Role of Phosphatidic Acid in High Temperature Tolerance in Maize

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ABSTRACT
Maize (Zea mays L.) germplasm exhibits large genetic variations in tolerance to high temperature (HT) stress under field conditions, but the mechanisms underlying this variation are largely unknown. Based on many years of field observation, maize inbred line B76 consistently displays better tolerance to HT than B106. Heat waves during growing season cause leaf firing in developing leaves and tassel blasting in B106 but not in B76. The difference in HT tolerance between the two inbred lines was confirmed in growth chambers under controlled conditions. The two inbred lines showed similar level in the induction of heat shock proteins and a maize chloroplast protein synthesis elongation factor (EF-Tu), two mechanisms known to involve in HT tolerance in maize. A drastic decrease in photosynthetic system II (PSII) quantum efficiency occurred at 34 to 35°C in B106; in B76, it occurred at a much higher temperature (>38°C). Cell membranes of B76 appeared to be more stable under HT than those of B106 based on electrolyte leakage analysis. Lipid profiles of young developing leaves by lipidomics showed that, among all lipids detected, only phosphatidic acid (PA) exhibited significant higher level in B76 than in B106 under both normal and HT stressed conditions (p < 0.02). Moreover, PA was the only lipid that was significantly increased by HT treatment (p < 0.05). Our results suggest that membrane thermostability is essential to HT tolerance and that PA may play an important role in imparting membrane thermostability, and, hence, HT tolerance in maize.

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Abbreviations: CI, class 1; CII, class 2; EF-Tu, maize chloroplast protein synthesis elongation factor; Fv/Fm, steady state photosynthetic system II quantum yield; HSP, heat shock protein; HT, high temperature; KLRC, Kansas Lipidomics Research Center; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PSII, photosynthetic system II; RI, relative injury; sHSP, small heat shock protein.

Maize is the number one grain crop in the United States, serving as a major staple food for human consumption and feed for animals. The demand for maize production has increased in recent years due to world population increase and the growing need for maize as a resource for bioenergy. However, drought and high temperature (HT), two major environmental factors, severely limit maize production in the United States and worldwide. The optimum growth temperature for most maize lines ranges from 28 to 32°C (Ellis et al., 1992). On the southern plains, sporadic heat waves above 37°C can occur at any development stage during the growing season. While integrated crop management such as irrigation can alleviate water stress and minimize crop losses due to drought, it cannot reduce the negative
impacts of HT stress. High temperature affects essentially all aspects of plant growth and development, from gene expression, translation, enzyme activity, metabolism, and organelle structure to cell growth and tissue development (Al-Khatib and Paulsen, 1999; He and Huang, 2007; Quinn, 1988; Weis and Berry, 1988). In maize, temperatures above optimum have detrimental effects on vegetative growth, pollination, seed set, and kernel development (Cantarero et al., 1999; Cheikh and Jones, 1994; Dupuis and Dumas, 1990; Wilhelm et al., 1999). High temperature stress occurring at pollination, silking, and early kernel development stages can result in significant reduction in grain yield and quality (Cantarero et al., 1999; Comnuri and Jones, 1999; Wilhelm et al., 1999).

Many years’ field observations show that maize plants in the field become particularly sensitive to HT after the eighth leaf stage (V8) when tassel begins to develop rapidly and stalk has started rapid elongation (http://www.extension.iastate.edu/hancock/info/corn.htm [verified 14 Aug. 2010]). Heat waves during V10 to V14 stage can cause massive tissue damage to young developing leaves on upper part of plants of sensitive lines and the damaged leaf tissues die quickly, a phenomenon called leaf firing. The male reproductive tassels are also very sensitive to HT. A heat wave during tassel development often results in abnormal tassel development, reduction in pollen production, and even total sterility. It can also cause drying out of tassel tissues, a phenomenon known as tassel blasting. Leaf firing and tassel blasting are the most visible phenotypes of HT-induced tissue injuries in the field. Plants with severe leaf firing and tassel blasting lose considerable photosynthetic area, shed little or no pollen, and produce small ears with reduced kernel set.

High temperature tolerance in plants is complex, involving multiple pathways and many biological, biochemical, and physiological processes (Burke and Chen, 2006; Iba, 2002; Kotak et al., 2007; Larkindale et al., 2005). Several mechanisms of HT tolerance have emerged from studies in model organisms and crop plants. Induction of heat shock proteins (HSPs) is a well known mechanism by which plants acquire enhanced tolerance to extreme HT in response to a brief exposure to sublethal elevated temperature (Gurley, 2000; Hong and Vierling, 2000; Nieto-Sotelo et al., 2002). Many HSPs function as molecular chaperones and enable organisms to survive brief exposures to extreme HTs by preventing aggregation of denatured proteins and repairing misfolded proteins (Lee et al., 2005; Vierling, 1991; Wang et al., 2004). In maize, induction of HSP101, up-regulation of a maize chloroplast protein synthesis elongation factor (EF-Tu), and enhanced thermostability of rubisco activase have all been implicated in HT tolerance (Bhadula et al., 2001; Crafts-Brandner and Salvucci, 2002; Momcilovic and Ristic, 2007; Nieto-Sotelo et al., 2002; Vargas-Suarez et al., 2004). The maintenance of proper membrane stability and fluidity through adjustments to membrane lipid composition and fatty acids saturation levels is another well characterized adaptive response to stresses associated with both high and low temperatures (Alfonso et al., 2001; Chen et al., 2006b; Falcone et al., 2004; Gorver et al., 2000; Marcum, 1998; Sung et al., 2003). Other responses, such as the production of antioxidants and modification of protein properties or enzyme activities, also play roles in protecting plants from extreme temperature stresses (Burke and Chen, 2006; Chen et al., 2006a; Larkindale and Huang, 2004; Wang et al., 2006a). Despite these recent advances, our understanding of HT tolerance mechanisms in maize, is very limited.

Maize inbred lines display large variations in HT tolerance under field conditions, but little is known about the mechanisms underpinning these variations. In this report, two maize inbred lines—B76 (tolerant) and B106 (susceptible)—displaying drastic difference in HT tolerance under both field and controlled conditions were studied to identify biological components critical to HT tolerance in maize. Several mechanisms known to be involved in HT tolerance were examined under controlled conditions. Our results reveal that levels of phosphatidic acid (PA) in developing leaves are associated with HT tolerance in these two inbred lines.

MATERIALS AND METHODS

Field Phenotype Evaluation

Two maize inbred lines with distinct HT tolerance phenotypes, B76 and B106 (developed by Iowa State University, Ains, IA), were used in this study. B76 was released in 1974 as germplasm to resist European corn borer (Russell and Hallauer, 1974). The original study indicates that B76 is more tolerance to heat and moisture stress than inbred line B37, the backcross parent used to produce B76. When subjected to such stress, B76 showed much less delay of silk emergence and slightly better pollen production than B37 (Russell and Hallauer, 1974). B106 was released in 1997 for insect resistance and staygreen trait (Hallauer et al., 1997). Both lines have excellent combining ability to Mo17. Maize inbred lines were planted in mid April yearly at Texas A&M AgriLife Research and Extension stations in Halfway (34°19′ N, 101°95′ W, and 1072 m elevation) and Lubbock (33°59′ N, 110°89′ W, and 982 m elevation). The soil is a Pullman clay loam at Halfway Station and a Sherman clay loam at Lubbock Station. The HT tolerance of the inbred lines was evaluated as part of maize breeding program from 2000 to 2007. The HT tolerance evaluation process was triggered when a moderate heat wave occurred (moderate heat stress is defined as a daily air high temperature exceeded 37°C for a few hours within a day and it lasts 1 to 2 d while a severe stress will last more than 3 d and/or temperature exceeded 39°C for a few hours within a given day) for the first time during the growing season and whenever a moderate or severe heat wave occurred thereafter. The inbred lines were inspected visually 5 to 7 d after onset of the stress for symptoms of HT-induced injuries. Due to variations in weather conditions among years (Supplementary Fig. S1), the developmental stages at
which HT tolerance were evaluated under field conditions varied from year to year. B73 and Mo17 were included as controls every year in germplasm evaluation. These two lines display moderate to susceptible in HT tolerance under field conditions in the southern Plains. Based on several years’ observation in the fields, the inbred line B76 consistently displays better tolerance to sporadic heat waves than the control lines, while B106 is more susceptible to heat stress at vegetative and reproductive development stages under both limited irrigation and fully irrigated conditions. This difference in HT tolerance was carefully re-evaluated in the USDA-ARS research field (33° 35.62′ N, 101° 54.19′ W, 982 m elevation, and soil type Amarillo sandy loam) in Lubbock in 2006 and 2007 following periodic occurrence of heat waves.

High Temperature Treatments under Controlled Environments

Three seeds were planted in 10-L plastic pots (23 cm diam., 24 cm high) containing Sunshine #1 growth mix (Sun Gro Horticulture, Bellevue, WA) and were thinned to 1 plant per pot at the two-leaf stage. The two inbred lines were grown side by side in a greenhouse to various developmental stages. The temperature in greenhouse was controlled in square wave fashion. The temperature was set at 25/21°C day/night, which was controlled by an external light sensor. The light intensity was 400 to 550 μmol quanta m−2 s−1 during the day. Plants were fertilized once a week with diluted Peters Excel Fertilizer 21–5–20 (N–P–K; Scotts-Sierra Horticultural Products, Marysville, OH), and the pots were rotated twice a week to ensure uniformity.

A split block design was used for HT treatment with temperature regime as main effect. Plants at the desired stages were randomly assigned to treatment groups. The plants in the control group remained in the greenhouse; the plant in the HT treatment group were transferred to a temperature controlled walk-in growth chamber set at 38/30°C day/night with a 14/10 h photoperiod with similar light intensity as in the greenhouse. To avoid water stress, plants were watered twice a day and the relative humidity was controlled to around 50%. The HT treatments were always initiated at 1200 h (a 8 h exposure to 38°C in light followed by 10 h at 30°C in dark and then by a 14/10 h day/night cycle at 38/30°C). For phenotype evaluation, the HT treatment was applied to maize plants at set developmental stages and HT tolerance phenotypes were evaluated visually. Phenotypical evaluation experiment was repeated three times. Means separation was performed on the temperature treatments and lines using the MIXED procedure in SAS (version 9.1.3) (SAS Institute, 2004) for all variables except for protein quantification. The data was analyzed as a split block design with temperature treatment and plant line as fixed effects and replicate block as a random effect. The LSMEANS option was used to calculate Tukey’s Honestly Significant Difference (HSD) groupings of treatments and lines.

Maize plants used for physiological and biochemical analyses were allowed to grow under normal conditions to a stage where 2 to 5 cm long tassel tissues became visible. At this stage, the top four leaves from the apex were not fully expanded (young developing leaves) whereas the leaf sheath of the fifth leaf from the apex was clearly visible and the ones below it were mature leaves with a visible leaf collar. All leaf samples were collected from the middle one-third section of the leaf blade. Leaf samples from HT-treated plants were collected at 1, 2, and 3 d after the initiation of HT treatment. Leaf samples from four plants in an experiment were pooled for analysis and the subsamples were treated as such in the corresponding statistical analyses. Each experiment was repeated two times. In this particular study, the leaves were numbered from the apex where the flag leaf was designated as the first leaf.

Electrolyte Leakage Measurements

Electrolyte leakage was measured as relative injury (RI) according to a procedure described by Warren et al. (1996) with minor modification. Ten leaf discs (1 cm diameter) from each of the second, third, fourth, fifth, and sixth leaves were collected and placed immediately into separate wells of a 24-well plate filled with distilled water. Leaf discs collected from the leaves at the same position but on different plants were combined, washed three times, and used for electrolyte leakage and cell viability measurements. For electrolyte leakage measurement, leaf discs were placed into 50 mL screw-cap glass tubes containing 5 mL aliquots of distilled water: 5 discs per tube and 4 tubes per line per treatment. Tubes were then transferred to a 25°C water bath for 15 min and incubated at room temperature for 16 h. Before measurement, the tubes were gently shaken for 15 min. Electrical conductivity was measured with an Oakton 510 conductivity meter (Eutech Instruments, Singapore). These values were designated as T1. The tubes were then autoclaved for 15 min to release all electrolytes. After cooling to room temperature, the tubes were briefly vortexed and the conductivity measured as the total ion content (T2). Average conductivity of distilled water from 10 tubes served as the base ion content (T0). The RI due to HT treatment was calculated by (T1 – T0)/(T2 – T0) × 100. At the same time, reduction of 2,3,5-triphenyltetrazolium chloride (TTC) was also measured according to procedures described by Porter et al. (1994) using 5 discs per tube and 4 tubes per line per treatments.

Lipid Extraction and Analysis

Leaf punches (0.5 cm diameter) from each of the second, third, and fourth leaves of four plants were randomly pooled as young developing leaf samples, and punches from the sixth leaf were pooled as mature leaf samples. Leaf punches from HT-stressed and nonstressed plants were harvested concurrently and immediately immersed in 6 mL of 75°C isopropanol with 0.01% butylated hydroxytoluene (Sigma, St. Louis, MO). Total lipids in leaf tissues were extracted according to the procedures described by Chen et al. (2006b). Lipid extracts were shipped on dry ice to Kansas Lipidomics Research Center (KLRC; http://www.k-state.edu/lipid/lipidomics/index.htm [verified 14 Aug. 2010]) at Kansas State University in glass tubes filled with nitrogen gas. Lipid profiles in the leaf samples were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in the KLRC analytical laboratory according to standard protocols (Welti et al., 2002).

Chlorophyll Fluorescence

The changes in steady state photosynthetic system II (PSII) quantum efficiency ($F_v/F_m$) in response to temperature increase were measured for the second and third leaves at temperatures ranging from 26 to 43.5°C using an OS1-FL modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, MA).
The temperature treatment of leaf tissues was performed on an electronically controlled eight position thermal plate system (Celtec) (Burke and Mahan, 1993). The eight Celtec thermoplates were set at different temperatures and covered with a layer of moistened filter paper and then with cellophane (Glad Cling wrap, First Brands Corporation, Danbury, CT) to retain moisture but allow air exchange. A 12 cm × 2 cm leaf tissue from mid leaf blade was harvested from each maize plant grown under normal condition and then cut into 8 pieces of 1.5 × 2 cm leaf sections, which were immediately placed on moistened filter paper and covered with Glad Cling wrap. After 30 min conditioning at room temperature (22–23°C), the Fv/Fms of each leaf section was measured (0 min). Then each leaf section was placed on top of the filter paper that overlaid on a Celtec thermo-plate and covered immediately with Glad Cling wrap, one leaf section per plate. The actual temperature that each leaf sample experienced was monitored by placing a thermocouple of a microprocessor thermometer (HH21, Omega, Stamford, CT) next to the leaf tissue. The kinetic changes in chlorophyll fluorescence of each leaf section at a set temperature were monitored by measuring Fv/Fms every 3 to 4 min over a 36 to 37 min incubation time. The changes in Fv/Fms of the leaf tissues in response to different temperature exposure were measured after 18 min incubation at set temperatures. The steady state PSII quantum yield was calculated according to the equation after 18 min incubation at set temperatures. The steady state in response to different temperature exposure were measured.Fv/Fm = (Fm – F)/Fm, where F is the fluorescence emission of a light-adapted plant under steady state conditions and Fm is the fluorescence emission under a saturation light flash.

Western Blot Analysis
For protein analysis, leaf tissues were harvested from non-stressed (0 h) and 2 h and 8 h HT-stressed maize plants at 38°C. Leaf tissues from the second, third, and fourth leaves were pooled together and those from the fifth and sixth leaves from the apex were pooled. Total protein was extracted according to procedures described previously (Chen et al., 2006b). Fifty μg of total protein was separated on 8% (for HSP101 and EF-Tu) or 15% (for class I and class II small HSPs [sHSPs]) SDS/PAGE (w/v) and then blotted to a Polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Western blot analyses were performed individually using anti-HSP101 (1:1,000, v/v), sHSP17.6 (1:1,000), sHSP17.4 (1:1,000), and EF-Tu (1:2000) antisera and visualized using an immune-blot kit (Bio-Rad, Hercules, CA). The identity of each class of proteins was identified by strong and specific band(s) at appropriate size on western blot as showed in previous reports. The abundance of each protein was estimated the relative intensity of the band as described (Momcilovic and Ristic, 2007).

RESULTS AND DISCUSSION
Difference in High Temperature Tolerance in Maize Inbred Lines B76 and B106 under Field Conditions
Observation in breeding fields from 2000 to 2007 consistently showed that heat waves occurred at vegetative stages caused injury to young developing leaves of B106 plants, resulting in leaf firing symptoms (Fig. 1a). Heat waves that occurred at the transitional stage from vegetative to reproductive growth caused both leaf firing and tassel blasting symptoms in B106 plants (Fig. 1b). Sporadic HTs at these and early reproductive stages caused significant reduction in photosynthetic tissues, pollen shed, and pollination efficiency to B106 plants, resulting in significant decreases in kernel set and even total loss of yield, whereas B76 showed no symptoms of injury under the same heat waves. We observed that the severity of HT-induced injury depended on temperature and duration of the heat waves and the relative humidity in the air. In 2006 and 2007, the environmental conditions in the south Plains differed dramatically between the two growing seasons. From May to July, the weather was mostly hot and dry in 2006 while it was cool and wet in 2007 (Supplementary Fig. S1). As a result, field crops in 2006 experienced severe drought stress combined with recurrent heat waves whereas crops in 2007 experienced neither drought nor severe HT stress during early to mid growing season. In the season of 2006, the mid to upper portions of B106 plants died completely in both irrigated and partially irrigated fields whereas B76 plants grew normally. Figure 1d shows the comparison of accumulated HT-induced tissue damage in B106 and B76 in irrigated plots at the middle of the growing season.

In the early season of 2007, no difference between B76 and B106 plants were observed in both growth and development. A brief heat wave occurred on June 18th in 2007 with air temperature exceeding 35°C over a 5-h period (Supplementary Fig. S1b) and the next morning, signs of heat injury were already visible on young developing leaves of B106. Later that day an additional 3.5-h period with temperatures slightly above 35°C occurred, and leaf injury symptoms became clearly visible on most of the B106 young leaves. At the same time, all tissues of the B76 plants remained healthy (Fig. 1c). The observed phenotype differences between B76 and B106 plants in 2007 were likely attributed to a difference in HT tolerance since there was no soil moisture shortage during the periods of HT stress. Our systematic evaluation confirmed the previously observation from many years’ of breeding experience that B76 is more tolerant to HT than B106 under field conditions.

 Confirmation of the High Temperature Tolerance Phenotypes under Controlled Environments
Evaluation of HT tolerance under field conditions is probably the most relevant approach in maize breeding program to identify germplasm and breeding lines tolerant to HT stress. However, field evaluation of HT tolerance is extremely difficult because it calls for heat waves to occur at a specific developmental stage with the right intensity and duration. To overcome this inherent difficulty, we evaluated the HT tolerance of B76 and B106 inbred lines under controlled environments.
Similar to the field observations, B76 plants displayed a high degree of temperature tolerance at all developmental stages examined under controlled environment (Fig. 1e, i, k) whereas B106 plants were highly sensitive to HT at both vegetative and reproductive stages (Fig. 1e–h, j). Phenotypes of HT-induced injury comparable to those observed in the field (e.g., leaf firing, abnormal tassel development, and tassel blasting) were observed in the HT-stressed B106 plants (Fig. 1e–h, j). B106 plants were found to be most sensitive to HT stress during the transition stage from vegetative to reproductive growth and at the early reproductive stages. In addition, we observed that the thermosensitivity of B106 plants appeared to be developmentally regulated. The young developing leaves of B106 were very sensitive to HT treatment while the fully developed leaves (leaf collar visible) were less sensitive, displaying no sign of injury under similar HT stresses (Fig. 1c, f–h). Our result, thus indicate that under controlled environmental conditions, HT tolerance of maize inbred lines can be evaluated in growth chambers.

**Similar Induction Levels for Heat Shock Proteins and EF-Tu in B76 and B106 by High Temperature Treatment**

Recently, HSP101 and EF-Tu have been shown to contribute to HT tolerance in maize (Bhadula et al., 2001; Momcilovic and Ristic, 2007; Nieto-Sotelo et al., 2002). To test if the thermosensitivity of B106 plants is due to misregulation of these known components of HT tolerance, we examined the changes in HSP101 and EF-Tu protein levels in young developing and mature leaves of B76 and B106 before and after HT treatments (38°C at 0, 2, and 8 h). Both young and mature leaves of B76 and B106 grown at optimal temperature contained similar levels of EF-Tu (Fig. 2). High temperature increased the expression of HSPs but slightly reduced the expression of EF-Tu in both lines. The induction patterns of HSP101 by HT in developing leaf tissues of both inbred lines were similar (Fig. 2). The levels of small HSPs (sHSPs) in those leaf tissues were also examined against *Arabidopsis* antisera of class I (CI) and class II (CII) sHSPs and results showed significant inductions of sHSPs in 2h at 38°C stressed young and mature leaf tissues of the sensitive and tolerant lines (Fig. 2). No difference in either the amount or induction pattern of the CI sHSP was observed between young leaves of B76 and B106 while genotype associated apparent differences were detected for CII sHSPs. In B76, one CII sHSP was detected while two CII sHSPs were detected in B106. However, the induction patterns of CII sHSP (and other HSPs examined) in young (sensitive) and mature (tolerant) leaves of B106 were similar.

Our results showed that despite of the dramatic difference in HT tolerance under both field and controlled conditions, B76 and B106 had similar levels of HSP101, EF-Tu, and other HSPs before stress and similar inductions by HT stress. Our results suggest the mechanisms
Different mechanisms to develop tolerance to HT.

CROP SCIENCE, VOL. 50, NOVEMBER–DECEMBER 2010  WWW.CROPS.ORG 2511

was found among the different leaves and between the two
tative to reproductive transitional stage. No difference in RI
taken from control and HT-stressed plants at the vegeta-
table electrolyte leakage measurements on leaf discs
aging leakage of cell membranes. To test this hypothesis, we
observed in B106 plants, it appeared that HT might dam-
Based on the symptoms of HT-induced leaf tissue injury
that impart the HT tolerance in B76 and B106 are inde-
pendent of the induction of HSPs and EF-Tu. Our results
Figure 2. Western blots of young developing and mature leaf
tissues of B76 and B106 plants against antibodies of small heat
shock protein (sHSP) Class I, sHSP Class II, heat shock protein
HSP101, and EF-Tu. Rbcl, Rubisco large subunit.

Difference in Cell Membrane Stability
at High Temperature

Based on the symptoms of HT-induced leaf tissue injury
observed in B106 plants, it appeared that HT might dam-
age cell integrity in developing tissues, presumably caus-
leakage of cell membranes. To test this hypothesis, we

Fig. 2. Western blots of young developing and mature leaf
tissues of B76 and B106 plants against antibodies of small heat
shock protein (sHSP) Class I, sHSP Class II, heat shock protein
HSP101, and EF-Tu. Rbcl, Rubisco large subunit.

that HT-stressed plants, indicating that, even with the shortest
exposure tested, a low level of cell membrane damage had
ocurred. Similar results were observed in an independent
cell viability assay based on reduction of 2,3,5-triphenyltet-
razolium chloride (TTC) (data not shown).

Our result is consistent with previous observations that
plant cell membranes are a primary site for damages
associated with temperature stresses (Falcone et al., 2004; Horvath
et al., 1998; Quinn, 1988). The difference in RI suggests
that thermostability of cell membranes in young developing
leaves of B106 plants is much lower than that in similar
leaves of B76 plants and more mature leaves of both lines.
We postulate that cell membranes in young leaves of B106
are very susceptible to HT, become less stable as temperature
increases, and eventually lose viability, resulting massive
leakage of ions from the damaged cells. On the other hand,
cell membranes of B76 tissues are stable and able to maintain
normal function at HT. The correlation between membrane
stability at HT and HT tolerance in B76 and B106 inbred
lines indicates that the thermostability of plant cell mem-
branes plays an important role in coping with the negative
impact of HT stress in maize and provides a possible target
for improving HT tolerance in maize.

Sensitivity of Photosynthetic System of B106
to High Temperature Treatments

Chlorophyll fluorescence is often used to evaluate the health
of photosynthetic system in chloroplast membranes under
various stresses. Figure 4a shows the dynamic changes of $F_{\text{v}}/F_{\text{m}}$ of second leaf sections over a 36 min incubation period
at the indicated temperatures. Below 30°C, the $F_{\text{v}}/F_{\text{m}}$
remained stable over time in both B76 and B106 leaf tissues,
although $F_{\text{v}}/F_{\text{m}}$ values of B106 were somewhat lower than
those of B76. However, when the temperature exceeded
30°C, $F_{\text{v}}/F_{\text{m}}$ started to decline over time indicating a nega-
tive impact of HT applied on chloroplast membranes and the
photosynthetic system (Fig. 4a). We then conducted detailed
studies on the effects of temperature on $F_{\text{v}}/F_{\text{m}}$ from 26 to
41°C at 1 to 2°C increments with a set incubation time of 18
min. Figure 4b shows the results of measurements from leaf
samples of the second and third leaves. In general, $F_{\text{v}}/F_{\text{m}}$
started a gradual decline when the temperature reached 30°C
followed by a continued gradual reduction as the tempera-
ture increased but at a different pace. In B106, $F_{\text{v}}/F_{\text{m}}$ began
a continuously decline at about 30°C while in B76 $F_{\text{v}}/F_{\text{m}}$
remained somewhat steady between 30 to 35°C and then
decreased gradually with increasing temperature (Fig. 4b).
Eventually, a clear transition to a more rapid decline of $F_{\text{v}}/
F_{\text{m}}$ was observed with both B76 and B106. In the develop-

In B106, the abrupt decline in $F_{\text{v}}/F_{\text{m}}$ occurred at
34 to 35°C, while it occurred at a much higher temperature
(38–39°C) in B76 (Fig. 4b).

Reduction in $F_{\text{v}}/F_{\text{m}}$ under stress conditions is often
considered an indication of damage to photosynthetic system
(Baker et al., 1988; Chen et al., 2006a; Crafts-Brandner and
Salvucci, 2002; Pastenes and Horton, 1996). The differences
in the temperature at which the decline in $F_{\text{vs}}/F_{\text{ms}}$ occurs indicated that the chloroplast membranes and photosynthetic system in the developing leaves of B76 plants were more tolerant to HT than those of B106. Based on the nature of HT stress applied in this assay (detached leaf sections were directed placed on thermo plates set at specific temperatures, and the $F_{\text{vs}}/F_{\text{ms}}$ values were obtained within 18 min), the HT (heat shock effect) -induced structural changes in thylakoid membranes
are likely a major cause of the abrupt decreases in $F_{v}/F_{m}$ in both B76 and B106 (Fig. 4b). This result agrees with previous studies that chloroplast membranes, especially thylakoid membranes, are extremely sensitive to HT stress (Armond et al., 1980; Chen et al., 2006b; Crafts-Brandner and Salvucci, 2002; Mohanty et al., 2002; Weis and Berry, 1988).

### Levels of Phosphatidic Acid

Phenotypic observation and electrolyte leakage data (Fig. 1 and 3) suggest that plant membrane stability play a role in HT tolerance in maize inbred lines B76 and B106. Membrane lipid composition is known to affect membrane stability under temperature stresses (Alfonso et al., 2001; Falcone et al., 2004; Gorver et al., 2000; Quinn, 1988). Lipid profiles of developing leaf tissues by lipidomics showed that, under normal condition, the young leaves of B76 plants contained more total lipid than those of B106 plants (104 vs. 91 nmol mg$^{-1}$ dry wt.) (Fig. 5). In relative abundance, the developing leaf of B106 and B76 contained similar levels of major chloroplast membrane lipids—monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol (PG)—and major cellular lipids—phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Note: PG is not considered here because most PG comes from chloroplast membranes). However, the amount of PA in young developing leaves of B76 was much higher than that of B106 (Fig. 5). In B76 plants, the PA content was 0.88 nmol mg$^{-1}$ dry wt., a level that is consistent with those reported in many other biological systems (Wang et al., 2006b). Surprisingly, in B106 leaves, PA content was only 0.05 nmol mg$^{-1}$ dry wt., a level considered exceptionally low. Of all the detectable lipids, PA was the only lipid in young developing leaves that showed a significant difference ($p < 0.02$) between B76 and B106 plants grown under normal condition (Fig. 5; ck [control]). Recent studies have showed that PA plays important roles in regulating plant growth and developments such as membrane tethering during cell development and structural effects on cell membranes (Wang et al., 2006b). The correlation between membrane thermosensitivity and lack of normal level of PA in the young leaves of B106 may be a factor accounting for the HT sensitivity observed.

Consistent with its increased HT tolerance, the PA level in mature leaves (sixth leaf) of B106 increased to 0.14 nmol mg$^{-1}$ dry wt., about three fold that in developing leaves of B106 (Supplementary Table S1). The PA level in mature leaves of B76 decreased. The PA levels were similar in the mature leaves of B106 and B76 (Supplementary Table S1).

### Effect of High Temperature Treatment on Levels of Phosphatidic Acid in Developing Leaves

Figure 5 showed changes in lipid composition within developing leaf tissues in response to HT treatment. While the total lipid content of B76 remained similar throughout the treatments, it was noticeably reduced in 2 d and 3 d HT-stressed B106 plants (Supplementary Fig. S2). The difference in total lipid content between B76 and B106 plants was evident (108.4 vs. 76.7 nmol mg$^{-1}$ dry wt.) after even a 1 d exposure to HT. At the end of the 2 d treatment, the total lipid content in B106 decreased to 47% of that in unstressed B106 plants. The difference became even larger after 3 d of HT stress. The dramatic decrease in total lipid content seen in young developing leaves of 2 d and 3 d stressed B106 plants is likely due to cell injury caused by the HT treatment, as indicated by increased electrolyte leakage (Fig. 3) and
visible symptoms of tissue injury seen in those leaves (Fig. 1). Therefore, only samples from the 1 d HT-stressed plants were used to compare how the two inbred lines actively adjust their lipid profiles in response to HT stress. The most notable change was the increase in level of PA in the developing leaves of both B76 and B106 (Fig. 5). In B76, PA content increased from 0.88 nmol to 1.56 nmol mg−1 dry wt, while in B106 it increased 0.05 nmol to 0.35 nmol mg−1 dry wt. The increase in PA was significant (p < 0.05) and the interaction between temperature treatment and line is not significant. Nevertheless, the absolute amount of PA in the developing leaves of B76 was still 3.5 times higher than that in B106 after 1 d HT exposure, despite the increase in B106. In both B76 and B106, the increase in PA content was concomitant with decreases in the amount of its substrates, mainly PC and PE. Overall, among all the lipids analyzed, PA was the only one that was significantly enhanced by HT exposure in the developing leaves of both inbred lines during the early stage of HT treatment.

In addition to its structural roles in cell development, PA also serves as a lipid signaling molecule regulating the responses of plants to chilling, freezing, wounding, abscisic acid, and biotic stresses (Mane et al., 2007; Testerink and Munnik, 2005; Wang et al., 2006b; Zhang et al., 2005). The levels of PA can change rapidly in response to various stresses such as cold stress, oxidative stress, and drought stress in plant (Wang, 2005; Wang et al., 2006b; Zhang et al., 2005). This study demonstrates, for the first time, that HT stress also induces a significant increase in PA production (Fig. 5), suggesting that PA might be involved in adaptation response to HT stress in maize. Since HT sensitivity was coupled with high electrolyte leakage from cellular membranes (Fig. 3), the low PA content in young developing leaf tissues of B106 plants may account for the membrane thermo-instability of these tissues at HT. The association revealed between PA content in the developing tissues of maize plant and thermostability of plant membranes at HT suggests that PA might play an important role in maintaining normal membrane function and stability under HT. Further studies are required to elucidate how PA mediates membrane thermostability, hence HT tolerance, in maize.

CONCLUSIONS

This study has showed that B76 and B106 differ in HT tolerance under both field and controlled growth chamber conditions. This difference is likely due to mechanisms that are independent of the induction of HSPs and EF-Tu. The phenotypic symptoms and electrolyte leakage and Fv/Fm results indicate that membrane stability under HT was the major factor accounting for the difference in HT sensitivity of developing leaves between the two inbred lines. Lipid profiling data have shown that, among all lipids analyzed, PA was the only lipid that differed in developing leaves of B76 and B106 plants under both normal and HT stress conditions. Phosphatidic acid was also the lipid that showed the greatest difference between young and mature leaves of B106 plants grown under normal condition. It is evident that the amount of PA in young developing leaves of maize plants is closely associated with membrane thermostability and the HT tolerance of these tissues. In summary, our results suggest that PA may play an important role in HT tolerance in maize, reflected in an increase in PA levels in maize tissues on HT treatment.

Supplemental Information Available

Supplemental material associated with this article is available free of charge online at http://www.crops.org/publications/cs.

Acknowledgments

The authors thank James Golden and Dr. John Stout for the collection of the meteorological data. The authors thank KLRC at Kansas State University for lipid analysis and following laboratories for providing antibodies: Dr. Elizabeth Vierling (sHSP class I and II); Dr. Jorge Nieto-Sotelo (HSP 101) and the late Dr. Zoran Ristic (EF-Tu). The authors are grateful to Dr. Jeff Velten and Dr. M. B. Kirkham for critical reading of the manuscript and Kathleen Yeater for statistical analysis.

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