**Two phases of response to long-term moderate heat: variation in
thermotolerance between *Arabidopsis thaliana* and its relative *Arabis
paniculata***

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Abstract

Long-term moderate heat is often experienced by plants and will become even more common in the future due to global warming. However, the responses of plants to this stress have not been characterised. In the present study, growth between *Arabidopsis thaliana* and its relative *Arabis paniculata* upon long-term exposure to moderate heat was compared. It was found that the latter was more tolerant than the former, and the patterns of physiological and biochemical responses of both plants presented two phases. The early phase involved no significant visible morphological and physiological changes. It occurred during the first third of the heat treatment and was extended when the stress was attenuated. During the later phase, the plants died or were damaged. Heat shock proteins were dramatically induced at the early phase and gradually decreased at the later phase in *A. thaliana*. By contrast, the levels were induced and maintained in *A. paniculata*. Profiling of membrane lipids found that the two plants exhibited opposite patterns of lipid remodelling at the early phase: *A. paniculata* synthesised phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol and showed a rapid decrease in the degree of lipid unsaturation, while *A. thaliana* degraded its lipids at the early phase and showed an accelerated degradation at the later phase. These biochemical adjustments during the early phase could favor the thermotolerance of *A. paniculata*. These results suggest that this species could thus be a model for the study of resistance to long-term moderate heat, through a strategy by which plants can adapt to long-term moderate heat.

Key-words: *Arabis paniculata*; *Arabidopsis thaliana*; *Cruciferae*; long-term moderate heat; photosynthesis; heat shock protein; membrane lipids

Abbreviations list:

DAG, diacylglycerol;

DBI, double bond index;

DGDG, digalactosyldiacylglycerol;

ESI-MS/MS, electrospray ionization tandem mass spectrometry;

F_v/F_m , the maximal photochemical efficiency;

HSPs, heat shock proteins;

MDA, malondialdehyde;

MGDG, monogalactosyldiacylglycerol;

PA, phosphatidic acid;

PC, phosphatidylcholine;

PCA, principal components analysis;

PE, phosphatidylethanolamine;

PG, phosphatidylglycerol;

PI, phosphatidylinositol;

PS, phosphatidylserine;

$Y(II)$, the actual photochemical efficiency;

PS II, photosystem II;

ROS, reactive oxygen species;

$Y(NPQ)$, the yield of regulated energy dissipation.

1. Introduction

Environmental heat stress is usually defined as a temperature that is 10–15°C above the optimal level for plant development and growth (Wahid et al., 2007). Heat stress can be classified into acute and chronic types according to the extent and rate of the change in temperature, as well as its duration (Blum, 1988). Under acute heat stress (heat shock), plants experience rapid dramatic changes in temperature and undergo three phases of stress response: triggering, maintenance, and recovery, within a period of hours (Al-Whaibi, 2011). In contrast, chronic heat stress (long-term moderate heat) lasts for days, weeks, or even months, and involves slow moderate increases in temperature. Chronic heat stress is commonly experienced in nature and is one of the major adverse factors that cause serious loss of yield in crops (Devasirvatham et al., 2012). Considering the impact of global warming and the expectation that the number of days of moderate heat will increase in the future (Song et al., 2010; Wagner, 1996), it is ecologically and agriculturally important to perform broad investigations of the strategies by which plants adapt to chronic heat stress and to examine wild species with genetic elements involved in tolerance to moderate heat.

Common responses of plants to heat stress include the inhibition of growth and photosynthesis, the reduction of water content and membrane stability, increases in reactive oxygen species (ROS) and heat shock proteins (HSPs), and changes in hormone levels and microtubules (Wahid et al., 2007). However, the responses to heat shock and long-term moderate heat involve different mechanisms and plants use different adaptation strategies to cope with them (Blum, 1988). For example, heat shock can cause the collapse of cellular organisation, which results in cell death within minutes (Schöffl F, 1999). In contrast, moderately high temperatures result in the gradual denaturation and aggregation of proteins and cell death only occurs eventually after long-term exposure (Howarth, 2005). Heat shock leads directly to severe damage to photosystem II (PSII) (Long et al., 1988; Sharkey, 2005; Yamane et al., 1998), whereas moderate heat stress reduces the activity of PSII while enhancing

the cyclic flow of electrons in PSI (Bukhov et al., 1999; Pastenes and Horton, 1996; Sharkey, 2005; Zhang and Sharkey, 2009). In *Wedelia trilobata*, a tropical invasive species, the photosynthetic apparatus remains stable and energy partitioning in PSII is maintained effectively under long-term moderately high temperatures (Song et al., 2010).

Our understanding of the responses of plants to long-term moderate heat is still relatively limited compared to those involved in heat shock. Plants have two phases during their heat-shock response: first, they acclimate at non-lethal heat doses and then tolerate to lethal heat (Wahid et al., 2007). HSPs are one of the major tools used by plants to resist heat stress and they basically function in the folding and assembly of proteins (Queitsch et al., 2000). During heat shock, the levels of HSPs fluctuate in accordance with rapid changes of temperature over the course of a day, which provides spatial and temporal protection for proteins and membranes against heat-induced damage (Latijnhouwers et al., 2010; Nakamoto and Hiyama, 1999; Nieto-Sotelo et al., 2002; Queitsch et al., 2000; Schöffl F, 1999). However, given that the synthesis of HSPs is energy-intensive (Al-Whaibi, 2011), it is challenging for plants to use HSPs continuously for a long period, such as days or weeks. In fact, few studies have evaluated the pattern of expression of HSPs during long-term exposure to moderate heat. One specific question that arises in this context is whether the expression of HSPs is induced and maintained for the entire period of exposure to moderate high temperature, or whether their levels decrease some time before the temperature returns to normal.

Membrane stability is another vital factor that affects plant thermotolerance (Blum, 1988). Plants remodel the composition of their membrane lipids to adapt to heat stress. For example, they increase the level of the plastidic lipid digalactosyldiacylglycerol (DGDG) (**1-19**) and the ratio of DGDG to monogalactosyldiacylglycerol (MGDG) (**20-38**) to resist both heat shock (Chen et al., 2006) and long-term moderate heat (Su et al., 2009). The induction of high levels of

phosphatidic acid (PA) (**58-76**) is associated with thermostability in maize (Chen et al., 2010). A low level of unsaturated membrane lipids also promotes resistance to heat shock in tobacco (Murakami et al., 2000). However, unsaturated lipids seem to have conflicting roles in different species in terms of the response to long-term moderate heat. An increase in unsaturated lipids has a negative effect on thermotolerance in turfgrass (Su et al., 2009), but a positive effect in *Arabidopsis* (Routaboul et al., 2012). Consequently, it is particularly important to characterise the dynamic changes in membrane lipids that occur during long-term exposure to moderate heat and determine whether they affect thermotolerance.

A. paniculata is a typical alpine plant and is a perennial relative of annual *A. thaliana* (Bohlenius et al., 2006; Koch et al., 1999). It was found that *A. paniculata* has a very strong tolerance to heat shock and can survive exposure at 52°C for two hours (Tang et al., 2014). In the current study, resistance to long-term moderate heat between *A. paniculata* and *A. thaliana* was characterised and compared. Both species exhibited two clear phases, in terms of damage symptoms and changes in photosynthesis, lipid peroxidation, soluble sugars and HSP levels, during long-term moderate heat stress. Though *A. paniculata* was much more thermotolerant than *A. thaliana*, a symptom difference in heat resistance was observed only during the later phase. Nevertheless, further investigation on lipid molecular species showed that the two species had different remodelling of membrane lipids during the early phase and the remodelling patterns significantly correlated with their thermotolerance. These results reveal a strategy by which plants can adapt to long-term moderate heat, and suggest that *A. paniculata* could be a model plant to study resistance to chronic heat stress.

2. Results and Discussion

2.1 Resistance to long-term moderate heat in *A. thaliana* and *A. paniculata*

To investigate the resistance of *A. thaliana* and *A. paniculata* during long-term

exposure to moderate heat, growth between these two species was compared at 38°C and 35°C. The leaf temperature of both species was slightly lower than air temperature and the leaf temperature of *A. paniculata* was slightly higher than that of *A. thaliana* (Table S1). During exposure to 38°C (Fig. 2), the leaves of *A. thaliana* exhibited no observable symptoms after one day and only slight wilting after two days. However, the leaves were shrivelled markedly at 4 d and had died by 8 d. In contrast, the leaves of *A. paniculata* were not affected until 4 d, then were shrivelled at 8 d, and had become more severely so at 12 d. At 35°C (Fig. 3), the leaves of *A. thaliana* began to show yellowing at 8 d and they were severely withered at 14 d. At 22 d, *A. thaliana* had died. In contrast, *A. paniculata* showed slight yellowing at the edges of their leaves at 8 d and survived until 22 d. These results indicate that *A. thaliana* is more sensitive and *A. paniculata* is more tolerant to long-term moderate heat stress and suggest that these plants have two phases of response to moderate heat, although their durations differ. The early phase takes approximately one third of the duration. During the early phase, no visible symptoms of stress appear; the duration of this phase becomes longer as the stress is attenuated. During the later phase, plants start to wilt and/or die; the duration of this phase is reduced as the stress is increased.

2.2 Two phases of response to long-term moderate heat in *A. thaliana* and *A. paniculata*

To characterise further the responses of *A. thaliana* and *A. paniculata* to long-term moderate heat, photosynthesis in plants grown at 35°C for 17 d was examined. During the first four days, the levels of photosynthesis were the same and normal, namely, the value of F_v/F_m of both species was approximately equal to 0.8 (Fig. 4). In addition, the actual photochemical efficiency ($Y(II)$) and the electron transfer rate (ETR) and the yield of regulated energy dissipation [$Y(NPQ)$], showed no apparent difference between the two species and was equal to that measured under non-stressed conditions. In other words, during the first 4 d at 35°C, photosynthesis was not affected. These results indicate that the physiological status of these two species was normal and the same during this phase, which is consistent with the phenotypic

observations mentioned above (Fig. 3). However, after exposure to heat stress for 4 d, the levels of photosynthesis in *A. thaliana* and *A. paniculata* started to diverge (Fig. 4). Photosynthesis in *A. thaliana* declined dramatically after 4 d and almost completely ceased to function at 10 d; in contrast, photosynthesis in *A. paniculata* remained unchanged until 8 d and then declined significantly. For example, in *A. thaliana*, the value of F_v/F_m decreased to 0.3 and eventually reached almost zero, whereas in *A. paniculata* it remained at 0.8 from 0 d until 8 d and then decreased from 0.8 to zero at 17 d. The change trend of $Y(II)$ and ETR of both plants was generally similar with F_v/F_m . In addition, $Y(NPQ)$ basically declined in *A. thaliana* from 6 d until 17 d, whereas the level was maintained in *A. paniculata* over this course. Given that a high value of $Y(NPQ)$ indicates effective heat dissipation in plants (Kato et al., 2003; Korniyev et al., 2004), this suggests that *A. paniculata* showed a strong capacity to protect its photosynthetic apparatus during the later phases of long-term exposure to moderate heat. All of these results confirm that *A. paniculata* is more tolerant to moderate heat than *A. thaliana*, and that there are two physiological phases in terms of the responses of these plants to such stress. In these phases, physiological damage is exhibited mainly during the later phase, and the early phase is longer in plants that are more tolerant to heat.

Given that heat stress is associated closely with oxidative, osmotic, and other environmental stresses under field conditions (Song et al., 2010), the changes in membrane lipid peroxidation and soluble sugars that occurred in *A. thaliana* and *A. paniculata* were then examined during exposure to moderate heat (Fig. 5). The levels of both malondialdehyde (MDA) (172) and soluble sugars (173) were the same in these two species and did not change during the first 4 d at 35°C. However, the levels of MDA (172) and soluble sugars (173) increased at 6 d in *A. thaliana*, but not until 10 d in *A. paniculata*. These results indicate that *A. paniculata* was less stressed than *A. thaliana* and again confirmed the presence of two phases of biochemical responses to long-term moderate heat. Overall, these lines of evidence support all of the

conclusions that were derived from the observation of plant growth and the physiological analysis.

2.3 The induction and maintenance of HSPs during exposure to moderate heat in *A. thaliana* and *A. paniculata*

The expression of HSP101 and HSP70 at a high temperature of 35°C were measured (Fig. 6). The two HSPs play major roles in the ability of plants to tolerate heat stress (Korotaeva and G B, 2001; Nakamoto and Hiyama, 1999). Under normal growth conditions, the levels of expression of the two proteins were low. Upon heat stress, the levels of both HSPs increased and plateaued at the early phase and then decreased gradually at the later phase in *A. thaliana*. The levels of the HSPs increased and were maintained in *A. paniculata*. The remarkable loss of HSPs at the end of later phase in *A. thaliana* could be due to their degradation as the plants were going to die. The long maintenance of high HSP levels in *A. paniculata* verified its tolerance to moderate heat. These results suggest that HSPs are induced and reach their highest levels at early phase and that HSPs can be maintained during whole period of long-term moderate heat.

2.4 Changes in membrane lipid composition during long-term exposure to moderate heat in *A. thaliana* and *A. paniculata*

To investigate the basis of the difference in resistance to long-term exposure to moderate heat between *A. thaliana* and *A. paniculata* and the roles of the two physiological phases, the dynamic changes in membrane lipids that occurred during long-term exposure to moderate heat were determined. The molecular species of membrane glycerolipids in the leaves of *A. thaliana* and *A. paniculata* that had been grown at 35°C for 22 d were thus profiled. 171 molecular species of membrane glycerolipids were identified, which belonged to two classes of galactolipid, six classes of phospholipid, and one class of neural glycerolipid diacylglycerol (DAG) (153-171) (Fig1, Fig. 7 and Table S2). There were significant differences in lipid species between both species and among treatments. For examples, PG (39-57)

composition showed that the two most abundant species were 34:1 (**39**) and 34:2 (**40**) in *A. paniculata* but were 34:4 (**42**) and 34:3 (**41**) in *A. thaliana*; plastidic lipids MGDG (**20-38**) and DGDG (**1-19**) levels also dramatically decreased as moderate heat progressed.

Next, a principal component analysis (PCA) on the relative contents (mol%) of the nine classes of lipid was conducted (Fig. 8). The changes in composition were extracted into two principal components, which represented 85.65% of the variance (Supplementary Table S3 and S4). Principal component 1 explained 57.02% of the variance and it was mainly contributed to by phosphatidylcholine (PC) (**77-95**) and phosphatidylinositol (PI) (**134-152**). By contrast, principal component 2 explained 28.63% of the variance and it was mainly contributed to by plastidic lipids, phosphatidylglycerol (PG) (**39-57**), and DGDG (**1-19**). Score plots of the two components were then used to determine the changes in lipid composition that occurred in response to long-term exposure to moderate heat (Fig. 8 and Supplementary Table S5). Under normal growth conditions, the plotted points associated with the two plant species were separated by both principal components 1 and 2, which indicated that the initial composition of membrane lipids differed between *A. thaliana* and *A. paniculata*. In both species, the trends in the changes in composition showed a clear turning point. This indicated that the changes in lipid composition showed two phases in both *A. thaliana* and *A. paniculata*. The timing of these two phases matched those of the physiological phases described above. In the principal component plots, the orientation of the plotted points that represented the compositional changes in the two plant species were opposite, i.e. from upper right to lower left in *A. thaliana*, meaning its composition of PC (**77-95**), PI (**134-152**), PG (**39-57**) and DGDG (**1-19**) decreased, while from the lower left to upper right in *A. paniculata*, meaning composition of PC (**77-95**), PI (**134-152**), PG (**39-57**) and DGDG (**1-19**) increased. This indicates that, during the early phase, both *A. thaliana* and *A. paniculata* remodelled their lipid composition to cope with moderate heat, but in distinct ways. In the principal component plots, the points representing the

composition of 0 d and 22 d were less separated in *A. paniculata* than that in *A. thaliana*. This indicates that, over the course of the experiment, the lipid composition was basically maintained in *A. paniculata*, but was altered dramatically in *A. thaliana*. These results also suggest that, in response to moderate heat, *A. thaliana* and *A. paniculata* adopted different strategies in terms of modifying their lipid compositions during the early phase, and these strategies had different consequences on the maintenance of their lipid compositions during the later phase. Specifically, the former fails to adapt successfully to moderate heat, but the latter is successful.

2.5 Changes in individual membrane lipids during long-term exposure to moderate heat in *A. thaliana* and *A. paniculata*

Next, the changes in the absolute levels (nmol/mg dry weight) of individual membrane lipids during long-term exposure to moderate heat were investigated (Supplementary Table S6). The fold changes relative to levels at 0 d are shown in Fig. 9. In *A. thaliana*, levels of all lipid classes declined throughout the entire period of exposure to stress, except for PA (**58-76**) and DAG (**153-171**), which increased dramatically during the later phase. Combining the fact that the majority of lipids decreased during both phases, and that PA (**58-76**) and DAG (**153-171**) are intermediates of lipid degradation and their increase reflects lipid degradation (Testerink and Munnik, 2005), it is not surprising that the latter occurs during the later phase. This agrees with the observation that *A. thaliana* was damaged seriously or fatally during the later phase. In *A. paniculata*, the changes in the levels of all the individual lipids differed from those in *A. thaliana*, except for MGDG (**20-38**). For example, during the early phase, the levels of PG (**39-57**), PC (**77-95**), phosphatidylethanolamine, PI (**134-152**), and phosphatidylserine (**115-133**) increased in *A. paniculata*, in contrast to their decrease in *A. thaliana*. This suggests that lipid synthesis took place during the early phase in *A. paniculata*. During the later phase, PA (**58-76**) and DAG (**153-171**) were maintained at low levels, which indicated that no lipid degradation occurred. Overall, these results show that, during long-term exposure to moderate heat, *A. thaliana* and *A. paniculata* exhibited differences in their

lipid metabolism. Specifically, in *A. paniculata*, synthesis was performed during the early phase and no degradation occurred during the later phase. This could ensure that membranes in this species are tolerant to long-term exposure to moderate heat.

2.6 Changes in the degree of lipid unsaturation during long-term exposure to moderate heat in *A. thaliana* and *A. paniculata*

Then, the degree of unsaturation of membrane lipids was calculated (Supplementary Table S7), as indicated by the DBI (double bond index) (Rawyler et al. 1999), to examine how it responded to long-term exposure to moderate heat (Fig. 10). Under normal growth conditions, the DBIs of the two species were the same. Upon exposure to moderate heat, during the early phase, the DBI of the lipids, except for DAG (**153-171**), basically decreased in both species, but the decrease was significantly greater in *A. paniculata* than in *A. thaliana*. During the later phase, the DBI of the lipids continued to decrease in *A. paniculata*, but in *A. thaliana*, this decrease stopped or the value even increased. For example, the DBI of PG (**39-57**) decreased, that of PC (**77-95**) was maintained, and that of PE (**96-114**) increased in *A. thaliana*. This diversity in the trends in changes in DBI could be due to lipid degradation. These results indicate that both species lower the degree of lipid unsaturation in response to long-term moderate heat and suggest that the adjustment of the degree of lipid unsaturation occurs earlier and more intensively in *A. paniculata* than in *A. thaliana*. This could be one of the reasons for the higher thermotolerance of the former as compared with the latter.

3 Conclusions

The different speed of stresses results in different cellular responses in plants. According to this view, acute and chronic heat stress are two distinct processes. Long-term moderate heat progresses slowly enough that adaptation and resistance mechanisms in plants can follow. Therefore, characterization of the dynamic changes

in the process at physiological and biochemical levels is particularly important to understand the nature of long-term moderate heat. Using the model plant *A. thaliana* and its relative *A. paniculata* as examples, the responses of plants to long-term moderate heat step by step in terms of growth, and at the physiological and biochemical levels were comparatively examined. Our results show that plants undergo complex changes in order to adapt to long-term exposure to moderate heat. The two species differed in terms of their adaptive mechanisms, which may result in different levels of resistance.

Our major conclusions are as follows:

- 1) *A. paniculata* was more tolerant than *A. thaliana* to long-term exposure to moderate heat.
- 2) The responses of *A. thaliana* and *A. paniculata* to long-term exposure to moderate heat exhibited two phases. The characteristics of these phases were related to the thermotolerance of these plants. In particular, a longer early phase was associated with greater thermotolerance.
- 3) The levels of expression of HSP101 and HSP70 showed an increase, maintenance, and then a decrease during long-term exposure to moderate heat. In *A. paniculata*, the HSPs were maintained at the higher level for longer than in *A. thaliana*.
- 4) *A. paniculata* and *A. thaliana* exhibited different strategies in terms of the adjustment of their membrane lipid composition in response to long-term moderate heat. The former was able to maintain its lipid composition, whereas the latter showed lipid degradation. The membrane lipids were adjusted mainly during the early phase.
- 5) Both plant species reduced the degree of lipid unsaturation in response to long-term moderate heat. However, this occurred more rapidly and for longer in *A. paniculata* than in *A. thaliana*.
- 6) *A. paniculata* could be a suitable model plant for the study of the consequences of long-term exposure to moderate heat.

The intriguing questions raised from our results are: first, why do plants have two phases to respond to long-term moderate heat, and what are the roles of these two

phases. In nature, the responses of plants to temperature stress are usually exhibited in multiple phases. For example, in response to the progress of low temperature stress, plants experience three phases: cold-acclimation at chilling temperature during autumn, freezing at sub-zero temperature during winter, and de-acclimation when weather becomes warm in spring (Thomashow, 2001; Streb et al., 2003). In response to high temperature shock, plants first acclimate at non-lethal heat and then tolerate extreme heat (Wahid et al., 2007). The role of the first phase in these responses is acclimation, which is thought to be an active process because plants spontaneously accumulate resources to resist severe temperature stress in later phase (Larkindale et al., 2005; Cook et al., 2004). Our results establish that during long-term moderate heat, plants have adjustments in early phase and the adjustments positively correlate to their thermotolerance. Therefore, plants might have two phases in active and passive responses to long-term moderate heat, respectively. The response in the first phase could function as acclimation.

The thermotolerance to acute and chronic heat stress can be characterized by survival rate, photosynthesis, HSP expression and changes of membrane lipids, but their molecular mechanisms are thought to be different (Blum, 1988). The characterization in our previous and present studies demonstrate that *A. paniculata* is able not only to tolerate to extreme high temperature shock (Tang et al., 2014) but also to long-term moderate heat. Thereby questions left open are whether and how the thermotolerance of acute and chronic heat stress in *A. paniculata* are mechanically linked. Future exploration of these questions will promote *A. paniculata* to be a model plant for studying the mechanism of plant thermotolerance.

4 Experimental

4.1 Ethics statement

The habitat of *A. paniculata* used in this study is neither a site of conservation of the natural environment nor a private location. Access to this area did not require

permission, and the species is not protected by Chinese law up to now.

4.2 Plant materials and heat treatment

Arabis paniculata, a relative of the model plant *A. thaliana* (Koch et al., 1999), grows on Yulong Snow Mountain in Lijiang, Yunnan, China. Seeds of *A. paniculata* were collected from its original habitat (according to voucher specimens Kun0495309, Herbarium, Kunming Institute of Botany). Seeds of *A. thaliana* were of the Columbia ecotype and collected from plants bred in our experimental lab. For surface sterilisation, the seeds of these two species were washed with EtOH: H₂O (75:25 v/v) and NaOCl (5% v/v) for 2 min each, followed by three washes with sterile distilled H₂O. The sterilised seeds were kept at 4°C for 5 d and then sown on 1/2 Murashige and Skoog medium for two weeks at 22°C under 120 $\mu\text{mol}\cdot\text{photons m}^{-2}\cdot\text{s}^{-1}$ light. Subsequently, seedlings were moved to boxes with soil for another three week. Seedlings that had been grown in soil for three weeks were placed in a growth chamber at 35°C or 38°C under 80 $\mu\text{mol}\cdot\text{photons m}^{-2}\cdot\text{s}^{-1}$ light (12 h light/12 h dark) for long-term moderate heat treatment.

4.3 Measurement of chlorophyll fluorescence

Chlorophyll (Chl) fluorescence parameters were measured at room temperature at 0, 1, 4, 6, 8, 10, 12, and 17 d during exposure to moderately high temperature using an IMAGING-PAM chlorophyll fluorometer and the Imaging Win software application (Walz, Effeltrich, Germany) in accordance with Woo et al., 2008. A dark–light induction curve was established to assess parameters associated with adaptation to dark and light conditions. The value of F_v/F_m was obtained after leaf samples had been allowed to adapt to the dark for 15 min and were then exposed to a saturating pulse ($>1,800 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). False-colour images of the F_v/F_m parameter were produced using Imaging Win software. This was followed by a 40-second exposure to darkness, and then 6 min of actinic illumination ($111 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) with saturating flashes at 20-second intervals. This actinic intensity simulated growth conditions. The actual quantum yield of PSII photochemistry [$Y(II)$], the yield

of regulated energy dissipation [$Y(NPQ)$], and the electron transfer rate (ETR) were obtained after allowing the plants to adapt to the light conditions for 6 min before performing the final measurements.

4.4 Detection of lipid peroxidation and soluble sugars

The level of MDA (**172**) has been used routinely to indicate the extent of lipid peroxidation in leaves. After heat treatment, samples were collected and the MDA (**172**) level was measured as described previously with slight modification (Mishra and Singhal, 1992). Briefly, leaves that had been frozen in N_2 were ground, homogenised with 5 ml of 10% (w/v) trichloroacetic acid (TCA), and centrifuged at 4226 g for 10 min. An aliquot of the supernatant (1 ml) was mixed with thiobarbituric acid solution (1 ml, 0.6% [w/v] in 10% TCA). The mixture was incubated at 95°C for 20 min, cooled quickly in an ice bath, and then centrifuged at 4226 g for 10 min. The absorbance of the supernatant was measured at 450 nm, 532 nm, and 600 nm. The concentration of MDA was calculated as follows: $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$. The concentration of soluble sugars (**173**) was calculated as follows: $11.71 \times A_{450}$.

4.5 Protein extraction and immunoblotting of HSPs

Leaf samples were obtained at intervals of two to three days from seedlings that had been grown in soil for three weeks at 35°C. Total protein from *A. paniculata* and *A. thaliana* leaves was isolated with protein extraction buffer 0.5 ml (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with beta-mercaptoethanol 2 μ l. After centrifugation at 5752 g for 10 min, the concentration of protein in the supernatant was determined by measuring the binding of Coomassie Brilliant Blue at 595 nm (Fan et al., 1997). For each sample, the same amount (15 μ g/lane) of total protein was separated by dodecyl sulphate sodium salt-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride filters. These filters were incubated with antibodies against HSP101 and HSP70, and then incubated with a secondary antibody conjugated to

alkaline phosphatase. The HSP-alkaline phosphatase conjugates were visualised by staining the blot for phosphatase activity. Actin was used as loading control and its second antibody was horse radish peroxidase conjugated goat anti-mouse and detected by an enhanced chemiluminescent kit (Thermo Fisher Scientific, USA).

4.6 Lipid extraction, ESI-MS/MS analysis, and data processing

Lipids were extracted in accordance with a previous report with minor modification (Wolti et al., 2002). Leaves of five different plants were harvested at the indicated times and then immersed immediately in *i*-PrOH 3 ml with 0.01% butylated hydroxytoluene at 75°C in a water bath to inhibit lipolytic activity. After 15 min, CHCl₃ (1.5 ml) and H₂O (0.6 ml) were added to glass tubes, which were then shaken for 1 h. The leaves were extracted with CHCl₃: MeOH (2:1) and then agitated gently for 12 h. This procedure was repeated three times until the leaves became white. The remaining leaf materials were heated overnight at 105°C and weighed to give the dry weight of the plants. The extracts were combined and evaporated under N₂. A triple quadrupole MS/MS equipped for ESI was used to analyse the lipid samples. The data were processed as described previously (Wolti et al., 2002). The lipids of each lipid class were quantified by comparison to two internal standards for that class. Five replicates of each treatment were analysed for each plant. Data from discordant samples were removed using the Q-test carried out on the total amount of lipid in each class (Wolti et al., 2002). The data were analysed by one-way analysis of variance with SPSS 19.0. Statistical significance was detected by Fisher's least significant difference method. Double bond index (DBI) was calculated using the following formula: $DBI = (\sum [N \times \text{mol \% fatty acid}]) / 100$, where N indicates the number of double bonds in each fatty acid molecule, in accordance with a previously described method (Zheng et al., 2011). Principal component analysis (PCA) was conducted with "Factor Analysis" after "Data Reduction" using Statistical 13 Product and Service Solutions (SPSS) version 19.

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Figure Legends

Fig. 1. Representative structures from each lipid class. Provided for each compound is the lipid class (total acyl carbons: total double bonds) [Lipid class (sn-1 acyl carbons: sn-1 double bonds/sn-2 acyl carbons: sn-2 double bonds)] *individual compound number* (refer to Supplementary Table S2)

Fig. 2. Effects of moderate heat stress on the growth/development of *A. thaliana* and *A. paniculata*. Photographs of 3-week-old soil grown seedlings were taken before heat treatment (0 d) and after 1 d, 2 d, 4 d, 8 d, and 12 d at 38 °C. Each measurement was repeated independently at least three times.

Fig. 3. Effects of moderate heat stress on the growth/development of *A. thaliana* and *A. paniculata*. Photographs of 3-week-old soil grown seedlings were taken before heat treatment (0 d) and after 2 d, 8 d, 14 d and 22 d at 35 °C. Each measurement was repeated independently at least three times.

Fig. 4. Changes in the maximal photochemical efficiency of PSII (F_v/F_m), the actual photochemical efficiency ($Y(II)$), the yield of regulated energy dissipation($Y(NPQ)$) and the total electron transport rate (ETR) of *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. After treatment for 1 d, 4 d, 6 d, 8 d, 10 d, 12 d and 17 d, the leaves were measured by IMAING-PAM chlorophyll fluorometer for photosynthetic activity assay. There were five replicates for each soil pot, and the experiment repeated at least three times. Values are mean $\pm$ S.D, $n = 15$.

Fig. 5. The content of MDA (**172**) and soluble sugar (**173**) of *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. After treatment for 1 d, 4 d, 6 d, 8 d, 10 d, 12 d and 17 d, the leaves were harvested for biochemical analysis. There were five replicates for each soil pot, and the experiment repeated at least three times. Values are mean $\pm$ S.D, $n = 15$.

Fig. 6. The accumulation of HSPs of *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. Total proteins from 3-week-old seedlings grown in the soil exposed to 35 °C for 2 d, 4 d, 6 d, 8 d, 12 d, 16 d and 20 d or 32 d were electrophoretically separated on dodecyl sulphate sodium salt-polyacrylamide gel, transferred onto polyvinylidene difluoride filters, and reacted with antibodies against HSP101 and HSP70. The lanes of HSPs were visualized by staining the blot for phosphatase activity. Actin was used as loading control. Each experiment was repeated at least three times.

Fig. 7. Changes in the molecular species of membrane lipids in *A. thaliana* and *A. paniculata* under long term moderate high temperature. After treatment for 2 d, 8 d, 14 d and 22 d at 35°C, the leaves were harvested for lipidomic analysis. The values are means $\pm$ S.D. ($n = 3, 4$ or 5). “*” indicate the content of molecular species of membrane lipids under moderate heat is significantly different from control.

Fig.8. Principal component analysis (PCA) score plot of the membrane lipid classes in *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. After treatment for 2 d, 8 d, 14 d and 22 d, the leaves were harvested for lipidomic analysis. “AT” stands for *A. thaliana*, and “AP” stands for *A. paniculata*.

Fig. 9. The changes of membrane lipid levels in *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. After treatment for 2 d, 8 d, 14 d and 22 d, the leaves were harvested for lipidomic analysis. The fold changes were calculated using the average amount of total membrane lipids of each class with the formula: $(A2-A1)/A1$, where A2 is the total amount of membrane lipids at a particular day at 35°C and A1 is the total amount of membrane lipids before moderate heat treatment. Solid line stands for *A. thaliana*, and dot line stands for *A. paniculata*. “*” indicate the content of membrane lipids under moderate heat is significantly different from control

in *A. thaliana*. “^” indicate the content of membrane lipids under moderate heat is significantly different from control in *A. paniculata*.

Fig. 10. The changes of double-bond index (DBI) of the membrane lipids in *A. thaliana* and *A. paniculata* during moderate heat treatment at 35°C. After treatment for 2 d, 8 d, 14 d and 22 d, the leaves were harvested for lipidomic analysis. $DBI = (\sum[N \times \text{mol\% lipid}])/100$, where N is the total number of double bonds in the two fatty acid chains of each glycerolipid molecule. The fold changes were calculated using the average DBI of total lipids of each class with the formula: $(A2-A1)/A1$, where A2 is DBI of membrane lipids at a particular day at 35° C and A1 is DBI of membrane lipids before moderate heat treatment. Solid line stands for *A. thaliana*, and dot line stands for *A. paniculata*. “*” indicate the DBI of membrane lipids under moderate heat is significantly different from control in *A. thaliana*. “^” indicate the DBI of membrane lipids under moderate heat is significantly different from control in *A. paniculata*.

Figure 1

DGDG(36:6) [DGDG(18:3/18:3)] 11

PG(34:2) [PG(18:2/16:0)] 40

PC(34:2) [PC(16:0/18:2)] 78

PS(36:4) [PS(18:2/18:2)] 123

DAG(34:1) [DAG(18:1/16:0)] 153

MGDG(34:6) [MGDG(18:3/16:3)] 25

PA(36:5) [PA(18:2/18:3)] 67

PE(42:2) [PE(24:0/18:2)] 112

PI(34:2) [PI(16:0/18:2)] 135

MDA 172

Glucose 173

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10