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Developmental Changes in Photosynthetic Pigments and Chlorophyll Fluorescence in Etiolated Rice Seedlings During Greening

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Development of photosynthetic pigments and changes in chlorophyll fluorescence of dark-grown rice seedlings were studied during greening. Light-illumination stimulated accumulations of total chlorophylls and carotenoids in leaves of etiolated seedlings, accompanied by a decrease in the ratio of chlorophyll *a* to chlorophyll *b*. When the composition of carotenoids was analyzed, violaxanthin level was shown to increase up to 24 h after the beginning of light illumination, followed by a subsequent decline. In contrast to this, zeaxanthin level increased consistently with progress of deetiolation. The role of zeaxanthin is discussed in relation to chlorophyll fluorescence quenching. A study on chlorophyll fluorescence kinetics of the rice seedlings being deetiolated showed a time-dependent increase in Fv/Fm (yield of variable fluorescence/maximum yield of fluoresecnece) ratios, indicating that greening is responsible for the activation of photochemical reaction centers of the photosystem. When chlorophyll fluorescence quenching was examined, qNP (nonphotochemical quenching) and qE (energy-dependent quenching) exhibited a time-dependent decline with progress of greening. The presented results indicate that greening-induced development of the photosynthetic machinery is associated the conversion of the carotenoid violaxanthin to zeaxanthin, suggesting that zeaxanthin synthesized in the illuminated leaves may provide the protection from the damage when etiolated plants are exposed to light.

Keywords : rice (*Oryza sativa* L.), greening, photosynthesis, xanthophyll cycle, zeaxanthin, chlorophyll fluorescence quenching, Fv/Fm

Plant leaves change their xanthophyll composition when exposed to light. In the illuminated leaves, the ratio of violaxanthin to lutein plus zeaxanthin is about one-fourth of that in dark-grown leaves (Dorothea *et al.*, 1978). Thayer and Björkman (1990) also observed that the size of the xanthophyll cycle pool (violaxanthin+antheraxanthin+zeaxanthin) was about four times greater in sun-grown leaves than in shade-grown ones in a number of plant species. Moreover, the increase in the amount of xanthophylls was dependent on light-intensity.

Transitions from low to high light intensity induce photoinhibition in which the levels of components of the xanthophyll cycle, particularly zeaxanthin, increase significantly. The level of violaxanthin declines in contrast to an increase in zeaxanthin by the action of violaxanthin deepoxidase. The carotenoid zeaxanthin plays an important role in dissipating excessive light energy in the photosystems (Demmig-Adams and Adams III, 1992).

In relation to the role of excessive energy dissipation, xanthophylls are correlated with non-photochemical quenching of chlorophyll fluorescence (Adams III *et al.*, 1990; Gilmore and Yamamoto, 1993; Falbel *et al.*, 1994). Non-photochemical quenching (qNP) is associated with deepoxidation of violaxanthin to zeaxanthin, which, in turn, depends on transthylakoid pH, in high light or at low temperature, and in this way, it acts as a mechanism of regulating PS II efficiency (Noctor *et al.*, 1991).

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In the present study, we report that chloroplast development is associated with conversion of the carotenoid violaxanthin to zeaxanthin, and that zeaxanthin synthesized in the illuminated leaves may provide the protection from the damage when etiolated plants are exposed to light.

MATERIALS AND METHODS

Plant material and treatments

Seeds of rice (*Oryza sativa* L. cv. Dongjin) were obtained from Yeongnam Crop Experiment Station, Rural Development Administration, Korea. After dark-germination, seedlings were grown at 25°C in darkness for 2 weeks with supply of full-strength Hoagland solution. Then, the etiolated seedlings were exposed to light at an intensity of 100 μmol quanta $m^{-2} s^{-1}$ at 25°C to induce deetiolation.

Pigment analysis

Two grams of leaf segments were first homogenized in a mortar in liquid nitrogen followed by homogenization in cold 10 ml of acetone. The extracts were centrifuged at 3,000×g for 10 min and the supernatant was applied for TLC. Silica gel G-60 was used for the supporting medium of TLC, and n-hexane:acetone (4 : 2) for the solvent of development. After completion of TLC, each of the colored bands was scraped off and extracted with ethanol for determining the respective amount of carotenoids according to Jensen and Jensen (1971).

Pigments in the extracts of the leaves were seperated by reversed-phase HPLC using a Millenium Liquid Chromatography System (Waters, Millipore, LC Module I, U.S.A.) following the procedure of Wright and Shearer (1984). Pigments were eluted with a linear gradient of ethyl acetate (20-100%) in acetonitrile-water (9 : 1, v/v) on a 5 μm Zorbax ODS column (250×4.6 mm, Dupont Co., U.S.A.). Flow rate of elution was 1 ml min^{-1}. The eluted pigments were detected by monitoring absorbance at 445 nm and identified by their visible absorption spectra and retention times.

Chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence emission from the upper surface of leaves was routinely measured at room temeperature in modulated light using a PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) as described previously (Chun *et al.*, 1994). After dark adapation for 10 min at room termperature, the leaves were placed on foam rubber and the upper surface was pressed against a Perspex window adjacent to the end of the fiber-optic probe (Walker, 1987).

The initial level (Fo) of modulated chlorophyll fluorescence was elicited by a weak red light (655 nm, 1 μmol $m^{-2} s^{-1}$ modulated at 1.6 kHz) and was measured at wavelength higher than 700 nm with a photodiode. The maximal fluorescence (Fm) was induced by a short pulse (1 s) of intense white light (4,000 μmol $m^{-2} s^{-1}$). Variable fluorescence (Fv) is the difference between Fm and Fo. The quantum yield of PS II photochemistry in the dark-adapted leaves was calculated by the ratio Fv/Fm (Kitajima and Butler, 1975).

Quenching characteristics of fluorescence induction were measured according to Schreiber *et al.* (1986). Photochemical quenching (qQ) and nonphotochemical quenching (qNP) at the steady state of photosynthesis were recorded in normal air, at PFD (photon flux density) of 300 μmol $m^{-2} s^{-1}$, white light. Energy-dependent quenching (qE) was defined as the part of nonphotochemical quenching that was reversible within 10 min of darkness. The amount of chlorophyll was determined according to Arnon (1949). After the extraction of the pigments from the leaves with 80% acetone, the absorbance of the pigment extract were measured spectrophotometrically at 663 nm and 645 nm.

RESULTS AND DISCUSSIONS

Changes in photosynthetic pigments

Table 1 shows light-induced accumulations of chlorophylls and carotenoids in leaves of the dark-grown rice seedlings. The amounts of chlorphyll *a*, chlorophyll *b* and carotenoids increased rapidly with time of illumination. The rate of accumulation of chlorophyll *b* was found to be almost two times faster than that of chlorophyll *a*, leading to the decrease in the ratio of chlorophyll *a*/*b* after prolonged light illumination. The ratio of chlorophyll *a*/*b* was 2.7, which was similar to the characteristic value of higher plants grown at a low level of irradiance.

A significant amount of carotenoids was found to be already present in etiolated leaves even before the onset of illumination. Biosynthesis of chlorophylls is crucial to the formation of the chlorophyll *a*/*b*-binding proteins that are the predominant com-

Table 1. Changes in the contents of chlorophylls and total carotenoids in the etiolated rice leaves during greening

Duration of Greening	Total Chl	Chl *a*	Chl *b*	Chl *a*/*b*	Total Carotenoids
0 h	-	-	-	-	26±5
12 h	201±14	168±16	33±2	5.1	55±7
24 h	497±17	393±26	105±9	3.7	117±42
48 h	674±5	526±9	143±2	3.7	137±66
Mature	1213±136	882±101	331±35	2.7	170±58

The amount of the pigments is shown in the unit of μg/g fresh wt. 'Mature' represents the intact leaves that had been grown for two weeks under continuous light. The values were obtained from the results of three independent experiments. ± represents the standard deviation.

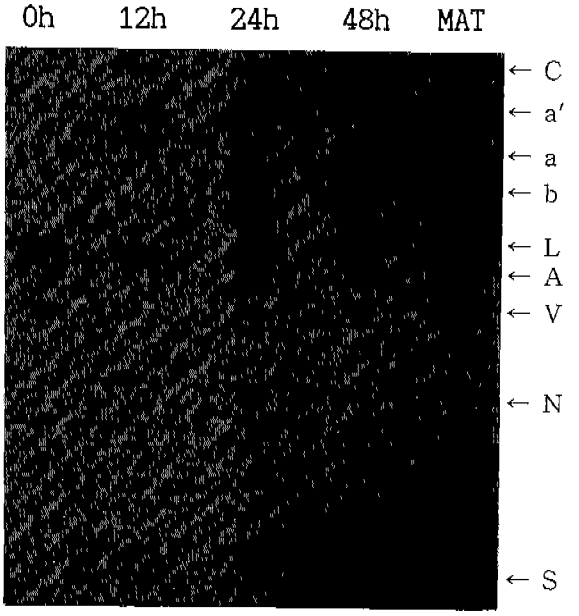

Fig. 1. TLC chromatogram showing solvent-development patterns of carotenoids and chlorophylls in the extracts of rice leaves during deetiolation. a, chlorophyll *a*; a', unidentified pigment; A, antheraxanthin; b, chlorophyll *b*; C, β-carotene; L, lutein; MAT, mature leaf; N, neoxanthin; S, starting point; V, violaxanthin.

ponents of thylakoid membranes (Thornber, 1986).

Carotenoids in leaves are synthesized in plastids and are essential for the formation of the light-harvesting and photosynthetic reaction center complexes (Peter and Thornber, 1991), and thus we examined the composition of carotenoids more closely. To compare the relative ratios of the pigments that were accumulated during greening, we tried to seperate the pigment mixtures into the respective components. But the problem arose when the pigments were seperated by using TLC, because zeaxanthin

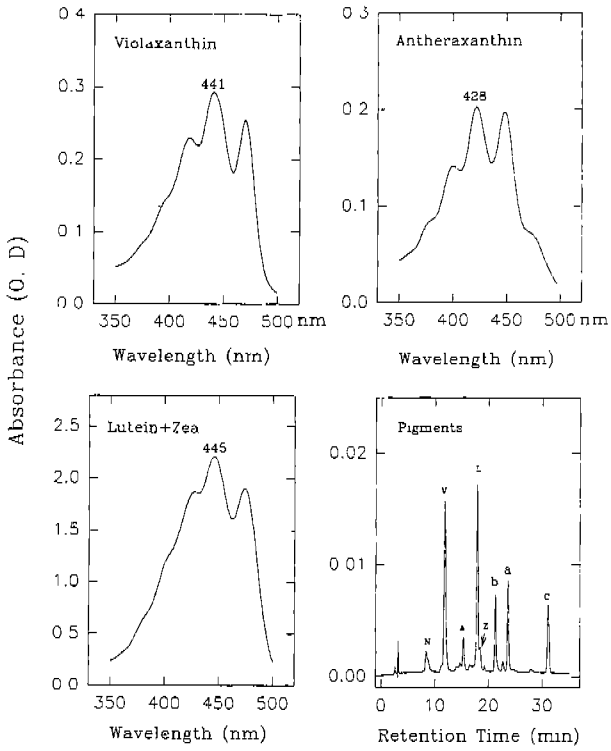

Fig. 2. The absorption spectra of carotenoids, and HPLC chromatogram showing elution pattern of chlorophylls and carotenoids in the extracts of the rice leaves that had been deetiolated for 12 h. a, chlorophyll *a*; b, chlorophyll *b*; A, antheraxanthin; c, β-carotene; L, lutein; N, neoxanthin; V, violaxanthin; Z or Zea, zeaxanthin.

was indistinguishable from lutein on TLC chromatogram (Fig. 1). Therefore, we separated these two pigments by using HPLC (Fig. 2). Furthermore, we identified the nature of each pigment by obtaining the absorption characteristics of each component of the carotenoids, and also by comparing the retention times of the pigment elution of HPLC with those cited in the literatures (Fig. 2). Chlorophylls *a* and *b*, β-carotene, lutein, neoxanthin increased with time of greening (Fig. 1). However, the levels of the individual components of the xanthophyll cycle changed quite differently from one another during greening (Fig. 3).

Before the illumination of etiolated plants, the content of violaxanthin was high in contrast to that of zeaxanthin. When the dark-grown leaves were exposed to light, the levels of all the components of the xanthophyll cycle increased up to 24 h after illumination. However, after light exposure for 24 h, the contents of violaxanthin and antheraxanthin declined, while that of zeaxanthin increased consistently from the beginning of illumination. The com-

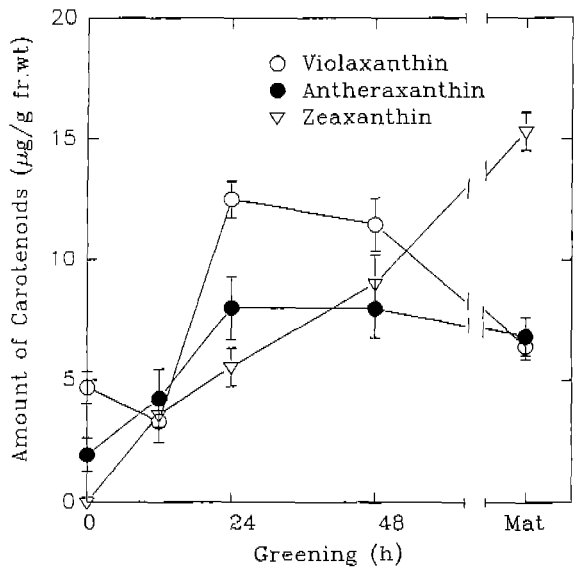

Fig. 3. Light-induced changes in the amount of individual components of xanthophyll cycle in rice leaves during deetiolation. 'Mat' represents the intact mature leaves that had been grown for two weeks under continous light.

pensatory changes in the levels of these pigments might be due to the operation of xanthophyll cycle (Fig. 3).

Zeaxanthin is enzymatically de-epoxidized from violaxanthin when exposed to light and converted back to violaxathin in darkness, constituting a xanthophyll cycle, in chloroplast membranes of higher plants (Yamamoto, 1979). The role of this cycle is associated with protection of chloroplast against the harmful effects of excessive light energy (Demmig-Adams and Adams III, 1992). In connection with the photoprotection, the carotenoid zeaxanthin is involved in the amplification of the formation of the pH-related quenching of PSII chlorophyll fluorescence (qE) (Noctor *et al.*, 1991).

To further examine the role of zeaxanthin during prolonged period of greening, we analyzed chlorophyll fluorescence kinetics and fluorescence quenching phenomena from rice leaves that were being deetiolated.

Changes in chlorophyll fluorescence

Table 2 shows changes in Fv/Fm (yield of variable fluorescence/maximum yield of fluorescence) of etiolated rice leaves during illumination. The ratio of Fv/Fm, an indicator of the photochemical efficiency of PS II (Kitajima and Butler, 1975), reached 0.79 after greening for 48h, indicating that light-induced

Table 2. Changes in chlorophyll fluorescence parameters, measured at room temperature, in the rice leaves being deetiolated

Duration of Greening	Fluorescence Intensity (Arbitrary Units)			
	Fo	Fm	Fv	Fv/Fm
0 h	394±133	538±206	144±83	0.27
12 h	720±50	2180±285	1460±240	0.67
24 h	589±54	2658±77	2069±84	0.78
48 h	618±10	2990±178	2372±169	0.79
Mature	555±22	3342±94	2787±76	0.83

'Mature' represents the intact leaves that had been grown for two weeks under continuous light. The values were obtained from the results of six independent experiments. ± represents the standard deviation.

development of photosynthetic machinery is accompanied by the increase in Fv/Fm. The changes in Fm, Fv, and Fo throughout the period of prolonged illumination were similar to those reported elsewhere for barley (Park and Chung, 1996).

The light-induced increase in Fv/Fm which is observed during greening might be due to increased production of chlorophylls, which is essential to carry out photochemical activity. The low value of Fo and Fm in the initial phase of greening might be the result of the incomplete build-up of antennae (Kitajima and Butler, 1975).

On the other hand, the increase in Fo, that had been observed after further greening, might indicate that the probability of energy transfer from the antenna chlorophyll to the reaction center decreases as the size of the PS II unit increases. This observation can be inferred from the fact that the increase in the size of the PS II can lead to the increase in the number of antenna chlorophyll which is further away from the reaction center (Akoyunoglou, 1978).

Fig. 4 shows changes in various quenching components of chlorophyll fluorescence from leaves of etiolated rice plants that had been being deetiolated. The oxidation state of Q_A (primary quinone acceptor of photosystem II), as monitored in terms of qQ, remained relatively stable up to 48h of greening, after which it decreased markedly. Both qNP and qE also decreased with time of greening.

Despite of limited light absorption during the early stage of greening, high qNP was observed. When we consider that high qNP indicates the low emission level of chlorophyll fluorescence from the leaves that are performing steady state photosynthesis, and that the levels of chlorophylls are relatively low at the initial greening stage, it might be possible that plants at the early phase of greening

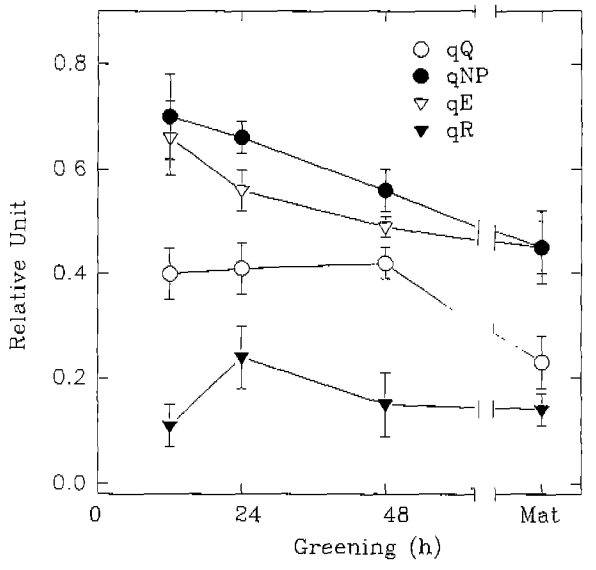

Fig. 4. Changes in the parameters of chlorophyll fluorescence quenching in rice leaves during deetiolation. 'Mat' represents the intact mature leaves that had been grown for two weeks under continous light. qP, photochemical quenching; qNP, nonphotochemical quenching; qE, energy-dependent quenching; qR, fluorescence quenching that is not reversed by DCMU treatment.

utilize light energy quite efficiently. Furthermore, high qE that had been observed during the initial phase of greening indicates that energy production of the rice plants during this stage is also occurring rather efficiently.

In particular, qE was shown to occupy the entire part of qNP in mature plants (Fig. 4). These results suggest that qE becomes significantly dominant in qNP formation in the mature chlroplast, because of the efficient build-up of pH gradient across the thylakoid membrane during electron transport, possibly by light-stimulated assembly of the thylakoid membrane components after greening.

Chloroplast development in leaves that are being deetiolated is accompanied by marked changes in membrane composition and structure, the light harvesting pigment complex, and the capacity for electron transport (Popovic *et al.*, 1984; Dreyfuss and Thornber, 1994). These changes would certainly affect various components of chlorophyll fluorescence quenching. Energy-dependent quenching which is strongly affected by the formation of zeaxanthin does occur in leaves that are exposed to various environmental conditions where linear photosynthetic electron transport is inhibited, thus preventing sustained inactivation of photochemistry by excessive light (Adams III *et al.*, 1990).

Although little is known about correlation between the carotenoid zeaxanthin and nonphotochemical fluorescence quenching during greening, it seems likely that the increased zeaxanthin represents a further extension of the overall protective function of zeaxanthin during exposure to light of the etiolated plants.

Based on the presented results, we suggest that there is an efficient utilization of light energy in leaves being dectiolated. The results also suggest that the carotenoids biosynthesized play an important role in the protection of the photosystems against damage from excessive light during dectiolation, possibly by operating xanthophyll cycles.

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