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# Seasonal changes of excitation energy transfer and thylakoid stacking in the evergreen tree *Taxus cuspidata*: How does it divert excess energy from photosynthetic reaction center?

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**Abstract**

Photosystems must efficiently dissipate absorbed light energy under freezing conditions. To clarify the energy dissipation mechanisms, we examined energy transfer and dissipation dynamics in needles of the evergreen plant *Taxus cuspidata* by time-resolved fluorescence spectroscopy. In summer and autumn, the energy transfer processes were similar to those reported in other higher plants. However, in winter needles, fluorescence lifetimes became shorter not only in PSII but also in PSI, indicating energy dissipation in winter needles. In addition, almost the same fluorescence spectra were obtained with different excitation wavelengths. In contrast, the fluorescence spectrum showed a large difference due to excitation wavelength in spring needles. The fluorescence spectrum of spring needles in 550-nm excitation showed similar spectra to that of winter needles, however, red-chlorophyll fluorescence was not observed in chlorophyll excitation. These observations suggest that some complexes with some kind of red-shifted carotenoid and red-chlorophyll unlink from the core complex in spring. Seasonal changes of excitation energy dynamics are also discussed in relation to changes in thylakoid stacking.


*Keywords:* Seasonal change; Energy transfer; Quench; Time-resolved fluorescence; Evergreen plant; Thylakoid stacking

## 1. Introduction

In the natural environment, plants encounter stressful conditions such as high light, drought, salt and low temperatures. Under these conditions, photosynthetic activity decreases due to the inactivation of $CO_2$ fixation enzymes and photodamage of photosystems [1–3]. Decrease in photosynthetic activity must be accompanied by dissipation of absorbed light energy to protect against further photodamage [4].

Evergreen plants retain chlorophyll (Chl) under freezing conditions. Under these conditions, light energy is harvested by chlorophyll but the energy cannot be used for photosynthesis [5]. Therefore they protect themselves against freezing and photodamage using many strategies, including dissipation of light energy channeled into Chls depending on seasons [6]. Several mechanisms to prevent photodamage in winter have been reported previously. Chloroplasts of *Taxus* congregated together in the centers of the cells during winter, whereas they were localized adjacent to plasma membranes in summer, which may serve to control the amount of light absorption [7]. In case the excess light energy induces formation of reactive oxygen species, evergreen plants minimize damage using the antioxidants glutathione and α-tocopherol [8]. Dissipation of absorbed light energy before it arrives at reaction centers is especially important for evergreen plants because $CO_2$ fixation is completely inhibited and absorbed light energy cannot be used for photosynthesis. Ottander et al. focused on photosystem II (PSII) organization and pigment composition in the evergreen *Pinus sylvestris*, and revealed the losses of chlorophyll, of the D1-protein of the PSII reaction center (RC) and of PSII light-

*Abbreviation:* PSI, photosystem I; PSII, photosystem II; RC, reaction center; LHC, light-harvesting complex; Chl, chlorophyll; FDAS, fluorescence decay-associated spectrum; ELIP, Light-induced stress protein; TRFS, time-resolved fluorescence spectra

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harvesting-complex (LHCII) proteins in winter. Much of the remaining chlorophyll was reorganized in aggregates that quench excitation energy efficiently [9]. Steady-state fluorescence spectra revealed the presence of the 705-nm and 715-nm fluorescent forms in winter leaves of *Eucalyptus pauciflora* and *Amyema miquelii* [10,11]. These forms coincide structurally with a loss of Chl and an increase in energy-dissipating carotenoids and were assumed to be involved in energy dissipation. However, the details of energy transfer and quenching processes in evergreen species are still unknown.

Time-resolved fluorescence spectroscopy is a useful tool to reveal these processes because energy transfer is expressed as peak-shifts or peak-alternations of fluorescence spectra [12], and the energy quenching can be monitored by the rapid dissipation of fluorescence peaks [13]. In this study, we examined the seasonal changes in excitation relaxation dynamics in needles of *Taxus cuspidata* by means of a picosecond time-resolved fluorescence spectroscopy. PSII fluorescence of winter needles was quite small and its lifetimes were twice shorter than that of summer needles, indicating the efficient quenching of excitation energy and low PSII level. PSI was also modified to dissipate absorbed light energy. Energy migration and dissipation are discussed in relation to the structural change in chloroplast.

## 2. Materials and methods

### 2.1. Preparation of samples

Second-year needles were sampled from *T. cuspidata* growing on the campus of the Hokkaido University and used for electron microscopy as previously reported [7]. For fluorescence measurements, sampling was carried out on July 31 (hereafter, referred to as 07/31 or summer), November 7 (11/07 or autumn), December 30 (12/30 or winter), and March 31 (03/31 or spring) and the needles were frozen in liquid nitrogen immediately after harvest, and stored at −70 °C until measurements. Fluorescence spectra of intact needles were measured by the surface-reflection technique to prevent changes in spectral properties during chloroplast isolation, because winter chloroplasts have specific intraorganelle conditions such as redox state [14,15].

### 2.2. Fluorescence spectroscopy

Steady-state fluorescence spectra were measured using a fluorescence spectrometer (Hitachi F-4500, Japan). Time-resolved fluorescence spectra (TRFS) and fluorescence decay curves were measured with a picosecond time-correlated single-photon counting system [16]. The light source was a Ti:Sapphire laser (Coherent MIRA 900, USA) and the excitation wavelength was 425 nm, which excites both Chl *a* and Chl *b*. We employed a microchannel plate photomultiplier (Hamamatsu R3809, Japan) as a detector, combined with a monochromator (Nikon P-250, Japan). The time step was 2.6 ps/ch or 52 ps/ch. Excitation laser intensity was set to give fluorescence signals of less than 10,000 c/s around fluorescence peak wavelengths, and in this condition samples did not suffer damage from the laser excitation with a repetition rate of 2.9 MHz. All measurements were carried out at −196 °C with a custom-made Dewar system. After deconvolution with an instrument function, the time resolution was improved to approximately 3 ps.

### 2.3. Electron microscopy

Electron microscopy was performed following procedures described in a previous paper [7]. Sampling was carried out on October 28, November 22, December 6, December 16 of 2004, and January 6, February 1, March 7, March 14, March 17, March 22, March 25, March 28, April 18, May 16, June 20, July 19, August 22, and September 12 of 2005. About 10 micrographs of chloroplasts (magnification 5000-10,000) were used to obtain an average stacking number of thylakoids on each date. Grid lines with an interval of 550 nm were drawn perpendicular to the plane of thylakoid membrane and the number of membranes per grana stacks was counted along the grid lines. These values were distributed from 1 to more than 10, and reflect overall thylakoid stacking in chloroplasts. We calculated an average stacking number on each date.

## 3. Results and discussion

### 3.1. Steady-state fluorescence spectra

To reveal the energy migration and quenching efficiency, we first measured the steady-state fluorescence spectra of the needles in various seasons. The solid lines in Fig. 1 show steady-state fluorescence spectra when the needles were excited at 440 nm (Chl *a* excitation) at −196 °C. For summer and autumn needles, three fluorescence peaks at approximately 685,

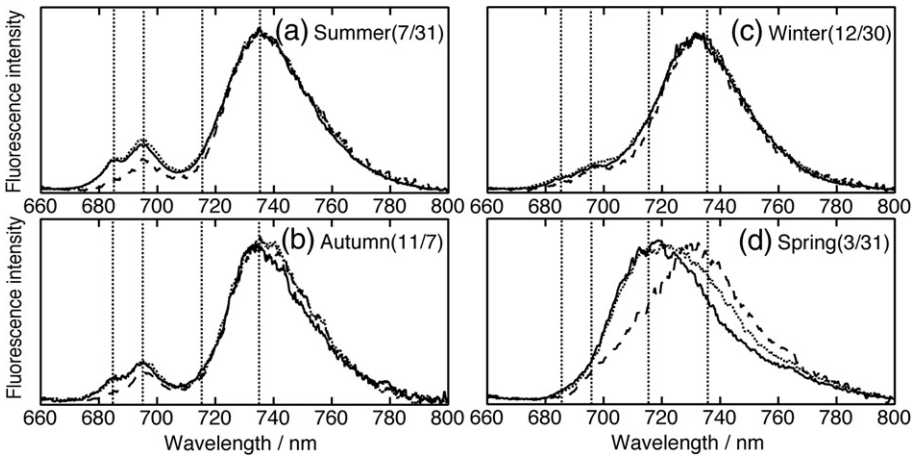


Fig. 1. Steady-state fluorescence spectra of intact needles normalized at peak intensities. Excitation was performed at 440 nm for Chl *a* excitation (solid line), 480 nm for Chl *b* excitation (dotted line), 550 nm for carotenoid excitation (broken line). All measurements were performed at −196 °C. Vertical dotted lines indicate 685, 695, 715, and 735 nm.

695, and 735 nm were observed (Fig. 1a, b). The 685-nm fluorescence might derive from CP43 and PSII RC [17,18]. The 695-nm peak was assigned to fluorescence from CP47 of PSII [19]. The 735-nm peak is derived from red-chlorophyll (red-Chl) in photosystem I (PSI) [20]. These peak positions were similar to those of intact leaves (unpublished data) or isolated thylakoid membranes of *Arabidopsis* [16]. In winter, the 735-nm peak blue-shifted to 732 nm, which indicates seasonal change in PSI. PSII fluorescence around 685–695 nm was low. Because all spectra were normalized at the PSI fluorescence peak, one can assume that the smaller intensity of PSII might come from larger intensity of PSI fluorescence in winter. However, as shown later (Section 3.3), both PSI and PSII fluorescence decayed faster in winter than in summer and autumn, indicating lower fluorescence quantum yield in winter. Therefore, the low intensity of PSII fluorescence in winter should be due to the high quenching efficiency in PSII [8,11] and/or to the low level of PSII [9]. Compared to the fluorescence spectra in summer and autumn (Fig. 1a, b), the spectra in winter showed clear shoulder around 715 nm (Fig. 1c), which is consistent with the previous report [11]. The 715-nm form was reported to be correlated to an increase in energy-dissipating carotenoids [10]. In spring, the needles excited at 440 nm (Chl *a* excitation) showed a peak at around 720 nm.

Both PSI and PSII contain Chl *a*, Chl *b*, and various carotenoids as chromophores. However, the constituent ratios of these chromophores and the energy transfer efficiency among them are different between PSI and PSII [21]. Most carotenoids in the photosystems have a pronounced maximum at around 500 nm [22]. However, the fluorescence spectra of the needles excited at 500 nm showed spectral shapes similar to those excited at 480 nm (data not shown) probably due to an overlap of the Chl *b* absorption [23]. On the other hand, the 550-nm excitation caused different spectra compared to the Chls excitation (Fig. 1a, b, and d), indicating that pigments other than Chls contribute to light absorption at 550 nm. Previous report suggested that zeaxanthin environment and configuration changed dramatically during quenching, while the absorption spectrum of zeaxanthin red-shifted toward 525 nm and the absorbance change centered around 535 nm [24]. Siphonaxanthin also exhibited a characteristic in vivo absorption band at 535 nm as a new electronically excited state, which also due to a specific interaction with amino acids [25]. Since the absorption spectra of isolated Lhcb proteins showed lowest intensity around 550 nm [26], it is likely that some kind of red-shifted carotenoids with protein interactions are predominantly excited at 550 nm in *T. cuspidata*.

For summer and autumn needles, fluorescence of PSII was low when needles were excited at 550 nm. The low intensity of PSII fluorescence by the 550-nm excitation indicates the low efficiency of energy transfer from red-shifted carotenoids to Chl (high thermal energy dissipation) and/or smaller amount of red-shifted carotenoids in PSII. On the other hand, the fluorescence spectra of winter needles were almost independent of the excitation wavelengths (Fig. 1c). These results suggest that energy migration occurs between PSII and PSI complexes in winter needles. Previous report suggested that winter-induced aggregates in Scots pine contain LHCII and PSI-LHCI complexes [6]. It is, therefore, likely that *Taxus* may also form aggregates including LHCII and PSI-LHCI with red-Chls, and excited energy migration occurs in the aggregates. Another explanation for the excitation wavelength independence of fluorescence spectra in winter is the larger contribution of red-shifted carotenoids in PSII.

In spring, the spectra showed drastic changes depending on the excitation wavelength. The needles excited at 550 nm showed a

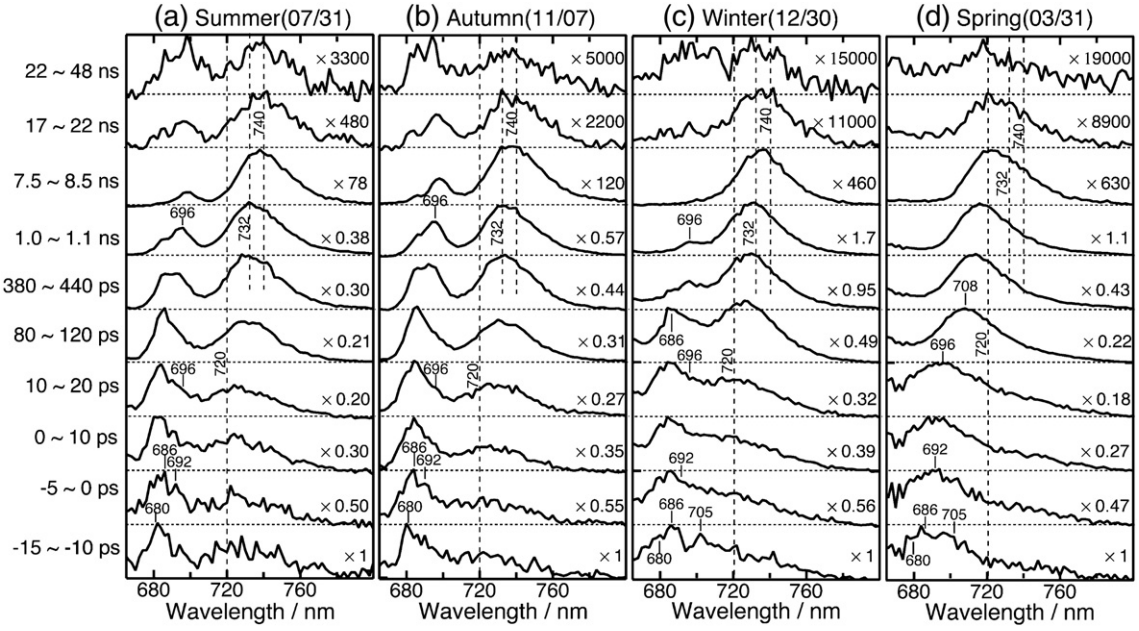


Fig. 2. Time-resolved fluorescence spectra of intact needles before deconvolution with an instrument function. Each spectrum was normalized at peak intensity. Excitation was performed at 425 nm for Chl excitation. All measurements were performed at −196 °C.

fluorescence peak at around 732 nm that is similar to the peak in winter. When the needles were excited at 440 nm (Chl *a* excitation), a peak at 720 nm and shoulders at around 690 nm and 732 nm were observed. The 720-nm peak was assigned to fluorescence from the PSI core [27]. The excitation energy captured by Chl *a* is mainly transferred to core complexes. The fluorescence spectrum obtained by Chl *b* excitation showed similar spectra to that obtained by Chl *a* excitation, except the increase in fluorescence around 730 nm. The increase in fluorescence by Chl *b* excitation might be due to the presence of Chl *b* in LHCI that contains red-Chl with 730-nm fluorescence maxima [28]. The excitation dependency of the peak position observed with spring needles indicates that major part of bulk Chl *a* are energetically unlinked from red-Chl in LHCI. Upon 550-nm excitation, the spring needles exhibited almost the same fluorescence spectra with winter needles, although the 715-nm fluorescence was increased in its intensity in spring. These results indicate that some complexes with red-shifted carotenoid and red-Chl (LHCI) are energetically unlinked from the major part of bulk Chl *a* in spring. To dissipate energy in winter needles, energy migrates among the PSI core complex, LHCI, and LHCII, and is finally transferred to red-Chls. However, in spring, light energy captured by Chl *a* is mainly transferred to core complexes, where red-Chl (LHCI) and red-shifted carotenoid are energetically uncoupled with the major part of bulk Chl.

### 3.2. Time-resolved fluorescence spectra (TRFS)

Time-resolved fluorescence measurements were performed to reveal the energy transfer and quenching processes in more detail. Fig. 2 shows TRFS of intact needles at −196 °C upon Chls excitation. At the very beginning, the spectra in summer and autumn showed a peak at 680 nm. Neither isolated PSII core nor PSI-LHCI showed fluorescence maxima at 680 nm even at early times before deconvolution [18,20], therefore the 680-nm peak at the very beginning mainly comes from LHCII [29,30]. The LHCII peak decreased quickly and a peak at around 686 nm became dominant. When increasing the time delay, the 686-nm peak disappeared while the 692-nm peak shifted to 696 nm. These changes were due to energy transfer from LHCII to CP43,

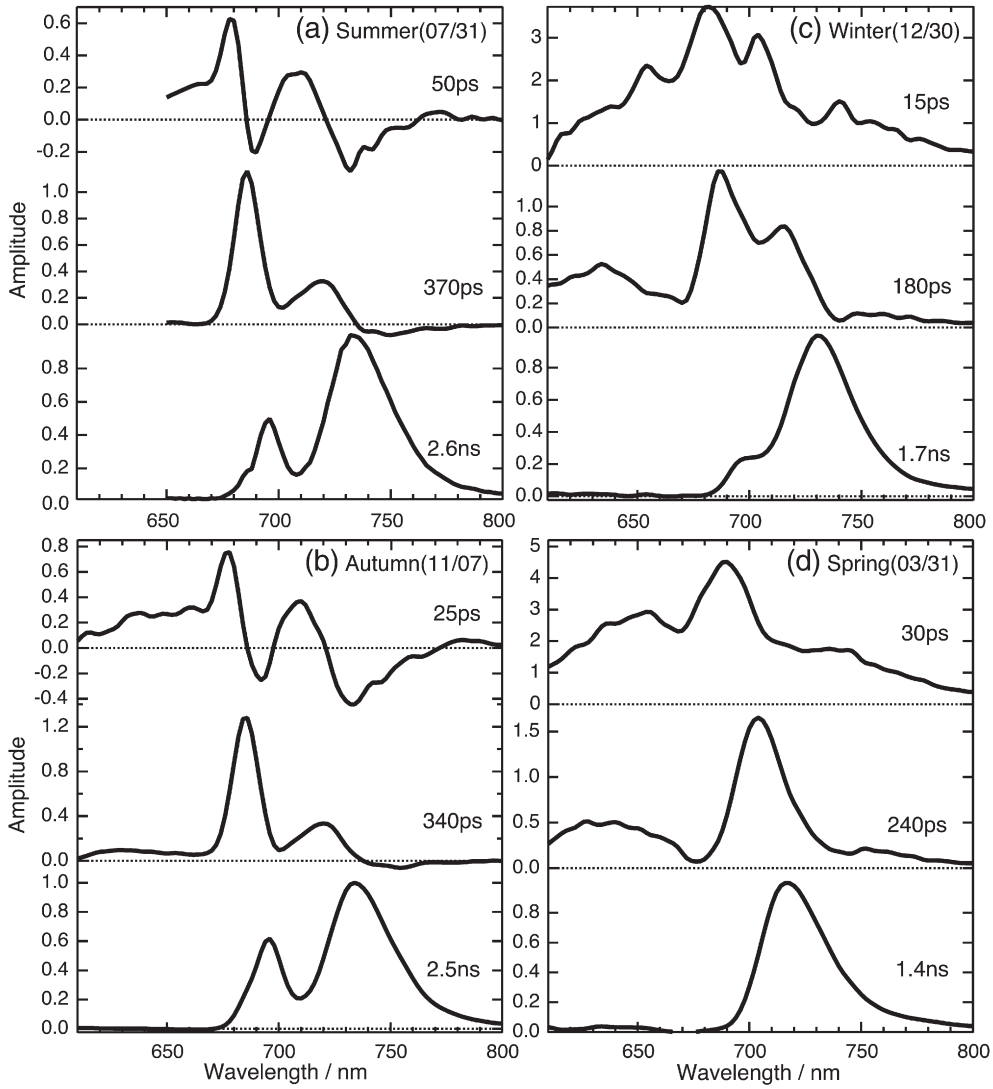


Fig. 3. Fluorescence decay-associated spectra (FDAS) of intact needles after deconvolution with an instrument function. Analysis was carried out at 2 nm intervals.

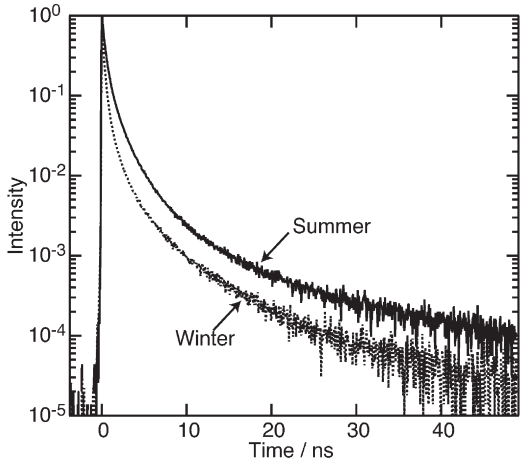


Fig. 4. Normalized fluorescence decay curves observed at 685 nm. Solid line and dotted line represent the decay curve of summer and winter, respectively.

CP47, and PSII RC [18]. In the PSI fluorescence, a peak at 720 nm decreased and a fluorescence at 732 nm increased, which comes from the PSI core and PSI red-Chl, respectively [20]. These TRFS profiles were similar to those of isolated thylakoids of *A. thaliana* [16], however at the later time stage, the PSI fluorescence at 740 nm became dominant, which was not found in *A. thaliana*. Some interactions between Chls of red-Chl might differ slightly between the two species.

In winter and spring (Fig. 2c and d), the LHCII peak at 680 nm showed lower intensity than the 686-nm peak even at the very beginning, which indicates low levels of LHCII. The 692-nm peak shifted to 696 nm as observed in summer and autumn, but the 686-nm and the 696-nm peaks decreased in their intensities at approximately double the rate of those in summer and autumn, indicating that excitation energy did not remain in RC or core antennae of PSII but transferred to quenching components. In addition, a peak at 705 nm was observed immediately after excitation and disappeared quickly in winter (Fig. 2c), whereas it remained until several hundred picoseconds in spring (Fig. 2d). The 705-nm form existed both in winter and spring, however it attached with quenching components in winter.

In winter, the PSI fluorescence showed a similar time-course to those of summer and autumn except for faster decay of the 740-nm form. This reflects the existence of a quenching pathway for the 740-nm form. In spring, both the 732-nm and 740-nm forms were absent in TRFS, resulting in the blue-shifted steady-state fluorescence by Chl *a* excitation (Fig. 1d). This indicates that the quenching pathway in spring is different from those in other seasons; the 740-nm red-Chls might not work as an energy reservoir in spring.

### 3.3. Fluorescence decay-associated spectra

To resolve TRFS into spectral forms with the same rise and decay profiles, we employed the global analysis method [31]. Fig. 3 shows FDAS of intact needles at −196 °C. Positive and negative peaks correspond to fluorescence decay and rise components, respectively. A number of lifetime components for each sample were determined by plotting the eigenvalues as a function of component numbers as described previously [32]. Three components, tens of ps (hereafter, short-lived component), hundreds of ps (medium-lived component), and longer than 1 ns (long-lived component) were necessary to fit fluorescence rise and decay profiles.

The kinetics of the short-lived components reflects fast energy transfer between chromophores. In summer and autumn needles, the short-lived components possessed two sets of positive and negative peaks at 680–690 nm and 710–730 nm, indicating the energy transfer in PSII and PSI, respectively. In contrast, the short-lived components of winter needles showed only positive peaks around 680 nm, 705 nm and 740 nm, which indicates that the energy acceptor dissipates its excited energy at a similar rate to energy-accepting. Therefore, excited energy captured by Chl that fluoresces around 680 nm, 705 nm or 740 nm was immediately quenched in winter needles. Carotenoids may play the role of acceptor because the lifetime of the lowest electronically excited singlet state ($S_1$) is less or equal to tens of ps [33]. The energy transfer from Chl to the Chl-zeaxanthin heterodimer, which then undergoes charge separation, is also proposed as the quenching mechanism [34]. These successive processes take tens of ps, including the net dynamics of the Chl that transfer to Chl-zeaxanthin. Red-Chl with carotenoids may also relate to the quenching [35]. The quencher is most possibly related to carotenoids, although no carotenoid fluorescence was recognized due to the forbidden $S_1$–$S_0$ transition [36]. The other possibility of the quencher is chromophore aggregations. The interaction between tetraphenylporphyrin accelerated the fluorescence decay, but the decay time is rather slow even after aggregation (~100 ps) [37]. If strong Chl–Chl interaction is achieved in pigment-protein complexes of winter and spring needles, fluorescence might be immediately quenched.

In spring, the 705-nm form gave a positive peak in the medium-lived component, not in the short-lived component, which indicates the energy quencher could not accept energy from the 705-nm form. In addition, a positive peak at 715 nm

Table 1
Fluorescence lifetimes and their amplitudes at 685 nm

| Season | $T_{mean}$ | $T_1$ (amp) | $T_2$ (amp) | $T_3$ (amp) | $T_4$ (amp) | $T_5$ (amp) | $T_{DF}$ (amp) |
|---|---|---|---|---|---|---|---|
| Spring | 0.96 ns | 25 ps (78.7%) | 180 ps (16.8%) | | 990 ps (4.1%) | 3.7 ns (0.4%) | 16 ns (<0.05%) |
| Summer | 1.2 ns | | 130 ps (53.5%) | 420 ps (27.2%) | 1.3 ns (14.4%) | 3.8 ns (1.4%) | 18 ns (3.5%) |
| Autumn | 1.2 ns | 29 ps (−19.0%) | 130 ps (52.5%) | 630 ps (23.4%) | 1.4 ns (11.0%) | 3.1 ns (3.0) | 12 ns (0.1%) |
| Winter | 0.51 ns | 40 ps (66.2%) | 160 ps (27.5%) | | 740 ps (6.1%) | 3.2 ns (0.2%) | 15 ns (<0.05%) |

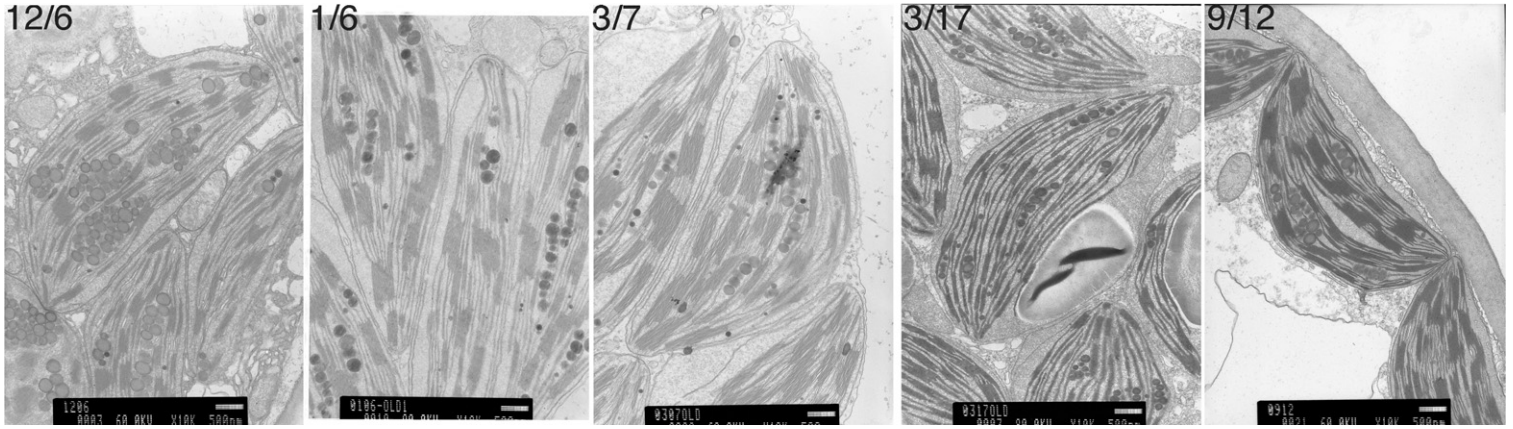


Fig. 5. Typical examples of electron micrographs of chloroplasts in each season (Bars = 500 nm). The upper left numbers indicate date (month/date).

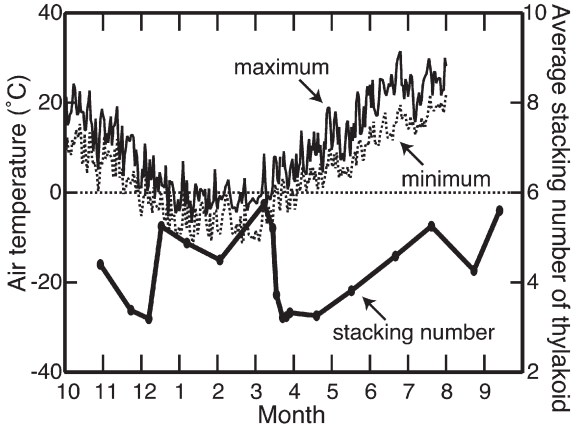


Fig. 6. Seasonal changes of average number of stacked thylakoids. Maximum and minimum air temperatures recorded at the Sapporo Station of the Meteorological Agency are also plotted.

appeared in the long-lived component, which is a major fluorescent form of the steady-state fluorescence spectra. The slow fluorescence decays of the 705-nm and 715-nm forms indicate low energy transfer efficiency from these forms in spring. As discussed based on the steady-state fluorescence measurements, some complexes with carotenoid and red-Chl unlinked from the winter-aggregates in spring. This unlinking of the quencher may be useful for rapid switching from the quenching mode to the light-harvesting mode.

### 3.4. Delayed fluorescence

Delayed fluorescence in PSII comes from charge recombination between the primary electron donor and the primary electron acceptor [18]. Its lifetime is in the order of 10 ns, and is therefore easily distinguished from prompt fluorescence. Fig. 4 shows fluorescence decay curves at 685 nm in a longer time region. In winter, the intensity of fluorescence in the later time region was one digit smaller than that in summer. Instead, a fast decay time appeared in winter (40 ps) and spring (25 ps) (Table 1). This indicates that most of the excitation energy captured by PSII antennae is quenched before it reaches the RC. It should be noted that some energy arrived at the RC in spite of the dissipation by antennae in winter. RC-based quenching was also reported in Scots pine previously [7,14].

### 3.5. Seasonal changes of ultrastructure of chloroplasts

Next, we examined whether seasonal changes in spectral properties are correlated with chloroplast structures. Fig. 5 shows examples of micrographs in each seasons. Thylakoids and plastoglobules were observed in every season, however the amount of thylakoid stacking changed with the season. The average stacking number of thylakoids and air temperature were plotted in Fig. 6. Maximum air temperature remained above 0 °C until early December. The maximum temperatures were below or around 0 °C from mid-December until mid-March and then rose gradually. The changes in temperatures were well collated to the structural changes in chloroplasts (Fig. 6). The stacking number increased rapidly in December when maximum temperature was below 0 °C. On March 17, the stacking number suddenly decreased and remained at a low level into April, where the highest temperature was always above 0 °C and the lowest temperature stayed at around 0 °C. It was reported that PSII-associated LHC proteins participate in thylakoid stacking [38,39] and protein composition such as LHCII, ELIPs and PsbS changed depending on the season [6,40]. These changes in protein composition might relate to changes in thylakoid stacking [38,41]. The drastic change in typical properties of fluorescence in spring might be related to reorganization of thylakoid membranes and photosystems. After May 18, the average stacking number gradually increased (Fig. 6), where the PSII fluorescence band appeared clearly in summer and autumn (Fig. 1). Previous report suggests that LHCII and D1-protein of *P. sylvestris* recovered in May [9]. Therefore, the increase of thylakoid stacking in average number after May 18 might be also related to accumulation of PSII-associated proteins.

Fig. 7 shows schematic models for stacked and unstacked regions of thylakoids membranes. PSI RCs are found predominantly in the unstacked region, while PSII RCs are located mainly in the stacked region [42,43]. Previous reports suggest that winter-induced aggregates contain PsbS, LHCII, PSI, and some low-molecular weight polypeptides [6,9]. These reports are consistent with our present results of energy migration between LHCII and PSI. On the other hand, PSII fluorescence of mistletoe's winter leaves showed a mean lifetime half as long as that of mistletoe's summer leaves [11]. Our TRFS measurement and global analysis revealed that both PSII and PSI fluorescence showed fast quenching in winter. This indicates the presence of quenchers for PSII and PSI. Although quenching molecules or mechanisms in winter needles have not been fully identified, many mechanisms have been proposed. Xanthophyll cycle-dependent energy dissipation was reported in *Taxus* [8]. PsbS is reported to bind two molecules of zeaxanthin and is believed to be essential for rapid quenching [44]. One recent study suggested multiple locations and lateral mobility of PsbS in the thylakoid membrane, where PsbS can interact both with PSII and PSI [45]. Accumulation of ELIPs in winter also relates to

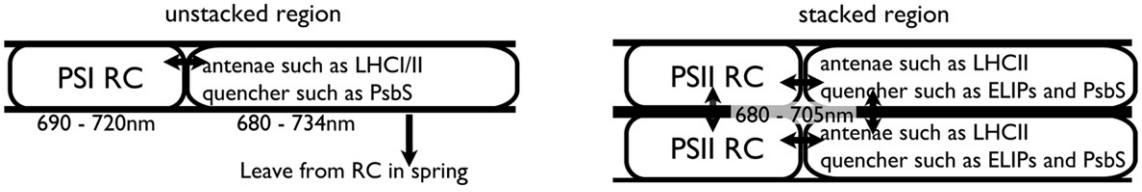


Fig. 7. Schematic model for winter-aggregates in stacked and unstacked thylakoid membranes.

PSII efficiency [40,46,47]. Aggregation of LHCII may also help efficient quenching [48–50]. These quenchers probably relate to fast quenching in winter.

In this report, we used fluorescence spectroscopy to reveal seasonal changes of excitation energy transfer and quenching in *T. cuspidata*. In winter needles of *T. cuspidata*, excited energy was quenched efficiently before it reached the PSII RC. In addition, PSI red-Chls also exhibited a fast fluorescence decay signal. When photosynthesis started in spring, some components with red-shifted carotenoid and red-Chl (LHCI) separated from the RC, which may decrease quenching efficiency. Analysis of electron micrographs confirmed rapid changes in thylakoid stacking before and after the winter season, which may relate to changes in composition and movement of proteins.

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