Ultrafast Excitation Relaxation Dynamics of Lutein in Solution and in the Light-Harvesting Complexes II Isolated from *Arabidopsis thaliana*

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Ultrafast excitation relaxation dynamics and energy-transfer processes in the light-harvesting complex II (LHC II) of *Arabidopsis thaliana* were examined at physiological temperature using femtosecond time-resolved fluorescence spectroscopy. Energy transfer from lutein to Chl *a* proceeded with a rate constant of $k_{ET} = 1.8-1.9 \times 10^{13}$ s^{-1} and a yield of approximately $\Phi_{ET} = 0.70$, whereas that from neoxanthin to Chl *a* had a rate constant of $k_{ET} = 6.5 \times 10^{11}$ s^{-1} and a yield at the most of $\Phi_{ET} = 0.09$. Fluorescence anisotropic decay of lutein in LHC II showed a value larger than 0.4 at the initial state and decayed to approximately 0.1 in 0.3 ps, indicating that two lutein molecules interact with each other in LHC II. In solution, anisotropy of lutein remained constant (0.38) independent of time, and thus a new excited state inferred between the S_2 ($1B_u$) state and the S_1 ($2A_g$) state was not applicable for lutein in solution. Energy migration processes among Chl *a* or Chl *b* molecules were clearly resolved by kinetic analysis. On the basis of these results, relaxation processes and energy-transfer kinetics in LHC II of *A. thaliana* are discussed.

1. Introduction

Excited-state relaxation dynamics of carotenoids continue to receive considerable interest because of their biological functions. Carotenoids play important roles in photoprotective activities, radical scavenging, singlet oxygen trapping, and so on.[1−3] In photosynthesis, carotenoids have an additional function: they absorb light energy and transfer it to nearby chlorophyll molecules, followed by an energy flow toward a photochemical reaction center where a light-induced electron-transfer reaction takes place.[4]

In general, carotenoids are classified into polyenes which belong to the point group C_{2h} in which two energetically low-lying singlet states are expected in the near-UV to visible region; one is closely related to the $2A_g$ (S_1) state which is dipole forbidden from the ground ($1A_g$ or S_0) state by parity, and the other is related to the $1B_u$ (S_2) state which is allowed for one-photon excitation.[5] According to these assignments, the following relaxation processes of carotenoids are expected: the optical excitation to the S_2 state induces an internal conversion to the S_1 state followed by relaxation to the ground state. Because of the presence of two singlet excited states, two energy-transfer pathways can occur in the photosynthetic antenna system: one from the S_2 state of the carotenoid to the S_2 state of (bacterio)chlorophyll ((B)Chl) and the other from the S_1 state of the carotenoid to the S_1 state of (B)Chl. However, carotenoids loose the ideal symmetry because of the presence of substituent groups such as methyl, allene, β-end, and keto-carbonyl groups. With respect to the conjugation system, two kinds of carotenoids are found in photosynthetic organisms; one consists of conjugated polyenes ($-(C{=}C)_n-$) and the others contain a keto carbonyl group ($>C{=}O$) in the conjugated double-bond system. When a conjugation system contains a keto-carbonyl group, excitation relaxation dynamics within the carotenoid, internal conversion and vibrational relaxation, dramatically change.[6] In pigment-protein complexes, the break in symmetry is further induced because of the distortion of the carotenoid molecule and the specific carotenoid−protein interaction. Therefore, besides the existence of energy transfer to chlorophyll, excited-state relaxation dynamics of carotenoids in pigment-protein complexes are expected to be much different from those in solutions.

Initial relaxation processes of carotenoids have been recently investigated in solution and in pigment-protein complexes from several points of view by ultrafast time-resolved fluorescence.[7−15] Fluorescence up-conversion is a selective method to detect signals from excited states. Fleming and co-workers examined the internal conversion and energy-transfer dynamics of spheroidene in solution and in LH 1 and LH 2 complexes from photosynthetic bacteria[7] and those of lutein in solution and in LHC II complexes.[8] Macpherson and Gillbro investigated solvent dependence of the $S_2 \rightarrow S_1$ internal conversion rates of β-carotene.[9] We have previously examined (1) the effects of molecular structures on the S_2 state dynamics of carotenoids and its carotenoid analogues,[6,10−12] (2) the vibrational relaxation within the S_2 state of carotenoids,[12,13] and (3) the relaxation dynamics of carotenoids containing a keto-carbonyl group,

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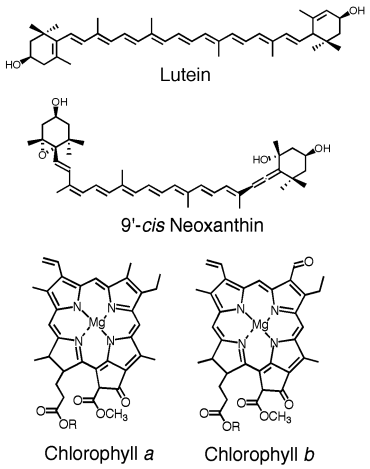
SCHEME 1: Molecular Structures of the Pigments in *A. thaliana* LHC II[a]



[a] R stands for phytol chain.

peridinin,[14] and siphonaxanthin[15] in pigment-protein complexes. We showed that (1) rate constants of the $S_2 \rightarrow S_1$ internal conversion were not monotonically related to the conjugation length of linear carotenoids,[11] (2) the minimum rate constant value was around the conjugation number of 9−10, and (3) the conjugation of a keto-carbonyl group shortens the S_2 lifetime and prolongs the S_1 lifetime.[6]

It is well-known that carotenoids containing a keto-carbonyl group, for example, siphonaxanthin, fucoxanthin, and peridinin, work as efficient antenna pigments in specific oxygenic photosynthetic organisms.[16,17] In a previous study, we reported ultrafast excitation relaxation dynamics and energy-transfer processes of siphonaxanthin in chloroplasts of the siphonaxanthin-containing green alga *Codium fragile*.[15] Fluorescence anisotropy decay and fluorescence lifetimes clearly indicated that an efficient siphonaxanthin to Chl *a* energy transfer was achieved via the S_1 state of siphonaxanthin and that siphonaxanthin in chloroplasts exhibited a new excited state (S_x) between the S_2 and S_1 states. Among the polyene-type carotenoids, the carotenoids containing 9−10 conjugated double bonds are expected to function as efficient antenna because of their relatively long S_2 lifetimes.[11] In the present study, we examined relaxation dynamics of a typical polyene-type carotenoid lutein (Scheme 1), which consists of nine conjugated double bonds with an additional conjugation by the β-end group, in solution and in LHC II complexes isolated from *Arabidopsis thaliana* by means of femtosecond time-resolved fluorescence spectroscopy.

2. Materials and Methods

2.1. Isolation of Lutein from Spinach Leaves. In spinach leaves, three kinds of carotenoids functioning in the violaxanthin cycle are present: violaxanthin, antheraxanthin, and zeaxanthin. Under strong light conditions, amounts of zeaxanthin are high. Since it is known that the chromatographic behavior of zeaxanthin is similar to that of lutein, it is a better option to reduce the content of zeaxanthin. To achieve this condition, spinach leaves were left in the dark for 1 h at 4 °C. Pigments were extracted with an acetone/methanol (7:2 v/v) mixture, evaporated to dryness, and dissolved in *n*-hexane/acetone (1:1 v/v). Pigments were loaded onto a DEAE-Toyopearl 650M column (Tosoh, Japan). All carotenoids were eluted with *n*-hexane/acetone (1:1 v/v) but Chl and polar lipids remained on the column.[18,19] A carotenoid fraction was then applied to the normal-phase chromatography column (Wakosil C-300). The column was developed with *n*-hexane/acetone (7:3 v/v), and a fraction rich in lutein was recovered. Finally, lutein was purified by high-performance liquid chromatography (HPLC) (JASCO, 900 series, Japan) equipped with a reverse-phase column (NUCLEOSIL 5C18, 7.5 × 150 mm, Chemco, Japan). Elution patterns were monitored with a photodiode-array detector (JASCO 915, Japan).

2.2. Isolation of LHC II from *Arabidopsis thaliana*. LHC II was isolated from *A. thaliana* leaves according to Burke et al.[20] Spectroscopic measurements of LHC II were carried out in 100 mM HEPES (pH 7.6) containing 0.06% dodecyl-β-D-maltoside.

2.3. Steady-State and Time-Resolved Spectroscopy. Absorption and steady-state fluorescence spectra were recorded using a spectrophotometer (Hitachi U-3010) and a fluorescence spectrometer (Hitachi F-4500), respectively. Fluorescence rise and decay curves were measured by the time-correlated single-photon-counting (SPC) system and the femtosecond fluorescence up-conversion system as previously reported.[13,21] The second harmonic of Ti:Sapphire laser (Spectra-Physics Tsunami, U.S.) was used for excitation (425 nm). Each rise and decay curve was analyzed using an iterative deconvolution method.[13] The fitting procedure for polarization fluorescence components is described in section 3.4 and that for total fluorescence was the same except that each fitting was independently carried out. Width of instrumental response function was obtained to be 30 ps for the SPC method and 220 fs for the up-conversion method; therefore, time resolution after the deconvolution analysis was estimated to be 3 ps and 20 fs, respectively.[22] Time-resolved fluorescence spectra were reconstructed from the rise and decay curves following procedures reported previously.[12,13] For time-dependent fluorescence anisotropy, polarization components of the up-conversion signals, parallel ($I_{\parallel}(t)$) and perpendicular ($I_{\perp}(t)$) components of polarization to the excitation laser pulse, were measured alternately,[15] and the fluorescence anisotropy $r(t)$ was calculated as $r(t) = (I_{\parallel}(t) - I_{\perp}(t))/(I_{\parallel}(t) + 2\, I_{\perp}(t))$. To improve the signal-to-noise ratio of $r(t)$ for pigments in the *A. thaliana* LHC II, the $I_{\parallel}(t)$ and $I_{\perp}(t)$ profiles of carotenoid fluorescence were independently measured three times with at least 25 measurements for each, and for Chl fluorescence, two independent measurements were carried out with at least two measurements for each, and data were finally summed up. All experiments were carried out at room temperature (approximately 22 °C). Spectral-grade solvents including *n*-hexane, benzene, methanol, and acetonitrile were purchased from Kanto Chemical Co. (Japan) and were used for measurements without further purification.

3. Results

3.1. Steady-State Absorption and Fluorescence Spectra. Figure 1 shows the absorption spectrum and the fluorescence spectrum of the *A. thaliana* LHC II. In the absorption spectrum, three peaks were discernible at 438, 471, and 673 nm, corresponding to the Soret band of Chl *a*, the Soret band of Chl *b*, and the Q_y band of Chl *a*, respectively. A remarkable shoulder at approximately 652 nm was assigned to the Q_y band of Chl *b*. Carotenoid absorption bands were not discriminated because of the overlap of Soret bands of Chl *a* and Chl *b*, however, they were located in wavelength regions shorter than 530 nm.[23]

Upon excitation at 425 nm, where Chl *a*, Chl *b*, and carotenoids were simultaneously excited, only a single fluorescence peak from Chl *a* ($\lambda_{max} = 681$ nm) was observed, resulting

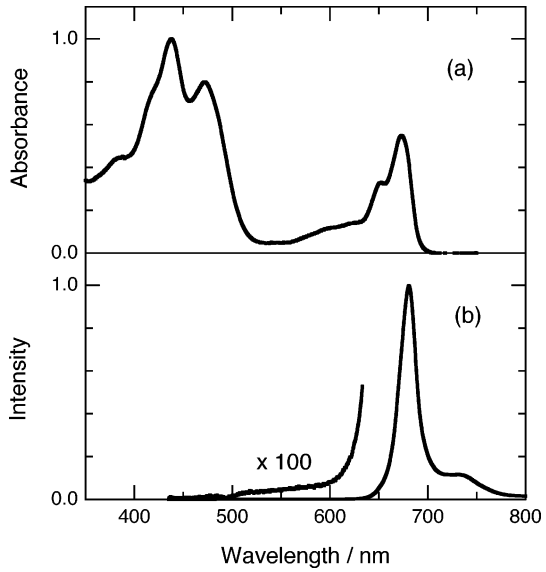


Figure 1. Steady-state spectra of *A. thaliana* LHC II. (a) Absorption and (b) fluorescence spectra excited at 425 nm.

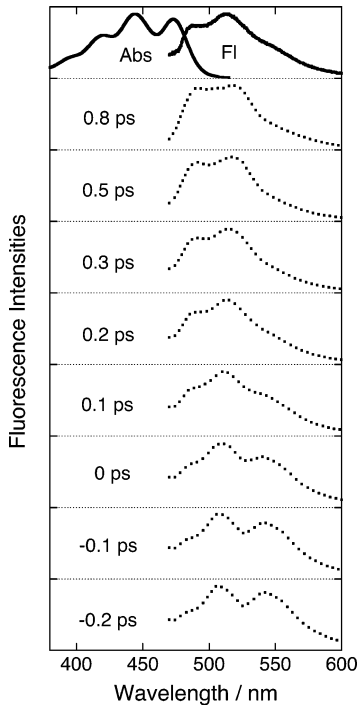


Figure 2. Time-resolved fluorescence spectra of lutein in *n*-hexane. Intensities of individual spectra are normalized to the maximum of spectrum. Steady-state absorption (Abs) and fluorescence (Fl) spectra are shown in the top window.

from the efficient energy transfer from carotenoids or Chl *b* to Chl *a*. A low but significant intensity was clearly detected from 500 nm to the blue edge of the strong Chl *a* fluorescence. It was assigned to the carotenoid fluorescence in the short wavelength region and the Chl *b* fluorescence in the long wavelength region (approximately from 600 nm), since, in solution, no fluorescence signal was detected for Chl *a* or Chl *b* in the wavelength region shorter than 600 nm.

3.2. Fluorescence Kinetics of Lutein in Solution. Time-resolved fluorescence spectra of lutein in *n*-hexane are shown in Figure 2, together with absorption and steady-state fluorescence spectra in *n*-hexane. To avoid polarization effects, the angle between the polarizations of excitation and the probe laser beams was set to the magic angle. In the very beginning, the

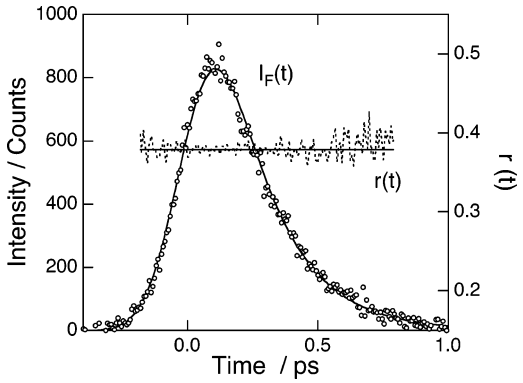


Figure 3. Fluorescence kinetics of carotenoids in *n*-hexane monitored at 560 nm: a rise and decay curve, $I_F(t)$, and an anisotropy, $r(t)$. Time interval was set to 6.6 fs per channel. Solid lines represent the best-fit functions for the rise and decay curve and the anisotropy ($r(t) = 0.379$).

TABLE 1: Fluorescence Lifetimes and Anisotropy Values of Lutein in Different Solutions

solvent	wavelength (nm)	rise time (fs)	decay time (fs)	anisotropy
n-hexane	480	100	185	0.375
	505	85	210	0.379
	540		200	0.379
	570		225	0.381
benzene	585		175	0.383
methanol	570		165	0.380
acetonitrile	574		145	0.380

spectra exhibited significant intensities at blue and red sides of fluorescence, and two peaks were recognized around 505 and 545 nm. An energy difference of these two peaks was estimated to be 1450 cm^{-1}, and this was responsible for the stretching vibration of CC bond as revealed by Raman study.[24] When increasing the delay time, the spectra became narrower and diffuse, indicating that an excess energy of approximately 2400 cm^{-1} was efficiently transferred from the Franck−Condon active mode to the dark low-frequency modes and that the magnitude of the displacement of the potential surface between S_0 and S_2 states increased with time. These behaviors are essentially the same as observed for neurosporene in *n*-hexane[13,25] and β-carotene in 2-methyltetrahydrofuran.[12]

Figure 3 shows typical fluorescence decay and fluorescence anisotropy decay curves of lutein in *n*-hexane, and the fluorescence kinetic parameters resolved by convolution procedures are listed in Table 1. Fluorescence lifetimes were dependent upon the wavelength monitored; the lifetimes were shorter than 190 fs at the blue edge of the fluorescence spectra, 190−210 fs at 480−540 nm, and 215−225 fs at longer than 550 nm. Besides the decay component, a rise component was resolved for the best fit to the fluorescence decay curves at particular wavelengths; rise times of 100 and 85 fs were necessary at 480 and 505 nm, respectively. The wavelength dependence of lifetime and the presence of the rise time were consistent with previous reports for β-carotene[12] and neurosporene.[13] When increasing solvent polarity, lifetimes at the center of the fluorescence spectra became shorter: 175 fs in benzene, 155 fs in methanol, and 145 fs in acetonitrile. The fluorescence anisotropy did not change with time and showed a constant value of 0.38, which was independent of the wavelengths monitored or the solvents used.

3.3. Fluorescence Rise and Decay Curves of the *A. thaliana* LHC II. Figures 4 and 5 show the fluorescence rise and decay curves of the *A. thaliana* LHC II, which were obtained as $I_{\parallel}(t) + 2I_{\perp}(t)$. Fluorescence lifetimes were dependent upon the

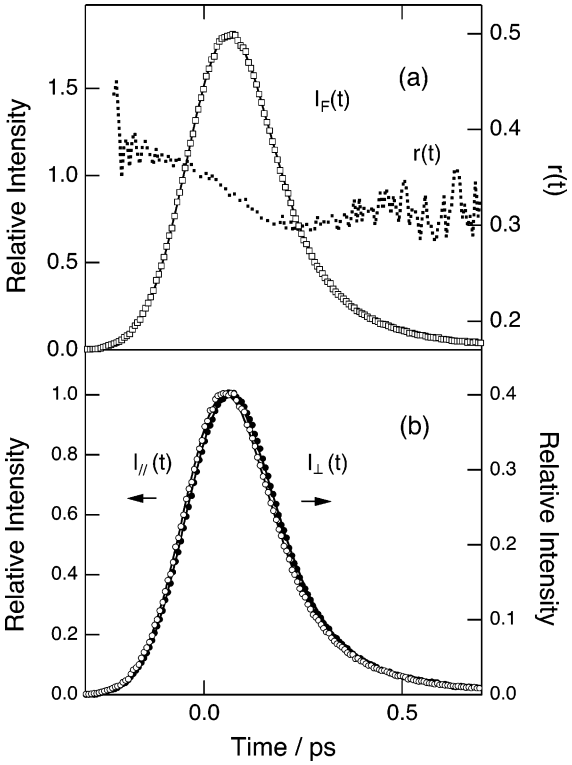


Figure 4. Fluorescence kinetics of carotenoids in *A. thaliana* LHC II monitored at 560 nm: (a) a rise and decay curve, $I_F(t)$, and an anisotropy curve, $r(t)$, and (b) polarization components of the up-conversion signals parallel ($I_{//}(t)$, open circle) and perpendicular ($I_\perp(t)$, closed circle) components of polarization to the excitation laser pulse. Time interval was set to 6.6 fs per channel. The solid line represents the best-fit function for the rise and decay curve.

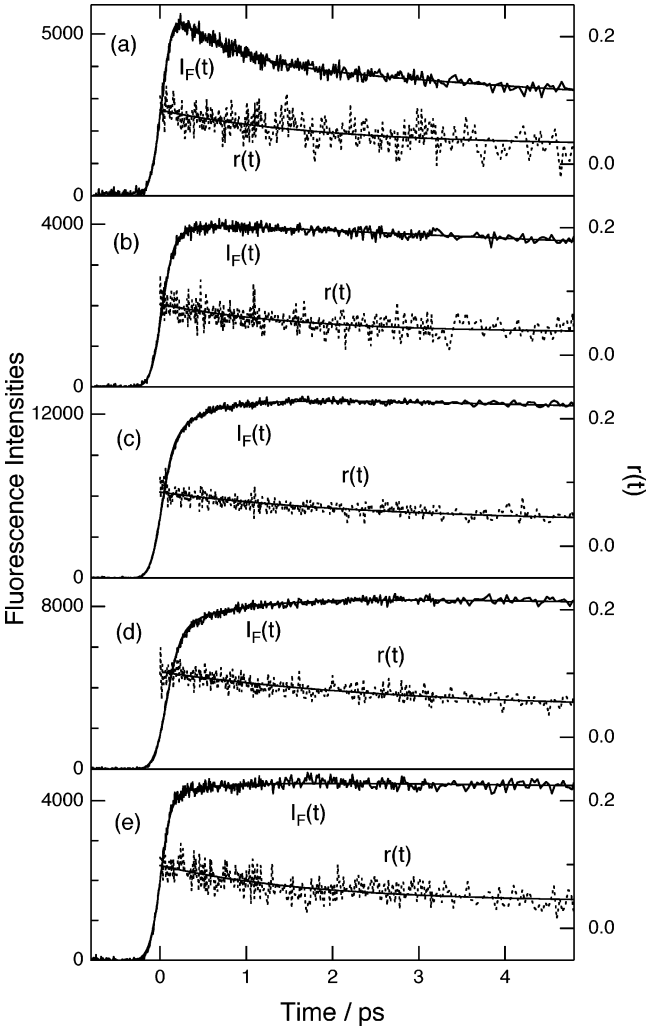


Figure 5. Fluorescence kinetics of chlorophylls in *A. thaliana* LHC II. Monitoring wavelengths were at 645 nm (a), 655 nm (b), 665 nm (c), 680 nm (d), and 690 nm (e). $I_F(t)$ and $r(t)$ stand for fluorescence rise and decay curves and fluorescence anisotropy curves, respectively.

wavelengths monitored (Table 1), reflecting the excitation dynamics of pigment molecules, that is, internal conversion and energy transfer. Two decay components, 40 fs (76%) and 145 fs (24%), were resolved for the fluorescence kinetics at 560 nm. All pigments were simultaneously excited at 425 nm; however, we assigned the decay seen at 560 nm to carotenoid fluorescences, because no signal was detected in this wavelength region for Chl *a* or Chl *b* in solution. In the LHC II trimer prepared from *A. thaliana*, lutein and neoxanthin are the main carotenoids, and the lutein/neoxanthin ratio was reported to be 2.2.[26] On the basis of spectral analysis by Croce et al.,[23] contributions of lutein and neoxanthin to the carotenoid absorption at 425 nm in LHC II are estimated to be 70% and 30%, respectively. These values were in good agreement with the amplitudes obtained through our lifetime measurements. Therefore, we assigned the lifetimes of 40 and 145 fs to those of lutein and neoxanthin, respectively. We estimated the averaged lifetime of carotenoids to be 96 fs, which is identical to the reported S_2 lifetime of carotenoids, 95 fs, in the spinach LHC II after an excitation at 489 nm where fractions of initially excited carotenoids are 2:1 (lutein:neoxanthin).[27]

Fluorescence kinetics of Chl *a* and Chl *b* were strongly dependent on monitoring wavelengths. For the Chl *b* fluorescence (645 nm), a rise component of 60 fs and a decay of 675 fs were resolved in the analyses in the femtosecond time range, and for the Chl *a* fluorescence (665, 680, and 690 nm), two rise components of 100 fs and 535−750 fs were necessary. Only one rise of 120 fs was observed at 655 nm. In the pico- to nanosecond region, Chl *a* and Chl *b* exhibited triple exponential decay components; the fastest two became longer on going toward longer wavelengths, whereas the longest one was a constant value of 3.5−3.7 ns. These results indicate that there is an excitation energy transfer to long-wavelength forms of Chl molecule, followed by equilibrium among Chl molecules.

TABLE 2: Fluorescence Lifetimes of LHC II as a Function of Observed Wavelengths[b]

wavelength (nm)	lifetime				
560	40 fs	145 fs			
645	60 fs[a]	670 fs	20 ps	350 ps	3.5 ns
655	120 fs[a]		40 ps	500 ps	3.5 ns
665	125 fs[a]	535 fs[a]	90 ps	870 ps	3.7 ns
680	125 fs[a]	705 fs[a]	120 ps	1.3 ns	3.6 ns
690	125 fs[a]	675 fs[a]	180 ps	1.4 ns	3.7 ns

[a] Rise component. [b] Lifetime values longer than 20 ps were independently measured with a time-correlated single-photon-counting method.

3.4. Fluorescence Anisotropy Decays of the *A. thaliana* LHC II. Fluorescence anisotropy decays of the *A. thaliana* LHC II are depicted in Figures 4 and 5, and their decay time constants are summarized in Table 2. When delay time increased, the anisotropy of carotenoids decreased from 0.37 to 0.30 until 0.25 ps, slightly increased again during 0.25−0.60 ps, and finally showed a constant value of 0.33 after 0.60 ps. This behavior has never been observed before and was in striking contrast with that in solution (Figure 2) and that of siphonaxanthin in

chloroplasts of *Codium fragile*, where the anisotropy of the siphonaxanthin fluorescence at 560 nm in chloroplasts showed a constant value of 0.30, independent of the delay time.[15]

To analyze the time evolution of the anisotropy decay of carotenoids in the *A. thaliana* LHC II, we globally evaluate the time constants for $I_{||}(t)$, $I_{\perp}(t)$, and $r(t)$ as follows.

$$I_{||}(t) = \sum_i A_i \int_0^t f(t') \exp\left(-\frac{(t-t')}{\tau_i}\right) dt' \quad (1)$$

$$I_{\perp}(t) = \sum_i B_i \int_0^t f(t') \exp\left(-\frac{(t-t')}{\tau_i}\right) dt' \quad (2)$$

$$r(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)} \quad (3)$$

Here, $f(t)$ is an instrumental response function, and A_i and B_i are preexponential factors for τ_i in $I_{||}(t)$ and $I_{\perp}(t)$, respectively. For the instrumental response function, a Gaussian function of 220 fs fwhm was used.[15] We found that the fluorescence kinetics, $I_{||}(t) + 2I_{\perp}(t)$, was represented as a double exponential decay function. However, at least four constants, τ_i ($i = 1-4$), were necessary to simultaneously fit the observed rise and decay curves of $I_{||}(t)$, $I_{\perp}(t)$, and r(t) by eqs 1−3. The resolved time constants were $\tau_1 = 20$ fs, $\tau_2 = 40$ fs, $\tau_3 = 70$ fs, and $\tau_4 = 145$ fs with respective preexponential factors (Figure 6 and Table 3). The longest time constant, $\tau_4 = 145$ fs, was in good agreement with the lifetime of neoxanthin in the *A. thaliana* LHC II; therefore, it was assigned to the decay constant of neoxanthin. The amplitude of the 145-fs component obtained as $(A_4 + 2B_4)/(\sum A_i + 2\sum B_i)$ was 23.7%, which was identical to that analyzed from the fluorescence decay (24%). This confirms that the 145-fs component is due to neoxanthin and that the other three are due to lutein. From the amplitudes in $I_{||}(t)$ and $I_{\perp}(t)$, A_4, and B_4, the anisotropy of this 145-fs component was found to be 0.33. On the other hand, only the 40-fs component was consistent with the fluorescence decay of lutein in the *A. thaliana* LHC II, and components corresponding to the 20-fs and 70-fs decays were not resolved in the fluorescence decay, indicating that the latter two were responsible for the time-dependent changes in fluorescence polarization of lutein in the *A. thaliana* LHC II, which should be coming from an interaction between two lutein molecules as analyzed later. Figure 7 illustrates the reconstructed fluorescence anisotropy decays for lutein and neoxanthin in the *A. thaliana* LHC II. As for the lutein fluorescence, the anisotropy was resolved to 0.52 at $t = 0$ and decreased to 0.12 within 0.3 ps. Neoxanthin showed a constant anisotropy of 0.33. The anisotropy decays were obtained from a combination of the 20-fs, the 40-fs, and the 70-fs components for lutein and the 145-fs component for neoxanthin. The 20-fs component was required especially to fit the anisotropy decay in the initial time region. The 70-fs component was also necessary to represent curvature of $r(t)$ in the recovery region.

The fluorescence anisotropy of Chl *a* and Chl *b* decayed with time constants of 1.7−2.9 ps (Table 4). Since these components were not observed in solution, they were assigned to the energy migration among Chl *a* and Chl *b* pigment pools, respectively, in the *A. thaliana* LHC II. These components were not observed in the fluorescence rise and decay curves, as similar to the 20-fs and 70-fs components of lutein. Any anisotropy signal originating from carotenoid S_1 fluorescence was not resolved, because lutein lost its fluorescence polarization in the highly excited states.

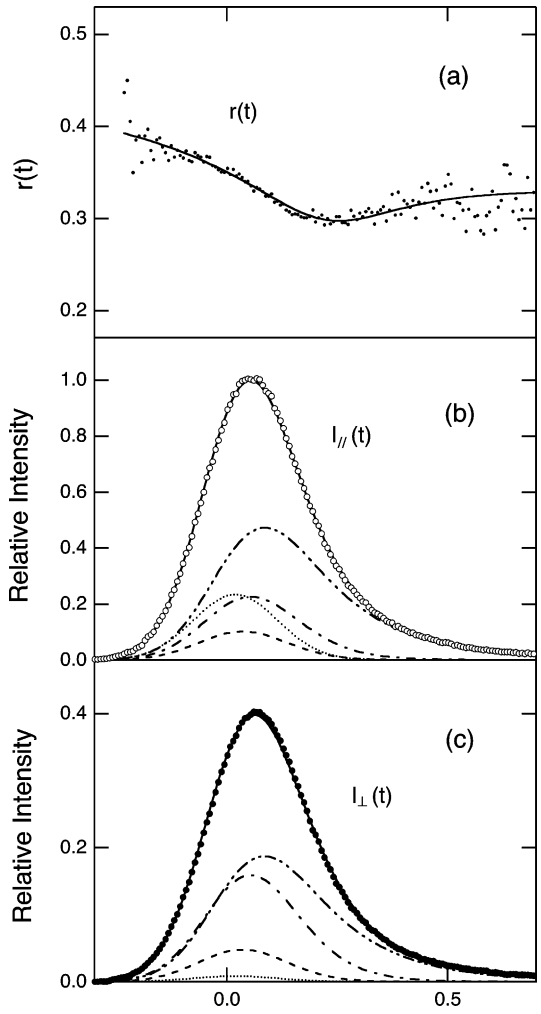


Figure 6. Results of analyses for the polarized fluorescence kinetics of carotenoids in *A. thaliana* LHC II monitored at 560 nm: (a) $r(t)$, (b) $I_{||}(t)$, and (c) $I_{\perp}(t)$. Time interval was set to 6.6 fs per channel. Solid lines are the best-fit functions to each component analyzed by using eqs 1−3. In a and b, contributions of the 20-fs (dotted line), the 40-fs (dashed line), the 70-fs (dot−dashed line), and the 145-fs (two dot−dashed line) components are shown.

TABLE 3: Parameters for Global Fits of the Fluorescence Anisotropic Decays in LHC II

	20 fs	40 fs	70 fs	145 fs
A for $I_{||}(t)$[a]	0.335	0.077	0.107	0.131
B for $I_{\perp}(t)$	0.012	0.036	0.075	0.053
$A + 2B$	0.358	0.149	0.256	0.237

[a] Results of analyses for $I_{||}(t)$, $I_{\perp}(t)$, and $r(t)$ by using eqs 1−3. Contributions of the amplitudes for each time constant are listed. $\sum A_i + 2\sum B_i$ is normalized to 1.

4. Discussion

4.1. Relaxation Dynamics of Lutein in Solutions. Recently, it was reported that in a few carotenoids, an additional excited state (S_x) exists between the S_2 and S_1 states even in solution, on the basis of an ultrafast transient absorption measurement.[28] We carried out global analyses of the time-resolved fluorescence spectra of lutein in *n*-hexane to obtain decay-associated spectra but could not obtain any convergent results that suggested an $S_2 \rightarrow S_x$ internal conversion, because the rise times were only resolved in the short wavelength region of the fluorescence

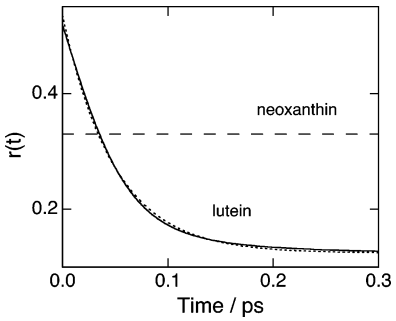


Figure 7. Fluorescence anisotropy curves after deconvolution analyses: lutein (solid line) and neoxanthin (broken line). The dotted line shows the best-fit function by a single-exponential decay (49 fs) to the deconvoluted fluorescence anisotropy of lutein.

TABLE 4: Analyzed Decay Time Constants for the Fluorescence Anisotropy Decay in the Chl Regions of the LHC II

wavelength (nm)	lifetime	assignment
645	1.9 ps	Chl *b*
655	1.7 ps	Chl *b*, Chl *a*
665	2.6 ps	Chl *a*
680	2.9 ps	Chl *a*
690	1.8 ps	Chl *a*

maximum. The time-dependent spectral changes reflect not only the internal conversion but also the dissipation processes of excess energy within the electronically excited state. Fluorescence kinetics observed for lutein in solution were essentially the same as reported for those assigned to the vibrational energy relaxation for other organic molecules, that is, Coumarin 153,[29] zinc tetraphenyl porphyrin,[30] and perylene.[31] Therefore, we concluded that changes in the time-resolved fluorescence spectra of lutein in solution reflected the dissipation processes of excess energy within the particular electronically excited state (S_2 state). There are two possibilities of the electronically excited state from which we observed fluorescence: the S_2 state or the S_x state. Since fluorescence anisotropy measurement of lutein did not indicate the existence of an additional excited state (S_x) (Figure 3), the spectral change was assigned to vibrational relaxation in the S_2 state. When the latter case is applicable, the S_2-S_x internal conversion should be an ultrafast process with a time constant shorter than 10 fs as reported for lycopene and β-carotene by Cerlullo et al.[28] and hence could not be detected by our system. According to Cerlullo et al.'s assignment, the spectral change is due to vibrational relaxation in the S_x state, however, this was less probable on the basis of our analysis.

4.2. Fluorescence Anisotropy Decays of Carotenoids in the *A. thaliana* LHC II. In the *A. thaliana* LHC II, fluorescence anisotropies $r(t)$ of carotenoids exhibited different features from those in solution. The anisotropy of lutein showed $r(0) = 0.52$ just after excitation and decreased to $r(t) = 0.12$ within 0.3 ps, whereas that of neoxanthin was of a constant value of $r(t) = 0.33$. The behavior of lutein anisotropy could not be explained by the relaxation dynamics within one lutein molecule, because the $r(0)$ value was larger than 0.4. The theory for molecular dimers discussed by Wynne and Hochstrasser[32] and by Knox and Gulen[33] would account for an anisotropy larger than 0.4 in terms of dephasing of the initial dimer electronic coherence induced by the absorbed photon. When two energetically degenerated states, whose transition dipole moments are orthogonal in their orientations, are coherently excited, a fluorescence anisotropy is predicted to show 0.7 just after excitation and decrease to 0.1 with a damping oscillation.[32] According to the theoretical treatment by Wynne and Hochstrasser,[32] the $t \rightarrow \infty$ limiting anisotropy is obtained as a function of an angle between the transition dipoles corresponding to each molecule (θ),

$$r(\infty) = 0.1 + 0.3 \cos^2 \theta \qquad (4)$$

By substituting $r(\infty) = 0.12$, the angle between the dipole moments of two lutein molecules was estimated to be 75° or 105°. It is, therefore, reasonable that the initial anisotropy of lutein in the *A. thaliana* LHC II showed a value less than 0.7, since $r(0)$ becomes 0.7 under the condition that $\theta = 90°$. Since time evolution of the anisotropy strongly depends on the term of the damping oscillation, it is necessary to observe oscillating behavior with a better time resolution before precise discussion for $r(0)$.

The structure of LHC II has been determined by electron micrograph crystallography[34] and recently by X-ray crystallography.[35] Two lutein molecules are located in the center, being bound on both sides of the supercoil (helices A and B) to form a cross-brace. The polyene chains of two lutein molecules are inclined with respect to the membrane normal by angles of about 59° and 62°.[35] Therefore, the angle between the polyene chains is 121°. Since it is inferred that the direction of S_2-S_0 transition dipole moment of carotenoid slightly deviates from the molecular long axis,[36] our estimation (105°) was in good agreement with that estimated from the crystal structure. This leads us to conclude that two lutein molecules form a dimer in LHC II and that interaction between the two is significant. The anisotropy decay of lutein in LHC II does not clearly suggest the presence of an underdamped oscillatory component. When $\cos^2 \theta \approx 0$, the fluorescence anisotropy exhibits one or two decay time constants.[32] The deconvoluted anisotropy deviated from the best-fit function of a single-exponential decay, in which the time constant was obtained to be 49 fs (Figure 7), and this situation was not improved through a fitting with a double-exponential decay (not shown). This suggests that an oscillatory component might be present; however, it might be resolved with a better time resolution. When an anisotropy decays without an underdamped oscillatory component, polarization fluorescence should be represented by two decay components; the long-lived one is responsible for the $t \rightarrow \infty$ limiting anisotropy, and the short-lived one is responsible for the time evolution of the anisotropy. In this sense, the presence of the three components in the polarization fluorescence of lutein might be a sign of the existence of an underdamped oscillatory component.

The fluorescence anisotropy of lutein in LHC II changed from 0.52 to 0.12 within 0.3 ps, but this was not the case for siphonaxanthin that contains a keto-carbonyl group in its conjugation system in *Codium fragile* chloroplasts.[15] This difference suggests that the interaction between carotenoid molecules is more significant for lutein in pigment-protein complexes. Although crystal structure of LHC II from green algae has not yet been revealed, it is probable that siphonaxanthin occupies the central part of LHC II instead of lutein, because the amino acid sequence of LHC II in the siphonaxanthin-containing green alga *Bryopsis maxima* is very similar to that of the higher plant LHC II.[37,38] Therefore, the difference in strength of the carotenoid−carotenoid interaction might not be due to the geometry between the carotenoid molecules but to the carotenoid properties. In pigment-protein complexes, carotenoids containing the keto-carbonyl group, that is, siphonaxanthin and fucoxanthin, exhibit an absorption band at approximately 530 nm.[15] This is explained by the strong interaction between the keto-carbonyl group and the protein.[15] Since lutein has no group that could strongly interact with

proteins in its conjugation system, the carotenoid−protein interaction should not be significant for lutein, at least not enough to make the S_x-S_0 transition occur. It is, therefore, likely that the carotenoid−protein interaction is less operative for lutein compared with the carotenoid−pigment interaction in the pigment-protein complex. This was confirmed by circular dichroism (CD) spectrum where lutein showed a small but significant CD signal in the pigment-protein complex,[39] whereas siphonaxanthin showed no CD signal even in chloroplasts.[15] Since the energy level of the Soret band of Chl is very close to the energy level of the S_2 state of lutein, it is expected that carotenoid−Chl interactions also affect the time evolution of the fluorescence anisotropy of lutein. On the basis of this assumption, recurrence motion of excitation should occur between the S_2 state of lutein and the Soret band of Chl. However, it is not detectable, since fluorescence from the Soret band was not observed because of ultrafast internal conversion from the Soret band to the lower excited states in Chl. Therefore, it is probable that the carotenoid−carotenoid interaction is the dominant factor for the observed anisotropy decay in lutein.

The anisotropy of neoxanthin was 0.33 in the *A. thaliana* LHC II, being independent of delay time. This behavior was almost the same as observed for siphonaxanthin in the *Codium fragile* chloroplasts except for the anisotropy value (0.30).[15] In chloroplasts of Chl *a*/*b* containing organisms, only 9′-cis neoxanthin was found.[40] Although 9′-cis neoxanthin deviates from the C_{2h} symmetry, no difference was observed between the relaxation dynamics of the S_2 state of the 9′-cis and all-trans forms.[10] Thus, it seems that the reduced anisotropy value of 9′-cis neoxanthin was not due to differences in the conjugation structure but to the carotenoid−protein interaction although it might be weak compared to the case of siphonaxanthin in chloroplasts.

4.3. Energy Transfer in the *A. thaliana* LHC II. The rate constant of energy transfer from a specific pigment to another k_{ET} can be calculated from the formula, $k_{ET} = k_{DA} - k_D$, where k_{DA} is the rate constant with energy transfer and k_D is the rate constant without energy transfer. k_D in the pigment-protein complexes can be adopted with the value in solvents of high polarity or polarizability, because in pigment-protein complexes, the protein is thought to produce highly polarizable environment. By using the abovementioned rate constants, an energy-transfer efficiency Φ_{ET} is obtained as $\Phi_{ET} = k_{ET}/(k_{ET} + k_D)$. In the *A. thaliana* LHC II, the lifetimes of lutein and neoxanthin were 40 and 145 fs, respectively. On the other hand, the lifetimes in solution were 175 fs for lutein in benzene, 145 fs for lutein in acetonitrile, 130 fs for lutein in benzyl alcohol,[41] and 160 fs for neoxanthin in benzene.[10] As a result, the rate constants of the carotenoid-to-chlorophyll energy transfer were calculated as $k_{ET} = 1.8-1.9 \times 10^{13}\ s^{-1}$ and $\Phi_{ET} = 0.69-0.77$ for lutein and $k_{ET} = 6.5 \times 10^{11}\ s^{-1}$ and $\Phi_{ET} = 0.09$ for neoxanthin. For more accurate estimation, it is desirable to use lifetimes in a more polarizable solvent. However, it can be concluded that the energy transfer from neoxanthin is less efficient.

The ultrafast relaxation dynamics of carotenoids in the LHC II were previously studied by two different techniques, that is, the pump−probe method to observe transient absorption spectra[23,27,42] and the fluorescence up-conversion method.[8] In a recent fluorescence study by Holt et al.,[8] the S_2 fluorescence lifetime of carotenoids in the *A. thaliana* LHC II was 28 fs (0.92) and 114 fs (0.08) after fitting by a double-exponential decay and 57 fs by a single-exponential decay. These values are shorter than those found in the present study. Here, we reported values of 40 fs (76%) and 145 fs (24%) for the best fit and 96 fs for the averaged lifetime. This discrepancy probably results from differences in the direction of laser polarization. With respect to the polarization of the gate pulse, that of the excitation pulse was parallel in Holt et al.'s study[8] but was at the magic angle in our present study. As listed in Table 3, the contribution of the component with the shortest lifetime is larger in $I_{||}(t)$ than in $I_{||}(t) + 2I_{\perp}(t)$; therefore, the lifetime values are estimated to be shorter when $I_{||}(t)$ is used as the fluorescence rise and decay kinetics. From the femtosecond transient absorption studies, the S_2 lifetime of carotenoids in the LHC II was 95 fs for the mixture of lutein and neoxanthin with a fraction of 2:1[27] and 50−70 fs for lutein.[23] These values are in good agreement with our estimation of the averaged fluorescence lifetime and the fluorescence lifetime of lutein. Although transient absorption and the transient fluorescence studies show almost the same kinetics, they are not necessarily in agreement on the energy transfer from neoxanthin to Chls. The lifetime of neoxanthin in LHC II was estimated to be 90 fs by transient absorption[23] and 145 fs by fluorescence up-conversion (the present study). Since signals originating from neoxanthin are smaller than those from lutein, it might be difficult to exactly resolve kinetic parameters of neoxanthin especially when signals from Chls overlap.

With a laser excitation at 425 nm, Chl *a*, Chl *b*, and carotenoids are simultaneously excited, therefore, the internal conversion within the chlorophyll, the energy transfer from carotenoid to chlorophyll, and the energy transfer from Chl *b* to Chl *a* are included in the fluorescence rise and decay kinetics of chlorophylls. The decay time constant of 675 fs observed for the Chl *b* fluorescence (645 nm) was in good agreement with the rise time constant of 535−750 fs for the Chl *a* fluorescence (665, 680, and 690 nm in Table 2), indicating an energy transfer from Chl *b* to Chl *a*. Rise times of 60 fs (Chl *b*) and 120−125 fs (Chl *a*) seem to correlate with decay times of 40 fs (lutein) and 145 fs (neoxanthin), respectively; however, this possibility was excluded because the Chl species surrounding lutein are Chl *a* and those surrounding neoxanthin are Chl *b*.[27,35] Hence, rise times of 60 fs and 120−125 fs were assigned to the internal conversion within Chl *b* and Chl *a*, respectively. The carotenoid-to-chlorophyll energy transfer was directly estimated by the decays in the short wavelength region under this excitation condition.

5. Summary

Energy-transfer processes in LHC II of *Arabidopsis thaliana*, especially from carotenoids to Chl, were resolved by fluorescence up-conversion and anisotropy measurements. The energy-transfer efficiency from the S_2 states of carotenoids to Chl was approximately $\Phi_{ET} = 0.70$ for lutein and at the most $\Phi_{ET} = 0.09$ for neoxanthin. Fluorescence anisotropic decay of lutein in LHC II showed a value larger than 0.4 just after excitation and decreased to approximately 0.1 within 0.3 ps, whereas that of neoxanthin in LHC II did not change with time ($r(t) = 0.33$). These results indicate that the interaction between two lutein molecules is significant and that the carotenoid−protein interaction is dominant for neoxanthin. Time constants of the energy transfer between Chl molecules were approximately 700 fs for the Chl *b*−Chl *a* transfer and 1.7−2.9 ps for the energy migration among Chl *a* and Chl *b* pigment pools.

In solution, anisotropy of lutein showed a constant value of 0.38 independent of time, and the transient fluorescence spectrum decreased in width as the delay time increased. These behaviors were interpreted as the dissipation process of an excess energy within an electronically excited state. Therefore, a new

excited state inferred between the S_2 ($1B_u$) state and the S_1 ($2A_g$) state was not recognized for lutein in solution, which was also the case for β-carotene in 2-methyltetrahydrofuran,[12] neurosporene in *n*-hexane,[13] and siphonaxanthin in benzene.[15]

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