Respiration responses of wheat seedlings to treatment with trehalose under heat stress

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Abstract

Heat stress limits wheat production and trehalose can improve stress tolerance. How trehalose affects wheat respiration is unclear. In this study, we investigated the effects of exogenous trehalose on the respiration of wheat seedlings during heat stress and the subsequent recovery period. Trehalose pretreatment significantly increased the expression of the alternative oxidase genes AOX1a and AOX1c under heat stress, indicating that trehalose pretreatment increased the capacity of the alternative respiration pathway (AP) in response to heat stress. Trehalose pretreatment also enhanced the activity of the malate-oxaloacetate (Mal-OAA) shuttle and ameliorated the decrease in photosynthetic activity caused by heat stress. However, when the AP was inhibited by salicylhydroxamic acid under heat stress, both Mal-OAA shuttle activity and photosynthetic efficiency were substantially reduced in the control and trehalose pretreatment groups. In addition, trehalose pretreatment helped to maintain inner mitochondrial respiratory activity and the activity of Complex II during heat stress, particularly the coupling of oxidative phosphorylation with the Complex II electron transport chain, thereby mitigating heat-related damage to the cytochrome pathway (CP). Taken together, these results suggest that exogenous trehalose enhanced the AP and reduced damage to the CP under heat stress in wheat seedlings, thus maintaining cellular energy metabolism. Up-regulation of the AP by trehalose pretreatment may improve the heat tolerance of wheat seedlings by dissipating excess reducing equivalents transported through the Mal-OAA shuttle, thereby protecting photosynthetic performance.

Keywords: alternative respiration pathway, heat stress, malate-oxaloacetate shuttle, photosynthetic efficiency, trehalose, *Triticum aestivum*, wheat.

Introduction

During this century, ambient temperatures have increased steadily and are expected to rise continuously due to climate change (Intergovernmental Panel on Climate Change 2008). Heat stress has therefore become a global problem for organisms. This is especially true for plants such as wheat because heat stress affects its photosynthesis, respiration, and mineral absorption, ultimately reducing its yield and quality. According to one report, heat disasters occur frequently in the north of China and the Yangtze River region, reducing wheat production by 10 - 20 % (Jin 1983). Increasing the thermal tolerance of wheat is an important challenge for continued wheat cultivation under

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Abbreviations: AOX - alternative oxidase; AP - alternative pathway; CK - control; Complex I - NADH dehydrogenase complex; Complex II - succinate dehydrogenase complex; CP - cytochrome pathway; ETC - electron transport chain; F0 - minimal fluorescence yield of the dark-adapted state; Fm - maximum fluorescence; Fv/Fm - variable to maximum fluorescence (maximum efficiency of photosystem II photochemistry); LED - light-emitting diode; Mal - malate; NAD-MDH - NAD-malate dehydrogenase; NADP-MDH - NADP-malate dehydrogenase; OAA - oxaloacetic acid; RCR - respiration control rate; ROS - reactive oxygen species; SHAM - salicylhydroxamic acid; TRE - trehalose.

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the present conditions of global climate change.

Respiration and photosynthesis, two basic metabolic processes in plants, are easily affected by environmental conditions (Wahid et al. 2007). Under stress conditions, excess of reducing equivalents in the chloroplasts can cause over-reduction of the photosynthetic electron transport chain (ETC) and the accumulation of reactive oxygen species (ROS), leading to photoinhibition (Zhang et al. 2012, Wang et al. 2016). However, the relationships between respiration and plant stress resistance are not fully understood. It has been reported that heat-tolerant wheat cultivars showed greater respiratory homeostasis than heat-susceptible cultivars under heat stress, while heat-susceptible cultivars showed significant increases in respiration (Almeselmani and Viswanathan 2012). Stress can directly or indirectly affect plant mitochondrial function, inhibiting many important enzymes in the mitochondrial ETC and resulting in the uncoupling of electron transport from oxidative phosphorylation (Zhang et al. 1990). In plant mitochondria, the ETC is mainly divided into the cytochrome pathway (CP) and the alternative pathway (AP), whose electron acceptor is the alternative oxidase (AOX) (Vanlerberge and McIntosh 1997). The AP can be activated rapidly when plants such as tobacco are affected by adverse conditions (Yip and Vanlerberge 2001), and changes in AOX activity in cauliflower mitochondria were reported to result from the effects of temperature stress on mitochondrial respiratory chain activity and ATP synthesis rate (Rurek et al. 2015). Møller (2001) proposed that the AP is related to plants’ responses to oxidative stress because it reduces ROS production under oxidative stress, and Wang et al. (2016) suggested that the AP is an important means for dissipation of excess reducing equivalents from the chloroplast. The malate-oxalacetic acid (Mal-OAA) shuttle is thought to be the means by which excess reducing equivalents are moved from the chloroplast to the mitochondrion under heat stress, where they are consumed by increased activity of the AP (Meng et al. 2013).

Trehalose, a non-reducing disaccharide, is widespread throughout a variety of organisms. When plants such as rice and wheat seedlings are exposed to stress conditions, trehalose protects their cellular components, including biological macromolecules (e.g., proteins) and cell membranes, due to its unique physical properties (Luo et al. 2010, Abdallah et al. 2016). Previously, we found that exogenously supplied trehalose protected the photosynthetic apparatus and photosynthetic activity in multiple ways under heat stress, including protecting the thylakoid membrane structure, improving the activity of the antioxidant system, lowering the malondialdehyde content, and reducing ROS production (Luo et al. 2008, 2010). Trehalose is therefore considered to be a type of stress protectant (Sampedro et al. 2001, Luo et al. 2008). Because the AP can dissipate excess reducing equivalents transported through the Mal-OAA shuttle and thereby reduce ROS production, we wondered whether exogenously supplied trehalose could protect photosynthesis by increasing the AP capacity and the activity of the Mal-OAA shuttle. In addition, trehalose may also play a role in relieving heat-induced reductions in respiration by stabilizing the enzymes of the mitochondrial inner membrane and matrix. In the present work, we investigated how exogenously supplied trehalose affects the respiration of wheat seedlings under heat stress and how trehalose influences the relationship between chloroplasts and mitochondria.

Materials and methods

Plant growth and treatments: According to our previous methods (Luo et al. 2008), seedlings of wheat (Triticum aestivum L.) cv. Ning 13 grew in vitro in Hoagland solution, under a 13-h-photoperiod, an irradiance of 300 μmol(photon) m−2 s−1, and a temperature of 25 °C. When the second leaves were fully expanded, wheat seedlings were root-supplied with Hoagland solution containing 1.5 mM trehalose (TRE) for 72 h, with pure Hoagland solution as control (CK). After that, the seedlings were subjected to heat stress (40 °C for 24 h), and then they were kept at room temperature for another 24 h which was taken as recovery (R24).

Respiration rate measurement: The respiration rate was measured by a Clark oxygen electrode (Chlorololab-2, Hansatech, Norfolk, UK). The third leaves of wheat seedlings were cut into small pieces. After vacuuming the reaction medium (50 mM Tris-HCl buffer, pH 7.5), the small pieces of leaves were suspended in the oxygen electrode cuvette at 25 °C. All the respiration rates were measured and calculated according to Lei et al. (2010) with some modifications. The total respiratory rate (Vt) is defined as the O2 uptake rate without any inhibitors. Residual respiration (Vres) is defined as O2 uptake by leaves in the presence of both NaN3 (inhibitor for the CP) and salicylhydroxamic acid (SHAM, inhibitor for the AP). CP capacity (VCOP) is defined as the O2 uptake rate in the presence of 2 mM SHAM minus Vres. AP capacity (VAOX) is defined as Vt minus the O2 uptake rate in the presence of SHAM.

Extraction of mitochondria: Based on the method mentioned by Erdal et al. (2015), the extraction of mitochondria was improved slightly. Fresh leaves (5 g) were homogenized in 20 cm3 of extraction buffer containing 0.33 M sorbitol, 1 mM MgCl2, 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)/KOH (pH 7.8), 2 mM EDTA, 10 mM dithiothreitol (DTT), 0.1 % (m/v) bovine serum albumin (BSA), 5 mM sodium ascorbate, and 0.1 % (m/v) polyvinylpyrrolidone. The homogenate was filtered through four layers of gauze. Then the filtrate was centrifugated at 2 000 g for 10 min. The supernatant portion was taken and centrifuged at 10 000 g for 30 min. The pellet was homogenized in a wash buffer (0.3 M sorbitol, 1 mM MgCl2, 50 mM HEPES/KOH (pH 7.8), and 2 mM EDTA) and then centrifuged at 15 000 g for 15 min. The pellet was a crude mitochondrial pellet that was then gently homogenized in the wash buffer. The mitochondrial protein concentration was determined.
Mitochondria respiration measurement: The measurement of steady-state respiration rate was carried out by a Clark oxygen electrode (Chlorolab-2): 1 cm³ of mitochondrial proteins and 1 cm³ of wash buffer were added into the oxygen electrode cuvette at 25 °C to determine the respiration parameters. Based on the method mentioned by Liu et al. (2014), some modifications were made in the isolation protocols of mitochondria. Firstly, 10 mM (final concentration) α-ketoglutarate was added as the respiration substrate for Complex I to measure the State 4 respiration rate (S4), then 1 mM ADP (final concentration) was added to measure the State 3 respiration rate (S3). Thus, the respiration control rate (RCR) of Complex I was defined as S3/S4. Next, a final concentration of 5 mM succinic acid (respiration substrate for Complex) was added to measure the State 4 respiration rate (S4) of Complex II (S4'), then ADP was added to measure the State 3 respiration rate (S3'). The RCR of Complex II was defined as S3'/S4'.

Total RNA extraction and the synthesis of cDNA: According to the manual of the total RNA extraction kit (TakaRa, Dalian, China), the second fresh wheat seedling leaves were used for the total RNA extraction. The synthesis of cDNA was carried out in turn according to the manual of the cDNA synthesis kit (Yeason, Shanghai, China).

Quantitative real-time PCR (qPCR): According to the published mRNA sequence of wheat AOX1a (GenBank Accession AB078882.1) and AOX1c (GenBank Accession AB078883.1), a pair of specific primers were designed in the conserved region respectively (Table 1 Suppl.) and were synthesized by Songon corporation (Shanghai, China).

The qPCR was carried out in a real-time PCR system (CFX96, BioRad, Hercules, USA) according to the manual of the Hieff™ qPCR SYBR® Green Master Mix kit (Yeason). The amplification was carried out by a three-step procedure (pre-denaturation at 95 °C for 5 min; denaturation at 95 °C for 10 s; renaturation at 57 °C for 20 s; extension at 72 °C for 20 s) and this cycle was repeated for 40 times. Each template should have three repetitions. After this reaction, the dissociation curve was measured and analyzed. According to the Cq value, a 2^{-ΔΔCT} method was used to calculate the relative expression quantity of genes in each treatment group.

Alternative oxidase inhibition: Before high temperature (40 °C) treatment, the second and third leaves of wheat seedlings were harvested. The different volume of SHAM mother liquor (100 mM) was added to the Hoagland solution and Hoagland solution with trehalose to get the final concentrations of 0, 1, and 2 mM. After applying the solution from the roots for 4 h, the wheat leaves were exposed to a high temperature (40 °C) for 24 h. The SHAM inhibition concentration was determined by measuring Vt and VAOX of differently treated leaves (Wang et al. 2016).

Chlorophyll fluorescence measurements were carried out using Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany) and the parameters were assessed using the induction curve recording mode in the Dual-PAM software. A 620 nm light-emitting diode (LED) light and a 460 nm LED blue actinic light were delivered to the wheat seedlings. Additionally, saturation pulses of 10 000 μmol m⁻² s⁻¹ for 600 ms were applied to wheat seedlings dark-acclimated for 30 min and the minimal fluorescence (F₀), the maximum fluorescence (F₅₀), and variable fluorescence Fv (F₅₀ - F₀) were detected. The maximum efficiency of photosystem II was determined as Fv/F₅₀ (Kramer et al. 2004, Schreiber 2004).

Determination of NAD-malate dehydrogenase (NAD-MDH) and NADP-MDH activity: The extraction and activity determination of NAD-MDH and NADP-MDH was performed according to Millar and Leaver (2000) and Dutilleul et al. (2003) with some modification. Leaves (0.5 g) were homogenized in 25 mM HEPES-KOH (pH 7.5) buffer containing 10 mM MgSO₄, 1 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 % (m/v) insoluble polyvinylpyrrolidone, and 0.05 % (v/v) Triton X-100 at 4 °C. After centrifugation at 14 000 g for 5 min, the initial NAD-MDH and NADP-MDH activity in the supernatant were measured at 340 nm. The assay of NAD-MDH activity was performed in the reaction solution containing 25 mM Bicine-KOH (pH 8.0), 150 mM MgCl₂, 5 mM DTT, 0.2 mM NADH, and 2 mM oxalacetic acid (OAA). The assay of NADP-MDH activity was performed in the reaction solution containing 25 mM Tricine-KOH (pH 8.3), 150 mM KCl, 1 mM EDTA, 5 mM DTT, 0.2 mM NADPH, and 2 mM OAA.

Statistical analysis: All comparisons were analyzed by one-way analysis of variance (ANOVA), LSD, and Tukey’s test of multiple comparison analysis for the independent samples. In all cases, the confidence coefficient was set at 0.05. Values were expressed as means ± SEs from at least three independent experiments.

Results
Although no change in Vt was observed in control plants under heat stress (Fig. 1A), V₅ₒₓ was decreased and V₅ₒₓ was increased (Fig. 1B,C). When compared to heat-stressed seedlings without trehalose pretreatment, heat-stressed seedlings pretreated with trehalose exhibited higher Vt, V₅ₒₓ, and V₅ₒₓ (Fig. 1).
To further investigate the molecular mechanisms by which trehalose influenced respiration rate, we measured the transcription of AOX1a and AOX1c, two non-homologous genes in the wheat AOXI gene family that encode AOX proteins. Fig. 2 shows that trehalose pretreatment reduced the expression of AOX1a but increased the expression of AOX1c under normal temperature conditions. Under heat
stress, compared with the control, the expression of \textit{AOX1a}
in the trehalose-pretreated plants enhanced slightly, while the expression of \textit{AOX1c} was still significantly increased (Fig. 2).

The mitochondrial ETC has two transmission modes: Complex I/III/IV and Complex II/III/IV. Steady-state respiration results from isolated mitochondria suggested that trehalose pretreatment significantly enhanced the coupling process between mitochondrial ETC and oxidative phosphorylation under normal growth conditions (Complex I respiratory control ratio, RCR: 42.3 %; Complex II RCR: 21 % (Table 1). The inner respiration rate, the RCR of Complex I, and the RCR of Complex II were all decreased in control plants under heat stress (Table 1), consistent with the observed decline in Vcox (Fig. 1B). However, these parameters were significantly higher in the trehalose pretreated plants compared with the control plants under heat stress (Table 1). Similarly, during the subsequent recovery period, the degree of coupling between the ETC and oxidative phosphorylation was significantly enhanced in pretreated plants compared with controls.

After heat stress, \( \frac{F_v}{F_m} \) declined by 31.9 % in the control group and 22.9 % in the trehalose pretreatment group (Fig. 3), suggesting that exogenously supplied trehalose could partially alleviate the negative effects of heat stress on the photosynthetic activity of wheat seedlings. Moreover, when the AP of wheat seedling leaves was inhibited by SHAM (Fig. 1 Suppl.) under heat stress, \( \frac{F_v}{F_m} \) was markedly decreased and no significant differences were observed between the control and trehalose pretreatment groups (Fig. 3). These results indicate that the AP plays an important role in the maintenance of photosynthetic activity under heat stress and during the subsequent recovery.
The excess of reducing equivalents generated in chloroplasts under stress conditions can be transported to the mitochondria through the Mal-OAA shuttle (Noguchi and Yoshida 2008), in which NAD-MDH and NADP-MDH are the two key enzymes (Yoshida et al. 2007). After heat stress, the activities of NAD-MDH and NADP-MDH were increased by 65.4 and 84.5 % in the control group, and the trehalose-pretreatment group showed even greater increases (Fig. 4). After 24 h of recovery from heat stress, NAD-MDH initial activity was increased by 329.1 % in the heat-stressed controls compared with the unstressed controls, but trehalose pretreatment diminished this increase (Fig. 4). The increase in the initial activity of NADP-MDH in the control and trehalose pretreated plants was similar to the increase in NAD-MDH during the subsequent recovery period. When the AP was inhibited by SHAM under heat stress and during recovery, MDH initial activity was substantially reduced, suggesting that AP plays an important role in the regulation of NAD-MDH and NADP-MDH activities under heat stress (Fig. 4). Furthermore, in the trehalose-pretreated plants compared with the controls, MDH activity showed a greater decrease under heat stress after SHAM treatment (Fig. 4).

**Discussion**

**Trehalose and respiratory pathway changes:** Respiratory pathway changes have previously demonstrated the ability of the mitochondrial electron transport chain to switch flexibly from the phosphorylation pathway to the non-phosphorylation pathway under heat stress in order to alleviate excess reduction in the ETC and to reduce ROS production (Dinakar et al. 2016). This kind of flexibility was also observed in the present study. In particular, increased AP capacity (V_max) was accompanied by a decrease in CP capacity (V_OX) under heat stress (Fig. 1B,C). Compared with the control seedlings, trehalose pretreated wheat

Table 1. Effects of exogenous trehalose on inner respiration, steady-state respiration of Complex I and Complex II in wheat seedling leaves under different treatments. CK - control group; TRE - trehalose pretreatment; H - high temperature (40 °C) treatment for 24 h; HR24 - recovered for 24 h after high temperature; S3 and S4 - state respiration III and IV of Complex I; S3’ - state respiration III of Complex II; S4’ - state respiration IV of Complex I. Means ± SEs, different letters show significant differences (P < 0.05).

| Parameters | CK             | TRE            | CKH            | TREH           | CKHR24        | TREHR24       |
|------------|----------------|----------------|----------------|----------------|---------------|---------------|
| Inner respiration | 3.759±0.433b   | 4.653±0.544a   | 2.397±0.306c   | 4.414±0.321ab  | 4.111±0.497b  | 4.637±0.501a  |
| Complex I  |                |                |                |                |               |               |
| S4         | 3.805±0.399b   | 4.512±0.193a   | 4.256±0.522ab  | 3.859±0.584b   | 3.772±0.123b  | 4.174±0.469ab |
| S3         | 4.937±0.962ab  | 8.332±1.854a   | 4.789±0.892ab  | 6.992±2.301ab  | 3.813±0.157b  | 7.495±2.286ab |
| S3/S4      | 1.298          | 1.847          | 1.124          | 1.812          | 1.011         | 1.796         |
| Complex II |                |                |                |                |               |               |
| S4’        | 2.977±0.818ab  | 4.207±1.843a   | 2.836±0.512d   | 5.187±1.418a   | 3.494±0.847abc| 4.857±0.534ab |
| S3’        | 4.930±0.888bc  | 7.854±2.801a   | 2.889±0.546d   | 7.461±0.168ab  | 4.072±0.168ab | 6.538±1.071ab |
| S3’/S4’    | 1.541          | 1.867          | 1.019          | 1.439          | 1.165         | 1.346         |

Fig. 3. Effects of exogenous trehalose and SHAM on the maximum efficiency of photosystem II (F_v/F_m) in wheat seedling leaves under different treatments. The leaves were treated with 2 mM SHAM for 4 h under a light. CK - control group; TRE - trehalose pretreatment; H0 - without high temperature; H - high temperature (40 °C) treatment for 24 h; HR24 - recovery 24 h from high temperature (40 °C) treatment. Means ± SEs, n = 3; different letters indicate significant difference at P < 0.05.
seedlings showed less CP and more AP capacity under heat stress (Fig. 1B, C), which was confirmed by higher expression of AOX1 (Fig. 2). These results were similar to those of Rurek et al. (2015), who suggested that heat stress can increase the abundance of AOX protein and AOX1a mRNA. Because trehalose can act as a signaling molecule (Schluepmann et al. 2004), we propose that exogenous trehalose may affect the expression of AOX1 genes through a specific signaling pathway, thereby resulting in increased AP capacity and alleviating excess reduction in the ETC. Stress adaptation is an energy-consuming process in plants, and it is closely related to changes in the metabolic processes for which the CP provides energy. Therefore, it is reasonable to suppose that trehalose supply can ameliorate the decrease in CP capacity and promote wheat seedlings’ adaptation to heat stress so as to reduce the negative effects of stress.

Relationships between trehalose, the alternative pathway, and photosynthetic activity: The effects of trehalose on the relationship between mitochondria and chloroplasts under stress have not been investigated previously. To alleviate over-reduction of the chloroplast, excess NADPH produced during linear electron flow can be transferred to the mitochondria by the Mal-OAA shuttle and dissipated by AP (Hoefnagel et al. 1998, Zhang et al. 2012). Fig. 3 shows that Fv/Fm decreased sharply under heat stress and during recovery when the AP was inhibited by SHAM, indicating that the AP plays an important role in maintaining photosynthetic performance under heat stress. The higher Fv/Fm of the trehalose pretreatment group under heat stress also decreased dramatically after AP inhibition, dropping to a level similar to that of the control group (Fig. 3). These results show that exogenous trehalose can alleviate stress-related damage to chloroplast photosynthetic performance through its effects on AP.

In accordance with results in cucumber (Meng et al. 2013), MDH activities in our study were also enhanced under heat stress and during the subsequent recovery period but decreased significantly when the AP was inhibited by SHAM (Fig. 4). This result indicates that increased AP activity under heat stress effectively consumed the excess of reducing power transported by the malate-OAA shuttle and that inhibition of AP may lead to feedback inhibition of the malate-OAA shuttle and aggressive photoinhibition in the chloroplasts. Similarly, it was reported that the Mal-OAA shuttle was activated and the initial activity of NADP-MDH was promoted by low nitrogen stress in barley (Wang et al. 2016). Therefore, increased AP capacity under heat stress helped to dissipate excess reducing power and
prevent over-reduction of the chloroplast electron transport chain. In the present study, we observed higher \( V_{\text{ox}} \) and higher NAD-/NADP-MDH activities in the trehalose-pretreatment group than in the control group under heat stress (Fig. 4). Both enzyme activities were significantly reduced in trehalose-pretreated seedlings when the AP was inhibited with SHAM (Fig. 4). Meanwhile, the reduction of MDH activity in the trehalose-pretreatment group was greater than that of the control group under heat stress and SHAM treatment. These results indicated that trehalose pretreatment may increase MDH activity and promote the operation of the Mal-OAA shuttle through its effects on the AP.

Thus, we concluded that the application of exogenous trehalose increased the activity of the AP under heat stress and effectively consumed excess reducing power transported by the Mal-OAA shuttle, ultimately alleviating heat stress, which can cause photo inhibition in the chloroplasts.

**Trehalose pretreatment alleviates heat stress damage to the cytochrome respiratory pathway:** Stress-induced oxidative damage influences the structure and function of numerous biological macromolecules and can lead to inhibition of many important enzymes in the mitochondrial respiratory chain, including the NADH dehydrogenase complex (Complex I), the succinate dehydrogenase complex (Complex II), and ATP synthase (Zhang et al. 1990). State respiration can dynamically reflect the coupling process between electron transport and oxidative phosphorylation in mitochondria (Liu et al. 2014). Valent et al. (2007) showed that NADH-dependent respiration is markedly inhibited in tobacco BY-2 cells after heat stress but that succinate-dependent respiration is less inhibited, indicating that heat stress can damage the activity of Complex I. Furthermore, in a study of cauliflower mitochondria, heat stress has negative effects on ATP synthase, Complex I, and Complex IV (but not Complex II) and decreases the efficiency of oxidative phosphorylation (Rurek et al. 2015). In the present study, trehalose pretreatment appeared to protect mitochondrial respiration against heat stress by enhancing the activity of Complex II and the degree of coupling between electron transport and oxidative phosphorylation (Table 1), thus ensuring adequate energy supply to maintain cellular metabolism and reduce heat stress damage to mitochondria and the cytochrome respiratory pathway of wheat seedlings.

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