High-temperature induced Alteration of ABA and Polyamine Contents in Leaves and its Implication in Thermal Acclimation of Photosynthesis in Cucumber (*Cucumis sativus* L.)

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**Summary**

The possible involvement of ABA and polyamines in the thermal acclimation process of photosynthesis in cucumber (*Cucumis sativus* L., cv. Nankyou No. 2) was investigated. Photosynthetic activity was measured in the photosynthetic oxygen evolution rate and photosystem (PS) II chlorophyll fluorescence yield (Fv/Fm). Raising the growth temperature from 25/25 °C to 38/38 °C (day/night) caused gradual reduction in heat damage to photosynthesis from the prior exposure of intact leaves to 45 °C. Thylakoids isolated from acclimated leaves were more thermostable than those from non-acclimated leaves, as judged by a lesser degree of reduction in PS II and PS I electron transport activity and the loss of 33-kDa polypeptides and manganese after exposure of isolated thylakoids to 40 °C. The enhanced thermostability of the photosynthetic apparatus is probably attributable to the decrease in lipid unsaturation of thylakoid membranes. Exposing the plants to 38 °C caused a rapid decrease in ABA content in leaves, which may exclude the possible involvement of ABA in increased thermostability of photosynthesis during the acclimation treatment. However, acclimation treatment caused a gradual increase in spermidine and spermine titers in leaves. Application of 5 mM spermidine or spermine to non-acclimated leaves made the photosynthetic apparatus more thermostable. Spermine treatment to isolated thylakoids from non-acclimated leaves also alleviated the heat-inactivation of photosystems. The results suggest that polyamines play a role in thermal acclimation of photosynthesis in cucumber.

**Key Words:** cucumber, heat stress, photosynthesis, polyamine, thermal acclimation.

**Introduction**

Plant behavior under the influence of high temperature is related to the irreversible inactivation of photosynthesis. There is unequivocal evidence that in higher plants exposed to heat stress, the photosynthetic apparatus is irreversibly damaged prior to impairment of other cellular functions (Berry and Björkman, 1980). This indicates that heat-damage to photosynthesis is a major cause of growth inhibition at supraoptimal temperature. Irreversible heat-damage to photosynthesis includes inhibition of various photochemical and enzyme functions in chloroplasts. Among the photosynthetic membranes, the donor side of PS II, i.e. the oxygen evolving complex, is considered to be most susceptible to heat-damage (Enami et al., 1994). It is also considered that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase is particularly labile to heat (Law and Crafts-Brandner, 1999).

Exposing the plants to a moderately high temperature for several days may result in reduced susceptibility of photosynthesis to heat damage (Yordanov et al., 1986).

This phenomenon, known as thermal acclimation of photosynthesis, is characterized by an upward shift in the optimal temperature for photosynthesis and the threshold temperature above which the photosynthetic apparatus is irreversibly heat-damaged (Berry and Björkman, 1980; Havaux, 1993). It is not uncommon that plants experience supraoptimal temperature particularly in the protected cultivation of vegetable crops in a hot summer. From the agricultural viewpoint, therefore, it is important to elucidate the physiological factors involved in thermal acclimation of photosynthesis.

Abass and Rajasheker (1993) observed that heat-tolerance induction in grape leaves during exposure to 38 °C corresponded with an accumulation of ABA in the leaves. We found previously that foliar application of 1 mM ABA was effective in inducing the thermostability of the photosynthetic apparatus in cucumber (Li et al., 2003). ABA has been suggested to be a common mediator for environmental stress because of its rapid response to various environmental stresses (Dai and Campbell, 1981). In view of these results and suggestions, we are interested in a possibility that ABA is involved in the thermal acclimation process of the photosynthetic apparatus in cucumber.

We are also interested in a possible involvement of
polyamines in thermal acclimation of photosynthesis. Polyamines such as putrescine (Put), spermidine (Spd) and spermine (Spm) are small molecular aliphatic amines that occur in all living cells. They are believed to play vital roles in growth, development and environmental stress tolerance in plants (Bouchereau et al., 1999; Cohen, 1998; Tachibana, 2000). Generally, stress–tolerant species and cultivars have high capacity to enhance polyamine biosynthesis in response to various kinds of environmental stresses (He et al., 2002a; Krishnamurthy and Bhagwat, 1984; Shen et al., 2000).

Although the implication of polyamines in heat tolerance of plants has not been well documented, Roy and Ghosh (1996) found that heat tolerance of rice cultivars was closely correlated with the level of polyamine accumulation in heat–stressed leaves.

Thus, the aim of this study was to examine the possibility that ABA and polyamines are involved in the thermal acclimation process of photosynthesis. For thermal acclimation treatment, cucumber plants were exposed to 38 °C because this temperature exerted little influence on photosynthesis in cucumber leaves (Wang and Tachibana, 1996).

Materials and Methods

Plant materials

Seedlings of cucumber (Cucumis sativus L., cv. Nankyoku No. 2) were grown in the glasshouse in 10-cm clay pots filled with commercial nursery soil. When the second leaves were fully expanded, the plants were transferred to a growth chamber operating at 25/25 °C (day/night) with a photoperiod of 12-hr under a light intensity of 300 μmol photon·m⁻²·s⁻¹. After 3 days, one set of plants was transferred to another growth chamber kept at 38/38 °C with other conditions being the same as those in the original growth chamber. Another set of plants, kept in the original growth chamber, served as the control. Relative humidity (RH) in the growth chamber was controlled at 85% to prevent the transpirational cooling of leaves. Thus, leaf temperature was the same as air temperature in both treatments.

Measurement of thermostability of photosynthesis

Plants were removed from the growth chambers at the middle of the light period before and 2 and 4 days after the thermal acclimation treatment. Then, all expanded leaves were heat–stressed by dipping them in a dark water–bath kept at 45 °C for 10 min as described previously (Li et al., 1996). Dipping the leaves in 25 °C–water for 10 min did not affect photosynthesis in cucumber. Thermostability of photosynthesis was expressed in terms of photosynthetic oxygen evolution rates and Fv/Fm of heat–stressed leaves in percent of those before stress. Photosynthetic oxygen evolution rates and PS II chlorophyll fluorescence yield (Fv/Fm) of the second leaves were measured at 25 °C with a Clark–type oxygen electrode (Rank Brothers, Cambridge, UK) and a chlorophyll fluorometer (MINI–PAM, Heinz Walz, Effeltrich, Germany), respectively, as described previously (Li et al., 2003).

Measurement of thermostability of photosynthetic electron transport in isolated thylakoids

Thylakoids were isolated from the second leaves as described by Li et al. (2003). To impose heat stress on isolated thylakoids, an aliquot of thylakoid suspensions (0.5 mg Chl·ml⁻¹) was placed in a micro–tube, and the tube was dipped in a dark water–bath at 40 °C for 5 min. During this heat treatment, the photosynthetic electron transport through PS II and PS I was measured at 1-min intervals at 25 °C in the light of 1.1 mmol photon·m⁻²·s⁻¹, as described previously (Li et al., 2003). Chlorophyll was quantified by the method of Arnon (1949).

To quantify the amount of proteins lost from isolated thylakoids during heat stress, thylakoid suspensions were heated at 40 °C for 3 min as above. Then, they were centrifuged at 42,000 × g for 10 min, and proteins in the supernatant were quantified by the protein–dye method (Bradford, 1976). The amount of proteins lost was estimated by the increase in proteins due to heat treatment. To carry out the electrophoresis of released proteins, proteins in the supernatant were precipitated with cold acetone, and the precipitate dissolved in sample buffer consisting of 62.5 mM Tris–HCl (pH 6.8), 2.3% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) β-mercaptoethanol and 10% (w/v) glycerol (Laemmli, 1970). A 20 μl aliquot of polypeptide solution, corresponding to 10 μg Chl of thylakoids, was subjected to SDS–PAGE using a 12% (w/v) polyacrylamide gel. The gel was stained with Silver Stain Kit Wako (Wako Pure Chemical Ind., Osaka, Japan).

The amount of heat–induced loss in manganese was estimated as follows: thylakoids were heated for 3 min as above, and transferred to a porcelain dish and evaporated to dryness on a hot plate. After dry–ashing in an electric furnace at 550 °C, the ash was dissolved in 1 N HCl. An atomic absorption spectrophotometer (AA–6200, Shimadzu, Kyoto, Japan) was used to determine manganese concentrations. The amount of manganese lost from thylakoids was estimated by the decrease in manganese contents in the thylakoids after heat stress.

Lipid analysis

Total lipids in thylakoid membranes were extracted with chloroform–methanol–water (1:2:0.8, v/v) (Bligh and Dyer, 1959). The lipids were fractionated into neutral lipids, glycolipids, and phospholipids using a Sep–Pak silica cartridge (Lynch and Steponkus, 1987). Then, fatty acids in glycolipids and phospholipids were derivatized to methyl esters with 5% (w/v) HCl in methanol. Fatty acid methyl esters were quantified via GLC–FID (GC–7A, Shimadzu, Kyoto, Japan) as described previously (Li et al., 2003). The degree of fatty
acid unsaturation was expressed as a molar ratio of unsaturated to saturated fatty acids.

**ABCA analysis**

ABCA was extracted at room temperature with 80% methanol (pH 7.0) containing 0.1 M acetic acid, 200 mg · liter⁻¹ of butylated hydroxytoluene and 100 mg · liter⁻¹ of sodium ascorbate (Huber and Reid, 1980). After the addition of trans-trans ABCA as an internal standard, the extract was purified by passing through LC-18 and LC-NH₂ columns (SUPELCO, Bellefonte, USA) (Guinn et al., 1986). Then, ABCA was transmethlated with diazomethane and quantified via GLC-ECD.

**Polyamine analysis**

Polymamines were extracted with 5% (v/v) perchloric acid (5 ml · g⁻¹ FW). After centrifugation, free polymamines in the supernatant were dansylated with dansyl chloride (10 mg · ml⁻¹ acetone) and dansyl polymamines extracted in toluene. Dansyl polymamines were analyzed via HPLC (LC-10AT VP, Shimadzu, Kyoto, Japan) as described by Song et al. (2002).

**Results**

**Thermostability of photosynthesis**

In plants grown under control conditions (25/25 °C), heat treatment of intact leaves at 45 °C in the dark for 10 min caused a severe loss in photosynthetic oxygen evolution rate and PS II chlorophyll fluorescence (Fv/Fm) (Fig. 1). These results indicate a rapid development of heat damage to the photosynthetic apparatus. When the plants were exposed to acclimation temperature (38/38 °C), the photosynthetic apparatus was made less susceptible to heat damage. This acclimatory effect of sub-lethal high temperature was increased with the increase in the duration of acclimation treatment.

![Fig. 1. Effect of growth temperature on thermostability of photosynthesis in cucumber leaves. Thermostability of photosynthesis was estimated by photosynthetic oxygen evolution rates and Fv/Fm remaining after heat in percent of those before heat (190–195 μmol · dm⁻² · hr⁻¹ and 0.82–0.83, respectively). ○, 25 °C; ●, 38 °C. Vertical bars indicate SE (n=3).](image1)

**Thermostability of photosynthetic electron transport in isolated thylakoids**

Since the Fv/Fm ratios reflect the energy-trapping efficiency of PS II (Havaux, 1993), the above results indicate the increased thermostability of PS II. To confirm this, thylakoids were isolated from acclimated and non-acclimated leaves and heated at 40 °C in the dark for 5 min, during which the PS II and PS I electron transport activities were measured at 25 °C at 1-min intervals. Thylakoids were heated at 40 °C instead of 45 °C because at 45 °C the thylakoids isolated from both treatments lost PS II activity completely within 1 min. Compared with thylakoids from control leaves, those from acclimated leaves showed a significant decrease in heat-induced loss of electron transport through both PS II and PS I (Fig. 2), indicating an increase in thermostability of photosystems. PS I was less thermostable than PS II and acquired higher thermostability especially after 4 days of acclimation.

The amount of proteins lost from isolated thylakoids during exposure to 40 °C for 3 min was smaller in acclimated than non-acclimated leaves (Fig. 3A). This effect of acclimation increased with increase in the duration of acclimation treatment. SDS-PAGE analysis of proteins in the incubation medium of thylakoids...
revealed that there were several polypeptides that showed an increase in band intensity after heat treatment of thylakoids (Fig. 3B). Among them, the band intensity of a polypeptide with a molecular mass of 33–kDa increased significantly in thylakoids from non-acclimated leaves, but only slightly in thylakoids from acclimated leaves. Since the amount of polypeptides loaded on the gel was correspondent to unit weight of thylakoids, the band intensity increases in proportion to the amount of proteins lost from unit weight of thylakoids. Thus, the thylakoids from non-acclimated leaves lost a substantial amount of 33–kDa polypeptides during heat treatment, which was alleviated in thylakoids from acclimated leaves.

The 33–kDa polypeptide is assumed to originate from manganese–stabilizing proteins in the oxygen–evolving complex of PS II (Enami et al., 1994). Our analysis shows that the thylakoids isolated from non-acclimated leaves lost 9–10 µmol manganese·mmol⁻¹ Chl during heat stress for 3 min. This heat–induced loss in manganese from thylakoids was reduced by the acclimation treatment to intact leaves to 7.3 at day 2 and further to 5.8 µmol·mmol Chl at day 4 (Fig. 4).

**Fatty acid composition of thylakoid membrane lipids**

The fatty acid composition of thylakoid membrane lipids extracted from plants exposed to 25 and 38 °C revealed that glycolipids were highly enriched with...
Fig. 6. Effect of growth temperature on the degree of lipid unsaturation of thylakoid membranes in cucumber leaves. The degree of lipid unsaturation is expressed in the molar ratio of unsaturated to saturated fatty acids in the lipids. □, 25°C; ■, 38°C. Vertical bars indicate SE (n=3).

Fig. 7. Effect of changes in growth temperature from 25°C to 38°C on ABA content in cucumber leaves. Vertical bars indicate SE (n=3).

Linolenate (18:3) while phospholipids were enriched with linolenate and palmitate (16:0). The acclimation treatment did not affect the concentrations of these polar lipids in thylakoid membranes (data not shown), but induced a significant alteration in their fatty acid composition (Fig. 5). In glycolipids, linolenate was decreased significantly at 38°C with a concomitant increase in palmitate, stearate (18:0) and linoleate (18:2). In phospholipids, high growth temperature caused a decrease in palmitoleate (16:1) and oleate (18:1) and an increase in palmitate. As a result, the degree of fatty acid unsaturation of thylakoid membrane lipids progressively declined as the duration of acclimation treatment was lengthened (Fig. 6). This decline of lipid unsaturation was more noticeable in glycolipids than in phospholipids.

ABA and polyamine contents in leaves

When plants with three expanded leaves were transferred from the 25°C to 38°C chambers, the ABA and polyamine contents in the second leaves fluctuated during the first 2 days. ABA content markedly decreased on plants exposed to 38°C to about a half of the original level in the first 6 hr; it remained at this low level thereafter (Fig. 7).

Polyamine content in leaves was affected by the acclimation treatment, depending on the species of polyamines. Put content decreased rapidly to less than a third of the original level while Spd and Spm contents increased gradually to about 140% of their original levels at the end of the daytime in the second day (Fig. 8). Foliar polyamine levels displayed diurnal fluctuations. In the control leaves, Put decreased during the day and increased at night, while Spd and Spm contents were constant throughout the 24 hr. In contrast, in the acclimated leaves, Put content did not fluctuate once it rapidly decreased after 6 hr while Spd and Spm contents tended to increase during the day and decrease at night.

Effect of exogenous polyamines on thermostability of photosynthesis

Aqueous solutions of 5 mM Spd or 5 mM Spm containing 0.01% (v/v) Tween 20 were sprayed on leaves of non-acclimated plants at the start of the 12-hr
alleviated the heat-induced decrease in photosynthetic electron transport rates through both PS II and PS I (Fig. 10). Spd also exhibited a similar protective effect but to a lesser extent than did Spm.

**Discussion**

Caldwell (1993) found that acute heat stress at 45°C to detached leaves of 25°C-grown cucumber plants did not affect the viability of leaf cells, determined by ion leakage, unless the duration of stress exceeded 45 min. In contrast, photosynthetic activity of non-acclimated cucumber leaves, measured by oxygen evolution in the light and PS II chlorophyll fluorescence (Fv/Fm), was severely damaged by heating the intact leaves at 45°C for only 10 min (Fig. 1), indicating the thermolabile nature of the photosynthetic apparatus.

Heat treatment of isolated thylakoids from non-acclimated leaves caused severe loss of PS II electron transport activity (Fig. 2). In addition, thylakoids lost significant amounts of 33-kDa polypeptides and manganese during heat stress (Figs. 3, 4). That the PS II is the most heat-sensitive of the thylakoid membrane complexes involved in photosynthetic electron transport and ATP synthesis is well-documented (Berry and Björkman, 1980; Havaux, 1993). The heat-inactivation of PS II corresponds with the release of 33-kDa manganese-stabilizing proteins in the oxygen-evolving complex (Enami et al., 1994). Thylakoids deficient in manganese evolve less oxygen and exhibit decreased chlorophyll fluorescence (Okada and Asada, 1983; Nash et al., 1985). Our results do not prove that the 33-kDa polypeptide is derived from the oxygen-evolving complex, but the loss of manganese from isolated thylakoids may indicate some heat damage to the oxygen-evolving complex. Thus, we conclude that heat damage to the oxygen-evolving complex of PS II contributes largely to the heat-inactivation of photosynthesis in cucumber leaves.

Raising the growth temperature to 38°C resulted in a gradual reduction of heat damage to photosynthetic oxygen evolution activity and PS II Chl fluorescence (Fig. 1). Heat inactivation of PS II and PS I electron transport (Fig. 2) and heat-induced loss of 33-kDa polypeptides and manganese (Figs. 3, 4) in isolated thylakoids were also alleviated by the acclimation treatment to intact plants. These results indicate that during exposure of intact leaves to 38°C, various components of photosystems in thylakoids, including the oxygen-evolving complex, gradually became more heat-tolerant.

It seems that the heat-induced increase in loss of thylakoid proteins was caused by excess fluidity of the membranes. Excessive fluidity of thylakoid membranes may result in a break of interactions between the lipid bilayer and intrinsic and extrinsic protein complexes of thylakoids, which in turn, may lead to loss of proteins from the membranes and eventual degradation of photo-
systems (Vigh et al., 1994; Murakami et al., 2000). In general, the level of membrane fluidity at a given temperature is high in the membranes with high degrees of lipid unsaturation compared to those with low degrees of lipid unsaturation (Raison et al., 1979). Therefore, the decrease in lipid unsaturation of thylakoid membranes may be of major importance for keeping the membrane fluidity in the normal range at elevated temperatures. In fact, Thomas et al. (1986) found that saturation of thylakoid membrane lipids by catalytic hydrogenation increased thermostability of the membranes. In our study, thylakoid membranes underwent a gradual decrease in lipid unsaturation during exposure of intact leaves to acclimation temperature; this decrease was more prominent in the glycolipid, a major lipid species in thylakoid membranes (Figs. 5, 6). Thus, it is most likely that the decreased lipid unsaturation of thylakoid membranes is a major factor contributing to the increased thermostability of the photosynthetic apparatus.

Previously, we found that foliar application of 1 mM ABA induced thermostability of photosynthesis in cucumber leaves (Li et al., 2003). This observation led us to speculate that ABA might play a role in the thermal acclimation process of the photosynthetic apparatus in cucumber. However, exposure of plants to 38 °C caused a marked decrease in foliar ABA content by the end of the 2-day thermal acclimation treatment (Fig. 7), when the photosynthetic apparatus became considerably heat-tolerant (Fig. 1).

There is evidence in the literature that exposure to high temperature causes ABA accumulation in plants (Daie and Campbell, 1981; Talanova et al., 1992). Abass and Rajasheker (1993) suggested that this increase in ABA is a factor in thermal acclimation and heat-tolerance induction in grape leaves. However, it should be noted that an elevation of leaf temperature may cause increased loss of water from leaves; thus, water-stress-induced increase in ABA synthesis may take place (Hiron and Wright, 1973). In our study, humidity in the chamber during the acclimation treatment was kept at 85% RH to minimize transpirational cooling of leaves. Under such conditions, high ambient temperature inhibits the synthesis of ABA (Eze et al., 1983; Du and Tachibana, 1995). Thus, the increase in heat tolerance due to high temperature treatment, observed in the literature, could be attributable to water-stress-induced increase in ABA, thus enhancing thermostability of whole cells and the photosynthetic apparatus (Bassiri-Rad and Radin, 1992; Li et al., 1996, 2003). Based on our present results and above discussions, we conclude that ABA does not play any role in the increase in thermostability of photosynthesis caused by exposure of leaves to acclimation temperature.

Nevertheless, plants grown at 38 °C exhibited a significant increase in Spd and Spm contents and a decrease in Put content in leaves as compared with those grown at 25 °C (Fig. 8). Furthermore, both in vivo and in vitro experiments showed that exogenous Spm was effective in alleviating heat damage to the photosynthetic apparatus (Figs. 9, 10). These results strongly suggest that Spm plays an important role in the thermal acclimation process of photosynthesis in cucumber.

Chloroplasts are enriched with polyamines and their biosynthetic enzymes (Borrell et al., 1995). In addition, polyamine content in chloroplasts increases notably in response to environmental stress (He et al., 2002a). Because of the polyacidic nature at a physiological pH, polyamines can bind strongly to cellular constituents such as nucleic acids, proteins and membranes (Cohen, 1998). Polyamine binding to proteins is catalyzed by transglutaminase, which is also contained in chloroplasts in large amounts (Del Duca et al., 1994). Besford et al. (1993) and He et al. (2002a, 2002b) have suggested that such interaction with macromolecules is an important function of polyamines in protecting the thylakoid protein complexes and Rubisco against stress-induced degradation. Pretreatment of isolated thylakoids with Spm was effective in alleviating the heat-inactivation of photosystems (Fig. 10), suggesting that protein complexes in thylakoids were made more stable to heat due to their binding to Spm. However, the thermostability of Spm-pretreated thylakoids was lower than that of thylakoids in thermal acclimated leaves, particularly in the PS I photochemistry (compare Figs. 2 and 9). Further study is necessary to examine the role of polyamines in thermal acclimation of photosynthesis.

Recently, an ecological method was exploited for the disease and pest control in protected cultivation of cucumber in summer (Satoh, 2003). In this method, diseases and insect pests can be exterminated by raising the room temperature in the greenhouse to about 45 °C in the early morning for several days. With this method, however, plants and especially the photosynthetic apparatus have to be sufficiently thermostable before being exposed to such extreme temperatures. Our results suggest that this can be achieved by foliar application of Spm. This also requires further investigation.

**Literature Cited**


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高温遭遇によるキュウリ葉の ABA およびポリアミン含量の変化と光合成器官の高温順化との関係

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摘要
キュウリ（品種：南極2号）の葉の光合成器官の高温順化過程に ABA とポリアミンが関与しているかどうかについて調べた。光合成活性は光下での酸素放出速度と光化学系IIのクロロフィル蛍光収率 (Fv/Fm) の差で評価した。植物を 25/25℃（昼/夜）から 38/38℃に移すと、45℃遭遇による光合成活性の低下が観察された。また、高温順化葉から単離したチラコイドは非順化葉から単離したチラコイドよりも少ない。40℃遭遇による光合成電子伝達活性の低下や 33 kDa タンパク質およびマンガンの解離が少なくな、高温順化処理によりチラコイドの熱安定性が高まることが示された。このような光合成器官の高温順化にはチラコイド膜の脂質不飽和度の低下が関与していると考えられた。植物を 38℃に遭遇させると、葉の ABA 含量が顕著に低下した。このことは、ABA は光合成器官の高温順化過程には関与していないことを示している。一方、葉のスパルミンおよびスペルミン含量は高温順化処理中に増加した。また、非順化葉に 5 mM のスペルミンまたはスペルミンを葉面散布すると光合成器官の熱安定性が高まり、非順化葉から単離したチラコイドに 5 mM スペルミンを添加すると光化学系の熱失活が軽減された。これらのことは、ポリアミンが光合成器官の高温順化に何らかの役割を果たしていることを示唆する。