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LOW DISSIPATION OF EXCITATION ENERGY IN THE PHOTOSYNTHETIC MACHINERY OF CHILLING-SENSITIVE PLANTS DURING LOW-TEMPERATURE PHOTOINHIBITION

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Abstract – Using a squash plant, a chilling-sensitive species, and a spinach plant, a chilling-resistant one, effects of chilling temperature on the photosynthetic machinery were studied in terms of chlorophyll fluorescence. When thylakoid membranes were isolated and subjected to incubation at different temperatures, spinach showed stable photosystem II activity at the low temperature side, in contrast to squash which showed quite severe inactivation at low temperature. When parameters of chlorophyll fluorescence were examined, chilling in darkness did not affect either Fv/Fm or photochemical and non-photochemical quenching, in both types of plants. However, chilling of squash plants under irradiance of medium intensity caused a specific decrease in Fv/Fm accompanied by a decline in energy-dependent quenching. Contrastingly, photosystem II of spinach plants were not much affected by light-chilling. When the pool size of zeaxanthin was examined after exposure to high light at different temperatures, squash plants was shown to have a much lower content of antheraxanthin + zeaxanthin, as compared to spinach plants, during low-temperature photoinhibition. These results suggest that chilling-sensitive plants have low capacity to dissipate excitation energy nonradiatively, when they are exposed to low-temperature photoinhibition, and, as a consequence, more vulnerable to photoinhibitory damage to the photosynthetic apparatus.

INTRODUCTION

Exposure of plants to light in excess results in a reduction in photosynthsis, which is termed photoinhibition. Photoinhibition is accelerated by the interaction between light and additional environmental stresses such as low temperature.[1]

In contrast to chilling-resistant plants, species from tropical or subtropical origin are very sensitive to low, but non-freezing temperatures below about 12°C.[2,3] Susceptibility to chilling limits the season of growth, geographic distribution as well as storage and transport conditions of many economically important crop plants.

One of the most sensitive metabolic processes to chilling is photosynthesis. In particular, chilling enhances photoinhibition of photosynthesis through accelerating damage to and degradation of the D1 protein of photosystem (PS) II. This phenomenon of low-temperature photoinhibition induces accumulation of reduced form of primary electron acceptors of PS II (Q_A), and it leads to the disruption of energy dissipation mechanism at PS II, which is manifested by an alteration of chlorophyll fluorescence.[4]

Chlorophyll (Chl) fluorescence can be used as a probe for detecting the physical state of chloroplast membrane lipids, because red fluorescence emitted from Chl molecules reflects changes in the stability of chloroplast membrane components. Moreover, Chl fluorescence is quenched by using the light energy in photochemistry (qQ), or by non-photochemical mechanisms. Several factors can contribute to non-photochemical quenching. One major component involves energy-dependent quenching (qE), which is thought to reflect a mechanism for energy dissipation at PS II, triggered by a rising pH gradient across thylakoid membranes or, more precisely,

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† *Abbreviations*: A, antheraxanthin; Chl, chlorophyll; Fm, maximal fluorescence from dark-adapted sample; F'm, maximal fluorescence from light-adapted sample; Fo, initial fluorescence; Fp, chlorophyll fluorescence at the maximum of the induction kinetics at non-saturating light condition; Ft, measured fluorescence yield from light-adapted leaves at any given time; Fv, variable fluorescence; P_{680}, reaction center chlorophyll of photosystem II; PBQ, phenyl-*p*-benzoquinone; PS, photosystem; Q_A, primary quinone electron acceptor of photosystem II; qE, coefficient for energy-dependent quenching of fluorescence; qNP, coefficient for non-photochemical quenching of fluorescence; qQ, coefficient for photochemical quenching of fluorescence; V, violaxanthin; Z, zeaxanthin.

an increasing proton concentration in the thylakoid lumen.[5]

Carotenoids that are present in thylakoid membranes are known to protect the photosynthetic machinery against the damaging effects of light and oxygen.[6] When plants are subjected to strong light, violaxanthin shows a light-dependent conversion to zeaxanthin through intermediate antheraxanthin. This process is reversed in the dark and is called the xanthophyll cycle. The xanthophyll cycle is present in thylakoid membranes and is finely regulated to respond to the balance of electron transport and CO_2 fixation.[7] Increased levels of zeaxanthin in the antenna have been shown to protect PS II when plants are exposed to high light by dissipating excess absorbed light energy as heat in higher plants[8] and algae.[9] Thus inhibition of zeaxanthin formation leads to enhanced sensitivity to photo-inhibition.

In the present study, we report that sensitivity of plants to chilling is strongly dependent on the capacity of plants to dissipate excess energy of PS II *via* zeaxanthin-related quenching of fluorescence.

MATERIALS AND METHODS

Plant materials and growth conditions. Seeds of squash (*Cucurbita moschata* var. Shirakikuza) and spinach (*Spinacia oleracea* var. glabra) were germinated and planted in the medium of vermiculite. After growth for about two weeks, seedlings of each plant were transferred to a liquid medium of Hyponex (Hyponex Co., Ohio, U.S.A.). Squash and spinach plants were grown at 25°C and 22°C, respectively, at an intensity of 100 μmol m^{-2} s^{-1}.

Isolation of thylakoid membranes and measurement of PS II-mediated electron transport activity. Isolation of thylakoid membranes and measurement of oxygen evolution was carried out as described in Moon *et al.* (1995).[10]

Extraction of pigments. Two leaf disks (8 mm dia.) were ground in liquid nitrogen in a darkened room and the ground powder was solubilized in 2.5 mL of 85% acetone. The extract was divided into two microcentrifuge tubes and spun with a microcentrifuge at 4°C for 3 min at a speed of 12,000 rpm. The supernatants were removed and the pellets extracted again with 1.25 mL of 100% acetone each at room temperature for 5 min, with occasional vortex mixing before spinning for 3 min. The supernatants were pooled and filtered through 0.4-μm syringe filters. The pigment extracts were analyzed immediately by carrying out HPLC.

Liquid chromatography. The chromatographic system was a Waters gradient liquid chromatograph (Millipore, Milford, MA, U.S.A.) equipped with a Waters 990 photodiode array detector. All solvents were HPLC grade. Zorbax ODS columns (5-μm particle size, 250 mm × 4.6 mm I.D.) were from Rockland Technologies, PA, U.S.A.

The flow rate for all seperations was 1 mL/min and all sample injections were 20 μL. Solvent mixtures were: A, acetonitrile-methanol (85 : 15); B, methanol-ethyl acetate (66 : 34). Solvent A was run isocratically for 14.5 min, followed by a 2 min linear gradient to 100% solvent B. The columns were re-equilibrated between samples for a minimum of 10 min with solvent A. All runs were performed at 30°C.

Measurement of Chl fluorescence. Chl fluorescence was measured at room temperature using a pulse amplitude modulation fluorometer (model PAM-2000; H. Walz, Effeltrich, Germany) according to Schreiber *et al.* (1986).[11] Measuring red light of low average intensity pulsed at a frequency of 600 Hz was used to probe the initial fluorescence yield, Fo, when all reaction centers are open, from the dark-adapted leaves. Fm, the maximal fluorescence yield from the dark-adapted leaves, when all reaction centers are closed, was determined by exposure to photosynthetically saturating light in addition to the measuring light. After measuring Fo and Fm, leaves were illuminated with 655 nm non-modulated actinic light (~ 112 μmol m^{-2} s^{-1}) superimposed by a series of 0.8 s pulses of super-saturating white light (~ 4,000 μmol m^{-2} s^{-1}) fired at 30 s intervals. The non-actinic modulated measuring beam was used at 20 kHz in the light and was switched to 600Hz in the dark. The intensity of measuring beam used in the dark was less than 0.1 μmol m^{-2} s^{-1} and during actinic illumination was 1.5 μmol m^{-2} s^{-1}. Results were registered on a recorder with time compensation. Fluorescence quenching coefficients were calculated according to Schreiber *et al.* (1986).[11]

To determine the fluorescence at the maximum of the induction kinetics, Fp at non-saturating light conditions, leaf disks which had been dark-adapated for at least 2 h were placed in a temperature-controlled chamber for 10 min for the equilibrium of temperature. Then, chlorophyll fluorescence was induced by 10 μmol m^{-2} s^{-1} actinic light from a light emitting diode and measured by PAM-2000 (H. Walz, Effeltrich, Germany).

RESULTS AND DISCUSSION

PS II-mediated electron transport of thylakoid membranes during storage at different temperatures

Fig. 1 compares the profiles of inactivation at various temperatures of the oxygen evolving activity when isolated thylakoid membranes were incubated in darkness at different temperatures. When the membranes from spinach were incubated for 12 h, inactivation was observed only at the high temperature side of 40°C. However, when the membranes from squash were incubated, inactivation was observed at both the low-temperature and the high-temperature of 0°C and 40°C, respectively. The results indicate that photosynthetic machinery of squash is quite sensitive to chilling, in contrast to spinach.

Chlorophyll fluorescence during chilling of detached leaves in darkness

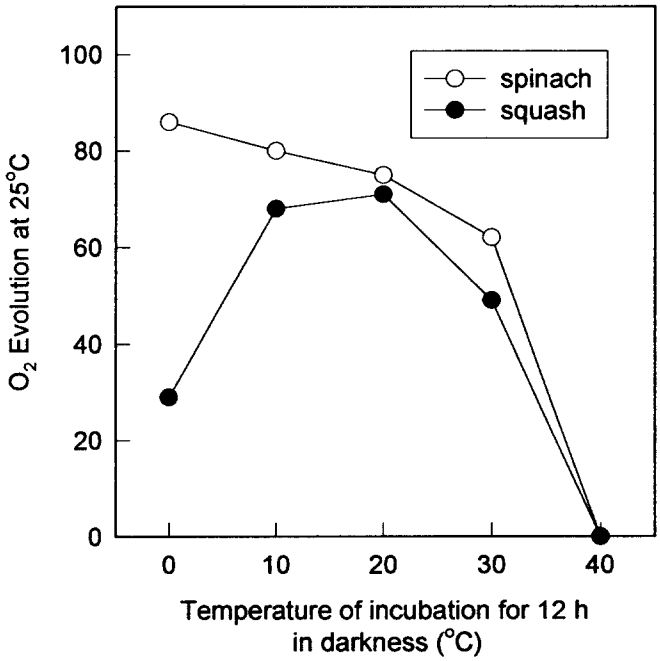

Figure 1. Profiles of the inactivation of PS II-mediated transport of electrons during incubation in darkness of thylakoid membranes from spinach leaves and squash cotyledons at various temperatures. The thylakoid membranes (10 μg of Chl per mL) were incubated at the designated temperature in darkness for 12 h in 1.0 mL of 50 m*M* soidum/potassium phosphate buffer (pH 7.4) that contained 200 m*M* NaCl, 300 m*M* sucrose, and 1.0 *M* betaine. The activity of PS II-mediated transport of electrons from H_2O to PBQ was measured at 25°C as described in Moon et al (1995). The activities before incubation were taken as 100 arbitrary units, which corresponded to 320 ± 10 and 200 ± 7 (μmol O_2) (mg of Chl)$^{-1}$ h^{-1} for thylakoid membranes from spinach and squash plants, respectively. The values were calculated from results of three independent experiments. The deviation of values was less than 8% in each case.

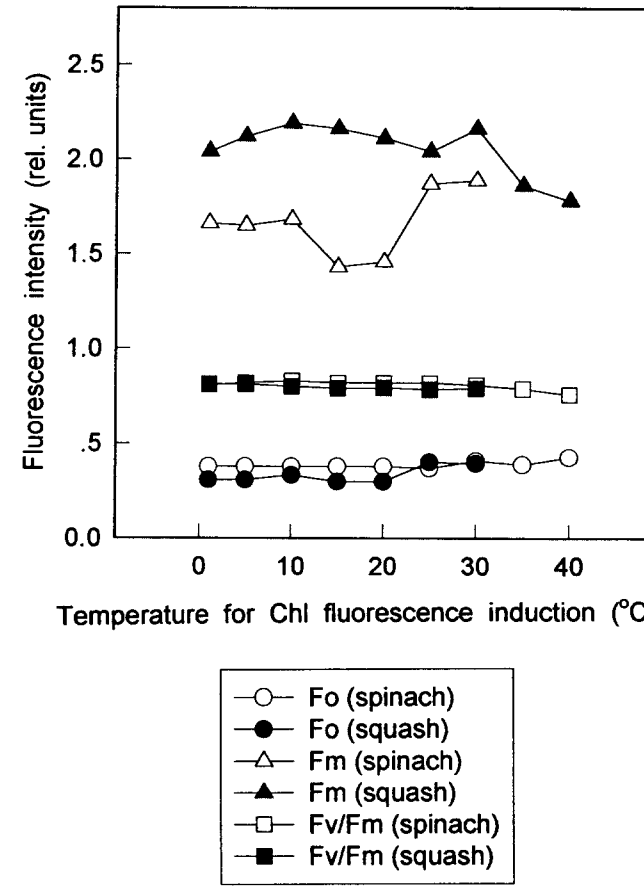

Figure 2. Time course of the changes in Fo, Fm and Fv/Fm in detached leaves of spinach and squash that had been incubated in darkness at 5°C. Fluorescenc was measured by the saturating-flash method at 25°C, following temperature adjustment for 10 min in darkness.

Fig. 2 shows the time course of changes in the level of Fo, Fm and Fv/Fm of leaves of spinach and squash during chilling in darkness. When detached leaves of spinach and squash were chilled at 5°C for 24 h in darkness and subsequently subjected to room temperature induction of chlorophyll fluorescence, they showed little change in the level of Fo, Fm and Fv/Fm. Since the efficiency of excitation energy capture by open PS II reaction centers is defined by Fv/Fm,[12, 13] it seems that dark-chilling did not affect the potential qunatum efficiency of PS II photochemistry in the two types of plants.

Fig. 3 shows the time course of changes in photochemical quenching (qQ) and non-photochemical quenching (qNP) during chilling of leaves at 5°C in darkness. When detached leaves of spinach and squash were chilled in darkness up to 24 h, they showed almost no changes in qQ and qNP. Since qQ results from electron transfer from P_{680} to Q_A and qNP is attributed to increase in the rate of dissipation of excitation energy by non-radiative processes,[14, 15] the above results suggest that low temperature in the absence of light was not sufficient to induce any perturbations in PS II photochemistry for excitation energy.

In addition, when the effective quantum yield of PS II was examined and monitored in terms of (F'm-Ft)/Fm, no apparent differences were observed between spinach and squash plants (Fig. 5A). Since (F'm-Ft)/Fm is a measure of quantum efficiency of PS II photochemical energy conversion for the leaf under the given conditions, it can be regarded as electron transport activity *in vivo* under steady-state photosynthesis.[16] The results suggest that dark-chilling had no effect on the overall photosynthetic electron transport system.

Based on the observation that Fv/Fm implicates the efficiency of excitation energy capture by open PS II reaction centers, and that qQ is a measure of the proportion of oxidized PS II reaction centers, Genty *et al.* (1989)[16] have proposed that the effective quantum yield of PS II electron transport is defined by Fv/Fm ×

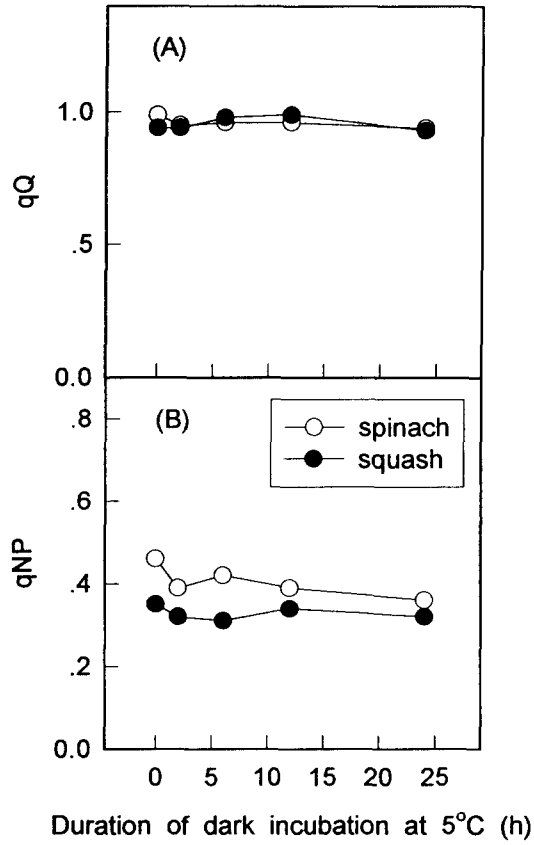

Figure 3. Time course of the changes in photochemical quenching qQ (A) and non-photochemical quenching (B) in detached leaves of spinach and squash that had been incubated in darkness at 5°C. Fluorescenc quenching was analyzed by the saturating-flash method at 25°C, following temperature adjustment for 10 min in darkness.

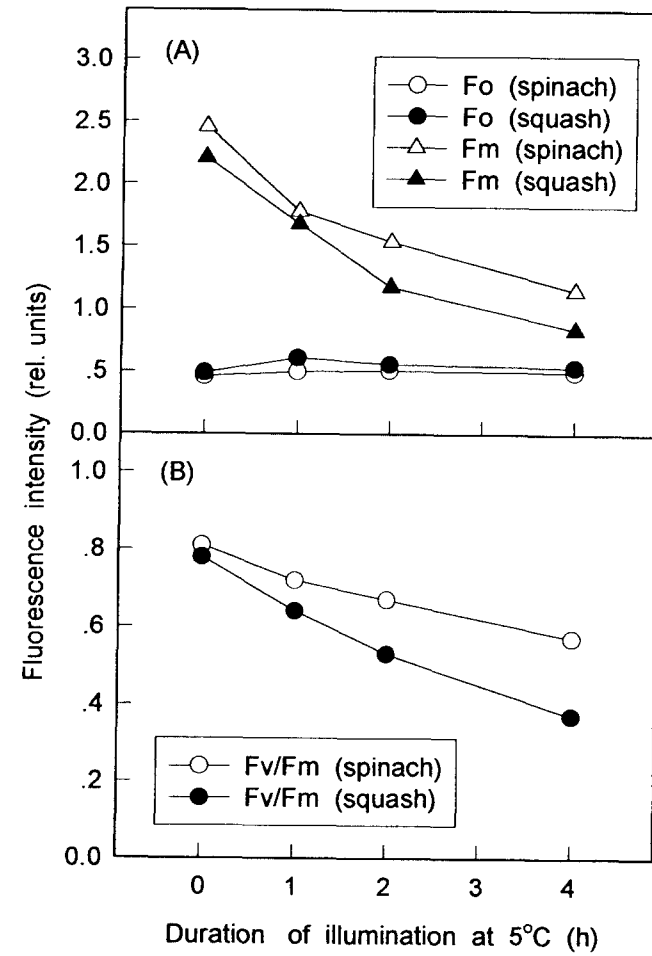

Figure 4. Time course of the changes in Fo, Fm (A) and Fv/Fm (B) in detached leaves of spinach and squash that had been exposed to a white light at an intensity of 200 μmol m^{-2} s^{-1} at 5°C. Fluorescenc measurements were done at 25°C, following temperature-adjustment for 10 min in darkness. Fm, maximal fluorescence from dark-adapted sample; Fo, initial fluorescence; Fv, variable fluorescence.

qQ. The ineffectiveness of dark-chilling upon the fluorescence characteristics of spinach and squash, as observed from the above results, indicates that chilling alone, at least for the exposure time of 24 h, is not strong enough to induce damages to PS II reaction centers.

Chlorophyll fluorescence during chilling of leaves in light

To examine the interaction of chilling and an additional environmental stress, *i.e.*, light, on the photosynthetic performances of spinach and squash plants, detached leaves were pretreated for the designated periods at 5°C under white light at an intensity of 200 μmol m^{-2} s^{-1} for 4 h. Then, following 10 min-period of dark adaptation, room-temperature chlorophyll fluorescence was induced from the upper surface of the leaves. Fig. 4 shows the time course of changes in the levels of Fo, Fm and Fv/Fm, measured by the saturating-flash method at room temperature from leaves under light-chilling. When squash leaves were light-chilled for 4 h, they showed a marked decrease in Fv/Fm, in contrast to spinach which showed a relatively minor decline in the ratio (Fig. 4B). The decline in Fv/Fm was shown to be due to the decrease in the level of Fm of squash leaves under light-chilling at 5°C (Fig. 4A). The controls kept in darkness showed no change in Fv/Fm (Fig. 2).

Fig. 5B shows the time course of changes in the level of effective quantum yield of PS II from leaves under light-chilling at 5°C. Exposure of squash leaves to light (200 μmol m^{-2} s^{-1}) at a chilling temperature was accompanied by a decrease in the level of (F'm-Ft)/Fm in contrast to spinach, suggesting that low-temperature induced inhibition of photosynthetic electron transport was more exacerbated in squash than in spinach.

Effects of induction temperature on chlorophyll fluorescence

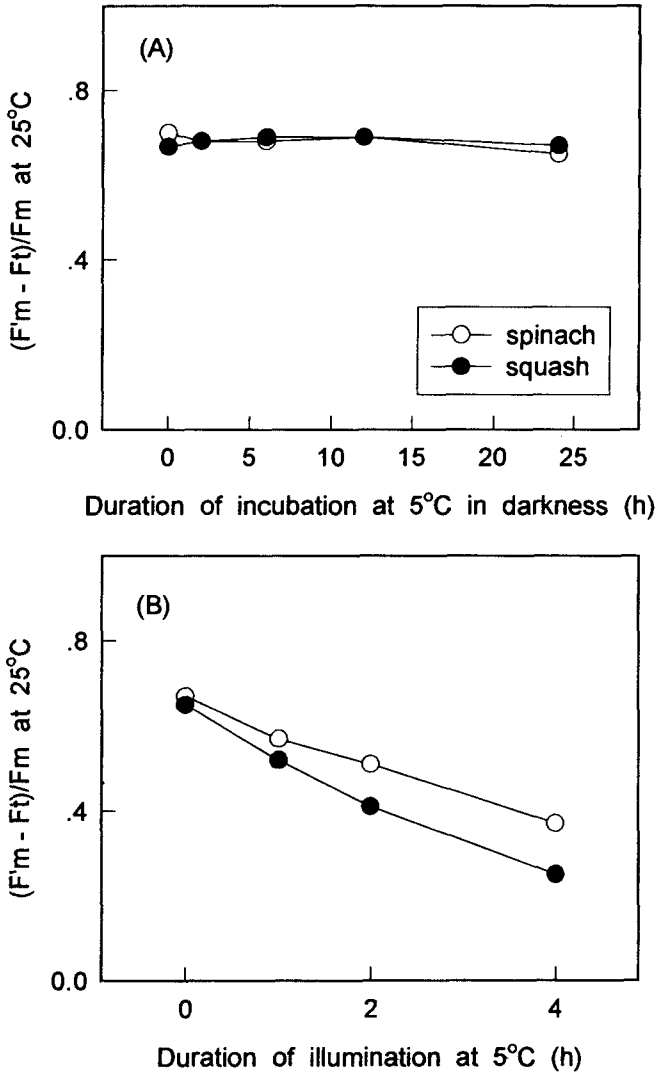

Figure 5. Time course of the changes in the effective quantum yield of photosystem II, (F'm-Ft)/Fm, in detached leaves of spinach and squash that had been incubated at 5°C in darkness (A), and that had been exposed to a white light at an intensity of 200 μmol m^{-2} s^{-1} at 5°C (B). Fluorescence measurements were done at 25°C, following temperature-adjustment for 10 min in darkness. Fm, maximal fluorescence from dark-adapted sample; F'm, maximal fluorescence from light-adapted sample; Ft, fluorescence yield from light-adapted leaves measured at any given time.

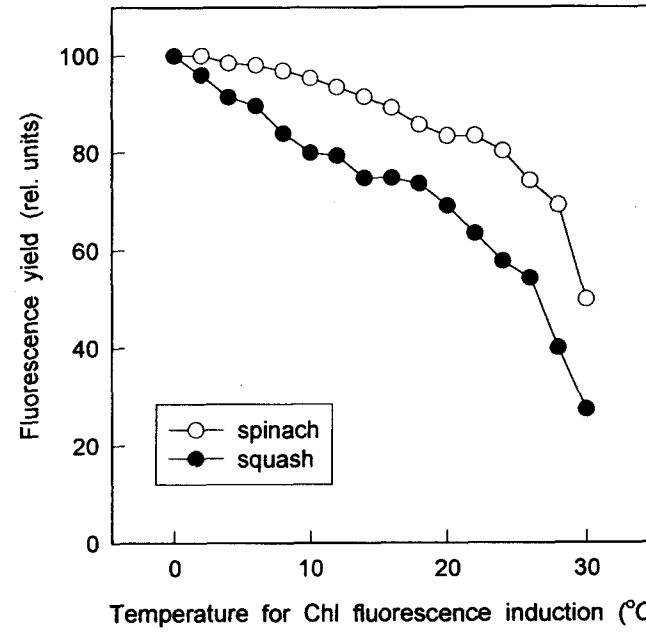

Figure 6. Chlorophyll fluorescence at the maximum of the induction kinetics, Fp of leaf disks from spinach and squash plants at non-saturating light conditions. Chlorophyll fluorescence was induced by 10 μmol m^{-2} s^{-1} actinic light and measured at decreasing temperatures from 30°C to 0°C. Chlorophyll fluorescence values were normalized to the highest value (100%) found at 0°C.

We attempted to examine initial change in chlorophyll fluorescence during its induction at the start of chilling in darkness of leaf disks. For this purpose, leaf disks were placed in a temperature-controlled chamber and the fluorescence at the maximum of the induction kinetics (Fp) at non-saturating light conditions (10 μmol m^{-2} s^{-1}) was determined at different temperatures.

Fig. 6 shows temperature-dependence of the first maximum in chlorophyll fluorescence induction, Fp, of leaf disks of spinach and squash. In spinach plants, Fp increased slowly as temperature decreases from 20°C to around 0°C accompanied by a rapid decrease from 20°C to around 30°C. In squash plants, Fp increased slowly as temperature decreases from 18°C to around 0°C accompanied by a rapid decrease from 18°C to around 30°C. When the effect of induction temperature on Fp was examined in the range from around 20°C to 0°C, squash showed a more rapid increase than spinach.

Since the actinic light intensity was very low and Fp was reached within seconds after a dark-period of 10 min,[17] it can be assumed that the thylakoid membranes were not energized. Consequently, effects of qNP can be neglected[18] and thus Fp is only controlled by the reduction state of Q_A, the primary electron acceptor of PS II.[5] At Fp, maximal Q_A reduction reflects an equilibrium of PS II reducing activity and PS I oxidizing activity, which can be affected by low temperature through limited electron transport by a decreased lateral diffusion of plastoquinone/plastoquinol at low temperature.[19, 20] The results indicate that squash plants are more susceptible to low temperature-induced alteration of photosynthetic electron transport.[21]

Susceptibility to chilling stress is suggested to be associated with high Q_A^- concentrations at low temperatures. As leaf temperatures drops, chilling-sensitive plant species are unable to balance the redox state of the electron transport carriers in order to maintain a constant and low level of reduced Q_A.[22] Photochemical quenching qQ is dependent on the redox

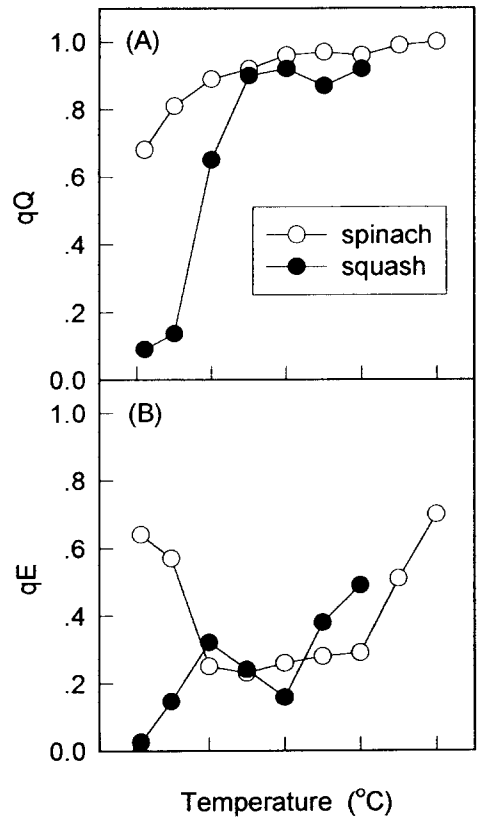

Figure 7. Photochemical quenching qQ (A) and energy-dependent quenching qNP (B) as a function of temperature in leaf disks of spinach and squash photosynthesizing under steady-state conditions. The intensity of actinic light given for steady-state photosynthesis was about 110 μmol m^{-2} s^{-1}.

state of Q_A. In Fig. 7A, the temperature-dependence curves of qQ in spinach and squash plants are shown.

Chilling-induced reduction of qQ was much more marked in squash than in spinach. Exposure to 1°C of squash leaves resulted in almost 90% inhibition of the photochemical quenching whereas chilling-resistant spinach was characterized by a much smaller inhibition (around 32%).

Chilling in darkness of leaf disks from spinach and squash was not related to a decrease in PS II efficiency as no change in Fv/Fm was seen (Fig. 2). It has been shown that chilling stress affects certain light-regulated enzymes of the photosynthetic carbon reduction, and thus the depression of CO_2 fixation might be related to an impairment of light activation of the Calvin-cycle.[23] As CO_2 is the final electron acceptor in the photosynthetic electron transport system, inhibited CO_2 assimilation could be expected to affect the redox state of the PS II electron acceptor Q_A, leading to alteration of photochemical quenching, qQ.

However, in the state of incipient chilling stress, excess energy seems to be dissipated *via* the energy-dependent quenching, qE mechanism. Figure 7B shows temperature-dependence of qE in spinach and squash. With decreasing temperatures from 30°C to 1°C, qE decreased with a brief increase from around 15°C to 10°C in squash whereas in spinach qE increased both at the low-temperature and high-temperature side. The initial inhibition of qE by chilling temperatures, as observed in squash, is suggested to be originated from losing a fluidity dynamics of the thylakoid membranes which is responsible for the regulation of the qE-mechanism.[24] Energy dependent quenching qE is related to the trans-thylakoid proton gradient.[25] Therefore, it can be assumed that lower chilling temperatures inhibited the qE capacity of chilling-sensitive squash plants, resulting in the decreased thermal deactivation and therefore in the depressed dissipation of excess excitation energy. Thus, we can speculate that the accumulation of excess energy in PS II has led to closure of reaction centers, seen as decline in qQ at chilling temperatures (Fig. 7A).

Temperature-dependent changes in zeaxanthin formation during exposure to strong light

Zeaxanthin is thought to play an essential role in the photoprotective thermal dissipation of excitation energy in the antennae, effective when the rate of light absorption of excitation energy in the light-harvesting complexes exceeds the maximum rate of photosynthesis. It has also been suggested that the synthesis of carotenoids zeaxanthin (Z) and anthraxanthin (A), when the plants are exposed to strong light, could directly quench excitation energy in PS II.[8, 26] To investigate the role of the carotenoids A and Z in low-temperature photoinhibition, we attempted to analyze relative concentrations of each carotenoid species in leaf disks of spinach and squash which had been exposed to strong light at different temperatures. Figs. 8 and 9 show the HPLC profiles of carotenoid pigments extracted from leaf disks which had been exposed to strong light, at an intensity of 1,500 μmol m^{-2} s^{-1}, for 3 h at 25°C and 10°C, respectively. The results indicate that conversion of violaxanthin (V) into antheraxanthin (A) and zeaxanthin (Z) is strongly dependent on temperature, as well as on light. Fig. 10 shows that exposure of both types of plant leaves to strong light at 25°C was accompanied by a rapid and massive conversion of V to A and Z, increasing (A + Z)/V ratio from zero to 1.8 in spinach, and from zero to 1.2 in squash plants, respectively. However, low temperature (10°C) notably inhibited synthesis of A and Z, with the steady-state value of (A + Z)/V being approximately 38% and 40% in spinach and squash, respectively, lower than that reached in leaves illuminated at 25°C. The correlation between zeaxanthin (+ antheraxanthin) formation and decrease in PS II efficiency has suggested that zeaxanthin (and antheraxanthin) has a protective function. Zeaxanthin has also been suggested to amplify the formation of ΔpH-related quenching of PS II chlorophyll fluorescence (qE), presumably by activation

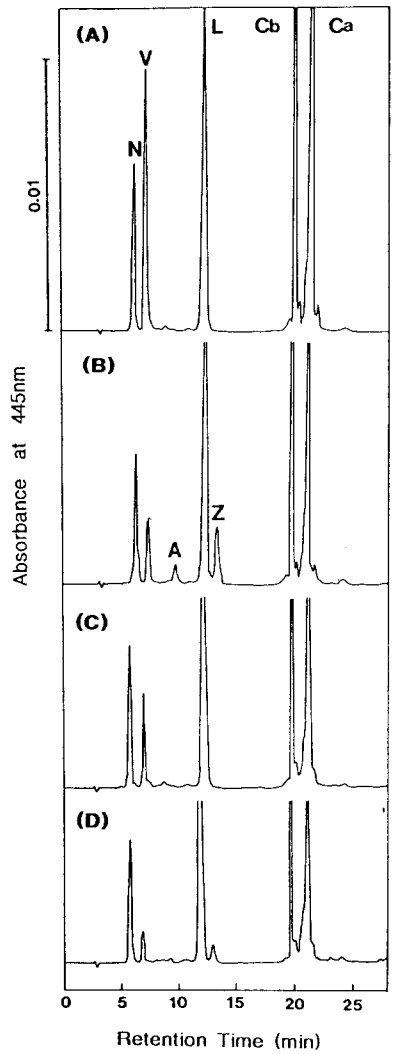

Figure 8. The HPLC elution profiles of carotenoids extracted from leaf disks of spinach (A, B) and squash (C, D) that were exposed, for 3h, to a strong white light at an intensity of 1,500 μmol m^{-2} s^{-1} at 25°C. A and C, leaf disks that had been incubated in darkness for 3 h at 25°C; B and D, leaf disks that had been exposed to strong light for 3 h at 25°C; A, antheraxanthin; Ca, chlorophyll a; Cb, chlorophyll b; L, lutein; N, neoxanthin; V, violaxanthin; Z, zeaxanthin.

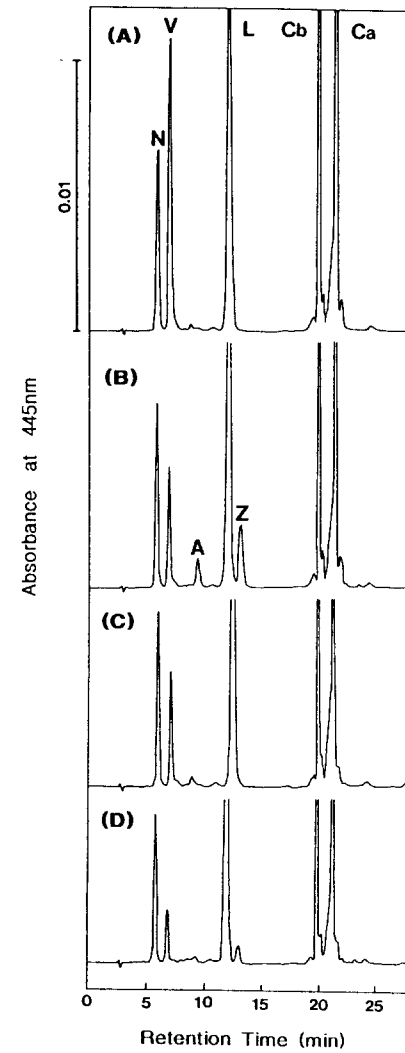

Figure 9. The HPLC elution profiles of carotenoids extracted from leaf disks of spinach (A, B) and squash (C, D) that had been exposed, for 3h, to a strong white light at an intensity of 1,500 μmol m^{-2} s^{-1} at 10°C. A and C, leaf disks that were incubated in darkness for 3 h at 10°C; B and D, leaf disks that were exposed to strong light for 3 h at 15°C; A, antheraxanthin; Ca, chlorophyll a; Cb, chlorophyll b; L, lutein; N, neoxanthin; V, violaxanthin; Z, zeaxanthin.

of the LHC II aggregation facilitated by protonation of lumen-exposed residues of LHC II proteins.[27, 28] Zeaxanthin is important in maintaining the light-generated trans-thylakoid pH which has been reported to rapidly collapse during photoinhibitory illumination of thylakoids.[29]

Excess light energy is dissipated as heat in the light-harvesting antenna of PS II. This dissipation is under complex influence of photosynthetic events at the chloroplast level, but is primarily controlled by the trans-thylakoid pH gradient. The heat dissipation competes for excitation energy and lowers (quenches) both the Chl fluorescence and PS II photochemical yields. The lowering of the Chl fluorescence yield is termed nonphotochemical quenching (qNP).

Fig. 7B shows that chilling of squash plants in light has induced marked decline in qE, indicating that dissipation of excess excitation energy was inhibited primarily by chilling-induced collapse of trans-thylakoid pH gradient. In relation to qE, Fig. 10 shows that pool size of A and Z relative to V was much lower in squash plants, in contrast to spinach at 10°C as well as at 25°C . The results suggest that the senstivity of squash plants to light-chilling is originated, in part, from the inactivation of thermal dissipation of excess excitation energy in the light-harvesting antenna of PS II, resulting from the

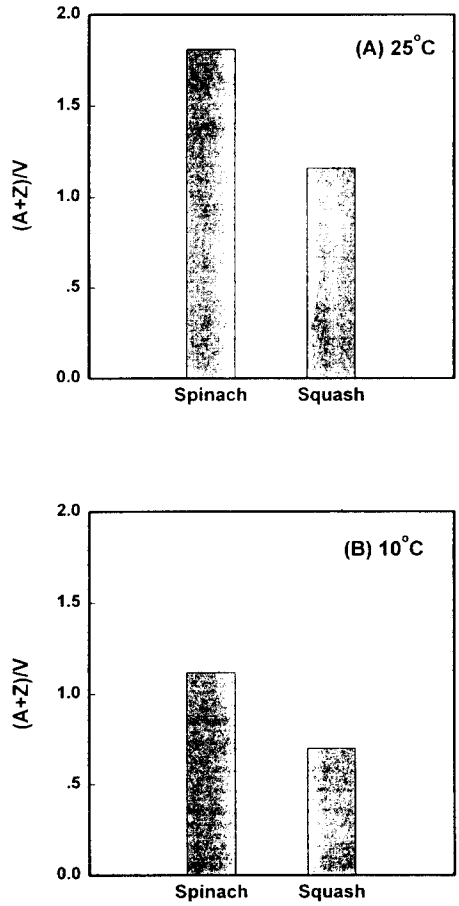

Figure 10. Ratio of antheraxanthin plus zeaxanthin to violaxanthin of leaf disks that had been exposed, for 3 h, to a strong white light at an intensity of 1,500μmol m^{-2} s^{-1} at 25°C (A) and 10°C (B). A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.

content of zeaxanthin and antheraxanthin. Therefore, we conclude that low amount of xanthophyll cycle components during chilling stress which leads to depression of excess energy dissipation in the photosynthetic machinery is largely responsible for the chilling sensitivity of squash plants.

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