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Glucose application protects chloroplast ultrastructure in heat-stressed cucumber leaves through modifying antioxidant enzyme activity

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Abstract

To elucidate a physiological mechanism of heat stress mitigation by exogenous glucose, seedlings of *Cucumis sativus* cv. Jinchun No. 4 were pretreated with glucose and then exposed to normal (25/18 °C) and elevated (42/38 °C) temperatures. We investigated whether glucose can protect cucumber plantlets and chloroplast ultrastructure from heat and whether this protection is associated with antioxidant enzymes, proline, and soluble sugars. Heat inhibited plant growth, disorganized membranes of 86.33 % of chloroplasts, and elevated the content of malondialdehyde (MDA), superoxide radical ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2). An optimum concentration of glucose was 30 mM as it significantly alleviated plant growth inhibition and obviously reduced the content of MDA, $O_2^{\cdot-}$, and H_2O_2 under the heat stress. The pretreatment with 30 mM glucose mitigated heat-induced damage of chloroplast ultrastructure and changes in leaf morphology more than 30 mM mannitol suggesting that glucose did not act only as osmolyte. Moreover, the glucose pretreatment increased activities of some antioxidant enzymes and enhanced the content of proline and soluble sugars under the heat stress, as well as the transcriptions of *Cu/Zn-superoxide dismutase*, *Mn-superoxide dismutase*, *catalase*, and *glutathione reductase* genes. We conclude that the pretreatment with 30 mM glucose protected chloroplast ultrastructure and enhanced heat tolerance of the seedlings by the increased activites of antioxidants and the content of proline and soluble sugars, and repressed accumulation of reactive oxygen species.

Additional key words: catalase, *Cucumis sativus*, glutathione reductase, H_2O_2, invertase, malondialdehyde, proline, soluble sugars, superoxide dismutase.

Introduction

Heat is harmful to crops in many areas of the world (Luo *et al*. 2014). It causes oxidative stress due to the overproduction of reactive oxygen species (ROS) including superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Almeselmani *et al*. 2006). The chloroplast is a major site for generating ROS in plants (Xu *et al*. 2006). The accumulation of ROS causes membrane lipid peroxidation and can result in cell death (Molassiotis *et al*. 2006). To protect cells from damage, plants evolve enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and non-enzymatic antioxidants including ascorbate (AsA) and reduced glutathione (GSH) (Wahid *et al*. 2007).

Glucose and other sugars are important nutrients, parts of structure of living cells, and also signaling molecules (Hirabayashi 1996, Seckin *et al*. 2009). The application of exogenous glucose affects photosynthesis-related characteristics in water-stressed wheat seedlings (Hu *et al*. 2009). A pretreatment with glucose decreases the extent of membrane lipid peroxidation and preserves quality of watermelon seedlings under low-temperature storage (Jiang *et al*. 2012). Moreover, exogenous glucose regulates activities of antioxidant enzymes and enhances tolerance of plants to dehydration (Huang *et al*. 2013) and salt stress (Hu *et al*. 2012).

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; CAT - catalase; DHAR - dehydroascorbate reductase; GR - glutathione reductase; GSH - reduced glutathione; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; NI - neutral invertase; $O_2^{\cdot-}$ - superoxide radical; ROS - reactive oxygen species, SAI - soluble acid invertase; SOD - superoxide dismutase.

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To the best of our knowledge, it has not been reported that pretreatment with glucose alleviates heat stress in cucumber. It is well known that heat increases ROS accumulation and causes oxidative damage in chloroplasts, however, we hypothesize that pretreatment with glucose might increase the activities of antioxidants and thus reduce ROS content and membrane lipid peroxidation. Therefore, we examined whether glucose application can protect membranes of chloroplasts and improve cucumber growth under heat stress due to increased antioxidant enzyme activities. It has been reported that proline (Zhang *et al*. 2014) and soluble sugars (Santarius 1973) are able to mitigate stress, and soluble acid invertase (SAI) and neutral invertase (NI) are important enzymes in sugar metabolism in plants (Batta and Singh 1986). Thus, we also investigated whether proline, soluble sugars, SAI, and NI are involved in heat stress mitigation after glucose pretreatment.

Materials and methods

Seedlings of cucumber (*Cucumis sativus* L. cv. Jinchun No. 4) were planted in plastic pots filled with sand moistened with a Hoagland nutrient solution (Liu *et al*. 2010) under day/night temperatures of 25/18 °C, a relative humidity of 75 %, a 12-h photoperiod, and an irradiance of 300 μmol m^{-2} s^{-1}. At the 2-leaf stage, 32 cucumber seedlings (8 plants per group) were separately watered for 3 d with the Hoagland nutrient solution containing different concentrations (0, 20, 30 and 40 mM) of glucose, and subsequently exposed to a heat stress (day/night temperatures of 42/38 °C) for 2 d. Three different sets of plants grown at different times were used. Based on the results of these preliminary experiments, 48 cucumber seedlings were divided into 6 groups. Two groups of seedlings were watered with the Hoagland nutrient solution only, and two groups were watered with the Hoagland nutrient solution containing the optimum concentration of glucose (30 mM), and two groups were watered with the Hoagland nutrient solution containing 30 mM mannitol. After three days, all the seedlings were rinsed six times with water and six times with the Hoagland nutrient solution. Then, one group from each pretreatment was exposed to the normal temperatures (25/18 °C) and one group to the high temperatures (42/38 °C). After 2 d, the second leaves (which became wilted in some seedlings of the heat stressed plants) were harvested from all the treatment groups. Plant growth parameters were determined according to Sun *et al*. (2012).

Transmission electron microscopy (TEM) was performed following the procedures of Helliot *et al*. (2003) and modified by Xu *et al*. (2008). Chloroplasts which had the same ellipsoidal shapes as those in the control group were classified as normal ones. Otherwise, they were classified as abnormal.

Membrane lipid peroxidation in leaves was detected according to malondialdehyde (MDA) content (Dhindsa *et al*. 1981). Absorbances were determined at 450, 532 and 600 nm using a spectrophotometer *TU-1810* (*Purkinje General Instrument,* Beijing, China), and the MDA content was estimated by the formula: $MDA = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$ (Sun *et al*. 2012). The formation rate of $O_2^{\cdot-}$ was measured according to Elstner and Heupel (1976). When powdered in liquid nitrogen, 0.2 g of leaves was mixed with 2 cm^3 of a 65 mM phosphate buffer (pH 7.8) and centrifuged at 4 °C and 5 000 *g* for 10 min to extract $O_2^{\cdot-}$. After measuring the absorbance at 530 nm, the formation rate of $O_2^{\cdot-}$ was calculated from a standard curve of a $NaNO_2$ reagent. H_2O_2 content in leaves was determined according to the method of Bernt and Bergmeyer (1974). When the absorbance was measured at 436 nm, the content of H_2O_2 was calculated from a standard curve of a H_2O_2 reagent.

Leaves were powdered in liquid nitrogen and used to extract antioxidant enzymes according to Sun *et al*. (2012). The activity of SOD was measured at 560 nm (Hwang *et al*. 1999) and one unit of SOD activity was defined as the amount of enzyme that caused a 50 % inhibition of the rate of nitroblue tetrazolium reduction. The activity of CAT was determined according to decomposition of H_2O_2, and the absorbance was measured at 240 nm (Pereira *et al*. 2002). The activity of DHAR was determined based on the formation of AsA (Doulis *et al*. 1997) by measuring the absorbance at 265 nm. The activity of MDHAR was measured by monitoring the oxidation of NADPH and determining the absorbance at 340 nm (Hoque *et al*. 2007). The activity of GR was assayed by measuring the absorbance at 340 nm due to the oxidation of NADPH (Foyer and Halliwell 1976). To assess the activity of APX, the H_2O_2-dependent oxidation of AsA was used and the absorbance was measured at 290 nm (Zhu *et al*. 2004).

After homogenization with 10 % (m/v) trichloroacetic acid, the content of reduced glutathione (GSH) was analyzed by measuring the absorbance at 412 nm (Guri 1983). The proline content was determined by Bates *et al*. (1973) and the absorbance was measured at 520 nm. Soluble sugars in leaves were quantified by the anthrone-sulfuric acid assay according to Yemm and Willis (1954), and their amounts were determined through measuring the absorbance at 620 nm and using a standard curve of glucose reagent.

To determine the glucose and fructose content, samples (0.3 g) of leaves were homogenized in 6 cm^3 of boiling ethanol (80 %, v/v) for one hour. After cooling, the homogenate was centrifuged at 8 000 *g* for 10 min. The process was repeated three times for complete extraction of glucose and fructose. Then, the alcoholic

extracts were evaporated to dryness and dissolved in sterile water. Subsequently, the glucose or fructose content was determined using high-performance liquid chromatography according to Ozaki *et al*. (2009).

The activities of SAI and NI were measured according to Batta and Singh (1986) and the absorbance was determined at 520 nm. The protein content in the enzyme extract was measured according to Bradford (1976).

For estimation of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, and *GR* gene expressions, total RNA was extracted from leaves with a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA) and then was reverse-transcribed to cDNA using a *Quantscript RT* kit (*Cwbio*, Beijing, China). Forward and reverse primers of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, *GR*, and *actin* are listed in Table 1. The reaction mixture contained 10 mm^3 of a 2 × *ultraSYBR* mixture (with *Rox*) (*Cwbio*), 2 mm^3 of template cDNA, and 0.8 mm^3 of each 5 pM forward and reverse primers in a 20-mm^3 reaction system. PCR parameters included an initial denaturation step at 95 °C for 10 min; followed by amplification (40 cycles) at 95 °C for 15 s and at 62 °C for 1 min. The expressions of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, and *GR* were normalized against the *actin* expression by using the $2^{-\Delta\Delta Ct}$ comparative method. PCR amplification was conducted with a *CFX96*^{TM} real-time system (*Bio-Rad*, Hercules, CA, USA), and the results were analyzed using the *Bio-Rad CFX* manager software.

The data were expressed as means ± standard errors and compared by one-way *ANOVA* and the least significant difference (LSD) test. *P*-values < 0.05 were considered to be significant.

Table 1. Primers used for real-time PCR assays.

Gene	Acc. No	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse	Product size [bp]
Cu/Zn-SOD	EF121763	GACTGGGCCACATTTCAACC	GCCTTGCCATCTTCACCAA	108
Mn-SOD	EF203086	CAATGGCGGAGGTCACATTA	AGAGCAAGCCACACCCATC	195
CAT	EF468517	AATGGCCGGAGGATGTGA	CCAACGACATAGAGAAAGCCAAC	111
GR	EF530128	TGATGAGGCTTTGAGTTTAGAGGAG	AACTTTGGCACCCATACCATTC	109
actin	AB010922	GGTCGTGACCTTACTGATGC	CAATAGAGGAACTGCTCTTTGC	166

Results

Under the heat stress, the highest fresh and dry masses of the second leaves, roots, and shoots were after the pretreatment with 30 mM glucose when compared to the other glucose concentrations (Table 2). The pretreatment with 30 mM glucose resulted in the lowest content of MDA, $O_2^{\cdot-}$, and H_2O_2, and the highest activities of SOD and CAT in the heat-stressed leaves. Therefore, 30 mM glucose was found to be the optimum concentration to mitigate the heat stress.

In comparison to the control, the fresh and dry masses of the second leaves, shoots, and roots were significantly decreased under the heat stress but less in the glucose-pretreated group (Table 3). When compared the mannitol-pretreated group with the untreated group, the fresh and

Table 2. Changes in plant growth, content of MDA, $O_2^{\cdot-}$, and H_2O_2, and activities of SOD and CAT in the second leaves after glucose (0 - 40 mM) pretreatments and a heat stress. Means ± SE, $n = 3$. Different letters indicate statistically significant differences between treatments at $P < 0.05$.

Parameters	0	20	30	40 mM glucose
Leaf fresh mass [g]	1.19±0.02b	1.22±0.00b	1.49±0.05a	1.27±0.03b
Leaf dry mass [g]	0.13±0.00c	0.14±0.00b	0.16±0.00a	0.12±0.00d
Root fresh mass [g]	1.51±0.01d	1.73±0.02b	1.97±0.00a	1.63±0.02c
Root dry mass [g]	0.06±0.00c	0.10±0.00a	0.12±0.01a	0.08±0.00b
Shoot fresh mass [g]	2.44±0.04b	2.60±0.05b	2.87±0.00a	2.60±0.00b
Shoot dry mass [g]	0.22±0.00c	0.21±0.00d	0.25±0.00a	0.24±0.00b
MDA [nmol g^{-1}(f.m.)]	14.24±0.03a	12.45±0.12c	10.86±0.17d	13.61±0.10b
$O_2^{\cdot-}$ [nmol g^{-1}(f.m.) min^{-1}]	5.28±0.11a	3.41±0.06c	3.01±0.06d	4.10±0.06b
H_2O_2 [nmol g^{-1}(f.m.)]	301.02±7.98a	265.19±2.48b	220.76±2.87d	246.56±3.79c
SOD [U mg^{-1}(protein)]	31.44±0.20d	33.95±0.49c	39.32±0.44a	36.02±0.35b
CAT [μmol mg^{-1}(protein) min^{-1}]	0.08±0.00c	0.11±0.00b	0.12±0.00a	0.11±0.00b

dry masses of the second leaves and shoots, and the fresh mass of roots significantly decreased, but the dry mass of roots significantly enhanced. Under the heat stress, the leaf margins of 87.50 ± 0.07 % of leaves wilted in the untreated group which was significantly more than in the glucose-pretreated group (31.25 ± 0.04 %) and the mannitol-pretreated group (56.25 ± 0.07 %; Fig. 1). Thus, the heat stress significantly inhibited plant growth, and the glucose-pretreatment alleviated the growth inhibition more than mannitol.

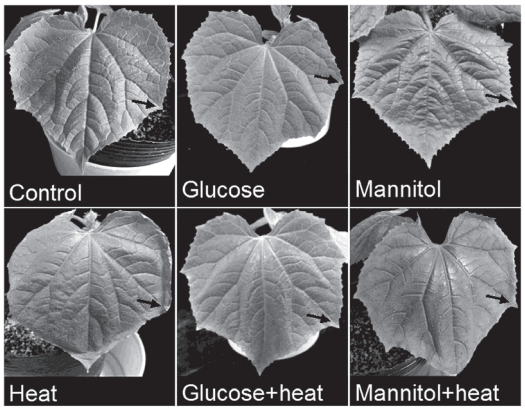

Fig. 1. The effects of glucose pretreatment and heat on morphology of the second leaves of cucumbers.

Under the control conditions, similar ellipsoid chloroplasts were found in the untreated and glucose and mannitol pretreated groups (Fig. 2). However, 86.33 ± 0.3 % of chloroplasts in the heat stressed untreated plants were abnormal and swollen to various degrees. After the glucose- or mannitol-pretreatment, the percentage of abnormal chloroplasts was only 37.33 ± 0.7 and 56.00 ± 1.2 %, respectively. The chloroplast thylakoids were well organized in the control, and under the heat stress only in the glucose or mannitol pretreated plants (Fig. 3). However, in the untreated heat-stressed plants, the grana lamellae were disorganized. So, the heat stress damaged chloroplast ultrastructure, whereas glucose mitigated the heat-stress caused injury better than mannitol.

The content of MDA, $O_2^{\cdot-}$, and H_2O_2 in leaves significantly decreased after the glucose-pretreatment compared to the control. After the mannitol-pretreatment, the content of MDA and H_2O_2 enhanced, but the $O_2^{\cdot-}$ content was reduced as compared to the untreated group. The lipid peroxidation and content of ROS significantly enhanced in the heat-stressed plants, but significantly less in the glucose-pretreated plants (Table 3). However, the mannitol pretreatment reduced only the formation rate of $O_2^{\cdot-}$.

In comparison to the control, the activities of SOD, CAT, APX, MDHAR, and GR in leaves of the glucose-pretreated plants significantly increased, and the activity of DHAR did not change (Table 3). In the mannitol-pretreated plants, the activities of SOD, APX, DHAR, and GR significantly enhanced, the activity of CAT decreased and the activity of MDHAR did not change. Compared to the control, the heat stress significantly enhanced the activities of SOD, CAT, APX, DHAR, MDHAR, and GR in leaves. In comparison to the heat-stressed group, the glucose pretreatment further increased the activities of all these enzymes, whereas, the mannitol pretreatment increased the activities of SOD, CAT, APX, and GR but decreased the activity of MDHAR and did not alter the activity of DHAR.

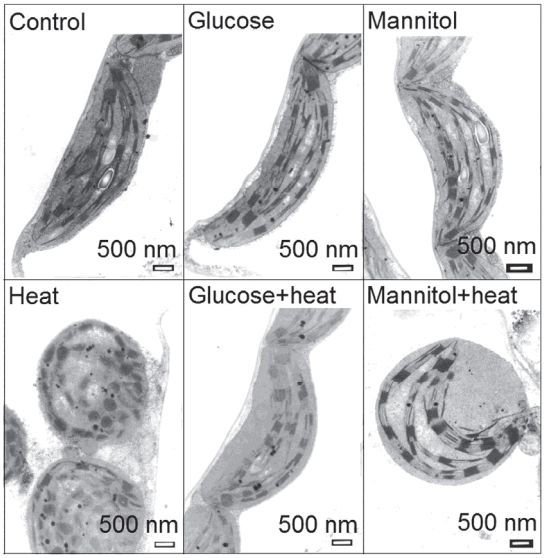

Fig. 2. Chloroplasts in the first layer palisade parenchyma of cucumber leaves.

To analyze the effects of glucose on the GR activity under the heat stress, the GSH content in leaves was investigated (Table 3). In comparison to the control, the content of GSH significantly enhanced in the glucose pretreated plants, whereas decreased in the mannitol pretreated plants. Under the heat stress, the content of GSH was higher in the glucose- or mannitol-pretreated plants than in non-treated ones.

In comparison to the control, the expressions of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, and *GR* genes significantly increased in the glucose-pretreated plants (Table 3). When compared the mannitol-pretreated plants with the control, the expressions of *Cu/Zn-SOD*, *Mn-SOD*, and *CAT* significantly decreased and the expression of *GR* enhanced. Compared to the heat-stressed untreated plants, the amounts of mRNA of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, and *GR* significantly increased in the glucose-pretreated plants. However, in the mannitol-pretreated plants, the expressions of *Cu/Zn-SOD*, *Mn-SOD* and *CAT* genes significantly decreased, whereas the expression of *GR* increased.

When compared to the control, the content of proline, total soluble sugars, glucose, and fructose was significantly elevated in the glucose-pretreated plants and decreased in the mannitol-pretreated plants (Table 3). Compared to the heat treatment group, the content of proline, soluble sugars, endogenous glucose, and fructose significantly enhanced in the glucose + heat treatment group and significantly decreased in the mannitol + heat treatment group.

Compared to the heat-stressed untreated plants, the activities of SAI and NI in leaves significantly increased after the glucose-pretreatment or the mannitol-pretreatment (Table 3).

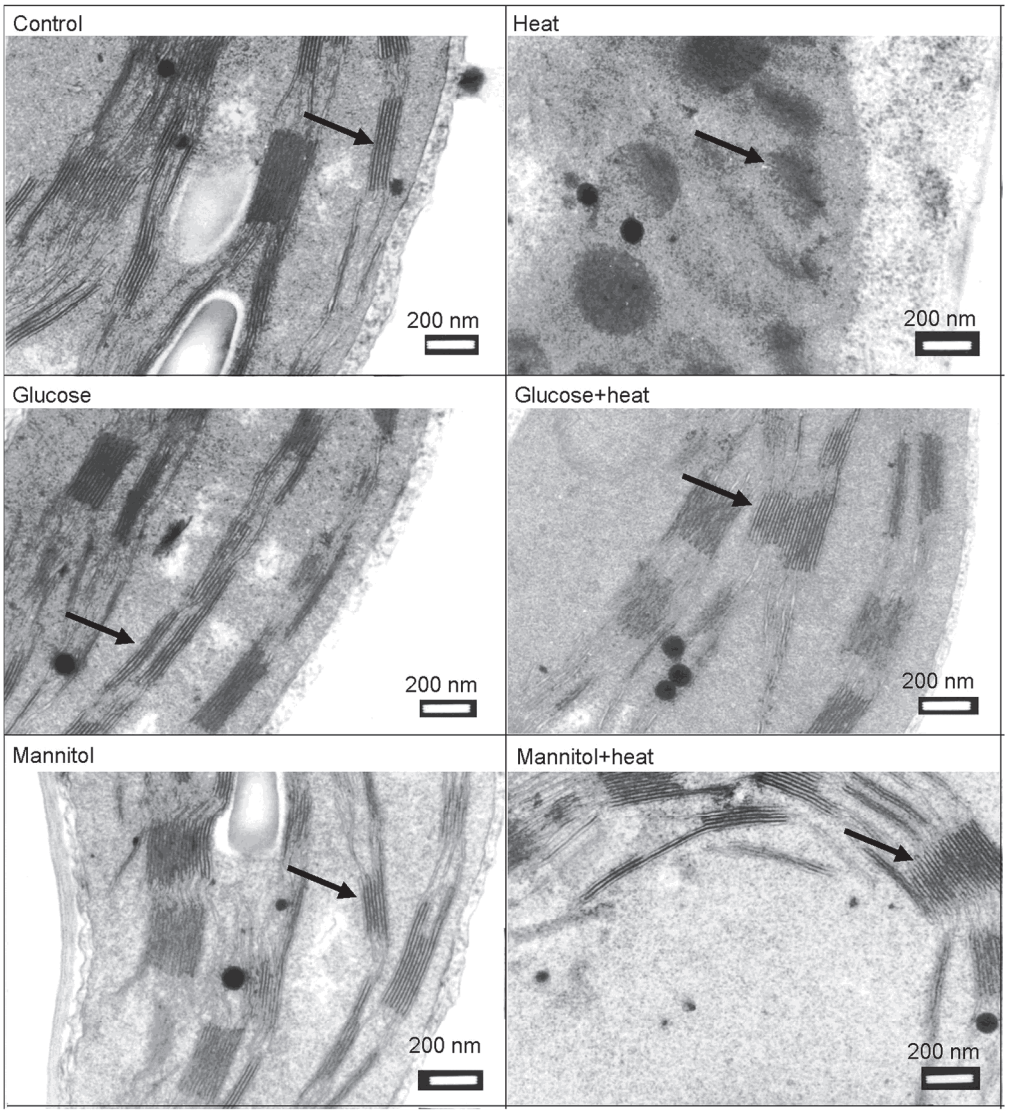

Fig. 3. Chloroplast thylakoids in the first layer palisade parenchyma of cucumber leaves.

Discussion

Heat stress inhibits cucumber growth (Baninasab and Ghobadi 2011). The same result was obtained in this study. When cucumber seedlings were pretreated with 30 mM glucose and then subjected to heat in the current experiment, the growth inhibition caused by the heat was alleviated indicating that the pretreatment with glucose at a certain concentration enhanced the ability of cucumber seedlings to resist the heat stress. This can be demonstrated also by less changes of chloroplast ultrastructure under the heat stress in the glucose-pretreated than in untreated plants observed in this study.

MDA is a product of membrane lipid peroxidation

Table 3. Changes in plant growth parameters [g], content of glucose and fructose [mg g^{-1}(f.m.)], MDA and H_2O_2 [nmol g^{-1}(f.m.)], and GSH [mg g^{-1}(f.m.)], $O_2^{\cdot-}$ production [nmol g^{-1}(f.m.) min^{-1}], activities of SOD [U mg^{-1}(protein)], CAT, APX, MDHAR, DHAR, and GR [μmol mg^{-1}(protein) min^{-1}], relative expression of *Cu/Zn-SOD*, *Mn-SOD*, *CAT* and *GR* genes, content of proline [μg g^{-1}(f.m.)] and soluble sugars [mg g^{-1}(f.m.)], and activities of SAI and NI [nmol mg^{-1}(protein) min^{-1}] in the second leaves after a glucose or mannitol pretreatment and heat stress. Means ± SE, n = 3. Different letters indicate statistically significant differences between treatments at $P < 0.05$.

Parameters	Control	Glucose	Mannitol	Heat	Glucose+heat	Mannitol+heat
Leaf fresh mass	1.56±0.00b	1.79±0.01a	1.21±0.01c	1.19±0.02c	1.49±0.05b	0.95±0.01d
Leaf dry mass	0.15±0.01c	0.18±0.02a	0.13±0.01d	0.13±0.01d	0.16±0.02b	0.10±0.02e
Root fresh mass	1.72±0.00c	2.19±0.01a	1.42±0.01e	1.51±0.01d	1.97±0.01b	1.28±0.01f
Root dry mass	0.07±0.02e	0.20±0.01a	0.14±0.01b	0.06±0.01f	0.12±0.01c	0.09±0.02d
Shoot fresh mass	2.78±0.01c	3.02±0.02a	2.50±0.01d	2.44±0.04d	2.87±0.01b	2.21±0.01e
Shoot dry mass	0.26±0.01b	0.28±0.01a	0.23±0.00d	0.22±0.01e	0.25±0.01c	0.21±0.01f
Glucose	0.16±0.01e	0.33±0.00d	0.00±0.00f	1.68±0.00b	4.47±0.00a	1.01±0.00c
Fructose	0.05±0.00e	0.18±0.00c	0.00±0.00f	0.28±0.00b	0.35±0.00a	0.08±0.00d
MDA	5.86±0.08e	5.51±0.11f	7.38±0.09d	14.24±0.03b	10.86±0.17c	16.78±0.29a
H_2O_2	94.63±3.80f	73.13±1.43e	149.09±2.48d	301.02±7.98b	220.76±2.87c	338.29±6.57a
GSH	0.83±0.00e	0.95±0.01d	0.82±0.00f	1.28±0.01b	1.34±0.01a	1.04±0.01c
$O_2^{\cdot-}$	3.87±0.03b	2.55±0.09d	2.65±0.09d	5.28±0.11a	3.01±0.06c	3.93±0.07b
SOD	26.10±0.14e	34.50±0.36c	31.83±0.33d	31.44±0.20d	39.32±0.44a	36.02±0.35b
CAT	0.05±0.00d	0.08±0.00c	0.04±0.00e	0.08±0.00c	0.12±0.00a	0.11±0.00b
APX	0.10±0.01f	0.43±0.01d	0.29±0.00e	0.69±0.02c	1.07±0.01a	0.82±0.02b
MDHAR	0.06±0.02e	0.08±0.01d	0.05±0.02e	0.15±0.02b	0.17±0.01a	0.12±0.01c
DHAR	0.06±0.01d	0.06±0.01d	0.07±0.02c	0.16±0.03b	0.21±0.02a	0.15±0.02b
GR	0.78±0.00e	2.24±0.00d	2.25±0.00d	4.76±0.22c	7.12±0.14a	5.75±0.12b
Cu/Zn-SOD	0.37±0.02e	0.53±0.02d	0.13±0.01f	1.72±0.06b	3.47±0.20a	1.33±0.04c
Mn-SOD	0.85±0.08e	2.16±0.04b	0.56±0.00f	1.77±0.06c	3.00±0.13a	1.24±0.04d
CAT	0.54±0.04d	1.19±0.04b	0.35±0.03e	1.12±0.05b	1.34±0.02a	0.91±0.02c
GR	0.56±0.04f	1.50±0.12d	1.00±0.09e	2.08±0.06c	4.26±0.41a	2.70±0.09b
Proline	18.51±0.06e	21.45±0.16d	12.14±0.11f	50.02±0.11b	66.57±0.11a	32.18±0.11c
Soluble sugar	4.99±0.02e	7.06±0.01c	2.92±0.01f	7.48±0.11b	11.42±0.02a	5.91±0.02d
SAI	5.17±0.17f	15.67±0.01d	8.50±0.17e	17.50±0.17c	32.67±0.17a	19.67±0.01b
NI	101.33±0.33f	156.50±0.17d	126.33±0.33e	171.33±0.83c	232.17±0.17a	201.83±0.17b

caused by ROS and is often regarded as indicator of stress damage (Dhindsa *et al*. 1981). Heat elevates MDA content in *Cucumis melo* (Zhang *et al*. 2014) and also in cucumber leaves in this study. A pretreatment with α-tocopherol decreases MDA content in heat-stressed wheat leaves (Kumar *et al*. 2013). Similarly, exogenous glucose reduced the MDA content in cucumber leaves under the heat stress in this study. This is consistent with the changes of $O_2^{\cdot-}$ and H_2O_2 content in the glucose-pretreated heat-stressed seedlings (Table 3) and indicates that the pretreatment with glucose decreased the ROS accumulation and thus mitigated damage by heat to some extent.

As major site for generating ROS, chloroplasts were studied in the current experiment. The cucumber chloroplasts became round and thylakoids were disorganized under the heat stress similarly as observed by Gao *et al*. (2010). Li *et al*. (2012) found that a methyl jasmonate pretreatment helps to prevent chloroplast membrane fusion in leaves subjected to heat stress. Similarly in this study, the pretreatment with glucose decreased the percentage of abnormal chloroplasts and protected the chloroplast ultrastructure under the heat-stress, which coincided with a less ROS accumulation.

SOD dismutates $O_2^{\cdot-}$ into H_2O_2, whereas CAT and APX regulate H_2O_2 content (Shigeoka *et al*. 2002). Moreover, APX needs AsA as substrate and that can be regenerated by MDHAR or GSH-dependent DHAR, being coupled with GR (a key enzyme for GSH regeneration). As adaptive response to stress environments, the activity of SOD in plants under chilling (Lee and Lee 2000), and the activities of CAT, APX, DHAR, MDHAR, and GR under heat-stress (Dai *et al*. 2012) increase. Similarly in this study, the heat treatment elevated the activities of SOD, CAT, APX, DHAR, MDHAR, and GR in cucumber. When the glucose-pretreated seedlings were exposed to the heat, the activities of SOD, CAT, APX, MDHAR, and GR increased more than in those treated with the heat alone. Similarly, the combination of 24-epibrassinolide pretreatment and high temperature increases some antioxidant activities more than high temperature alone

(Zhang *et al*. 2014). In the glucose + heat treatment, the enhanced activities of antioxidant enzymes were accompanied by the elevated transcription of the *Cu/Zn-SOD*, *Mn-SOD, CAT*, and *GR* genes and the increased content of GSH. This suggests that exogenous glucose regulated the accumulation of ROS and decreased membrane lipid peroxidation in heat-stressed cucumbers through inducing some antioxidant enzymes. Similarly, a cinnamic acid pretreatment alleviates growth inhibition of cucumber under chilling stress through the enhancement of antioxidant enzyme activities (Li *et al*. 2011).

Exogenous uniconazole enhances the content of proline and soluble sugars and induces the tolerance of cucumber seedlings to drought stress (Zhang *et al*. 2007). In this study, we got similar results.The pretreatment with glucose increased the content of proline and soluble sugars under the heat stress. This was confirmed by the increased content of endogenous glucose and fructose and by the enhanced activities of sugar metabolism-related enzymes including SAI and NI (Table 3). The simultaneously decreased content of $O_2^{\cdot-}$, H_2O_2, and MDA indicates that proline and soluble sugars might play roles in the inhibition of ROS accumulation under the heat stress due to the glucose pretreatment.

In the current experiments, exogenous mannitol increased the activities of some antioxidant enzymes and mitigated the growth inhibition and chloroplast damage to some extent under the heat stress. Similarly, the application of mannitol alleviates salt stress by enhancing antioxidant enzyme activities (Seckin *et al*. 2009) and protects chloroplast under cold stress (Stoyanova-Koleva *et al*. 2013). However, the mannitol pretreatment mitigated the heat-induced growth inhibition and chloroplast damage less than glucose at the same concentration in this study. The reason may be that mannitol is not as easy metabolized as glucose (Zhifang and Loescher 2003), and exogenous glucose does not act only as osmotic regulator when it enhances heat tolerance of seedlings.

In conclusion, the pretreatment with 30 mM glucose alleviated the damage by heat to plant growth and chloroplast membranes more than 30 mM mannitol. The glucose pretreatment increased the activities of SOD, CAT, APX, DHAR, MDHAR, and GR under the heat stress together with the increased transcriptions of *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, and *GR* genes and also the increased content of GSH, proline, and soluble sugars. Thus, the glucose pretreatment decreased the ROS accumulation and membrane lipid peroxidation to some extent in heat-stressed cucumber, and enhanced heat tolerance of the cucumber seedlings.

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