INTRODUCTION

Future global temperature change, with predicted 1.5–5.8 °C increases in temperatures by 2100, will cause increased heat stress to plants and create threats to agricultural production (Rosenzweig et al., 2001). The increasing threat of temperature change is already having a substantial impact on agricultural production worldwide as heat waves cause significant yield losses posing great risks for future food security for humankind (Christensen and Christensen, 2007). The unfavorable effects of heat stress can be mitigated by developing crop plants with improved thermotolerance using an assortment of genetic approaches. For this reason, it is crucial to have a thorough understanding of the physiological responses of plants to high temperatures and their mechanisms of heat tolerance, as well as to formulate possible strategies for improving crop thermotolerance.

Photosynthesis is one of the most sensitive physiological responses in plants to heat stress. Thus, it is important to maintain high photosynthetic activity for heat stress tolerance in plants (Berry and Björkman, 1980). When plants are subjected to high temperatures, carbon dioxide (CO₂) fixation, oxygen (O₂) evolution, and photophosphorylation are restrained rapidly (Berry and Björkman, 1980). The limit of CO₂ fixation by high temperature occurs simultaneously with the inactivation of ribulose 1, 5 bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) activase, which leads to the activation of RuBisCO (Feller et al., 1998; Salvucci et al., 2004). In the thylakoid membrane, the most sensitive component element to high temperature is photosystem II (PSII). Heat stress may suppress the light-absorption capacity of the plant owing to the dissolution of the O₂ evolution apparatus (Mamedov et al., 1993; Nash, et al., 1985; Tompson et al., 1989).

Many studies have shown that the instantaneous response of leaf carbon exchange to temperature depends on the temperature experienced by the plant over longer time periods, a response termed temperature acclimation (Atkin et al., 2005; Atkin and Tjoelker, 2003; Berry and Björkman, 1980; Smith and Dukes, 2013; Way and Yamous, 2014; Yamori et al., 2014). Temperature acclimation can be observed through a change in the parameters that define the instantaneous temperature response curve as a result of changes previously experienced by the plant or the acclimated temperature (Atkin and Tjoelker, 2003). Hikosaka et al. (2006) indicated that changes in the photosynthesis-temperature curve with long-term thermal acclimation are attributable to four factors: intercellular CO₂ concentration, activation energy of the maximum rate of RuBP carboxylation (Vcmax), activation energy of the rate of RuBP regeneration (Jmax), and the ratio of Jmax to Vcmax. These results suggest that the quantitative intensification of RuBisCO by thermal acclimation makes up for its decreased activation state and leads to the recovery of photosynthetic potential after inhibition from heat stress.

Keywords: chlorophyll fluorescence, gas exchange rate, nitrogen nutrient, RuBisCO activase
activation energy of Vcmax. Therefore activation energy of Vcmax which contributes the content of RuBisCO is common important factor for both long and short term thermal acclimation.

Our previous study (Li et al., 2003) indicated that, although the maximum quantum yield of PSII (Fv/Fm) in cucumber (Cucumis sativus L., cv. Suyo) leaves was subjected to extensive suppression on heat stress treatment of 45 °C leaf temperatures for 10 minutes, these suppressions were alleviated by subjecting the plants to thermal acclimation treatment at growth temperatures of 38 °C for 4 days before heat stress. This enhancement of PSII tolerance through thermal acclimation may be involved in an increased stability in the O2 evolution apparatus, along with a decrease in the unsaturation grade of the thylakoid membrane lipids (Li et al., 2003). As mentioned above, it is suggested that photosynthesis improvement through thermal acclimation may induce a modification of RuBisCO.

However, information on the photosynthetic response to thermal acclimation in cucumber leaves is only exhibited in PSII (Li et al., 2003) and not in the CO2 fixation system.

In this study, to clarify the enhancement mechanism of heat stress tolerance by thermal acclimation, we investigated the CO2 fixation responses to thermal acclimation and heat stress in cucumber leaves.

MATERIALS AND METHODS

Plant cultivation and the thermal acclimation treatment
Cucumber plants were germinated in the dark at 25°C for 2 days and then sowed onto a vermiculite tray in a glass greenhouse. When the cotyledons were fully expanded, they were transplanted into a clay pot filled with small gravel supplied with half-strength (0.5) Hoagland nutrient solution. When the first leaves were fully expanded, they were transferred to a growth chamber (FLI-301NH; EYE-LA, Tokyo, Japan) with 25/20 °C, 60 % relative humidity (RH), and a photoperiod of 14 hours illuminated at photosynthetic photon flux density (PPFD) of 150 μmol m−2 s−1. The thermal acclimation treatment (38/38 °C, day/night temperatures, 85 % RH) was started when the second leaves were fully expanded and continued for 4 days. The second leaves were used for photosynthesis measurement after 0, 2, and 4 days of the thermal acclimation treatment and stored at −80°C for RuBisCO analysis after photosynthesis measurement.

Photosynthetic parameter measurement in cucumber leaves before and after heat stress treatment
Heat stress treatment was conducted by dipping the aboveground plant into a water bath at 45°C for 10 minutes under illumination at a PPFD of 90 μmol m−2 s−1 (Nada et al., 2020). The gas exchange rate was measured on the fully expanded second leaves before heat stress treatment using a portable gas exchange measurement system (LCA-4; Shimadzu, Kyoto, Japan). The CO2 concentration and RH of the fully expanded second leaves (leaf area 6.25 cm2, 2.5×8.0×0.7 cm) placed in a small assimilation chamber (PLC-4; Shimadzu, Kyoto, Japan) were measured using LCA-4. The air flowing into the assimilation chamber, set at 400 mL min−1, was considered to be the atmosphere and RH was adjusted to 30 % using a desiccant (Drierite, W.A. Hammond Drierite Co., Ltd., USA). The chamber temperature was set at 25 °C and the PPFD at 1,100 μmol m−2 s−1 using a metal halide lamp (LS-M180; Sumita Optical Glass, Inc., Japan). Net photosynthetic rate, respiration rate, stomatal conductance (Gs), and transpiration rate (TR) were calculated as described by von Caemmerer and Farquhar (1981), and the gross photosynthetic rate (PG) was calculated by adding the respiration rate and the net photosynthetic rate. Each parameter was expressed as the mean of four individuals. After heat stress treatment, the water adhering to the plant was wiped using Kimwipes (Nippon Paper Creica Co., Ltd., Tokyo, Japan). The plant was then returned to the growth chamber at 25 °C and 60 % RH for 20 minutes. The gas exchange rate of leaves with dry surfaces was measured again at chamber temperatures of 25 °C. Their effective quantum yield of PSII (ΦPSII) was determined using a chlorophyll fluorometer (PAM-2000; Walz, Germany) simultaneously with gas exchange rate under PPFD of 1,100 μmol m−2 s−1. To measure the maximum quantum yield of PSII (Fv/Fm), leaves were dark-acclimated using a leaf clip holder for 30 minutes before the first gas-exchange measurement. The Fv/Fm value was determined at the end of the first gas-exchange measurement. Subsequently, the leaves underwent dipping treatment for heat stress for 10 minutes with a leaf clip holder, followed by induction processing at 25 °C for 20 minutes (i.e., dark-acclimated for 30 minutes); Fv/Fm was determined again before the second gas-exchange measurement. Each parameter was expressed as the mean of four individuals.

Ribulose 1, 5 bisphosphate carboxylase/oxygenase activity and content analysis in cucumber leaves
Three leaf discs (0.15 cm) were obtained before and after the dipping treatment of the leaves that had been used to measure gas exchange and chlorophyll fluorescence, and were immediately placed in liquid nitrogen (N), and stored at −80°C for analysis of RuBisCO activity. The residual leaf (1 g) was stored at −30°C for RuBisCO content analysis.

RuBisCO activity was analyzed based on the method described by Nada et al. (2020). To measure the initial activity of RuBisCO, leaf discs stored at −80°C were ground in liquid N, to which extraction buffer [100 mM Tricine-potassium hydroxide (KOH) (pH 7.8), 5 mM dithiothreitol (DTT), 5 mM magnesium chloride (MgCl2), 1 mM disodium ethylenediaminetetraacetic acid (Na2-EDTA), 0.2 % bovine serum albumin (BSA) (w/v)], 0.5 mM PMSF, and 20 % PVPP (w/v) were added, and the sample was mixed using a metal halide lamp (LS-M180; Sumita Optical Glass, Inc., Japan) and then centrifuged (at 4°C and 2,500×g for 30 seconds). The supernatant and analysis buffer [100 mM Tricine-KOH (pH 7.8), 5 mM DTT, 20 mM MgCl2, 1 mM Na2-EDTA, 5 mM potassium chloride (KCl)], 2.5 mM ATP, 10 mM sodium bicarbonate (NaHCO3), 0.2 mM nicotinamide adenine dinu-
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cleotide (NADH), 5 mM Creatin-P, 1.2 mM RuBP, 6.4 U GAPDH, 9.0 U PGK, and 20 U PCK were mixed and the attenuation rate was measured at 340 nm using a spectrophotometer (UV-1200; Shimadzu, Japan) at 25°C. The time required to add the extraction buffer solution at the beginning of measuring absorbance was almost 180 seconds. For measurement of the total activity of RuBisCO, the residual of the supernatant in the first extraction was again centrifuged at 4°C and 10,000g for 10 minutes, after which the analysis buffer, except RuBP, was added to it and the solution was incubated in a spectrophotometer at 25°C for 2 minutes. The attenuation rate at 340 nm was measured by adding RuBP (1.2 mM). The initial and total activity of RuBisCO (μmol·m⁻²·s⁻¹) was expressed as the mean of four individuals.

RuBisCO content was analyzed based on the method described by Nada et al. (2020). Leaves (1 g) stored at −30°C were ground in liquid N using a pestle. Proteins were extracted on ice using an extraction buffer (100 mM sodium phosphate buffer pH 7.0, 2 mM MgCl₂, 1 mM Na₂EDTA, 12.5 % glycerol, 1 % 2-mercaptoethanol), 0.1 mM PMSF, and 20 % PVP (w/v). The solution was centrifuged at 10,000g for 10 minutes. The solution thus produced (ashing solution) was sealed tightly and stored. One milliliter of the ashing solution was added to the magnetic plate and evaporated to dryness on a hot plate set to 180°C, after which 100 mg of leaf powder was placed in a muffle furnace for 10 minutes at 400°C. Then, 40 mL of Laemmli buffer [100 mM Tris-hydrochloride (HCl), pH 6.8, 4 % sodium dodecyl sulphate (SDS) (w/v), 12 % 2-mercaptoethanol (w/v), 20 % glycerol (w/v), 0.2 % BBP] and 8 μL of 2-mercaptoethanol were added to 40 μL of protein extracts, and heated at 100°C for 5 minutes. After cooling to room temperature, 10 μL of the sample was loaded onto an SDS-polyacrylamide gel (resolving gel, 12.5 % polyacrylamide; stacking gel, 4.5 % polyacrylamide) filled with electrophoresis buffer (25 mM Tris, 192 mM glycine, 0,1 % SDS (w/v)). Electrophoresis was performed using power supply equipment (CONSTAPOWER 3500; ATTO, Japan). An electric current was set at 20 mA in the stacking gel and at 40 mA in the resolving gel. After electrophoresis, the gel was stained using the Coomassie brilliant blue (CBB) solution [0.1 % CBB-R250 (w/v), 25 % ethanol (w/v), 7 % acetic acid (w/v)]. After decoloring treatment, bands of 55 kDa, which corresponded to the large subunit of RuBisCO, were photographed using FAS III (Toyobo, Japan), and quantified using NIH-Image (free software). The RuBisCO content (mg g⁻¹FW) was expressed as the mean of four individuals.

Mineral content analysis

For potassium (K), magnesium (Mg), and phosphorus (P) analysis, organic matter was removed from the dry matter of leaves using the dry-ashing method. The freeze-dried, fully expanded, second leaves were ground to a powder, after which 100 mg of leaf powder was placed in a magnetic plate and heated at 550°C in a muffle furnace for 10 hours. Thereafter, 5 mL of 6 N hydrochloric acid (HCl) was added to the magnetic plate and evaporated to dryness on a hot plate set to 180°C. Then, 5 mL of 1 N HCl was added to the dried matter and warmed on the hot plate for 10 minutes. The solution thus produced (ashing solution) was sealed tightly and stored. One milliliter of the ashing solution was added to 2 mL of lanthanum chloride solution (La: 25,000 ppm) and its K and Mg content was measured using atomic absorption spectrometry (AA-6200; Shimadzu, Japan). For measurement of P content, 5 mL of the ashing solution was mixed with 5 mL of the coloring reagent vanadomolybdate-nitric acid, after which the absorbance of the mixture was measured at 430 nm using a spectrophotometer (UV-1200; Shimadzu, Japan). The K, Mg, and P content per dry weight (mg g⁻¹DW) was calculated using a standard curve derived from the absorbance of each ion standard solution and expressed as a mean of four individuals.

Nitrogen analysis was performed using the Kjeldahl method. Leaf powder (100 mg) was placed in a Kjeldahl flask to which 4 mL sulfuric acid was added. The Kjeldahl flask was placed in a fume hood overnight. Then, the Kjeldahl flask was incubated at 440°C for 4 minutes using a Digested digestion apparatus (Echo, USA), after which 10 mL of hydrogen peroxide (H₂O₂) was added to it and the mixture was heated again for 1 minute. After cooling to room temperature, the pyrolysis liquid in the Kjeldahl flask was diluted to 100 mL using distilled water. Then, 25 g diluting solution and 4 mL of 40 % sodium hydroxide (NaOH) were put in a distillatory apparatus and distilled for 8 min. Ammonia (NH₃) that was obtained from distillation was titrated using dilute sulfuric acid. The N content per gram of dry weight (mg g⁻¹ DW) was calculated based on the titration value and expressed as the mean of four individuals.

Statistical analysis

The data were analyzed using the Excel statistics software (Exsum, Japan). Significant differences (P < 0.05) among treatments were determined using the Tukey-Kramer test.

RESULTS

Photosynthetic parameters, RuBisCO activity and content, and mineral content in cucumber leaves after thermal acclimation

Although the PG, Fv/Fm, and ΔF/ΦPSII of thermally acclimated and non-acclimated leaves did not differ significantly, the TR and GS of thermally acclimated leaves were significantly higher compared to those of non-acclimated leaves (Table 1). The initial activity of RuBisCO also showed no significant difference in between thermally acclimated and non-acclimated leaves, whereas the total activity of thermally acclimated leaves was significantly higher compared to that of non-acclimated leaves. The ratio of initial to total activity in non-acclimated leaves was higher than 90 %, but that of thermally acclimated leaves decreased significantly. The RuBisCO content showed the same tendency as total activity, i.e., it increased after thermal acclimation treatment.

The N content of non-acclimated leaves increased once on 2d, but returned to the level before the treatment on 4d, whereas that of acclimated leaves also increased on 2d of the thermal acclimation treatment and remained at a high level for the duration of the treatment (Table 2). The K content of thermally acclimated leaves was lower than...
Heat stress and remained the same during treatment. The initial activity of RuBisCO in non-acclimated leaves decreased to 46 % by heat stress and remained almost the same during treatment, whereas that of thermally acclimated leaves on 4d of treatment recovered to 69 % of those before the heat stress (Fig. 3). The total activity of RuBisCO in both thermally acclimated and non-acclimated leaves did not have an influence on heat stress, i.e., these values were approximately equal to those before heat stress. The ratio of initial to total activity of RuBisCO in both thermally acclimated and non-acclimated leaves decreased to about 50 % by heat stress and remained the same during treatment.


different letters indicate significant differences at \( P < 0.05 \) using Tukey-Kramer’s test.

DISCUSSION

In the photosynthetic responses to heat stress in cucumber leaves, PG and Fv/Fm decreased to 13 and 28 %, respectively, due to heat stress at leaf temperature 45°C for 10 minutes (Fig. 1, 2). These severe inhibitions by heat stress were alleviated by thermal acclimation at a growth temperature of 38°C for 4 days. The PG and Fv/Fm recovered to 92 and 88 % of those before exposure to heat stress, respectively. The photosynthetic enhancement of heat tolerance in cucumber was also confirmed by the O₂ evolution rate and the electron transport rate of PSI and PSII using the O₂ electrode method (Li et al., 2003). Li et al. (2003) also indicated that thermal acclimation induced stabilization of the thylakoid membrane by increasing its lipid saturation, thus resulting in the enhancement of heat tolerance of photosystem in cucumber.

Smith and Duked (2017) indicated that the thermal acclimation of photosynthesis occurred by changing the temperature only at 7 days (i.e., after mechanisms) when provided with high levels of water and N. The plant cultivation conditions in the present research were appropriate to allow “the fast mechanisms” function. In addition, it was observed that the decrease in a nutrient solution in thermally acclimated seedlings was faster than that in non-acclimated seedlings. The reason for this phenomenon seems to be an increase in absorption of a nutrient solution with an increase in TR of thermally acclimated leaves (Table 1). As a result, the mineral nutrient status of thermally acclimated leaves was progressive, i.e., N and Mg content of thermally acclimated leaves was higher than that of non-acclimated leaves (Table 2).

We found that PG, Fv/Fm, and \( \Phi PSII \) did not differ...
significantly between thermally acclimated and non-acclimated leaves (Table 1). Our previous research indicates that the photosynthetic parameters in cucumber leaves do not decrease even at growth temperatures of 40 °C because of a decrease in leaf temperatures with an increase in TR. The thermal acclimation temperature of 38 °C was within the temperature range studied in the previous research and did not decrease the photosynthetic function of cucumber leaves. The total activity and content of RuBisCO in thermally acclimated leaves increased significantly compared to that in non-acclimated leaves (Table 1). This could be related to the increase in N content of leaves (Table 2). Hikosaka et al. (2006) have indicated that the activation energy of Vcmax is the most important factor for long-term thermal acclimation of photosynthesis. Smith and Dukes (2017) have also suggested that “fast mechanism” of thermal acclimation may be attributable to activation energy of Vcmax, which contributes the total activity and content of RuBisCO. Therefore, it may be important for the increase in heat tolerance that thermal acclimation enhance RuBisCO synthesis, as shown in the present research. Thermal acclimation had a negative effect on the initial activity of RuBisCO in spite of an increase in total activity, implying that the activation state of RuBisCO (i.e., activity of RuBisCO activase; Salvucci and Crafts-Brandner, 2004) decreased during thermal acclimation treatment, as shown by the decrease in the ratio of initial to total activity (Table 1). After heat stress treatment, the ratio of initial to total activity remained at a low level in both thermally acclimated and non-acclimated leaves; however, initial activity increased only in thermally acclimated leaves. These results suggest that the quantitative intensification of RuBisCO by thermal acclimation makes up for a functional decline of RuBisCO activase and leads photosynthetic potential to recover from the inhibition of heat stress.

We propose that the enhancement of heat tolerance in photosynthesis by thermal acclimation in cucumber leaves occurs due to the stabilization of the thylakoid membrane by an increase in its lipid saturation, as shown by Li et al. (2003), and by the quantitative intensification of RuBisCO with an increase in N content of leaves, as shown in this research. The prerequisite for a thermal acclimation
response is the presence of high levels of water and N (Smith and Dukes, 2017); however, these conditions are not necessarily guaranteed under natural conditions. Therefore, we should accumulate information on tolerance mechanisms in plants against multiple stresses, e.g., high temperature, water stress, and deficit of N.

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REFERENCES

Atkin, O. K., Bruhn, D., Hurry, V. M., Tjoelker, M. G. 2005. The hot and cold: unravelling the variable response of plant respiration to temperature. Funct. Plant Biol. 32: 87-105.

Atkin, O. K., Tjoelker, M. G. 2003. Thermal acclimation and dynamic response of plant respiration to temperature. Trends Plant Sci. 8: 343-351.

Berry, J., Björkman, O. 1980. Photosynthetic response and adaptation to temperature in higher plants. Annu. Rev. Plant Physiol. 31: 491-543.

Christensen, J. H., Christensen, O. B. 2007. A summary of the PRUDENCE model projections of changes in European climate by the end of this century. Clim. Change 81: 7-30.

Feller, U., Crails-Brandner, S. J., Salvucci, M. E. 1998. Moderately high temperatures inhibit ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activate-mediated activation of Rubisco. Plant Physiol. 116: 539-546.

Gunderson, C. A., O’hara, K. H., Campion, C. M., Walker, A. V., Edwards, N. T. 2010. Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate. Glob. Chang. Biol. 16: 2272-2286.

Hikosaka, K., Ishikawa, K., Borjigida, A. Muller, O., Onoda, Y. 2006. Temperature acclimation of photosynthesis: mechanisms involved in the changes in temperature dependence of photosynthetic rate. J. Exp. Bot. 57: 291-302.

Li, Z., Nada, K., Tachibana, S. 2010. High-temperature-induced alteration of ABA and polyamine contents in leaves and its implication in thermal acclimation of photosynthesis in cucumber (Cucumis sativus L.). J. Jpn. Soc. Hort. Sci. 72: 393-401.

Mamedov, M., Hayashi, H., Murata, N. 1993. Effects of glycinebetaine and unsaturation of membrane lipids on heat stability of photosynthetic electron-transport and phosphorylation reactions in Synechocystis PCC6803. Biochem. Biophys. Acta 1142: 1-5.

Nada, K., Mukoh, S., Hiratsuka, S. 2020. Evaluation of photosynthetic responses to heat stress in cucumber leaf treated by dipping into heating water under illuminated condition. J. Sci. High Technol. Agric. 32: 214-220.

Nash, D., Miyao, M., Murata, N. 1985. Heat inactivation of oxygen evolution in photosystem II particles and its acceleration by chloride depletion and exogenous manganese. Biochim. Biophys. Acta 807: 127-133.

Rosennweig, C., Iglesias, A., Yang, X. B., Epstein, P. R., Chivian, E. 2001. Climate change and extreme weather events. Implica-
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Salvucci, M. E., Crafts-Brandner, S. J. 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. Physiol. Plant. 120: 179–186.

Smith, N. G., Dukes, J. S. 2013. Plant respiration and photosynthesis in global-scale models: incorporating acclimation to temperature and CO₂. Global Change Biol. 19: 45–63.

Smith, N. G., Dukes, J. S. 2017. Short-term acclimation to warmer temperature accelerates leaf carbon exchange processes across plant types. Global Change Biol. 23: 4840–4853.

Tompson, L. K., Blaylock, R., Sturtevant, J. M., Brudvig, G. W. 1989. Molecular basis of the heat denaturation of photosystem II. Biochemistry 28: 6686–6695.

von Caemmerer, S., Farquhar, G. D. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153: 376–387.

Way, D. A., Yamori, W. 2014. Thermal acclimation of photosynthesis: on the importance of adjusting our definitions and accounting for thermal acclimation of respiration. Photosyn. Res. 119: 89–100.

Yamori, W., Hikosaka, K., Way, D. A. 2014. Temperature response of photosynthesis in C3, C4, and CAM plants: temperature acclimation and temperature adaptation. Photosyn. Res. 119: 101–117.
