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Wheat leaf lipids during heat stress: I. High day and night temperatures result in major lipid alterations

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Abstract

Understanding how wheat (*Triticum aestivum* L.) plants under high temperature (HT) regulate lipid composition is critical to developing climate-resilient varieties. We measured 165 glycerolipids and sterol derivatives under optimum and high day and night temperatures in wheat leaves using electrospray ionization-tandem mass spectrometry. Levels of polar lipid fatty acyl chain unsaturation were lower in both heat-tolerant genotype Ventnor and susceptible genotype Karl 92 under HT, compared to optimum temperature. The lower unsaturation was predominantly due to lower levels of 18:3 and higher levels of 18:1 and 16:0 acyl chains. Levels of 18:3-containing triacylglycerols increased 3-fold/more under HT, consistent with their possible role in sequestering fatty acids during membrane lipid remodeling. Phospholipids containing odd-numbered or oxidized acyl chains accumulated in leaves under HT. Sterol glycosides (SG) and 16:0-acylated sterol glycosides (ASG) were higher under HT than optimum temperatures. Ventnor had lower amounts of phospholipids with oxidized acyl chains under HT and higher amounts of SG and 16:0-ASG than Karl 92. Taken together, the data demonstrate that wheat leaf lipid composition is altered by HT, that some lipids are particularly responsive to HT, and that two wheat genotypes, chosen for their differing physiological responses to HT, differ in lipid profile under HT.

Keywords

wheat; high day temperature; high night temperature; direct infusion automated electrospray ionization-tandem mass spectrometry; lipid unsaturation; lipid remodeling; triacylglycerols; sterol glycosides; ox-lipids; odd-chain lipids

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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 temperature 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 the 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 as much as 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 in order 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 and floret fertility are well documented (Prasad and Djanaguiraman, 2014; Narayanan et al., 2015), and membranes are among the major targets of high temperature stress. 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). High day and night temperatures increase reactive oxygen species (ROS) content in wheat leaves (Narayanan et al., 2015), leading to peroxidation of lipids, resulting in the production of malondialdehyde (MDA) (Bowler et al., 1992; Liu and Huang, 2000; Mène-Saffrané et al., 2009; Ayala et al., 2014). Both ROS and MDA are recognized as toxic by-products of aerobic metabolism and, at the same time, as mediators of signaling stress response. The thylakoid membranes, which are the sites of light-dependent reactions of photosynthesis, are particularly sensitive to high temperature stress (Allen and Forsberg, 2001; Sharkey, 2005). Recently, Vu et al. (2012, 2014) found that mechanical, biotic, and low temperature stresses led to increases in many membrane lipids with oxidized acyl chains (ox-lipids) in *Arabidopsis thaliana*. Ox-lipids may be produced enzymatically through the action of lipoxygenase as well as non-enzymatically through the action of ROS (Zoeller et al., 2012). Like ROS and MDA, ox-lipids may act as signaling molecules that initiate stress responses in plants (Andersson et al., 2006).

In addition to acyl oxidation, plants regulate other aspects of membrane lipid composition in response to changes in temperature, in order to maintain optimum fluidity and integrity of membranes (Larkindale and Huang, 2004; Chen et al., 2006; Zheng et al., 2011). It is well established that plants decrease lipid unsaturation levels at high temperatures and increase them at low temperatures to maintain optimal membrane fluidity (Larkindale and Huang, 2004; Chen et al., 2006; Welti et al., 2002, 2007). Additionally, plants regulate the ratio of bilayer to non-bilayer-forming lipids to maintain a stable membrane as the temperature changes (Chen et al., 2006; Webb and Green, 1991; Welti et al., 2007).

Currently, roles of the major phospholipids and galactolipids, such as maintaining membrane stability, have been partially elucidated, but the biological significance of other plant lipids,

such as triacylglycerols (TAG), sterol derivatives (sterol glycoside [SG] and acylated sterol glycoside [ASG]), and specific ox-lipids is just beginning to be uncovered. In the current work, we take advantage of an automated direct infusion electrospray ionization-triple quadrupole mass spectrometry (ESI-MS/MS) approach to quantitatively profile a wide range of plant lipid molecular species from wheat leaves under high day and night temperature stresses. 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, in terms of yield and physiological traits, and to identify corresponding lipid changes, including those that may be related to the high temperature tolerance or susceptibility of the wheat genotypes.

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, Hummert International, Topeka, KS, USA) was fertilized with Osmocote, a controlled-release fertilizer with 19:6:12 N:P₂O₅:K₂O, 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: optimum temperature (OT; 25/15°C, maximum/minimum; Al-Khatib and Paulsen, 1984), high night temperature (HN; 25/24°C; Prasad et al., 2008), high day temperature (HD; 35/15°C; Al-Khatib and Paulsen, 1984), and high day and night temperature (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 Supporting Information Fig. 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 reactive species and MDA contents. The remaining five plants were used to collect leaf samples for lipid extraction. For each measurement, five plants were individual replicates and the final number of samples (5 plants x 2 experiments) was n=10 for each genotype. 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 (Supporting Information Fig. 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, Bourne, MA, USA). The photoperiod was 16 h, and photon flux density (400 to 700 nm) provided by cool fluorescent lamps was about $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 15 cm away from the lamps and $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ 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 on the 12th day of stress (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, Hudson, NH, USA). The minimum fluorescence (F_o) and maximum fluorescence (F_m) were measured on 60-min dark-adapted flag leaves. Thylakoid membrane damage was estimated as the ratio of F_o to F_m (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 daytime growth temperature and ambient CO_2 conditions ($390 \mu\text{mol mol}^{-1}$). The internal light-emitting diode (LED, the light source) in the LI-COR 6400 was set at $1600 \mu\text{mol m}^{-2} \text{s}^{-1}$ to have a constant and uniform light across all measurements.

Reactive species (ROS and reactive nitrogen species [RNS]) content was estimated using an OxiSelect in vitro ROS/RNS assay kit (Cell Biolabs, San Diego, CA, USA). This assay is based on a proprietary quenched, ROS/RNS-specific fluorogenic probe, dichlorodihydrofluorescein 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 reactive species content within the sample (Wang and Joseph, 1999). To estimate reactive species content, leaf samples (~ 50 mg) were collected from flag leaves of five plants per genotype from each temperature regime on the 12th day of stress and stored at -80°C until processing. Weighed leaf samples were pulverized by grinding using a Retsch Mixer Mill MM 400 (Verder Scientific Inc., Newtown, PA, USA) for 2 min at 30 Hz. The pulverized leaf tissues were used for the estimation of reactive species 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 96-well microplate and incubated for 5 min at room temperature with a catalyst (1X). Freshly prepared DCFH

solution (100 μ L) 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, Waltham, MA, USA). The reactive species content of samples was determined by comparison with the predetermined DCF standard curve and expressed as nmol DCF per g of leaf tissue (fresh weight basis).

Malondialdehyde content in leaf samples was measured using an OxiSelect thiobarbituric acid reactive substances (TBARS) assay kit (Cell Biolabs, San Diego, CA, USA) 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 MDA:TBA (1:2) 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 on the 12th day of stress and stored at -80°C until processing. Weighed leaf samples were pulverized by grinding using a Retsch Mixer Mill MM 400 (Verder Scientific Inc., Newtown, PA, USA) 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 μ L of 100X butylated hydroxytoluene (BHT), and centrifuged at 10000 g for 5 min. The supernatant (100 μ L) was incubated with 100 μ L of sodium dodecyl sulfate lysis solution for 5 min at room temperature. Thiobarbituric acid (250 μ L) 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 μ L) was transferred to a 96-well microplate, and the absorbance was read at 532 nm using an Epoch spectrophotometer (BioTek, Winooski, VT, USA). The MDA content of samples was determined by comparison with a predetermined MDA standard curve and expressed as μ mol 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 on the 12th day of stress. At sampling, the middle one third of flag leaves was 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, Inc., Waltham, MA, USA). 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., Berlin, MA, USA). 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, Mettler Toledo International, Inc., Columbus, OH, USA), 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 phosphatidylcholine (PC) (di12:0), 0.6 nmol PC(di24:1), 0.6 nmol lysophosphatidylcholine (LPC) (13:0), 0.6 nmol LPC(19:0), 0.3 nmol phosphatidylethanolamine (PE) (di12:0), 0.3 nmol PE (di23:0), 0.3 nmol lysophosphatidylethanolamine (LPE) (14:0), 0.3 nmol LPE(18:0), 0.3 nmol phosphatidylglycerol (PG) (di14:0), 0.3 nmol PG (di20:0(phytanoyl)), 0.3 nmol lysophosphatidylglycerol (LPG) (14:0), 0.3 nmol LPG(18:0), 0.23 nmol phosphatidylinositol (PI) (16:0–18:0), 0.16 nmol PI(di18:0), 0.2 nmol phosphatidylserine (PS) (di14:0), 0.2 nmol PS(di20:0(phytanoyl)), 0.3 nmol phosphatidic acid (PA) (di14:0), 0.3 nmol PA(di20:0(phytanoyl)), 0.31 nmol TAG(tri17:1), 0.36 nmol digalactosyldiacylglycerol (DGDG) (16:0–18:0), 0.95 nmol DGDG(di18:0), 1.51 nmol monogalactosyldiacylglycerol (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, Framingham, MA, USA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 $\mu\text{L min}^{-1}$.

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 or acyl fragment. The scan mode and adduct used to detect the lipid species are given in Supporting Information Table S1. 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 Supporting Information Table 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 was 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 Supporting Information Table 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, 300:665:35, v / v / v) in 1.4 mL. The QC mass spectrometry samples were stored at -80°C and brought to room temperature 1 h before analysis. Each set of 80 analytical samples and 20 QC samples was arranged in VT 54 racks as shown in Supporting Information Table 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 (Supporting Information Table 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 CoV (standard deviation divided by mean of the amount of the analyte in the QC samples; Supporting Information Table S3) was greater than 30% (107 analytes) were removed from the data, except we retained PA(36:6) (CoV of 0.32) and PA(36:5) (CoV of 0.34). These two lipid species were retained in the data because they were present in relatively large amounts in samples and are potentially metabolically important. 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 correction. After calculation of the lipid levels in each sample, the values were divided by the fraction of the original sample that was analyzed and the dry weight of the original sample to present 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 nmol 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 normalized 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 the greater the unsaturation index, the greater is the unsaturation of that lipid. The unsaturation index of each lipid molecular species was calculated as the product of the 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 the 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. Treatment means were compared using least significant difference (LSD) ($P < 0.05$). A supervised classification method,

Random Forests (Breimen, 2001), offered by MetaboAnalyst (metabolanalyst.ca; Xia et al., 2009), was used to identify the most important lipid species that differentiate the three high temperature regimes (HN, HD, and HDN) from OT, and that differentiate the two genotypes from each other.

RESULTS

High day and night temperatures affect yield and physiological traits

Genotypes Ventnor and Karl 92 were grown at OT (25/15°C) 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. 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. 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. 2A). Photosynthesis decreased at HN, HD, and HDN in both genotypes (Fig. 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 (ROS and reactive nitrogen species [RNS]) content and MDA content increased to a similar extent under HN, HD, and HDN in Karl 92 (Fig. 2, C and D). High temperature did not affect reactive species and MDA contents of Ventnor, indicating that this genotype showed tolerance to high temperature stress in terms of reactive species and MDA contents.

ESI-MS/MS profiling and quantification of plant lipids

An ESI-MS/MS approach was used for lipid profiling. For lipid extraction, leaf samples were collected from five plants per genotype from each temperature regime on the 12th day of stress or at the same time point for plants at optimum temperature. 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 (Supporting Information Table 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 (QC) samples were prepared by pooling

an aliquot from each leaf sample, and were analyzed recurrently among the experimental samples (Supporting Information Table S2). The intensity of each lipid species in the experimental samples was normalized using the QC analyte intensities, as described in “Materials and Methods”. Lipid analytes in the QC samples in which the CoV was greater than 30% (107 analytes) 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 165 retained lipid analytes from 16.1% (before QC correction) to 13.7% (after QC correction) (Supporting Information Table S3). Data are presented as normalized intensity per mg of leaf dry weight (Supporting Information Table S4).

High temperature alters lipid composition and unsaturation levels in wheat

The total amount of DGDG, MGDG, PG, SQDG, PC, and PE generally decreased under high temperature stress, whereas the total amount of ASG, SG, TAG, and ox-lipids generally increased under high temperature stress in both genotypes (Fig. 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 (PG and SQDG); (2) some also decreased at high temperatures but decreased more in Ventnor than in Karl 92 (PC and PE); (3) some increased at high temperatures and increased more in Karl 92 than in Ventnor (ox-lipids); and (4) some increased at high temperatures but increased more in Ventnor than in Karl 92 (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 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. 3). The percentage changes under HD and HDN were larger for ASG (16 and 28% in Karl 92 and 54 and 113% in Ventnor, respectively), SG (74 and 129% in Karl 92 and 68 and 123% in Ventnor, respectively), TAG (200 and 98% in Karl 92 and 205 and 196% in Ventnor, respectively), and ox-lipids (92 and 126% in Karl 92 and 67 and 108% in Ventnor, 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.

High temperature resulted in significant changes in the diacyl lipid species composition of plastidic classes DGDG, MGDG, PG, and SQDG (Fig. 4) and extraplastidic classes phosphatidylinositol (PI), PE, and PC (Fig. 5). 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 containing two polyunsaturated acyl chains, such as 36:5- (which is an 18:2/18:3 combination, based on Table 1 of Devaiah et al., 2006) or 36:6- (which is a di18:3 combination) DGDG, MGDG, PG, SQDG, PE, and PC, decreased at HN, HD, and HDN (Figs. 4 and 5). On the other hand, the amount of less unsaturated species, such as 34:1- (largely 16:0/18:1) MGDG, PG, PI, PE, and PC increased at HN, HD, and HDN (Figs. 4 and 5). 34:1-DGDG, MGDG, PI, PE, and PC 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) 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. 6). However, 36:4- (likely 18:3/18:1) DGDG increased at HD and HDN in both genotypes, the relevance of which cannot be deduced from the present data.

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. 7). 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 increases the amounts of phospholipids containing acyl chains with odd numbers of carbons

ESI-MS/MS revealed unannotated phospholipid species which were highly responsive to high temperature stress in wheat plants. These phospholipid species were subjected to product ion analysis using quadrupole time-of-flight mass spectrometry (Q-TOF MS) to determine the formula of the acyl chains, as described by Vu et al. (2012). Q-TOF MS analysis identified the fatty acids in the phospholipids as having odd numbers of carbons (Supporting Information Table S5). PC, PE, PI, and PG lipids containing 33:3, 33:2, 35:4, 35:3, and/or 35:2 acyl chains were identified. In general, the odd fatty acyl chain components were 15:0, 17:0, 17:1, and 17:2. The amounts of phospholipids containing acyl chains with odd numbers of carbons increased under high temperature stress in both genotypes (Fig. 8).

Sterol derivatives respond significantly to high temperature stress in wheat

ASG and SG responded significantly to high temperature stress in plants (Fig. 9). Sitosterol, campesterol, and stigmasterol derivatives were found in the wheat genotypes. Palmitic acid (16:0) and linolenic acid (18:3) were detected in ASG. ASG containing 16:0 [Campesterol-Glc(16:0), Stigmasterol-Glc(16:0), and Sitosterol-Glc(16:0)] increased under HD and HDN treatments in both genotypes (Fig. 9, 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. 9, 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 three species of SG at optimum temperature as well as at 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. 10). The 18-carbon oxidized acyl chain in these lipids is abbreviated as 18:3-2O, to indicate three double bond equivalents and two oxygen atoms beyond the carbonyl group. “18:3-2O” is consistent with a hydroperoxide or a ketol derived from 18:3. 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 the oxidized chain, 18:3-2O.

High temperatures, HD and HDN, significantly increased all the 18:3-2O-containing species of PE and PC in Karl 92 (Fig. 10). 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 reactive species content reported in Fig. 2C (increased reactive species content at high temperatures in Karl 92, while Ventnor maintained normal levels of reactive species at high temperatures). Taken together, these results are consistent with the possibility that reactive species-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 of 165 lipid species levels. Random Forests identified 50 lipid species, based on their ability to differentiate OT from HN, HD, or HDN (data not shown); a subset including lipid species that differentiated all three high temperature regimes from OT is given in Table 1. This subset includes plastidic and extraplastidic diacyl species with 18:3, 18:1, and/or 16:0 acyl chains. Among them, lipid species with 18:3 acyl chains (36:6 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 under 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 (Figs. 1 and 2). 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 than in Ventnor. This suggests that Ventnor has greater tolerance to high temperature stress than Karl 92. In addition, reactive species and MDA contents significantly increased under HN, HD, and HDN in Karl 92, but did not show any change in Ventnor. Malondialdehyde (MDA), an end product of lipid peroxidation that mainly occurs through the action of reactive species, 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. In addition, Karl 92 showed inferior performance, compared to Ventnor, in terms of all yield traits except individual grain weight (Fig. 1) under all four temperature regimes. Collectively, these results indicate that Karl 92 would not be a good choice for heat-stress-prone areas unless it is chosen for its disease resistance, hard wheat milling, and bread-making qualities (Sears et al., 1997).

High temperature caused significant changes in the lipid profiles of wheat genotypes. Although, as documented, some damage had occurred by the 12th day of stress, both genotypes tried to adapt to high temperature stress by lipid remodeling and decreasing the level of lipid unsaturation. Lipid remodeling refers to decreases in the amounts of certain lipids and increases in others (Zheng et al., 2011). For example, the amounts of plastidic glycolipids (DGDG, MGDG, and SQDG), plastidic phospholipids (PG), and extraplastidic phospholipids (PC and PE) decreased under high temperature stress, while the amounts of sterol lipids (SG and ASG), 18:3-acyl-containing TAGs, and ox-lipids increased (Fig. 3). Lipid remodeling is likely to prevent the phase transition of membranes from a liquid crystalline phase to a 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; Vikström 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 (Supporting Information Fig. S2) to maintain membrane fluidity, presumably avoiding high-temperature-induced non-bilayer phase formation. However, both genotypes underwent a decrease in PG and SQDG (Fig. 3), 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. 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. 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. 6). 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, 5, 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. 7) suggest that these TAGs might have been synthesized from the 18:3 fatty acids released from membrane lipids. Hernández et al. (2012) reported that a soluble diacylglycerol acyltransferase (*DGAT3*) is responsible for recycling 18:3 fatty acids into TAGs. Fan et al. (2013) reported that *DGAT1* and phospholipid:diacylglycerol acyltransferase (*PDAT1*) are involved in TAG synthesis; however, *PDAT1* is more important than *DGAT1* in growing leaves, whereas the opposite is the case in senescing leaves. In addition, it has been reported that the expression of *PDAT* and *DGAT* is increased under heat stress (Higashi et al., 2015). 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).

Perhaps unexpectedly, because wheat is an “18:3 plant” and is not known to produce 16:3 through plastidic glycerolipid metabolism (Heinz and Roughan, 1983), very low levels of MGDG(34:6), which is known to be MGDG(18:3/16:3) in “16:3 plants”, were detected by the ESI-MS/MS analysis (Fig. 4; Supporting Information Table S4). The very low levels (approximately 1/10,000th of the levels of MGDG(36:6)) did not allow us to confirm the fatty acyl composition of this lipid. One possible explanation is that a low level of 16:3 is produced due to the ability of a 16-carbon substrate to be desaturated to 16:3 by the enzymes that predominantly act on 18-carbon substrates in the plastid and endoplasmic reticulum.

Precursor scanning by ESI-MS/MS and product ion analysis by Q-TOF MS revealed phospholipids containing 15:0, 17:0, 17:1, or 17:2 acyl chains; these increased under high temperature stress in wheat plants (Fig. 8; Supporting Information Table S5). Odd-chain lipids have been reported in humans, animals, and microorganisms, and very rarely in plants (Sperl et al., 2000; ezanka 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 the biosynthesis of odd-chain fatty acids, and excess propionyl-CoA leads to enhanced synthesis of 15- and 17-carbon fatty acids in humans. Further, odd-chain fatty acids were reported as biomarkers for coronary heart disease risk and type II diabetes mellitus risk in humans (Jenkins et al., 2015). In fungi, odd-chain fatty acids, such as 15:0, 17:0, and 17:1, were produced under alcoholic and hypoxic stress conditions (Jeennor et al., 2006). Taken together, these reports lead to the hypothesis that the increased formation of phospholipids with odd-chain fatty acyl components under high temperature stress might be an indication of the extent of stress damage. The incorporation of the three-carbon species (propionic acid) into fatty acids could be regulated by altered enzymatic specificity as a function of temperature.

Compared to the heat-susceptible genotype Karl 92, the heat-tolerant genotype Ventnor may be more efficient in lipid remodeling to maintain membrane bilayer structure under 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. 9). 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 in wheat. Ventnor had greater amounts of all the SG species than Karl 92 at HN, HD, and HDN (Fig. 9, C and D). The total amounts of SGs were 23, 28 and 29% greater in Ventnor, compared to Karl 92 at HN, HD, and HDN, respectively (Fig. 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 three SG species were ranked among the top 50 at HD and HDN (Supporting Information Table S6). Thus, our results suggest that SGs could be potential biomarkers for heat tolerance (under HN, HD, and HDN) 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 results in 'ox-lipid signatures' in plants that reflect their physiological status. For example, high temperature caused oxidation of lipids originating in the endoplasmic reticulum (ox-PCs and ox-PEs) (Fig. 10). The nonenzymatic oxidation of membrane lipids is thought to prevent oxidative damage that could happen elsewhere in the cell (Mène-Saffrané et al., 2009). In the present study, the oxidized fatty acid species in the detected ox-lipids was 18:3, a 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 (Mène-Saffrané 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 (Mène-Saffrané 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. 10). 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 in Ventnor at HD and HDN (Fig. 10). The total amounts of ox-lipids were 57 and 55% greater in Karl 92, compared to Ventnor at HD and HDN, respectively (Fig. 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 reactive species, MDA (Fig. 2), and ox-lipid (Fig. 10) contents of the susceptible genotype Karl 92 at HD and HDN suggest 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 the 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. 3–10).

This is consistent with the effects of HN, HD, and HDN on yield and physiological traits (Figs. 1 and 2). Interestingly, SGs showed similar response to HN and HD (Fig. 9). 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 profiles 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 and increases in the amounts of less unsaturated lipid species, SGs, and ASGs with 16:0 acyl chains. The lower level of lipid unsaturation under high temperature stress was predominantly due to lower levels of 18:3 and higher levels of 18:1 and 16:0 fatty acyl chains. 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 phospholipids containing acyl chains with odd numbers of carbons in both genotypes, suggesting the importance of including these lipids in 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. It will be interesting to examine additional wheat genotypes to determine whether these lipids may serve as biomarkers for heat tolerance and susceptibility in wheat.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY

Understanding how wheat plants under high temperature regulate lipid composition is critical to developing climate-resilient varieties. Lipid remodeling under high temperature stress in heat-tolerant and susceptible genotypes resulted in decreases in the amounts of more unsaturated lipid species and increases in the amounts of less unsaturated lipid species, sterol glycosides, 16:0-acylated sterol glycosides, 18:3-acyl-containing triacylglycerols, and phospholipids containing odd-numbered or oxidized acyl chains. The two analyzed wheat genotypes, chosen for their differing physiological responses to high temperature, differed in lipid profiles under high temperature.

Figure 1.

Effects of temperature 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 $\pm$ SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (LSD) test at $P < 0.05$. Percentage decreases in various traits due to high temperature as compared with optimum temperature are indicated on the bars. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 2.

Effects of temperature on physiological traits of wheat genotypes Karl 92 and Ventnor. Values shown are mean $\pm$ SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (LSD) test at $P < 0.05$. Percentage changes in various traits under high temperature stress as compared with optimum temperature are indicated on the bars. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high

day and night temperature; Fo, minimum fluorescence; Fm, maximum fluorescence; DCF, 2', 7'-dichlorodihydrofluorescein; FW, fresh weight.

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Figure 3.

Effects of temperature on total amount of lipids in various head group classes of wheat genotypes Karl 92 and Ventnor. Values shown are mean $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 4.

Effects of temperature on plastidic diacyl lipid molecular species of wheat genotypes Karl 92 (A, C, E, and G) and Ventnor (B, D, F, and H). Values shown are mean $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 5.

Effects of temperature on extraplastidic diacyl lipid molecular species of wheat genotypes Karl 92 (A, C, and E) and Ventnor (B, D, and F). Values shown are mean $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 6.

Effects of temperature on unsaturation index of various lipid classes of wheat genotypes Karl 92 and Ventnor. The unsaturation index of each lipid molecular species was calculated as the product of the 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 the 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 $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least

significant difference (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. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 7.

Effects of temperature on triacylglycerols (TAG) of wheat genotypes Karl 92 and Ventnor. Values shown are mean $\pm$ SE; n=10 (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 8.

Effects of temperature on phospholipids containing odd-chain fatty acids in wheat genotypes Karl 92 and Ventnor. Values shown are mean $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 9.

Effects of temperature 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 $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

Figure 10.

Effects of temperature 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, 18:3-20 (three double bond equivalents and two oxygen atoms beyond the carbonyl group). Values shown are mean $\pm$ SE; $n=10$ (two experiments and five replications (plants) of each genotype). Means with different letters are significantly different according to the least significant difference (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. Breaks on the y axis indicate a

change in scale. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

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Table 1

Lipid species that differentiate high night (HN), high day (HD), and high day and night (HDN) temperature samples from optimum temperature (OT) samples. These 20 lipid species were identified by Random Forests, a supervised classification method.

Lipid species	Direction of significant changes ^a			Relative contribution ^b		
	HN	HD	HDN	HN	HD	HDN
DGDG(34:1)	Up	Up	Up	2	3	14
DGDG(34:2)	Up	Up	Up	31	16	9
DGDG(36:5)	Up	Up	Up	38	4	13
MGDG(34:1)	Up	Up	Up	16	45	20
MGDG(36:3)	Up	Up	Up	47	14	3
MGDG(36:5)	Up	Up	Up	21	10	4
PC(34:1)	Up	Up	Up	37	2	7
PC(33:3)	Up	Up	Up	20	5	33
PE(33:3)	Up	Up	Up	24	18	30
PE(33:2)	Up	Up	Up	49	15	39
PG(34:1)	Up	Up	Up	1	29	25
PI(34:1)	Up	Up	Up	7	7	6
PI(33:3)	Up	Up	Up	15	12	18
Stigmasterol-Glc	Up	Up	Up	3	28	10
MGDG(34:6)	Down	Down	Down	45	32	34
MGDG(36:6)	Down	Down	Down	12	24	16
PE(36:5)	Down	Down	Down	6	20	11
PE(42:2)	Down	Down	Down	5	17	17
PG(34:4)	Down	Down	Down	25	11	8
SQDG(36:6)	Down	Down	Down	28	1	1

^aIndicates whether the lipid species went up or down at HN, HD, and HDN, compared to OT.

^bIndicates ranks given by Random Forests showing the relative ability of lipids to differentiate HN, HD, and HDN from OT.