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PLANT-ENVIRONMENT INTERACTIONS

Exogenous trehalose differently improves photosynthetic carbon assimilation capacities in maize and wheat under heat stress

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1. Introduction

The enhancement of the greenhouse effect in recent years has resulted in the increased occurrence of abnormal high-temperature weather. Furthermore, the global temperature is expected to rise by 1.5–2°C by 2100 (Masson-Delmotte et al. 2021). High temperatures strongly affect plant growth and development. For example, yields of three major grain crops (maize, wheat and rice) can potentially decrease by 10% – 25% with a 1°C rise in global temperature (Deutsch et al. 2018). In particular, photosynthesis is extremely sensitive to heat, with high temperatures damaging photosynthetic mechanisms, including chlorophyll biosynthesis, photochemical reactions, electron transport and carbon assimilation (Alemu 2020).

Maize and wheat are the most important food crops worldwide. Maize is a C4 plant, while wheat is a C3 plant. Both of their photosynthetic carbon assimilation processes include the Calvin cycle, whose key enzyme is Ribulose-1,5-bisphosphate carboxylase (RuBisCO). However, in C4 plants, prior to the Calvin cycle, a 4-stage specialized CO2 concentrating mechanism (carboxylation, reduction, decarboxylation and regeneration) is necessary. Phosphoenol pyruvate carboxylase (PEPC), NADP-malate dehydrogenase (NADP-MDH), NADP-malic enzyme (NADP-ME), and pyruvate phosphate dikinase (PPDK) are the key enzymes in the CO2 concentrating mechanism, and are commonly known as the C4 pathway enzymes (Ashton et al. 1990). The standard temperature response of net photosynthetic rate (Pn) in C4 plants is similar to that in C3 plants, while the former has a higher optimum temperature (Yamori et al. 2014). This can be attributed to the CO2 concentrating mechanism of C4 plants, which is able to abate the photosynthetic inhibition by heat stress typically observed in C3 plants under ambient conditions (Crafts-Brandner and Salvucci 2002). However, as the temperature increases, the photosynthesis of C4 plants can also be perturbed. After 40°C, the photosynthetic rate of maize leaves will decrease sharply; at 45°C, only 40% of the maximum photosynthetic rate can be retained (Rotundo et al. 2019).

Trehalose, a non-reducing disaccharide that consists of two units of glucose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside), plays an important physiological role as an abiotic stress protectant for a large number of organisms, including bacteria, yeasts and plants. Several recent studies have demonstrated the involvement of trehalose in numerous signaling and metabolic pathways in plants, as well as its vital role in plant growth and development and the achievement of stress (e.g. drought, salinity and temperature) tolerance (Kosar et al. 2019; Onwe et al. 2022). The exogenous application of trehalose has been able to effectively elevate the carbon assimilation, and are commonly known as the C4 pathway enzymes (Ashton et al. 1990). The standard temperature response of net photosynthetic rate (Pn) in C4 plants is similar to that in C3 plants, while the former has a higher optimum temperature (Yamori et al. 2014). This can be attributed to the CO2 concentrating mechanism of C4 plants, which is able to abate the photosynthetic inhibition by heat stress typically observed in C3 plants under ambient conditions (Crafts-Brandner and Salvucci 2002). However, as the temperature increases, the photosynthesis of C4 plants can also be perturbed. After 40°C, the photosynthetic rate of maize leaves will decrease sharply; at 45°C, only 40% of the maximum photosynthetic rate can be retained (Rotundo et al. 2019).

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enhance the level of internal trehalose content, improving heat resistance in wheat (Luo et al. 2014), drought resistance in maize (Ali and Ashraf 2011), and protecting maize from salt stress and phosphorus deficiency (Rohman et al. 2019). In addition, research has shown that trehalose can efficiently stabilize dehydrated enzymes and scavenge reactive oxygen species under heat stress (Fernandez et al. 2010). Trehalose can interact with water molecules and phospholipid bilayer to form a highly structured layer on the surface of the cell membrane, thus improving the stability of the biofilm (Kumar et al. 2020). Furthermore, by relieving the increase in electrolyte leakages and malondialdehyde accumulation, trehalose can improve the heat resistance of maize seedlings (Li et al. 2014). Exogenous trehalose can also help to maintain cellular energy metabolism and enhance the heat resistance of wheat seedlings by increasing alternative respiratory pathway and the coupling between oxidative phosphorylation and the Complex II oxidative respiratory chain (Luo et al. 2021c).

High temperature (>42°C) mainly affects the photosynthesis of crops through increasing reactive oxygen species generation, inactivating the oxygen evolving complex of photosystem II (PSII) and inhibiting the carbon assimilation process (Slattery and Ort 2019). Trehalose can act as an antioxidant or stimulant of other antioxidants to scavenge reactive oxygen species (Kosar et al. 2019). Studies have also shown that application of exogenous trehalose can accelerate photosynthetic electron transport in heat-stressed maize and wheat by protecting the oxygen evolving complex and the PSII reaction center (Mamedov et al. 2015; Yanykin et al. 2015; Luo et al. 2021a). However, whether exogenous trehalose can alleviate the inhibition of photosynthetic carbon assimilation in maize and wheat seedlings under heat stress remains to be determined. Considering that affecting the carbon assimilation process is the major reason for high temperature to inhibit the photosynthesis of both C3 and C4 crops (Slattery and Ort 2019), there is a clear need to clarify the effect of exogenous trehalose on photosynthetic carbon assimilation capacities of maize and wheat under heat stress. In this work, we investigated the impact of specific concentration of exogenous trehalose on carbohydrate content, gas exchange parameters, as well as activities and transcript levels of key enzymes in carbon assimilation in maize and wheat seedlings under heat stress, so as to study the effect and potential mechanism of trehalose on their photosynthetic carbon assimilation. The reported results can be applied to improve the yield of heat-stressed crops in the context of global warming.

2. Material and methods

2.1 Culture of plants

Healthy and plump seeds of maize (Zea mays L. cv ‘Shenkenuo 1’) and wheat (Triticum aestivum L. cv ‘Yang 18’) were selected, then covered with four layers of gauze soaked with water to promote germination for 2–3 days. The seeds in good germination condition were placed in trays (25 × 18 × 5 cm) with small holes and cultured in half-strength Hoagland solution at a temperature of 25/20°C (day/night), photoperiod of 16 h (irradiance: 800 μmol m⁻² s⁻¹ for maize, 120 μmol m⁻² s⁻¹ for wheat) and relative humidity of 70% for about 14 days.

2.2 Determination of heat treatment temperature

On the 14th day after germination, some seedlings were kept at 25/20°C (day/night) for 1 d, as the ‘no high temperature treatment group’ (C). The other part of the seedlings was put into light incubators and treated with different degrees of high temperature stress (38, 40, 42 and 45°C; humidity: 38%) for 1 d (day 13 h, night 11 h), as the ‘high temperature treatment for 1 d group’ (HR0). Then, some of the heat-stressed seedlings were placed at the temperature of 25/20°C (day/night) for 1 d as the ‘recovery group after high temperature treatment’ (HR1). Heat stress can damage the efficiency of PSII, leading to a decline in the maximal photochemical efficiency of PSII (Fv/Fm) (Crafts-Brandner and Salvucci 2002), with greater rates of decline associated with more serious heat stress levels. Therefore, the degree of heat stress used in subsequent experiments was decided by measuring the Fv/Fm ratio of the third leaves with different treatments. The temperature that could significantly reduce but would not completely eliminate the Fv/Fm ratio was the appropriate level of heat stress.

2.3 Determination of trehalose treatment concentration

On the 14th day after germination, the seedlings were treated with half-strength Hoagland solution containing 0.5, 1.5 and 10 mM trehalose for 3 days as the T0.5, T1.5 and T10 groups, respectively. Seedlings grown in half-strength Hoagland solution without trehalose served as control group (CK). A part of the seedlings treated with the above different concentrations of trehalose were placed at 25/20°C (day/night) for 1 d as the ‘no high temperature treatment group’ (C). Other parts of the seedlings were put into a light incubator (temperature: 42°C; humidity: 38%) and treated for 1 d (day 13 h, night 11 h), as the ‘high temperature treatment for 1 d group’ (HR0). Then, some of the heat-stressed seedlings were placed at the temperature of 25/20°C (day/night) for 1 d as the ‘recovery group after high temperature treatment’ (HR1). The degree of trehalose concentration used in subsequent experiments was determined by measuring the Fv/Fm ratio of the third leaves with different treatments. Trehalose concentration, which could most significantly alleviate the decrease of Fv/Fm ratio caused by high temperature, was the optimal degree. 2.4 Measurement of chlorophyll fluorescence parameters

The Chlorophyll fluorescence was measured by a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany) and the parameters were assessed using the induction curve recording mode in the DUAL-PAM software. After the maize and wheat seedlings were dark-acclimated for 30 min, the minimal chlorophyll fluorescence (F₀) and the maximal chlorophyll fluorescence (Fm) of dark adapted were detected. The Fv/Fm ratio was calculated according to the formula: (Fm−F₀)/Fm (Kitajima and Butler 1975).

2.5 Determination of carbohydrate content

Determination of carbohydrate content was performed according to Jang et al. (2003) with some modifications. The 5 g leaves were ground in liquid nitrogen and extracted for 15 min at 100°C with 15 mL water. The extract was centrifuged for 10 min at 3000 × g, and the supernatant was
filtered through a 0.45 μm filter unit. Quantitative carbohydrate analysis was carried out by HPLC (Agilent 1260, USA) with a Shodex Asahipak NH2P-50 4E column (4.6 × 250 mm) and evaporative light scattering detector. The sample injection volume was 10 μL, using commercially available trehalose, fructose, glucose and sucrose (Sigma, St. Louis, USA) as the standard.

2.6 Measurements of gas exchange parameters
Gas exchange parameters were measured with a portable photosynthesis system (LI-6400, Li-COR, USA) at a temperature of 25°C, photosynthetic photon flux density (PPFD) of 800 μmol m−2 s−1 (for maize) and 120 μmol m−2 s−1 (for wheat) and a CO2 concentration ranging from 400 to 420 μmol mol−1.

2.7 Extraction of PEPC, NADP-MDH, NADP-ME, PPDK and RUBPCase
Extraction procedures of PEPC (EC 4.1.1.31), NADP-MDH (EC 1.1.1.82), NADP-ME (EC 1.1.1.40), PPDK (EC 2.7.9.1) and RUBPCase (EC 4.1.1.39) were performed according to Ashton et al. (1990) with some modifications. 0.5 g leaves were fully ground with liquid nitrogen, and then homogenized with 25 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 10 mM NaHCO3, 1 mM PMSF, 5% (w/v) insoluble polyvinylpyrrolidone and 0.05% (v/v) Triton X-100 at 4°C. The homogenates filtered through 2 layers of gauze were centrifuged at 14,000 × g for 5 min and the supernatants were immediately transferred to clean centrifuge tubes and kept on ice.

2.8 Determination of PEPC, NADP-MDH, NADP-ME, PPDK and RUBPCase activity
The activities of PEPC, NADP-MDH, NADP-ME, PPDK and RUBPCase were asayed at 340 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech, Germany) according to Christelle et al. (2003) with some modifications. The assay of PEPC activity was performed in the reaction solution containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM MgCl2, 10 mM NaHCO3, 5 mM DTT, 5 mM glucose-6-phosphate, 5 mM PEP, 0.2 mM NADH and 2 U MDH. The determination of NADP-MDH activity was performed in the reaction solution containing 25 mM Tris-HCl (pH 8.3), 1 mM EDTA, 5 mM DTT, 2.5 mM malic acid, 0.25 mM NADP+ and the reaction was initiated by adding 5 mM MgCl2. The PPDK activity was obtained in the reaction solution containing 25 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 10 mM NaHCO3, 5 mM DTT, 8 mM MgSO4, 2 mM pyruvate, 1 mM ATP, 5 mM (NH4)2SO4, 1 mM glucose-6-phosphate, 2.5 mM K2HPO4, 0.2 mM NADH, 0.5 U PEPC and 2 U MDH. The RUBPCase activity was analyzed in the reaction solution containing 25 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 10 mM NaHCO3, 5 mM DTT, 20 mM MgCl2, 1 mM RuBP, 0.2 mM NADH, 5 mM ATP, 5 mM creatine phosphate, 2 U creatine phosphokinase, 2.8 U glyceraldehyde-3-phosphate dehydrogenase and 2 U phosphoglycerate kinase.

2.9 Total RNA extractions and quantitative real time polymerase chain reaction (qRT-PCR) analysis
The total RNA was extracted using RNAiso Plus (Takara, Japan) following the manufacturer’s instructions. Then, cDNAs were synthesized according to the manual of cDNA Synthesis Kit (Yeason, Shanghai).

The resultant cDNA was diluted 10-fold and was used as a template for qRT-PCR according to the manual of HiFi™ qRT-PCR SYBR® Green Master Mix (No Rox Plus) Kit (Yeason, Shanghai) and run on a qRT-PCR machine (CFX96, Bio-Rad, USA). The qRT-PCR steps were carried out as follows: pre-denaturation at 95°C for 5 min; then 40 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s. The maize actin2 gene was used as a reference for maize, and ubiqutin gene was used as a reference for wheat. According to the Cq value, 2−ΔΔCt method was used to calculate the relative expression quantity of genes. Assays were conducted with three samples from each treatment. Primer sets for qRT-PCR analysis were listed in Table 1.

2.10 Statistical analysis
The full data were statistically analyzed using package SPSS version 26.0 (SPSS, Chicago, USA) and the comparison of averages of each treatment was based on the analysis of variance (one-way ANOVA) according to Tukey’s test. Statistical significance is attained at the P ≤ 0.05 or P ≤ 0.01 level. Values were presented as mean ± SE from at least three independent experiments.

3. Results
3.1 Chlorophyll fluorescence responses in maize and wheat seeding leaves to heat stress
The decline in Fv/Fm ratio can be used to reflect the degree of heat stress. Our results show that, both in maize (Figure
1A) and wheat (Figure 1B) seedling leaves, there was no observed effect on Fv/Fm at 38°C, while the ratio gradually reduced as temperature increased, and was almost undetected at 45°C. Therefore, 42°C was used in the subsequent analysis.

H38°C, H40°C, H42°C and H45°C represent seedlings treated at 38°C, 40°C, 42°C and 45°C for 1 d, respectively. C: No high temperature treatment; HR0: High temperature treatment for 1 d; HR1: Recovery group after high temperature treatment. *represents $P < 0.05$; **represents $P < 0.01$.

### 3.2 Effects of exogenously-supplied trehalose concentration on chlorophyll fluorescence in maize and wheat seedling leaves

As it shows in maize (Figure 2A) and wheat (Figure 2B) seedling leaves, under heat stress, the Fv/Fm ratios in the 0.5 and 1.5 mM exogenously-supplied trehalose pretreatment groups improved. Moreover, in maize seedlings, following heat stress recovery, the Fv/Fm ratio sharply increased in the 0.5 mM trehalose-pretreated group relative to the control group (Figure 2A). In wheat seedlings, the recovery effect of Fv/Fm ratio in the 1.5 mM trehalose-pretreated group was the best. This indicates that the 0.5 and 1.5 mM trehalose-pretreatment aided in the heat stress recovery of the maize and wheat seedlings respectively. Thus, 0.5 mM (for maize) and 1.5 mM (for wheat) exogenously-supplied trehalose were used in the subsequent analysis.

CK, T0.5, T1.5 and T10 represent 3 days of culture with nutrient solutions containing 0 mM (control group), 0.5 mM, 1.5 mM and 10 mM trehalose, respectively. C: No high temperature treatment; HR0: High temperature treatment (42°C) for 1 d; HR1: Recovery group after high temperature treatment. Different letters represent significant differences ($P < 0.05$).

### 3.3 Effects of exogenously-supplied trehalose on carbohydrate content in maize and wheat seedling leaves

The untreated maize seedlings accumulated approximately 334.68 μg g$^{-1}$(f.m.) trehalose, 234.35 μg g$^{-1}$(f.m.) fructose, 645.08 μg g$^{-1}$(f.m.) glucose and 1274.57 μg g$^{-1}$(f.m.) sucrose. These values represent a 32%, 68%, 12%, 94% and 88% increase, respectively, in the trehalose-pretreated maize (Table 2). Furthermore, heat stress markedly decreased carbohydrate levels in maize seedling leaves (Table 2). However, trehalose pretreated maize plants exhibited a great improvement in carbohydrate levels, with 91% more trehalose compared to the control group (Table 2).
The effects of exogenous trehalose on the carbohydrate content in wheat seedlings were more obvious. Compared with the control group, the content of trehalose, fructose, glucose and sucrose in wheat pretreated with trehalose increased by 779%, 93%, 330% and 289%, respectively (Table 2). Heat stress reduced the content of glucose and sucrose in wheat seedling leaves, but increased the levels of trehalose and fructose, suggesting that the latter two may play important roles in the heat shock response of wheat (Table 2). Similar to maize, the trehalose level of wheat seedlings pretreated with exogenous trehalose increased under heat stress. However, the content of fructose decreased, and the content of glucose and sucrose remained unchanged (Table 2).

### 3.4 Effects of exogenously-supplied trehalose on gas-exchange parameters in maize and wheat seedling leaves

Gas-exchange parameters in maize and wheat seedling leaves were measured in order to investigate whether trehalose pretreatment is able to substantially improve carbohydrate content by effectively elevating the capacity of photosynthetic carbon assimilation. Figure 3 shows that prior to the heat stress treatment, no differences were observed in gas-exchange parameters between the control and trehalose pretreatment maize and wheat plants. Besides, in the control wheat group, Pn (Figure 3A2), Gs (Figure 3B2), Tr (Figure 3D2) and WUE (Figure 3F2) decreased under heat stress, while Ci and Ci/Ca increased (Figure 3C2, E2). The changes in the control maize were similar except for Tr (Figure 3D1). Furthermore, under heat stress, there were no differences in Gs and Tr (Figure 3B, D), while higher Pn and WUE and lower Ci and Ci/Ca levels were observed in the trehalose pretreatment groups compared to the control groups both in maize and wheat (Figure 3A, C, E, F).

### 3.5 Effects of exogenously-supplied trehalose on key enzymes in carbon assimilation in maize and wheat seedling leaves

We investigated the activity changes of key enzymes for photosynthetic carbon assimilation in maize and wheat seedling leaves before and after heat treatment. Figure 4 shows that in maize seedling leaves, prior to the heat stress, no differences in the activities of PEPC, NADP-MDH, NADP-ME, PPDK and RUBPCase were observed between the control and trehalose pretreatment plants. Under heat stress, however, all enzyme activities (with the exception of PPDK) rose sharply in the trehalose pretreated plants (Figure 4A-E). Moreover, the activity of RUBPCase, the key enzyme for carbon assimilation in wheat seedlings, in trehalose pretreated plants was higher than that of non trehalose treatment under heat stress (Figure 4F).

### 3.6 Effects of exogenously-supplied trehalose on the relative transcript abundance of key enzymes in carbon assimilation in maize and wheat seedling leaves

We performed qRT-PCR experiments in order to investigate whether trehalose affected the transcript levels of these enzymes. Figure 5 demonstrates that prior to heat stress, the trehalose pretreatment was able to improve the expression of PPDK and RuBisCO-LSU in maize seedling leaves (Figure 5D, E). Under heat stress, the expressions of PEPC, PPDK and RuBisCO-SSU were markedly reduced (Figure 5A, D, F), while NADP-ME clearly increased (Figure 5C) and NADP-MDH and RuBisCO-LSU remained unchanged (Figure 5B, G). Meanwhile, under heat stress, PEPC, NADP-MDH, NADP-ME, PPDK and RuBisCO-SSU expressions were observed to greatly increase in trehalose pretreated maize plants (Figure 5A-D, F). Note that this was not the case for RuBisCO-LSU (Figure 5E). These results suggest that exogenously-supplied trehalose is able to alleviate the damage of heat stress on maize seedlings by altering the expression of PEPC, NADP-MDH, NADP-ME, PPDK and RuBisCO-SSU in photosynthetic carbon assimilation.

For wheat seedling leaves, after heat treatment, the expression of RuBisCO-LSU and RuBisCO-SSU in the control group also decreased (Figure 5G, H). However, their levels didn’t increase in the trehalose pretreatment group (Figure 5G, H). These results suggest that trehalose pretreatment can not improve the heat resistance of wheat seedlings by up-regulating the transcript level of the key enzymes in Calvin cycle.

### 4. Discussion

High temperatures greatly affect plant growth and development, which subsequently decreases crop yield. This phenomenon has led to a considerable amount of attention being focused on the physiological and molecular responses of crops to heat stress (Crafts-Brandner and Salvucci 2002; Wahid et al. 2012; Jang et al. 2003). Studies have shown that trehalose can improve the tolerance of plants to abiotic stress (Garg et al. 2002; Abdallah et al. 2016). In the current study, 0.5 and 1.5 mM exogenous trehalose alleviated the decrease of Fv/Fm ratio caused by high temperature of 42°C.
Figure 3. Effects of exogenously-supplied trehalose on gas-exchange parameters in maize (A1-F1) and wheat (A2-F2) seedling leaves under different treatments. (A) Pn: Net photosynthetic rate; (B) Gs: Stomatal conductance; (C) Ci: Intercellular CO2 concentration; (D) Tr: Transpiration rate; (E) Ci/Ca: The ratio of intercellular to ambient CO2 partial pressures; (F) WUE: Water-use efficiency. CK: Control group; T: Trehalose pretreatment (maize: 0.5 mM, wheat: 1.5 mM) group; C: No high temperature treatment; HR0: High temperature treatment (42°C) for 1 d. Different letters represent significant differences (P < 0.05).

Figure 4. Effects of exogenously-supplied trehalose on the key enzymes in photosynthetic carbon assimilation of maize (A-E) and wheat (F) seedling leaves under different treatments. (A) PEPC: Phosphoenol pyruvate carboxylase; (B) NADP-MDH: NADP malate dehydrogenase; (C) NADP-ME: NADP-malic enzyme; (D) PPDK: Pyruvate phosphate dikinase; (E) RUBPCase: Ribulose-1,5-bisphosphate carboxylase; CK: Control group; T: Trehalose pretreatment (maize: 0.5 mM, wheat: 1.5 mM) group; C: No high temperature treatment; HR0: High temperature treatment (42°C) for 1 d. Different letters represent significant differences (P < 0.05).
C in maize and wheat seedlings, respectively, indicating that exogenous trehalose can promote photosynthesis of maize and wheat under heat stress. Exogenous trehalose has been found to notably increase the soluble sugar content and improve stress resistance in Arabidopsis thaliana (Bae et al. 2005; Ranwala and Miller 2009). In this work, we found that 0.5 mM exogenously-supplied trehalose heightened endogenous trehalose and improved other carbohydrate content in maize seedling leaves under different treatments (Table 2). In wheat seedlings, 1.5 mM exogenously-supplied trehalose also increased the content of endogenous trehalose under heat stress, but the levels of other carbohydrates showed different trends: the content of fructose decreased, and the content of glucose and sucrose remained unchanged (Table 2). Our previous study has proved that trehalose pre-treatment increased the abscisic acid content in wheat seedlings recovered from heat stress (Luo et al. 2014). According to Kumar et al. (2012), exogenously-supplied trehalose increased endogenous trehalose levels, thereby promoting the growth of heat-stressed plants by functioning downstream of abscisic acid. Therefore, trehalose may interact with the abscisic acid signaling pathway and act as a central coordination variable in the synthesis and recycling of carbohydrates through a series of downstream reactions, hence affecting the content of other carbohydrates in plants. Besides, Photosynthetic carbon assimilation is an important process for plants to generate carbohydrates. We can therefore assume that exogenously-supplied trehalose was able to effectively influence the assimilation of carbon.

In order to evaluate the effects of exogenous trehalose on carbon assimilation, we performed gas-exchange measurements, which, due to their direct link to net productivity, are considered as a vital indicator of the growth of plants (Ashraf 2004; Piao et al. 2008). In the current study, under heat stress conditions, the control maize and wheat plants exhibited a decline in \( P_n \), accompanied by a decrease in \( G_s \), \( T_r \), WUE and an increase in \( C_i \) and \( C_i/C_a \) (Figure 3), which reveals that non-stomatal limitation is the principle factor inhibiting the photosynthetic capacity of control plants. This result is consistent with the report of Gerganova et al. (2016). However, the trehalose-pretreated maize and wheat seedlings showed little change in \( C_i \) and \( C_i/C_a \) under high temperature (Figure 3), which indicates that the decrease of \( P_n \) caused by heat stress in them was restricted by both stomata and non-stomatal factors (Flexas et al. 2004; Lu et al. 2017). Because the more intense the stress levels, the smaller the number of stomatal limitation components. The above results suggest that exogenous trehalose alleviated stress-induced damage. The role of trehalose behind this impact is two-fold. First, trehalose is able to eliminate reactive oxygen species under heat stress (Luo et al. 2014). Second, it can stabilize biological macromolecules (e.g. key carbon assimilation enzymes) in the folded state under stress conditions, as these enzymes play key roles in

![Figure 5](image-url). Effects of exogenously-supplied trehalose on expression of the key enzymes in photosynthetic carbon assimilation in maize (A–F) and wheat (G–H) seedling leaves under different treatments. (A) PEPC: Phosphoenol pyruvate carboxylase; (B) NADP-MDH: NADP-malate dehydrogenase; (C) NADP-ME: NADP-malic enzyme; (D) PPDK: Pyruvate phosphate dikinase; (E) RuBisCO-LSU: Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; (F) RuBisCO-SSU: Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; CK: Control group; T: Trehalose pretreatment (maize: 0.5 mM, wheat: 1.5 mM) group; C: No high temperature treatment; HR0: High temperature treatment (42°C) for 1 d. Different letters represent significant differences (\( P < 0.05 \)).
the response of plants to heat stress. In particular, any inhibition in the activities of these enzymes will disturb the CO₂-concentrating mechanism, thus slowing down the rate of photosynthesis in heat-treated maize and wheat (Crafts-Brandner and Salvucci 2002; Pushpalatha et al. 2008).

We subsequently investigated the effects of high temperature on key enzymes of carbon assimilation in maize and wheat, and whether the application of exogenous trehalose could protect these enzymes from heat. Our results show that exogenous trehalose is able to protect the key enzymes under heat stress in both C4 plants (maize) and C3 plants (wheat), but the mechanisms are slightly different.

In maize seedlings, as Kelly (2006) reported, we observed that heat stress inhibited the carboxylation and regeneration of PEP by decreasing PEPC and PPDK activities. Similarly, the activity of all other C4 pathway enzymes also decreased (Figure 4B-D). Meanwhile, high temperature down-regulated the expression of PEPC and PPDK, and up-regulated the expression of NADP-ME, while the expression of NADP-MDH remained constant (Figure 5A-D). Therefore, heat stress can perturb the CO₂-concentrating mechanism in maize by affecting these C4 pathway enzymes, and the inhibition of NADP-MDH and NADP-ME activities may be due to changes in the protein level rather than insufficient transcript level. Similarly, heat stress signal down-regulated the expression of Rubisco-SSU (Figure 5F) and inhibited the activity of Rubisco (Figure 4E), slowing down the Calvin cycle process and eventually leading to a decline in the photosynthetic rate (Figure 3A1).

Compared with the control group (with no exogenous trehalose), exogenously-supplied trehalose improved the activities of PEPC, NADP-MDH, NADP-ME and Rubisco in heat-stressed maize. However, this was not the case for PPDK (Figure 4), which may be attributed to the greater sensitivity of PPDK to heat stress compared to the other four enzymes. Correspondingly, the activity of PPDK was also the lowest under heat stress (Figure 4D). Trehalose can act as an elicitor of genes involved in detoxification and stress responses (Bae et al. 2005). This may explain why exogenously-supplied trehalose improved the transcript levels of all C4 pathway enzymes in maize under heat stress (Figure 5A-D). We also found that, under heat stress, the expression of Rubisco-SSU remained unchanged, and exogenously-supplied trehalose was only able to up-regulate the transcript level of Rubisco-SSU (Figure 5E-F). This suggests that the Rubisco-SSU in maize is more sensitive to heat stress, which is consistent with the results of Lu et al. (2017) for tomato crops. Therefore, exogenously-supplied trehalose attenuated the adverse effects of high temperature on carbon assimilation enzymes differently at protein and transcription levels, further enhancing CO₂ fixation efficiency and reducing C4 level (Figure 3C).

In wheat seedlings, we observed a decrease in the activity of Rubisco under heat stress, accompanied by a decrease in the transcript levels of Rubisco-SSU and Rubisco-SSU (Figure 4F, Figure 5G, H), which is in accordance with the conclusions of Pushpalatha et al. (2008). Wheat is a C3 plant and does not need to go through the carbon fixation process of C4 plants. CO₂ can be fixed through the Calvin cycle directly, in which Rubisco is the key rate-limiting enzyme. Therefore, high temperature can affect the process of Calvin cycle by down-regulating the activity and amount of Rubisco, and ultimately inhibit the photosynthesis of wheat.

Consistent with that in maize, trehalose pretreatment also increased the activity of Rubisco in wheat under heat stress. However, exogenous trehalose did not help to restore the decreased expression levels of Rubisco-SSU and Rubisco-SSU caused by heat stress, which indicates that trehalose can only improve Pn of wheat by increasing the functional activity of Rubisco rather than its transcriptional level.

Maize is a typical C4 plant, and wheat is a C3 plant. The initial product of CO₂ fixation in C4 plants is not 3-phosphoglycerate in the Calvin cycle, but the four-carbon compound oxaloacetate. This reaction occurs in the unique mesophyll cells surrounding the vascular bundles of C4 plants, and is catalyzed by PEPC. Compared with Rubisco, the affinity of PEPC with CO₂ is higher, so it can fix CO₂ more effectively. Generally, in high temperature environments, plants will close the stomata of the leaves to reduce water loss, resulting in a notable drop in the CO₂ concentration in the leaves. At this time, C4 plants can use the low content of CO₂ in the intercellular spaces of leaves for photosynthesis, while C3 plants cannot. Therefore, C4 plants are generally more tolerant to high temperature than C3 plants. However, as leaf temperature increases, photosynthesis in C4 plants will also be disrupted. For example, PEPC activity and regeneration can decrease markedly at elevated temperatures, and the activation state of Rubisco may also fall for temperatures above 32.5°C, with almost complete inactivation at 45°C (Crafts-Brandner and Salvucci 2002; Kelly 2006). In this work, we used maize (C4 plant) and wheat (C3 plant) as research materials and found that in both crops, exogenous trehalose could protect the carbon assimilation process at high temperature by maintaining the activity and transcript level of some key enzymes. Studies have shown that trehalose has a good protective effect on biological macromolecules through the two mechanisms of water replacement and vitreous. Vitreous trehalose plays a key role in protecting molecular functional activities by resisting dehydration and denaturation caused by the separation of biological macromolecules (Crowe 2007; Elbein et al. 2003). Therefore, we speculate that trehalose may prevent the inactivation of some enzymes (PEPC, NADP-MDH, NADP-ME and Rubisco) at high temperature in this way, thereby maintaining the carbon assimilation process. Due to the different protein structures and thermal sensitivities of the enzymes, the stabilizing effects of trehalose on various enzymes are different, which may explain why the protection of trehalose on maize PPDK is not obvious in this study. On the other hand, trehalose-6-phosphate (T6P) is an intermediate in the process of trehalose synthesis in plants and can serve as an essential signaling molecule for plant growth and development (Schluepmann et al. 2004). Studies have further shown that T6P can inhibit the catalytic activity of SnRK1 (SNF1-related kinase 1) (Schluepmann and Paul 2009). SnRK1 is an important regulatory hub in plant growth and stress response, and is involved in the regulation of carbon metabolism and energy supply (Emanuelle et al. 2016). Accordingly, trehalose may affect SnRK1 through T6P and further affect the transcript level of some enzyme genes during carbon assimilation in maize and wheat.

In the majority of higher plants, Trehalose exists in very low concentrations, and its content is not enough to alleviate the adverse effects of stress (Garg et al. 2002). In the current study, exogenously-supplied trehalose notably enhanced...
endogenous trehalose levels in maize and wheat seedling leaves (Table 2). It has been reported that exogenously-supplied trehalose can be easily absorbed by leaf tissues and roots, allowing for its subsequent transportation to other parts of the plant (Smith and Smith 1973). Thus, the increased endogenous trehalose in seedling leaves in this study is assumed to be absorbed through roots.

5. Conclusion

In our study, heat stress of 42°C was used, at which the Pn of maize and wheat were both inhibited. These effects were partly caused by down-regulating the key enzyme activities and transcript levels in the process of photosynthetic carbon assimilation. However, the application of exogenous trehalose can alleviate the decrease in Pn caused by high temperature to a certain extent. Our results show that, both in maize and wheat seedlings, exogenous trehalose can increase the activity of RUBPCase, thereby promoting the Calvin cycle. However, in maize seedlings, in addition to the increase in enzyme activity, trehalose can also promote the transcript level of RuBiCO-SSU, hence increasing the amount of RUBPCase. What’s more, the application of exogenous trehalose can also increase the activity of some C4 pathway enzymes in maize (including PEPC, NADP-MDH, NADP-ME and RUBPCase) and their transcript levels (including PEPC, NADP-MDH, NADP-ME, PPDK and RuBiCO-SSU) to promote carbon assimilation. Taken together, our study demonstrates the beneficial effects of exogenous trehalose on photosynthetic carbon assimilation in maize and wheat under heat stress. These results can provide guidance for improving heat-stressed maize and wheat yields under the trend of global warming. However, the molecular mechanism and signaling pathway by which trehalose enhanced the enzyme activity and transcript level remain to be further studied.

Disclosure statement

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