FEBS Letters 353 (1994) 147–150

FEBS 14641

The protective function of the xanthophyll cycle in photosynthesis

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Received 25 July 1994; revised version received 30 August 1994

Abstract The rapid conversion of the carotenoid violaxanthin to zeaxanthin via antheraxanthin (xanthophyll cycle) in potato leaves exposed at 23°C to a strong white light of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ was associated with a slight inhibition of photosynthetic electron transport (as estimated from chlorophyll fluorescence measurements) and a low lipid peroxidation (as estimated from ethane measurements). When the xanthophyll cycle was blocked by dithiothreitol (3 mM) or low temperature (3°C), photoinhibition of electron transport was exacerbated and pronounced lipid peroxidation occurred concomitantly. Accumulation of zeaxanthin and antheraxanthin in potato leaves by a non-photoinhibitory light treatment at 23°C (900 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 1 h) considerably reduced the level of lipid peroxidation during subsequent light stress at 3°C. The presented results indicate that one of the functions of the xanthophyll cycle could be the protection of thylakoid membranes against lipid peroxidation, suggesting that zeaxanthin and antheraxanthin synthesized in strong light are present as free pigments in the membrane lipid bilayer.

Key words: Xanthophyll cycle; Lipid peroxidation; Photosynthetic electron transport; Photoprotection

1. Introduction

The carotenoid composition of the chloroplast membranes rapidly responds to changes in the plant's light environment, with zeaxanthin (Z) being rapidly formed in bright light by violaxanthin (V) de-epoxidation via the intermediate antheraxanthin (A) and reconverted to V in low light [1]. Although it seems established that this reversible formation of Z (termed 'xanthophyll cycle') is aimed at protecting chloroplasts against the harmful effects of excessive light [2], the mechanism by which Z could exert its protective role is still a matter of debate. It has been suggested that the synthesis of Z and A in strong light could: (i) regulate the reduction state of the photosynthetic electron-transport chain [1]; (ii) directly quench excitation energy in photosystem (PS) II [2,3]; (iii) promote the formation of an aggregated state of the light harvesting complexes (LHC) of PSII [4]; (iv) modulate the thylakoid membrane fluidity [5,6]; or (v) protect membrane lipids from photodestruction [7]. Some of those suggestions are not mutually exclusive. This report provides experimental evidences supporting hypothesis (v): the presence of A and Z was observed to determine the lipid peroxidation status of illuminated potato leaves.

2. Materials and methods

2.1. Plant material and treatments

Intact leaves picked from 4-week-old potato plants (*Solanum tuberosum* L., cv. Haig) were used. Plants were grown in a growth chamber under controlled conditions of temperature, light and air humidity, as described in [8]. Detached leaves placed on moist filter paper were exposed at 23 or 3°C to a white light of 900 or 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (at the leaf surface) produced by a halogen metal halide lamp (Osram Powerstar HQI-TS 150 W/NDL) equipped with two infra-red suppressor filters. Photon flux densities were measured with a Li-Cor quantummeter (model Li-185B/Li-190SB). Leaf temperature, monitored with a thermistor thermometer, was maintained constant during the light treatment as previously described [8]. In some experiments, leaves were pretreated with dithiothreitol (DTT) as described by Bilger et al. [9]: cut leaves were kept at a photon flux density of 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 2 or 3 h with their petiole in a solution of 3 mM DTT or in distilled water (controls).

2.2. Pigment analysis

Leaf discs (of 1.13 cm^2) were frozen in liquid nitrogen and kept at −80°C before pigment analysis. Pigments were extracted in dim light at 3°C with pure acetone. Insolubles were removed by centrifugation (14,000 × *g* for 1.5 min) and the supernatant was passed through a 0.2 μm PTFE filter (Alltech). Pigments were separated by reversed-phase HPLC using a Shimadzu chromatograph (model LC-6A) following the protocol of Thayer and Björkman [10] with some modifications. Pigments were eluted with a mixture of acetonitrile and methanol (84:16, v/v) on a 5 μm Spherisorb ODS-1 column (250 × 4.6 mm, Alltech) protected by a C18 Adsorbosphere guard column (Alltech). Flow rate of elution was 2 $ml \cdot min^{-1}$. The eluted pigments were detected using a Shimadzu SPD-M6A diode-array detector, monitoring at 445 nm. Pigments were identified by their visible absorption spectra and their retention times (3.2 min, 4.1 min and 5.5 min for V, A and Z, respectively) compared with standards. Purified Z was obtained from Extrasynthèse (Genay, France) whereas V and A were prepared by TLC with *n*-hexane:isopropanol (100:10, v/v) as solvent system. Xanthophylls were quantified using published extinction coefficients [11].

2.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence emission from the upper surface of the leaves was measured in modulated light using a Walz fluorometer (model PAM 2000). After the strong light treatment, leaves were adapted to darkness for 15 min and then illuminated for 15 min with a red light (655 nm) of 130 $\mu E \cdot m^{-2} \cdot s^{-1}$. The steady-state level (F_s) of modulated chlorophyll fluorescence was elicited by a weak red light (655 nm, 3 $\mu E \cdot m^{-2} \cdot s^{-1}$) modulated at 20 kHz and was measured at wavelengths higher than 700 nm with a photodiode. The maximal fluorescence level (F_m) was induced by a short pulse (800 ms) of intense white light (4000 $\mu E \cdot m^{-2} s^{-1}$). The quantum yield ($\phi_P$) of PSII photochemistry in the light-adapted leaves was calculated by the ratio $(F_m - F_s)/F_m$ which is closely correlated with the quantum yield of photosynthetic electron transport as determined from O_2 or CO_2 exchange measurements [12].

2.4. Ethane assay

The leaf (approximately 250 mg) was placed into a 16-ml flask and sealed under nitrogen atmosphere. In situ decomposition of ω-3 unsaturated hydroperoxy fatty acids (linolenic acid hydroperoxide) into

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Abbreviations: ϕ_P, quantum yield of photosynthetic electron transport; V, A and Z, violaxanthin, antheraxanthin and zeaxanthin, respectively; DTT, dithiothreitol; PS, photosystem; SOD, superoxide dismutase; LHC(II), light harvesting chlorophyll *a/b*–protein complexes (of PSII).

SSDI 0014-5793(94)01028-5

ethane was accelerated by a brief heat treatment (90 s) of the samples in a microwave oven, as described elsewhere [13]. After cooling the flask at 20°C for 10 min, 1 ml of the gas phase was withdrawn and injected in a GC (Delsi Di200) for ethane determination, following the protocol of Degousée et al. [13].

3. Results

3.1. Light stress at 23°C and at 3° C

Exposure of intact potato leaves to a strong white light of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the growth temperature (23°C) resulted in a reduction of the quantum yield (ϕ_P of photosynthetic electron transport (Fig. 1A). After 3 h at 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$, ϕ_P was decreased by about 30%. Photoinhibition of electron transport was exacerbated when the light treatment was imposed at a low temperature of 3°C, as previously observed [8]. At 3°C, a 3-h light-treatment caused a marked decrease in ϕ_P of ca. 70%. As shown in Fig. 1B, exposure of potato leaves to the strong white light at 23°C was accompanied by a rapid and massive conversion of V into A and Z, increasing the (A+Z)/V ratio from nearly 0 to about 1. Low temperature notably inhibited the synthesis of A and Z, with the steady-state value of (Z+A)/V being approximately 3 times lower than that reached in leaves illuminated at 23°C. In Fig. 1C, the lipid peroxidation status of the leaves was monitored during light stress at 23°C and at 3°C by measuring the amount of ethane generated by the samples [13,14]. Strong light stress at 23°C did not change appreciably the level of ethane production by potato leaves (which remained close to around 90 pmol $\cdot g^{-1}$ F.W.). In striking contrast, ethane production was observed to drastically increase during light stress at 3°C, thus indicating the occurrence of lipid peroxidation. Chilling-induced photooxidative lipid peroxidation was previously observed by Wise and Naylor [15] in chilled cucumber leaves.

3.2. Effect of DTT on light stress at 23° C

Dithiothreitol (DTT) is known to be a potent inhibitor of the V-to-Z conversion [9]. As expected, the xanthophyll cycle was almost completely blocked in intact potato leaves infiltrated with 3 mM DTT prior to exposure to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 23°C (Fig. 2A). Conversely, infiltration with water had no effect on the synthesis of A and Z. Reduction of ϕ_P by the strong light stress was somewhat accelerated in DTT-poisoned leaves as compared to water-infiltrated leaves (Fig. 2B). Very interestingly, DTT-treated leaves showed a marked and rapid increase in their ethane production under light stress whereas the amount of ethane generated by water-infiltrated leaves remained low throughout the light treatment. One can then conclude that the absence of Z and A in illuminated leaves favored lipid peroxidation. It was also observed that DTT (in the absence of light stress) had no effect on ϕ_P, dark respiration and net photosynthetic O_2 evolution in both limiting- and saturating-light conditions as measured with a Clark-type O_2 electrode (data not shown).

3.3. Effect of preillumination at 23°C on light stress at 3° C

In the experiments shown in Table 1, lipid peroxidation was measured in leaves exposed for 1 h to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 3°C before and after pre-accumulating zeaxanthin in moderate light of 900 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 23°C. This latter treatment brought about synthesis of A and Z without appreciable photoinhibition of

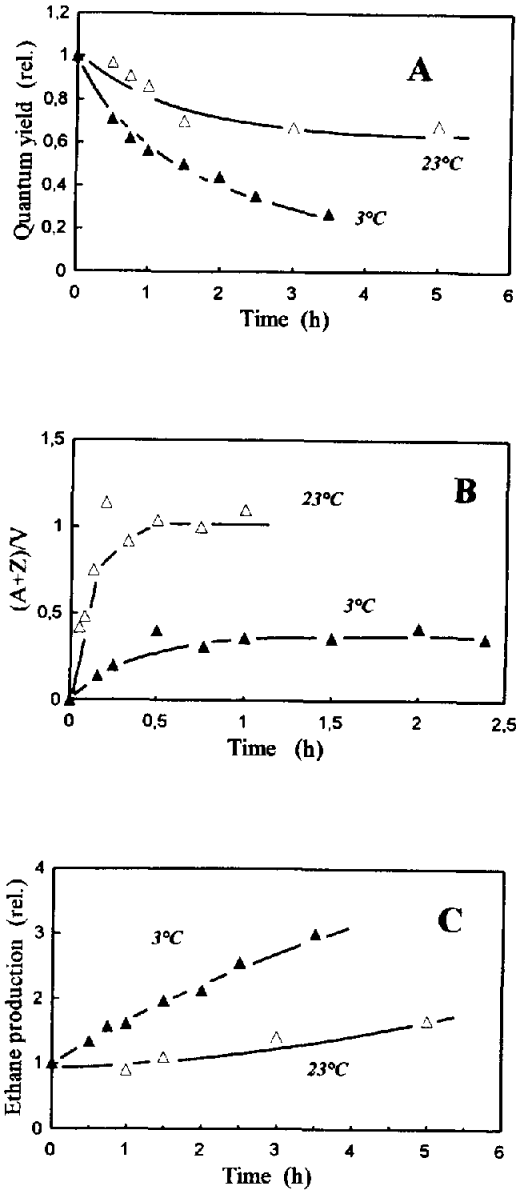

Fig. 1. ϕ_P (panel A), (A+Z)/V ratio (panel B) and ethane production (panel C) of potato leaves exposed to a strong white light of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 23°C (open triangles) or 3°C (closed triangles). ϕ_P and ethane-production data are expressed as fractions of the values measured before light stress, with: $I = 0.66 \pm 0.02$ for ϕ_P and $I = 90 \pm 19$ pmol $\cdot g^{-1}$ F.W. for the ethane production. The sum V+A+Z remained virtually constant during the light treatments. Each point is the mean value of 5–10 separate experiments.

photosynthetic electron transport or lipid peroxidation. Leaves preilluminated at 23°C exhibited a much lower production of ethane after light stress at 3°C as compared to non-preilluminated samples, indicating strong reduction of lipid peroxidation. Parallely, photoinhibition of ϕ_P was slightly alleviated.

4. Discussion

A previous study of light-treated potato leaves [8] has shown that photoinhibition of photosynthetic electron transport at 23°C (growth temperature) and at 3°C are different phenom-

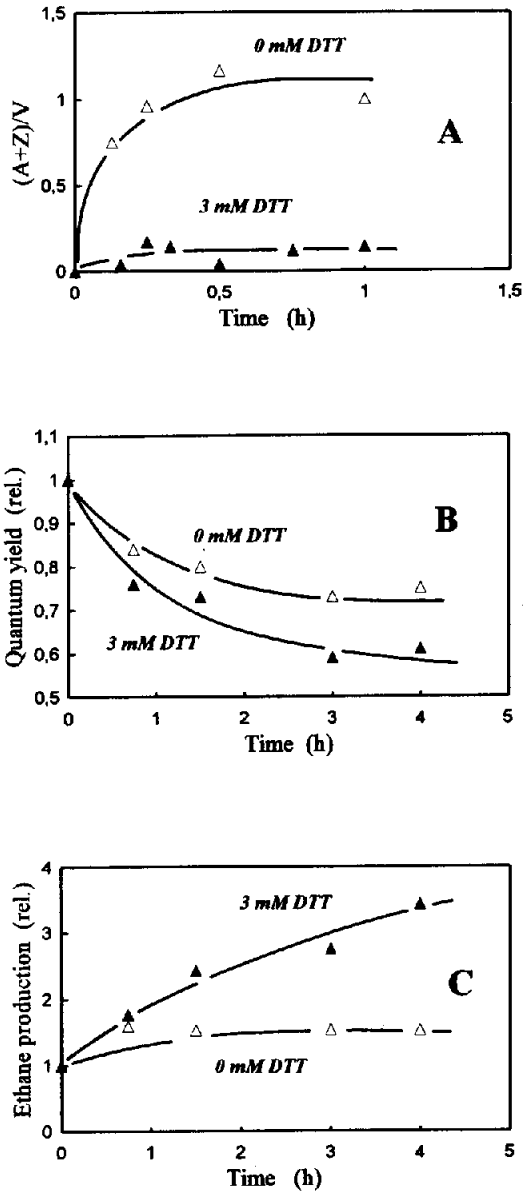

Fig. 2. (A + Z)/V ratio (panel A), ϕ_P (panel B) and ethane production (panel C) of potato leaves exposed at 23°C to a strong white light of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ after pre-infiltration with distilled water containing 0 or 3 mM DTT (open and closed triangles, respectively). ϕ_P and ethane data are expressed as fractions of the values measured before light stress: $I = 103 \pm 18$ pmol·g^{-1} F.W. for the ethane production and $I = 0.59 \pm 0.05$ for ϕ_P in both DTT- and water-infiltrated leaves. Each point is the mean value of 5–10 separate experiments.

ena. This is confirmed by the data of Fig. 1: light-induced loss of electron transport at 23°C proceeded independently of lipid peroxidation whereas chilling stress in the light simultaneously caused photosynthesis inhibition and lipid photooxidation. Moreover, strong light stress at 3°C was previously observed to cause an O_2-dependent selective loss of PSI function which was mimicked at 23°C by illuminating leaves infiltrated with a SOD inhibitor [8], suggesting that O_2^- is involved in the lipid peroxidation phenomena reported here. The present study (Table 1, Fig. 2) shows that low lipid peroxidation at 23°C is attributable to the rapid and massive conversion of V into A and Z, supporting our previous suggestion [6,7] that the xanthophyll cycle is involved in the photoprotection of membrane lipids. Thylakoid membranes are rich in polyunsaturated fatty acids and are thus sensitive targets for photodestruction by activated O_2 species produced in strong light. Therefore, rapid appearance (within a few minutes, see Fig. 1B) of xanthophylls in the lipid bilayer of the thylakoid membrane under potentially harmful light conditions will be highly beneficial for the chloroplast, reinforcing the action of α-tocopherol, the only photoprotective compound that is constitutively located in the lipid matrix of thylakoid membranes [16]. As a corollary, our data indirectly suggest that A and Z are present in strong light as free pigments in the lipid phase of the thylakoid membrane. In fact, this location is corroborated by a series of previous observations. First, the transmembranous organization of the enzymes involved in the xanthophyll cycle [1] implies Z to be present as a mobile pigment in the membrane lipid matrix. In agreement with this idea is also the observation that the purified de-epoxidase of V is active in vitro in the presence of thylakoid lipids only [17]. Secondly, a high proportion of V is found in the free-pigment fraction after polyacrylamide gel electrophoresis of chlorophyll–protein complexes [18,19], indicating that V is loosely bound to the light-harvesting complexes of the thylakoid membranes, presumably allowing rapid conversion to Z in strong light. Thirdly, completely etiolated tissues lacking any active photosynthetic apparatus have been shown to possess a functional xanthophyll cycle, suggesting a role in membrane structure and formation [20]. Finally, light- and ascorbate-induced accumulations of Z have been reported to affect the in vivo physical properties of the thylakoid membrane lipids [5,6].

The possible modes of action of the xanthophyll-cycle pigments as lipid photoprotectors in light-stressed leaves can be inferred from previous in vitro studies. When incorporated into liposomal membranes, Z and some other carotenoids protect against dye-sensitized lipid peroxidation [21]. This effect can be explained by the well-recognized activity of carotenoids as

Table 1
Effects of a non-photoinhibitory light treatment at 23°C (900 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 1 h) and/or a strong light stress at 3°C (2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 1 h) on the ϕ_P, the ethane production and the (A+Z)/V ratio of potato leaves

Treatments	ϕ_P	Ethane production (pmol · g^{-1} F.W.)	(A + Z)/V
Control before treatments	0.66 ± 0.03 (n = 8)	85 ± 16 (n = 12)	0.07 ± 0.02 (n = 5)
Light-stressed at 3°C	0.37 ± 0.04 (n = 7)	182 ± 56 (n = 11)	0.31 ± 0.04 (n = 3)
Illuminated at 23°C	0.60 ± 0.04 (n = 6)	88 ± 18 (n = 7)	0.97 ± 0.19 (n = 5)
Preilluminated at 23°C and light-stressed at 3°C	0.44 ± 0.03 (n = 7)	118 ± 21 (n = 12)	0.93 ± 0.19 (n = 3)

Data are mean values of n independent experiments ± S.D.

'preventive' antioxidants that deactivate singlet oxygen [21] and free radical scavengers [22,23]. In chloroplast membranes, the major part of the antiradical activity has been attributed to unsaponifiable lipids, with the main contribution being made by α-tocopherol and dihydroxycarotenoids [24]. Very recently, Lim et al. [25] have demonstrated that Z acts as a chain breaking antioxidant in peroxyl radical-mediated peroxidation when incorporated into membrane phospholipids. Our study provides in vivo data that support this conclusion. In vitro studies have also pointed to the particular orientation of Z in lipid bilayers, with the long axis of the carotenoid being almost perpendicular to the membrane surface with two polar groups anchored in the head group region on both sides of the membrane [26,27]. The consequences of such an orientation are a marked reduction of lipid fluidity, a general rigidification of the membrane and a decrease in membrane permeability to small molecules [27,28,29]. There is experimental evidence that Z-induced decrease in lipid fluidity occurs also in vivo [5,6]. This effect could be of importance with respect to the photoprotection of chloroplastic membranes, e.g. by lowering the penetration of reactive O_2 species inside the thylakoid membranes or maintaining the light-generated transthylakoid pH which has been reported to rapidly collapse during photoinhibitory illumination of thylakoids [30]. As the competing reactions of V de-epoxidation and Z epoxidation occur simultaneously in the light [31], it has been suggested that the photoprotective action of the xanthophyll cycle could involve epoxidation of Z by singlet O_2 showing up in strong light [31,32].

Our data and interpretations are compatible with the recent suggestion of Horton and coworkers that Z amplifies the formation of the ΔpH-related quenching of PSII-chlorophyll fluorescence (q_E), presumably by activation of the LHCII aggregation [4,33]. As V is an important structural component of PSII that stabilizes the LHC conformation [34], high-light-induced release of V from PSII-pigment complexes (as suggested here) is likely to affect the LHC conformation, possibly rendering PSII more sensitive to the pH of the thylakoid lumen (as suggested in [4,33]). Therefore, when the q_E phenomenon is interpreted in terms of V disappearance (working synergistically with protonation) rather than in terms of Z accumulation, q_E-mediated modulation/protection of the PSII activity [2,3,4,33] and inhibition of lipid peroxidation can be seen as two complementary aspects of the xanthophyll-cycle activity.

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