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Redox Reactions between Kaempferol and Illuminated Chloroplasts

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ABSTRACT

Bleaching of kaempferol by illuminated chloroplasts was observed at 380 nanometers. The photobleaching was stimulated by methyl viologen and suppressed by superoxide dismutase indicating the participation of O_2^- in the reaction. An electron transfer inhibitor on the oxidizing side of photosystem II, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), stimulated the photobleaching and 3-(3,4-dichlorophenyl)-1,1-dimethylurea partially suppressed it. The stimulation by CCCP suggests that kaempferol is also bleached on the oxidizing side of photosystem II. The spectrum of kaempferol bleaching in the presence of methyl viologen was the same as that in the presence of CCCP having a maximum in absorbance decrease at around 380 nanometers. When kaempferol was oxidized by $KMnO_2$ or KO_2, the oxidized minus reduced difference spectra had also a negative peak at about 380 nanometers. The results suggest that kaempferol was oxidized by illuminated chloroplasts.

The rate of kaempferol photooxidation increased as its concentration was increased from 1 to 100 micromolar. The rate of quercetin photooxidation also increased as its concentration was increased from 1 to 100 micromolar. Concentration of quercetin glycosides higher than 10 micromolar was required to detect their photobleaching by illuminated chloroplasts. From these results, it is postulated that flavonols function as antioxidants in chloroplasts.

Flavonols are widely distributed pigments in vascular plants. Their subcellular localization has been investigated in many plants, and the kaempferol and quercetin glycosides have been found to be localized in chloroplasts (13, 14, 19). Arntzen *et al.* (2) have discussed a function of flavonols in chloroplasts in relation to energy transduction reactions. The binding of kaempferol to chloroplast coupling factor has been confirmed by Cantley and Hammes (4). Energy conservation reactions in plant mitochondria were also affected by flavonols (9, 16). Recently, it has been reported that kaempferol inhibited electron transfer reactions of mitochondria from potato (12).

On the other hand, it has been reported that carotenoid photobleaching, which is induced when electron transfer was blocked on the oxidizing side of PSII (20, 21), was suppressed by flavonols (17). The suppression was not due to the inhibition of electron transfer on the reducing side of PSII nor due to donation of electrons to PSII by flavonols; electron transfer inhibitors like as DCMU and electron donors to PSII have been reported to suppress the carotenoid photobleaching (20, 21). To elucidate the mechanism of the suppression of carotenoid photobleaching by flavonols, it is necessary to investigate redox reactions between flavonols and illuminated chloroplasts. This paper deals with oxidation of flavonols by O_2^- formed on the reducing side of PSI (3, 6, 7), and by oxidized equivalents accumulated on the oxidizing side of PSII by illumination.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach obtained from a local market. Depetiolated leaves were homogenized in 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 330 mM sorbitol, and 25 mM Mes-NaOH (pH 6.3) for 5 s at 4°C, and filtered through eight layers of gauze. The filtrate was centrifuged at 3,000 rpm for 1 min. The sediment was washed once with the isolation medium and suspended in a buffer solution which contained 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 330 mM sorbitol, and 50 mM Hepes-NaOH (pH 6.7) and centrifuged again at 500 rpm for 30 s to remove cell debris. The isolated chloroplasts were resuspended in a large volume of cold distilled H_2O to rupture them, collected by centrifugation at 10,000 rpm, and suspended in 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes-NaOH (pH 7.6). The obtained thylakoid membranes were used for experiments.

Light-induced *A* changes at 380 nm were measured with the double-beam mode using a Hitachi 557 spectrophotometer at 25°C. Actinic light was provided by a tungsten lamp (30 w) passing through glass filters (Corning CS 2-64 and Hoya HA-30) and a layer of water (5 cm). Light intensity at the surface of a cuvette was about 300 w/m^2. The light-path of the measuring beam was 4 mm. A blue glass filter (Corning CS 4-96) was placed in front of a photomultiplier to protect it from actinic light. The reaction mixture (0.6 ml) contained chloroplasts equivalent to 20 μg of Chl/ml, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes-NaOH (pH 7.6). Illumination was started 5 min after preparing the reaction mixtures. Oxidized minus reduced extinction coefficient of kaempferol was estimated to be 8×10^3 M$^{-1}\cdot$cm^{-1} at 380 nm oxidizing it by $KMnO_4$.

CCCP[1] and kaempferol were obtained from Sigma. Rutin, quercitrin, and quercetin were from Wako Pure Chemical Industry, Osaka. Catalase was from Boehringer Mannheim GmbH.

RESULTS

Figure 1 shows time courses of light-induced *A* changes at 380 nm in the presence and absence of kaempferol. In the absence of kaempferol, light-induced absorbance increase was observed and a steady-state was reached within 1 min illumination. The light-induced absorbance increase was stimulated by MV (Fig. 1B, trace 1). When kaempferol was added, light-induced absorbance decrease was observed (Fig. 1A, trace 2). The photobleaching of kaempferol was stimulated by MV (Fig. 1B, trace 2). The rate of kaempferol photobleaching in the presence and absence of MV varied from sample to sample in the range from 21 to 36 and from 7 to 11 μmol/mg Chl·h, respectively. The light minus dark difference spectra in the presence of kaempferol had a negative

[1] Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; MV, methyl viologen; SOD, superoxide dismutase; O_2^-, superoxide anion radical.

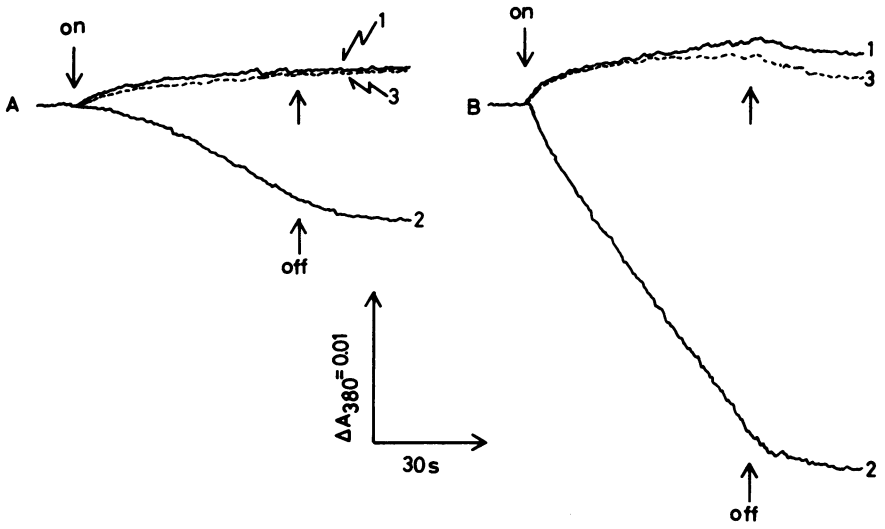

FIG. 1. Time courses of light-induced *A* changes at 380 nm in the presence (B) and absence of (A) of 100 μM MV. Reaction mixture was described in "Materials and Methods." Trace 1, no addition; trace 2, 17 μM kaempferol; trace 3, 17 μM kaempferol plus 3.3 μg SOD/ml.

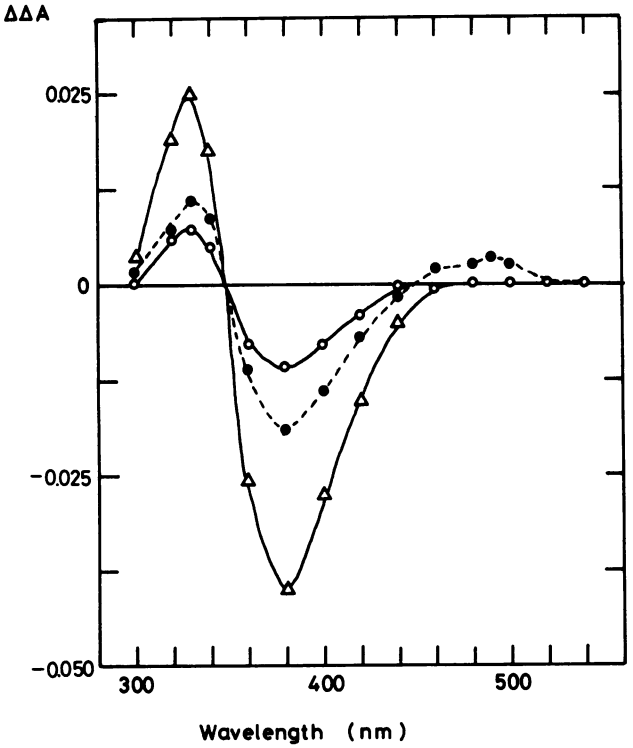

FIG. 2. Difference spectra between light minus dark difference spectra in the presence and absence of kaempferol. Reaction mixture was the same as that in Figure 1. Samples were illuminated for 1 min. (○——○), 17 μM kaempferol; (●——●), 17 μM kaempferol plus 100 μM CCCP; (△- - -△), 17 μM kaempferol plus 100 μM MV.

and a positive peak at about 380 and 330 nm, respectively (Fig. 2), suggesting the *A* decrease at 380 nm was due to the bleaching of kaempferol. This suggestion is supported by the observation that kaempferol has an absorption maximum at about 380 nm in this experimental condition (Fig. 6). The lag of the absorbance decrease after turning on the light (Fig. 1A, trace 2) might be due to the transient absorbance increase of chloroplasts which was opposite to the absorbance change of kaempferol. The light-induced absorbance change in the absence of kaempferol was considered to be due to the change of light scattering property of chloroplasts because DCMU suppressed the absorbance change

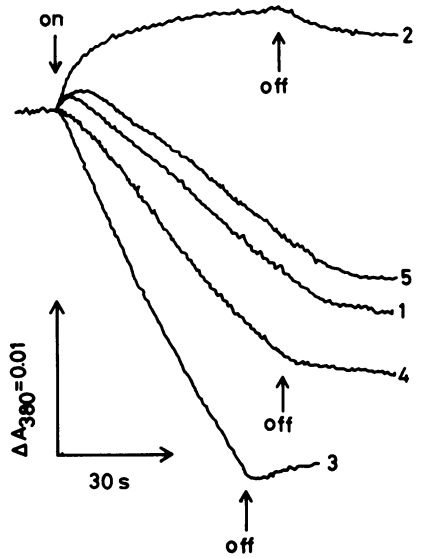

FIG. 3. Time courses of light-induced *A* changes of kaempferol at 380 nm. Reaction mixture was the same as that in Figure 1. Trace 1, 17 μM kaempferol; trace 2, 17 μM kaempferol plus 1 mM sodium ascorbate; trace 3, 17 μM kaempferol plus 100 μg catalase/ml; trace 4, 17 μM kaempferol plus 1 mM mannitol; trace 5, 17 μM kaempferol plus 1 mM H_2O plus 1 mM NaN_3.

(Fig. 4). The light-induced change of the light scattering has been reported in relation to the change of energy state of chloroplasts (5).

Effects of SOD, ascorbate, catalase, mannitol (a hydroxyl radical quencher), and deuterium oxide on the photobleaching of kaempferol were examined in the presence of MV. SOD (Fig. 1) (10) and ascorbate (Fig. 3; Ref. 7), which stimulate the consumption of O_2^-, completely suppressed the photobleaching. The production of O_2^- in illuminated chloroplasts has been reported (3, 6, 7). Catalase and mannitol stimulated the photobleaching and H_2O_2 slightly suppressed it (Fig. 3). Deuterium oxide, which prolongs the lifetime of singlet molecular oxygen and enhances singlet molecular oxygen-dependent reactions (11), suppressed the photobleaching (data not shown). These results indicate that O_2^-, but not H_2O_2, hydroxyl radical, and singlet molecular oxygen, participates in kaempferol photobleaching.

Effects of electron transfer inhibitors on kaempferol photo-

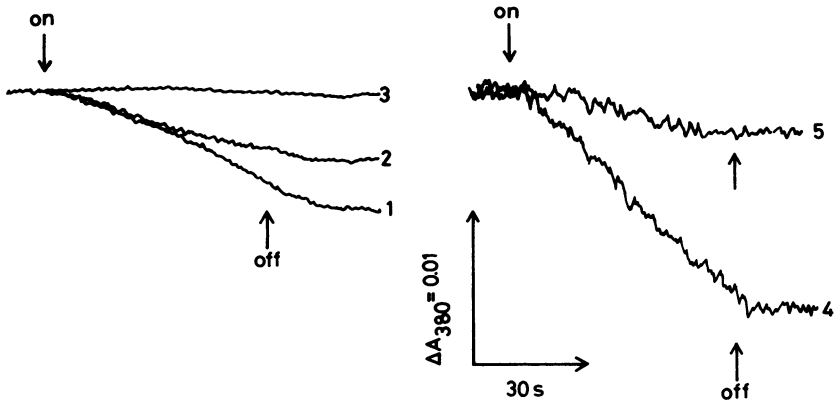

FIG. 4. Effects of DCMU and CCCP on kaempferol photobleaching. Reaction mixture was the same as that in Figure 1. Trace 1, 17 μM kaempferol; trace 2, 17 μM kaempferol plus 20 μM DCMU; trace 3, 20 μM DCMU; trace 4, 17 μM kaempferol plus 100 μM CCCP; trace 5, 100 μM CCCP.

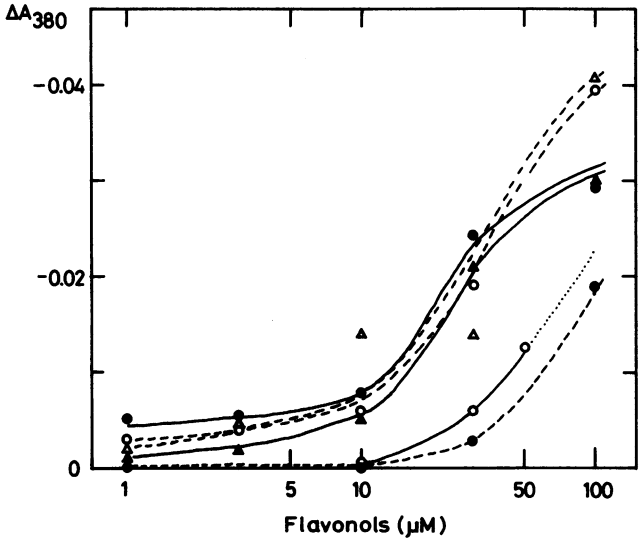

FIG. 5. Effects of flavonol concentration on their photobleaching by chloroplasts. Reaction mixture was similar to that in Figure 1. Samples were illuminated for 1 min and the extent of the absorbance decrease was plotted. (●, ▲), in the presence of 100 μM CCCP; (○, △), in the presence of 100 μM MV. (○- - -○) and (●——●), photobleaching of kaempferol; (△- - -△) and (▲——▲), photobleaching of quercetin; (○——○) and (●- - -●), photobleaching of quercitrin.

bleaching were also examined (Fig. 4). DCMU partially suppressed the photobleaching (Fig. 4, trace 2). CCCP, an electron transfer inhibitor on the oxidizing side of PSII, stimulated kaempferol photobleaching (trace 4) although the light-induced *A* decrease at 380 nm was observed even in the absence of kaempferol in CCCP-poisoned chloroplasts. The rate of kaempferol bleaching was about 17 μmol/mg Chl·h. The photobleaching in the absence of kaempferol may be due to the oxidation of CCCP (15) or the oxidation of carotenoids (20, 21). A maximum and a minimum in the absorbance change were observed at about 380 and 330 nm, respectively, in the presence of both CCCP and kaempferol (Fig. 2). The absorbance increase around 490 nm was due to the suppression by kaempferol of CCCP-induced carotenoid photobleaching (17). SOD partially inhibited the kaempferol photobleaching in the presence of CCCP (data not shown). Deuterium oxide suppressed the photobleaching of kaempferol in the presence of CCCP (17). The results suggest that singlet molecular oxygen does not participate in the reaction in the presence of the electron transfer inhibitor and that the photobleached product of kaempferol in the presence of the inhibitor is the same as that in the presence of MV. Kaempferol suppressed the photobleaching of carotenoids and Chl in CCCP-poisoned chloroplasts (17). The suppression was not due to inhibition of electron transfer nor donation of electrons to PSII by kaempferol (17). Arntzen *et al.* (2) have already reported that kaempferol did not affect noncyclic electron transfer in isolated chloroplasts.

Concentration dependences of kaempferol on its photobleaching by chloroplasts were examined in the presence of MV and CCCP (Fig. 5). In the two experimental conditions, the concentration dependences were similar; photobleaching of kaempferol appeared at about 1 μM, and the photobleaching rate increased as concentration of kaempferol was increased up to 100 μM. It was also examined whether or not other flavonols, quercetin and its glycosides quercitrin and rutin, were bleached by illuminated chloroplasts. These flavonols were also photobleached. The bleaching was enhanced by CCCP and MV and suppressed by SOD and DCMU (data not shown). The concentration dependences of quercetin on its photobleaching by chloroplasts was similar to that of kaempferol; however, ten times higher concentration was required to observe photobleaching of quercetin glycosides (Fig. 5). Quercetin and its glycosides also suppressed carotenoid photobleaching in the presence of CCCP without affecting electron transfer reactions (17).

Figure 6 shows absorption spectra of kaempferol oxidized by KO_2, which produces O_2^- in water, and $KMnO_4$. After the oxidation, an absorption maximum at about 380 nm disappeared and an absorption maximum at about 330 nm was newly formed. Oxidized minus reduced difference spectra had a negative and a positive peak at about 380 and 330 nm, respectively, independent of the oxidants used. The similarity of the oxidized minus reduced difference spectra (Fig. 6) to the light minus dark difference spectra (Fig. 2) suggests that kaempferol is oxidized by illuminated chloroplasts. Quercetin as well as kaempferol was oxidized by KO_2 and $KMnO_4$.

DISCUSSION

In this paper, it has been shown that participation of O_2^- in the photooxidation of kaempferol by chloroplasts (Fig. 1). The fact that catalase and mannitol stimulated the photooxidation (Fig. 3) indicates that reaction between O_2^- and kaempferol competes with the reactions between O_2^- and H_2O_2 and between O_2^- and hydroxyl radical; elimination of H_2O_2 and hydroxyl radical will increase the lifetime of O_2^- to oxidize kaempferol. The redox reaction between O_2^- and H_2O_2 has been reported as the Haber-Weiss reaction. The redox reaction between O_2^- and hydroxyl radical has been proposed by Arneson (1). The result that SOD partially suppressed kaempferol photooxidation in CCCP-poisoned chloroplasts indicates that kaempferol was preferentially oxidized by oxidized components accumulated on the oxidizing

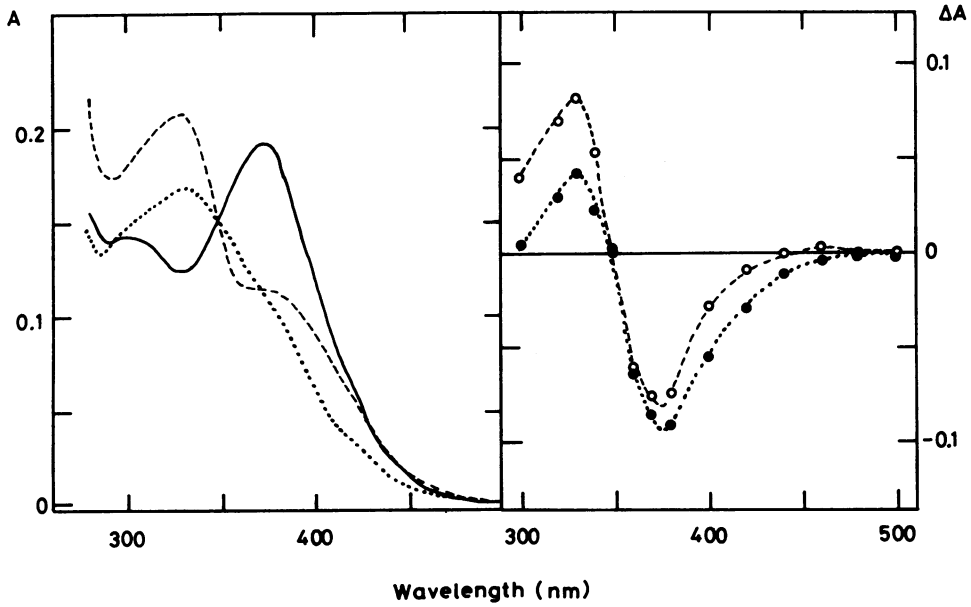

FIG. 6. Absorption (left) and oxidized minus reduced difference (right) spectra of kaempferol. Kaempferol (15 μM) was dissolved in 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes-NaOH (pH 7.6) and oxidized by adding 100 μM $KMnO_4$ or KO_2. A small grain of KO_2 was added to the above reaction mixture to oxidize kaempferol. Left: (——), kaempferol; (···), kaempferol oxidized by $KMnO_4$; (– – –), kaempferol oxidized by KO_2. Right: (O—O), KO_2-oxidized kaempferol minus kaempferol; (●···●), $KMnO_4$-oxidized kaempferol minus kaempferol.

side of PSII.

Light-oxidized minus reduced difference spectra in blue and near UV regions of kaempferol in the presence of MV was essentially the same as that in the presence of CCCP (Fig. 2). This suggests the oxidized product of kaempferol formed by illuminated chloroplasts was the same independent of the conditions under which kaempferol oxidation was performed. Kaempferol may be oxidized by donating reducing equivalents to O_2^- in the presence of MV and to oxidized components accumulated on the oxidizing side of PSII in the presence of CCCP.

The rates of kaempferol and quercetin photooxidation increased as their concentrations were increased from 1 to 100 μM. Concentrations higher than 10 μM were required to observe the photooxidation of quercetin glycosides (Fig. 5). Similar concentration dependences of flavonols were observed for the suppression of CCCP-induced carotenoid photobleaching (17). In plant cells, the concentration of flavonols has been calculated to be more than 1 mM by Arntzen *et al.* (2) based on the data reported by Furuya and Thomas (8). Recently, Vierstra *et al.* (18) reported that the concentration of flavonols in epidermal cells was 3 to 10 mM. The results obtained in this study and Reference 17 suggest that if flavonols in chloroplasts are above 10 μM, flavonols can function as antioxidants in chloroplasts. Flavonols have been reported to be localized in chloroplasts (13, 14, 19).

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