1-2018

Alterations in Wheat Pollen Lipidome during High Day and Night Temperature Stress

Sruthi Narayanan
Clemson University, skutty@clemson.edu

P.V. Vara Prasad
Kansas State University

Ruth Welti
Kansas State University

Title: Alterations in wheat pollen lipidome during high day and night temperature stress

Running Title: Heat induced alterations in wheat pollen lipidome

Authors: Sruthi Narayanan[1,2*], P.V. Vara Prasad[1*], Ruth Welti[3]

1. Department of Agronomy, 2004 Throckmorton Plant Sciences Center, Kansas State University, Manhattan, KS 66506, USA
2. Department of Plant and Environmental Sciences, 212 Biosystems Research Complex, Clemson University, Clemson, SC 29634, USA
3. Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, KS 66506, USA

*Correspondence: P.V. Vara Prasad. e-mail: vara@ksu.edu

Sruthi Narayanan.e-mail: skutty@clemson.edu

Summary Statement

Understanding the adaptive changes in wheat pollen lipidome during high temperature stress is critical to improving seed set and developing high temperature tolerant wheat varieties. We found that the most heat-responsive lipids in pollen were extraplastidic phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylserine (PS). Comparison of the present results on wheat pollen with results of our previous research on wheat leaves suggests that similar lipid changes contribute to high temperature adaptation in both leaves and pollen, though the lipidomes have inherently distinct compositions.

ABSTRACT

Understanding the adaptive changes in wheat pollen lipidome under high temperature (HT) stress is critical to improving seed set and developing HT tolerant wheat varieties. We measured 89 pollen lipid species under optimum and high day and/or night temperatures using electrospray ionization-tandem mass spectrometry in wheat plants. The pollen lipidome had a distinct composition compared to that of leaves. Unlike in leaves, 34:3 and 36:6 species dominated the composition of extraplastidic phospholipids in pollen under optimum and HT conditions. The most HT-responsive lipids were extraplastidic phospholipids, PC, PE, PI, PA, and PS. The unsaturation levels of the extraplastidic phospholipids decreased through the decreases in the levels of 18:3 and increases in the levels of 16:0, 18:0, 18:1, and 18:2 acyl chains. PC and PE were negatively correlated. Higher PC:PE at HT indicated possible PE-to-PC conversion, lower PE formation, or increased PE degradation, relative to PC. Correlation analysis revealed lipids experiencing coordinated metabolism under HT and confirmed the HT-responsiveness of extraplastidic phospholipids. Comparison of the present results on wheat pollen with results of our previous research on wheat leaves suggests that similar lipid changes contribute to HT adaptation in both leaves and pollen, though the lipidomes have inherently distinct compositions.

Key words: Pollen lipids; wheat; high temperature stress; direct infusion automated electrospray ionization tandem mass spectrometry; lipid remodeling; lipid unsaturation; extraplastidic phospholipids; phosphatidylcholine; phosphatidylethanolamine; lipid co-occurrence.

INTRODUCTION

High temperature is a major abiotic stress in wheat (*Triticum aestivum* L.) growing regions of the world. Lobell and Field (2007) reported that wheat yields decreased by 3.2 – 8.4 % for every 1°C increase in minimum and maximum temperatures from 1961 to 2002 around the globe. Controlled environmental research has shown that high night (HN), high day (HD), and high day and night (HDN) temperatures during flowering considerably decrease seed set (15 - 37 %), grain number (13 - 41 %) and grain yield (13 - 43 %) of wheat (Narayanan et al., 2015). The high temperature-induced yield decreases in wheat are 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). Tubiello et al. (2002) simulated the effects of climate change on crop production specifically in the U.S., and predicted that winter wheat production could be decreased by 30 – 40% in the rainfed areas of the U.S. due to climate change. Similarly, Asseng et al. (2015) reported that global wheat production will decrease by 6% for every further 1°C increase in global mean temperature, and will become more variable over time and space. In order to develop climate-resilient wheat varieties and to mitigate the impacts of climate change on wheat production, it is important to clearly elucidate the mechanisms of yield loss under high temperature stress.

Wheat yields are more sensitive to high temperature stress if the stress occurs during reproductive stages, compared to vegetative stages (Farooq et al., 2011). If high temperature occurs only during flowering, decreased grain yields are the result of decreased seed set since high temperature does not affect individual grain weight as stress is absent during grain filling (Narayanan et al., 2015). Seed set is primarily the result of floret fertility, which is defined by functionality of male and female gametes (pollen and ovule, respectively) (Prasad and

Djanaguiraman, 2014). Prasad and Djanaguiraman (2014) evaluated the effect of short episodes (5 days) of high temperature stress on wheat floret fertility between 15 days prior to flowering and 30 days after flowering, and identified that floret fertility showed maximum decrease when stress occurred 5 days prior to flowering and at the time of flowering. This period coincides with gamete formation and development, pollination, and fertilization in wheat (Saini and Aspinall, 1982). Pollen viability and performance are drastically affected if high temperature occurs during this highly sensitive period in wheat (Saini and Aspinall, 1982; Prasad and Djanaguiraman, 2014). Thus, reduced seed set in wheat due to high temperature stress can primarily be attributed to poor pollen performance, when stress occurs just before flowering or at the time of flowering.

Prasad and Djanaguiraman (2014) reported anatomical evidence explaining reasons for poor pollen performance under high temperature stress in wheat. For example, high temperature stress (35/25 °C) resulted in collapsed and desiccated pollen grains with deeply pitted and non-smooth surfaces. In addition, high temperature stress also decreased the number of pollen grains adhered to the stigmatic surface. Similarly, Saini and Aspinall (1982) and Saini et al. (1984) showed that high temperature causes structural abnormalities in wheat pollen, which lead to reduced pollen viability and performance. However, the metabolic changes leading to poor pollen performance are not clearly understood in wheat.

Only limited literature is available on metabolic changes leading to poor pollen performance in crops. Jain et al. (2007, 2010) reported that impaired sugar-starch metabolism in developing microspores leads to decreased pollen germination and viability in sorghum [*Sorghum bicolor* (L.) Moench]. Prasad and Djanaguiraman (2011) found that high temperature increases the amount of reactive oxygen species in pollen grains, leading to membrane damage, which results in reduced pollen viability and germination in the same species. Sakata et al.

(2010) reported that decreased endogenous levels of auxin in anther cells at high temperature cause impaired pollen development and pollen sterility in barley (*Hordeum vulgare* L.). Considering the unique lipidome that the pollen grains possess, Ischebeck (2016) reasoned that pollen lipid remodeling would be important for plants to cope with high temperature stress.

Recent studies have started investigating the role of lipid metabolic changes on pollen performance in crops. Prasad and Djanaguiraman (2011) found relatively increased phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and phosphatidylserine (PS) contents and decreased monogalactosyldiacylglycerol (MGDG), phosphatidylglycerol (PG), and phosphatidic acid (PA) contents associated with reduced pollen viability and germination under high temperature conditions in sorghum. Djanaguiraman et al. (2013) reported increased MGDG, digalactosyldiacylglycerol (DGDG), PC, phosphatidylinositol (PI), lysophosphatidylglycerol (LPG) levels and decreased PG and PA levels associated with reduced pollen viability and germination under high temperature conditions in soybean [*Glycine max* (L.) Merr.]. Potocky´ et al. (2003) found that PA produced by the action of phospholipase D is required for pollen tube growth in tobacco (*Nicotiana tabacum* L.).

Studies on Arabidopsis (*Arabidopsis thaliana*) reveal that silencing of phosphoethanolamine N-methyltransferase, a key enzyme involved in biosynthesis of PC, results in temperature-sensitive male sterility (Mou et al., 2002). Zienkiewicz et al. (2013) reported that phospholipase A (cleaves acyl side chains of phospholipids and releases lysophospholipids) and lipoxygenase (catalyzes fatty acid oxidation) are involved in oil body mobilization, which enables pollen germination in olive (*Olea europaea* L.). Lalanne et al. (2004) found that glycosylphosphatidylinositol (synthesized in the endoplasmic reticulum via the sequential

addition of monosaccharides, fatty acids, and phosphoethanolamines to PI) is required for pollen germination and tube growth in Arabidopsis. Zheng et al. (2003) found that membrane-bound glycerol-3-phosphate acyltransferase that mediates the initial step of glycerolipid biosynthesis in the extraplastidic compartments is essential for tapetum differentiation and pollen development in Arabidopsis. In spite of the importance of lipid metabolic changes and the enzymes involved in those pathways in pollen performance under optimum and high temperature stress conditions, no studies have investigated high temperature-induced perturbations in pollen lipidome and their role in high temperature adaptation of wheat plants.

In 2013-2014, we conducted a study, in which we grew plants of a heat tolerant wheat genotype Ventnor and a heat susceptible wheat genotype Karl 92 to evaluate the changes in the lipid profiles of leaf and pollen under high day and/or night temperatures and to relate that to the high temperature responses of these genotypes. The results of leaf lipid profiling are presented in Narayanan et al. (2016a, 2016b). The current paper is the third one in the series, and here we report the results of the pollen lipid profiling on the same plants, which were sampled for leaf lipid profiling. The objectives of the present research were to quantify the effects of high day and/or night temperatures during flowering on pollen lipid profile of wheat and identify the lipids that are associated with high temperature response of wheat plants. We hypothesized that the pollen lipidome will be altered by high temperature that some lipids will be particularly responsive to high temperature, and that some lipid responses will be associated with high temperature adaptation of wheat plants.

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. The experimental details and the plant material and growth conditions are described in Narayanan et al. (2016a). Briefly, wheat genotypes Ventnor (heat-tolerant) and Karl 92 (heat susceptible) were grown at optimum temperature conditions (OT; 25/15 °C, maximum/minimum) until the onset of flowering. At this point, the main spike (where flowering started) was tagged. 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, pollen grains were collected from five plants per genotype from each temperature regime. The five plants were individual biological replicates, and the final number of samples was n = 10 (5 plants × 2 experiments) for each genotype in each temperature regime.

Pollen collection and lipid extraction

Pollen grains were collected from the tagged main spike on each plant between 07:30 and 09:30 h on day 3 of stress or at the same time point for plants at OT. At sampling, pollen grains (0.3-6 mg dry weight) were dusted into 3 mL of isopropanol with 0.01% butylated hydroxytoluene 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, 1.5 mL of chloroform and 0.6 mL of water were added, and samples were stored at -80 °C until analysis. The lipid extraction procedure was carried out as described by Narayanan et al. (2016a) with slight modifications. Briefly, the lipid extract in isopropanol, butylated hydroxytoluene, chloroform, and water was shaken on an orbital shaker at room temperature for 1 h, centrifuged for 15 min (for pollen sedimentation), and transferred to a new glass tube using a Pasteur pipette, leaving the pollen grains at the bottom of the original tube. Four milliliters of chloroform:methanol (2:1) and 4 mL

of isopropanol (for pollen sedimentation) were added to the pollen grains, the samples were shaken on an orbital shaker at room temperature overnight, centrifuged for 15 min, and the solvent was transferred to the first extract. The addition, shaking (1 h), spinning, and transfer steps were performed four times until the pollen grains 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 pollen grains were dried in an oven at 105 °C overnight, cooled, and weighed to estimate the lipid content on a dry weight basis. Pollen 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 described by Vu et al. (2012).

Electrospray ionization-triple quadrupole mass spectrometry lipid profiling

An automated electrospray ionization-tandem mass spectrometry (ESI-MS/MS) approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Narayanan et al., 2016a). 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 [See Supporting Information Table S1 in Narayanan et al. (2016a)]. The goal of the quantification was to compare different pollen 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; Narayanan et al., 2016a). Quality control (QC) samples were prepared by pooling an aliquot from each pollen

sample and were analyzed recurrently among the experimental samples. The intensity of each lipid species in the experimental samples was normalized using the QC analyte intensities, as described in Narayanan et al. (2016a) (see 'Materials and Methods'). The lipid values were calculated as normalized intensity per mg pollen dry weight, where a value of one is the intensity of 1 nmol of internal standard. The lipid values were converted to percentage of total signal (as presented here), which was estimated as the signal for a lipid species x 100/total signal for that sample (Supporting Information Table S1). To maintain data quality, the following data were removed from the data set; (1) lipid analytes for which the amount (normalized mass spectral signal) per mg of pollen dry weight less than the limit of detection (LOD), (2) lipid analytes with coefficient of variation (CoV; standard deviation divided by mean of the amount of the analyte in the quality control samples) greater than 0.3, and (3) samples with total mass spectral signal less than 5 or greater than 100.

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 number of double bonds (degree of unsaturation) in 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 as the number of double bonds in the lipid molecular species divided 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; Narayanan et al., 2016a).

Production of heat maps and dendrograms

Utilities of the MetaboAnalyst web server (metabolanalyst.ca; Xia et al., 2009, 2012) were used to perform the autoscaling of lipid data (Supporting Information Table S2) and to produce the correlation tables (Supporting Information Tables S3 and S4) and heat maps. Autoscaling allows for easy comparison of lipid levels in different samples. The autoscaled value of a lipid in a sample is calculated as follows: [(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).

The lipid data of each genotype (Supporting Information Table S1) were uploaded to CLUSTER 3.0 (Open source software, Human Genome Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan) (Eisen et al., 1998) for determining lipid clusters. CLUSTER 3.0 generated lipid clusters for each genotype using a single-linkage hierarchical algorithm based on Spearman's correlation coefficient, ρ. The clustering output (.cdt file) of each genotype were viewed using 'Java Treeview' (Open source software, available at http://jtreeview.sourceforge.net) for identifying lipids with $\rho \geq 0.80$ with at least one other lipid. The data on these lipids (with $\rho \geq 0.80$ with at least one other lipid in either genotype) were again uploaded to CLUSTER 3.0, and the resulting output (.cdt file) for each genotype 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 files of each genotype were exported to Dendroscope (Huson et al., 2007; Huson and Scornavacca, 2012) to produce the dendrograms, which were modified in color.

Statistical analyses

The experimental design for plant husbandry 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 Ventnor and Karl 92). 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 9.4, 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) ($P<0.05$).

RESULTS

Electrospray ionization-triple quadrupole mass spectrometry profiling and quantification of wheat pollen lipids

The ESI-MS/MS approach quantified a total of 89 lipid analytes (Supporting Information Table S1). These included plastidic lipids [digalactosylmonoacylglycerol (DGMG), DGDG, monogalactosylmonoacylglycerol (MGMG), MGDG, PG, and sulfoquinovosyldiacylglycerol (SQDG)], extraplastidic phospholipids (PE, PC, PI, PA, and PS), lysolipids (LPG, LPC, and LPE), diacylglycerols (DAG), TAGs, and ox-lipids (lipids with oxidized acyl chains). Pollen lipid composition and lipid changes under high temperature stress are discussed in detail in the following sections. Since the composition and alteration of pollen lipidome were essentially the same for both the heat tolerant and susceptible genotypes, data for only the susceptible genotype are presented here; data for the tolerant genotype are given as Supporting Information Figs. S1-S6.

Composition of extraplastidic phospholipids in pollen differed from that in leaves

The composition of extraplastidic phospholipids in pollen was dominated by two species, 34:3 and 36:6, which contain linolenic acid (18:3) as 16:0/18:3 or as 18:3/18:3 (Fig.1a, c, e, g, and i). These species together made up >90, >85, and > 79% of total PC under OT and HN, HD, and HDN, respectively (Fig. 2). They were the predominant species in PE, PI, and PA, making up over 85% of these classes under optimum conditions and over 70% under high temperature stress conditions (Figs. 1 & 2). This is in contrast to the situation in leaves where 34:3 and 36:6 species made up <60% of each class for PC, PE, PI, and PA under any temperature regime (Figs. 1b, d, f, h, and j). Instead, leaf extraplastidic phospholipid compositions had large proportions of species containing linoleic acid (18:2), such as 34:2 (16:0/18:2), 36:5 (18:2/18:3), and 36:4 (likely primarily 18:2/18:2) (Figs. 1 & 2). Thus, wheat pollen grains contained the same extraplastidic phospholipid classes as leaves, but they had different compositions, compared to leaves, under optimum and high temperature stress conditions. Although the pollen lipids are somewhat altered in response to stress (Figs. 1 & 2 and following sections), the composition of the major extraplastidic phospholipids remain dominated by two molecular species. The following sections describe pollen lipid remodeling in wheat plants as they cope with high temperature stress.

High temperature alters composition and unsaturation levels of extraplastidic phospholipids in pollen

High day and/or night temperatures resulted in significant changes in the diacyl lipid species composition of extraplastidic classes PC, PE, PI, PA, and PS (Fig. 3). These are the phospholipids that are synthesized in the endoplasmic reticulum. High temperature caused decreases in the amounts of more unsaturated lipid species and increases in the amounts of less unsaturated lipid species. For example, lipid species containing two polyunsaturated acyl chains, such as 36:6- (which is a di18:3 combination) PC, PE, and PA, decreased at high temperatures

(Fig. 3). On the other hand, the amount of less unsaturated species, such as 34:1- (largely 16:0/18:1), 34:2- (largely 16:0/18:2), 36:4- (largely 18:2/18:2), 36:3- (largely 18:1/18:2), and 36:2- (18:1/18:1 or 18:0/18:2) PC, PE, and/or PI increased at high temperatures [Fig. 3; acyl composition is provided based on Table 1 of Devaiah et al. (2006)]. 34:2- PC had a threefold or greater increase at HD and HDN, compared with OT. In general, lipid species that had 18:3 acyl chains (e.g. 36:6 species) decreased during high-temperature stress, and lipid species that had 16:0, 18:0, 18:1, and/or 18:2 acyl chains (e.g. 34:1, 34:2, 36:4, 36:3, and 36:2 species) increased during high-temperature stress. This led to a decrease in unsaturation index of PE, PC, PI, PA, and PS at high-temperature stress (although the decrease was not statistically significant for PA and PS) (Fig. 4). Similar trends in the composition and unsaturation levels of extraplastidic phospholipids were noticed in the leaves of these plants (Narayanan et al., 2016a).

Interestingly, 34:3- [16:0/18:3; Devaiah et al. (2006)] PC, PI, and PS did not decrease under high temperature conditions (Fig. 3). This might be a reason for unsaturation index remaining high even when it decreased under high temperature stress conditions for these lipid classes (Fig. 4). Whether the 34:3 species contribute to any unique mechanism for high temperature adaptation in pollen grains needs to be investigated in future studies.

PC and PE species levels are negatively correlated

PC and PE species were negatively correlated in wheat pollen (Fig. 5a). In addition, a significant increase in PC:PE ratio was noticed at high temperatures (Fig. 5b). PC:PE ratio increased 96, 61, and 125 % at HN, HD, and HDN, respectively. Higher PC:PE ratios were also found in the leaves of these plants at high temperatures (Narayanan et al., 2016a).

Correlation analysis reveals co-occurring lipids under high temperature stress and confirms the high temperature responsiveness of extraplastidic phospholipids

Spearman's correlation coefficient (ρ), calculated among lipid analytes across all individual pollen samples (two experiments of five replicates each under four temperature regimes) revealed co-occurring lipids (lipids experiencing coordinated metabolism) under high temperature conditions. Spearman's correlation coefficient ranges from -1 (perfect negative correlation) to 1 (perfect positive correlation), and a value of zero indicates no correlation. Correlation analysis was performed on all 89 lipid analytes based on ρ (Supporting Information Table S3). A dendrogram was created by matching each lipid analyte with the one to which it was most highly correlated (Fig. 6; see Section on 'Materials and Methods'). The dendrogram includes groups of lipids (indicated by the red and blue bars on the dendrogram) in which every lipid is correlated with at least one other lipid within the group with $\rho \geq 0.85$. The arrows on the dendrogram indicate the directionality of differences in levels of each lipid under HDN compared with OT. The dendrogram of the wheat genotype Karl 92 included 10 lipid groups (Fig. 6). Each group comprises co-occurring lipids that are up-regulated or down-regulated together through time under various temperature treatments. The lipid groups detected in the present study mainly included ER synthesized phospholipids, which formed groups 1-7. This confirms that the pollen lipid changes, which are likely adaptive to the temperature increases, mainly included altered metabolism of ER synthesized phospholipids.

DISCUSSION

In the present study, we found that wheat pollen lipidome contained the same extraplastidic phospholipid classes as leaves, but their composition differed under optimum and high temperature stress conditions. Unlike in leaves, the composition of extraplastidic phospholipids in pollen was dominated by 34:3 and 36:6 species, which contain linolenic acid (18:3). This unique composition of the pollen lipidome might be important for the functioning of pollen

grains under optimum and stress conditions. Unlike in pollen, leaf extraplastidic phospholipid compositions had large proportions of 34:2, 36:5, and 36:4 species, which contain linoleic acid (18:2). Our results support Ischebeck (2016), who, after reviewing extensive data on the plant lipidome, commented that the pollen lipidome comprises the same lipid classes as other plant cells and tissues, but has different compositions, compared to other tissues.

The alterations in the composition and unsaturation levels of extraplastidic phospholipids in wheat pollen suggest that lipid remodeling and decreases in the level of lipid unsaturation are adaptive mechanisms in pollen under high temperature stress. However, some damage had occurred to pollen grains leading to their reduced performance, which was documented in terms of reduced seed set (Narayanan et al., 2016a). Lipid remodeling refers to decreases in the amounts of certain lipids and increases in others (Zheng et al., 2011). In the present study, the type of lipid remodeling, in terms of decreases in the amounts of extraplastidic phospholipids containing highly unsaturated acyl chains (e.g., 18:3) and increases in the amounts of extraplastidic phospholipids containing less unsaturated or saturated acyl chains (e.g., 16:0, 18:0, 18:1, and 18:2) (Fig.3), is consistent with the notion that it could prevent a phase transition of membranes from a liquid crystalline phase to a hexagonal II or cubic phase (corresponding to non-bilayer structure) at high temperatures.

Fatty acid double bonds largely have a *cis* configuration in plant lipids. The *cis* double bonds introduce bends in the fatty acid chains and thereby, reduce tight packing of adjacent lipid molecules. Decreasing the number of double bonds, and thus, the degree of unsaturation at high temperatures are adaptive mechanisms in plants to maintain the close packing of lipid molecules within the membranes, in order to maintain the optimal fluidity and integrity of membranes (Larkindale and Huang, 2004). In the present study, the degree of unsaturation of extraplastidic

phospholipids in wheat pollen decreased at high temperatures (Fig. 4). The decrease in unsaturation level was mainly due to the decrease in the polyunsaturated fatty acid, linolenic acid (18:3), and the increases in the less unsaturated fatty acids, oleic acid (18:1) and linoleic acid (18:2), and saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0) (Fig. 3). The same trend was also noticed in the leaves of these plants (Narayanan et al., 2016a). This suggests that similar lipid metabolic changes contributed to adaptive mechanisms under high temperature stress in wheat leaves and pollen.

In the present study, we found that PC and PE species were negatively correlated in wheat pollen such that a significant increase in PC:PE ratio was observed at high temperatures (Fig. 5). A larger PC:PE ratio at high temperatures indicates possible PE-to-PC conversion, reduced PE formation from PC, or PE degradation; the metabolic alterations are compensatory for the physical changes in membrane structure due to the temperature increases. Lipids such as PE tend to form hexagonal II phase or other non-bilayer phases, whereas PC forms bilayers (Seddon, 1990). Higher ratios of PC to PE reduce the propensity of membranes to form non-bilayer phases (Williams, 1998; de Vries et al., 2004). In the present study, increased PC:PE ratios in pollen grains at high temperatures would be consistent with maintenance of membrane fluidity, presumably avoiding high-temperature-induced non-bilayer phase formation. Higher PC:PE ratios were also found in the leaves of these plants at high temperatures (Narayanan et al., 2016a); again suggesting the same type of lipid remodeling as an adaptive mechanism under high temperature stress in wheat leaves and pollen.

The present study revealed lipid groups that included co-occurring lipids in pollen grains (Fig. 6). These lipids undergo co-metabolism and are up-regulated or down-regulated together through time under various temperature treatments. The strong metabolite correlations that form

363 the lipid groups could be attributed to strong mutual control by a single enzyme (Camacho et al.,
364 2005). Several lipid-metabolizing enzymes acting on multiple, related lipid substrates that
365 contain the same component acyl chain or head group are well known. Vu et al. (2014) reported
366 that co-occurring lipids could be the products of the same rate-limiting enzyme or downstream of
367 the rate-limiting enzyme(s) in a pathway. In the present study, the co-metabolism of the ER
368 synthesized phospholipids, which formed groups 1-7, comprises a possible upregulation of fatty
369 acid desaturase (FAD) 2 (which converts 18:1 PC to 18:2 PC), downregulation of FAD3 (which
370 converts 18:2 PC to 18:3 PC) and conversion of desaturated PCs to other phospholipids (such as
371 PI, PS, and PA).

372 The predominant appearance of PE and PC species in lipid groups (Fig. 6), negative
373 correlations among PE and PC molecular species (Fig. 5a), and the increased PC:PE ratios at
374 high temperatures (Fig. 5b), together indicate the role of PE and PC and PE-to-PC conversion,
375 reduced PE formation, or PE loss, relative to PC, in high temperature adaptive mechanisms in
376 pollen grains. The phospholipids, PC and PE, are abundant in plant tissues. PC accounts for 40 to
377 60% of lipids in extraplastidic membranes of plants (Moore, 1990; Bolognese and McGraw,
378 2000) and performs important functions required for reproductive success; e.g., diurnally
379 changing molecular species of PC bind with florigen FT protein to promote flowering
380 (Nakamura et al., 2014). PC can be synthesized in eukaryotic cells through the CDP-choline
381 pathway or phosphatidylethanolamine N-methyltransferase (PEMT) pathway. In the latter,
382 PEMT catalyzes three repeated methylation reactions with *S*-adenosylmethionine as the methyl
383 donor, converting PE to PC (Keogh et al., 2009). Another possible synthetic route is high
384 temperature induced activation of phospholipases, such as phospholipase C, which can convert
385 PE to DAG and phosphoethanolamine, and the subsequent trimethylation of

386 phosphoethanolamine to phosphocholine by *S*-adenosyl-L-methionine: phosphoethanolamine N-
387 methyltransferase (PMT) (Bolognese and McGraw, 2000). Thereafter, phosphocholine can be
388 converted to cytidine diphospho-choline by CTP:phosphorylcholine cytidylyltransferase
389 (Inatsugi et al., 2002), which is then incorporated into the DAG backbone by amino
390 alcohol:aminophosphotransferase to produce PC. Further research is needed to understand which
391 pathway and enzymes are activated under high temperature stress in pollen that results in co-
392 occurrence of PE and PC molecular species (lipid groups 1 and 2, respectively) and increased
393 PC:PE ratio.

394 Liu et al. (2015) reported that suppression of amino alcohol:aminophosphotransferases,
395 which catalyze the final step of PC synthesis resulted in pollen sterility in Arabidopsis. Mou et
396 al. (2002) reported that Arabidopsis plants in which the PMT gene is silenced failed to produce
397 functional pollen at high temperatures. This high temperature-induced male sterility is attributed
398 to defective biosynthesis of phosphocholine, and thus that of PC. This indicates a possible role of
399 PC in maintaining pollen performance under high temperature conditions. Thus, in the present
400 study, the increased co-metabolism of certain PC species (forming lipid group 2, Fig. 6) and the
401 increased PC:PE ratio at high temperatures might be adaptive mechanisms to minimize high
402 temperature induced pollen sterility.

403 **CONCLUSIONS**

404 Wheat pollen lipidome had a distinct composition compared to that of leaves. Unlike in leaves,
405 the composition of extraplastidic phospholipids in pollen was dominated by two species, 34:3
406 and 36:6, under optimum and high temperature stress conditions. High temperature stress altered
407 the composition and unsaturation levels of pollen lipids. Lipids that were most responsive to high
408 temperature stress were extraplastidic phospholipids (PC, PE, PI, PA, and PS). Lipid remodeling

and decreases in the level of lipid unsaturation were adaptive mechanisms in pollen grains under high temperature stress. The lipid remodeling included decreases in the amounts of more unsaturated extraplastidic phospholipids, and increases in the amounts of less unsaturated extraplastidic phospholipids. The lower unsaturation levels of extraplastidic phospholipids under high-temperature stress were predominantly due to lower levels of 18:3 fatty acyl chains and higher levels of 16:0, 18:0, 18:1, and 18:2 fatty acyl chains. Among extraplastidic phospholipids, PC and PE were particularly responsive to high temperature stress. PC and PE species had negative correlations. In addition, a significant increase in PC:PE ratio was noticed at high temperatures, indicating possible PE-to-PC conversion, lower PE formation, or increased PE degradation, relative to PC. In general, the changes in the extraplastidic phospholipid profile of wheat pollen were likely compensatory for the physical changes in membrane structure due to the temperature increases, i.e., to maintain membrane fluidity by avoiding high-temperature-induced non-bilayer phase formation. This could be an adaptive mechanism to prevent temperature-induced pollen sterility. Correlation analysis revealed co-occurring lipids (i.e., lipids experiencing coordinated metabolism) under high temperature stress and confirmed the high temperature responsiveness of extraplastidic phospholipids. Comparison of present results on wheat pollen with that of our previous research on wheat leaves suggests that similar lipid changes contribute to adaptive mechanism under high temperature stress in wheat leaves and pollen, though pollen and leaf lipidomes have inherently distinct compositions.

ACKNOWLEDGMENTS

We thank Triticeae Coordinated Agricultural Project Grant no. 2011–68002–30029 (Triticeae-CAP) from the USDA NIFA, United States Agency for International Development (USAID) Feed the Future Innovation Lab for Climate Resilient Wheat (Grant no. AID–0AAA–1300008),

and Kansas Wheat Alliance for financial support. We thank Prakarsh Tiwari, Predeesh Chandran and Aiswariya Deliephan for help in data collection. We are grateful to Mary Roth for excellent technical assistance with lipid profiling. The lipid analyses described in this work were performed at the Kansas Lipidomics Research Center Analytical Laboratory; instrument acquisition and lipidomics method development was supported by National Science Foundation (EPS 0236913, MCB 0920663, MCB 1413036, DBI 0521587, DBI 1228622), Kansas Technology Enterprise Corporation, K-IDeA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20GM103418), and Kansas State University. This publication is Contribution 18-127-J from the Kansas Agricultural Experiment Station. We declare that no conflict of interest exists regarding this paper.

REFERENCES

Asseng S., Ewert, F.W., Marte, P., Rotter, R.P., Lobell, D.B., Cammarano, D., …., Zhu, Y. (2015). Rising temperatures reduce global wheat production. *Nature Climate Change* 5, 143-147.

Bolognese C.P. & McGraw P. (2000) The isolation and characterization in yeast of a gene for Arabidopsis S-adenosylmethionine:phospho-ethanolamine N-methyltransferase. *Plant Physiology* 124, 1800–1813.

Camacho D., de la Fuente A. & Mendes P. (2005) The origin of correlations in metabolomics data. *Metabolomics* 1, 53–63.

Devaiah S.P., Roth M.R., Baughman E., Li M., Tamura P., Jeannotte R., …, Wang X. (2006) Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a PHOSPHOLIPASE Dα1 knockout mutant. *Phytochemistry* 67, 1907–1924.

Djanaguiraman M., Prasad P.V.V. & Schapaugh W.T. (2013) High day- or nighttime temperature alters leaf assimilation, reproductive success, and phosphatidic acid of pollen grain in soybean [*Glycine max* (L.) Merr.] *Crop Science* 53, 1594–1604.

Dunn W.B., Broadhurst D., Begley P., Zelena E., Francis-McIntyre S., Anderson N., …, Goodacre R. (2011) Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature Protocols* 6, 1060–1083.

Eisen M.B., Spellman P.T., Brown P.O. & Botstein D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America* 95, 14863–14868.

464 Farooq M., Bramley H., Palta J.A. & Siddique K.H.M. (2011) Heat stress in wheat during
465 reproductive and grain-filling phases. *Critical Reviews in Plant Sciences* 30, 491–507.
466 Hong M.Y., Chapkin R.S., Barhoumi R., Burghardt R.C., Turner N.D., Henderson C.E., …,
467 Lupton J.R. (2002) Fish oil increases mitochondrial phospholipid unsaturation,
468 upregulating reactive oxygen species and apoptosis in rat colonocytes. *Carcinogenesis*
469 23, 1919–1925.
470 Huson D.H., Richter D.C., Rausch C., Dezulian T., Franz M. & Rupp R. (2007) Dendroscope: an
471 interactive viewer for large phylogenetic trees. *BMC Bioinformatics* 8, 460.
472 Huson D.H. & Scornavacca C. (2012) Dendroscope 3: an interactive tool for rooted phylogenetic
473 trees and networks. *Systematic Biology* 61, 1061–1067.
474 Inatsugi R., Nakamura M. & Nishida I. (2002) Phosphatidylcholine biosynthesis at low
475 temperature: differential expression of CTP:phosphorylcholine cytidylyltransferase
476 isogenes in *Arabidopsis thaliana*. *Plant Cell Physiology* 43, 1342–1350.
477 Intergovernmental Panel on Climate Change (2013) *Intergovernmental Panel on Climate*
478 *Change Fifth Assessment Report: Climate Change 2013.*World Meteorological
479 Organization, Geneva, Switzerland.
480 Ischebeck T. (2016). Lipids in pollen - They are different. *Biochimica et Biophysica Acta* 1861,
481 1315–1328.
482 Jain M., Prasad P.V.V., Boote K.J., Allen Jr. L.H. & Chourey P.S. (2007) Effects of season-long
483 high temperature growth conditions on sugar-to-starch metabolism in developing
484 microspores of grain sorghum (*Sorghum bicolor* L. Moench). *Planta* 227, 67–79.
485 Jain M., Chourey P.S., Boote K.J. & Allen, Jr. L.H. (2010) Short-term high temperature growth
486 conditions during vegetative-to-reproductive phase transition irreversibly compromise

cell wall invertase mediated sucrose catalysis and microspore meiosis in grain sorghum. *Journal of Plant Physiology* 16, 578–582.

Keogh M.R., Polly D.C., Anthony J.K. & Ralph E.D. (2009) Functional characterization of phospholipid N-methyltransferases from Arabidopsis and soybean. *The Journal of Biological Chemistry* 284, 15439–15447.

Lalanne E., Honys D., Johnson A., Borner G.H.H., Lilley K.S., Dupree P., Grossniklaus U. & Twell D. (2004) SETH1 and SETH2, Two components of the glycosylphosphatidylinositol anchor biosynthetic pathway, are required for pollen germination and tube growth in Arabidopsis. *The Plant Cell* 16, 229–240.

Larkindale J. & Huang B. (2004) Changes of lipid composition and saturation level in leaves and roots for heat-stressed and heat-acclimated creeping bentgrass (*Agrostis stolonifera*). *Environmental and Experimental Botany* 51, 57–67.

Liu Y., Wang G. & Wang X. (2015) Role of aminoalcoholphosphotransferases 1 and 2 in phospholipid homeostasis in Arabidopsis. *Plant Cell* 27, 1512–1528.

Lobell D.B. & Field C.B. (2007) Global scale climate-crop yield relationships and the impact of recent warming. *Environmental Research Letters* 2,014002.

Moore T.S. (1990). Biosynthesis of phosphatidylinositol. In *Inositol metabolism in plants* (eds. D.J. Morre, W.F. Boss & F.A. Loewus), pp 107–112. Wiley-Liss, New York.

Mou Z., Wang X., Fu Z., Dai Y., Han C., Ouyang J., …, Li J. (2002) Silencing of Phosphoethanolamine N-methyltransferase results in temperature-sensitive male sterility and salt hypersensitivity in Arabidopsis. *Plant Cell* 14, 2031–2043.

508 Nakamura Y., Andrès F., Kanehara K., Liu Y.C., Dörmann P. & Coupland G. (2014).
509 Arabidopsis florigen FT binds to diurnally oscillating phospholipids that accelerate
510 flowering. *Nature Communications* 5, 3553.
511 Narayanan S., Prasad P.V.V., Fritz A.K., Boyle D.L. & Gill B.S. (2015) Impact of high night-
512 time and high daytime temperature stress on winter wheat. *Journal of Agronomy and*
513 *Crop Science* 201, 206–218.
514 Narayanan S., Tamura P.J., Roth M.R., Prasad P.V.V. & Welti R. (2016a) Wheat leaf lipids
515 during heat stress: I. High day and night temperatures results in major lipid alternations.
516 *Plant, Cell and Environment* 39, 787-803.
517 Narayanan S., Prasad P.V.V. & Welti R. (2016b) Wheat leaf lipids during heat stress: II. Lipids
518 experiencing coordinated metabolism are detected by analysis of lipid co-occurrence.
519 *Plant, Cell and Environment* 39, 608-617.
520 Potocky M., Elias M., Profotova B., Novotna Z., Valentova O. & Zarsky V. (2003) Phosphatidic
521 acid produced by phospholipase D is required for tobacco pollen tube growth. *Planta*
522 217, 122–130.
523 Prasad P.V.V. & Djanaguiraman M. (2011) High night temperature decreases leaf
524 photosynthesis and pollen function in grain sorghum. *Functional Plant Biology* 38, 993–
525 1003.
526 Prasad P.V.V. & Djanaguiraman M. (2014) Response of floret fertility and individual grain
527 weight of wheat to high temperature stress: sensitive stages and thresholds for
528 temperature and duration. *Functional Plant Biology* 41, 1261–1269.
529 Saini H.S. & Aspinall D. (1982) Abnormal sporogenesis in wheat (*Triticum aestivum* L.) induced
530 by short periods of high temperature. *Annals of Botany* 49, 835–846.

Saini H.S., Sedgley M. & Aspinall D. (1984) Developmental anatomy in wheat of male sterility induced by heat stress, water deficit or abscisic acid. *Australian Journal of Plant Physiology* 11, 243–253.

Sakata T., Oshino T., Miura S., Tomabechil M., Tsunaga Y., Higashitani N., Miyazawa Y., Takahashi H., Watanabe M. & Higashitani A. (2010) Auxins reverse plant male sterility caused by high temperatures. *Proceedings of National Academy of Sciences of the United States of America* 107, 8569–8574.

Seddon J.M. (1990) Structure of the inverted hexagonal (HII) phase, and nonlamellar phase-transitions of lipids. *Biochimica et Biophysica Acta* 1031, 1–69.

Tubiello F.N., Rosenzweig C., Goldberg R.A., Jagtap S., Jones J.W. (2002) Effects of climate change on US crop production: simulation results using two different GCM scenarios. Part I: Wheat, potato, maize, and citrus. *Climate Research* 20, 259–270.

de Vries A.H., Mark A.E. & Marrink S.J. (2004) The binary mixing behavior of phospholipids in a bilayer: a molecular dynamics study. *The Journal of Physical Chemistry* B 108, 2454–2463.

Vu H.S., Tamura P., Galeva N.A., Chaturvedi R., Roth M.R., Williams T.D., …, Welti R. (2012) Direct infusion mass spectrometry of oxylipin-containing Arabidopsis membrane lipids reveals varied patterns in different stress responses. *Plant Physiology* 158, 324–339.

Vu H.S., Shiva S., Roth M.R., Tamura P., Zheng L., Li M., …, Welti R. (2014) Lipid changes after leaf wounding in Arabidopsis thaliana: expanded lipidomic data form the basis for lipid co-occurrence analysis. *The Plant Journal* 80, 728–743.

Williams E.E. (1998) Membrane lipids: what membrane physical properties are conserve during physiochemically-induced membrane restructuring? *American Zoologist* 38, 280–290.

554 Xia J., Psychogios N., Young N. & Wishart D.S. (2009) MetaboAnalyst: a web server for
555 metabolomic data analysis and interpretation. *Nucleic Acids Research* 37, W652–W660.
556 Xia J., Mandal R., Sinelnikov I.V., Broadhurst D. & Wishart D.S. (2012) MetaboAnalyst 2.0-a
557 comprehensive server for metabolomic data analysis. *Nucleic Acids Research* 40, W127–
558 W133.
559 Zheng Z., Xia Q., Dauk M., Shen W., Selvaraj G. & Zou J. (2003) Arabidopsis AtGPAT1, a
560 member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is
561 essential for tapetum differentiation and male fertility. *Plant Cell* 15, 1872–1887.
562 Zheng G., Tian B., Zhang F., Tao F. & Li W. (2011) Plant adaptation to frequent alterations
563 between high and low temperatures: remodelling of membrane lipids and maintenance of
564 unsaturation levels. *Plant Cell and Environment* 34, 1431–1442.
565 Zienkiewicz A., Zienkiewicz K., Rejón J.D., Rodríguez-García M.I. & Castro A.J. (2013) New
566 insights into the early steps of oil body mobilization during pollen germination. *Journal*
567 *of Experimental Botany* 64, 293–302.

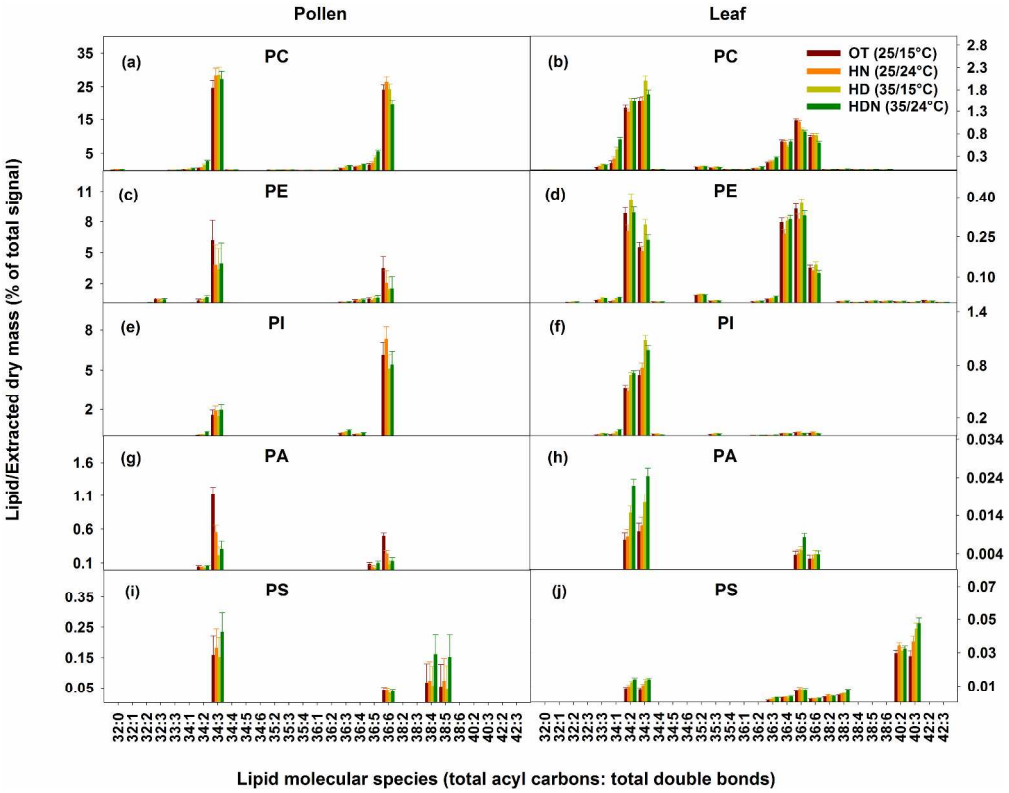

Figure 1. Composition of extraplastidic phospholipids of pollen grains (a, c, e, g, and i) and leaves (b, d, f, h, and j) of wheat genotype Karl 92. Leaf lipid compositions are reproduced from Narayanan et al. (2016a) for novel comparison with pollen lipid compositions. Values shown are mean ± SE; n = 10 [two experiments and five replications (plants)]. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

476x373mm (300 x 300 DPI)

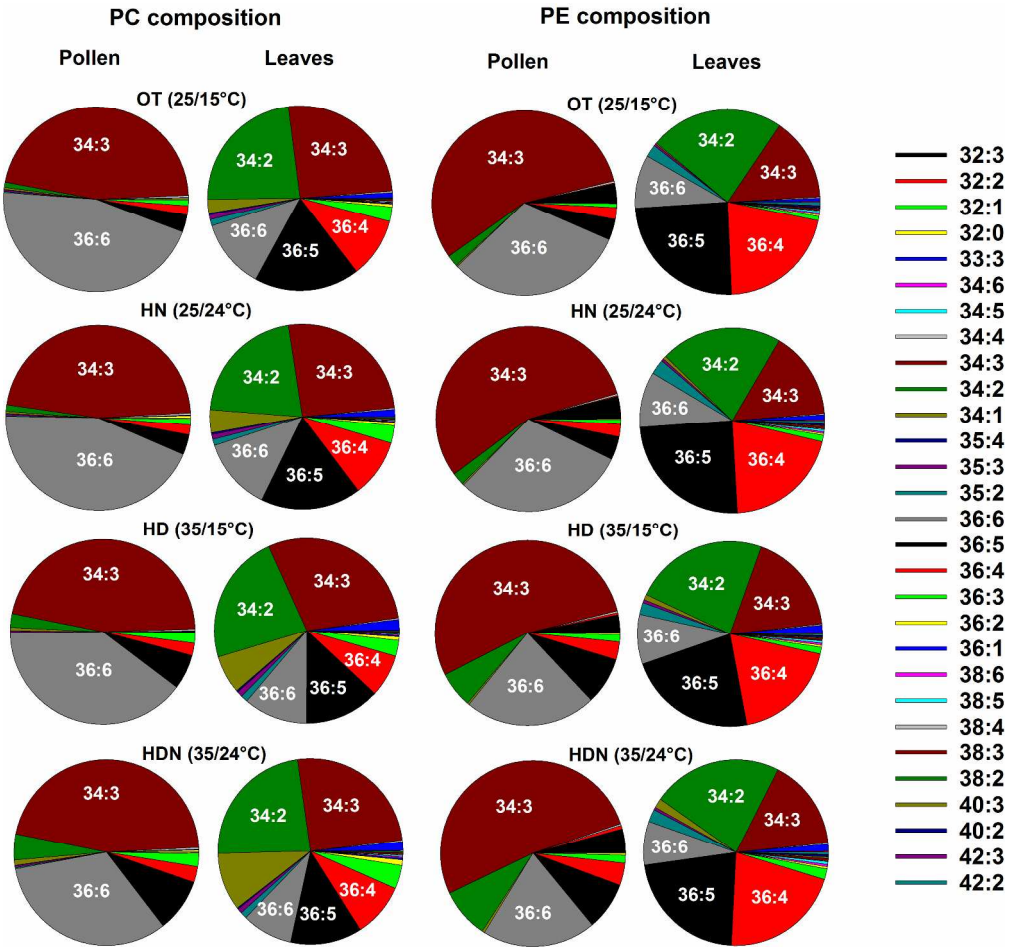

Figure 2. Composition of various lipid species as a percentage of total PC and PE of pollen grains and leaves of wheat genotype Karl 92. Leaf lipid compositions are reproduced from Narayanan et al. (2016a) for novel comparison with pollen lipid compositions. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

343x323mm (300 x 300 DPI)

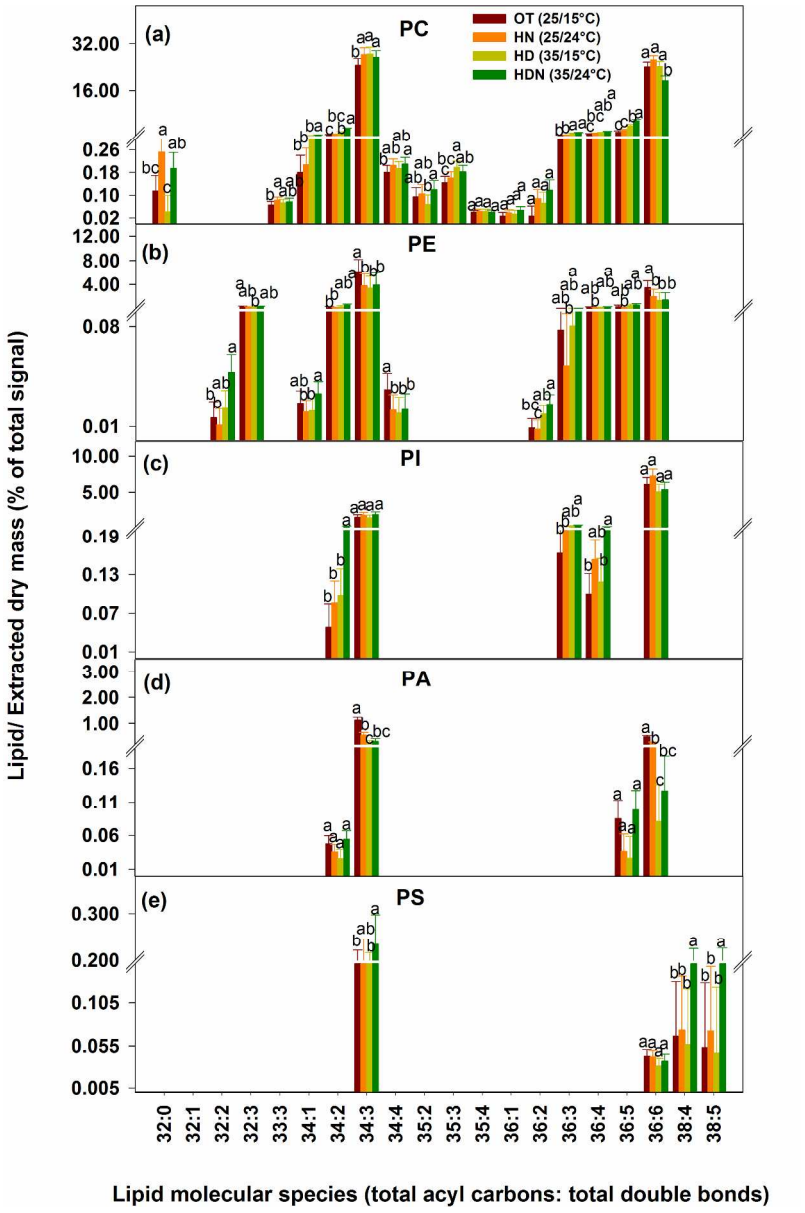

Figure 3. Effects of temperature on extraplastidic phospholipid molecular species of wheat genotype Karl 92. Values shown are mean ± SE; n = 10 [two experiments and five replications (plants)]. Means with different letters are significantly different according to the least significant difference (LSD) test at P<0.05. 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; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

233x351mm (300 x 300 DPI)

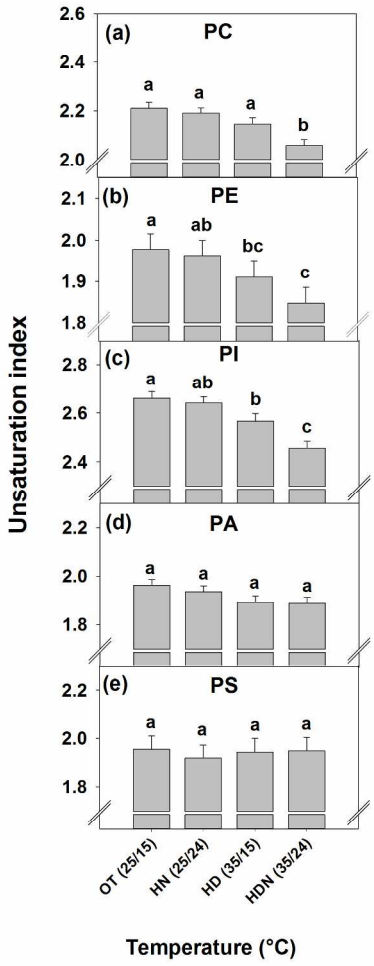

Figure 4. Effects of temperature on unsaturation index of extraplastidic phospholipid classes of wheat genotype Karl 92. The unsaturation index of each lipid molecular species was calculated as the product of the amount of that lipid molecular species and the average number of double bonds per acyl chain, 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 ± SE; n = 10 [two experiments and five replications (plants)]. Means with different letters are significantly different according to the least significant difference (LSD) test at $P<0.05$. OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

215x279mm (300 x 300 DPI)

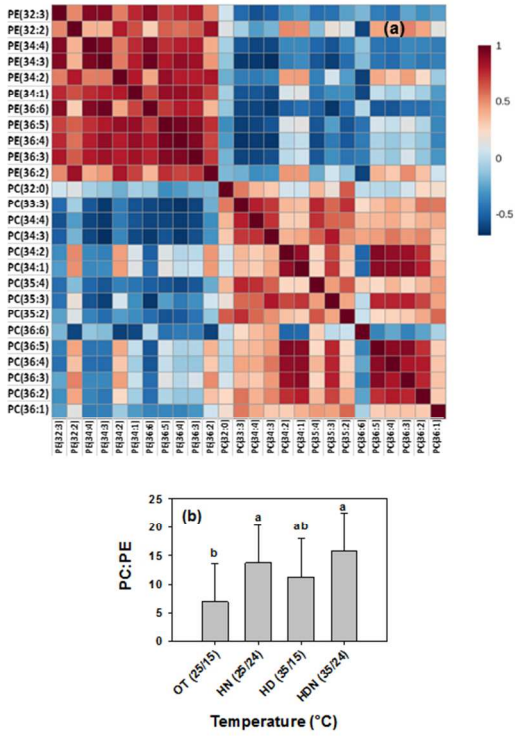

Figure 5. Heat map showing the correlation among phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species of wheat genotype Karl 92 (a) based on Spearman's correlation coefficient, ρ. Blue and red colors on the heat map indicate negative and positive correlations, respectively. Effects of temperature on PC:PE ratio (unitless) of Karl 92 (b). OT, optimum temperature; HN, high night temperature; HD, high day temperature; HDN, high day and night temperature.

254x190mm (96 x 96 DPI)

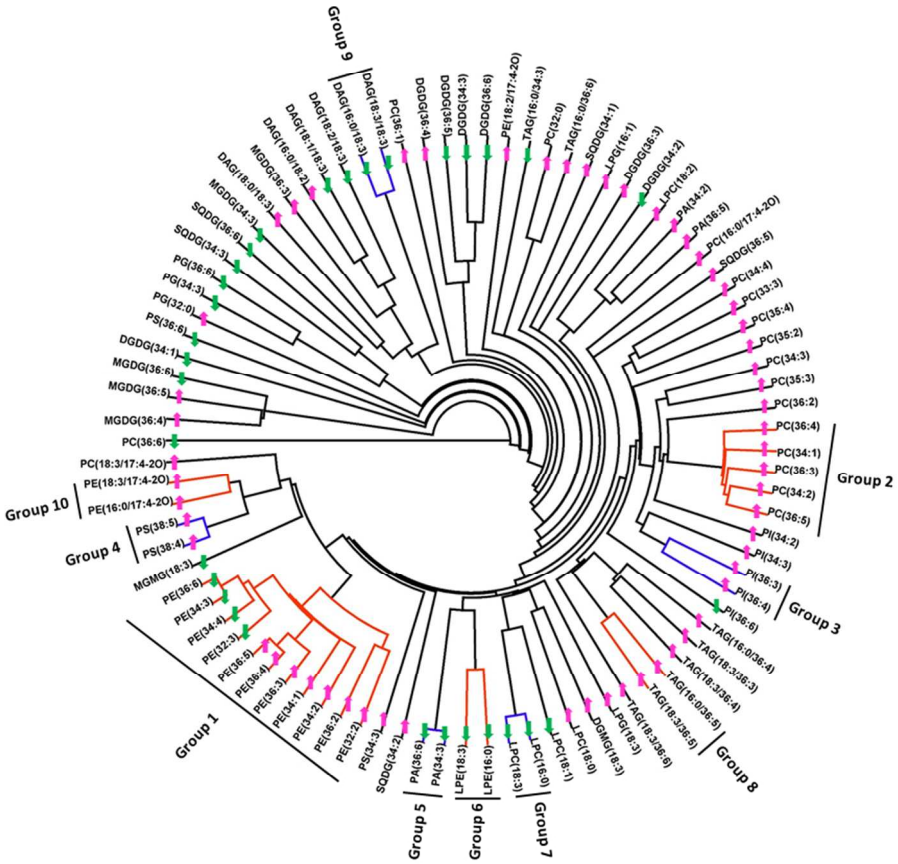

Figure 6. Lipid dendrogram of wheat genotype Karl 92. Eighty nine lipid analytes were clustered using a single-linkage hierarchical algorithm based on Spearman's correlation coefficient, ρ. Co-occurring lipid groups (Groups 1-10) with ρ ≥ 0.85 are indicated by red and blue bars on the dendrogram. The arrows on the dendrogram indicate the directionality of differences in levels of each lipid (based on % of total signal) under high day and night temperature stress conditions compared to optimum temperature conditions; lipids that decreased in amount are indicated by green-colored downward arrows, and lipids that increased in amount are indicated by pink-colored upward arrows.

254x228mm (96 x 96 DPI)