4]^+$ in positive ion mode with NL 277.0; PS, $[M + H]^+$ in positive ion mode with NL 185.0; PA, $[M + NH_4]^+$ in positive ion mode with NL 115.0; DGDG and digalactosylmonoacylglycerol, $[M + NH_4]^+$ in positive ion mode with NL 341.1; MGDG and monogalactosylmonoacylglycerol, $[M + NH_4]^+$ in positive ion mode with NL 179.1; SQDG, $[M - H]^-$ in negative ion mode with Pre 225.0; TAG(16:0-containing), $[M + NH_4]^+$ in positive ion mode with NL 273.2; TAG(18:3-containing), $[M + NH_4]^+$ in positive ion mode with NL 295.2; SG, $[M + NH_4]^+$ in positive ion mode with NL 197.1; ASG(16:0-containing), $[M + NH_4]^+$ in positive ion mode with NL 435.3; and ASG(18:3-containing), $[M + NH_4]^+$ in positive ion mode with NL 457.3. Ox-lipids were detected from vial 2 with the following scan: $[M - H]^-$ in negative ion mode with Pre 291.2, 293.2, and 295.2. The scan speed was 100 $u\ sec^{-1}$. The collision gas pressure was set at 2 (arbitrary units). Other mass spectral parameters are provided in Supplemental Table 4.S1. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, continuum scans were averaged in multiple channel analyzer

(MCA) mode. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas were integrated using a custom script and Applied Biosystems Analyst software. LipidomeDB Data Calculation Environment (Zhou et al., 2011; http://lipidome.bcf.ku.edu:9000/Lipidomics/) was used for isotopic deconvolution and quantification by comparison to the two internal standards of the same class (Brügger et al., 1997; Welti et al., 2002) when possible. Specifics are indicated in Supplemental Table 4.S1.

Quality control (QC) samples were prepared for data normalization by first pooling a volume, corresponding to 0.2 mg of dry weight, of extract from each sample to make a QC stock solution. Based on the leaf dry weight of the samples used to make the combined extract, the concentration was calculated to be 4 mg (of leaf dry weight) mL^{-1} (20 mL total volume). To prepare working QC samples, the internal standard mixture was added and the stock was diluted, so that each working QC sample contained lipid extract corresponding to 0.2 mg combined leaf dry weight (the same amount of internal standard mix used in the other samples) and mass spectrometry solvent (chloroform:methanol:300 mM ammonium acetate in water, 30:65:3.5, v / v / v) in 1.4 mL. The QC mass spectrometry samples was stored at -80°C, and brought to room temperature 1 h before analysis. Each set of 80 analytical samples and 20 QC samples were arranged in a VT 54 racks as shown in Supplemental Table 4.S2.

Once normalized intensities were calculated, the average level of the background, as indicated by the average of the "internal standard only" samples from that tray, was subtracted from every other sample in the tray. An adaptation of the method of Dunn et al. (2011), as used by Vu et al. (2014) was used to assure that the data could be compared throughout extended acquisition periods. The values for the first three QC samples in each set of analytes (Supplemental Table 4.S2) were eliminated, due to potential instrument instability when the instrument is first started. To correct for any drift during acquisition of each tray's data, a trend line was constructed of the intensity data for each lipid in the remaining 17 identical QC samples as a function of vial position number in the tray. The intensity of each lipid analyte in each analytical sample was multiplied by the average of that lipid's level in the QC samples on that tray divided by the level of the lipid on the QC trend line at the sample's vial position. To correct

for any variability across different trays (days), the trend-corrected value of each lipid in each sample was multiplied by the average of the QC values for that lipid from the entire acquisition process divided by the average of that lipid's level in the QC samples on the sample's own tray. Lipid analytes in which the coefficient of variation (CoV) (Supplemental Table 4.S3) was greater than 30% were removed from the data, except we retained PA(36:6) (CoV of 0.32) and PA(36:5) (CoV of 0.34). The average CoV of retained lipids was 16.1% before QC correction, 15.6% after trend correction only and 13.7% after trend correction and across-tray corrections. After calculation of the lipid levels in each sample, the values were divided by fraction of the original sample that was analyzed and the dry weight of the original sample to calculate the data in normalized units of each lipid/dry weight.

The lipid values are reported as normalized intensity per mg leaf dry weight, where a value of one is the intensity of 1 pmol of internal standard. Because the internal standards are not uniformly well-matched to the lipids analyzed (some differ in class; many differ substantially in *m/z*), the absolute values of the analytes provide only a rough guide to absolute amount of each lipid.

Unsaturation Index

Unsaturation index refers to the number of double bonds in a lipid such that greater the unsaturation index, greater is the unsaturation of that lipid or less is the saturation. Unsaturation index of each lipid molecular species was calculated as the product of amount of that lipid molecular species and the average number of double bonds per acyl chain, where the average number of double bonds per acyl chain was calculated by dividing the number of double bonds in the lipid molecular species by number of acyl chains. Finally, the unsaturation index of a lipid head group class was calculated as the sum of the unsaturation indices of individual lipid molecular species in that class (Hong et al., 2002).

Statistical Analyses

The experimental design was a randomized complete block with a split plot treatment structure. Temperature was the main plot factor and genotype was the split plot factor. The treatment factor, temperature had four levels (OT, HN, HD and HDN) and genotype had two levels (genotypes Karl 92 and Ventnor). There were five replications (five plants, biological replications) for the split plot treatment factor, genotype. The experiment was conducted two

times. MIXED procedure in SAS (Version 9.2, SAS Institute) was used to perform analysis of variance and to estimate means and standard errors. Separation of means was carried out using the LSD test ($P < 0.05$).

Results

High Day and Night Temperatures Affect Yield and Physiological Traits

High temperature (HN, HD and HDN) decreased seed set, grain number per spike, individual seed weight, grain yield per spike, grain yield per plant and harvest index in both genotypes (Fig. 4.1). The effects of HN and HD were similar on individual seed weight of both genotypes and on grain number per spike, grain yield per spike and harvest index of Ventnor. The effects of HN were not as large as that of HD for all other yield traits of both genotypes. The effects of HDN were greater than that of HD and HN on all yield traits of Ventnor and on grain number per spike, individual grain weight and grain yield per spike of Karl 92. The percentage decreases in seed set, grain number, grain yield and harvest index were generally greater in Karl 92, compared to Ventnor, indicating that Ventnor had greater tolerance to high temperature stress than Karl 92.

Significant thylakoid membrane damage was observed at HN, HD and HDN in Karl 92 and at HD and HDN in Ventnor (Fig. 4.2A). Photosynthesis decreased at HN, HD and HDN in both genotypes (Fig. 4.2B). Effects of HN and HD on thylakoid membrane damage and photosynthesis were similar in Karl 92, whereas the effects of HN were less than that of HD in Ventnor. The effects of HDN on thylakoid membrane damage and photosynthesis were greater than that of HN and similar to that of HD in both genotypes. Reactive species (RS, including ROS and RNS) content and MDA content increased under HN, HD and HDN in Karl 92 (Fig. 4.2, C and D). Effects of HN, HD and HDN on these traits were similar in Karl 92. High temperature did not affect RS and MDA contents of Ventnor, indicating that this genotype showed tolerance to high temperature stress in terms of RS and MDA contents.

ESI-MS/MS Profiling and Quantification of Plant Lipids

An ESI-MS/MS approach was used for lipid profiling. Data acquisition and analysis were carried out as described by Xiao et al. (2010) with modifications (See "Materials and Methods"). The lipid molecular species were identified by precursor or neutral loss scanning, and the lipids

in each head group class were quantified in comparison with internal standards of that class (Supplemental Table 4.S1). The goal of the quantification was to compare different leaf samples for the amount of each lipid molecular species, rather than to compare the absolute amounts of various lipid molecular species with each other. To assure that the data for each molecular species could be compared throughout long periods of mass spectral data acquisition, a quality controlled approach was employed (Dunn et al., 2011; Vu et al., 2014). Quality control samples were prepared by pooling an aliquot from each leaf sample, and were analyzed recurrently among the experimental samples (Supplemental Table 4.S2). The intensity of each lipid species in the experimental samples were normalized using the QC analyte intensities, as described in "Materials and Methods". Lipid analytes in which the CoV (standard deviation divided by mean of the amount of the analyte in the QC samples) was greater than 30% were removed from the data set. Normalization using QC intensities increased the analytical precision, as indicated by a decrease in the average CoV for the retained lipid analytes from 16.1% (before QC correction) to 13.7% (after QC correction) (Supplemental Table 4.S3). Using the above procedure, data on 165 lipid species were obtained and were presented as normalized intensity per mg of leaf dry weight (Supplemental Table 4.S4).

Changes in Lipid Composition and Unsaturation Levels under High Day and Night Temperatures

The total amount of lipids in the head group classes, DGDG, MGDG, phosphatidylglycerol (PG), sulfoquinovosyl diacylglycerol (SQDG), PC and phosphatidylethanolamine (PE) generally decreased under high temperature stress, whereas, levels of head group classes, ASG, SG, TAG and ox-lipids generally increased under high temperature stress in both genotypes (Fig. 4.3). Lipid classes responded in four different ways to high temperatures: (1) some lipid classes decreased at high temperatures and decreased more in Karl 92 than in Ventnor (e.g., PG and SQDG); (2) some also decreased at high temperatures but decreased more in Ventnor than in Karl 92 (e.g., PC and PE); (3) some increased at high temperatures and increased more in Karl 92 than in Ventnor (e.g. ox-lipids); and (4) some increased at high temperatures but increased more in Ventnor than in Karl 92 (e.g., ASG). These changes led to higher amounts of PG, SQDG and ASG and lower amounts of PC, PE and ox-

lipids in Ventnor than in Karl 92 at high temperatures. Ventnor had higher amounts of SG than Karl 92 at optimum temperature (OT) as well as high temperatures.

The impacts of HD and HDN were greater than that of HN on most lipid classes in both genotypes (Fig. 4.3). The percentage changes under HD and HDN were larger for ASG (16 and 28% in Karl 92 at HD and HDN, respectively and 54 and 113% in Ventnor at HD and HDN, respectively), SG (74 and 129% in Karl 92 at HD and HDN, respectively and 68 and 123% in Ventnor at HD and HDN, respectively), TAG (200 and 98% in Karl 92 at HD and HDN, respectively and 205 and 196% in Ventnor at HD and HDN, respectively) and ox-lipids (92 and 126% in Karl 92 at HD and HDN, respectively and 67 and 108% in Ventnor at HD and HDN, respectively), compared to other lipid classes. Among these four lipid classes (ASG, SG, TAG and ox-lipids), only SG showed a response to HN in both genotypes, increasing 54 and 38% in Karl 92 and Ventnor, respectively at HN.

High temperature resulted in significant changes in the diacyl lipid species composition of plastidic classes DGDG, MGDG, PG and SQDG and extraplastidic classes PE, PC and phosphatidylinositol (PI) (Fig. 4.4). High temperature caused a decrease in the amount of more unsaturated lipid species and an increase in the amount of less unsaturated lipid species. For example, many species that containing two polyunsaturated acyl chains, such as 36:5- or 36:6-DGDG, MGDG, PG, SQDG, PE and PC, decreased at HN, HD and HDN (Fig. 4.4). On the other hand, the amount of less unsaturated species, such as 34:1-MGDG, PG and PE increased at HN, HD and HDN (Fig. 4.4). 34:1-DGDG, MGDG, PE, PC and PI had a 3-fold or greater increase at HD and HDN, compared to OT in both genotypes. In general, lipid species that likely had 18:3 acyl chains (e.g., 36:6 and 36:5 species; the component fatty acid chains were determined based on Table 1 of Devaiah et al., 2006) decreased during high temperature stress, and lipid species that had 18:1 and/or 16:0 acyl chains (e.g., 34:1 and 36:3 species) increased during high temperature stress. This led to a decrease in unsaturation index of most lipid classes at high temperature stress in both genotypes (Fig. 4.5).

While there was a decline in the amount of plastidic and extraplastidic lipid species that contain 18:3 acyl chains at high temperatures, 18:3-acyl containing TAGs (18:3/36:6, 18:3/36:5, 18:3/36:4 and 18:3/36:3 species) increased at HD and HDN in both genotypes (Fig. 4.6). 18:3-acyl containing TAGs had a 3-fold or greater increase at HD and HDN, compared to OT in both genotypes. Triacylglycerols may act as buffers for cellular acyl lipids, and active recycling of

18:3 fatty acids may occur in TAGs (Hernández et al., 2012). This suggests a possible sequestration of 18:3 acyl chains from plastidic and extraplastidic lipid species into TAGs at high temperature.

High Temperature Increased the Amounts of Novel Odd-numbered Long-Chain Fatty Acid-containing Phospholipids

The ESI-MS/MS revealed certain novel phospholipid species, which were highly responsive to high temperature stress in wheat plants. These phospholipid species were further subjected to product ion analysis using quadrupole time-of-flight mass spectrometry (Q-TOF MS) to define the compounds, as described by Vu et al. (2012). The Q-TOF MS identified the novel phospholipid species as OLCFA-containing phospholipid species (Supplemental Table 4.S5). The OLCFA-containing phospholipid species included PC, PE, PI and PG containing 33:3, 33:2, 35:4, 35:3 and 35:2 acyl chains. In general, the novel phospholipid species had 15:0, 17:0, 17:1 or 17:2 acyl chains. The amounts of OLCFA-containing phospholipid species increased under high temperature stress in both genotypes (Fig. 4.7).

Sterol Lipids Respond Significantly to High Temperature Stress in Wheat

Our study revealed that sterol lipids, ASG and SG, which are likely to be biosynthetically interconvertible, respond significantly to high temperature stress in plants (Fig. 4.8). Derivatives with sitosterol, campesterol and stigmasterol were the sterol derivatives found in wheat genotypes. Palmitic acid (16:0) and linolenic acid (18:3) were found in ASG. The saturated species of ASG [Campesterol-Glc (16:0), Stigmasterol-Glc (16:0) and Sitosterol-Glc (16:0)] increased at HD and HDN in both genotypes (Fig. 4.8, A and B). Compared to Karl 92, Ventnor increased these saturated lipid species of ASG to a greater extent at high temperatures, resulting in higher amounts of these lipids at high temperatures in Ventnor than in Karl 92. Sterol glycosides (Campesterol-Glc, Stigmasterol-Glc and Sitosterol-Glc) increased at HN, HD and HDN in both genotypes (Fig. 4.8, C and D). They had a 2-fold or greater increase at HD and HDN, compared to OT in both genotypes. Impacts of HN and HD were similar on Campesterol-Glc and Sitosterol-Glc in Karl 92 and on Campesterol-Glc and Stigmasterol-Glc in Ventnor. This showed that SG responds in a similar way at HN and HD. Compared to Karl 92, Ventnor had greater amounts of all the three species of SG at OT as well as high temperatures.

High Temperature Increases the Amounts of Ox-lipids in the Susceptible Genotype

The present study also revealed that high temperature had significant impacts on the production of oxylipin-containing membrane lipids (ox-PE and ox-PC) (Fig. 4.9). The chemical formula of the oxidized acyl chain in these lipid species was $C_{18}H_{29}O_4$, which was abbreviated as 18:3-2O, to indicate three double bond equivalents and two oxygen atoms beyond the carbonyl group. Each detected ox-PE and ox-PC molecular species has a normal-chain fatty acid, 16:0, 18:3 or 18:2, in combination with an oxidized chain, 18:3-2O.

High temperatures, HD and HDN, significantly increased all the ox-lipid species of PE and PC in Karl 92 (Fig. 4.9). At these high temperatures, a 2-fold or greater increase was noticed for all ox-lipids in Karl 92, except for PC (18:2/18:3-2O) at HD. The impacts of HD and HDN on ox-lipids were similar in Karl 92. However, HN did not influence the amount of ox-lipids in Karl 92. Ventnor maintained the basal level of most ox-lipids at HD and HDN. In addition, the amounts of ox-lipids at HD and HDN were lower in Ventnor, compared to Karl 92. These trends in ox-lipid levels are consistent with RS contents reported in Fig. 4.2C (increased RS contents at high temperatures in Karl 92, while Ventnor maintained normal levels of RS at high temperatures). Taken together, these results are consistent with the possibility that RS-induced oxidation of lipids at high temperature contributed to increased amounts of ox-lipids in Karl 92; indeed, this genotype suffered from a greater degree of oxidative damage than Ventnor at high temperatures (HD and HDN).

Comparison of Lipid Changes under High Day and High Night Temperature Stress

To identify the most important lipid species differentiating each of the high temperature regimes (HN, HD and HDN) from OT, a supervised classification method, Random Forests (Breimen, 2001), offered by Metaboanalyst (metabolanalyst.ca; Xia et al., 2009), was employed on the data set including 165 lipid species. Random Forests identified 50 lipid species based on their contributions to differentiate OT from HN, HD or HDN (data not shown). The subset of this list that included the lipid species that differentiated all the three high temperature regimes from OT is given in Table 4.1. This subset included plastidic and extraplastidic diacyl species that had 18:3, 18:1 or 16:0 acyl chains. Among them, lipid species that had 18:3 acyl chains (36:6, 36:5 and 34:4 species) went down under high temperature stress, whereas, lipid species that are likely to have 18:1 and/or 16:0 acyl chains (34:1 and 36:3 species) went up at high temperature stress.

This result showed that a decrease in the amount of 18:3 acyl chains and an increase in the amount of 18:1 and 16:0 acyl chains are common mechanisms that occur at HN and HD to reduce the overall unsaturation level.

Discussion

High temperature (HN, HD and HDN) decreased all yield traits and photosynthesis and caused significant damage to thylakoid membranes in both genotypes. The effects of HN were less than or equal to that of HD on yield and physiological traits, whereas the effects of HDN were greater than that of HN and greater than or equal to that of HD. Even though HN, HD and HDN decreased all yield traits in both genotypes, the percentage decreases were greater in Karl 92, compared to Ventnor. This suggests that Ventnor has greater tolerance to high temperature stress than Karl 92. In addition, RS and MDA contents significantly increased under HN, HD and HDN in Karl 92, but did not show any change in Ventnor. Malondialdehyde, an end product of lipid peroxidation that mainly occurs through the action of RS is a well-defined marker of oxidative stress in cells (Heath and Packer, 1968; Kappus, 1985). Thus, our results indicate that the heat susceptible genotype, Karl 92 suffered from a greater degree of oxidative damage under high temperature stress than Ventnor.

High temperature caused significant changes in the lipid profile of wheat genotypes. Both genotypes tried to adapt to high temperature stress by lipid remodeling and decreasing the level of lipid unsaturation. Lipid remodeling refers to the decreases in the amount of certain lipids and increases in the amount of others (Zheng et al., 2011). For example, the amount of plastidic glycolipids (DGDG, MGDG and SQDG), plastidic phospholipid (PG) and extraplastidic phospholipids (PC and PE) decreased under high temperature stress, while the amount of sterol lipids (ASG and SG), 18:3-acyl containing TAGs and ox-lipids increased (Fig. 4.3). Lipid remodeling is likely to prevent the phase transition of membranes from a liquid crystalline phase to hexagonal II (HII) or cubic phase (corresponding to non-bilayer structure) at high temperatures. Lipids such as MGDG and PE tend to form HII phase or other non-bilayer phases, whereas DGDG, SQDG, PC and PG form bilayers (Shipley et al., 1973; Seddon, 1990; Hansbro et al., 1992; Vikstrom et al., 1999). Higher ratios of DGDG to MGDG and PC to PE reduce the propensity of membranes to form non-bilayer phases (Suss and Yordanov 1986; Webb and Green 1991; Williams, 1998; de Vries et al., 2004). In the present study, both genotypes

increased DGDG:MGDG and PC:PE ratios at high temperatures (Supplemental Fig. 4.S2) to maintain membrane fluidity, presumably avoiding high-temperature-induced non-bilayer phase formation. However, both genotypes underwent a decrease in PG and SQDG, which are integral parts of thylakoid membranes (Hagio et al., 2002; Sakurai et al., 2003; Sato, 2004). This may be a reason for the increased thylakoid membrane damage observed at high temperatures in both genotypes (Fig. 4.2).

In plant lipids, fatty acid double bonds are mainly in the *cis* configuration. The presence of *cis* double bonds introduces bends or kinks in the fatty acid chains and reduces tight packing of adjacent lipid molecules. Therefore, plants try to decrease the degree of unsaturation (number of double bonds) at high temperatures to maintain optimal fluidity and integrity of membranes (Larkindale and Huang, 2004). In the present study, both genotypes decreased the unsaturation level of plastidic and extraplastidic membrane glycerolipids at high temperatures (Fig. 4.5). As demonstrated in this work and by others, the decrease in unsaturation level was mainly due to the decrease in the polyunsaturated fatty acid, linolenic acid (18:3) and the increase in the less unsaturated fatty acid, oleic acid (18:1) and saturated fatty acid, palmitic acid (16:0) (Figs. 4.4 and 9, A and B; Larkindale and Huang, 2004). In addition, the increased amounts of 18:3-acyl containing TAGs under high temperature stress (Fig. 4.6) indicate that these TAGs might have synthesized from the 18:3 fatty acids released from membrane lipids. According to Hernández et al. (2012), a soluble diacylglycerol acyltransferase (*DGAT3*) is responsible for recycling 18:3 fatty acids into TAGs. Thus, our data suggest a possible role of TAGs in high temperature stress adaptation by sequestering the 18:3 fatty acids from the membrane lipids. This is also supported by a previous report on the role of leaf TAGs in leaf senescence by sequestering fatty acids that are de-esterified from thylakoid galactolipids (Kaup et al., 2002).

Precursor scanning by ESI-MS/MS and product ion analysis by Q-TOF MS revealed novel OLCFA-containing phospholipids, which increased under high temperature stress in wheat plants (Fig. 4.7). These novel phospholipids contained 15:0, 17:0, 17:1 or 17:2 acyl chains. The odd-chain lipids are generally reported in humans, animals and microorganisms, and very rarely in plants (Sperl et al., 2000; Rezanka and Sigler, 2009). The synthesis of odd-chain fatty acids might be through fatty acid oxidation or *de novo* synthesis (Hamberg et al., 2003; Jeennor et al., 2006). Wendel (1989) reported that propionyl-CoA acts as a primer in biosynthesis of OLCFA and excess propionyl-CoA leads to enhanced synthesis of 15 and 17 carbon fatty acids in

humans. Further, increased amounts of OLCFAs was found to reduce disease incidence in humans (Sperl et al., 2000; Jenkins et al., 2015). In fungi, odd-chain fatty acids, such as 15:0, 17:0 and 17:1 were found to be produced under alcoholic and hypoxic stress conditions (Jeennor et al., 2006). Taken together, these reports lead to hypotheses that the increased formation of OLCFA-containing phospholipids under high temperature stress, as observed in our study, might be an adaptation mechanism to this stress in plants and the incorporation of the three-carbon species (propionic acid) into fatty acids could be regulated by altered enzymatic specificity as a function of temperature. In addition, our results highlight the importance of including OLCFA-containing lipids into routine lipidomic analyses.

Compared to the heat susceptible genotype Karl 92, the heat tolerant genotype Ventnor was more efficient in lipid remodeling to maintain membrane bilayer structure at high temperature stress. For example, Ventnor had greater ability to increase the amounts of sterol derivatives (SGs and saturated species of ASGs) at high temperatures, compared to Karl 92 (Fig. 4.8). The sterol conjugates, SGs and ASGs are ubiquitous constituents of cells in vascular plants (Grille et al., 2010). In plants, SGs and ASGs function as membrane components, storage forms of sterols, transporters and signaling molecules (Grille et al., 2010). Sterol glycosides have a condensing effect on membranes that helps to eliminate phase transitions to non-bilayer phases at high temperatures (Muramatsu et al., 2000). In our study, 2-fold or greater increases were noticed for SGs in both genotypes at high temperatures, suggesting that SGs respond significantly to high temperature stress in wheat. Ventnor had greater amounts of all the SG species than Karl 92 at HN, HD and HDN (Fig. 4.9 C and D). The total amount of SGs was 23, 28 and 29% greater in Ventnor, compared to Karl 92 at HN, HD and HDN, respectively (Fig. 4.3). When we identified the most important lipid species that differentiated the two genotypes at high temperatures using Random Forests (metabolanalyst.ca; Breimen, 2001; Xia et al., 2009), all the three SG species were ranked as one among the top 50 (Supplemental Table 4.S6). Thus, our results suggest that SGs could be potential biomarkers for heat tolerance (under both HN and HD) in wheat, which needs to be verified by further studies including several genotypes with varying degrees of heat tolerance.

The present study is consistent with the notion that high temperature stress result in ‘ox-lipid signatures’ in the plant that reflect its physiological status. For example, high temperature caused oxidation of lipids originating in the endoplasmic reticulum (ox-PCs and ox-PEs) (Fig.

4.9). The nonenzymatic oxidation of membrane lipids is thought to prevent oxidative damage that could happen elsewhere in the cell (Me`ne-Saffrane´ et al., 2009). In the present study, the oxidized fatty acid species in the detected ox-lipids was 18:3 (trienoic fatty acid). It has been found that trienoic fatty acids act as a sink for ROS and 18:3 oxidation results in MDA as an end product (Me`ne-Saffrane´ et al., 2009). The nonenzymatic oxidation of trienoic fatty acids by ROS is a mechanism for immediately consuming ROS produced under stress conditions, without activating genes encoding ROS-catabolizing enzymes (Me`ne-Saffrane´ et al., 2009). Thus, the amount of ox-lipids could reflect the degree of oxidative stress a plant is experiencing.

In the present study, the ox-PCs and ox-PEs significantly increased (2-fold or greater) at HD and HDN in the susceptible genotype Karl 92 (Fig. 4.9). At the same time, the tolerant genotype Ventnor maintained the basal level of most ox-lipid species at HD and HDN. In addition, the amounts of ox-PCs and ox-PEs were significantly greater in Karl 92 than Ventnor at HD and HDN (Fig. 4.9). The total amount of ox-lipids were 57 and 55% greater in Karl 92, compared to Ventnor at HD and HDN, respectively (Fig. 4.3). These results indicate that the amount of ox-lipids differentiated the susceptible genotype Karl 92 from the tolerant genotype Ventnor at HD and HDN. The increased RS, MDA (Fig. 4.2) and ox-lipid (Fig. 4.9) contents of the susceptible genotype Karl 92 at HD and HDN suggests a possible mechanism of heat damage as the increased ROS under high temperature conditions caused nonenzymatic oxidation of trienoic fatty acids in the phospholipids of endoplasmic reticulum which led to the production of MDA and resulted in oxidative damage to the plant. Taken together, our results suggest that ox-lipids could be potential biomarkers for heat susceptibility (at HD and HDN) in wheat genotypes, which needs to be verified by further studies including several genotypes differing in heat response.

When the effects of HN, HD and HDN on glycolipids (DGDG, MGDG and SQDG), phospholipids (PG, PI, PE and PC), TAGs, ASGs, SGs and ox-lipids were considered, the effects of HN were generally less than or equal to that of HD, whereas, the effects of HDN were generally greater than that of HN and greater than or equal to that of HD (Figs. 4.4-9). This is consistent with the effects of HN, HD and HDN on yield and physiological traits (Figs. 4.1 and 2). Interestingly, SGs showed similar response to HN and HD (Fig. 4.8). In addition, decreasing the lipid unsaturation levels was a common adaptation mechanism at HN and HD.

Conclusions

High temperature (HN, HD and HDN) caused significant changes in the lipid profile of wheat genotypes Ventnor and Karl 92. Both genotypes tried to adapt to high temperature stress by lipid remodeling and decreasing the level of lipid unsaturation. The lipid remodeling included decreases in the amount of more unsaturated lipid species (e.g., 36:5 or 36:6 acyl containing glycerolipids) and increases in the amount of less unsaturated lipid species (e.g., 34:1 acyl containing glycerolipids), SGs and saturated species of ASG. The decrease in lipid unsaturation level under high temperatures was predominantly due to the decrease in the polyunsaturated fatty acid, linolenic acid (18:3) and the increases in the less unsaturated fatty acid, oleic acid (18:1) and saturated fatty acid, palmitic acid (16:0). The amounts of 18:3-acyl containing TAGs increased under high temperature stress, suggesting a possible role of TAGs in high temperature stress adaptation by sequestering the 18:3 fatty acids from the membrane lipids. High temperature increased the amounts of novel OLCFA- containing phospholipids in both genotypes, suggesting the importance of including these lipids into routine lipidomic analyses. The heat tolerant genotype Ventnor had higher amounts of SGs and saturated species of ASG and lower amounts of ox-lipids at high temperatures, compared to the susceptible genotype Karl 92. This result demands further studies including several genotypes with varying degrees of heat tolerance to confirm SGs and ox-lipids as potential biomarkers for heat tolerance and susceptibility, respectively in wheat.

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Figures and Tables

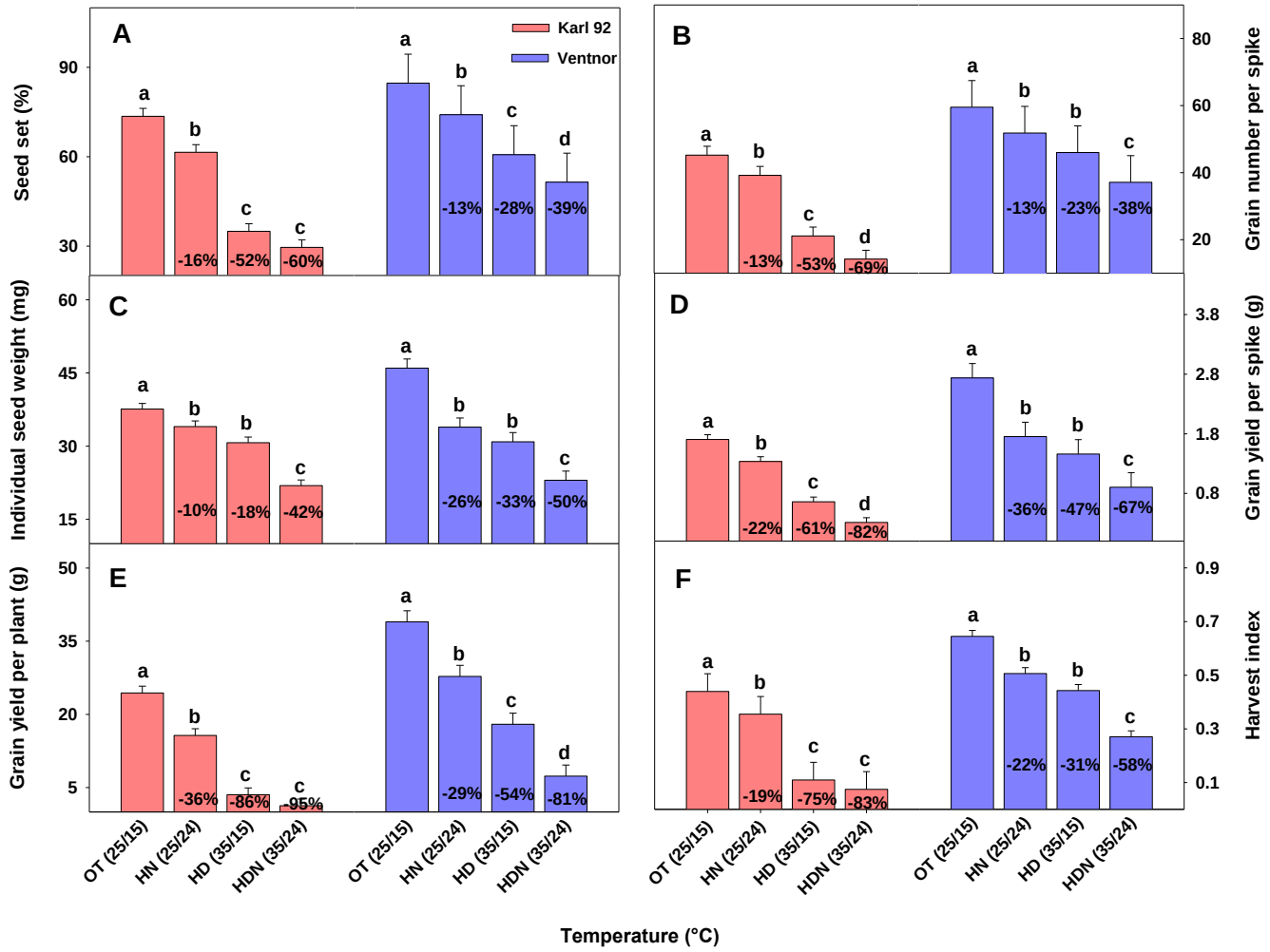


Figure 4.1. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on yield traits of wheat genotypes Karl 92 and Ventnor.

Grain number per spike and grain yield per spike denote grain number and yield from the main spike of wheat plants. Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. Percentage decreases in various traits due to high temperature as compared with OT are indicated on the bars.

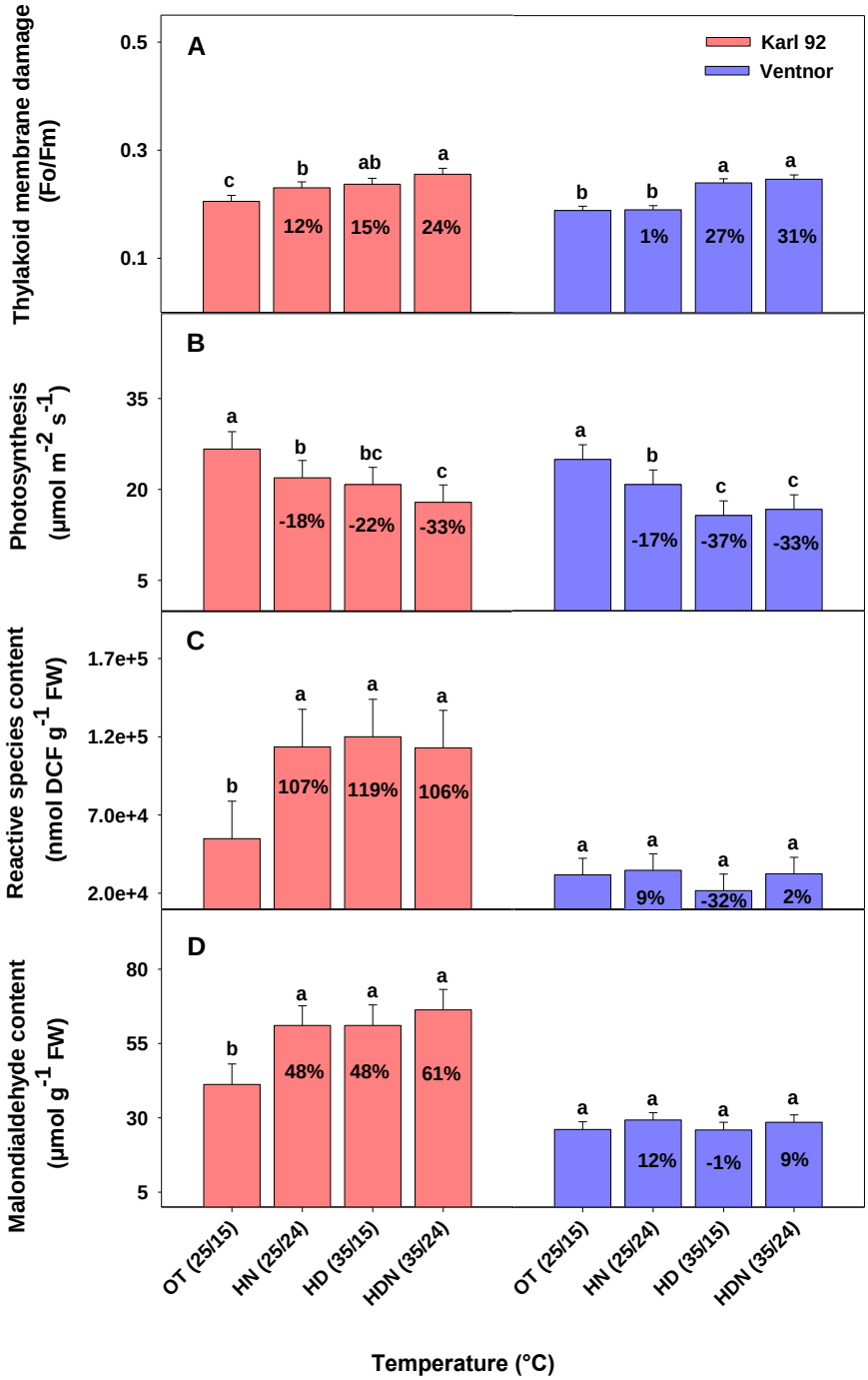


Figure 4.2. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on physiological traits of wheat genotypes Karl 92 and Ventnor.

Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. Percentage changes in various traits under high temperature stress as compared with OT are indicated on the bars. Fo, minimum fluorescence; Fm, maximum fluorescence; DCF, 2', 7'-dichlorodihydrofluorescein; FW, fresh weight.

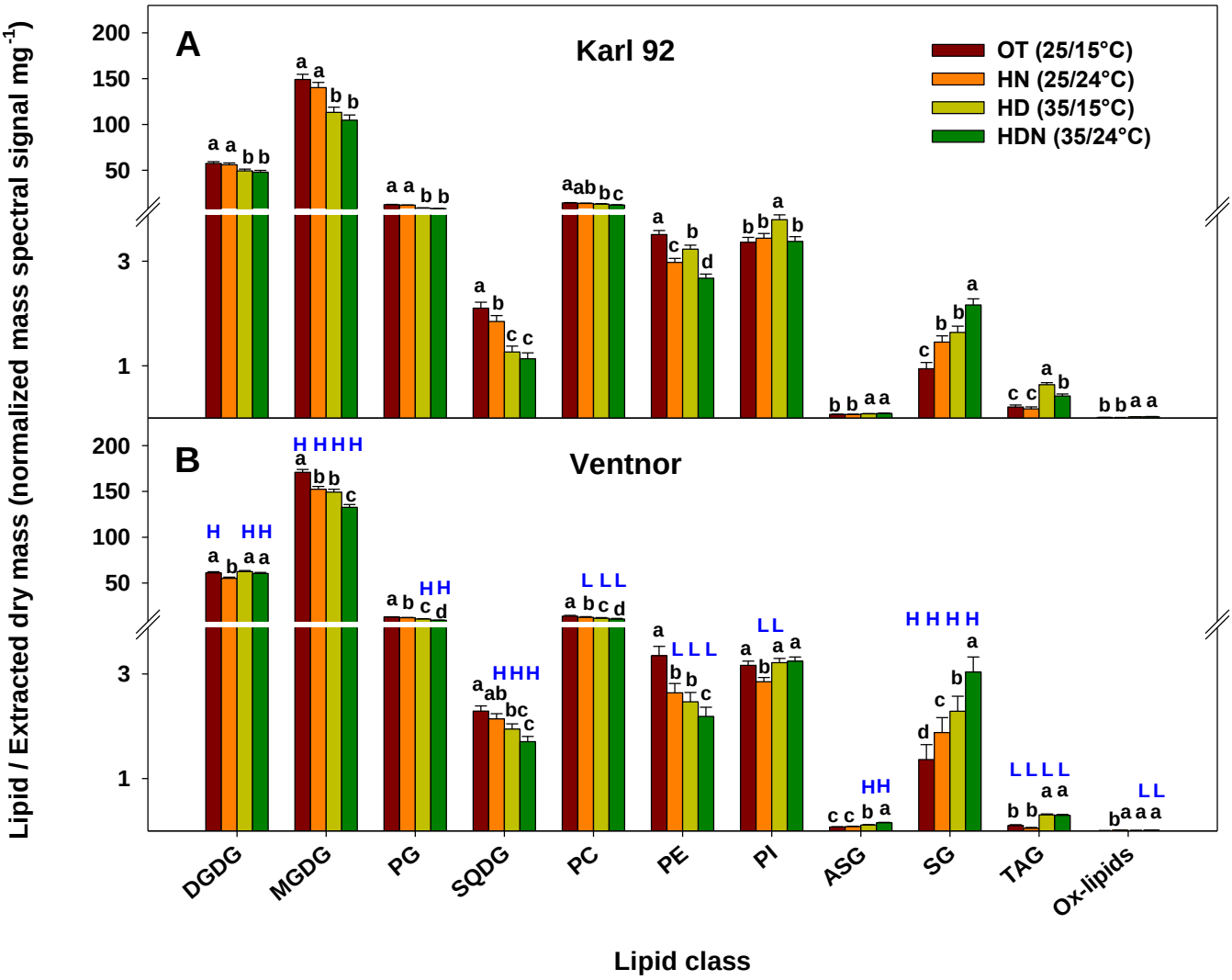


Figure 4.3. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on total amount of lipids in various head group classes of wheat genotypes Karl 92 and Ventnor.

Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.

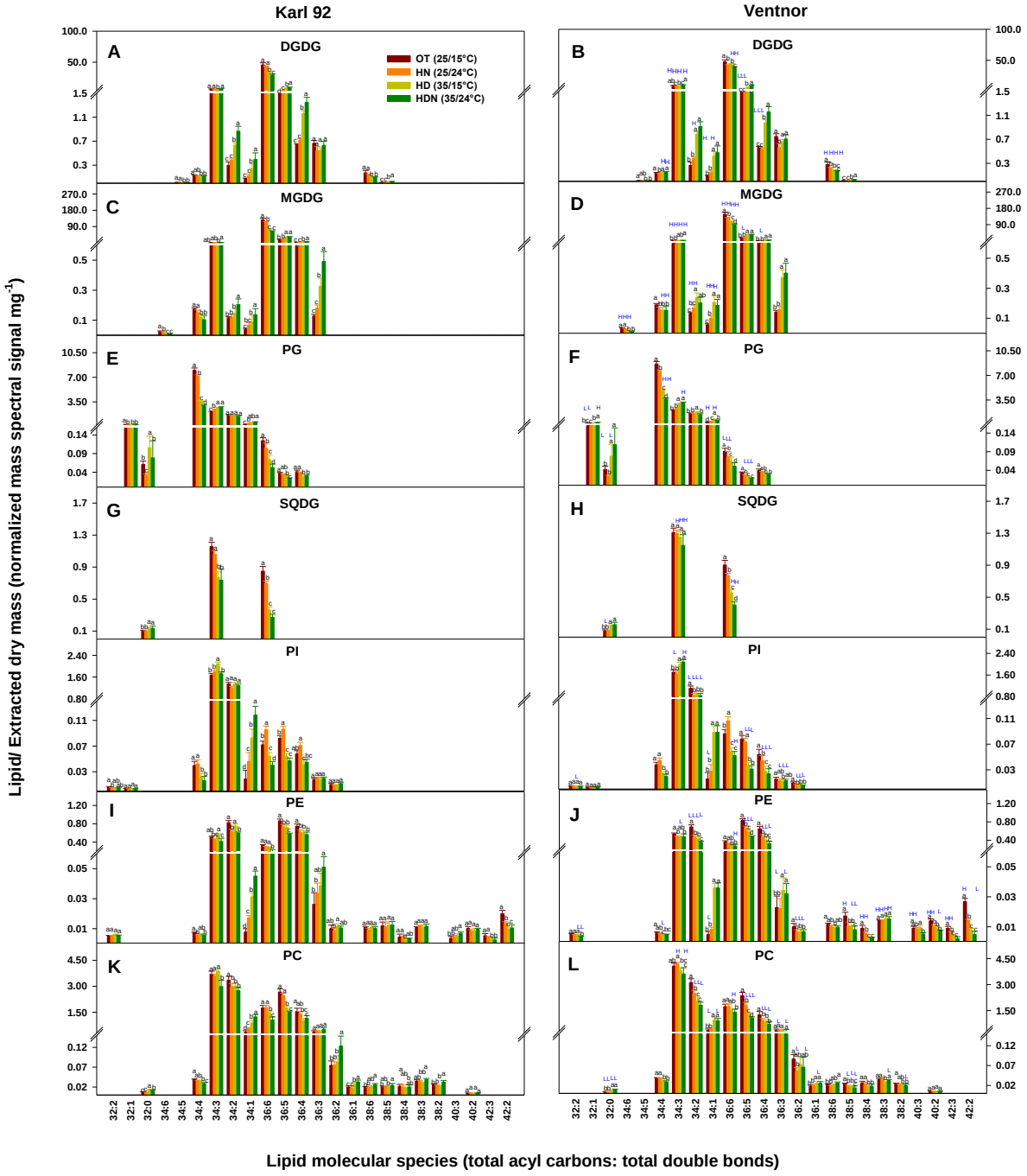


Figure 4.4. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on plastidic and extraplastidic diacyl lipid molecular species of wheat genotypes Karl 92 (A, C, E, G, I and K) and Ventnor (B, D, F, H, J and L).

Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.

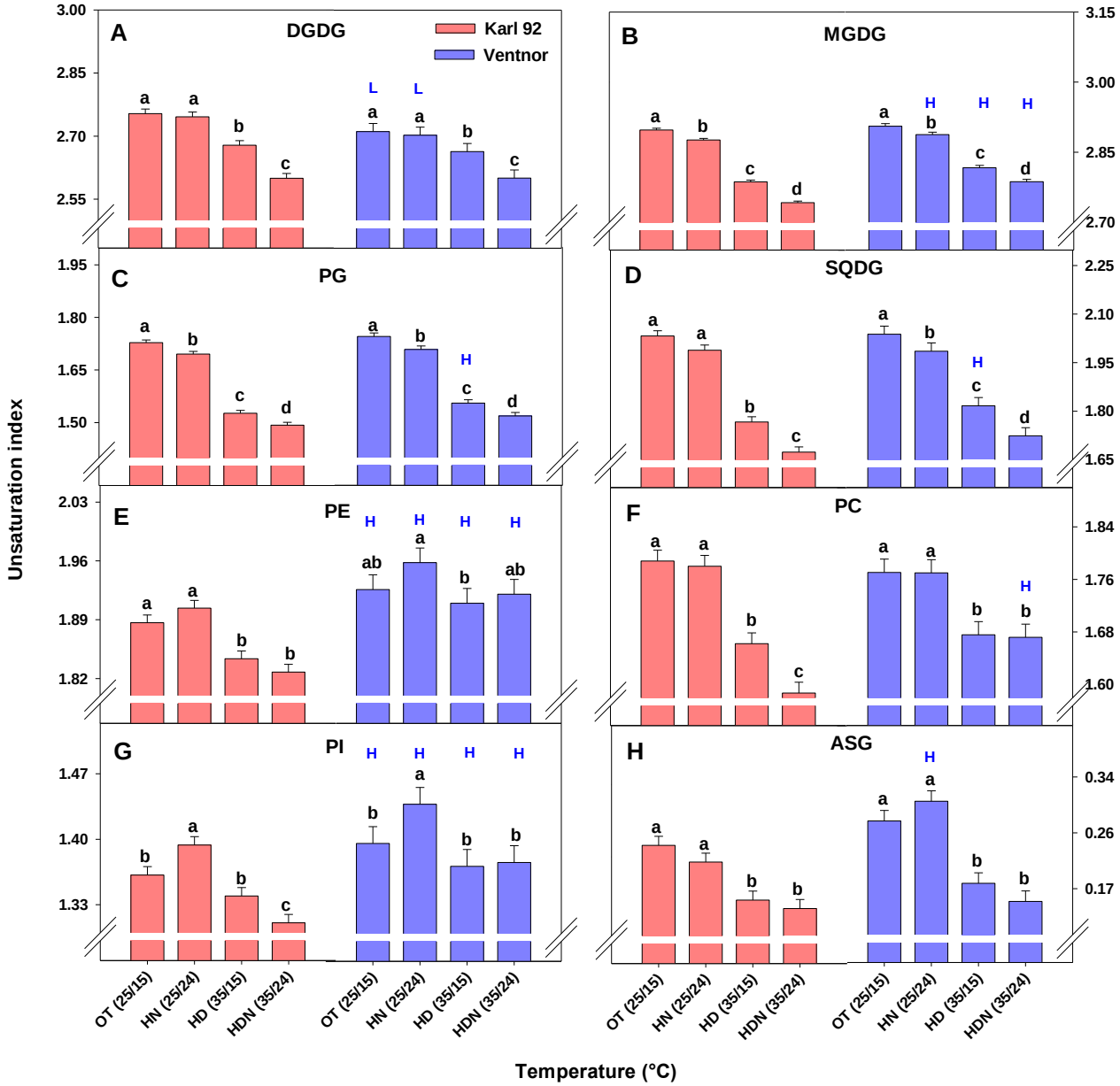


Figure 4.5. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on unsaturation index of various lipid classes of wheat genotypes Karl 92 and Ventnor.

Unsaturation index of each lipid molecular species was calculated as the product of amount of that lipid molecular species and the average number of double bonds per acyl chain, where the average number of double bonds per acyl chain was calculated by dividing the number of double bonds in the lipid molecular species by number of acyl chains. Finally, the unsaturation index of a lipid head group class was calculated as the sum of the unsaturation indices of individual lipid molecular species in that class. Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature.

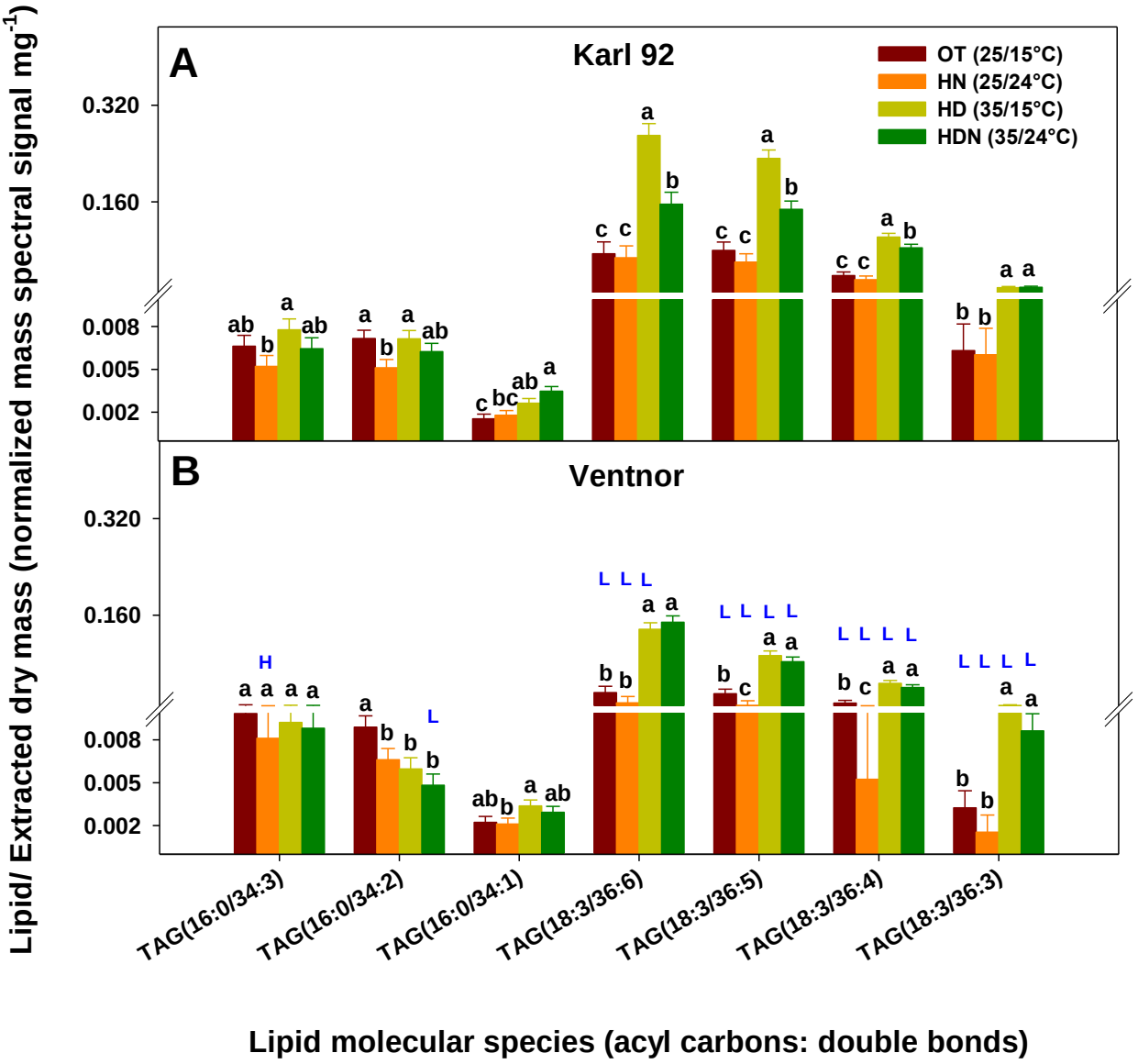


Figure 4.6. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on triacylglycerols (TAG) of wheat genotypes Karl 92 and Ventnor.

Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.

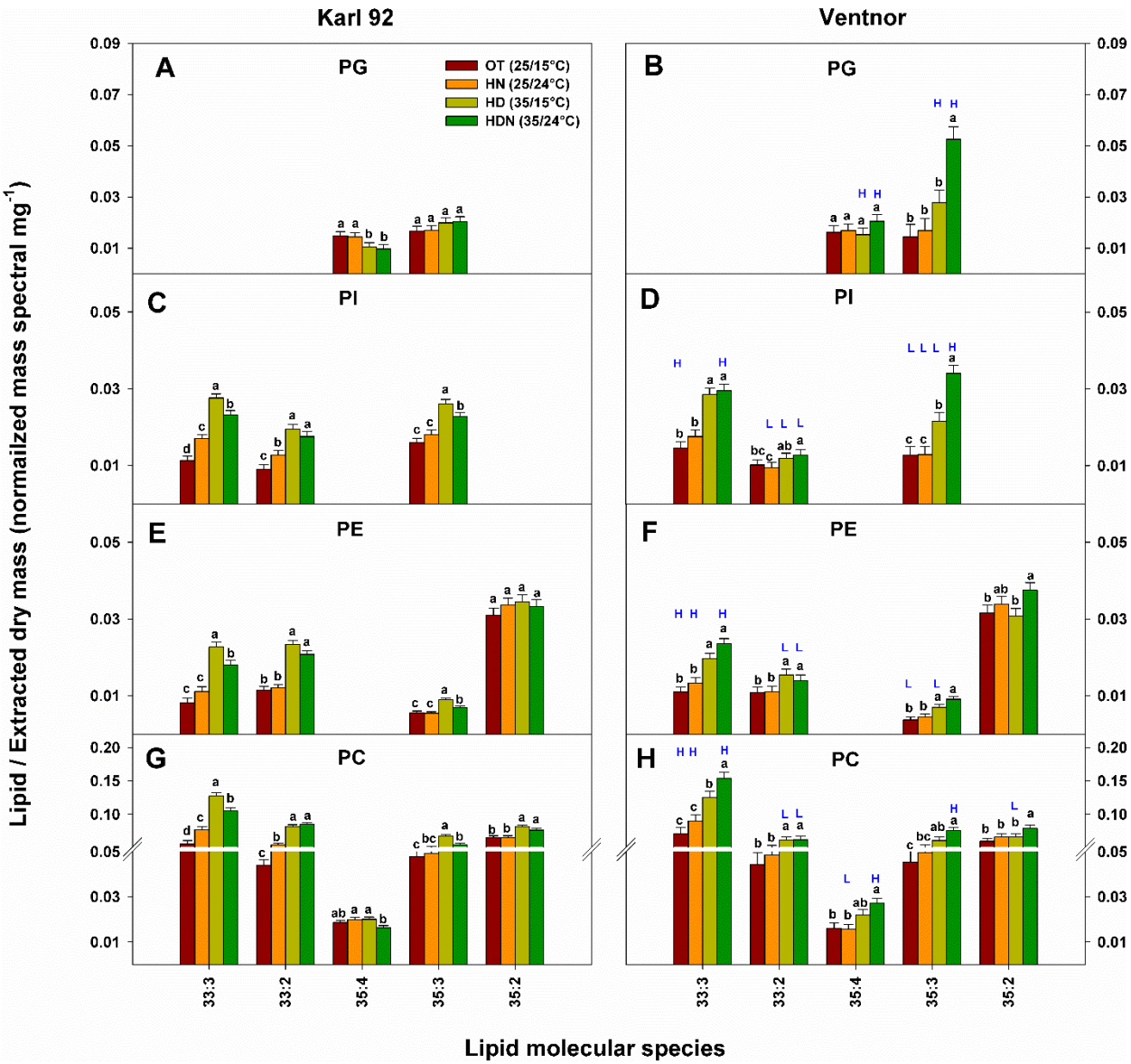


Figure 4.7. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on odd-numbered long-chain fatty acid-containing phospholipids of wheat genotypes Karl 92 and Ventnor.
Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.

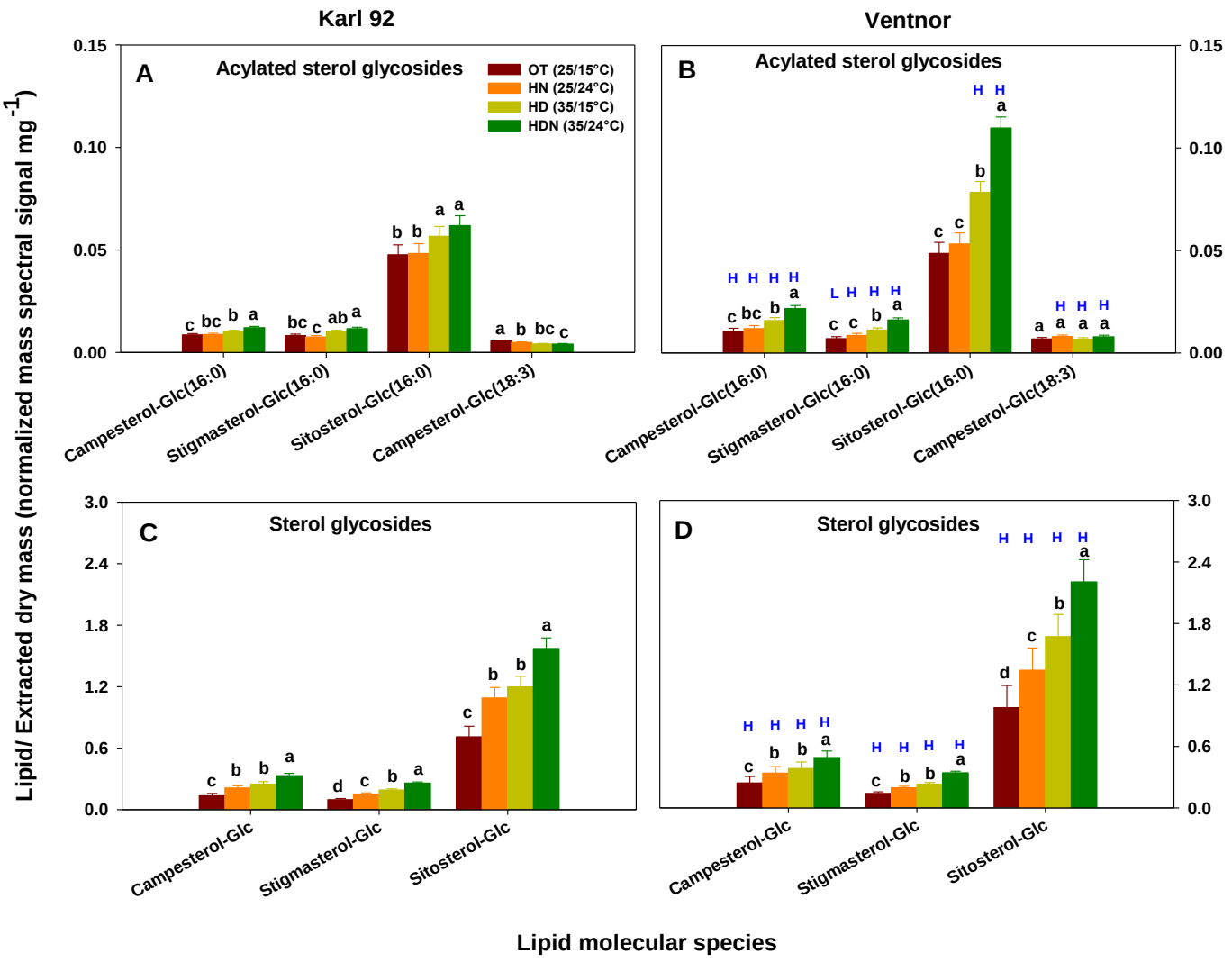


Figure 4.8. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on acylated sterol glycosides (A and B) and sterol glycosides (C and D) of wheat genotypes Karl 92 (A and C) and Ventnor (B and D).
Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature.

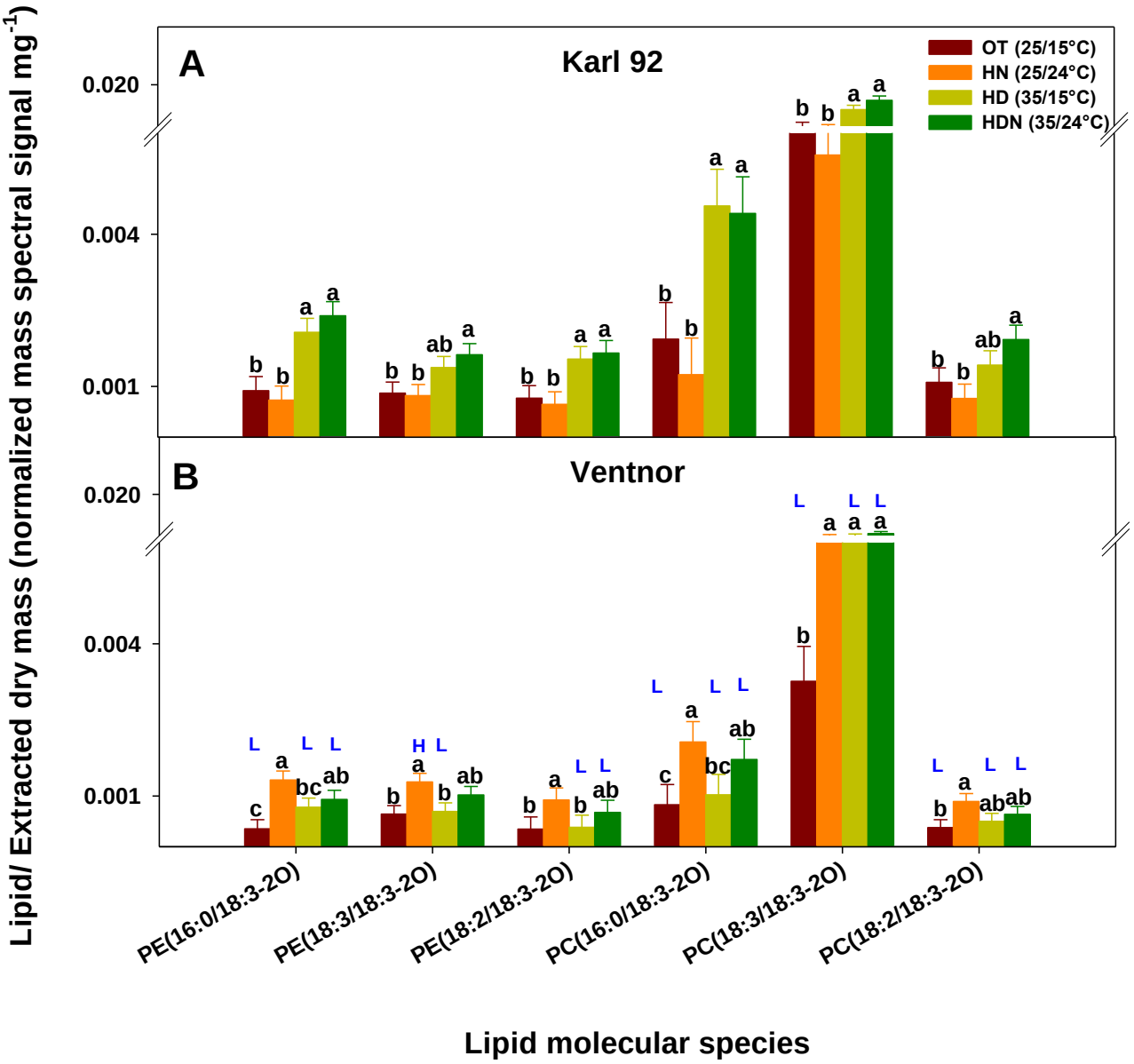
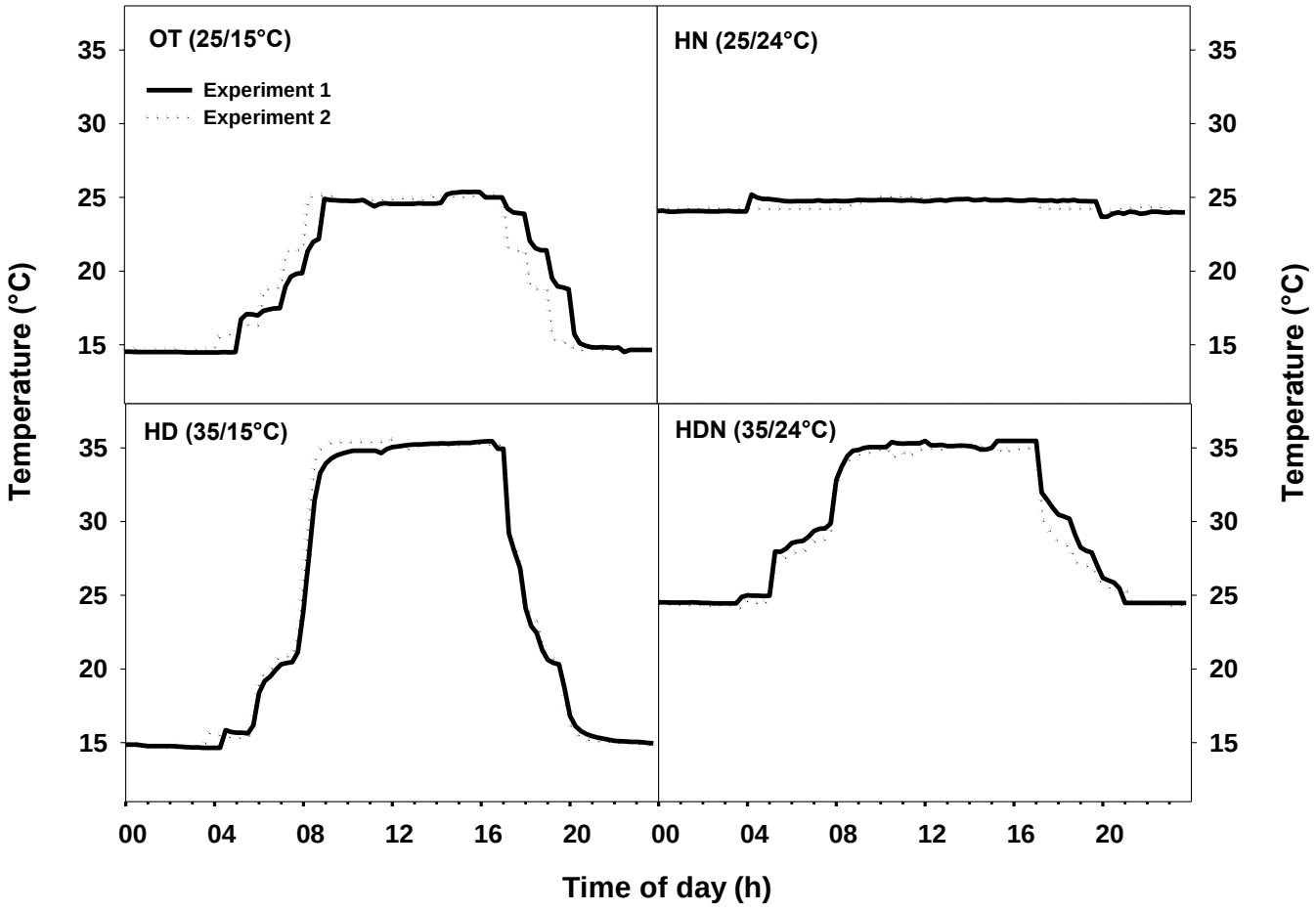


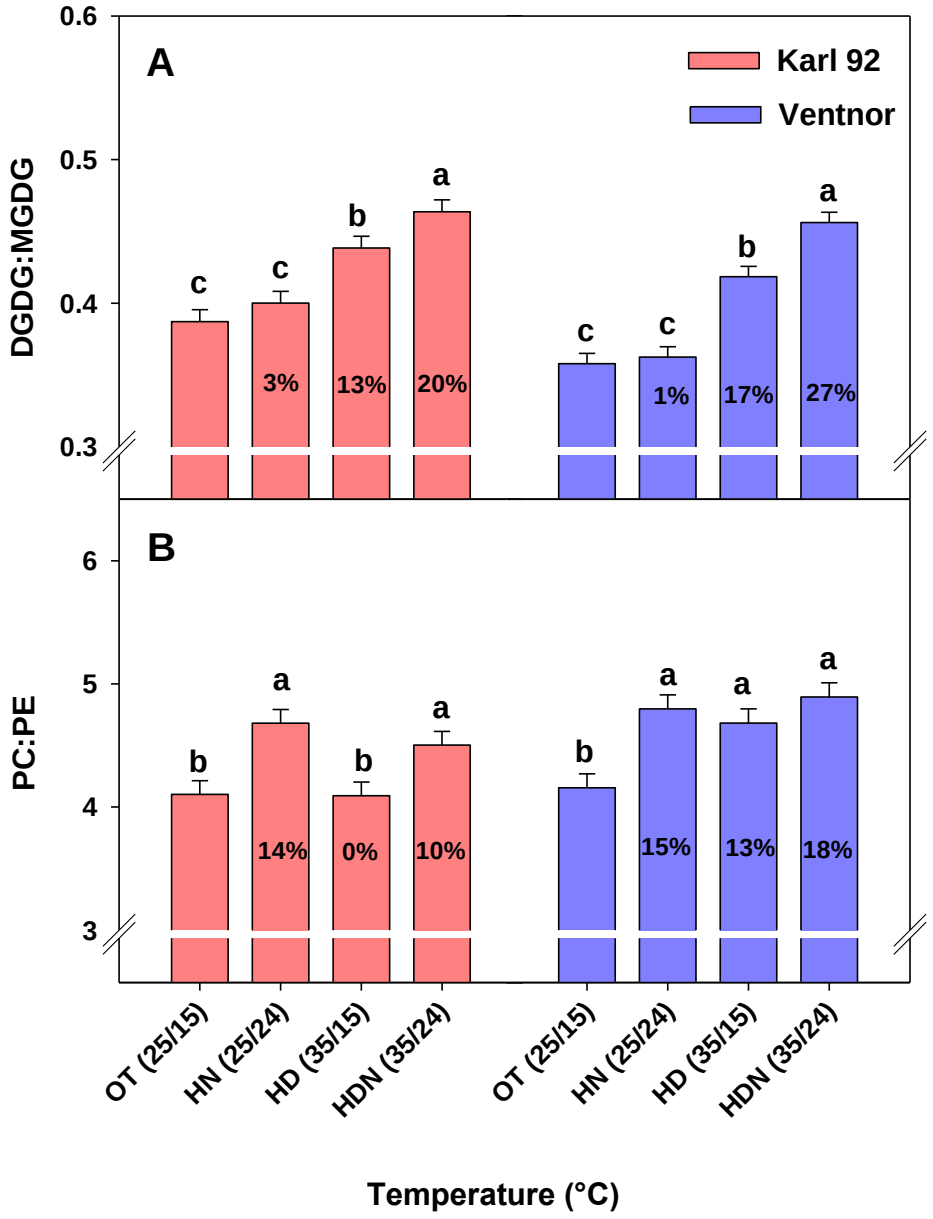
Figure 4.9. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on ox-lipids of wheat genotypes Karl 92 and Ventnor.

Ox-lipids are lipids that contain oxidized fatty acids. Each ox-lipid species in the above figure had a normal-chain fatty acid, 16:0, 18:3, or 18:2, in combination with an oxidized chain, 18:3-2O (three double bond equivalents and two oxygen atoms beyond the carbonyl group). Values shown are mean ± SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the LSD test at $P < 0.05$. The letters 'H' and 'L' indicate whether the amount of lipid was significantly higher or lower ($P < 0.05$), respectively in Ventnor, compared to Karl 92 at the corresponding temperature. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.



Supplemental Figure 4.S1. The observed air temperature inside the growth chambers at optimum temperature (OT), high night temperature (HN), high day temperature (HD), and high day and night temperature (HDN) regimes in experiment 1 (2013) and 2 (2014).

In all temperature regimes, daytime maximum temperature was held for 8 h from 09:00 h to 17:00 h. Similarly, night-time minimum temperature was held for 8 h from 21:00 h to 05:00 h.



Supplemental Figure 4.S2. Effects of temperature (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) on ratios of DGDG to MGDG and PC to PE of wheat genotypes Karl 92 and Ventnor.
Estimates with different letters are significantly different according to the LSD test at $P < 0.05$. Percentage changes in ratios under high temperature stress, compared with OT are indicated on the bars. Wherever there is a break on the y-axis, the different scales above and below the break are indicated.

Table 4.1. Lipid species that differentiated high night (HN), high day (HD) and high day and night (HDN) temperatures from optimum temperature (OT).

These 20 lipid species were identified by Random Forests, a supervised classification method that was offered by Metaboanalyst, based on their contributions to differentiate optimum temperature from high temperatures. The order in which lipids are listed in the table does not correspond to their ranks.

Lipid species	Direction of significant changes[a]		
	HN	HD	HDN
DGDG(34:1)	Up	Up	Up
DGDG(34:2)	Up	Up	Up
DGDG(36:5)	Up	Up	Up
MGDG(34:1)	Up	Up	Up
MGDG(36:3)	Up	Up	Up
MGDG(36:5)	Up	Up	Up
PC(34:1)	Up	Up	Up
PC(33:3)	Up	Up	Up
PE(33:3)	Up	Up	Up
PE(33:2)	Up	Up	Up
PG(34:1)	Up	Up	Up
PI(34:1)	Up	Up	Up
PI(33:3)	Up	Up	Up
Stigmasterol-Glc	Up	Up	Up
MGDG(34:6)	Down	Down	Down
MGDG(36:6)	Down	Down	Down
PE(36:5)	Down	Down	Down
PE(42:2)	Down	Down	Down
PG(34:4)	Down	Down	Down
SQDG(36:6)	Down	Down	Down

Chapter 5 - Lipid Groups Experiencing Coordinated Metabolism Can Be Detected by Analysis of Lipid Co-occurrence under High Day and Night Temperature Stress in Wheat

Abstract

It is well-established that lipid-metabolizing enzymes act on multiple, related lipid substrates that contain the same component acyl chain or head group. We hypothesized that co-occurring lipids, which are up-or-down-regulated together through time under high day and night temperature conditions in wheat (*Triticum aestivum* L.) represent groups that can be explained by co-metabolism. A direct infusion automated electrospray ionization-tandem mass spectrometry approach was used to quantitatively profile the lipid molecular species. Correlation analyses revealed 13 lipid groups that included co-occurring lipids under high day and night temperature stress. The lipid groups were broadly classified into five categories; (1) groups containing extraplastidic phospholipids, (2) plastidic glycerolipids, (3) oxidized glycerolipids, (4) triacylglycerols and diacylglycerols and (5) acylated sterol glycosides and sterol glycosides. Lipids in these groups either decreased or increased in amount under high temperatures stress, but the kinetic patterns of changes in lipid levels varied among the groups, with some groups being quicker to respond and whether or not they return to baseline or continue to change during the recovery period. Interpretation of lipid groups based on the current knowledge on lipid metabolism suggests that the lipid groups reflect metabolic relationships as the co-occurring lipids in each group are acted on by the same enzyme(s). Lipid co-occurrence in groups was primarily the result of desaturating, oxidizing, glycosylating and acylating activities of enzymes acting on lipids under high temperature stress. The results from this study indicate that lipid groups experiencing coordinated metabolism can be detected by analysis of lipid co-occurrence under high day and night temperature stress in wheat.

Introduction

Application of stress to a plant activates or suppresses multiple pathways involving various lipid metabolizing enzymes. For example, low temperature activates phospholipase A and phospholipase D in Arabidopsis (*Arabidopsis thaliana*) plants (Welti et al., 2002). In contrast, high temperature leads to reduced activity of digalactosyldiacylglycerol synthase 1 in the susceptible plants (reviewed by Welti et al., 2007). Wounding stress causes activation of phospholipase D, phospholipase A, oxidation of fatty acids on galactolipids, and head-group acylation of monogalactosyldiacylglycerols (MGDG; Narvaez-Vasquez et al., 1999; Ryu, 2004; Buseman et al., 2006; Zien et al., 2001; Vu et al., 2014). In addition, many of the lipid-metabolizing enzymes act on multiple, related substrates as many lipid species contain similar component acyl chains or head group. This is confirmed by gene knock-out or suppression studies in which individual lipid-metabolizing enzymes were found to act on multiple substrates and produce multiple products during stress conditions in plants (Welti et al., 2002; Peters et al., 2010). Taken together, these reports suggest that, under stress conditions, plant lipid composition will change due to activation of multiple enzymes, and activation of most of these enzymes will result in simultaneous changes in a group of lipids that are acted on by the same enzyme.

In the first paper of this series, we found that high day and night temperature stress caused significant changes in the lipid profile of wheat (*Triticum aestivum* L.) genotypes and plants tried to adapt to high temperature stress by lipid remodeling and decreasing the level of lipid unsaturation. Here, we hypothesize that co-occurring lipids, which are up-or-down-regulated together through time under high day and night temperature conditions represent groups that can be explained by co-metabolism. In the current work, we employed correlation analyses to detect the co-occurring lipid groups, as has recently been suggested by Vu et al. (2014) in case of wounding stress in Arabidopsis.

Wheat genotypes (Ventnor, heat tolerant and Karl 92, heat susceptible) were grown at optimum temperature (OT; 25/15°C, maximum/minimum) until the onset of flowering. Thereafter, plants were exposed to high night (HN; 25/24°C), high day (HD; 35/15°C), high day and night (HDN; 35/24°C) or optimum temperatures for 12 days. For lipid extraction, leaf samples were collected from five plants per genotype from each temperature regime at day of stress (DOS) 1, 6, 12 and 16 with DOS 16 indicating the recovery period. A direct infusion automated electrospray ionization-tandem mass spectrometry (ESI-MS/MS) approach was used

to quantitatively profile the lipid molecular species. Using tools developed for metabolomics and correlation analysis (Xia et al., 2009, 2012), lipidomic data were analyzed to reveal lipid groups defined by co-occurrence under high temperature conditions in wheat plants. The coordinated metabolism of lipids in the detected groups were examined.

Materials and Methods

Plant Material, Growth Conditions and Lipid Profiling

Plant material and growth conditions were essentially the same as described in the first series of this paper. Briefly, winter wheat genotypes (Ventnor, heat tolerant and Karl 92, heat susceptible) were grown at OT (25/15°C, maximum/minimum) until the onset of flowering. Thereafter, plants were exposed to HN (25/24°C), HD (35/15°C), HDN (35/24°C) or OT for 12 days. For lipid extraction, leaf samples were collected from five plants per genotype from each temperature regime between 13:00 and 15:00 h at DOS 1, 6, 12 and 16. The lipid extraction procedure and the ESI-MS/MS lipid profiling are described in the previous paper.

Data Analyses

Utilities of the Metabo-Analyst web server was used to perform the auto-scaling of lipid data (Supplemental Table 5.S2) and to produce the heat maps (Figs. 5.1 and 5.5) and correlation tables (Supplemental Tables 5.S3 and 5.S4) (metabolanalyst.ca; Xia et al., 2009, 2012). Auto-scaling allows for easy comparison of lipid levels in different samples. The autoscaled value of a lipid in a sample is calculated as: [(the amount of lipid in that sample) – (the average amount of that lipid among all samples)] divided by (the standard deviation for the amount of that lipid among all samples). Clustering of lipid data within the genotypes was performed using Cluster 3.0 (Eisen et al., 1997) and the clustering outputs (.gtr and .cdt files) were converted to NEWICK format (.nwk) using a Python script written by Haibao Tang (J. Craig Venter Institute, Rockville, MD, USA). The script can be obtained from the following link: https://github.com/tanghaibao/treecut/blob/master/scripts/eisen_to_newick.py. The NEWICK file was exported to Dendroscope (Huson et al., 2007; Huson and Scornavacca, 2012) to produce the dendrogram, which was modified in color.

Results

Variation in Lipid Analytes in Response to High Temperature Stress

An ESI-MS/MS approach was used for lipid profiling as described in the first paper of this series. The autoscaled lipid levels of individual samples (Supplemental Table 5.S2) are presented as a heat map in Fig. 5.1. Significant changes can be observed in the amount of various lipids under OT, HN, HD and HDN in genotypes Ventnor and Karl 92 (Fig. 5.1). Within each temperature regime, amount of different lipids varied at DOS 1, 6, 12 and 16 with DOS 16 indicating the recovery period, as stress was imposed for 12 days. Autoscaled levels of 13 lipid species in individual plant samples at various temperature regimes and time points are shown in Supplemental Fig. 5.S1. This figure shows that variation in lipid levels in samples subjected to different treatments (genotype, temperature and time) was generally more prominent than the variation in lipid levels in samples subjected to the same treatment (biological replications). To test the hypothesis that co-occurring lipids under high day and night temperatures represent groups that can be explained by co-metabolism, as has recently been suggested during wounding stress in Arabidopsis (Vu et al., 2014), correlations in the levels of the lipid analytes among individual plant samples were determined. Spearman's correlation coefficient (ρ) was calculated among lipid analytes across all 160 samples in each genotype (10 samples subjected to each temperature-by-DOS combination and 16 temperature-by-DOS combinations in total) (Supplemental Tables 5.S3 and 5.S4). Spearman's correlation coefficient ranges from -1 (perfect negative correlation) to 1 (perfect positive correlation) and a value of zero indicates no correlation.

By matching each lipid analyte with the one to which it was most highly correlated, dendrograms were created for genotypes Ventnor and Karl 92 (Figs. 5.2 and 5.3, respectively). These dendrograms include clusters of lipids in which every lipid is correlated with another lipid with $\rho \geq 0.80$. The dendrogram of Ventnor and Karl 92 included 12 (Fig. 5.2) and 13 clusters (Fig. 5.3), respectively, which are indicated by the red and blue bars on each dendrogram. Of the 165 lipids analyzed in our study, 48% were included in the clusters. Vu et al. (2014) reported that high analytical precision helps for the detection of clusters, while poor analytical precision or true lack of co-occurrence leads to lack of high correlations.

Comparison of clusters in Ventnor and Karl 92 allowed us to identify 13 lipid groups (Figs. 5.2 - 5.5). Each of these lipid groups represents a whole cluster, a part of that or a combination of two adjacent clusters. These lipid groups include lipids that are up-or-down regulated together through time under various temperature treatments and in at least one genotype (Fig. 5.4). These 13 lipid groups could be broadly classified into five categories; (1) groups containing extraplastidic phospholipids, (2) groups containing plastidic glycerolipids, (3) groups containing oxidized glycerolipids, (4) groups containing triacylglycerols (TAG) and diacylglycerols (DAG) and (5) groups containing acylated sterol glycosides (ASG) and sterol glycosides (SG). The kinetic changes in individual lipids in each of the 13 groups are presented in Supplemental Fig. 5.S2, whereas, the kinetic changes in lipid groups are presented in Fig. 5.6. The kinetic patterns of changes in individual lipid levels varied among and within the groups, with some lipids being quicker to respond and whether or not the lipids return to baseline or continue to change during the recovery period (Fig. 5.5 and Supplemental Fig. 5.S2). Genotypes differed/were similar in terms of these kinetic patterns of changes in lipid levels and the group compositions (Figs. 5.2 - 5.4). The details are given in the sections below.

Groups Containing Extraplastidic Phospholipids (Groups 1-6a)

Group 1 includes phosphatidylserine (PS) with an 18:3 acyl chain combined with a long acyl chain, whereas group 2 includes PS with an 18:2 acyl chain combined with a long acyl chain (Figs. 5.2 - 5.5). Group 3 includes extraplastidic phospholipids (phosphatidylethanolamine [PE], phosphatidylcholine [PC] and phosphatidylinositol [PI]) with 15:0 and 17:0 acyl chains. Group 4 primarily includes extraplastidic diacyl lipids with an 18:3 acyl chain combined with an 18:3, 18:2 or 16:0 acyl chain. These include normal-chain species of PE, PC and PI. Group 5 includes extraplastidic phospholipids (PE, PC and PI) with an 18:2 acyl chain. Group 6a includes extraplastidic diacyl species with 18:1 and 18:2 acyl chains. These include PE, PC, PI and DAG species with limited desaturation.

When comparing the amount of lipids under HDN with that of OT at DOS 12, groups 1, 3 and 6a include lipids that increased in amount under high temperature stress, whereas, groups 2 and 4 include lipids that decreased in amount under high temperature stress in both genotypes (Figs. 5.2, 5.3 and 5.5). In general, groups that contain structural (i.e., membrane) lipids with highly unsaturated acyl chains (e.g., 18:3; group 4) decreased under high temperatures stress and

groups that contain structural lipids with less unsaturated (e.g., 18:1; group 6a) or saturated (e.g., 15:0, 17:0; group 3) acyl chains increased under high temperatures stress.

The kinetic patterns of changes in lipid levels varied/were similar among and within various groups. For example, extraplastidic diacyl species with 15:0 and 17:0 acyl chains in group 3 and with 18:1 and 18:2 acyl chains in group 6a and PS in group 2 increased at DOS 6 and 12 and then decreased to baseline (or close to that level) at DOS 16 (Fig. 5.5 and supplemental Fig. 5.S2). On the other hand, most extraplastidic phospholipids with 18:2 acyl chains in group 5 decreased at DOS 6 and 12 and then increased to baseline at DOS 16. However, these groups (groups 2, 3, 5 and 6a) generally did not respond to high temperature stress at DOS 1.

The group compositions varied/were similar in the two genotypes (Figs. 5.2 - 5.4). For example, the lipids included in groups 1 and 3 were similar in both genotypes, whereas, composition of other groups varied between genotypes (Fig. 5.4). Group 5 was considerably larger in Ventnor (17 lipids), compared to Karl 92 (6 lipids). Lipids in group 5 decreased through time under high temperature stress in Ventnor, whereas, some of them (e.g., 36:2-PI and 36:3- and 36:2-PC) did not show a consistent decrease in Karl 92 (Figs. 5.2, 5.3 and 5.5 and Supplemental Fig. 5.S2).

Groups Containing Plastidic Glycerolipids (Groups 6b-8)

Group 6b includes plastidic diacyl species with 18:1 and 18:2 acyl chains (Figs. 5.2 - 5.5). These included digalactosyldiacylglycerol (DGDG), MGDG and phosphatidylglycerol (PG) species with limited desaturation. Groups 7 and 8 include plastidic monoacyl lipids and diacyl lipids, respectively with 18:3 acyl chains. These are normal-chain species of DGMG, MGMG (group 7), DGDG, MGDG, PG and sulfoquinovosyl diacylglycerol (SQDG) (group 8). Groups 7 and 8 include the completely desaturated galactolipids (18:3- DGMG and MGMG, 36:6- DGDG, MGDG and SQDG and 38:6-DGDG), while group 6b includes the incompletely desaturated galactolipids (34:2, 34:1, 36:5 and 36:4-DGDG and MGDG and 36:3-MGDG).

When comparing the amount of lipids under HDN with that of OT at DOS 12, group 6b included lipids that increased in amount under high temperature stress, whereas, groups 7 and 8 included lipids that decreased in amount under high temperature stress in both genotypes (Figs. 5.2, 5.3 and 5.5). Thus, the groups that contain plastidic lipids with highly unsaturated acyl

chains (e.g., 18:3; group 7 and 8) decreased under high temperatures stress and the group that contains plastidic lipids with less unsaturated acyl chains (18:1 and 18:2; group 6b) increased under high temperatures stress. The kinetic patterns of changes in lipid levels varied among groups 6b, 7 and 8; lipids in group 6b increased at DOS 6 and 12 and then decreased to baseline at DOS 16, whereas, lipids in groups 7 and 8 decreased at DOS 6 and 12 and then increased to baseline at DOS 16. All the three groups did not respond to high temperature stress at DOS 1.

Group Containing Oxidized Glycerolipids (Group 9)

Group 9 includes glycerolipids with oxidized acyl chains (ox-lipids) (Figs. 5.2 - 5.5). These include MGDG and PC containing a normal acyl chain and an oxidized acyl chain (18:3-2O; three double bond equivalents and two oxygen atoms beyond the carbonyl group). When comparing the amount of lipids under HDN with that of OT at DOS 12, all lipids in this group increased in amount under high temperature stress (Figs. 5.2, 5.3 and 5.5). Ox-PC in group 9 showed a consistent pattern of kinetic changes under high temperature stress as it increased at DOS 6 and 12 and then decreased to baseline at DOS 16. However, it did not respond to high temperature stress at DOS 1.

Groups Containing TAGs and DAGs (Groups 10-11)

Group 10 includes TAGs and a DAG with a 16:0 acyl chain, whereas, group 11 includes TAGs and a DAG with an 18:3 acyl chain (Figs. 5.2 - 5.5). When comparing the amount of lipids under HDN with that of OT at DOS 12, all lipids in group 11 increased in amount under high temperature stress (Figs. 5.2, 5.3 and 5.5). While all lipids in group 10 decreased under high temperature stress in Ventnor, they increased in Karl 92. Group 11 that included TAGs and a DAG with an 18:3 acyl chain increased under high temperature stress even on the first day of stress and returned to baseline during the recovery period (DOS 16) in both genotypes (Fig. 5.5 and supplemental Fig. 5.S2). In contrast, group 10 that included TAGs and a DAG with a 16:0 acyl chain increased during the recovery period (DOS 16).

Groups Containing SGs and ASGs (Groups 12-13)

Group 12 and 13 include ASGs and SGs, respectively (Figs. 5.2 - 5.5). When comparing the amount of lipids under HDN with that of OT at DOS 12, all lipids in groups 12 and 13

increased in amount under high temperature stress (Figs. 5.2, 5.3 and 5.5). Composition of both these groups were similar in Ventnor and Karl 92.

Groups 12 (ASGs) and 13 (SGs) showed a clear pattern of response to high temperature stress through time in both genotypes. Lipids in these groups increased under high temperature stress even on the first day of stress, continued to increase through time under stress and returned to baseline during the recovery period (DOS 16). Heat map of lipid groups showed that SGs (group 13) and ASGs (group 12) increased more in Ventnor than in Karl 92 under high temperature stress (Fig. 5.5 and Supplemental Fig. 5.S2). These results suggest a possible role of SGs and ASGs in heat tolerance in wheat. This is consistent with our previous report that sterol lipids were associated with heat tolerance in Ventnor.

Discussion

Co-occurrence analysis revealed 13 lipid groups that are up-or-down-regulated together through time under high day and night temperature stress in wheat. There were generally two types of lipid groups in both genotypes; groups that contain lipids that increased in amount under high temperature stress (groups 1, 3, 6a, 6b, 9, 11, 12 and 13) and groups that contain lipids that decreased in amount under high temperature stress (groups 2, 4, 7 and 8) (Figs. 5.5 and 5.6). In general, groups that contain extraplastidic or plastidic lipids with highly unsaturated acyl chains (e.g., 18:3; group 4, 7 and 8) decreased under high temperature stress and groups that contain extraplastidic or plastidic lipids with less unsaturated (e.g., 18:1; group 6a and 6b) or saturated (e.g., 15:0 and 17:0; group 3) acyl chains increased under high temperature stress. This is consistent with the findings in the first paper of this series that wheat plants decrease the degree of unsaturation in membrane lipids under high temperature stress by decreasing the amount of highly unsaturated fatty acids and increasing the amount of less unsaturated or saturated fatty acids. We also found that group 11 that included TAGs with 18:3 acyl chains increased under high temperature stress even on the first day of stress and returned to baseline during the recovery period (DOS 16). The 18:3-TAGs might have been synthesized from the 18:3 fatty acids released from the membrane lipids. This suggests that active recycling of 18:3 acyl chains in TAGs starts immediately when wheat plants are exposed to high temperature stress. In addition, group 10 that included TAGs with 16:0 acyl chains increased during the recovery period (DOS 16) suggesting that they might have synthesized from the 16:0 fatty acids released

from the membrane lipids when stress was relieved. Taken together, the changes in groups 10 and 11 under high temperature stress suggest a possible role of TAGs in high temperature stress response by sequestering the 18:3 (during the stress period) and 16:0 (during the recovery period) fatty acids from membrane lipids in order to adjust the degree of unsaturation in the membrane lipids.

The effects of HN, HD and HDN on the kinetic patterns of changes in lipid levels in various groups were different (Figs. 5.5 and 5.6 and Supplemental Fig. 5.S2). The effects of HN were generally less than or similar to that of HD, whereas, the effects of HDN were generally greater than that of HN and greater than or similar to that of HD. We also checked whether negative correlations exist among lipid analytes in wheat genotypes. There was only one negative correlation in Ventnor (between 36:6-SQDG and 34:2-DGDG) with $\rho \leq -0.80$ (Supplemental Table 5.S3). At the same time, no negative correlations existed among lipid analytes in Karl 92 with $\rho \leq -0.80$ (Supplemental Table 5.S4).

It is well-established that lipid-metabolizing enzymes act on multiple, related lipid substrates that contain the same component acyl chain or head group. Camacho et al. (2005) evaluated the origin of correlations in metabolomics data using metabolic control analysis and computer simulation of biochemical networks, and attributed strong metabolite correlations to the strong mutual control by a single enzyme. Vu et al. (2014) analyzed the lipid changes after leaf wounding in Arabidopsis and found that lipids that showed high correlations ($\rho > 0.96$) were the products of the same rate-limiting enzyme or were downstream of the rate-limiting enzyme(s) in a pathway. In the current work, we provide data supporting the hypothesis that correlation analysis of lipid molecular species can reveal groups of co-occurring lipids acted on by the same enzyme(s) in wheat plants subjected to high day and night temperature stress.

In Supplemental Fig. 5.S3, we have presented a dendrogram showing the correlation among lipids included in the groups in either Ventnor (Fig. 5.2) or Karl 92 (Fig. 5.3). This dendrogram was produced using pooled data across genotypes. Fig. 5.7 depicts the possible biosynthetic pathways for the lipids included in groups on the dendrogram presented in Supplemental Fig. 5.S3. As illustrated in Fig. 5.7, fatty acids, that are synthesized de novo in the plastid, can be transferred to the endoplasmic reticulum where the activated fatty acids are present as acyl Coenzyme A (CoAs; Ohlrogge and Browse, 1995). In the endoplasmic reticulum, these activated fatty acids can be transferred sequentially to glycerol-3-phosphate (G3P) to form

phosphatidic acids (PA), which are dephosphorylated to DAGs and are used to synthesize PCs. The PC species can then be desaturated by fatty acid desaturases (FAD), converting PC 34:1 sequentially to PC(34:3); PC(36:2) sequentially to PC(36:6); PC(38:2) to PC(38:3); and PC(40:2) to PC(40:3).

The PC species are also subjected to a process termed as "acyl editing," which is a deacylation-reacylation cycle of PC that exchanges the fatty acids on PC with fatty acids in the acyl-CoA pool (Bates et al., 2007, 2009). Thus, the PC acyl editing cycle involves release of a fatty acid (or acyl-CoA) and lysophosphatidylcholine (LPC). Reacylation of LPC with a different acyl-CoA from the pool completes the cycle. The released fatty acyl chain from PC may be polyunsaturated or used to acylate G3P (or lysophosphatidic acid). The incorporation of fatty acids released from PC into PA can lead to the formation of PE, PI and PS containing acyl chains with various degrees of unsaturation (Bates et al., 2009).

In the current work, group 5 included PC species that are acted on by FAD2 (converts 18:1 to 18:2) or FAD3 (converts 18:2 to 18:3) and PE and PI species that are produced through PC acyl editing (Fig. 5.7). Group 4 included PC species that are acted on by FAD3 and PE and PI species that are produced through PC acyl editing. Co-occurrence of acyl-identical (in terms of number of carbon atoms and degree of unsaturation) PC, PE and PI molecular species in the same groups (groups 3, 4, 5 and 6a) indicates that the rates of production of acyl-identical PCs, PEs and PIs are similar because of regulation upstream at steps involving fatty acid availability and incorporation. Previous reports suggest that PC and PE molecular species with the same acyl chains are unlikely to be formed initially by parallel pathways, instead, PEs are likely formed from PCs (Bates et al., 2007). In the current work, the ratios among acyl-identical PC, PE and PI species that are included in the same group (group 3, 4, 5 or 6a) did not show much variability among different plants (CoV < 30%), compared to the ratios among PC, PE and PI species included in different groups (CoV > 50%) (Supplemental Table 5.S5). Taken together, these results suggest that PC-PE or PC-PI conversions within the same groups were not the rate-limiting processes in formation of PE or PI molecular species under high day and night temperature stress. Instead, desaturase activity of FAD3 that leads to the formation of completely desaturated PCs of group 4 (that contains 18:3 acyl chains) from incompletely desaturated PCs of group 5 (that contains 18:2 acyl chains) was the rate-limiting step.

In the plastid, fatty acids, that are synthesized de novo, are sequentially transferred from acyl carrier protein (ACP) to G3P to form primarily PA(34:1) (18:1/16:0; reviewed by Ohlrogge and Browse, 1995). PA(34:1) can be used to form PG(34:1) (18:1/16:0), which can be desaturated sequentially to PG(34:3) through the action of FAD4 (converts 16:0 to 16:1) and FAD6 (converts 18:1 to 18:2) (Fig. 5.7). Plastidic lipids, MGDG and DGDG are formed from the acyl components of PC that are imported into the plastid (reviewed by Benning, 2009). DGDG (Group 6b) is formed from MGDG by a UDP-galactose-dependent DGDG synthase (DGDGS) (Kelly and D€ormann, 2002; reviewed by Li-Beisson et al., 2013). Fatty acyl chains can undergo desaturation on either MGDG or DGDG. Fatty acid desaturation of MGDG and DGDG species in group 6b involves the action of FAD5 (converts 16:0 to 16:1), FAD6 (converts 18:1 to 18:2), FAD7 and FAD8 (converts 18:2 to 18:3), whereas fatty acid desaturation of MGDG and PG species in group 8 involves the action of FAD7 and FAD8. Co-occurrence of acyl-identical MGDG and DGDG species in the same groups (groups 6b and 8) indicates that the rates of production of acyl-identical MGDG and DGDG species are similar because of regulation upstream. The ratios among acyl-identical MGDG and DGDG species in group 6b did not show much variability among different plants (CoV < 50%), compared to the ratios among MGDG and DGDG species included in different groups (groups 6b and 8) (CoV > 50%) (Supplemental Table 5.S5). Taken together, these results suggest that MGDG-DGDG conversions within the same groups were not rate-limited under high day and night temperature stress. Instead, desaturase activity of FAD7 and FAD8 that leads to the formation of completely desaturated MGDG and DGDG species of group 8 (that contains 18:3 acyl chains) from incompletely desaturated (that contains 18:2 acyl chains) MGDG and DGDG species of group 6b was rate-limited.

The ox-lipids in group 9 can be produced nonenzymatically through the action of reactive oxygen species (Zoeller et al., 2012) or enzymatically through the action of lipoxygenase. Nilsson et al. (2012) found that oxidized acyl chains are formed in lipids without release of the acyl chains from the lipid. The enzymatic formation of ox-lipids might involve oxidation and cyclization of the esterified fatty acyl chains, as suggested by Nilsson et al. (2012).

Sterol glycosides in group 13 (Fig. 5.7) are likely produced by the glycosylation of sterols by UDP-Glc:sterol glycosyltransferase(s) (DeBolt et al., 2009). Acyl sterol glycosides in group 12 are likely produced by acylation of SGs, though the acylating enzymes acting on SGs have

not been identified. The high correlation of SGs in group 13 (ρ, 0.90; Supplemental Fig. 5.S3) indicate that these species might have produced through parallel glycosylation of stigmasterol, sitosterol and campesterol. Further, it is possible that the three ASG species in group 12 were formed by parallel acylation of SGs, with the same acyl chain. Our data showed that groups 12 and 13 showed a clear pattern of response to high temperature stress as they increased under high temperature stress even on the first day of stress, continued to increase through time under stress and returned to baseline during the recovery period (DOS 16) (Figs. 5.5 and 5.6 and Supplemental Fig. 5.S2). In addition, SGs and ASGs increased more in Ventnor than in Karl 92 under high temperature stress. These results suggest that enzymes responsible for the formation of SGs and ASGs were highly responsive to high temperature stress, and their up-regulation might be associated with high temperature tolerance in Ventnor.

Triacylglycerols in groups 10 and 11 might have formed through the action of phospholipid:diacylglycerol acyltransferase (PDAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) (Zhang et al., 2009; Fan et al., 2013). Bates et al. (2012) reported that PC acyl editing and phosphocholine head group exchange between PC and DAGs are the major mechanisms responsible for directing polyunsaturated fatty acids into TAGs. Our data showed that PC species with 18:3 acyl chains (group 4) decreased under high temperature stress. This might be partly explained by PC acyl editing that involves removal of 18:3 acyl chains from PC, which was incorporated into TAGs. Our data also showed that group 11 that included TAGs with an 18:3 acyl chain was highly responsive to high temperature stress as it increased under high temperature stress even on the first day of stress and returned to baseline during the recovery period (DOS 16) in both genotypes (Figs. 5.5 and 5.6 and supplemental Fig. 5.S2). This suggests a possible role of PDAT in high temperature stress response through sequestering the 18:3 fatty acids from membrane lipids.

Conclusions

Correlation analysis revealed 13 lipid groups that are up-or-down-regulated together through time under high day and night temperature stress in wheat genotypes. Lipid groups that contain extraplastidic or plastidic lipids with highly unsaturated acyl chains (e.g., 18:3) decreased under high temperature stress and lipid groups that contain extraplastidic or plastidic lipids with less unsaturated (e.g., 18:1) or saturated (e.g., 15:0 and 17:0) acyl chains increased

under high temperature stress. The kinetic patterns of changes in lipid levels varied among the groups, with some groups being quicker to respond and whether or not they return to baseline or continue to change during the recovery period. Interpretation of lipid groups based on the current knowledge on lipid metabolism suggests that the lipid groups reflect metabolic relationships as the co-occurring lipids in each group are acted on by the same enzyme(s). Lipid co-occurrence in groups was primarily the result of desaturating, oxidizing, glycosylating and acylating activities of enzymes acting on lipids under high temperature stress. The results from this study indicate that lipid groups experiencing coordinated metabolism can be detected by analysis of lipid co-occurrence under high day and night temperature stress in wheat. Application of co-occurrence analysis to additional lipids, species and plants subjected to other stresses will provide further information on coordinated metabolism of plant lipids under stress conditions.

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Figures

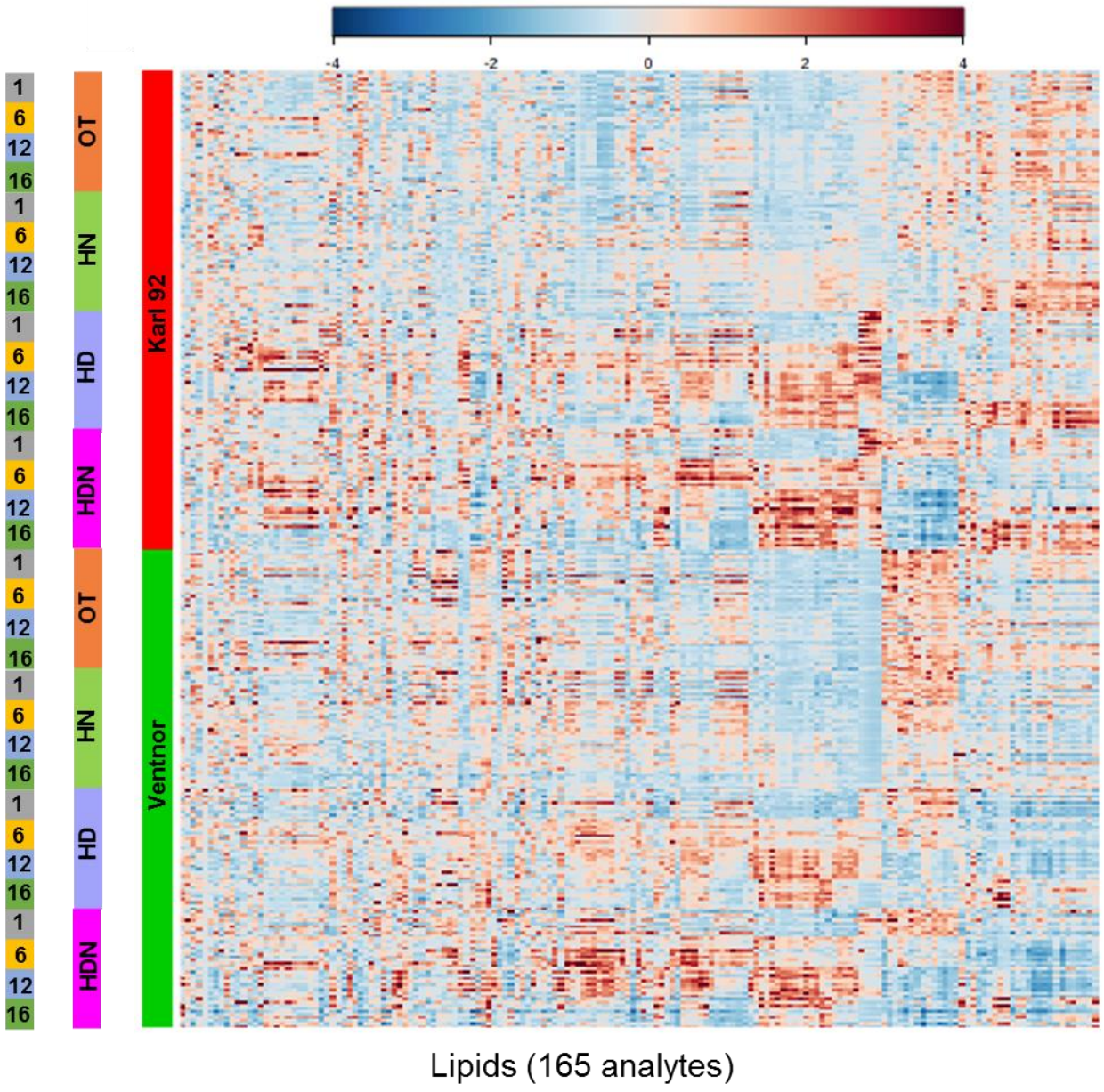


Figure 5.1. Heat map of autoscaled lipid levels under various temperature conditions in wheat genotypes.

Genotypes (Ventnor and Karl 92), temperature regimes (optimum temperature [OT; 25/15°C, daytime maximum/night-time minimum], high night temperature [HN; 25/24°C], high day temperature [HD; 35/15°C] and high day and night temperature [HDN; 35/24°C]) and days of

stress (DOS 1,6,12 and 16 with DOS 16 indicating the recovery period as stress was imposed for 12 days) are indicated on the left. Hundred and sixty five lipid analytes from 320 plant samples (two genotypes, four temperature regimes, four DOS, five replications [plants] and two experiments) are shown in the figure.

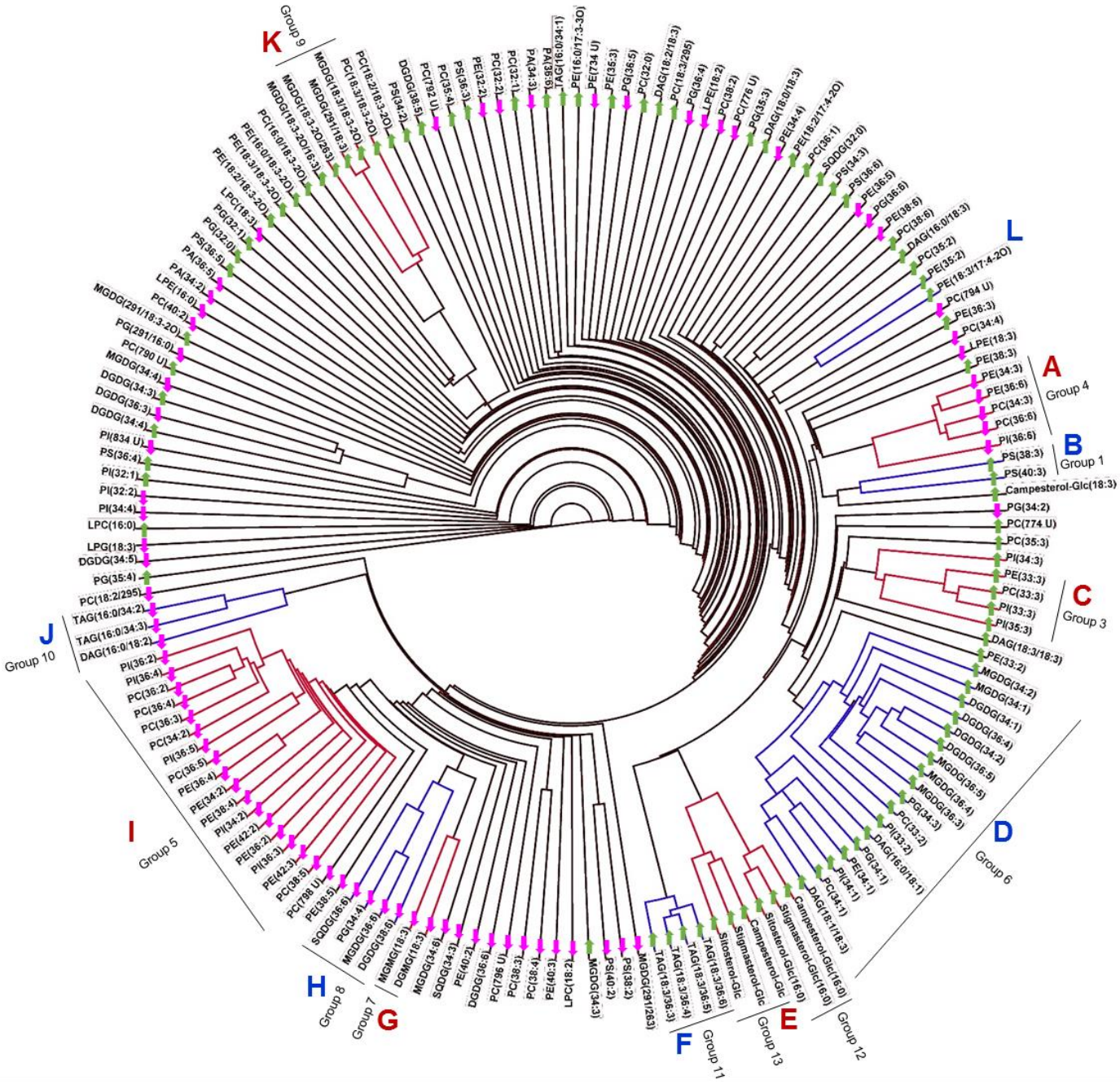


Figure 5.2. Lipid dendrogram of wheat genotype Ventnor.

Hundred and sixty five lipid analytes were clustered using a single-linkage hierarchical algorithm based on Spearman's correlation coefficient, ρ (Supplemental Table 5.S3). Twelve clusters with $\rho \geq 0.8$ are indicated by letters A through L in red and blue colors. Co-occurring lipid groups,

which were the whole clusters or a part of that, are marked on the dendrogram. Lipids that increased in amount under high day and night temperature stress (HDN), compared to optimum temperature (OT) on the last day of stress (day 12) are indicated by green colored upward arrows and lipids that decreased in amount under HDN, compared to OT on the last day of stress (day 12) are indicated by pink colored downward arrows on the dendrogram.

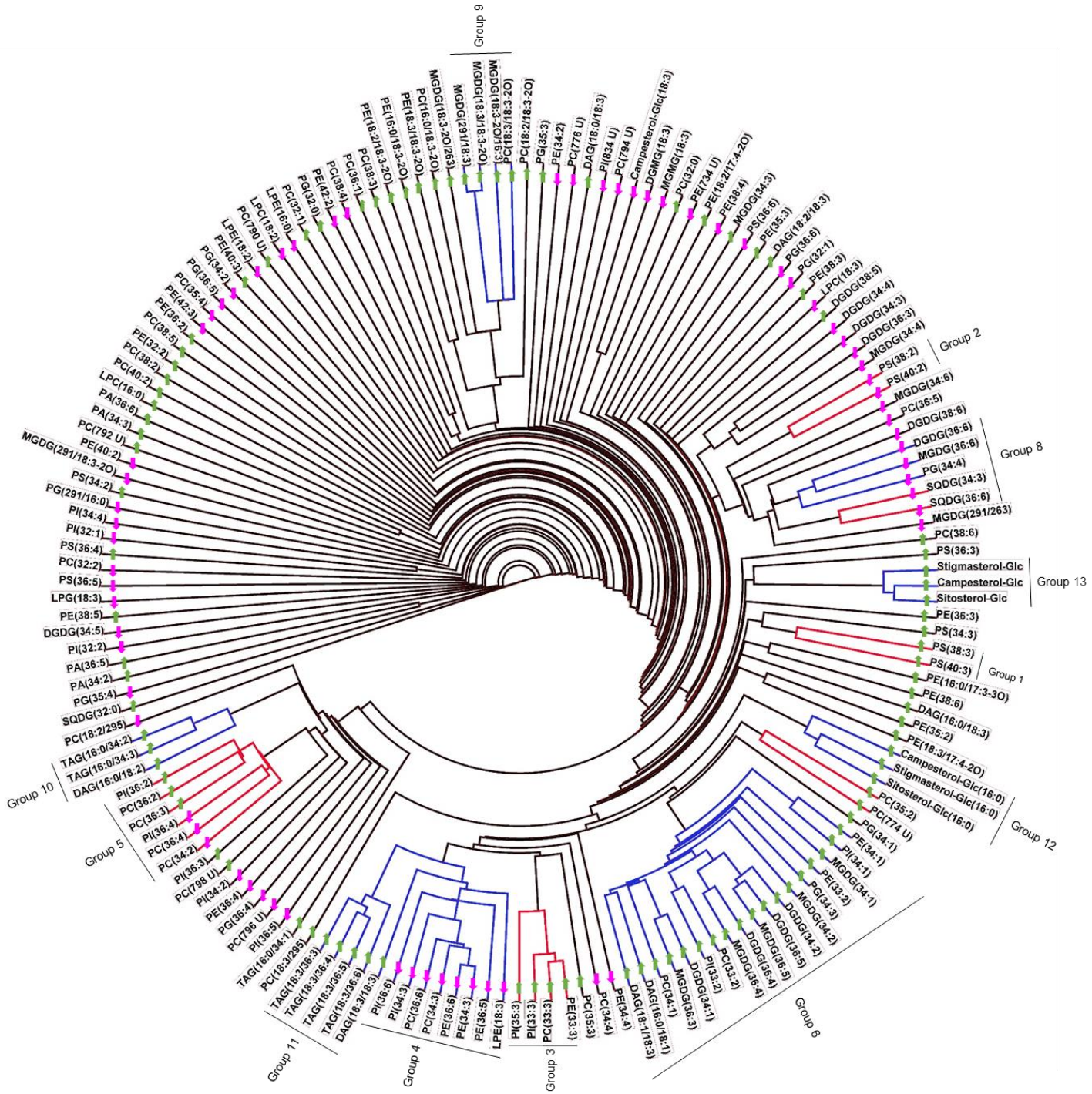


Figure 5.3. Lipid dendrogram of wheat genotype Karl 92.

Hundred and sixty five lipid analytes were clustered using a single-linkage hierarchical algorithm based on Spearman's correlation coefficient, ρ (Supplemental Table 5.S4). Thirteen clusters with

$\rho \geq 0.8$ are indicated by letters A through M in red and blue colors. Co-occurring lipid groups, which were the whole clusters, a part of that or a combination of two adjacent clusters, are marked on the dendrogram. Lipids that increased in amount under high day and night temperature stress (HDN), compared to optimum temperature (OT) on the last day of stress (day 12) are indicated by green colored upward arrows and lipids that decreased in amount under HDN, compared to OT on the last day of stress (day 12) are indicated by pink colored downward arrows on the dendrogram.

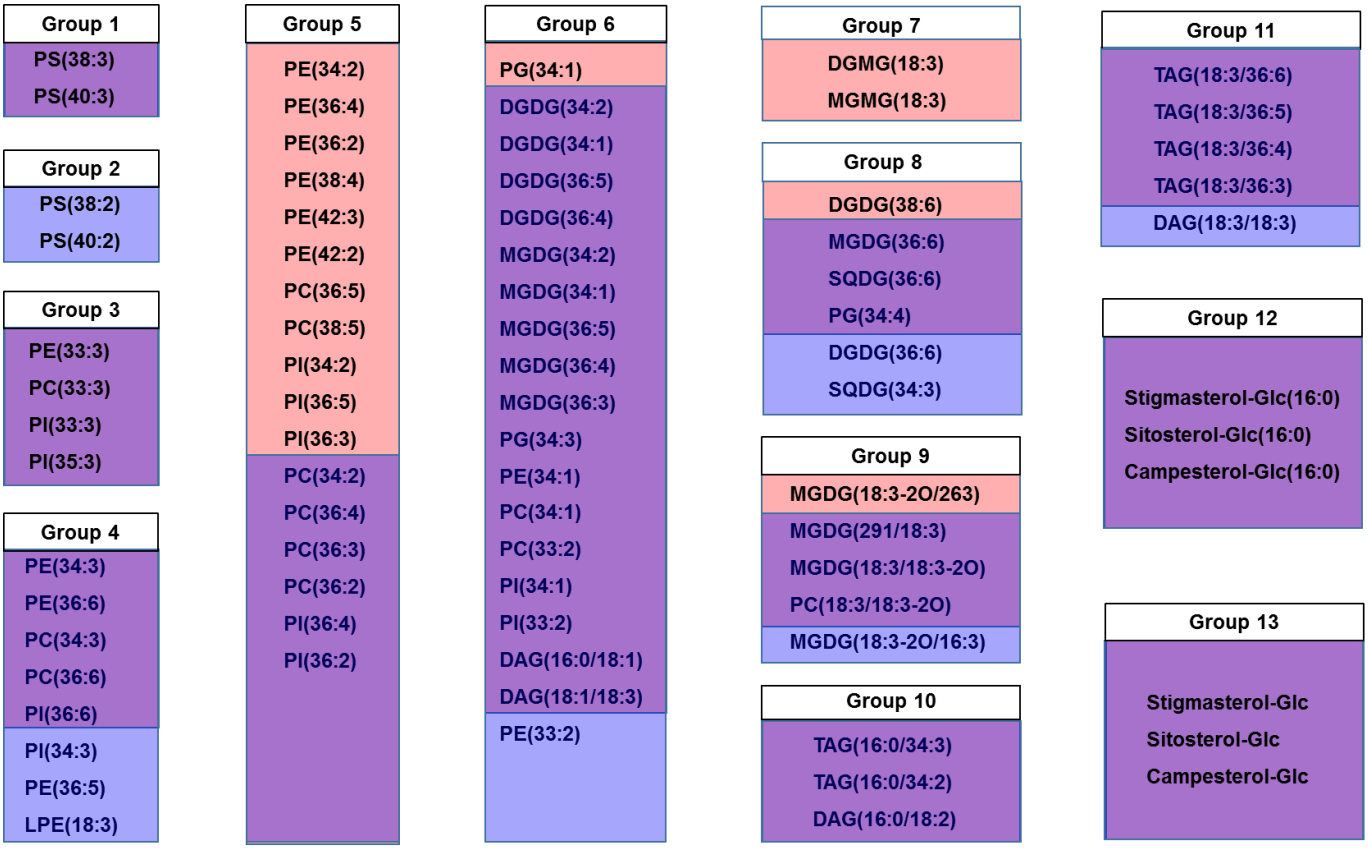


Figure 5.4. Comparison of lipid groups between genotypes Ventnor and Karl 92.
Red box indicates lipids that were included in groups only in Ventnor, blue box indicates lipids that were included in groups only in Karl 92 and lavender box indicates lipids that were included in groups in both genotypes. Group 6 is sub-divided into extraplastidic (PE, PC, PI and DAG; group 6a) and plastidic (DGDG, MGDG and PG; group 6b) groups.

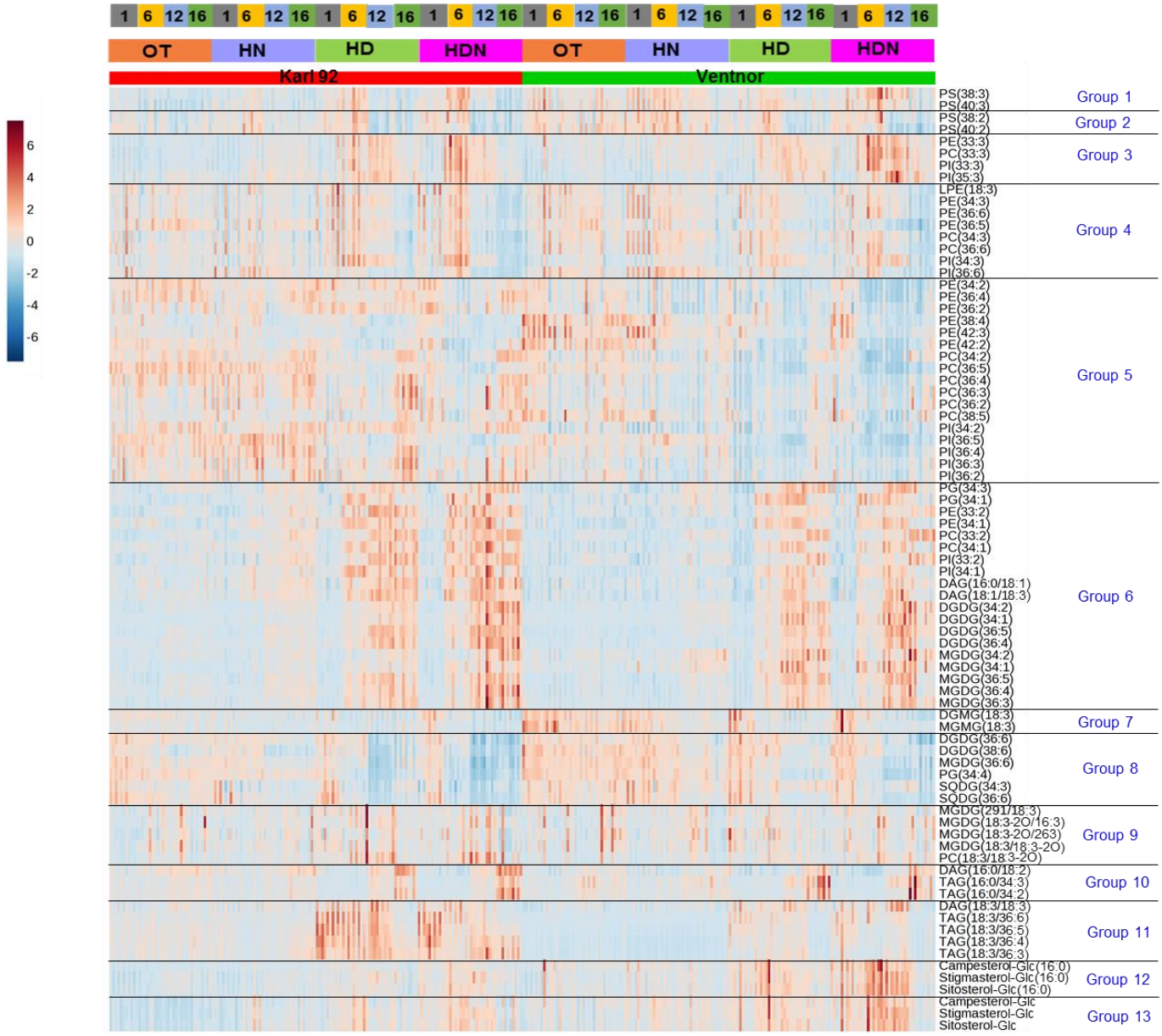


Figure 5.5. Heat map of lipid groups.

Genotypes (Ventnor and Karl 92), temperature regimes (optimum temperature [OT; 25/15°C, daytime maximum/night-time minimum], high night temperature [HN; 25/24°C], high day temperature [HD; 35/15°C] and high day and night temperature [HDN; 35/24°C]) and days of stress (DOS 1,6,12 and 16 with DOS 16 indicating the recovery period as stress was imposed only for 12 days) are indicated at the top. Data from 320 plant samples (two genotypes, four temperature regimes, four DOS, five replications [plants] and two experiments) are shown in the

figure. Group 1- PS with an 18:3 acyl chain and a long acyl chain, Group 2- PS with an 18:2 acyl chain and a long acyl chain, Group 3- Extraplastidic lipids with 15:0 and 17:0 acyl chains, Group 4- Extraplastidic lipids with an 18:3 acyl chain and an 18:3, 18:2 or 16:0 acyl chain, Group 5- Extraplastidic lipids with an 18:2 acyl chain, Group 6- Polar lipids with 18:1 and 18:2 acyl chains, Group 7- Plastidic monoacyl lipids with 18:3 acyl chains, Group 8- Plastidic diacyl lipids with 18:3 acyl chains, Group 9- Ox-lipids, Group 10- TAGs and DAGs with a 16:0 acyl chain, Group 11- TAGs and DAGs with an 18:3 acyl chain, Group 12- ASGs, Group 13- SGs.

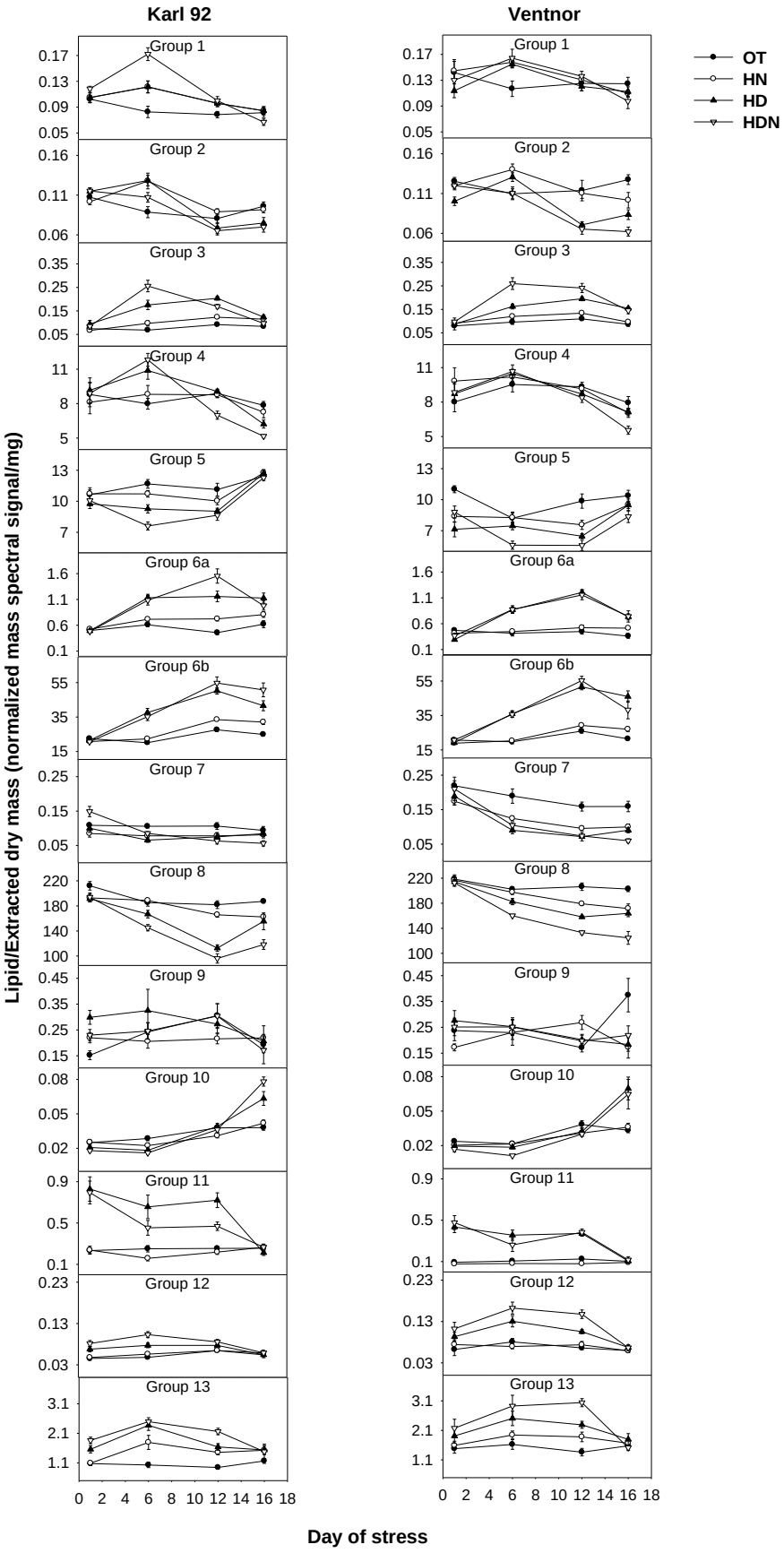


Figure 5.6. Kinetic changes in lipid groups under optimum temperature (OT; 25/15°C, daytime maximum/night-time minimum), high night temperature (HN; 25/24°C), high day temperature (HD; 35/15°C) and high day and night temperature (HDN; 35/24°C).

Days of stress (DOS 1,6,12 and 16) are indicated on the x-axis with DOS 16 indicating the recovery period as stress was imposed for 12 days. Group 1- PS with an 18:3 acyl chain and a long acyl chain, Group 2- PS with an 18:2 acyl chain and a long acyl chain, Group 3- Extraplastidic lipids with 15:0 and 17:0 acyl chains, Group 4- Extraplastidic lipids with an 18:3 acyl chain and an 18:3, 18:2 or 16:0 acyl chain, Group 5- Extraplastidic lipids with an 18:2 acyl chain, Group 6- Polar lipids with 18:1 and 18:2 acyl chains, Group 7- Plastidic monoacyl lipids with 18:3 acyl chains, Group 8- Plastidic diacyl lipids with 18:3 acyl chains, Group 9- Ox-lipids, Group 10- TAGs and DAGs with a 16:0 acyl chain, Group 11- TAGs and DAGs with an 18:3 acyl chain, Group 12- ASGs, Group 13- SGs.

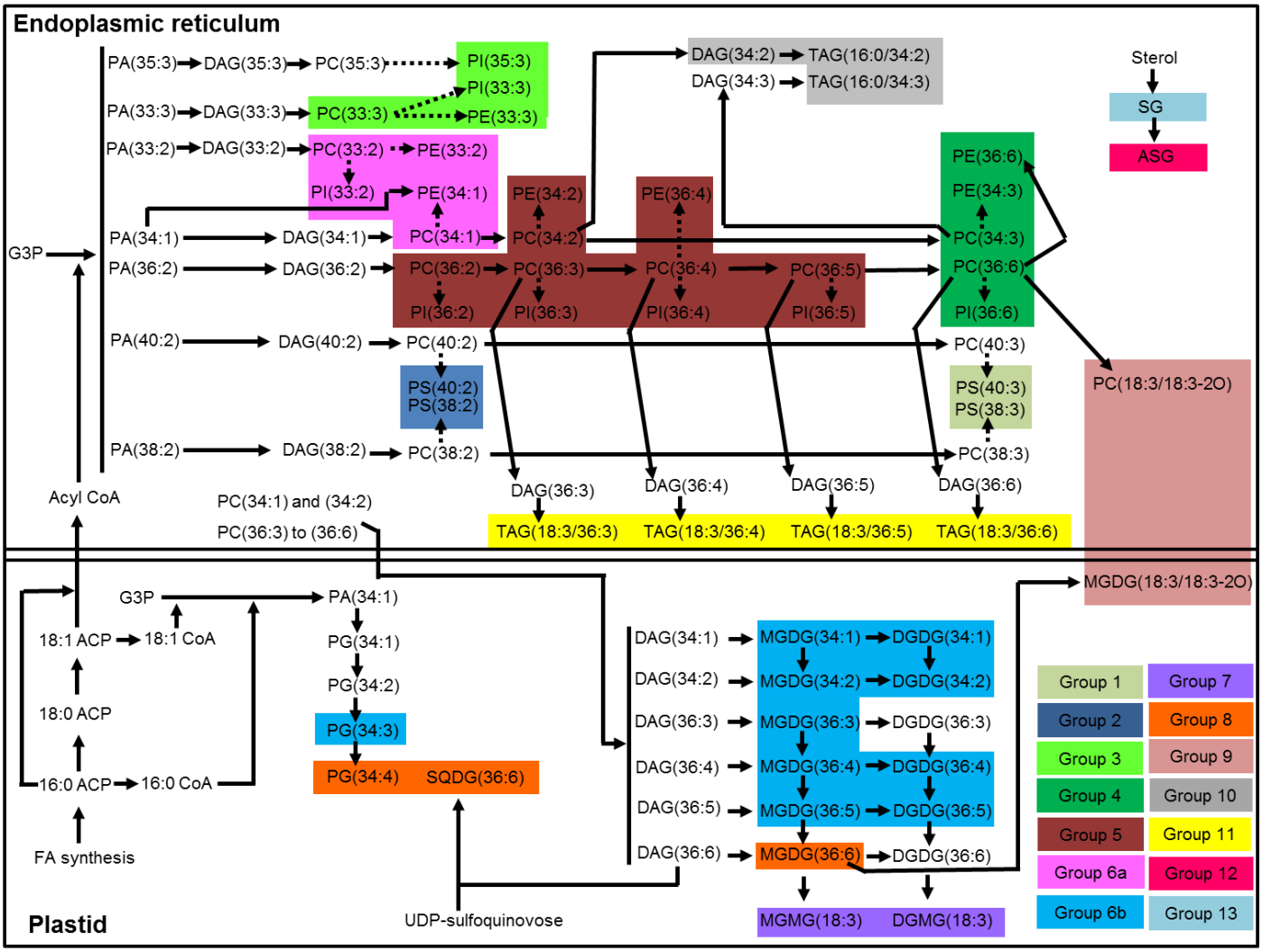
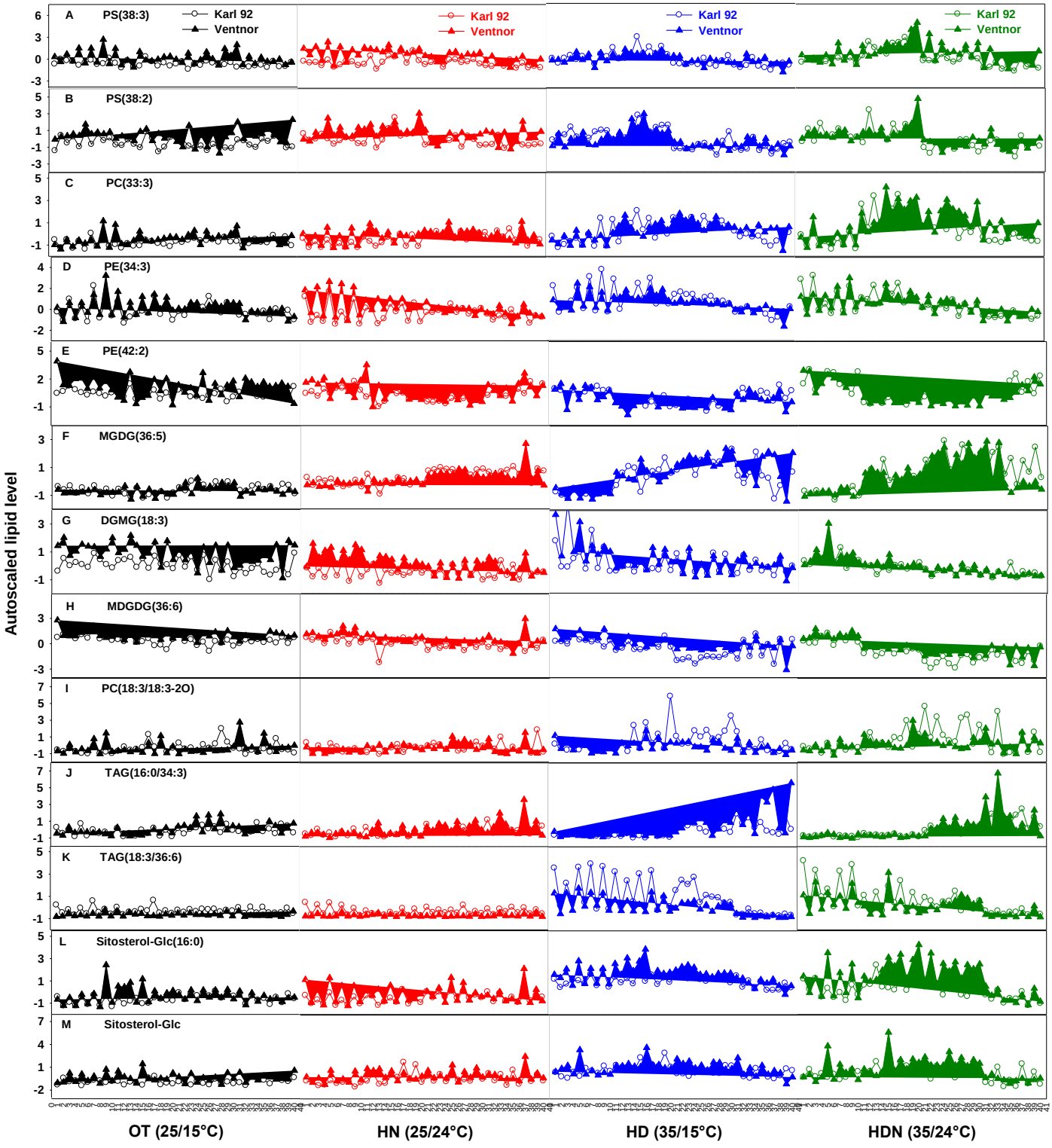
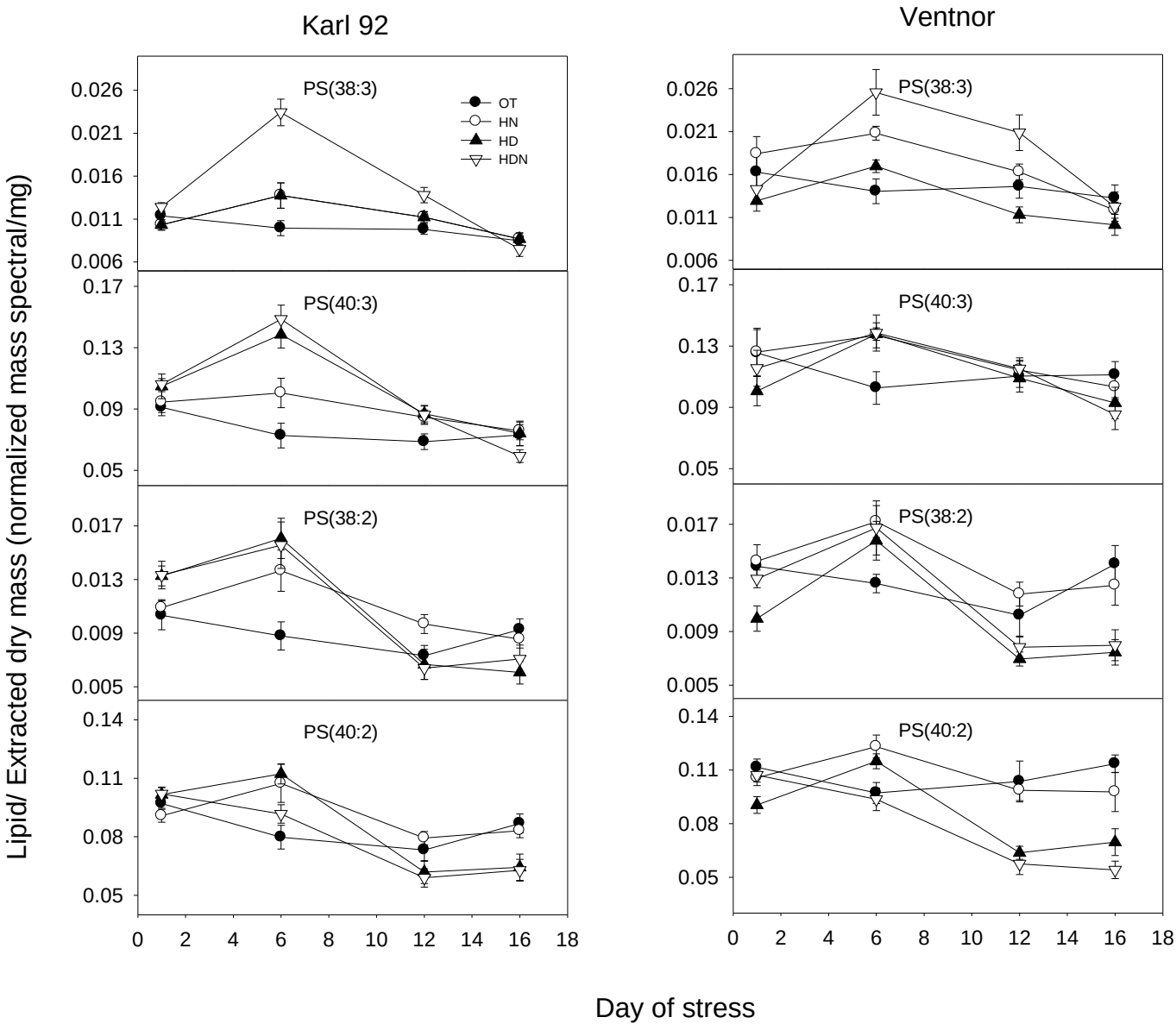


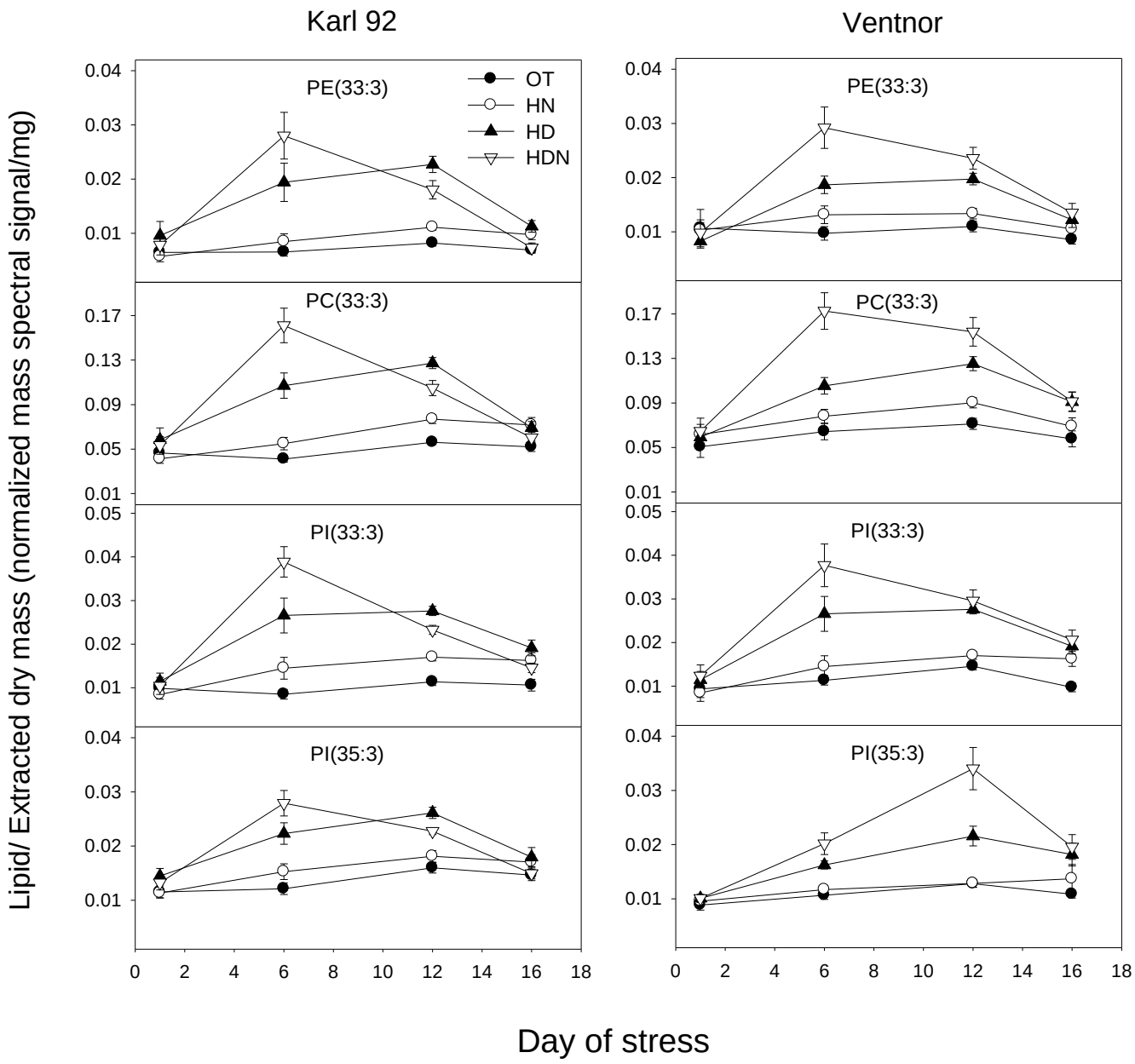
Figure 5.7. Metabolic pathway map showing the biosynthesis of lipids included in the lipid groups of wheat genotypes based on Ongun and Mudd (1970), Ohlrogge and Browse (1995), Bates et al. (2007,2009), Benning (2009), DeBolt et al. (2009) and Shimojima (2011). The dashed arrows between PC and other phospholipid species with similar acyl chains indicate that those conversions involve multiple steps in terms of metabolism. ACP - acylcarrier protein, CoA - Coenzyme A, FA – fatty acid, G3P - glycerol-3-phosphate and UDP - uridine diphosphate.



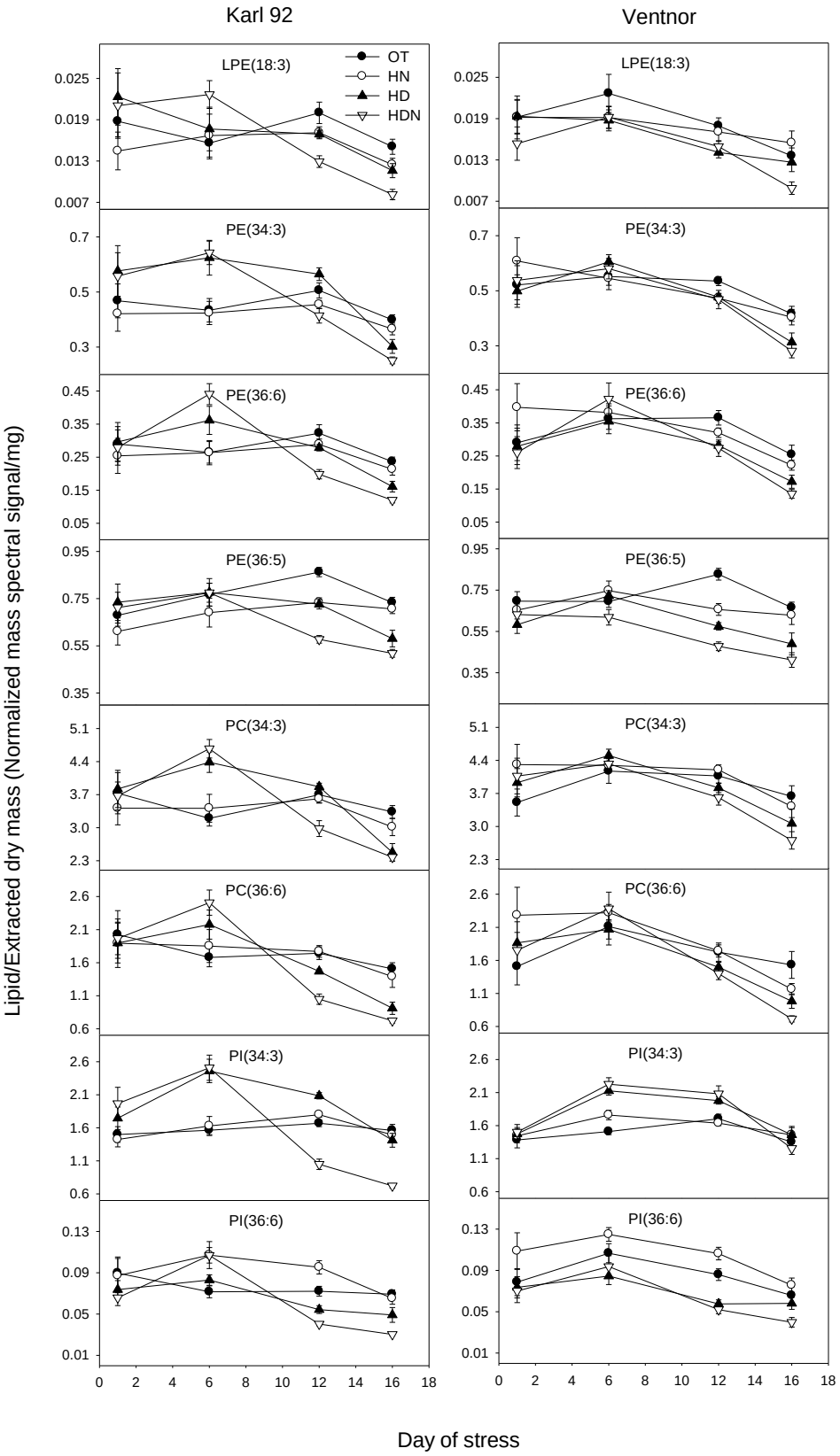
Supplemental Figure 5.S1. Autoscaled levels of representative lipid analytes in individual plants. Thirteen lipids presented in the figure represent 13 different lipid groups. Each temperature regime (optimum temperature [OT], high night temperature [HN], high day temperature [HD] and high day and night temperature [HDN]) includes 40 plants out of which plants 1-10 were sampled at day of stress (DOS) 1, plants 11-20 were sampled at DOS 6, plants 21-30 were sampled at DOS 12 and plants 31-40 were sampled at DOS 16 (during recovery period; stress was imposed for 12 days).



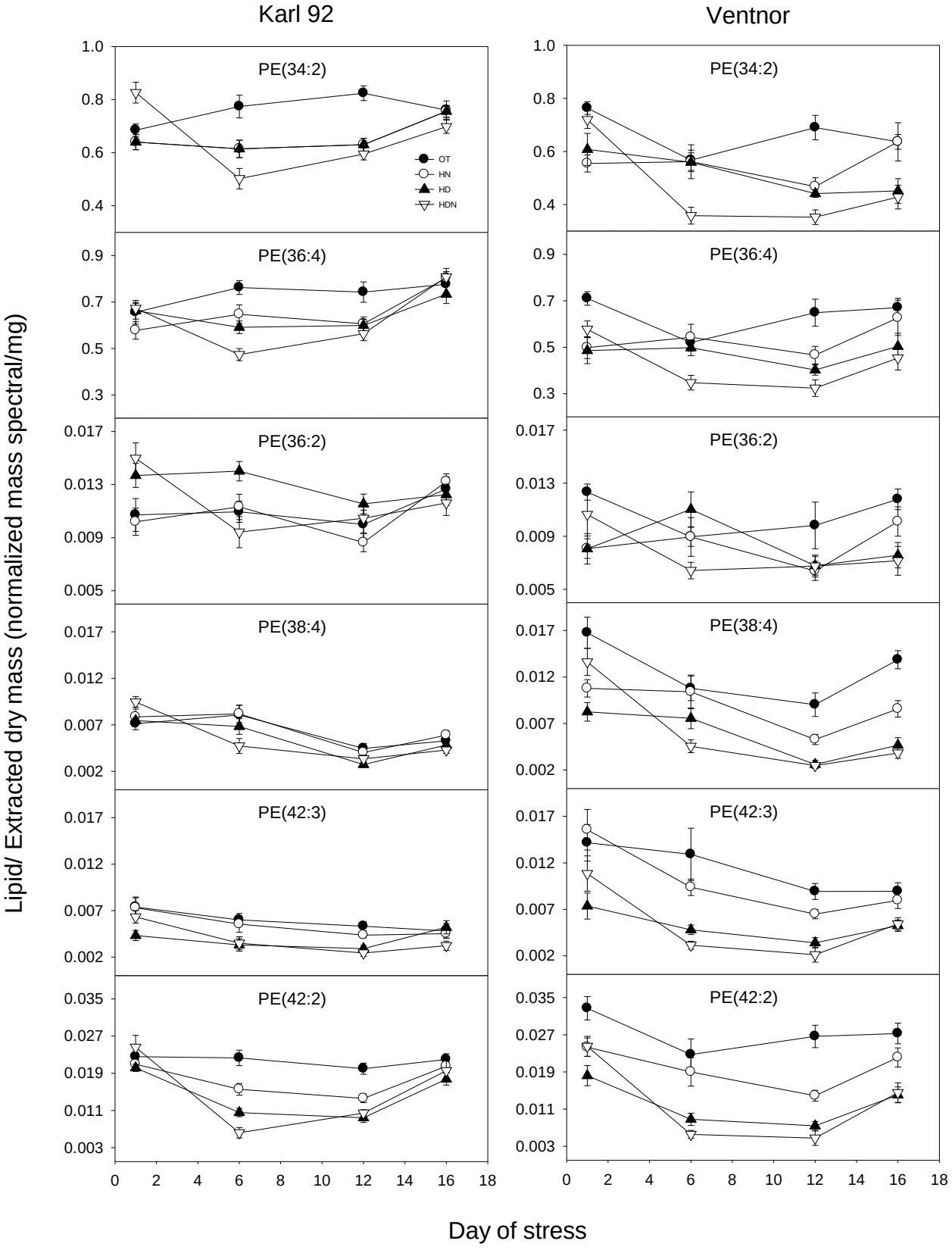
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (A) Lipid groups 1 (Phosphatidylserine [PS] with an 18:3 acyl chain and a long acyl chain) and 2 (PS with an 18:2 acyl chain and a long acyl chain).



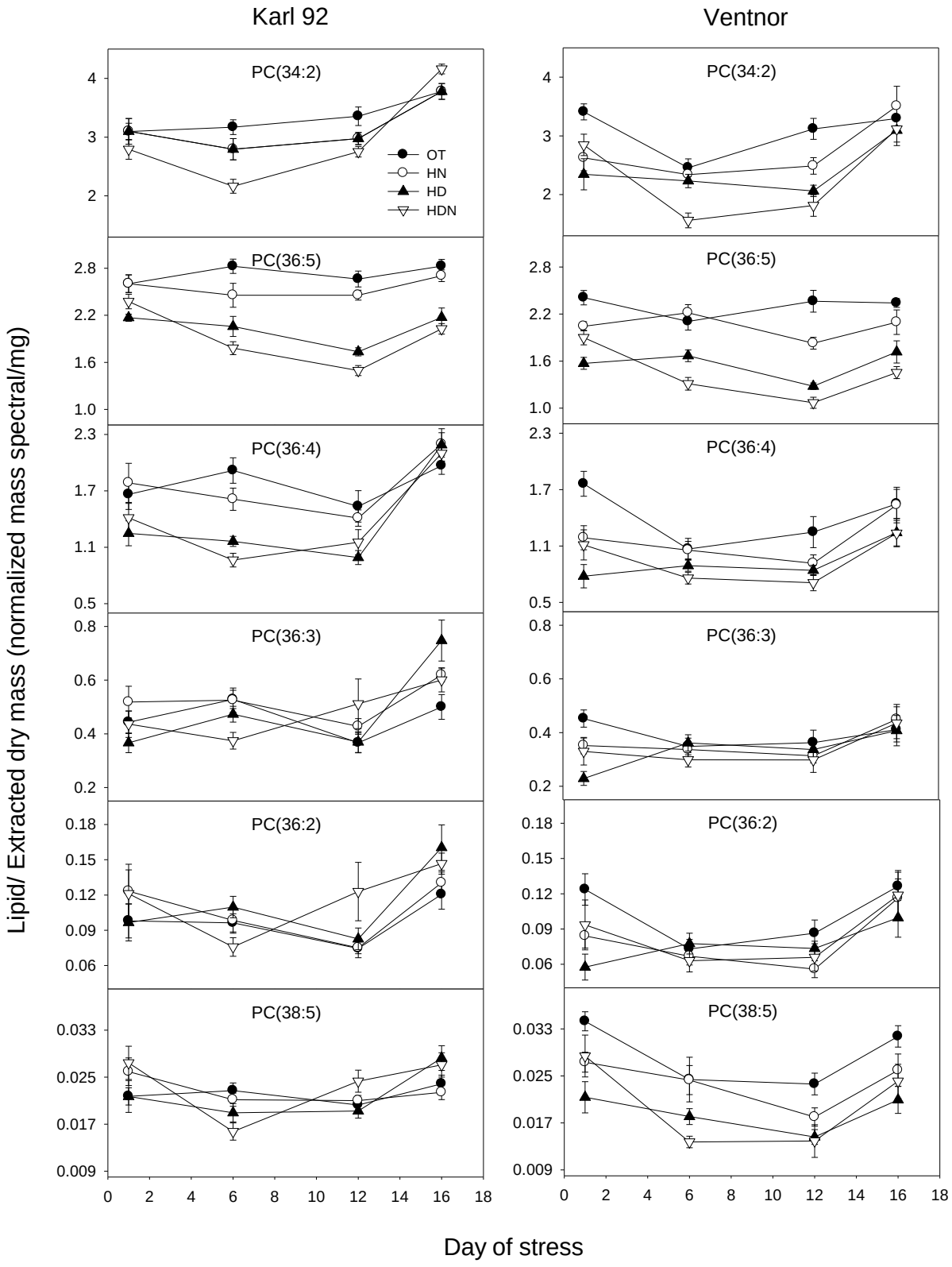
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (B) Lipid group 3- Extraplastidic lipids with 15:0 and 17:0 acyl chains.



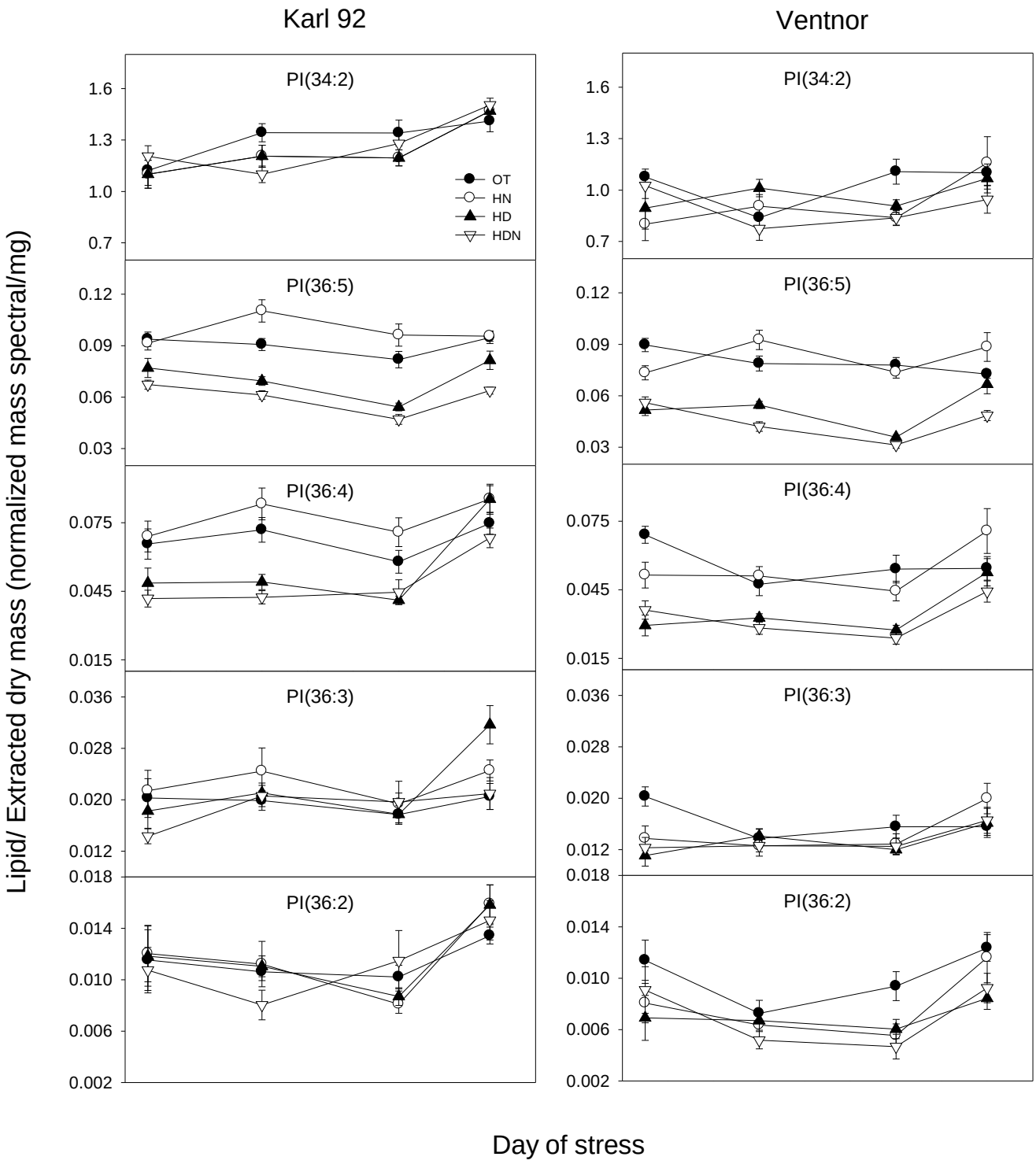
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (C) Lipid group 4- Extraplastidic lipids with an 18:3 acyl chain and an 18:3, 18:2 or 16:0 acyl chain.



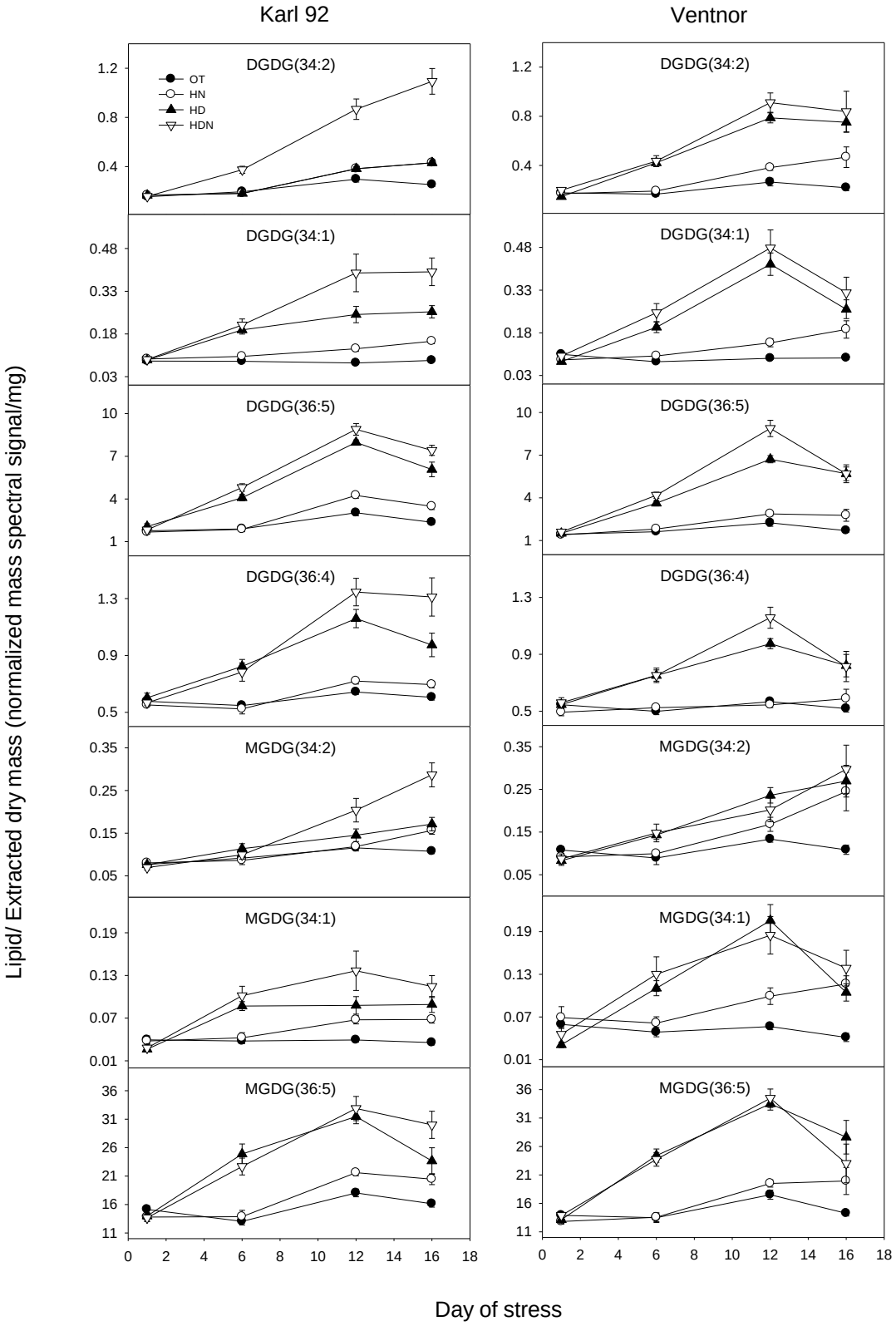
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (D) Lipid group 5- Extraplastidic lipids with an 18:2 acyl chain (continued on next page).



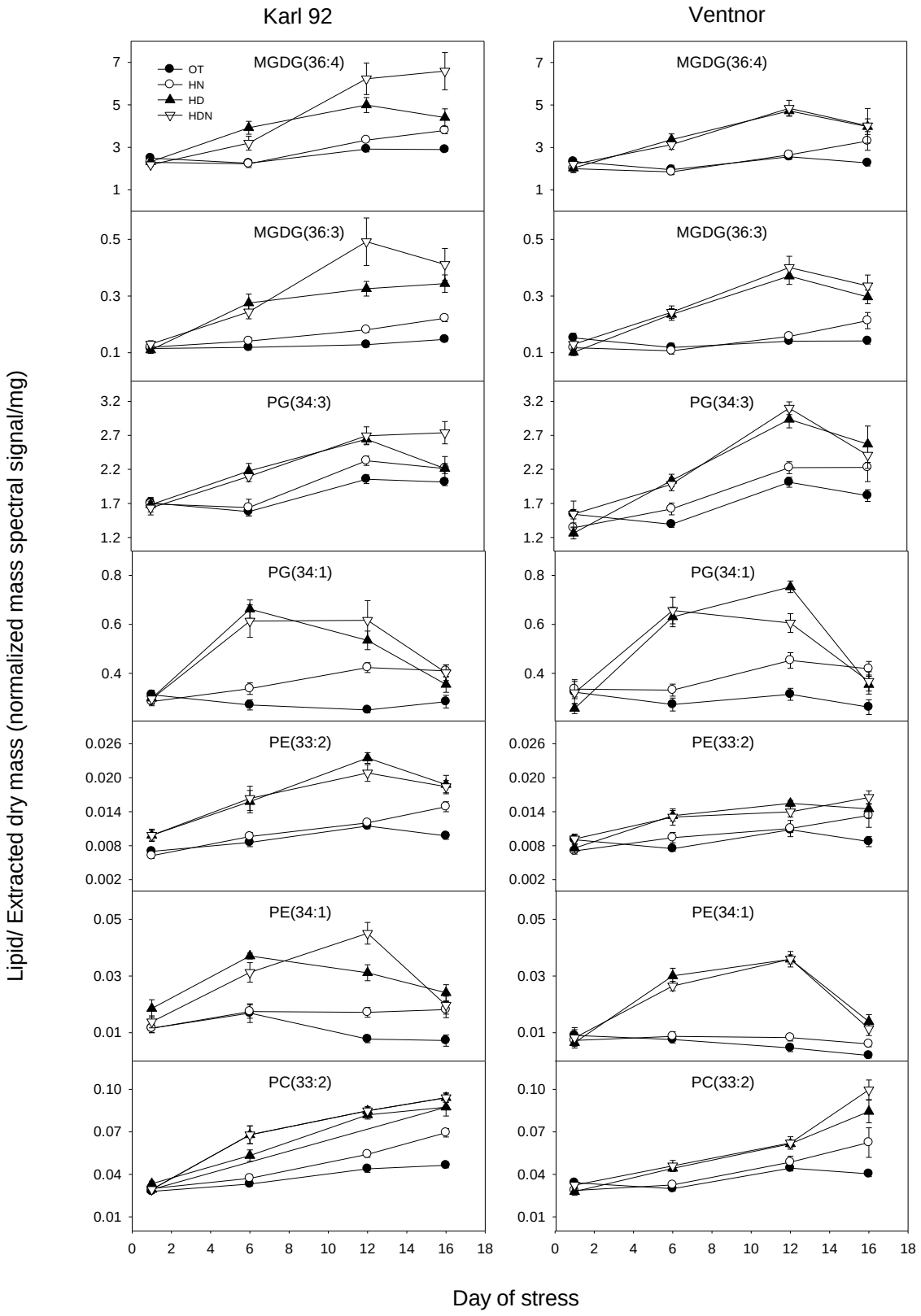
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (E) Lipid group 5- Extraplastidic lipids with an 18:2 acyl chain (Continued on next page).



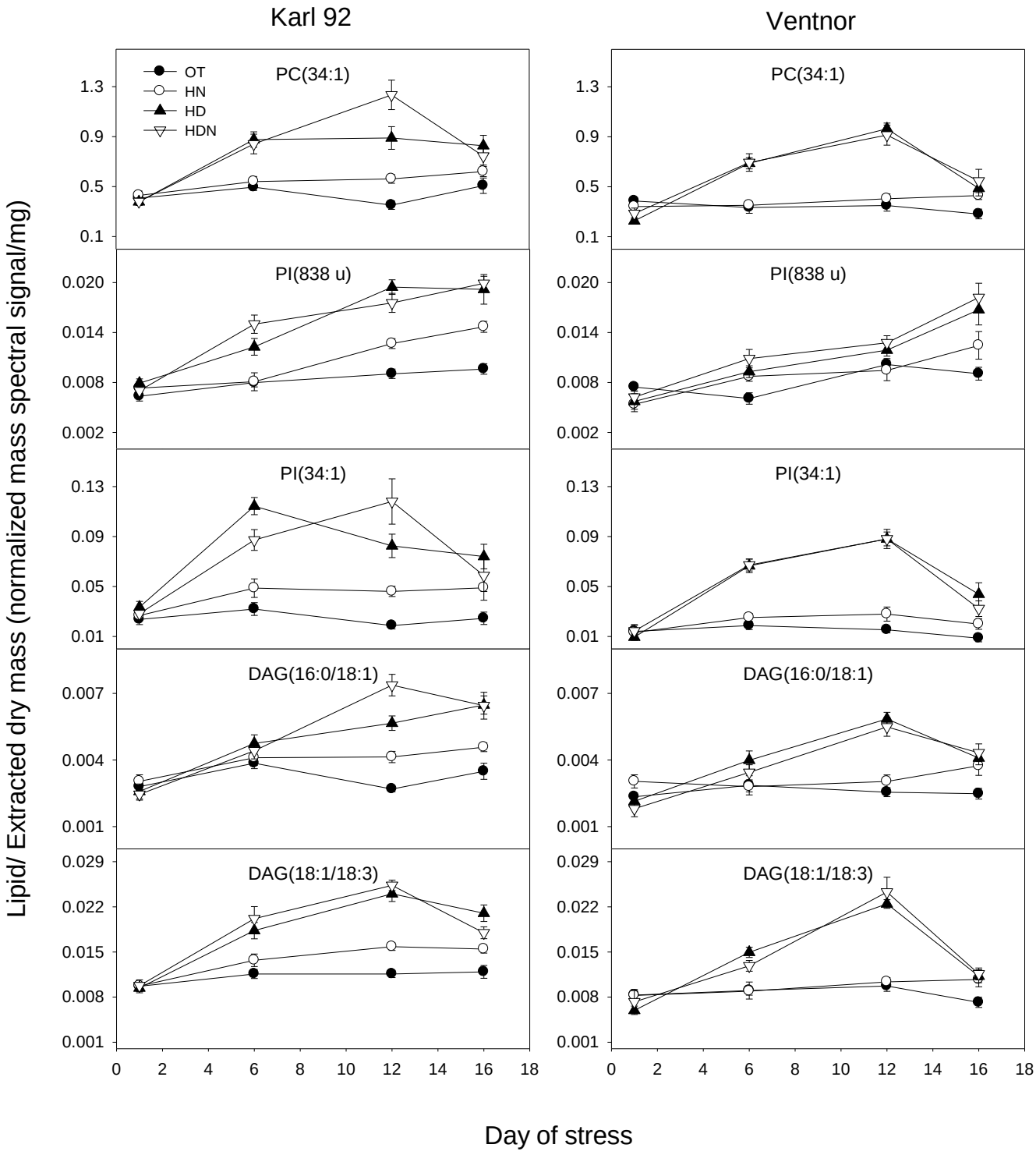
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (F) Lipid group 5- Extraplastidic lipids with an 18:2 acyl chain.



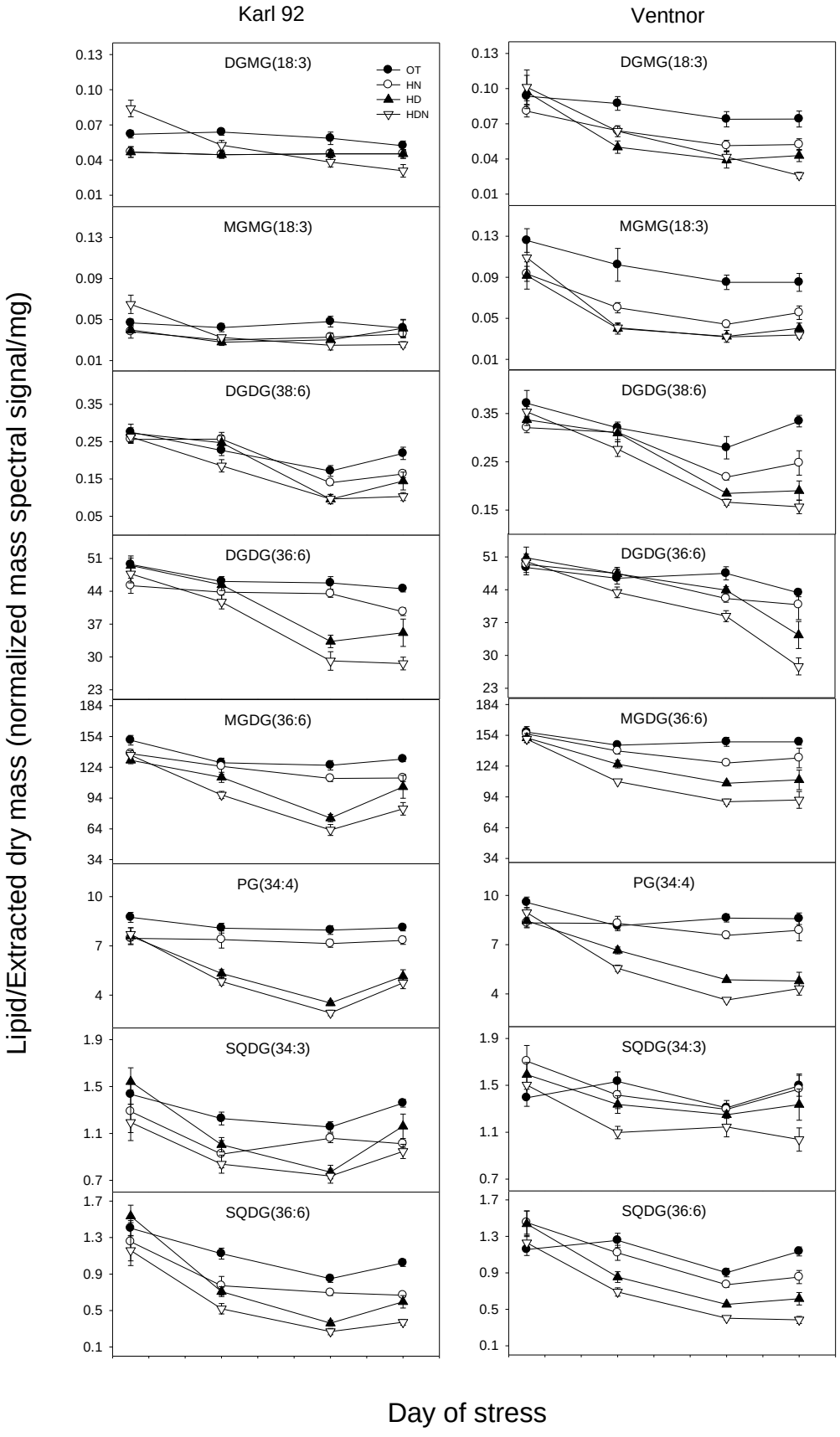
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (G) Lipid group 6- Polar lipids with 18:1 and 18:2 acyl chains (continued on next page).



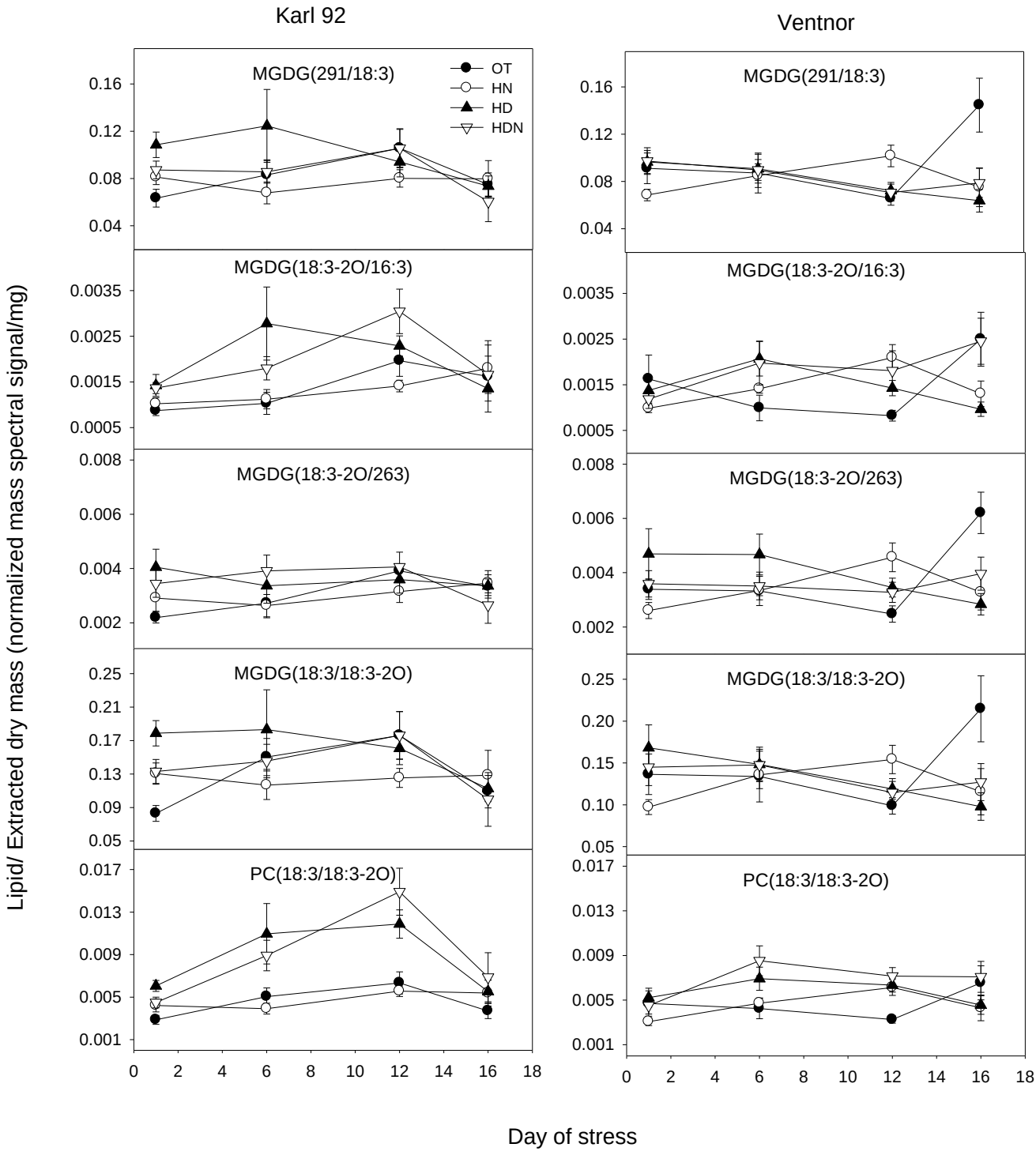
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (H) Lipid group 6- Polar lipids with 18:1 and 18:2 acyl chains (continued on next page).



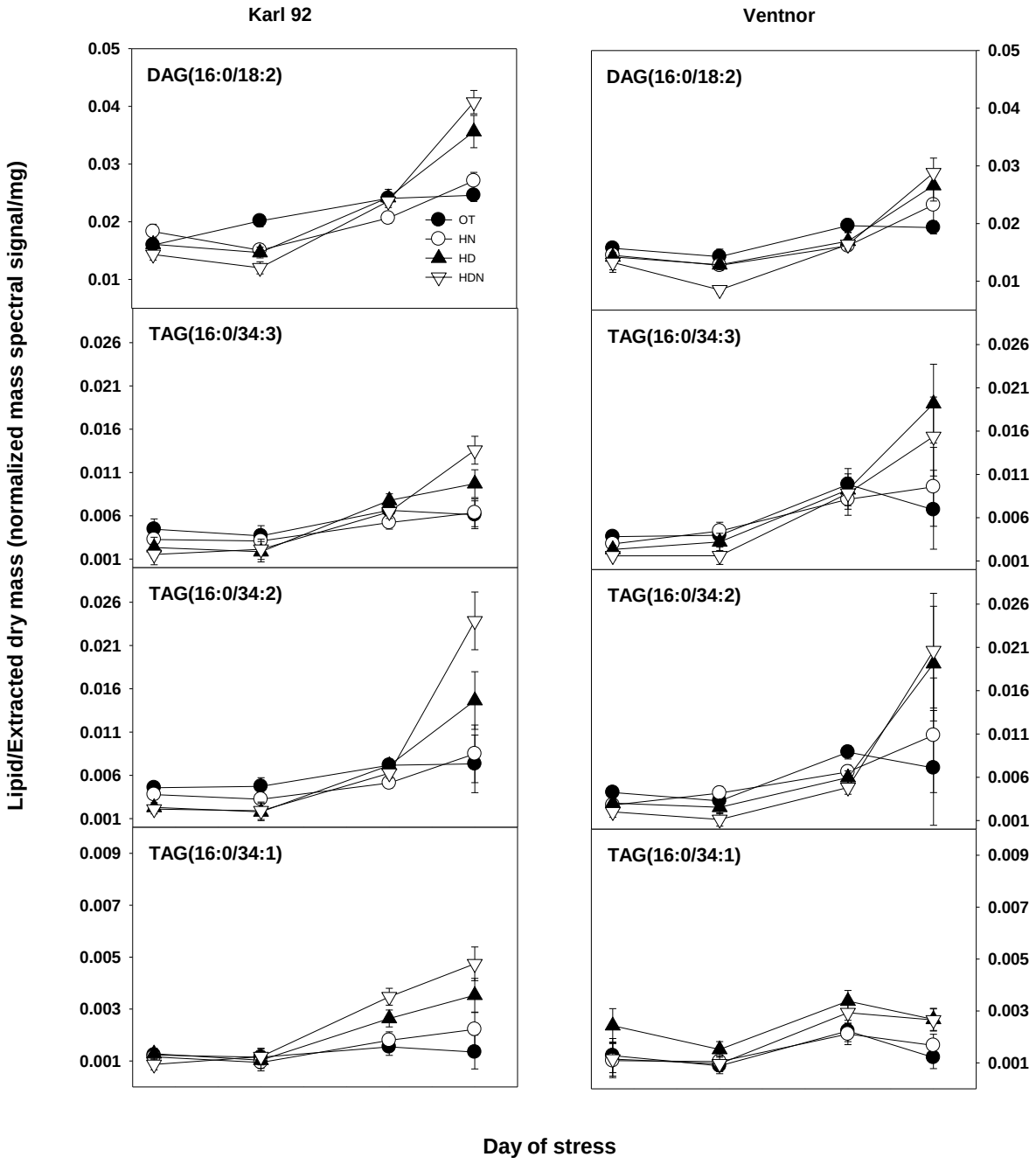
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (I) Lipid group 6- Polar lipids with 18:1 and 18:2 acyl chains.



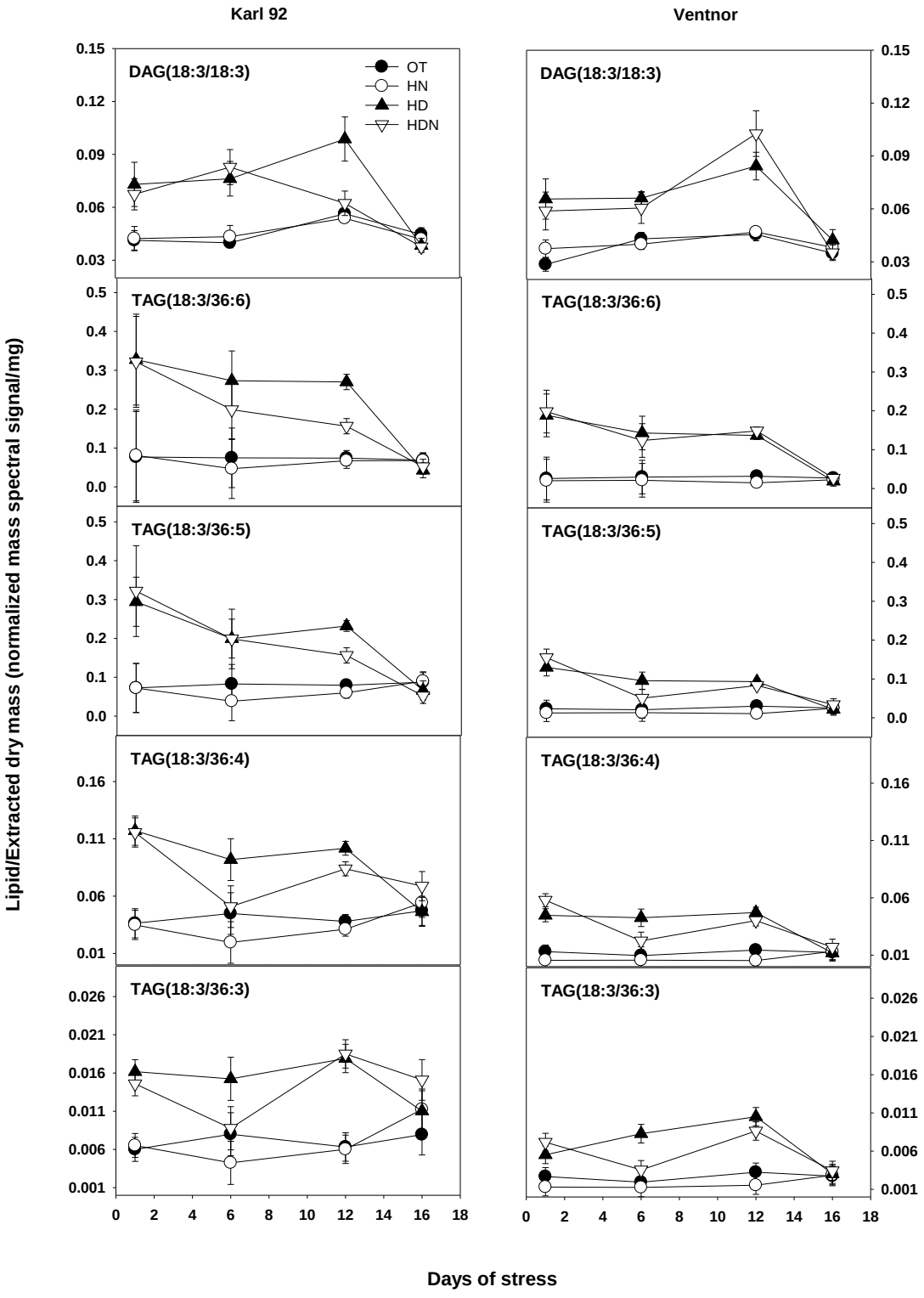
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (J) Lipid groups 7 and 8- Plastidic lipids with 18:3 acyl chains.



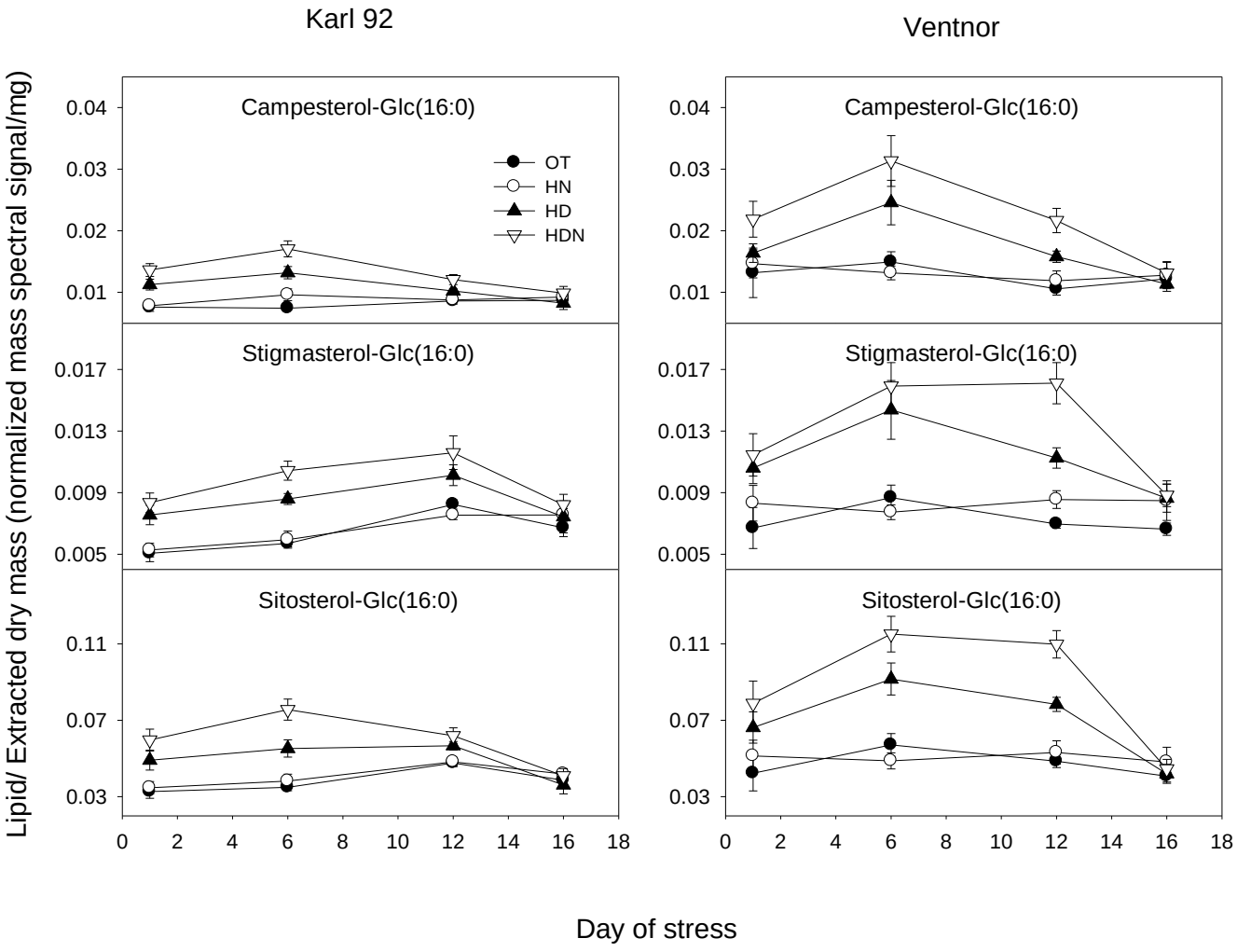
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (K) Lipid group 9- Ox-lipids.



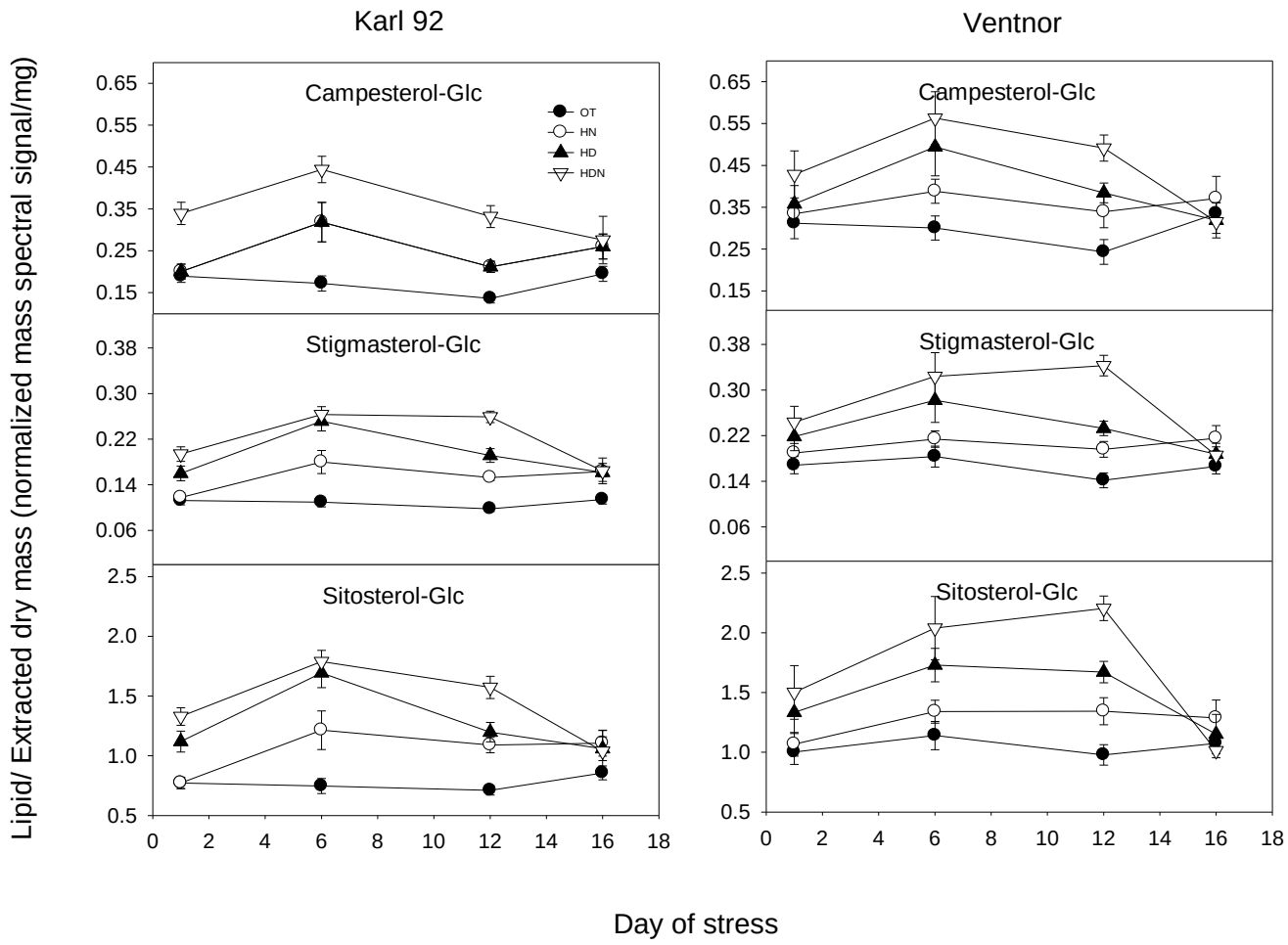
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (L) Lipid group 10- TAGs and DAGs with a 16:0 acyl chain.



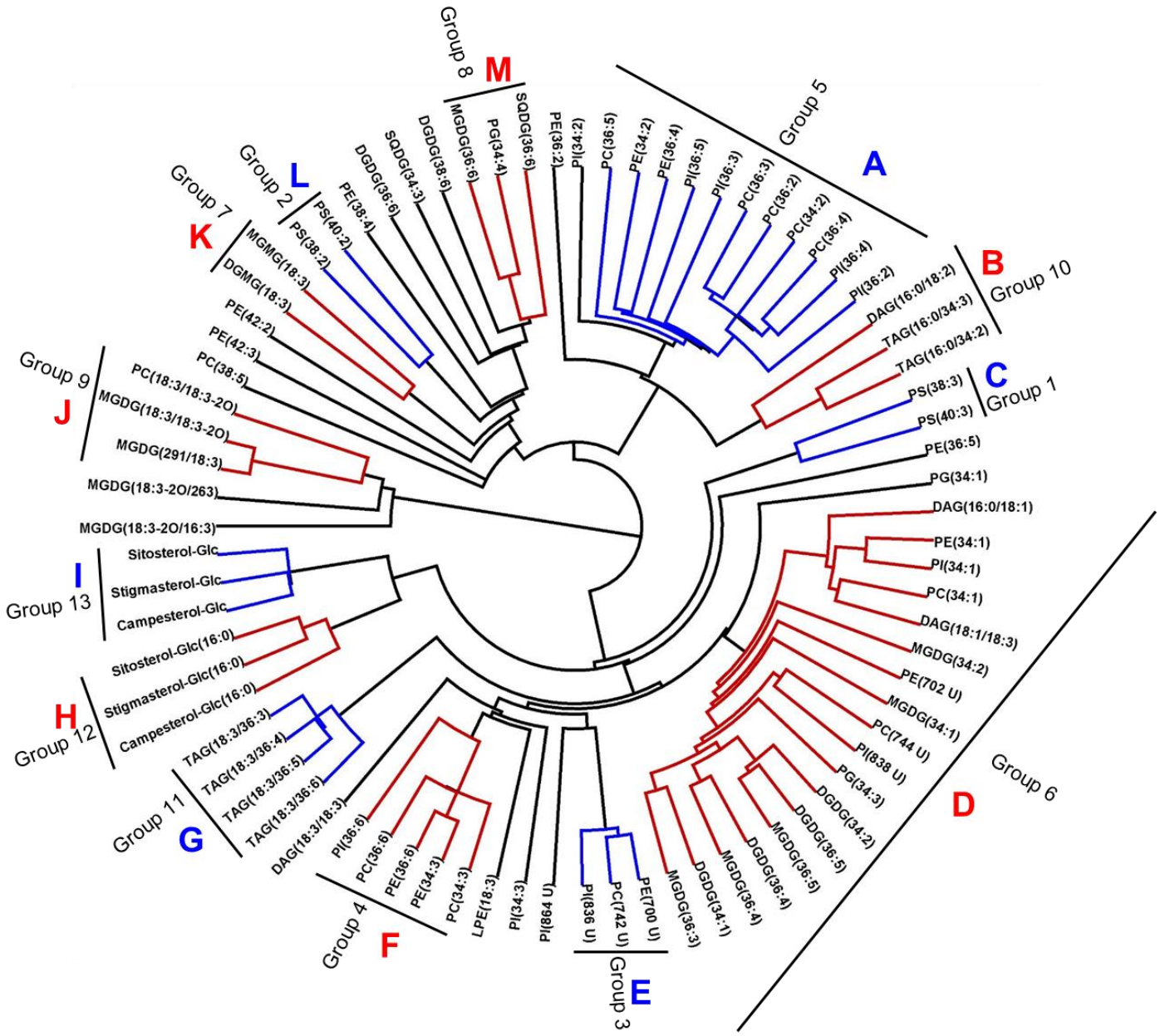
Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (M) Lipid group 11- TAGs and DAGs with an 18:3 acyl chain.



Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups (Continued on next page). (N) Lipid group 12- Acylated sterol glycosides.



Supplemental Figure 5.S2. The kinetic changes of individual lipid levels in the 13 lipid groups. (O) Lipid group 13- Sterol glycosides.



Supplemental Figure 5.S3. Dendrogram showing the correlation among lipids included in the groups in either Ventnor or Karl 92. This dendrogram was produced using pooled data across genotypes. Lipids were clustered using a single-linkage hierarchical algorithm based on Spearman's correlation coefficient (ρ) (Supplemental Table 5.S4). Thirteen clusters with $\rho \geq 0.8$ are indicated by letters A through M in red and blue colors. These clusters represent co-occurring lipid groups, which are marked as groups 1 through 13 on the dendrogram.

Chapter 6 - General Conclusions and Future Directions

General Conclusions

In this research, experiments were conducted with the objectives of quantifying the effects of high day and night temperatures following anthesis on physiological (chlorophyll fluorescence, chlorophyll concentration, leaf level photosynthesis, lipid peroxidation and membrane damage), biochemical (reactive oxygen species [ROS] concentration and antioxidant capacity in leaves), growth and yield traits and membrane lipid profile and identifying the lipids that are associated with high temperature response in wheat genotypes. Winter wheat genotypes Ventnor (heat tolerant) and Karl 92 (heat susceptible) were grown at optimum temperatures (25/15°C, maximum/minimum) until the onset of anthesis. Thereafter, plants were exposed to high night (HN, 25/24°C), high day (HD, 35/15°C), high day and night (HDN, 35/24°C) or optimum temperatures.

Compared with optimum temperature, HN, HD and HDN increased ROS concentration, lipid peroxidation and membrane damage and decreased antioxidant capacity, photochemical efficiency, leaf level photosynthesis, seed set, grain number and grain yield per spike. Impact of HN and HD was similar on all traits, when stress was imposed for seven days. However, HDN had greater impacts on yield traits, compared to HN and HD. These results suggested that HN and HD following anthesis cause damage of a similar magnitude to winter wheat, if stress is imposed for a short term (seven days).

High day and night temperatures resulted in significant changes in the amount of plastidic and extra-plastidic lipid species and lipids with oxidized acyl chains (ox-lipids) in both genotypes. The decrease in lipid unsaturation levels of complex lipids at high temperatures was predominantly due to decrease in 18:3 fatty acid and increase in 18:1 and 16:0 fatty acids. However, the amounts of 18:3-acyl containing triacylglycerols (TAG) increased under high temperature stress, suggesting a possible role of TAGs in high temperature stress adaptation by sequestering the 18:3 fatty acids from membrane lipids. Ventnor had higher amounts of sterol glycosides (SG) and saturated species of acylated sterol glycosides (ASG) and lower amounts of ox-lipids at high temperatures than Karl 92, suggesting that SGs and ox-lipids may be potential biomarkers for heat tolerance and susceptibility, respectively, in wheat. Our results indicate that

compositional changes in lipid profile in response to high temperature stress contribute to heat tolerance in wheat.

Correlation analyses revealed co-occurring lipid groups, which are up-or-down-regulated together through time under high day and night temperatures. The lipid groups were broadly classified into five categories; (1) groups containing extraplastidic phospholipids, (2) plastidic glycerolipids, (3) oxidized glycerolipids, (4) TAGs and diacylglycerols and (5) ASGs and SGs. Lipid co-occurrence in groups was primarily the result of desaturating, oxidizing, glycosylating and acylating activities of enzymes acting on lipids under high temperature stress. Interpretation of lipid groups based on the current knowledge on lipid metabolism suggests that the lipid groups reflect metabolic relationships as the co-occurring lipids in each group are acted on by the same enzyme(s). These results indicate that lipid groups experiencing coordinated metabolism can be detected by analysis of lipid co-occurrence under high day and night temperature stress in wheat.

Future Directions

- The high temperature regimes used in this research were 25/24°C (HN), 35/15°C (HD) and 35/24°C (HDN). Therefore, the high temperature effects quantified by this research refer to 10 and 9°C elevations for day maximum and night minimum temperatures, respectively, compared with an optimum temperature of 25/15°C. Further research is needed to evaluate the results over a wide range of day and night temperatures.

- The day/night temperatures for HN was 25/24°C in the current research. This means that the difference between day and night temperatures (diurnal temperature range) was 1°C. Further studies are required to quantify the impact of HN on wheat using wider diurnal temperature range.

- The diurnal temperature range for HN, HD and HDN was 1, 20 and 11°C, respectively in the current research. Therefore, the effects of HN observed on wheat genotypes may be a combined effect of a narrow diurnal temperature range (difference between the maximum and minimum temperature during a day) and the increase in minimum temperature. Further research is needed to separate the effects of these two stresses.

- In the current research, we imposed high temperature stress following flowering. Further research is needed to quantify the impact of stress during other growth stages of wheat such as prior-to-flowering or grain filling stages.

- We used only two genotypes in our study (a heat tolerant genotype and a heat susceptible genotype). Therefore, our conclusions are based only on these two genotypes. Further research is needed involving several genotypes with varying degrees of heat tolerance to validate our conclusions.

- Our conclusions are based only on the vegetative tissues since we did not evaluate the impacts of high temperature stress on pollen and ovule. However, reproductive tissues are more susceptible to high temperature stress than vegetative tissues. Therefore, further research is needed to quantify the impact of high temperature stress on physiological, biochemical and anatomical traits of pollen and to identify the changes in pollen lipid

profile in response to high temperature stress in heat tolerant and susceptible wheat genotypes.

- Further research is needed to identify genes or regulatory elements associated with high temperature tolerance in wheat genotypes.
- In the present research, we identified certain traits associated with high temperature tolerance. These traits could be utilized for targeted breeding for high temperature tolerance in wheat.