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CAROTENOID SCIENCE

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KWANSEI GAKUIN UNIVERSITY

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EDITOR

H. HASHIMOTO

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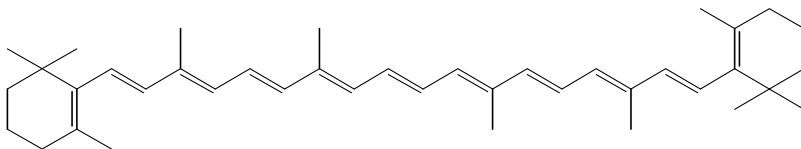
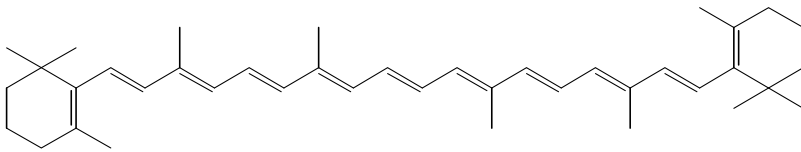
VOLUME 26 (2023)

Japanese Society for Carotenoid Research

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Japanese Society for Carotenoid Research

Preface for the 34th Annual Meeting

The 34th Annual Meeting of the Japanese Carotenoid Society took place at the Nishinomiya-Uegahara Campus of Kwansei Gakuin University in Hyogo Prefecture on September 17 – 18, 2022. This year's scientific program was a testament to the thriving field of carotenoid research, featuring three captivating invited lectures by distinguished speakers: Prof. Seiji Akimoto (Kobe University), Prof. Miho Takemura (Ishikawa Prefecture University), and Dr. Azusa Nishino. Additionally, we paid tribute to the late professors Yasushi Koyama and Takao Matsuno with heartfelt lectures by Prof. Hideki Hashimoto and Dr. Takashi Maoka. The event also featured 29 stimulating oral presentations, bringing together a total of 74 participants.

On the preceding day, September 16, we hosted the Annual Meeting of young carotenoid scientists, a forum that provided attendees with invaluable insights. This pre-meeting program featured three enlightening tutorial lectures delivered by Dr. Takashi Maoka (Research Institute for Production Development), Prof. Norihiko Misawa (Ishikawa Prefecture University), Prof. Hideki Hashimoto (Kwansei Gakuin University), and Prof. Tomohisa Hasunuma (Kobe University), complemented by five impressive poster presentations from emerging talents in the carotenoid field.

The presentations encompassed a diverse spectrum of research areas related to carotenoids and apocarotenoids, including physics, physical chemistry, synthetic and analytical chemistry, molecular biology, genetics, omics, pathway and metabolic engineering, as well as the nutritional and medical aspects of carotenoids. Collectively, these presentations vividly showcased the state of the art in carotenoid research, underscoring the remarkable growth and high standards upheld by Japanese carotenoid scientists.

I would like to extend my sincere gratitude to all the participants for their invaluable contributions, insightful comments, and for fostering a congenial atmosphere throughout the meeting. Additionally, I would like to express our deep appreciation to our generous sponsors:

- **Japan Business Association for Carotenoids (JBAC)**
- **San-Ei Gen F.F.I. Co., Ltd.**
- **AstaReal Co., Ltd.**
- **Fuji Chemical Industries Co., Ltd.**
- **Gulico Nutrition Co., Ltd.**

Over the years, this meeting has played a pivotal role in promoting cross-disciplinary collaboration among Japanese carotenoid researchers from both academic and industrial backgrounds. Let us ensure that this tradition continues to thrive! It is worth noting that this meeting marks a significant milestone, as it represents our triumphant return to in-person gatherings after overcoming the challenges posed by the COVID-19 pandemic. We offered a hybrid format, combining in-person and Zoom participation, to accommodate the diverse needs of our members.

We eagerly anticipate your participation in the 35th Annual Meeting of the Japanese Carotenoid Society, scheduled to be held at Japan Women's University on November 24 – 25, 2023.

Sincerely,

Hideki Hashimoto
Professor, Kwansei Gakuin University
Meeting Chair of the 34th Annual Meeting of the Japanese Carotenoid Society

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In Memory of Professor Emeritus Takao Matsuno

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We are deeply saddened by the passing of our former teacher, Professor Emeritus Takao Matsuno. Reminiscing about the path he traveled and his accomplishments, we would like to pay tribute to him and pray for him to rest in peace.



Professor Emeritus Takao Matsuno

1. Career of Professor Emeritus Takao Matsuno

1925 Born in Kyoto City, Japan.
1948 Graduated from the Department of Pharmacy, Faculty of Medicine, Kyoto University, Japan.
1949 Became a Lecturer at Kyoto Pharmaceutical University.
1956 Earned his doctorate at Kyoto University, with a Thesis titled “Studies on the Components of Citrus Species (Rutaceae).”
1957 Became an Associate Professor at Kyoto Pharmaceutical University.
1960–1962 Studied abroad at the New York State University, United States of America.
1964 Became a Professor at Kyoto Pharmaceutical University.

1990 Retired from Kyoto Pharmaceutical University as a Professor Emeritus.

1992 Received the Achievement Award of the Fisheries Society of Japan.

2005 Awarded the Order of the Sacred Treasure.

2022 Passed away at the age of 96.

2. Research activities

2-1. Essential oils and flavonoids in citrus

Professor Matsuno studied the structure of essential oils in citrus (auraptene) [1] and the chemical classification of flavonoids at the Kyoto University School of Medicine, Department of Pharmaceutical Pharmacognosy (Professor Tatsuo Kariyone), Japan. These studies earned him a doctorate in pharmacy. Subsequently, he began his research in the field of marine natural product chemistry, inspired by the discovery of cephalosporins in marine microorganisms (drugs from the sea).

2-2. Toxins of echinoderms, particularly sea cucumbers

Professor Matsuno isolated the first saponin of animal origin from a sea cucumber. His study is published in *Nature* [2]. He found that the amount of saponins in sea cucumbers increased during the spawning season and acknowledged that saponins play a role in the ecological defense of sea cucumbers. The results of his research have been reported in several international journals [3,4]. At the time, the only methods for structural analysis were crystallization, isolation, and purification using distillation, elemental analysis, chemical reactions, syntheses, mixed-fusion tests, ultraviolet and infrared absorption spectroscopy, and optical rotation measurements. Therefore, these structural analyses require significant effort. These studies are pioneering studies in Japanese marine natural product chemistry, particularly toxin research. During this period, many of the students who received

guidance on their graduate research from Professor Matsuno went on to become researchers at universities and companies.

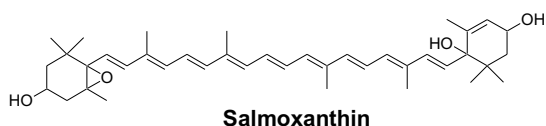
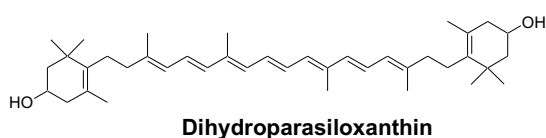
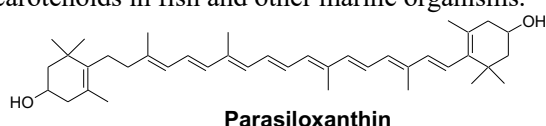
2-3. Carotenoid research

Professor Matsuno began his research on carotenoids in the 1970s. The motivation was that many species in the natural world, particularly aquatic animals, exhibit many different colors; he wanted to know why. He believed that colored substances would be easy to separate. Therefore, he started the research with the mindset that consistent study, even in a poorly equipped laboratory, can yield good results.

At the time, carotenoid research in Japan primarily focused on carotenoids from marine organisms. He was inspired by Dr. Masaru Yamaguchi's research on carotenoids with aromatic end groups in sea sponges and Dr. Shuichi Hirao's research on marine-fish carotenoids at the Tokai Fisheries Research Institute.

First, he studied the carotenoid components of plants and isolated lycopene, γ -carotene, and β -carotene from *Trichosanthes cucumeroides* Maxim [5]. Afterward, he isolated astaxanthin from the ovaries of sea cucumbers as a component of animal carotenoids [6] and conducted comparative biochemical studies on fish carotenoids.

Since the late 1970s, he has been elucidating the structures of the new carotenoids sequentially. His first carotenoid study was on the determination of the structures of parasiloxanthin and dihydroparasiloxanthin from catfish (1976) [7]. These carotenoids possess unique structures, wherein the double bonds at the 7,8 and 7',8' positions of the polyene chain are reduced. This discovery was followed by studies on the reductive metabolism of carotenoids in fish and other marine organisms.



In 1978, he made his international debut by presenting a paper on the structure of salmoxanthin [8], a novel carotenoid isolated from salmon, at the 5th International Conference on Carotenoids (Wisconsin, USA). In the 1980s, he determined the structure of new carotenoids by relying on his accumulated achievements and new technologies and became a leader in natural product organic-chemistry research on carotenoids. Furthermore, he elucidated the metabolic

pathways and physiological activities of carotenoids in aquatic animals. During this time, he trained many researchers through his research on carotenoids and nurtured many carotenoid researchers in Japan as well as at the Kyoto Pharmaceutical University.

To date, carotenoid research has been conducted separately in fields such as pharmaceuticals, fisheries, agricultural chemistry, photochemistry, and basic medicine. Following this and under the slogan "Let's create a gathering of Japanese carotenoid researchers; let's hold an international carotenoid conference in Japan," Professor Matsuno, Professor Tsukida (Kobe Women's Pharmaceutical University), Associate Professor Ito (Kobe Women's Pharmaceutical University), and Dr. Miki (University of Tokyo) led the first meeting to discuss carotenoid research. In 1987, the first carotenoid research conference was held, and people from various research fields gathered to discuss carotenoids.

In May 1990, the 9th International Conference on Carotenoids was held at the Hieizan Hotel with Professor Matsuno and Prof. Tsukida as chairpersons. An entire wall at the conference venue was furnished with 13 posters from Matsuno's laboratory and was titled "Matsuno's Wall" by Professor Liaaen-Jensen. Professor Matsuno was elected a Pioneer in Marine Carotenoids along with B.C.L. Weedon, C.H. Eugster, and M. Yamaguchi.

In July 1990, he retired from the Kyoto Pharmaceutical University. Thereafter, he participated in the International Carotenoid Research Society in Trondheim (1993) and Amsterdam (1996) and was active in the domestic carotenoid research society.

The following is a summary of Professor Matsuno's carotenoid research:

1. Structural studies of over 100 novel carotenoids [9, 10].
2. Comparative biochemistry and chemical classification of carotenoids [9, 10].
3. Metabolism research on animal carotenoids, particularly on elucidating the reductive metabolic pathways of carotenoids (leading to the utilization of carotenoids in fisheries) [10].
4. Research on provitamin A activity of xanthophylls (pioneering research on the biological activity of xanthophyll) [11].
5. Antioxidant and antitumor effects of carotenoids [12].

Professor Matsuno also undertook exploratory research in many areas of current carotenoid research. These studies have been published as original papers (165), reviews (22), and books (15).

3. Professor Emeritus Takao Matsuno's aspiration

His motto was "Life is an encounter." Many people who have met Professor Matsuno are actively researching carotenoids in universities, research institutions, companies, etc. His personality is demonstrated by the liveliness of the research side of the Carotenoid Research Discussion Meeting, which

continues to this day and the liveliness of the social gathering after the conference.

We wish to cherish the connections received from Professor Emeritus Takao Matsuno and continue to strive to develop carotenoid research.



First carotenoid research conference: Kyoto, 1987

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Ultrafast excitation relaxation in keto-carotenoids, probed by time-resolved fluorescence spectroscopy

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Abstract: Excitation relaxation processes of typical keto-carotenoids found in algae, siphonaxanthin, fucoxanthin, and peridinin, in solutions, and energy transfer processes involving these keto-carotenoids in light-harvesting pigment–protein complexes of a green alga *Codium fragile*, a diatom *Chaetoceros gracilis*, and a dinoflagellate *Symbiodinium* sp., respectively, are investigated by a femtosecond fluorescence upconversion method. In solutions, fluorescence kinetics of the keto-carotenoids examined here were analyzed by triple exponential decays, indicating the presence of an intermediate state between the 1^1B_u and 2^1A_g states. In the pigment–protein complexes, the time profiles of chlorophyll *a* Q_y fluorescence exhibit rise components, suggesting that the energy transfer from keto-carotenoid to chlorophyll *a* occurs involving their lowest excited states. This energy transfer path is also recognized by the fluorescence anisotropy measurements; the anisotropies of the pigment–protein complexes exhibit double decays around 690 nm, showing the energy transfer from ketocarotenoid to chlorophyll *a*.

1. Introduction

Carotenoids are generally classified into carotenes and xanthophylls; the former are hydrocarbon carotenoids, whereas the latter possess oxygen atoms particularly in the forms of hydroxyl, epoxide, and keto-carbonyl groups [1]. However, in terms of light absorption by carotenoids and subsequent excitation relaxation processes in carotenoids, another classification is available for carotenoids based on the existence of oxygen atom in the π conjugation systems: polyene-type carotenoids consisted of only C=C conjugations (for example, lutein and 9'-cis neoxanthin, Figure 1) and keto-carotenoids containing C=C and C=O conjugations (siphonaxanthin, fucoxanthin, and peridinin, Fig. 1).

In the visible region, the polyene-type carotenoids have at least two electronically excited states [2], among which the 1^1B_u (S_2) state is responsible for the strong absorption band [3, 4]. The longer π conjugation systems bring the lower energy levels of excited states, resulting in absorption spectra in the longer wavelength region [5]. The 1^1B_u lifetimes are longest for the polyene-type carotenoids possessing nine or ten C=C double bonds in the π conjugation systems [6], while the 2^1A_g (S_1) lifetimes become monotonously shorter with the longer π conjugation [7–9]. Contrary to the polyene-type carotenoids, the keto-carotenoids exhibit characteristic electronically excited states, such as the $1(n, \pi^*)$ state [10] and the intramolecular charge-

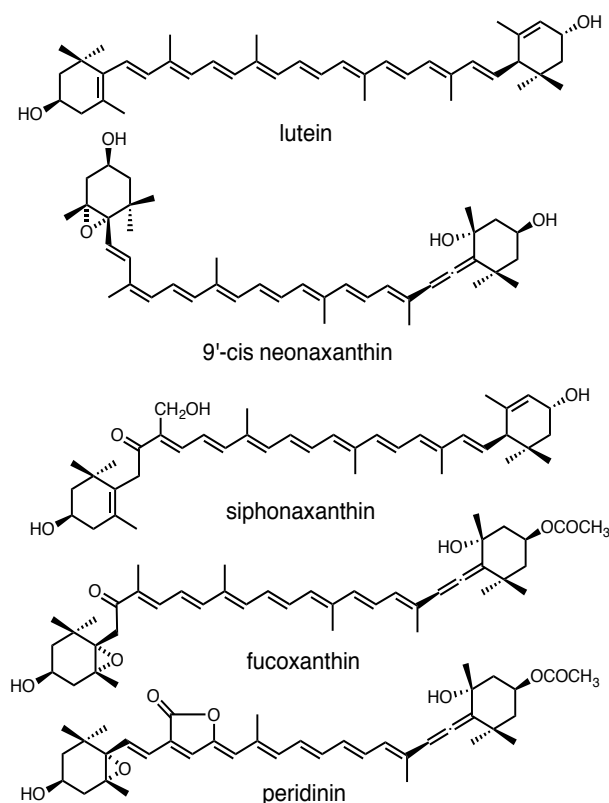


Fig. 1. Molecular structures of polyene-type carotenoids (lutein and 9'-cis neoxanthin) and keto-carotenoids (siphonaxanthin, fucoxanthin, and peridinin).

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transfer (ICT) state [11], because the existence of an oxygen atom brings lone pairs and breaks the ideal C_{2h} symmetry of the π conjugation system. It was reported that the $^1(n, \pi^*)$ state mediates the internal conversion from the 1B_u state to the $^2^1A_g$ state [12], whereas the ICT state affects the lifetime of the lowest excited state [11,13].

Fig. 2 show absorption spectra of the light-harvesting pigment–protein complexes isolated from a land plant (**Fig. 2a**) and algae (**Fig. 2b–d**). Besides the

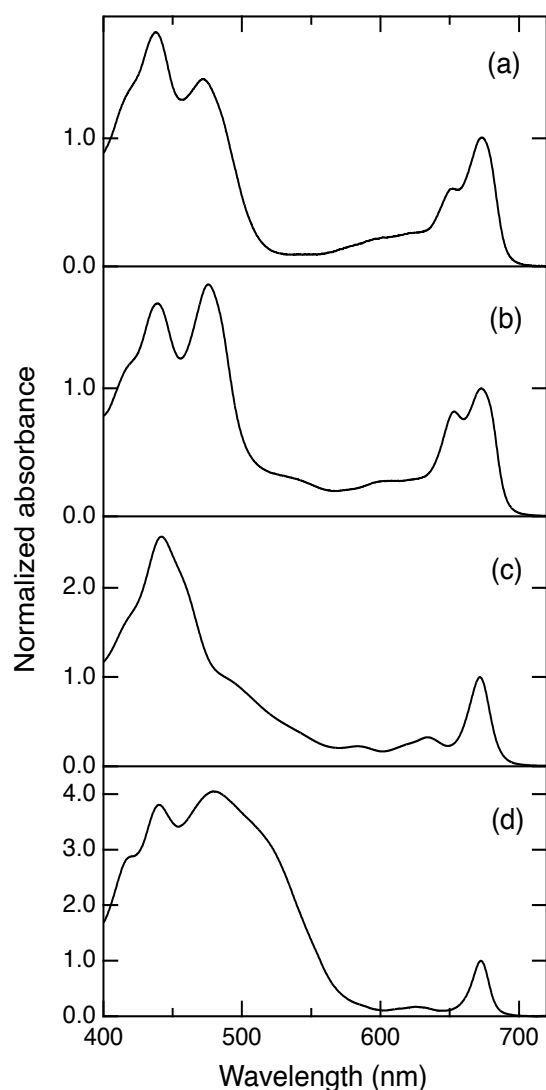


Fig. 2. Absorption spectra of the lutein chlorophyll *a/b*-binding protein complex (LHCII) isolated from a land plant *Arabidopsis thaliana* (**a**), the siphonaxanthin chlorophyll *a/b*-binding protein complex (SCP) from a green alga *Codium fragile* (**b**), the fucoxanthin chlorophyll *a/c*-binding protein complex (FCP) from a diatom *Chaetoceros gracilis* (**c**), and the peridinin chlorophyll *a*-binding protein complex (sPCP) from a dinoflagellate *Symbiodinium* sp. strain SGCH-03 (**d**). Each spectrum is normalized to 1.0 at the respective Q_y absorption band of chlorophyll *a*.

common pigment, chlorophyll *a*, the pigment–protein complexes possess their own characteristic carotenoids and chlorophylls [14–17]. Three types of keto-carotenoids, which have eight C=C bonds and one C=O bond in their π conjugation systems, are found in the oxygenic photosynthetic organisms (bottom three, **Fig. 1**): siphonaxanthin in the siphonaxanthin chlorophyll *a/b*-binding protein (SCP) complex of a green alga *Codium fragile*, fucoxanthin in the fucoxanthin chlorophyll *a/c*-binding protein (FCP) complex of a diatom *Chaetoceros gracilis*, and peridinin in the peridinin chlorophyll *a*-binding protein (water-soluble PCP, sPCP) complex or the peridinin chlorophyll *a/c*-binding protein (intrinsic PCP, iPCP) complex of a dinoflagellate *Symbiodinium* sp. In the light-harvesting pigment–protein complexes, these keto-carotenoids exhibit the unique function to capture the light energy especially in green region (500–550 nm) (**Fig. 2b–d**), due to their characteristic π conjugation systems. Contrary, the green absorption band does not occur in the light-harvesting pigment–protein complexes of a land plant *Arabidopsis thaliana* (**Fig. 2a**), LHCII, which accumulate only the polyene-type carotenoids, lutein, 9'-*cis* neoxanthin (top two, **Fig. 1**), and the xanthophyll cycle carotenoids of land plants [18].

Time-resolved fluorescence spectroscopy is a most useful technique to detect several ultrafast processes involving carotenoids: excitation relaxation within carotenoids and excitation energy transfer between carotenoids and (bacterio)chlorophylls. Because the fluorescence is emitted only from singlet electronically excited states, the assignment of the observed signals is straightforward; fluorescence signals from the higher excited states are observed in the shorter wavelength regions. In addition, the excitation laser pulses used for measurements are weak enough not to annihilate excitons or damage the samples. These points are advantageous against the transient absorption and Raman spectroscopies; in these methods, the signals from several states coexist in the same wavelength or wavenumber region, and the strong laser excitation is required. However, the fluorescence spectroscopy has difficulties to detect signals from the lowest excited state of carotenoid, because the ground (S_0) state is described as the 1A_g state due to the C_{2h} symmetry, thereby the one-photon transition between the lowest excited state and the ground state is forbidden for carotenoid.

In the present paper, the excitation relaxation processes of siphonoxanthin, fucoxanthin, and peridinin in solutions and the excitation energy transfer processes from these keto-carotenoids to chlorophylls in the respective light-harvesting pigment–protein complexes (SCP, FCP, and PCP), which were investigated by means of steady-state absorption and ultrafast time-resolved fluorescence spectroscopies [12, 19–21], are presented.

2. Measurements and analyses

To examine relaxation processes involving highly electronically excited states of carotenoids, fluorescence rise and decay curves were observed by the fluorescence upconversion system (Fig. 3), in which the second harmonic and the fundamental of a Ti:sapphire laser (Tsunami, Spectra-physics, US) were provided as excitation and gate pulses, respectively [22]. The second harmonic brings excitations at the $1^1B_u \leftarrow 1^1A_g$ absorption bands for carotenoids and at the Soret band for chlorophylls. By this system, the intensity of the sum frequency between the sample fluorescence and the gate pulse, which is generated by a BBO crystal, is monitored as a function of the delay time between the excitation and gate pulses.

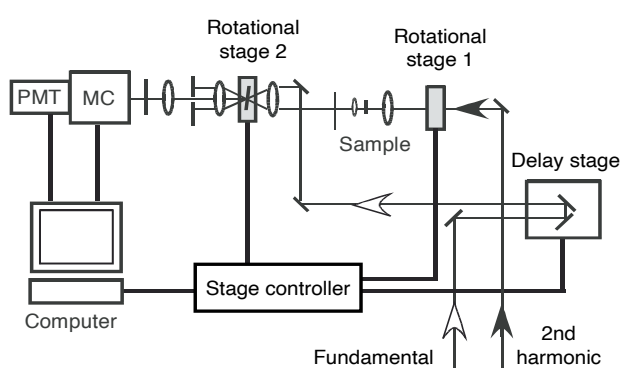


Fig. 3. Schematic drawing of the femtosecond fluorescence upconversion system. The light source is a Ti:sapphire laser. A wave plate set on the rotational stage 1 changes the angle between the polarizations of the excitation (2nd harmonic) and gate (Fundamental) pulses, while a BBO crystal on the rotational stage 2 generates the sum frequency of the sample fluorescence and the gate pulse. The delay time between the excitation and gate pulses is controlled by a pulse stage (Delay stage). MC: monochromator; PMT: photo-multiplier tube.

The fluorescence rise and decay curves were observed every 5–10 nm in the wavelength region of the highly excited states of keto-carotenoids, and were analyzed by the global analysis. In this analysis, a set of the fluorescence rise and decay curves ($I_F(t, \lambda)$) was analyzed by sums of exponential functions with the common fluorescence lifetimes (τ_i) independent of the observed wavelengths (λ):

$$I_F(t, \lambda) = \sum_{i=1}^n A_i(\lambda) \int_{-\infty}^t I_{IRF}(t') \exp\left(-\frac{t-t'}{\tau_i}\right) dt' \quad (1)$$

Here, $I_{IRF}(t)$ is an instrumental response function (IRF), and was a Gaussian function of ~200 fs full width at the half maximum for the IRF curves observed

by the fluorescence upconversion method [22]. The time-resolved fluorescence spectrum at time t is obtained by plotting $I_F(t, \lambda)$ against λ , whereas the i th fluorescence decay-associated (FDA) spectrum is constructed by plotting of $A_i(\lambda)$ against λ . For $A_i(\lambda)$, positive and negative values indicate the decrease and increase in fluorescence intensity, respectively, with time after excitation.

After alternatively measuring the fluorescence rise and decay curves with polarizations parallel ($I_{\parallel}(t, \lambda)$) and perpendicular ($I_{\perp}(t, \lambda)$) to the polarization of the excitation laser, time evolution of the fluorescence anisotropy ($r(t, \lambda)$) was calculated [22, 23]:

$$r(t, \lambda) = \frac{I_{\parallel}(t, \lambda) - I_{\perp}(t, \lambda)}{I_{\parallel}(t, \lambda) + 2I_{\perp}(t, \lambda)} \quad (2)$$

The denominator in the right-hand hand of Equation 2 is related to the isotropic fluorescence rise and decay curve, $I_F(t, \lambda)$.

Time-dependent changes in the fluorescence anisotropy are indicative of the internal conversion between excited states with transition dipole moments in different directions and the energy transfer between pigments whose transition dipole moments are not parallel in orientation. If the angle between the transition dipole moments of the excited states (or the pigments) absorbing and emitting photons is θ , the anisotropy after the internal conversion (or the energy transfer), r_A , is obtained by using the anisotropy before the internal conversion (or the energy transfer), r_B , as follows [22, 23]:

$$r_A = r_B \frac{3\cos^2\theta - 1}{2} \quad (3)$$

Rotational motion of the pigment–protein complex, which occurs in the longer time scale, does not affect the time-dependent change in anisotropy (Equation 2) in the time region examined here (up to 6 ps in Fig. 6).

3. Light absorption of keto-carotenoids

Polyene-type carotenoids such as β -carotene [24] and lutein (solid and dotted lines, Fig. 4a), exhibit absorption spectra having vibrational structures, independent of solvents. In contrast, the absorption spectrum of siphonoxanthin becomes broad and shows a long tail at the long wavelength side in methanol, a polar and hydrogen-donating solvent, (solid line, Fig. 4b), compared with those in n -hexane, a non-polar solvent (dotted line, Fig. 4b), and in acetonitrile, a polar but non-hydrogen-donating solvent [12]. This behavior clearly suggests that not the polarity of environment around siphonaxanthin, but the specific interaction between the C=O group of siphonaxanthin and the neighboring molecule(s) is responsible for the green-light absorption of siphonoxanthin in SCP. In fact, specific substitutions of amino acids close to siphonaxanthin are found for the *Codium fragile* SCP [25] and the SCP from a green alga *Bryopsis*

corticulans [26], compared with the amino acid sequences of the land plant LHCII. A recent study using cryogenic electron microscopy (cryo-EM) suggests a hydrogen bond between the C=O group of siphonaxanthin and the thiol group of Cys 67 in the *Codium fragile* SCP [27].

The interactions between fucoxanthin and the protein environment are also important to determine the energy levels and functions of fucoxanthin in FCP. In the FCP complexes isolated from a diatom *Cyclotella meneghiniana* [28] and the *Chaetoceros gracilis* FCP [19], fucoxanthin contributes to the light absorption in

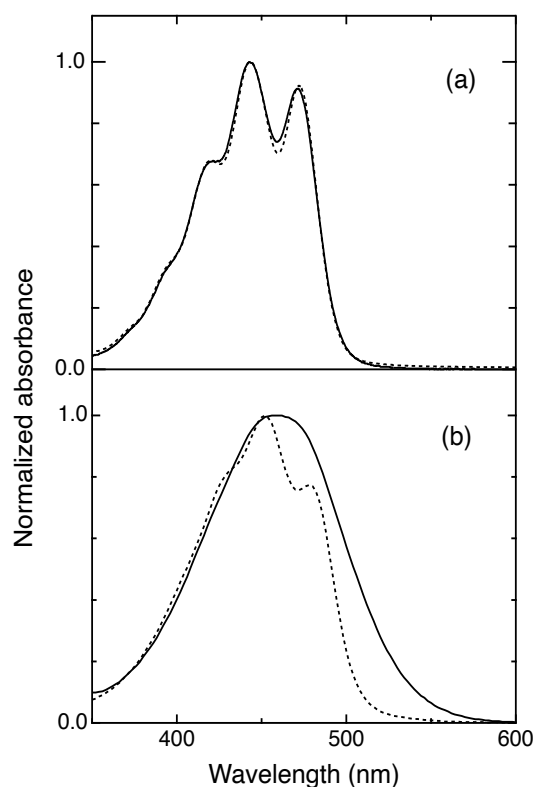


Fig. 4. Normalized absorption spectra of lutein (a) and siphonoxanthin (b) in *n*-hexane (dotted lines) and methanol (solid lines). Each spectrum is normalized by its maximum absorbance.

the wavelength region shorter than ~550 nm (Figure 2c), while a band around 500 nm is missing in the fluorescence excitation spectrum monitored by the chlorophyll *a* fluorescence. These results clearly indicate that fucoxanthin forms at least two energy forms in FCP, and that the lower-energy form responsible for the green absorption contributes to the energy transfer to chlorophyll *a*, whereas the other one with the higher energy (~500 nm) does not. The possible locations of different energy forms of fucoxanthin are proposed by a cryo-EM study on a photosystem II-FCPII complex isolated from *Chaetoceros gracilis* [29].

The absorption spectra of peridinin also show the solvent dependence [11], exhibiting a broad spectrum with a long-wavelength tail in ethanol (solid line, **Fig.**

5c). In the sPCP from a dinoflagellate *Amphidinium carterae*, four peridinin molecules surround one chlorophyll *a* molecule in close proximity to each other [30]. Besides the peridinin–protein interaction, the peridinin–peridinin and peridinin–chlorophyll *a* interactions are necessary to account for the absorption and circular dichroism spectra of the *Amphidinium carterae* sPCP; four energy forms of 18400, 18700, 19300, 20600 cm^{-1} were obtained to represent the spectra of sPCP [31]. In the *Amphidinium carterae* sPCP, the energy transfer efficiency from peridinin to chlorophyll *a* depends on the wavelength of absorbed light [32], suggesting that the different energy forms of peridinin exhibit different energy transfer efficiencies.

4. Excitation relaxation and energy transfer of keto-carotenoids, observed by time-resolved fluorescence

4.1. Excitation relaxation processes within keto-carotenoid in solution

The second harmonic of a Ti:sapphire laser (420–425 nm) brings the excitation of carotenoid to its 1^1B_u state, because of the broad absorption spectrum (**Fig. 4**). Therefore, the time-dependent changes in fluorescence signals contain information on the relaxation processes from the 1^1B_u state to the lower-lying states, including the relaxation processes occurring within each excited state. Three exponential functions (for $n = 3$ in Equation 1) were required to optimally analyze the fluorescence rise and decay curves of carotenoids [12, 19, 20, 33]. For all the keto-carotenoids examined here, all the values of $A_i(\lambda)$ were analyzed to be positive; only the decays of fluorescence were observed. This is in contrast to the fluorescence kinetics of the polyene-type carotenoids, neurosporene [34], lutein [35], and diadinoxanthin and diatoxanthin [33], in which negative $A_i(\lambda)$ values were resolved at several wavelengths, reflecting the intramolecular vibrational redistribution (IVR) in the 1^1B_u state. The lifetime values of keto-carotenoids were analyzed to be 30–80 fs for τ_1 and 180–230 fs for τ_2 , and the A_2 values (open circles, Figure 5) were much smaller than the respective A_1 values (closed circles, **Fig. 5**). Because the magnitude of amplitudes is related to the strength of the electronic transition to the ground state, the first and second components were assigned as the 1^1B_u state and the $^1(n, \pi^*)$ state, respectively. The 1^1B_u -state lifetimes of the keto-carotenoids (30–80 fs) are comparable to the time constants assigned to IVR in the polyene-type carotenoids [33–35]; thus, it appears that the internal conversion and IVR processes occur competing with each other in the keto-carotenoids. Therefore, it should be pointed out that the 1st and 2nd FDA spectra include fluorescence signals from the vibrationally excited state(s) in the 1^1B_u state and the $^1(n, \pi^*)$ state, respectively. The third component exhibits further smaller A_3 values with τ_3 longer

than a few ps, which is most probably assigned to vibrationally excited state(s) of the lowest excited state. Although the keto-carotenoids examined here possess the common π conjugation systems consisted of eight C=C bonds and one C=O bond, their excitation relaxations exhibit some differences. For siphonoxanthin, the τ_1 value is shortest (30 fs; closed circles, Figure 5a) and the relative values of A_2 to A_1 are smallest among three keto-carotenoids (open circles, Figure 5). Both the 1st and 2nd FDA spectra of peridinin (Figure 5c) are located in the longer wavelength region than the FDA spectra of siphonoxanthin (Figure 5a) and fucoxanthin (Figure 5b). Differences in π conjugation systems (Figure 1), such as the existence of an allene group at the end of π conjugation system of fucoxanthin, and the presence of the C=O group in a lactone ring of peridinin, might bring the excitation relaxation processes unique to each keto-carotenoid.

4.2. Energy transfer from keto-carotenoid to chlorophyll a in the light-harvesting pigment–protein complex

In the light-harvesting pigment–protein complexes, the excitation energy transfer from carotenoid to chlorophyll occurs, competing with the excitation relaxation within carotenoid. Therefore, the observed excited state lifetime (or the fluorescence lifetime in the case of fluorescence measurements) of carotenoid, τ_F , is expressed as a function of the rate constant of excitation energy transfer from carotenoid to chlorophyll, k_{EET} , and that of excitation relaxation within carotenoid, k_{Car} :

$$\tau_F = (k_{EET} + k_{Car})^{-1} \quad (4)$$

The efficiency of the energy transfer, Φ_{EET} , is defined as follows:

$$\Phi_{EET} = \frac{k_{EET}}{(k_{EET} + k_{Car})} \quad (5)$$

Therefore, the smaller k_{Car} , i.e., the longer lifetime of carotenoid excited state, is desirable to achieve the higher Φ_{EET} . In this sense, the lowest excited state is preferable to the highly excited state(s), because the former has a longer lifetime than the latter. However, the transition dipole moment between the lowest excited state and the ground state of carotenoid is much smaller than the moment(s) between the highly excited state(s) and the ground state; therefore, the smaller k_{EET} is expected for the energy transfer from the lowest excited state of carotenoid than its highly excited state(s), based on the Förster theory [36] with the dipole-dipole interaction between the energy donor and acceptor.

Fluorescence lifetime values of the highly electronically excited states of keto-carotenoids in the light-harvesting pigment–protein complexes are almost

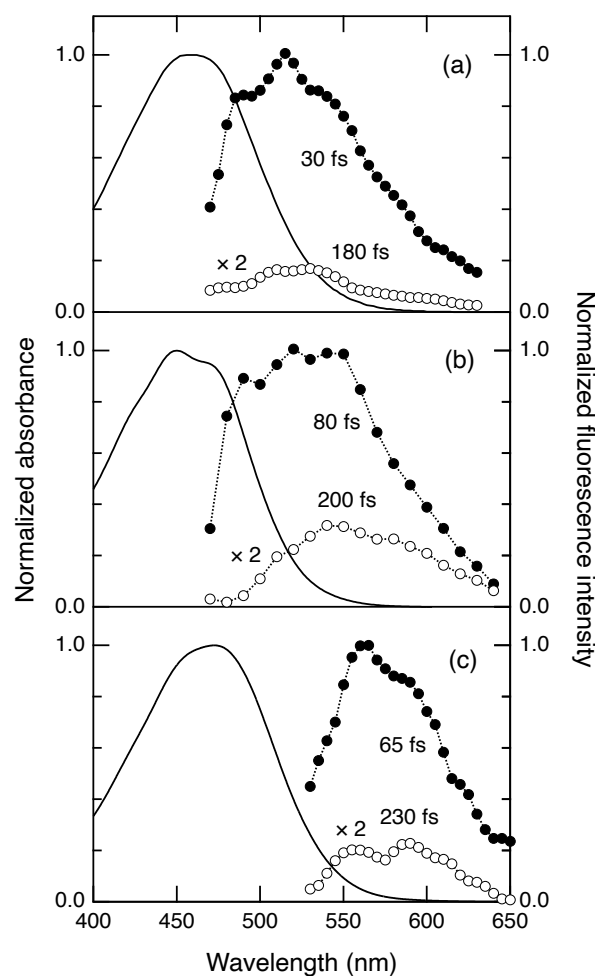


Fig. 5. Normalized absorption spectra (solid lines) and the 1st and 2nd fluorescence decay-associated (FDA) spectra (closed and open circles, respectively) of the keto-carotenoids in alcohol solutions: siphonoxanthin in methanol (a), fucoxanthin in ethanol (b), and peridinin in ethanol (c). Circles are plotted at observed wavelengths. Each pair of the 1st and 2nd FDA spectra is normalized to 1.0 by the maximum amplitude of the respective 1st FDA spectrum. After the normalization, the second FDA spectra are magnified by a factor of two in their intensities. Time constants of the FDA spectra are also listed. The excitation wavelength is 420 nm for (a) and 425 nm for (b) and (c).

the same as those in solutions, suggesting that the efficiencies of energy transfer from the highly excited states of keto-carotenoids to chlorophylls are low [19, 20]. In contrast to the keto-carotenoids, the polyene-type carotenoids, such as spheroidene [37] and lutein [35], exhibit shorter fluorescence lifetimes of the 1^1B_u state in the pigment–protein complexes, indicating the energy transfer from the highly excited states of polyene-type carotenoids. To achieve efficient energy transfer in the pigment–protein complexes including keto-carotenoids, the energy transfer efficiency from the lowest excited state of keto-carotenoids needs to be

high. Although fluorescence signals from the lowest excited state of keto-carotenoids are observable in solutions by both the steady-state measurements [17, 38] and the time-resolved measurements [12, 22], it is difficult to distinguish and detect the signals in pigment-protein complexes, suffering from the strong chlorophyll fluorescence. However, by analyzing the fluorescence kinetics of chlorophyll as an energy acceptor, the energy transfer from carotenoid to chlorophyll can be investigated. The fluorescence rise and decay curves ($I_F(t)$) of chlorophyll *a* show rise components of 380 fs in the *Codium fragile* SCP [39], 1.5 ps in the *Symbiodinium* sPCP [20], and 400 fs in the *Symbiodinium* thylakoid membranes including iPCP [20]. The time constants observed for the energy transfer from keto-carotenoid to chlorophyll *a* are comparable to those reported for the energy transfers from chlorophyll *b* to chlorophyll *a* [21, 35] and from chlorophyll *c* to chlorophyll *a* [19, 20]. These results indicate that energy transfers occur more efficiently from the lowest excited states of these keto-carotenoids than their highly excited states. Because the electronic transition between the lowest excited state and the ground state is forbidden, the energy transfer from the lowest excited state of carotenoid seems less efficient. However, a theoretical study suggests that a slight asymmetry in the carotenoid molecule can greatly enhance the rate of energy transfer from the lowest excited state of the carotenoid [40], which might be applicable to the keto-carotenoids having asymmetric π conjugation systems by the C=O group.

Fig. 6 shows the time-dependent fluorescence anisotropies ($r(t, \sim 690 \text{ nm})$) of the light-harvesting pigment-protein complexes. In the *Arabidopsis thaliana* LHCII (Figure 6a), the anisotropy value just after excitation ($r(0, \sim 690 \text{ nm})$) is ~ 0.1 , and the anisotropy value decreases over time, indicating the energy migration among chlorophylls. This behavior is independent of the observed wavelength including the chlorophyll *b* fluorescence region, showing the energy migration/transfer in chlorophyll pools [35]. On the contrary, the *Codium fragile* chloroplasts including SCP (Figure 6b), the *Chaetoceros gracilis* FCP (Figure 6c), and the *Symbiodinium* sp. thylakoid membranes (Figure 6d) show the $r(0, \sim 690 \text{ nm})$ values higher than 0.1 (up to ~ 0.15). The anisotropy exhibits a fast decay until ~ 1 ps, followed by a gradual decay over time. The slower decay exhibits almost the same time evolution to the anisotropy decay of *Arabidopsis thaliana* LHCII (Figure 6); therefore, it can be assigned due to the energy migration/transfer in chlorophyll pools. Time constants of the fast decays are analyzed to be 400 fs for the *Codium fragile* chloroplasts [21], 300 fs for the *Chaetoceros gracilis* FCP [19], and 210 fs for the *Symbiodinium* sp. thylakoid membranes. After keto-carotenoid and chlorophyll *a* are excited by the second harmonic of a Ti:sapphire laser, at least two internal conversions occur to emit the fluorescence from the lowest excited state: the $1^1B_u \rightarrow {}^1(n, \pi^*) \rightarrow$

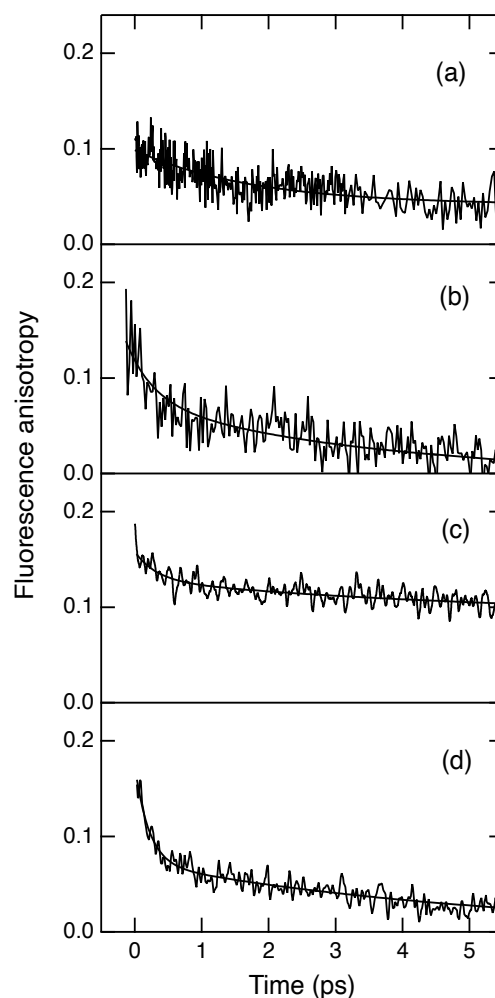


Fig. 6. Time evolution of fluorescence anisotropy after the pulse excitation for the *Arabidopsis thaliana* LHCII observed at 690 nm (a), the *Codium fragile* chloroplasts at 690 nm (b), the *Chaetoceros gracilis* FCP at 690 nm (c), and the *Symbiodinium* sp. thylakoid membranes at 688 nm (d). The excitation wavelength is 420 nm for (b) and 425 nm for (a), (c), and (d).

2^1A_g internal conversions for ketocarotenoid and the S_4/S_3 (Soret) $\rightarrow S_2(Q_x) \rightarrow S_1(Q_y)$ internal conversions for chlorophyll *a*. Upon excitation to the 1^1B_u state, the fluorescence anisotropy value of siphonoxanthin in benzene is ~ 0.32 for the 2^1A_g state [21]. On the other hand, the anisotropy of chlorophyll *a* Q_y fluorescence in castor oil shows negative values after excitation at the Q_x band [41], because the transition dipole moments due to the Q_y and Q_x bands are nearly orthogonal to each other (for $\theta \sim 90^\circ$ in Equation 3). Accordingly, if the energy transfer mainly occurs from the highly excited state of carotenoid to the S_2 state of chlorophyll *a*, the initial anisotropy value, $r(0, \lambda)$, of the chlorophyll *a* Q_y fluorescence should become smaller due to the $S_2 \rightarrow S_1$ internal conversion within chlorophyll *a* after the carotenoid-to-chlorophyll *a* energy transfer, while if the energy transfer to the S_1 state of chlorophyll *a* is the dominant energy-transfer

path, $r(0, \lambda)$ is expected to show a larger value. Therefore, the relatively smaller $r(0, 690 \text{ nm})$ value for the *Arabidopsis thaliana* LHCII (Figure 6a) is in good agreement with the observation that the energy transfer occurs from the 1^1B_u state of lutein to the S_2 state of chlorophyll *a* [34, 42]. On the other hand, the larger values of $r(0, \sim 690 \text{ nm})$ for the *Codium fragile* chloroplasts, the *Chaetoceros gracilis* FCP, and the *Symbiodinium* sp. thylakoid membranes (Figure 6b, 6c, and 6d, respectively) indicate that the energy transfers mainly occur from the lowest excited state of keto-carotenoid to the S_1 state of chlorophyll *a*.

The double decays of the fluorescence anisotropy were observed around 690 nm (Figure 6b, c, and d), but were not widely observed in all the chlorophyll *a* fluorescence region; the anisotropies around the steady-state fluorescence maxima (683 nm for the *Codium fragile* chloroplasts [21] and 677 nm for the *Chaetoceros gracilis* FCP [19]) exhibit single decays, as observed in the *Arabidopsis thaliana* LHCII (Figure 6a) [35]. Therefore, it appears that the energy transfers from the keto-carotenoids selectively occurs to the lower-energy form(s) of chlorophyll *a* ($\sim 690 \text{ nm}$), rather than the higher-energy form(s) ($\sim 680 \text{ nm}$) [19]. Following the energy transfer from fucoxanthin to the lower-energy chlorophyll *a*, an up-hill energy transfer from the lower-energy chlorophyll *a* to the higher-energy chlorophyll *a* was observed [19].

5. Summary

By the polarized and non-polarized time-resolved fluorescence measurements in the femtosecond to picosecond region after pulse-laser excitation [22], the excitation relaxation processes within the keto-carotenoids, siphonaxanthin, fucoxanthin, and peridinin, and the energy transfer processes in the light-harvesting pigment-protein complexes containing these keto-carotenoids, SCP, FCP, and PCP, respectively, were investigated [19–21, 39], in comparison with the processes in a polyene-type carotenoid, lutein, and the lutein-containing complex, LHCII [35]. The time evolution of fluorescence intensity, $I_F(t, \lambda)$, and that of fluorescence anisotropy, $r(t, \lambda)$, are different between the complexes possessing lutein and the keto-carotenoids; therefore, it appears that the energy transfer from keto-carotenoid to chlorophyll *a* mainly occurs through the lowest excited states of keto-carotenoids, and that the low-energy form(s) of chlorophyll *a* preferentially accept(s) the energy.

Possible electronically excited states occurring in polyene-type carotenoids and keto-carotenoids have been discussed [43, 44]. At the present stage, it is not necessarily clear by the fluorescence spectroscopy whether the lowest excited state of keto-carotenoid exhibits the ICT property in the pigment-protein complex, because it is difficult to separately observe the fluorescence from the lowest excited state of keto-

carotenoid in the pigment-protein complex. The contributions of the ICT state of keto-carotenoid to the energy transfer from keto-carotenoid to chlorophyll *a* were investigated with the transient absorption measurements by the pump-probe method for fucoxanthin in the FCP from a brown alga *Cladosiphon okamuranus* Tokida [45] and for peridinin in the *Amphidinium carterae* iPCP [46]. It was also reported that the four energy forms of peridinin in the *Amphidinium carterae* sPCP characterize the light harvesting by peridinin and the following energy transfer from peridinin to chlorophyll *a* [47]. For further studies, a combination of both the time-resolved absorption and fluorescence measurements with the theoretical investigations based on the structures of the pigment-protein complexes are required to fully understand the excitation relaxation dynamics within keto-carotenoids, the energy transfer processes involving keto-carotenoids, and the functions of keto-carotenoids with different energy forms in photosynthesis.

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Bioproduction of carotenoids using *Escherichia coli*

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Abstract: Several carotenoids extracted from plants, e.g., lycopene from tomato fruits, lutein from marigold flowers and capsanthin from paprika, have been utilized as natural colorants, functional foods, and cosmetics. In contrast, carotenoids rare in nature are difficult to prepare with large quantities. Thus, their biological functions have not been elucidated. For this solution, we have been developing methods to produce a great variety of carotenoids in recombinant *E. coli* cells. At the present time, we can produce not only typical C40-carotenoids including those of plants but also C30- and C20-carotenoids in *E. coli*. This bacterium cannot synthesize carotenoids naturally but produces FPP (farnesyl pyrophosphate) as the precursor. When introducing various genes derived from bacteria and plants (including algae) for carotenoid biosynthesis following FPP, *E. coli* is amenable to carotenoid production. We here describe methods effectively to produce representative carotenoid standards using *E. coli* as a heterologous platform.

1. Introduction

Carotenoids are isoprenoid pigments that are typically yellow, orange, and red, and contain more than 850 structurally distinct compounds. Carotenoids consist of two groups, which are called carotenes and xanthophylls. Carotenes are composed of only carbons and hydrogens, while xanthophylls further include other atoms, typically oxygens such as with hydroxy-, carbonyl- and epoxy-groups. The pigments are de novo synthesized in photosynthetic organisms, i.e., cyanobacteria and other photosynthetic bacteria, algae, liverworts, mosses, ferns, and higher plants, and some of non-photosynthetic bacteria, archaea, and fungi [1-6]. Among the non-photosynthetic bacteria, several species, e.g., those belonging to the phylum Firmicutes, have been found to produce acyclic carotenoids with the thirty-carbon skeleton (C30), instead of typical C40-carotenoids. Animals mostly cannot synthesize carotenoids de novo, and usually must obtain carotenoids from their diet [7,8]. However, a few animals (arthropods), e.g., aphids and spider mites, possess active genes for carotenoid biosynthesis [5].

Carotenoids have important physiological functions such as protection from oxidative damage in many

organisms [9], as well as light harvesting pigments in the photosynthetic organisms [10]. Several carotenoids of higher plants (or green algae) have been industrially produced, including β -carotene (mainly by organic synthesis), lycopene (from tomato fruits), lutein (from marigold flowers), zeaxanthin (from marigold flowers and other higher plants), capsanthin (from paprika), and astaxanthin (mainly from green alga *Haematococcus pluvialis*), and have been utilized as natural colorants, functional foods, and cosmetics [11]. Crocetin and its glycoside crocin with the twenty-carbon skeleton (C20; called apocarotenoids), which are contained in saffron stigmas, also have an attention as a functional food [5].

Carotenoids have widely been studied for their efficient production in heterologous hosts, due to their biological and industrial importance [5, 12]. Authentic carotenoid standards are desired to identify metabolized or biosynthesized carotenoids, but they are often too expensive and/or impure. An alternative method is to biosynthesize carotenoid standards in recombinant *E. coli* cells carrying appropriate plasmids. In this review, we describe methods effectively to produce representative carotenoid standards using *E. coli* as a heterologous platform.

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2. Materials and Methods

2.1 *E. coli* strains

For carotenoid biosynthesis, we have used strains JM109, JM101, JM101(DE3), and BL21(DE3) [13]. JM101(DE3) and BL21(DE3) are host strains for a plasmid that includes the T7 promoter.

2.2 Culture conditions

Transformed *E. coli* cells were typically precultured in 2YT medium (1.6% Bactotryptone, 1% yeast extract, 0.5% NaCl) containing (an) appropriate antibiotic(s) at 37°C. Then, this preculture was inoculated into the new 2YT medium with the same antibiotic(s), including 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultured at 20°C for 2 days.

2.3 Plasmid construction

In order to construct the plasmids, each gene was amplified by PCR and cloned into the vectors as described in the reference [14].

2.4 Carotenoid extraction and analysis

Carotenoids were extracted from *E. coli* and subjected to HPLC as described previously [15-17]. Individual carotenoids were fractionated by HPLC as needed, and analyzed by UV-VIS, FAB MS, and ¹H NMR (500 MHz, CDCl₃).

3. Results and Discussion

3.1 Carotenoids produced by *Escherichia coli* in our study

To produce diverse carotenoids in *E. coli*, we constructed plasmids that include various carotenoid biosynthesis genes (Table 1) [14]. We used vectors, pACYC184, pRK404, pUC18, pETDuet, pRSFDuet and CDFDuet as the backbones for the carotenoid-including plasmids. The carotenoid biosynthesis genes used here are derived from bacteria, *Pantoea ananatis*, *Pantoea agglomerans*, *Paracoccus* sp. strain N81106, *Brevundimonas* sp. strain SD212, *Xanthophyllomyces dendrorhous*, *Chlorolium phaeobacteroides*, *Rhodobacter capsulatus*, *Leuconostoc mesenteroides* and *Planococcus maritimus*, and alga *Haematococcus pluvialis*, fungus *Neurospora crassa*, aphid

Acyrtosiphon pisum, liverwort *Marchantia polymorpha*, and paprika *Capsicum annuum*.

We transformed the constructed plasmids into *E. coli*. Most of them have been deposited in Riken BRC (RIKEN BioResource Research Center).

Representative carotenoids that can be produced in the recombinant *E. coli* are listed in Table 1.

3.2 Factors that affect the productivity of carotenoids in *E. coli*

When we try to produce natural compounds including carotenoids in *E. coli*, various factors affect productivity. We will now discuss these factors, but there is not necessarily a rule, so trial and error should be needed.

As well known, host cells sometimes affect the expression of foreign genes and metabolic pathways [18-19]. For example, when we investigated the effect of *E. coli* strains as the hosts on the production of violaxanthin, not only the ratio of violaxanthin to total carotenoids but also the productivity of violaxanthin showed the highest level in the JM101 strain among four strains tested, JM101, JM109, JM101(DE3), and BL21(DE3) [13]. However, several *E. coli* should be tried, as which *E. coli* is best may depend on the carotenoids produced.

Ordinarily, we have carried out plasmid-mediated gene expression for carotenoid production. Each vector has a different replication origin and a different copy number in *E. coli*. So far, we compared productivity between the pUC vector, which confers a high copy number (approximately 100 copies/cell) to the host, and the pETD vector with a medium copy number (approximately 20 copies/cell) [13]. As a result, in some cases pUC showed higher activity than pETD, while in other cases the pUC and pETD vectors were about the same. Although it is expected that a higher copy number would result in higher gene expression and productivity, this is not the case. Therefore, it is better to try several vectors including ones with low-copy numbers such as pACYC184 and use the one that shows the highest productivity.

Factors involved in gene expression include promoters. It may be expected that the stronger a promoter is, the higher productivity is obtained, but it is also not the case. In fact, we have often used the IPTG-inducible T7 promoter (strong) and *lacUV5*

Table 1. Carotenoids that can be produced by recombinant *E. coli*.

Carotenoid	Plasmids
Carotene	
Phytoene (15-cis-Phytoene)	pACCRT-EB, pAHP-Phy, pAC-HIEB, pRK-HIEB
Neurosporene	pACCRT-EB-RcI
Lycopene (ψ,ψ -Carotene)	pACCRT-EIB, pAHP-Lyc, pAC-HIEBI, pRK-HIEBI
γ -Carotene (β,ψ -Carotene)	pAC-HIEBIA
β -Carotene (β,β -Carotene)	pACCAR16 Δ crtX, pAHP-Beta, pAC-HIEBIY, pRK-HIEBIY
α -Carotene [(6'R)- β,ϵ -Carotene]	pACCRT-EIB + pETD-MpLCYb/MpLCYe
β,γ -Carotene [(6'S)- β,γ -Carotene]	pAC-HIEBI-ApCrtYB3
3,4-Didehydrolycopene	pAHP-Phy + pUC-ApTor
Torulene	pAC-HIEBI-ApCrtYB1
Xanthophyll	
Zeaxanthin	pACCAR25 Δ crtX, pAHP-Zea, pAC-HIEBIYZ, pRK-HIEBIYZ
Canthaxanthin	pAC-Cantha
Astaxanthin	pACCAR25 Δ crtX + pUC-BreW, pAC-Asta, pAC-HIEBIYZW, pRK-HIEBIYZW
Nostoxanthin	pACCAR25 Δ crtX + pUCBew-O11, pAC-HIEBIYZG, pRK-HIEBIYZG
Zeinoxanthin	pACCRT-EIB + pETDuet-MpLCYb/MpLCYe + pRSFDuet-MpBHY
Lutein	pACCRT-EIB + pETDuet-MpLCYb/MpLCYe + pRSFDuet-MpBHY/MpCYP97C, pRKHIEBI-MpLCYb Δ TP-MpLCYe-Z-EPg and CDF-MpCYP97C-MpLCYe
Antheraxanthin	pAHP-Zea + pUC-CaZEP
Violaxanthin	pAHP-Zea + pUC-CaZEP
Capsanthin	pAHP-Zea + pUC-Ptac-CaCCSM40-CaZEP
Neoxanthin	pAC-Viol + pUC-PtVDL1
C₃₀-Carotenoid or Apocarotenoid	
Diapophytoene	pAC-HIM
Diaponeurosporene	pAC-HIMN
Crocetin dialdehyde	pAC-HIEBI-FhCCD2
Crocetin	pAC-HIEBI-FhCCD2-NcALD8

promoter (moderately strong) with induction by a very low concentration (0.05 mM) of IPTG for weak gene expression.

The SD sequence (RBS; ribosome binding site) determines translation efficiency. There are examples that production efficiency was increased by replacing the SD sequences [13, 20-21]. Previously, we designed the RBS sequences using the software “RBS calculator” [22-23]. The result indicated that RBS modification achieved higher productivity, while the effect of the RBS modification distinctively depended on the host strains.

Another factor affecting translation efficiency is codon usage. When prokaryotic genes are used, they are often functional with their original codon usage in *E. coli*. In fact, we have not changed the codon usage when we have used prokaryotic genes, e.g., those from *P. ananatis*, *R. capsulatus*, *L. mesenteroides*, and *P. maritimus*. On the other hand, if eukaryotic genes are used, their codon usage should be optimized to that of *E. coli*. For example, when the *MpLCYb* gene, i.e., the

lycopene β -cyclase gene from liverwort *M. polymorpha*, was codon-optimized, its activity became higher than that with the wild type gene [24].

In plant cells, carotenoids are produced in chloroplasts and chromoplasts. The carotenoid biosynthesis genes are encoded in the nucleus. Hence the translated proteins are needed to move to these intracellular organs. The transit peptide functions to transport a protein including itself at the N-terminus (typically), into chloroplasts, and after this transportation, it is cut away. Therefore, for the expression of a plant carotenogenic gene in *E. coli*, the transit peptide is often removed beforehand as previously reported [24-25]. However, some genes, e.g., *MpBHY*, are likely to be more active with the transit peptide [26], so it is better to try the gene both with and without the transit peptide.

The biosynthetic pathway of carotenoids sometimes comprises the redox reaction catalyzed by a cytochrome P450. Most of the P450s involved in the secondary metabolism require a FAD and FMN-

containing NADPH-P450 reductase to receive the electrons from NADPH and reduce P450 itself [27]. For the functional expression of such a P450 gene in *E. coli*, a gene for NADPH-P450 reductase is ordinarily required to be co-expressed [28-29]. However, interestingly, P450 genes involved in carotenoid biosynthesis, such as bacterial CYP175A1 and plant CYP9C7, often do not need the co-expression of any NADPH-P450 reductase gene for their functional expression in *E. coli* [26].

There are other methods to increase productivity that will not be discussed in detail in this paper. For example, the integration of carotenoid biosynthesis genes into the chromosome of *E. coli* is applied to the stable production [30-31]. The mevalonate pathway genes can also be added to increase the production of IPP (isopentenyl pyrophosphate) that is a substrate for carotenoids [28, 31-32].

After making the various improvements described above, the culture conditions should be examined. The culture conditions include culture medium, culture temperature, incubation time, etc [24, 33-34]. In addition, some genes (enzymes) may require metal ions such as iron, so it is better to add them to the medium [24].

4. Conclusion

In this review, we described the methods effectively to produce various carotenoids in *E. coli*. Until now, we can produce approximately more than fifty C40-carotenoids, ten C30-carotenoids, and three C20-carotenoids (apocarotenoids). Many of them are rare carotenoids in nature and not subjected to the test of biological activity. However, our studies made it possible to investigate the biological function of them. There are still some issues to be addressed, for example carotenoid yields. At this time, we achieved 11 mg/l as lutein yield using a mini jar which is lower than that of lycopene [24, 35]. The more complex the carotenoid, the lower the yield. Therefore, some improvements need to be made for high yield. Moreover, innovations will make it possible to produce a wider variety of carotenoids by *E. coli*.

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Functional properties of red paprika xanthophylls

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Abstract: Red paprika xanthophylls have been shown to reduce abdominal fat, improve endurance, reduce fatigue during excises, and reduce ultraviolet radiation-induced erythema and skin darkening. This article summarizes the mechanism and results of human studies on each of these effects.

1. Introduction

Carotenoids are classified as "carotenes" and "xanthophylls" and include more than 700 naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria [1,2]. These colorful molecules are the source of the yellow, orange, and red colors in many plants.

Tomatoes and carrots are known for their high carotenoid content and are mostly composed of carotenes. Red paprika contains a higher amount of carotenoids than tomatoes and carrots, and about 80% of its carotenoids are xanthophylls, including capsanthin, cucurbitaxanthin A (a xanthophyll not commonly found other foods except for pumpkin), capsanthin 3,6-epoxide, capsorubin, and cryptocapsin [3-7]. Xanthophylls have strong antioxidant activity and present various effects on human physiological functions. For example, astaxanthin improves brain and skin barrier function [8,9], and lutein accumulates in the eye and is effective in preventing age-related eye diseases [10]. Thus, xanthophylls have beneficial actions that support health.

Carotenes are hydrophobic (oil-soluble) substances composed only of carbon and hydrogen, whereas xanthophylls are amphiphilic substances with hydrophilic and hydrophobic portions. Dietary

carotenoids are absorbed in the small intestine and then transferred to the bloodstream, where they are distributed mainly as carotenes in plasma and xanthophylls in red blood cells; the subsequent distribution of the two substances is different because, as a result of their structure, xanthophylls have a higher affinity for cell membranes than carotenes [11]. These differences in distribution influence the antioxidant and other effects of the two substances. This review summarizes the results of three human studies that evaluated the effects of xanthophylls from red paprika.

2. Reduction of abdominal fat

2.1 Red paprika components increase levels of high-density lipoprotein cholesterol

Aizawa and Iwakuma reported on a study in which young male Wistar rats were fed diets containing red paprika powder, red paprika organic solvent extract, red paprika extract residue, and purified capsanthin to investigate the relationship between capsanthin, the major xanthophyll in red paprika, and lipid metabolism [12]. The study showed that 2 weeks' treatment with purified capsanthin significantly increased plasma high density lipoprotein (HDL) cholesterol and found a significant correlation between dietary capsanthin levels and plasma HDL cholesterol levels. Quantitative analysis of mRNA levels in the liver suggested that

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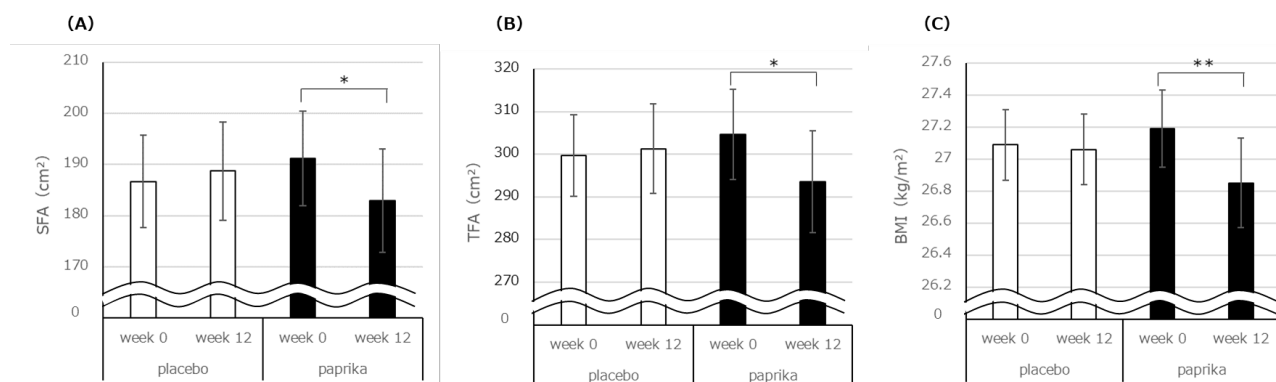


Fig. 1. Effect of red paprika xanthophyll intake on abdominal fat area and waist circumference and on body indices.

White and black bar in the graph indicate placebo group and red paprika xanthophylls group, respectively. Data are expressed as the mean±SEM. SFA: subcutaneous fat area, TFA: total fat area (paprika group: n=37, placebo group: n=38), BMI: body mass index (paprika group: n=41, placebo group: n=39). Differences between the two groups were determined with the unpaired t-test. Comparisons between week 12 and week 0 (baseline) were performed by using the paired t-test. *: $p < 0.05$, **: $p < 0.01$ vs. week 0. Analysis of visceral fat level data was performed by using the Wilcoxon rank sum test.

capsanthin treatment increases mRNA for apolipoproteinA5 and lecithin cholesterol acyltransferase; these effects may increase HDL cholesterol metabolism.

2-2. Red paprika extract increases levels of adiponectin, an adipocytokine

In lipid metabolism, adiponectin increases blood HDL cholesterol levels and improves blood lipid abnormalities and, consequently, can improve metabolic syndrome. Maeda et al. examined the effects of red paprika extract on adipocytokine secretion by investigating adipocyte cell differentiation and adiponectin secretion in 3T3-L1 mouse preadipocyte cells. They found that capsorubin, the characteristic xanthophyll in red paprika in red paprika improves lipid metabolism by activating peroxisome proliferator-activated receptor gamma, resulting in conversion of hypertrophied adipocytes to compact adipocytes [13].

2-3. Red paprika extract reduces abdominal fat

Kakutani et al. investigated whether intake of red paprika xanthophylls can decrease abdominal fat in healthy overweight volunteers [9]. In a randomized, double-blind, placebo-controlled, parallel-group study,

the researchers assigned 100 healthy volunteers with a body mass index (BMI) ranging from 25 to less than 30 kg/m² to 12 weeks' administration of oral capsules containing red paprika xanthophyll (9 mg) or placebo and investigated each abdominal visceral fat area (VFA) by computed tomography. They also evaluated changes in abdominal subcutaneous fat area (SFA), total fat area (TFA), and body mass index (BMI).

After 12 weeks, VFA was smaller in the paprika xanthophyll group than in the placebo group. The paprika xanthophyll group also showed a significant decrease in SFA, TFA, and BMI after 12 weeks compared with baseline; the reduction in these 3 variables was significantly greater in the paprika xanthophyll group than in the placebo group (Fig. 1). Intake of paprika xanthophyll capsules had no adverse effects. These results suggest that paprika xanthophyll is a safe food ingredient that improves lipid metabolism and reduces abdominal fat.

3. Increased endurance and reduced fatigue during exercise

3-1. Importance of oxygen during exercise and erythrocyte deformability

During exercise, active muscles have an increased need for oxygen. After oxygen enters the blood, it

combines with hemoglobin in erythrocytes and is transported to all parts of the body through the blood vessels. The peripheral capillaries in particular are very thin, so erythrocytes must change shape to pass through them [14]. Damage to the erythrocyte membrane by reactive oxygen species (ROS) may reduce the deformability of erythrocytes and their oxygen-carrying capacity [15]. Therefore, ROS must be removed to maintain the deformability of erythrocytes.

3-2. Red paprika has antioxidant potential by eliminating hydroxyl radicals

Nishino et al. showed that capsanthin and capsorubin, characteristic xanthophylls in red paprika, have high scavenging capacity for singlet oxygen and hydroxyl radicals. In addition, human studies showed that after oral supplementation of paprika carotenoids (15 mg/day of total carotenoids for 4 weeks), all paprika xanthophylls are found in plasma and preferentially in erythrocytes. Oral supplementation of paprika carotenoids resulted in a 2.2-fold increase in xanthophylls in erythrocytes, with xanthophylls making up over 80% of the erythrocyte carotenoids [16]. Therefore, because of their high antioxidant properties, continuous intake of red paprika

xanthophylls is expected to increase the distribution of xanthophylls in erythrocytes, inhibit damage caused by ROS, and improve the oxygen-carrying capacity of erythrocytes.

3-3. Red paprika improves endurance and reduces fatigue during exercise

A randomized double-blind, placebo-controlled, parallel-group clinical trial was performed in athletes to determine the effects of oral supplementation of red paprika xanthophylls on plasma carotenoid levels and oxygen uptake during exercise [17]. The study also assessed subjective levels of post-exercise fatigue with a visual analog scale (VAS). In the study, 14 athletes were assigned to receive either the beverage containing red paprika xanthophylls (9 mg) or a placebo beverage daily. Blood samples were collected to measure plasma carotenoid levels before and after a 4-week intervention period. Before and after the intervention period, athletes also exercised on a treadmill (at 12 km/h for 20 minutes), and respiratory parameters were analyzed with a respiratory gas analysis system that measured minute oxygen uptake ($V\text{-O}_2$), minute carbon dioxide production ($V\text{-CO}_2$), and minute ventilation ($V\text{-E}$).

After 4 weeks, the concentrations of total

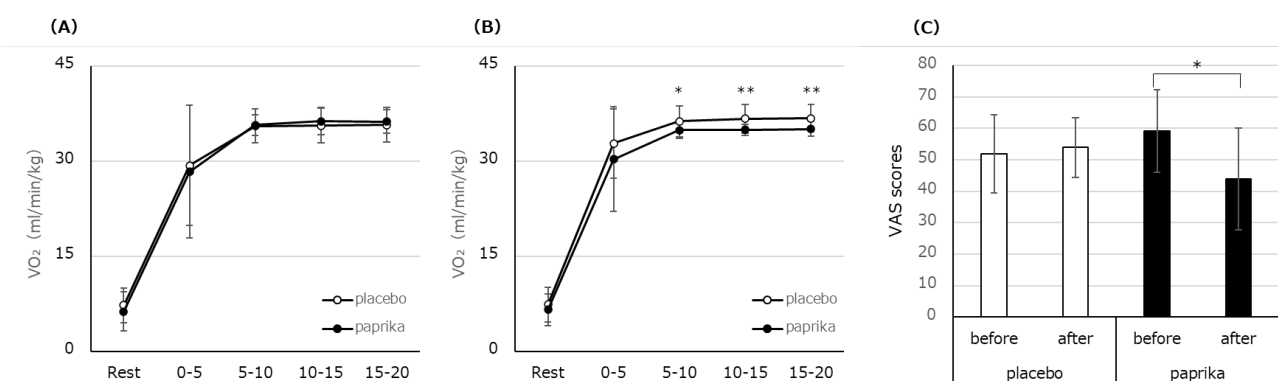


Fig. 2. Changes in minute oxygen uptake ($V\text{-O}_2$) during treadmill exercise and VAS evaluation of subjective fatigue before and after intervention.

(A) before intervention. (B) after the 4-week intervention. Placebo group ($\circ$: $n=7$) and red paprika xanthophylls group ($\bullet$: $n=7$). Data are expressed as the mean $\pm$ SD. * $p < 0.05$, and ** $p < 0.01$ vs. placebo group. Welch's t-test was used for between-group comparisons. (C) White and black bar in the graph indicate placebo group ($n=7$) and red paprika xanthophylls group ($n=7$), respectively. Data are expressed as the mean $\pm$ SD (mm). Differences between the red paprika and placebo groups were investigated with Welch's t-test. Comparisons between before treatment and after 4 weeks of treatment were performed by the paired t-test. *: $p < 0.01$ vs. before.

xanthophylls and total carotenoids in plasma were significantly higher in the red paprika xanthophylls group than in the placebo group, and V-O₂, V-CO₂, and V-E were significantly lower. These results suggest that oral supplementation of red paprika xanthophylls decreases the need for oxygen when athletes exercise at a set intensity. In addition, immediately after exercising on the treadmill, subjective fatigue as assessed by VAS scores was significantly lower in the red paprika xanthophylls group, clearly indicating that oral supplementation of red paprika xanthophylls reduces subjective fatigue (**Fig. 2**).

This study suggests that intake of red paprika xanthophylls may increase the oxygen-carrying capacity of erythrocytes and energy efficiency during endurance exercise, i.e., supplementation of red paprika xanthophylls may improve endurance performance.

4. Reduced ultraviolet radiation-induced erythema and skin darkening

4-1. Relationship between ultraviolet radiation and ROS

The skin is constantly exposed to ultraviolet (UV)

radiation. A major cause of skin disorders, including sunburn, is overexposure to UV radiation. The effects of UV on the skin are mediated by UV-induced oxidative stress, e.g., ROS formation. Among ROS, singlet oxygen is a major factor in light-induced skin damage [18-20]. UV-induced singlet oxygen induces the release of lipid peroxide and prostaglandin E₂, both of which are associated with skin inflammation and melanin synthesis [18]. Therefore, natural antioxidants that effectively eliminate ROS and singlet oxygen have the potential to prevent skin photodamage.

4-2. Antioxidant potential of red paprika in the skin

Nishino et al. showed that capsanthin and capsorubin exhibit strong singlet oxygen scavenging activity [21]. Furthermore, in a human study, oral supplementation of red paprika carotenoids increased plasma carotenoid concentrations [22,23].

Heinrich et al. also reported that daily oral supplementation with a mixture of carotenoids reduced the intensity of erythema induced by UV radiation [24]. Therefore, oral supplementation with red paprika xanthophylls was also expected to have anti-UV effects on the skin.

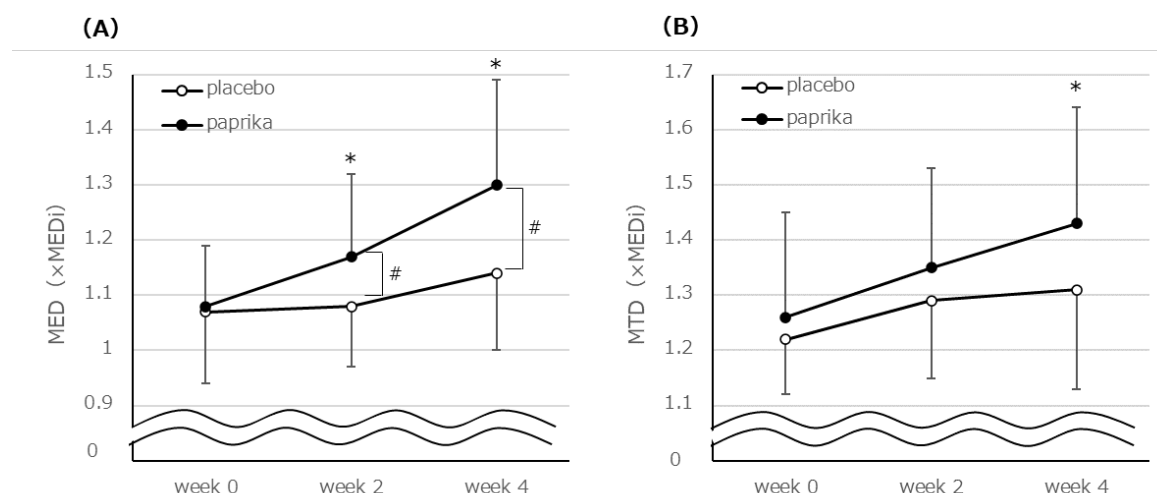


Fig. 3. Changes in minimal erythema dose (MED, (A)) and minimal tanning doses (MTDs, (B)) before and after ingestion of red paprika extract.

Placebo group (○ : n=21) and red paprika xanthophylls group (● : n=22). Data are expressed as the mean ± SE. *: p < 0.05 vs. 0week, #: p < 0.05 vs. placebo group. Within-group comparisons were performed by Wilcoxon signed-rank test with Holm's method, and between-group comparisons were conducted by Mann-Whitney U test.

4-3. Reduction of erythema and skin darkening by oral supplementation of red paprika

Nishino et al. evaluated the effects of oral supplementation of red paprika extract, which contains xanthophylls with high singlet oxygen scavenging activity, on UV-induced skin photodamage [25]. To do so, they performed a randomized, double-blind, placebo-controlled, parallel-group study in 46 healthy volunteers. Each individual's minimum erythematous dose (MED) was measured before the study, and capsules containing red paprika xanthophylls (9 mg) or placebo were subsequently administered daily for 5 weeks; the MED and minimum tanning dose (MTD) were assessed at weeks 0, 2, and 4.

Results showed that at 2 and 4 weeks after treatment, the MED of the red paprika xanthophylls group was significantly higher than that of the placebo group. In addition, the MTD of the red paprika xanthophylls group at 4 weeks was significantly higher than that of the placebo group in inhibiting UV-induced skin darkening (**Fig. 3**). These results indicate that oral supplementation of red paprika xanthophylls inhibits UV-induced skin damage.

5. Conclusion

As described above, red paprika xanthophylls have many beneficial effects on human health. Humans cannot synthesize these xanthophylls, but oral supplementation of red paprika xanthophylls can provide these benefits. Thus, foods or food ingredients containing red paprika xanthophylls can contribute to the maintenance of human health.

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Functional expression of the astaxanthin biosynthesis genes in *Freesia hybrida*

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Abstract: *Freesia* (*Freesia* × *hybrida*; the Iridaceae family) is a flowering plant popular as the cut flowers. We introduced the astaxanthin biosynthesis genes, which encoded the marine-bacterial CrtW, CrtZ, and Idi, into freesia cultivar ‘Ishikawa f6’ by *Agrobacterium*-mediated transformation under the control of the CaMV 35S promoter. The introduced genes were functionally expressed especially in the callus, which produced large amounts of astaxanthin including its esterified forms [63% of total carotenoids (32.2 µg/g fresh weight)] and free form (4.3%) in addition to other ketocarotenoids that contained fritschiellaxanthin (3%), 4-ketoantheraxanthin (1.6%), adonirubin (5.4%), adonixanthin (2%), and canthaxanthin (4.2%). The regenerated freesia plant exhibited bronze (reddish green) color, and its stems and (large) flower buds were found to accumulate astaxanthin esterified forms [50.3% and 35.3% of total carotenoids (83.1 and 21.6 µg/g fresh weight)], respectively. Whereas, the petals contained trace amounts of carotenoids, presumably because of rapid reduction and/or degeneracy of the chloroplasts (plastids).

1. Introduction

A terrestrial flowering plant freesia (*Freesia* × *hybrida*; subfamily, Ixioideae; family, Iridaceae) is originated in the Cape Province of South Africa. Many freesia cultivars have been generated worldwide by the traditional breeding from the original species such as *Freesia refracta* [1]. Nowadays, freesia establishes a position as one of the popular cut flowers, because of its flower forms, floral fragrance, and wide petal color variations [2]. This plant was also reported to be promising as the edible flowers [3].

Ishikawa prefecture (Department of Agriculture, Forestry and Fisheries, and the Ishikawa Agriculture and Forestry Research Center) has bred new varieties of freesia, ‘Ishikawa f1 ~ f11’ of the series named ‘Airy-Flora’, which are already distributed in market. The freesia flowers can generate not only pink and purple colors of flavonoids but also yellow and orange colors

of carotenoids in addition to white and red colors. Recently, we clarified that the yellow petals of ‘Ishikawa f2’ (and also cultivars ‘Aladin’ and ‘Kayak’) produced crocetin glycosides, crocetin neapolitanosyl ester and crocetin neapolitanosyl diester [3].

We constructed the *Agrobacterium*-mediated transformation system of freesia using cultivars ‘Mosera’ and ‘Ishikawa f3’ as the hosts [4]. In order to evaluate the productivity of astaxanthin (in freesia), which is the red carotenoid with strong anti-photooxidative activity, we have here tried to produce transgenic plants of ‘Airy-Flora’ cultivar ‘Ishikawa f6’, which expresses the astaxanthin biosynthesis genes from marine bacteria.

2. Materials and Methods

2.1 Plant materials

In this study, we used *Freesia* × *hybrida* cultivar ‘Ishikawa f6’ (‘f6’) generating white flowers with partially faint pink patterns (Fig. 1a) as the host. The

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bulb is also shown in **Fig. 1b** (left). The cormlets of 'f6' were sterilized as follows: first, they were immersed in 0.5% (w/v) Benlate T (thiuram 20% and benomyl 20%; Hokko Chemical Industry Co., Tokyo, Japan) for 20 min. Next, they were dipped briefly in 70% (v/v) ethanol and then immersed in a 5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 80) for 20 min. Finally, they were rinsed three times in sterile water. The surface-sterilized cormlets were immediately cut aseptically into ca. 5-mm cubes. The cubes were placed on the callus proliferation medium [MS (Murashige and Skoog) medium [5] containing 1 mg/L of thidiazuron (TDZ) (Wako Pure Chemical Industries, Osaka, Japan), 1 mg/L of 5,6-dichloro-1H-indole-3-acetic acid (Wako Pure Chemical Industries), 3% (w/v) sucrose, and 0.32% (w/v) gellan gum (Nacalai tesque, Kyoto, Japan)] [4]. All the media used in this study were adjusted to pH 5.8 prior to autoclaving at 120°C for 15 min. The cultures were maintained at 22±2°C in the dark.

2.2 Sensitivity of cormlets to hygromycin

To determine the optimum concentrations of hygromycin for the selection of transformed 'f6' cells, the cubes from the cormlets were placed on the callus proliferation medium containing different concentration of hygromycin (Hygromycin B; Wako Pure Chemical Industries) (0, 10, 25, and 50 mg/L). The cubes were incubated at 22±2°C in the dark. The ratio of callus formation was determined after a month of the cultures.

2.3 Construction of plasmids

The plasmid pUTR-crtZWidi (pZH2B) for plant transformation and expressing the astaxanthin biosynthesis genes were previously constructed as described [6,7]. This plasmid contained a binary vector pZH2B [8], in which three enzyme genes was inserted for β,β-carotenoid 3,3'-hydroxylase (CrtZ) and β,β-carotenoid 4,4'-ketolase (4,4'-oxygenase; CrtW) from a marine bacterium *Brevundimonas* sp. strain SD212, and isopentenyl diphosphate isomerase (type 2 Idi) from a marine bacterium *Paracoccus* sp. strain N81106, under the regulation of the CaMV 35S promoter and the transit peptide sequence of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small

subunit from pea. And the 5'-untranslated region (UTR) of the *Nicotiana tabacum* alcohol dehydrogenase gene (*NtADH*) was attached to the ATG of each gene, preceded with the transit peptide sequence.

2.4 Agrobacterium-mediated transformation

We carried out the Agrobacterium-mediated transformation. The cubes from the 'f6' cormlets were placed on the callus proliferation medium for 0 day, 7 day or 14 days. *Agrobacterium tumefaciens* strain EHA101, in which plasmid pUTR-crtZWidi (pZH2B) had been introduced, was cultured for 2 days at 26°C on LB medium solidified with 1.5% (w/v) agar containing 50 mg/L of kanamycin monosulfate (Wako Pure Chemical Industries, Osaka, Japan) and 50 mg/L of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan). This bacterial strain was collected and suspended in the co-cultivation medium [MS medium supplemented with 1 mg/L TDZ, 1 mg/L 5,6-dichloro-1H-indole-3-acetic acid, 3% (w/v) sucrose, 20 mg/L 3',5'-dimethoxy-4'-hydroxyacetophenone (acetosyringone) (Sigma-Aldrich, St. Louis, MO, USA), and 1% (w/v) glucose]. For the Agrobacterium inoculation, the bacterial suspension was adjusted to an OD600 of 0.15–0.20. The cubes were soaked in the bacterial suspension for 10 min. The pieces were removed from the suspension and blotted dry with sterile Kimwipes (Nippon Paper Crecia Company, Tokyo, Japan) to remove excess bacterial cells. Then, they were transferred onto the co-cultivation medium solidified with 0.32% (w/v) gellan gum, and co-cultivated at 23°C in the dark for 5 days. The cubes were then transferred onto the callus selection medium, which was the callus proliferation medium with the addition of 50 mg/L of meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan) and 15 mg/L of hygromycin B. The 'f6' cubes were cultured at 22±2°C in the dark onto the fresh selection medium, in which the meropenem trihydrate concentration was gradually decreased to 0 mg/L. The cubes were sub-cultured onto the fresh medium with every 3 weeks intervals.

After few months of the culture on the selection medium, the induced calli were transferred onto regeneration medium [MS medium containing 3 mg/L TDZ, 3% (w/v) sucrose, 15 mg/L hygromycin B,

0.32% (w/v) gellan gum], and cultured at $22\pm 2^{\circ}\text{C}$ under a 16-h photoperiod for a month. Calli and adventitious buds, which were formed from hygromycin-resistant tissues, were transferred onto the plant growth regulator (PGR)-free MS medium [MS medium containing 3% (w/v) sucrose, 15 mg/L hygromycin B and 0.32% (w/v) gellan gum] to induce plantlet regeneration.

2.5 PCR analysis

Genomic DNA was extracted from the transformed and wild-type plants of freesia 'f6' according to the method of the CTAB method [9]. PCR was performed using TaKaRa Ex Taq polymerase (Takara, Ohtsu, Japan). The PCR conditions were as follows: 98°C for 10 sec, 66°C for 30 sec, 72°C for 3 min; 35 cycles. Primer pairs were designed at the start codon of *crtZ* (5'-ATGGCTTGGCTTACTTGGATCGCTCTTTTCCT-3') and the stop codon of *crtW* (5'-TCAAGACTCTCCTCTCCAAAGTCTCCACCAAG-3'). PCR products were electrophoresed using a 1% (w/v) agarose gel.

2.6 Analysis of carotenoids

We carried out HPLC-PDA-MS/MS analysis on carotenoids that accumulated in the transformed and wild-type plants of freesia 'f6' based on the methods described [6,10]. An Acquity 1.7 μm BEH UPLC C18 (2.1 id $\times$ 100 mm) column (Waters Corporation, Milford, CT, USA) was used for the HPLC system, developed by acetonitrile : water (85:15) – acetonitrile : methanol (65:35) (linear gradient 0 to 15 min) as a mobile phase, at a flow rate of 0.4 mL/min. Carotenoids were identified by their UV-VIS (visible), and MS/MS spectral data as well as their retention time in HPLC by comparison with those of authentic standards.

3. Results and Discussion

3.1 Resistance experiment of the cormlets to hygromycin

Efficient selection is a necessary prerequisite for successful production of transgenic plants through the Agrobacterium-mediated method. Our result indicated that the formation of the callus from freesia 'f6' explants was extremely sensitive to hygromycin. When the cubes were cultured on the medium containing

more than 10 mg/L of hygromycin for 4 weeks, no calli were generated from the cubes (**Table 1**). Thus, 15 mg/L of hygromycin was used for the selection and maintenance of transformed 'f6' lines.

Table 1. Hygromycin sensitivity of freesia callus

Hygromycin concentration (mg/L)	0	10	25	50
Callus formation ratio (%) [*]	93	0	0	0

^{*}Thirty cubes from freesia cormlets were provided for each test.

3.2 Transformation of freesia 'f6'

After five days of the co-cultivation, the 'f6' cubes were transferred onto the callus selection medium. The cultures were incubated at $22\pm 2^{\circ}\text{C}$ in the dark. After around nine weeks of cultivation, formed hygromycin-resistant orange calli (called #21; indicated with arrow in **Fig. 1c**) were transferred onto the regeneration medium, and the cultures were incubated at $22\pm 2^{\circ}\text{C}$ under a 16-h photoperiod. After around 30 days of cultivation, calli containing formed adventitious buds were placed on the PGR-free MS medium (**Fig. 1d**) and cultivated. Consequently, three hygromycin-resistant plantlets (called #21-1, #21-2, and #21-3), which exhibited orange color systemically, were regenerated from the totals of 584 cubes [**Fig. 1e** (right), **Table 2**].

After acclimatization, these three transgenic plants (see **3.3**) were grown for five months in a biohazard greenhouse to form bulbs. Generated bulbs were kept at 30°C for 8 weeks to break dormancy [**Fig. 1b** (right)]. After this heat treatment, the bulbs were planted and raised in a greenhouse. The transgenic freesia plants exhibited bronze (reddish green) color [**Fig. 1fg** (left)]. Then, the freesia came into flower after the exposure of a low temperature in winter. According to the growth of the flower buds of the transgenic freesia, their bronze color gradually faded [**Fig. 1h** (right)].

Table 2. Transformation efficiency of freesia 'f6'

Test number	Pre-culture period	Number of tested callus	Number of hygromycin resistant plantlet	Number of PCR-positive plantlet	Transformation frequency (%)
1	7 days	123	1	1	0.8
2	14 days	128	1	1	0.8
3	14 days	111	1	1	0.9
4	—	222	0	0	0.0

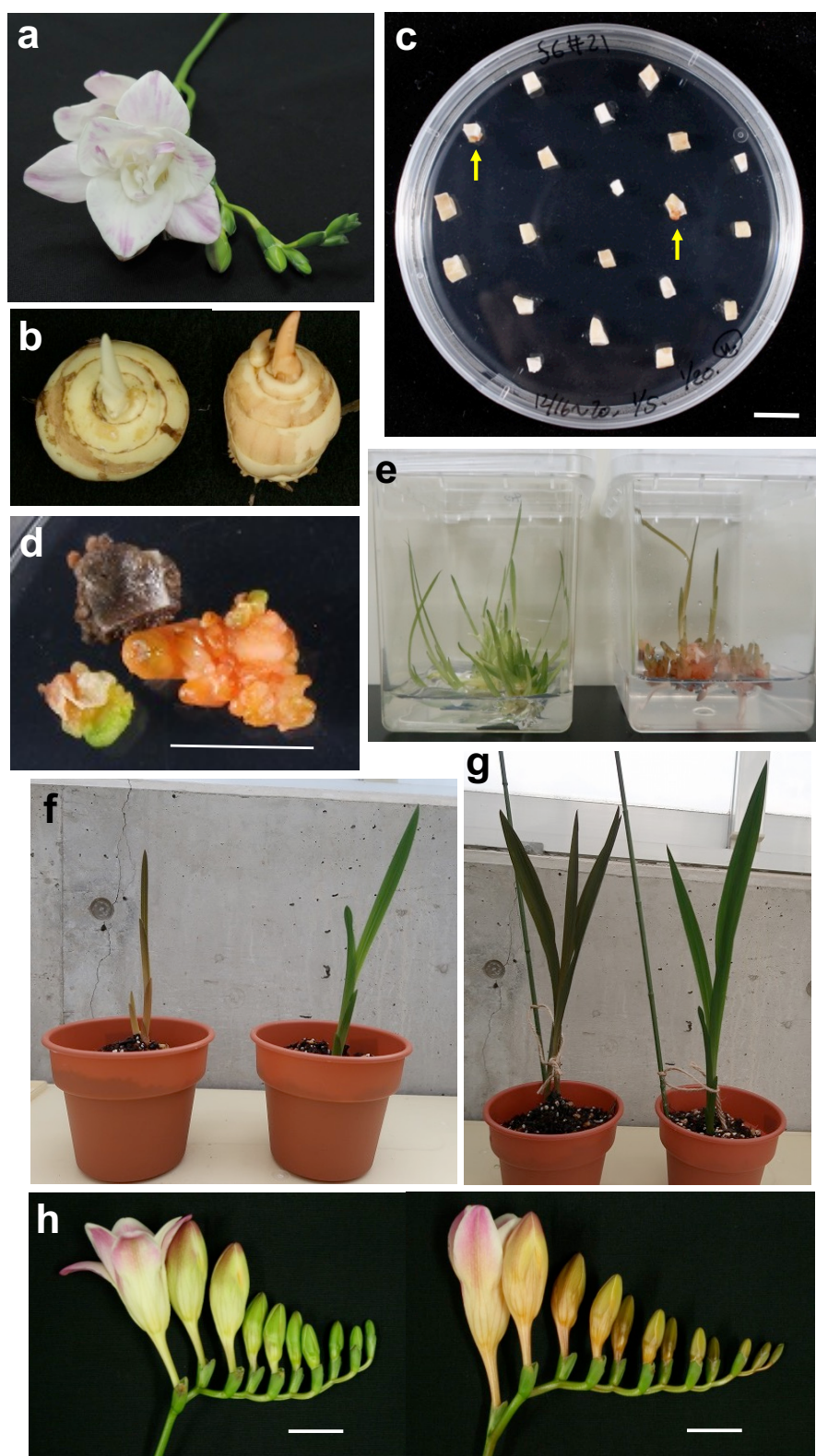


Fig. 1. Freesia 'Ishikawa f6' and construction of transgenic plants

a, the 'f6' flowers (wild type); **b**, the bulbs of the wild type (left) and transgenic (right) 'f6' plants; **c**, emergence of hygromycin resistant 'f6' calli (arrows) (bar =10 mm); **d**, hygromycin-resistant 'f6' calli containing adventitious buds (bar =10 mm); **e**, *in vitro* plantlets regenerated from the wild type (left) and hygromycin-resistant (right) 'f6' calli; **f** and **g**, appearance of the wild type (right) and transgenic (left) 'f6' plants after 3 weeks (**f**) and 5 weeks (**g**) of cultivation; **h**, the flowers of the wild type (left) and transgenic (right) 'f6' plants (bar =20 mm).

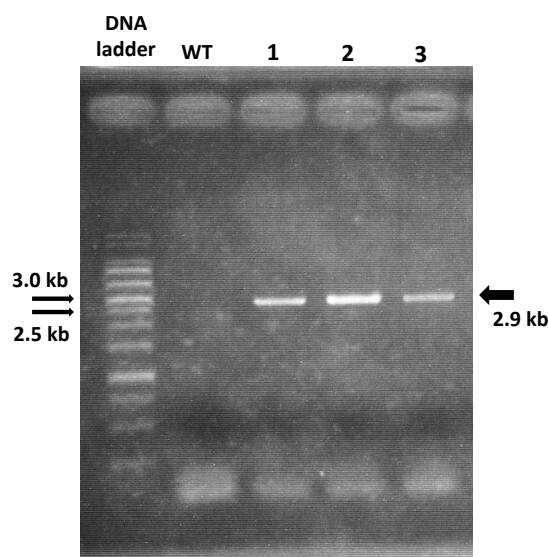


Fig. 2. PCR analysis for detection of the *crtZ* and *crtW* genes in three independent freesia 'f6' transformants lane WT, 'f6' wild type; lanes 1~3, independent 'f6' transformants #21-1, #21-2, and #21-3.

3.3 Confirmation of transgenic plants

Genomic DNA was extracted from the leaves of the non-transgenic plant and the regenerated hygromycin-resistant plants (#21-1, #21-2, and #21-3), which were regenerated from the orange callus. Using these extracts as templates, PCR was performed to detect the *crtZ* and *crtW* genes. Fragments, whose size is anticipated to be 2.9 kb, corresponding to the length from the *crtZ* start codon to the *crtW* stop codon, were detected from all the three putative transgenic plants (**Fig. 2**). It was thus confirmed that they were transgenic plants.

3.4 Analysis of carotenoids produced in the freesia 'f6' transgenic plants

Fig. 3 shows the result of HPLC analysis of the hygromycin-resistant callus [#21; callus in **Fig. 1e** (right)], i.e., cell cluster degenerated as the freesia 'f6' transformant. Interestingly, this callus was found to produce large amounts of astaxanthin including its esterified forms [63% of total carotenoids (32.2 µg/g fresh weight)] and free form (4.3%) in addition to other ketocarotenoids that contained fritschiellaxanthin (3%), 4-ketoantheraxanthin (1.6%), adonirubin (5.4%), adonixanthin (2%), and canthaxanthin (4.2%) (**Table 3**). Even the *in vitro* roots [roots in **Fig. 1e** (right)] accumulated high ratio of astaxanthin esters (**Table 3**).

The regenerated transgenic freesia plant exhibited bronze (reddish green) color, and its stems, infant and large flower buds were found to accumulate astaxanthin esterified forms [50.3%, 65.3% and 35.3% of total carotenoids (83.1, 17.9 and 21.6 µg/g fresh weight), respectively]. Especially, the ratios of astaxanthin diester were found to be higher levels in these organs of the regenerated plant than in those of the callus and *in vitro* roots, probably due to stronger endogenous esterification-enzyme activity.

On the other hand, the petals contained trace amounts of carotenoids (**Table 3**). It is presumably because of rapid reduction and/or degeneracy of the chloroplasts (plastids), in which carotenoids are produced.

Feasible carotenoid biosynthetic pathway in the freesia 'f6' #21 callus and the transgenic plant regenerated from this callus are shown in **Fig. 4**. Our study indicated that the carotenoid biosynthesis genes were efficiently functionally expressed in freesia plants, except for white color-base petals.

Freesia 'Ishikawa f2' is the variety with yellow flowers. Recently, we clarified that these yellow pigments are crocetin neapolitanosyl monoester and diester, which are synthesized from zeaxanthin [3]. In order to demonstrate the occurrence of flower color changes in freesia plants, we are now raising a transgenic freesia 'f2' plant, in which the astaxanthin biosynthesis genes were introduced and expressed.

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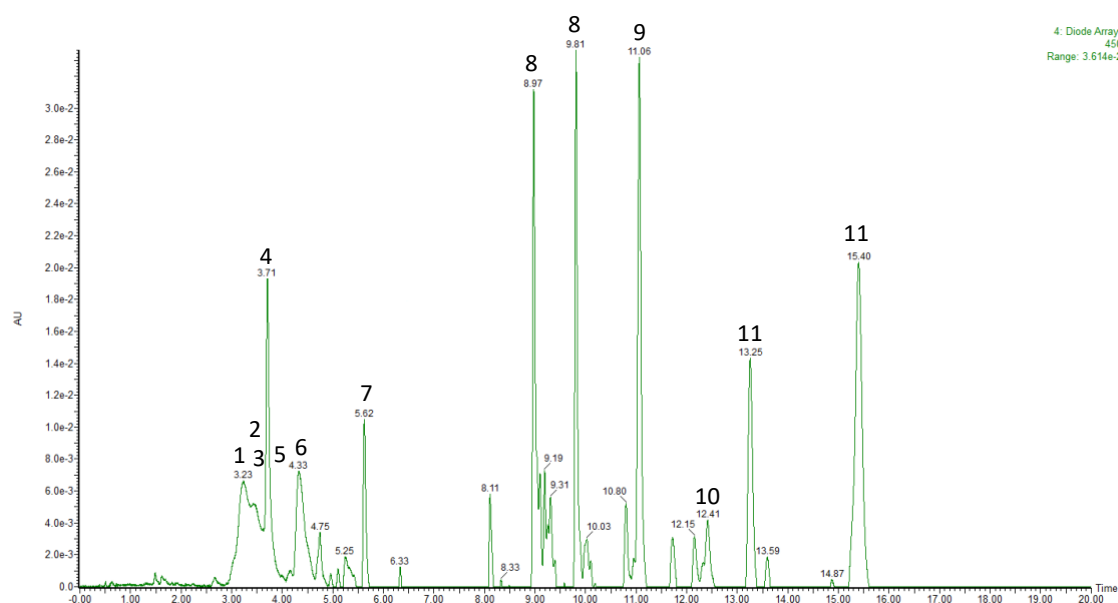


Fig. 3. HPLC chromatogram of the extract from freesia ‘f6’ transformant callus

1, astaxanthin (retention time, 3.23 min; per the total carotenoids, 4.3%); 2, fritschiellaxanthin (3.38 min; 3%); 3, adonixanthin (3.45 min; 2.0%); 4, lutein (3.69 min; 4.8%); 5, 4-ketoantheraxanthin (3.82 min; 1.6%); 6, adonirubin (4.33 min; 5.4%); 7, canthaxanthin (5.62 min; 4.2%); 8, astaxanthin monoesters (8.97 and 9.81 min; 32.5%); 9, chlorophyll (11.06 min); 10, β -carotene (12.41 min; 1.2%); 11, astaxanthin diesters (13.25 and 15.40 min; 30.5%).

Table 3. Carotenoid content and composition of the transgenic #21 and wild type plants

bioactive plant	transgenic 421 plant						wild type plant					
plant organ	callus	in vitro root	stem	infant flower bud	large flower bud	petal	callus	in vitro root	stem	infant flower bud	large flower bud	petal
total carotenoid content (µg/g FW)	32.2	2.3	83.1	17.9	23.6	trace ^a	0.89	trace ^a	111.2	132.4	81.1	trace ^a
carotenoid composition (%)												
β-Carotene	1.2		34.2	39.2	26.3		38.9		26.4	26.6	33.2	
Lutein	4.8	16.1	15.5	5.2	18.9		45.5	57.1	56.1	48.6		
β-Cryptoxanthin			2.1	2.2	4.1			7.2	7.9	2.1		
Neoxanthin		1.0					10.4					
Epoxy-carotenoids ^{ab}			2.1	1.2	5.2			6.7	2.5	11.6		
Isolarone			1.5	0.5	0.5							
Canthaxanthin	4.2	2.6	3.2	2.2	2.2							
Adonixanthin	2.0	4.5										
γ-Ironin	5.4	1.0										
Antoxanthin	4.3	4.2										
Antoxanthin nonoxides	32.5	30.2	15.2	25.5	9.2							
Antoxanthin diester	30.5	30.0	35.1	39.8	30.1							
Princhedioxanthin	5.0											
6-Ketocarotenoxanthin	1.6											
others	10.5	9.8	1.1	3.2	7.9		5.2		8.6	7.3	4.5	

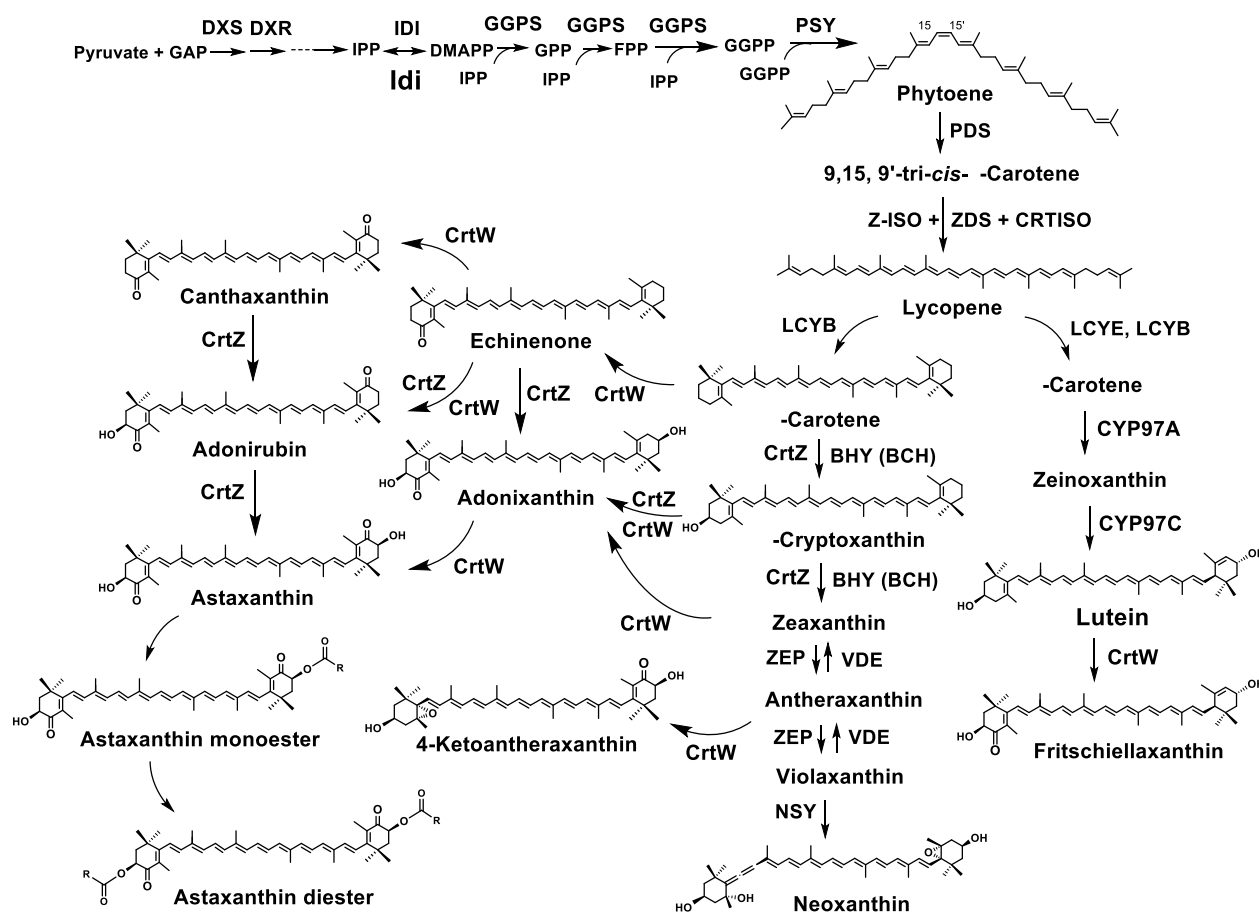


Fig. 4. Proposed carotenoid biosynthetic pathway in the freesia ‘f6’ transformed callus and transgenic plant.

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Oxidosqualene-based carotenoid pathway for the detection of triterpene cyclase activities

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Abstract: The carotenoid pathway is evolutionarily related to the triterpenoid pathway, and it is obvious that they diverged from the same ancestry. This indicates that they can potentially crosstalk with each other. Indeed, carotenoid pathway and triterpenoid pathway are spatially and/or temporally separated from each other in higher organisms. We have recently found that oxidosqualene, a common precursor of sterols and other compounds, can be a substrate for a carotenoid desaturase CrtN from *Staphylococcus aureus*, and co-expression of oxidosqualene pathway and CrtN result in the accumulation of yellow carotenoid pigments with novel structures. In this paper, we show that the additional expression of active oxidosqualene cyclases (OSCs) results in the consumption of oxidosqualene, resulting in the reduction of the level of the carotenoids accumulated in the cell. We also show that the extent of color recession was inversely correlated to the cellular activity of OSCs. This phenomenon could be potentially applied for the high-throughput screening for the genes encoding active OSCs.

1. Introduction

Most of the natural carotenoids have C40 backbone sizes, while some microbes produce carotenoids with a C30 backbone sizes [1]. The first committed step of the pathways for these carotenoids is the condensation of two molecules of farnesyl diphosphates (FPPs). The resultant 4,4'-diapophytoene or dehydrosqualene, is subjected to multi-step desaturation by CrtN, being converted into yellow pigments [2]. Previously, we have reported that squalene, the common precursor of >20,000 known triterpenoids [3], can be a substrate of CrtN from *Staphylococcus aureus* as well, and is converted into 4,4'-diaponeurosporene and 4,4'-diapolycopene [4]. Furthermore, we found that this enzyme (SaCrtN) is promiscuous enough that it can also convert oxidosqualene, the direct precursor of Eukaryote-type cyclic triterpenoids, into carotenoid pigments [5]. These results indicate that carotenoids and carotenoid-like pigments can be biosynthesized via multiple routes, and that carotenoid pathway can be established from at least three intermediates (FPP, squalene, and oxidosqualene) along the triterpene pathways as starters.

Sterols derived from animals exhibit various biological activities including antibiotic, anti-viral, and hormonal activities [6], while, saponins produced by plants exhibit other interesting functions as well, and are of industrial values as waxes, antibacterial, and anti-inflammatory drugs [7]. More than 100 unique

skeletal structures have been discovered from higher organisms, and all these structures are directly formed from a single substrate, oxidosqualene. The cyclization reactions of oxidosqualene are catalyzed by the enzymes, oxidosqualene cyclases (OSCs), and they proceed through a complex cascade of carbocation migration/sequestering/quenching. This single enzyme reaction result in the multiple formation of C-C bonds at a time, and the small variation in the shape and/or electrostatic microenvironment in the reaction pocket result in the drastic change in the cascade, yielding nearly 100 unique product types [8]. Despite half a century of research efforts on the mechanisms of this complex reaction cascade, it is currently extremely difficult, if not impossible, to predict how a particular product will be produced by given structure of OSCs. Currently, only a handful fraction of biosynthetic pathways of known cyclic steroids and saponins have been fully elucidated [9–11]. Indeed, even for the OSCs, the first committed enzyme of these pathways, very limited repertoire are available so far. While β -amyrin synthase and lupeol synthases have been found in many species, dammarenydiol synthase has been found only in ginseng [12]. OSCs with high product specificity are limited, and OSCs that specifically form α -amyrin and taraxasterol as a single product have not been found yet [7]. Even when available OSCs have been identified, it is not clear whether they function properly in heterologous cells such as *Escherichia coli*. The microbial production of dammarane reaches as much as 10 g/L [13], the production level of other triterpenoids remains traces.

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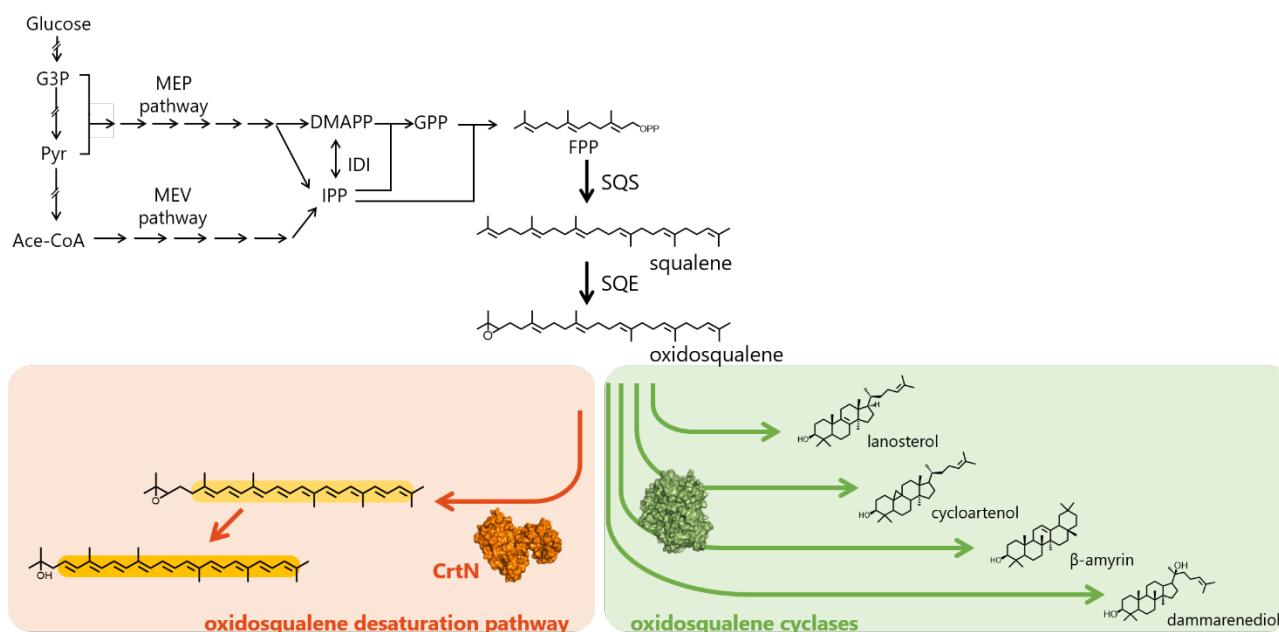


Fig. 1 Carotenoid pathway compete with OSCs for oxidosqualene precursor.

For the discovery, elucidation of the detailed cyclization mechanism, and improvement of the enzymatic properties of OSCs, it is desirable to perform high-throughput analysis of OSC candidates and/or variants. Because both of substrate (oxidosqualene) and products of OSCs are invisible, the enzymatic activity of OSCs must be analyzed by chromatography, thereby making it difficult to conduct high-throughput analysis of their activities.

In this paper, we report that the pigment pathway branched out from oxidosqualene competes with OSCs for the common precursor oxidosqualene. When co-expressed, this pathway reduces its level of pigmentation, according to the cellular activity of OSCs. By using this phenomenon as a screening principle, the cellular activity of OSCs, could have been visualized, for the first time, in a high-throughput manner without the use of chromatography.

2. Materials and Methods

2.1 Bacterial strains and plasmids used in this study

E. coli XL10-Gold (Stratagene) was used for cloning, while XL1-Blue (Stratagene) was used for production experiments. Reagents used were 50 mg/mL carbenicillin (carb), 30 mg/mL chloramphenicol (cm), and 20 mg/mL kanamycin (kan) purchased from Nacalai Tesque (Kyoto, Japan). LB-Lennox broth was purchased from Nacalai Tesque. Terrific Broth was purchased from BD Biosciences (NJ, USA).

Plasmids used in this study were showed in Table 1. ScLSS was PCR-amplified from the yeast genome and the resulting DNA fragment was cloned into the pJ404 vector [14], which contains the T5/lacO

promoter. PgDDS was purchased optimized for *E. coli* codons, and the two OSC genes (EtAS, AtCAS) were transferred to the above vector from sequences used in a previous report [15]. The coding regions of the *A. thaliana* LSS gene (AtLSS) were amplified by PCR with Arabidopsis stem cDNA as a template.

2.2 Culture medium and cultivation conditions

Plasmids were transformed into XL1-Blue cells, which were then plated on LB-Lennox agar plates and incubated at 37°C overnight. For liquid culture experiments, fresh colonies were inoculated with 2-mL LB-Lennox medium in 48-well (deep-well) plates and shaken at 37°C for 16 h. The cultures were then diluted 100-fold into 10 mL of fresh Terrific Broth medium in 50-mL flasks and shaken at 30°C at 200 rpm for 48 h. For cell pellet color development, fresh colonies were inoculated with 2-mL LB-Lennox medium in 48-well (deep-well) plates and shaken at 37°C for 16 h. The cultures were then diluted 100-fold into 10 mL of fresh Terrific Broth medium in 50-mL flasks and shaken at 30°C at 200 rpm for 40 h.

2.3 Triterpenoid extraction and HPLC analysis

7.5 mL of each cell culture were centrifuged at $3,300 \times g$ at 4°C for 15 min. The cell pellets were washed with 10 mL of 0.9% (w/v) NaCl and then re-pelleted *via* centrifugation. Carotenoids were extracted by adding 10 mL of acetone, followed by vigorous shaking. One milliliter of hexane and 35 mL of 1% (w/v) NaCl were then added, and the samples were centrifuged at $3,300 \times g$ for 15 min, after which the carotenoid-containing hexane phase was collected. The hexane was then allowed to evaporate *via* vacuum-drying. The dried samples were dissolved in 100 μ L of

Table 1 plasmids used in this study

name	Ori / Marker	construct
pSC- <i>sqs</i>	pSC101 /Kan ^R	P _{lac::lacO} - <i>sqs</i>
pAC- <i>idi-ispA-sqs-sqe</i>	P15A /Cm ^R	P _{lac::lacO} - <i>idi-ispA-sqs-P_{J23116}-sqe</i>
pAC-pJ23116- <i>sqe-crtN</i>	p15A /Cm ^R	P _{J23116} - <i>sqe-crtN</i>
pJ404-empty	pMB1/Amp ^R	
pJ404- <i>ScLss</i>	pMB1/Amp ^R	P _{T5/lacO} - <i>ScLss</i>
pJ404- <i>AtLss</i>	pMB1/Amp ^R	P _{T5/lacO} - <i>AtLss</i>
pJ404- <i>Atcas</i>	pMB1/Amp ^R	P _{T5/lacO} - <i>Atcas</i>
pJ404- <i>Etas</i>	pMB1/Amp ^R	P _{T5/lacO} - <i>Etas</i>
pJ404- <i>Pgdds</i>	pMB1/Amp ^R	P _{T5/lacO} - <i>Pgdds</i>

of (6:4) tetrahydrofuran/methanol immediately before HPLC analysis. An aliquot of 2 μ L of the final extract was analyzed using a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector and mass spectrometer. A Shim-pack FC-ODS column (75- $\times$ 2-mm I.D., 3- μ m particles; Shimadzu, Kyoto, Japan) was used. The mobile phase was methanol. The detector used was an APCI-MS. The MS was operated in selected ion monitoring mode at an interface temperature of 300°C, DL temperature of 300°C, interface voltage of $\pm$ 4,500 V, and neutral DL/Qarray with N₂ as the nebulizer gas.

2.4 carotenoid pigment analysis

7.5 mL of each cell culture were collected and centrifuged at 3,300 $\times$ g at 4°C for 15 min. The cell pellets were washed with 10 mL of 0.9% (w/v) NaCl and then re-pelleted by centrifugation. After brief vortex, 1 mL acetone was added to each of the cell pellets, and they were immediately vortexed for 10 min to extract carotenoids, followed by centrifugation. The absorbance spectra (350–550 nm at 5-nm intervals) were analyzed for acetone extracts using a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The total amount of carotenoid was calculated by measuring absorption at 440 nm, and using the molar extinction coefficient (ϵ) of C₄₀-neurosporene (147,000 M⁻¹ cm⁻¹ (1)). The production weights of carotenoids were then normalized to the dry cell weight (DCW) of each culture. The DCW was calculated using an OD₆₀₀-DCW calibration curve.

3. Results and Discussion

3.1 Cellular activities of Oxidosqualene cyclases.

Although various types of OSCs have been identified in nature, there are surprisingly few examples of successful expression of their functions in *E. coli* [13,15,16]. This is mainly because the *E. coli*

strain that feeds oxidosqualene, the substrate of this enzyme, has been unavailable. Based on the knowledge extracted from previous studies, we have developed a very robust and efficient producer strain of oxidosqualene [5].

In this study, we decided to use this strain to evaluate the cellular activity of five different OSCs from various sources. Included were lanosterol synthase from yeast (ScLSS), lanosterol synthase from Arabidopsis (AtLSS), cycloartenol synthase from Arabidopsis (AtCAS), β -Amyrin synthase from *Euphorbia tirucalli* (EtAS), and dammarenediol synthase (PgDDS) from *Panax ginseng*.

To feed substrates (oxidosqualene) to OSCs expressed in *E. coli*, it is necessary to express biosynthetic genes, squalene synthase (SQS) [17] and squalene epoxidase (SQE) [5,15], which is responsible for converting endogenous metabolite FPP to squalene and for squalene to oxidosqualene, respectively. In addition to the genes, we also decided to express genes for isopentenyl diphosphate isomerase (Idi) and farnesyl diphosphate synthase (IspA) from *E. coli*, both of which are known to increase the production level of carotenoids and terpenoids [18]. The cell expressing these four biosynthetic genes accumulated oxidosqualene almost as a sole product (**Fig. 2a**).

Five OSCs were independently expressed in this oxidosqualene-accumulating strain. From the extract from the cell expressing AtCAS, we observed a trace almost of oxidosqualene, and the new peak for cycloartenol appeared instead (**Fig. 2b**). From the extract of the cell expressing ScLSS, we observed small but distinct peak for remaining oxidosqualene, but together with the large peaks for lanosterol (**Fig. 2c**). The cell expressing AtLSS accumulated only a trace amount of lanosterol, and most of the oxidosqualene remained unconverted (**Fig. 2d**). From the cell expressing EtAS (**Fig. 2e**) and PgDDS (**Fig. 2f**), we observed no peaks for cyclic products (β -amyrin and dammarenediol, respectively).

It is interesting that the five tested OSCs exhibited this wide range of *E. coli* activities. In the previously reported work by Takemura et al. [15], the EtAS-expressing strains accumulated comparable amount of cyclic product with those expressing AtCAS expressing strains. Also, Li et al. successfully cyclized oxidosqualene using *E. coli*-expressed PgDDS [13]. In our construct, however, we could not detect any activity of EtAS (Fig. 2e) and PgDDS (Fig.2f). We do not exactly know where this discrepancy came from. But most likely, this could be due to that we adopted a weaker transcription promoter for the OSCs. In order to make the expression system compatible with cell growth, we chose the T5 promoter, while previous papers adopted viral (pT7) expression systems.

Whatever the reason is, our result indicated that the *E. coli* activity of OSCs are highly context-dependent and unpredictable.

3.2 Visualizing the cellular activities of OSCs

Previously, we reported that SaCrtN has desaturating activity not only on its cognate substrate diapophytoene but also on squalene. This promiscuous activity of SaCrtN enabled the yellow pigment formation from squalene [4]. Very recently, we found another promiscuous activity of SaCrtN; it can act on oxidosqualene and desaturated it to form yellow pigments with nine or ten conjugated double bonds (polyenes) (Fig. 1) [5].

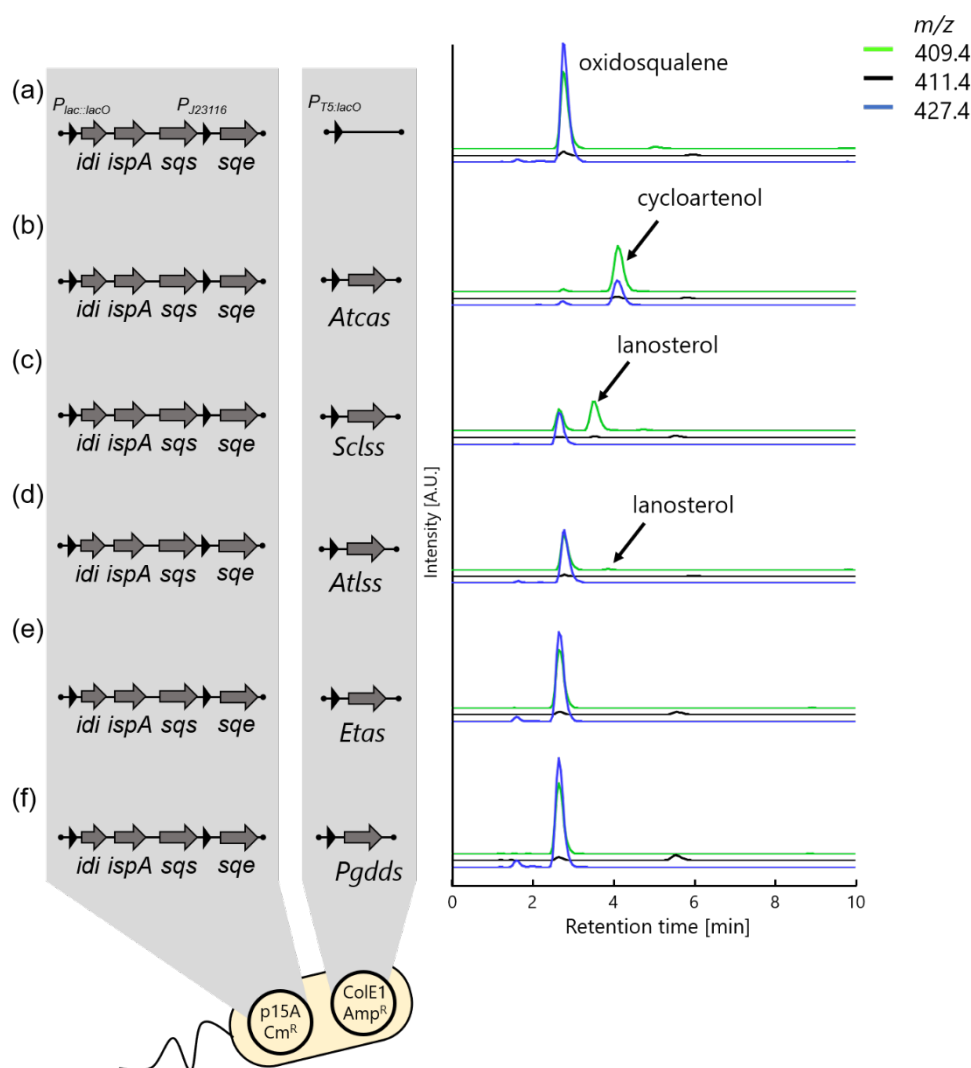


Fig. 2 In situ conversion of oxidosqualene by OSCs in *E. coli*. *E. coli* strain XL1 bule, expressing genes required for oxidosqualene accumulation, was transformed with either of (a) empty vector, (b) AtCAS-, (c) ScLSS-, (d) AtLSS-, (e) EtAS-, and (f) PgDDS-expressing plasmids. Each transformant cells were cultivated in TB medium for 48 h at 30°C, and the acetone-extracts were analyzed using HPLC/MS. Chromatograms in Green (m/z 409.4) corresponds to $[\text{sterol} + \text{H} - \text{H}_2\text{O}]^+$; those in black (m/z 411.4) for $[\text{squalene} + \text{H}]^+$; those in blue (m/z 427.4) for $[\text{oxidosqualene} + \text{H}]^+$.

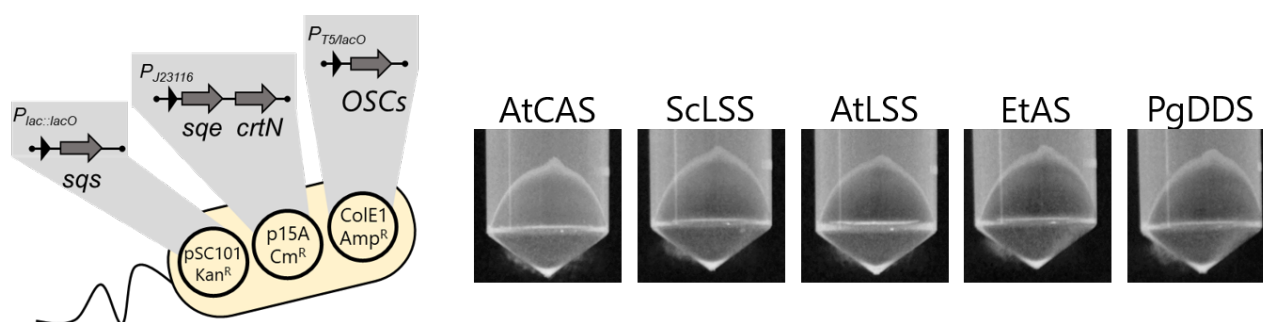


Fig. 3 The effects of the expression of oxidosqualene cyclase on the carotenoid production in *E. coli*. Cell pellet images were subdivided by the RBS channels, and the blue channel was shown.

We hypothesized that this desaturation activity of SaCrtN should compete for the common substrate oxidosqualene with eukaryotic OSCs, when co-expressed in the same cell. If the *E. coli* activity of OSCs is sufficiently high, the biosynthetic route from oxidosqualene to carotenoids should be reduced, which should be visualized as color recession of the host cells, regardless of the type of cyclization product. Visualizing substrate consumption using competition with carotenogenesis has been successfully implemented for the activity of Mono-, Sesqui-, and Diterpene synthases [19], and this system has been used as a screening principle to improve the activity of various terpene synthases/cyclases [14,20,21].

As a proof of principle, we examined how pigmentation by oxidosqualene-derived carotenoid pathway is affected by the co-expression of OSCs. To this end, we have constructed pAC-pJ23116-*sqa-crtN*. This plasmid was co-introduced with pSC-*sqs* and OSC-expressing plasmid into *E. coli* XL1Blue. Indeed, the resultant three-plasmid transformants exhibited a different level of pigmentation, depending on the OSC to be co-expressed (Fig. 3). AtCAS-expressing cells exhibited the faintest color, indicating that precursor for pigment formation was largely redirected to the formation of cycloartenol. The cell expressing ScLSS exhibited reduced color, too. The color of the cell expressing AtLSS, EtAS, and PgDDS was indistinguishable from the cell not expressing them. Note that these three OSCs are the ones that show little or no peaks for cyclized product of oxidosqualene (Fig. 2 d, e, f).

3.3 Visualizing the cellular activities of OSCs

Theoretically, the higher the activity of OSC, the less oxidosqualene remains and the lower the level of dye accumulation should be. Can we find a quantitative relationship between pigment production and OSC activity? We first measured the level of carotenoid pigments accumulated in cell harboring oxidosqualene-derived pigmentation pathway in the presence of the five OSCs (Carotenoid score). At the same time, we measured the production level of cyclic triterpenes accumulated in oxidosqualene biosynthesizing *E. coli*

upon expression of each of OSCs (OSC activity score). Carotenoid scores for the five OSCs are plotted against OSC activity scores (Fig. 4).

We observed a clear negative correlation between the carotenoid scores and the OSC activity scores. That is, carotenoid accumulation was significantly lower in the AtCAS-expressing strains, and its carotenoid accumulation level (carotenoid score) was about half that of those of the cells expressing EtAS and PgDDS, enzymes that failed to produce any cyclized products. A similar decrease in carotenoid score was observed also in the ScLSS-expressing strain, but the degree of pigmentation was intermediate between the AtCAS-expressing strain and the inactive OSCs-expressing strain. On the other hand, the expression of AtLAS, the enzyme that gave only trace amounts of cyclization products (Fig. 2d), showed only a slight, if any, decrease in carotenoid pigmentation.

4. Conclusions

The biosynthetic pathways of carotenoid pigments have long been valued as providers of valuable compounds [22], while they have also served as search tools for good host cells and/or screening probes carotenogenic pathways and enzymes with improved or altered specifications [1,23–25]. In addition, the carotenoid pathway has also been used for visualizing the enzyme activity of the neighboring pathways [19]. In this study, we showed, for the first time, that the carotenoid pathway via oxidosqualene can also be used for the activity probe of oxidosqualene cyclase.

To establish microbial production of valued triterpenoids biosynthesized by plants and animals, it is necessary to search for highly active and selective OSCs, either from databases or from libraries created in the lab. Until now, the activity of OSCs could be scored/estimated only by comparing the peak size in chromatographic data. Our system allows researchers to rapidly estimate the cellular activity of OSCs simply by measuring the carotenoids pigmentation level, either by visually (Fig. 3) or by measuring the absorption of lipid-soluble fractions (Fig. 4). Thanks to the omission of the chromatographic process, a dramatic increase in throughput of measuring OSC activity is expected.

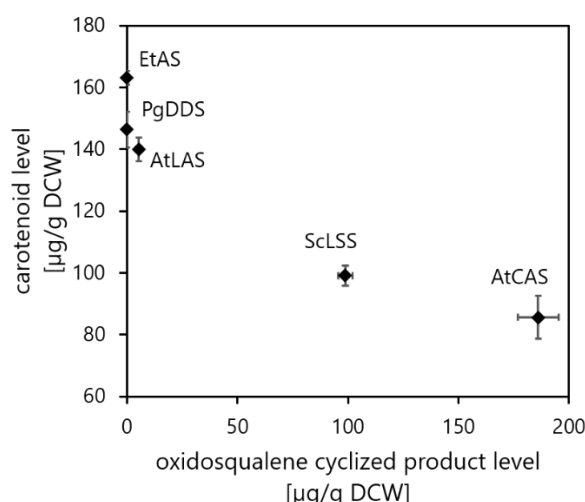


Fig. 4 Plot of carotenoid level versus oxidosqualene cyclized product level for OSCs.

Bars represent the average of three replicates; error bars represent $\pm$ standard deviation.

It should also be noted that this system is based on substrate consumption, not on the detection of a particular product. This visualization technology can be applied to any OSCs, regardless of the type of product. We expect that this system will greatly facilitate the discovery of new OSCs, improvement of their activities, and mutation analysis of this class of enzymes.

Finally, it should be noted that this screening system has some room for improvement. The pigmentation efficacy of oxidosqualene by CrtN was not very high, and we could not apply this system for eye-distinguishing colony colors of the cell harboring active/inactive OSCs. In order to develop a truly useful screening tool for OSCs, it is necessary to isolate CrtN homologues with higher oxidosqualene desaturating activity from databases or to create strong oxidosqualene-desaturating variants by evolution CrtN in the laboratory.

Acknowledgements

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Redesigning carotenoid-producing *Escherichia coli* to geranyl pyrophosphate accumulator

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Abstract: In attempt to establishing the pathways for carotenoids with smaller backbones, it is important to robustly accumulate geranyl pyrophosphate (GPP) in the cell. However, GPP is a good substrate of endogenous farnesyl pyrophosphate (FPP) synthase and is rapidly converted into FPP. Based on the previous report for yeast system, we designed and expressed genes for FPP synthase (IspA) mutants with both shifted size specificity and capacity to modulate pocket size of dimer partners in *Escherichia coli*. The *E. coli* strains expressing the *ispA* variant genes produced decreased amount of 4,4'-diaponeurosporene and lycopene, while increased level of monoterpene pinene, a metabolite of GPP.

1. Introduction

Most of the natural carotenoids are derived from phytoene, the condensation product of two molecules of geranylgeranyl pyrophosphates (GGPPs, C₂₀PP), while some bacterial carotenoids are synthesized *via* 4,4'-diapophytoene, which is made from two farnesyl pyrophosphates (FPPs, C₁₅PP). All the >750 natural carotenoids are biosynthesized from either of the above pathways. Using directed laboratory evolution, we have been expanding the repertoire of carotenoid backbones, especially toward those with larger sizes [1-4]. Encouraged by the success in creating numbers of new-to-nature carotenoids, we are turning our interest on establishing the pathways for carotenoids with smaller backbones (called apocarotenoids).

Before elaborating pathways to smaller carotenoids, we started to establish *Escherichia coli* strain that can robustly accumulate geranyl pyrophosphate (GPP, C₁₀PP). Unfortunately, however, this has been known to be difficult to achieve, because it is difficult to accumulate GPP; GPP is a good substrate of endogenous FPP synthase (IspA) and it is rapidly converted into FPP [5].

Indeed, this issue has been one of the biggest challenges for those who aim efficient production of monoterpenoids [6]. Knocking out of IspA is known to be lethal to the host cell, because FPP (the product of IspA) is essential precursor for ubiquinone and undecaprenol [7]. Attempts to reduce endogenous FPP synthase activity by introducing size-shifting mutations, but this did not lead to the increase in monoterpene production, probably due to the negative impact on

quinone- and cell wall-biosynthesis [8]. As of today, microbial monoterpene biosynthesis is attempted simply by maximizing metabolic flux to GPP [6,9,10-12], while preserving chromosomal IspA.

In an attempt to understand how product size specificity of FPPS is determined, Fernandez et al. identified numbers of amino acid substitutions that reduce the volume of reaction pocket of avian FPPS (AvFPPS) [13]. Among them, N144W was unique in that it does not decrease pocket size of its own but decrease the size of reaction pocket of the dimer-forming partner. Reaction pockets of prenyltransferases are known to be formed partially by residues provided by its binding partner [2][14].

Ignea *et al.* hypothesized that this provides golden opportunity to transiently controlling the size specificity of endogenous FPPS without modifying chromosomal FPPS gene. They showed plasmid expression of yeast FPPS (Erg20) with two mutations in yeast indeed lead to the threefold increase in the production of the monoterpene sabinene without affecting growth [15]. This FPPS mutant possesses two mutations F96W, N127W. Here, the former is for down-sizing reaction pocket of FPPS itself for converting it into GGPPs, while the latter mutation transiently decreasing the reaction pocket of dimer partner thereby converting the other side of heterodimer into GGPPs. By over-expressing this mutant, cells are dominated either by homodimer of Erg20 mutants and heterodimers of mutant/chromosomal Erg20s.

In this study, we tried to reproduce this brilliant strategy in *Escherichia coli*. We identified the amino acid mutation that does the same in IspA, thereby converting it as GGPPS as well as the transient specificity controller for endogenous, wildtype IspA (**Fig. 1**). Using this GPP-supplied *E. coli* strain, we confirmed

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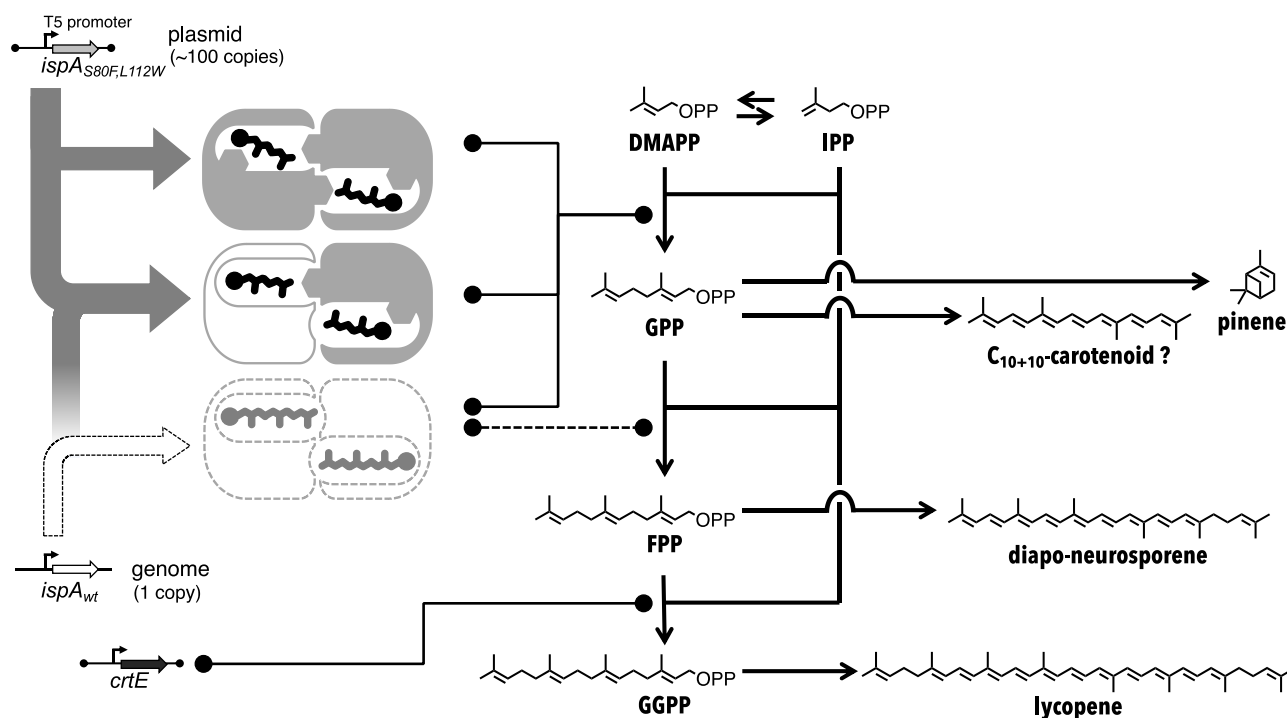


Fig. 1 Mutant of farnesyl pyrophosphate synthase (IspA) for accumulating GPP and for transiently altering specificity of endogenous IspA.

that the amounts of C₃₀-carotenoids was decreased, while the amounts of limonene (monoterpene) was increased.

2. Materials and Methods.

2.1 Bacterial strains and plasmids used in this study

E. coli XL1-Blue (Stratagene) was used for cloning and production experiments. Reagents used were 50 mg/mL carbenicillin (carb), 30 mg/mL chloramphenicol (cm), and 20 mg/mL kanamycin (kan) purchased from Nacalai Tesque (Kyoto, Japan). LB-Lennox broth was purchased from Nacalai Tesque. Terrific Broth was purchased from BD Biosciences (NJ, USA). For gene induction, 0.1 mM IPTG (Nacalai Tesque) was added to the culture media.

Plasmids used in this study were showed in Table 1. pJ404-*ispA*_{wt}, pJ404-*ispA*_{S80F}, pJ404-*ispA*_{S80F, L112W}, and pJ404-*Ptps*_{Q457L his-ispA}_{S80F, L112W} was newly constructed by PCR with pJ404-*Ptps*_{Q457L his-ispA}_{S80F} [17] as template. pJ211-MN were constructed by subcloning coding sequences for CrtM and CrtN from *Staphylococcus aureus* (codon-optimized for *E. coli*) into the pJ211 cloning vector by DNA 2.0 Inc. (Menlo Park, CA).

2.2 Analysis of carotenoid production.

Plasmids harboring *ispA* variants gene together with carotenoid-producing plasmids (pAC-LYC or

pJ211-MN) were transfected into XL1-Blue cells which were then plated on LB-agar plates and incubated at 37 °C for 16 h. Colonies were inoculated with 2-mL LB-Lennox medium in deep 48-well plate at 37 °C for 16 h. The cultures were then diluted 100-fold into 2 mL of fresh Terrific Broth medium in in deep 48-well plate and shaken at 30°C at 1000 rpm for 48 h. Cell were collected, washed with saline, centrifuged to obtain cell pellets (the supernatants were discarded). After vortexing, 1 mL acetone was added to each of the cell pellets, immediately followed by another vortex for 1 min and centrifugation. The absorbance spectra (300 nm-600 nm at 5 nm interval) of the resultant extracts were determined using a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, CA). The pigmentation level of each culture was determined from the lambda max (470 nm for C₃₀ carotenoids and 475 nm for C₄₀ carotenoids) of the resultant extract, using the molar absorption coefficients of diaponeurosporene (147,000 M⁻¹ cm⁻¹) or lycopene (185,000 M⁻¹ cm⁻¹).

2.3 Analysis of pinene production.

XL1-Blue was transformed with Pinene producing plasmids (pJ404-*Ptps*_{Q457L-his}, pJ404-*Ptps*_{Q457L-his-ispA}_{S80F}, or pJ404-*Ptps*_{Q457L-his-ispA}_{S80F,L112W}) and precursor booster plasmid (pMEV-idi) which were then plated on LB-agar plates and incubated at 37 °C for 24 h. Colonies were inoculated with 2-mL LB-Lennox medium in deep 48-well plate at 37 °C for 24 h. The cultures were then diluted 100-fold into 10 mL of fresh

Table 1 plasmids used in this study

name	Ori / Marker	construct	Ref
pJ404- <i>ispA</i> _{wt}	pMB1/Amp ^R	P _{T5/lacO} - <i>ispA</i> _{wt}	This study
pJ404- <i>ispA</i> _{S80F}	pMB1/Amp ^R	P _{T5/lacO} - <i>ispA</i> _{S80F}	This study
pJ404- <i>ispA</i> _{S80F, L112W}	pMB1/Amp ^R	P _{T5/lacO} - <i>ispA</i> _{S80F, L112W}	This study
pJ404- <i>Ptps</i> _{Q457L his}	pMB1/Amp ^R	P _{T5/lacO} - <i>Ptps</i> _{Q457L his}	[16]
pJ404- <i>Ptps</i> _{Q457L his-ispA} _{S80F}	pMB1/Amp ^R	P _{T5/lacO} - <i>Ptps</i> _{Q457L his-ispA} _{S80F}	[16]
pJ404- <i>Ptps</i> _{Q457L his-ispA} _{S80F, L112W}	pMB1/Amp ^R	P _{T5/lacO} - <i>Ptps</i> _{Q457L his-ispA} _{S80F, L112W}	This study
pJ404- <i>emp</i>	pMB1/Amp ^R	-	This study
pJ211-MN	P15A/Cm ^R	<i>crtM-crtN</i>	This study
pAC-LYC	P15A/Cm ^R	<i>crtE-crtI-crtB</i>	[17]
pMEV-idi	P15A/Cm ^R	<i>P_{lacUV/lacO}-atoB-hmgs-hmgr,</i> <i>p_{trc/lacO}-mk-pmk-pmd-idi</i>	[16]

Terrific Broth medium (4% glycerol) in 50-mL and shaken at 37°C at 200 rpm. Each culture was added with 100 µM IPTG when the cell density reached an OD₆₀₀ of ~0.4-0.7. Immediately after the induction, we overlaid 1 mL (10 v/v% of the original culture) of dodecane onto the cultures and shook them for an additional 24 h at 30 °C. Finally, 5 µL of dodecane fraction was collected and diluted in 495 µL of ethyl acetate.

Thus-obtained samples were analyzed by GC-FID (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) with an Rtx5 ms capillary column (30 m × 25 mm ID and 0.25 mm film thickness, Restek Corporation, Bellefonte, PA, USA). Split less injections (1 µL) were performed with an injector and an FID detector temperature of 250 °C and separated, respectively. GC oven-temperature program was: starting at 60 °C for 2 min, which was increased by 6 °C min⁻¹ up to 150 °C and then increased by 20 °C min⁻¹ until it reached 230 °C.

3. Results and Discussion.

3.1 Determination of amino acid substitution sites by sequence alignment and structural comparison

Eukaryotic FPP synthases are classified as type I synthases, while those from prokaryotes are classified as type II [18]. Indeed, the alignment of amino acid sequences (Fig. 2) showed that avian FPPS are far closer to other eukaryotic enzymes (including *Saccharomyces cerevisiae* Erg20) than those from prokaryotes (including *E. coli* IspA). Near the position corresponding to N144 in AvFPPS, we could not find N in the sequence of prokaryotic FPPSs. Because of that, we manually conduct gap refinement of the sequence alignment based on the structural alignment of each individual amino acids using AlphaFold2-generated model structure of IspA and previously reported crystal

structure of AvFPPS (PDB 1UBX) [19-20]. We estimated that the Leucine 112 of the IspA, would do the same role as specificity modulator of dimer partner (Fig. 3). The model structure shows that Leucine 112 forms the substrate pocket of the dimer partner. By substitution of Leucine 112 to Tryptophan in the dimer partner, IspA seemed to reduce its cavity volume. We constructed IspA mutant with this mutation (L112W) together with the other mutation S80F, which is a tried and proven mutation that reduces its own reaction pocket and convert FPPS into GPPS [21].

3.2 Effects of expressing *IspA*_{S80F, L112W} on *C*₃₀ carotenoid biosynthesis

If overexpression of *ispA*_{S80F,L112W} significantly reduces homodimer of wild-type IspA (Fig.1), thereby reducing the GPP-to-FPP activity in the cell, the biosynthesis of carotenoids should be reduced upon *ispA*_{S80F,L112W} expression. To test the hypothesis above, we examined the effect of expressing *IspA*_{S80F,L112W} on the biosynthetic pathway for 4,4'-diaponeurosporene: When *IspA*_{S80F, L112W} were co-expressed with *CrtM/CrtN*, the amount of 4,4'-diaponeurosporene was decreased by 47% compared to that without plasmid expressing IspA (Fig. 4a). These results indicate that the expression of *IspA*_{S80F, L112W} indeed decrease endogenous GPP-to-FPP activity.

Interestingly, we found that the expression of *IspA*_{S80F} also decreased 4,4'-diaponeurosporene. We found that the expression of *IspA*_{S80F} also decreased lycopene production. It is unlikely that dimer formation with *IspA*_{S80F} alters the size specificity of wildtype IspA. Rather, this reduction might be the result of down-regulation of total activity of wildtype-FPPS. Koyama *et al.* showed that the activity of the FPPS is decreased by dimerizing with variants with a mutation in the active site [12]. Most of the active site-mutations, including .

<i>Escherichia coli</i>	63	TNTLDAPAAAVECIHAYSLIHDDLPAMDDDDLRRLPTCHVKFGEAN-AILAGDALQTLAFSILSD	127
<i>Geobacillus stearothermophilus</i>	65	PAVGLPVACAIEMIHTYSLIHDDLPAMDNDLRRGKPTNHKVFGAM-AILAGDGLLTAFQLITE	129
<i>Staphylococcus aureus</i>	56	YELGMKSAIALEMIHTYSLIHDDLPAMDNDLRRGKLTNHKVYGEWT-AILAGDALLTKAFELIS	119
<i>Gallus gallus</i>	96	LRCALAVGWCIELFQAASLVADDI--MDQSLTRRGQLCWYKKEGVGLDAINDSFLESSVYRVLKK	159
<i>Saccharomyces cerevisiae</i>	79	YEKVAIILGWCIELLQAYFLVADDI--MDKSITRRGQPCWYKVPEVGEIAINDAFMLEAAIYKLLKS	142
<i>Rattus rattus</i>	82	LQRALMVGWCVELLQAFFLVDDI--MDSSHTRRGQICWYQKPGIGLNAINDALLLEACIYRLLKF	145
<i>Homo sapiens</i>	82	LQRAWTVGWCVELLQAFFLVADDI--MDSSLTRRGQICWYQKPGVGLDAINDANLLEACIYRLLKL	145
		Helix 3	Helix 4

Fig. 2. Multiple alignment of eukaryotic and prokaryotic FPPS. Gene bank numbers: HAN2190731 (*Escherichia coli*, *ispA*), WP_033016440 (*Geobacillus stearothermophilus*, *fds*), MBR9561365 (*Staphylococcus aureus*, *fds*), 1UBX_A (*Gallus gallus*, *fds*), NP_012368 (*Saccharomyces cerevisiae*, *erg20*), XP_032753921 (*Rattus rattus*, *fds*), 4DEM_F (*Homo sapiens*, *fds*). Asterisk indicates the partner-modulating mutations reported and those tested in this work (L122W).

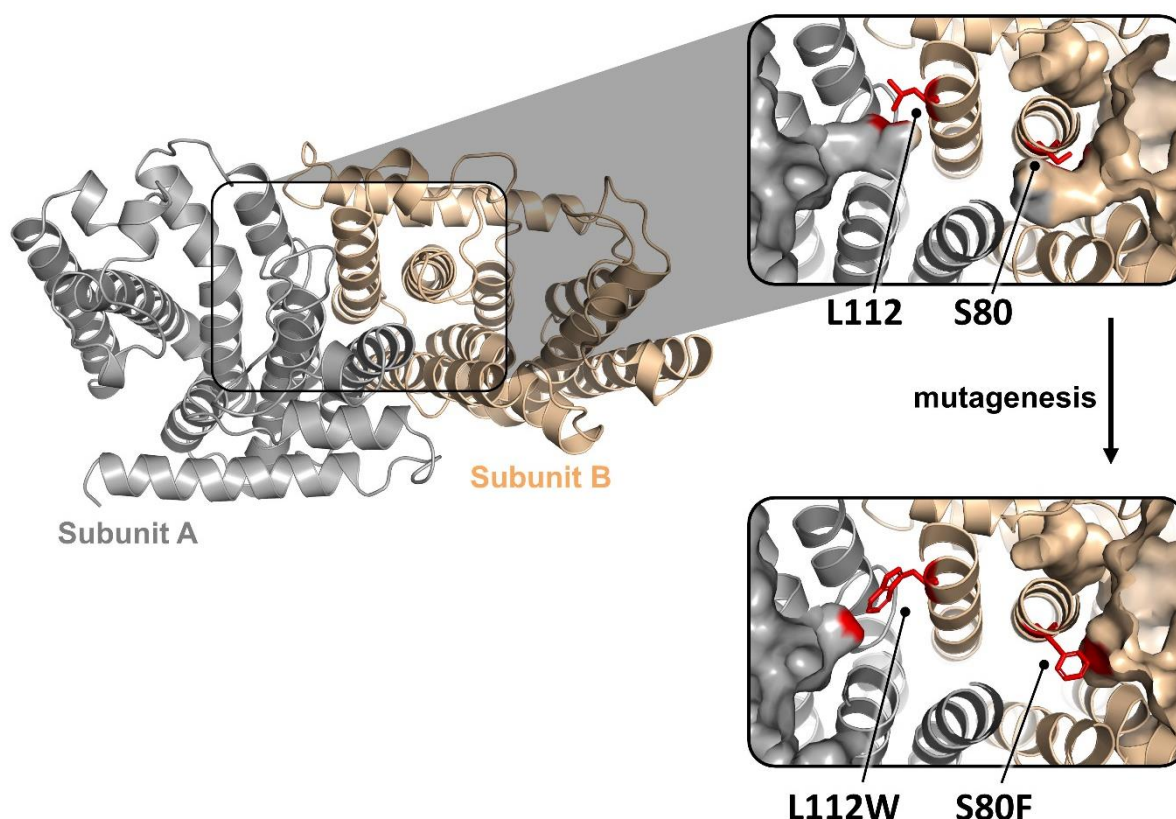


Figure 3. Modeled structure of IspA mutants and the putative effect of L112W. Model was constructed with Alpha Fold 2 [19-20]. L112 consists the floor of reaction pocket of the dimer partner. By replacing this leucine to tryptophan, pocket volume of the dimer partner decreased.

S80F, are de-stabilizing, and dimer formation with such de-stabilized partner should decrease the half-life of the individual IspA(wt) in the cell.

If mechanism shown in Fig.1 is on operation, transiently compromised activity for GPP-to-FPP should be complemented by heterologous expression of other prenyltransferases. Indeed, the degree of reduction of lycopene biosynthesis (CrtE-CrtB-CrtI, 24%) was not as significant as that for 4,4'-diaponeurosporene (47%) (Fig. 4b). CrtE is functionally annotated to be prenyltransferase responsible for the GGPP production from FPP, but it seems to partially complement GPP-to-FPP activity of the host cell impaired by expressing

IspA_{S80F, L112W} [22].

3.3 Effects of IspA_{S80F, L112W} expression on pinene biosynthesis

By decreasing the cellular activity to convert GPP into FPP, cellular availability of GPP (C₁₀PP) must be significantly elevated. To confirm this, we examined the productivity of biosynthetic pathway of pinene, the GPP-derived monoterpene, in the presence of IspA variants (Fig. 5). In the absence of IspA mutants, the production level of pinene was negligible. Pinene production was markedly increased by expressing either of IspA_{S80F} or IspA_{S80F, L112W}. When compared, pinene

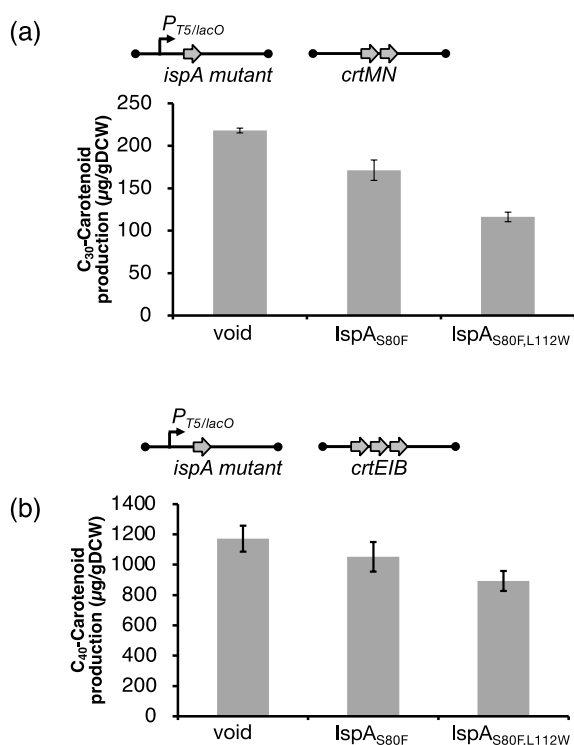


Fig. 4. Impact of the expression of IspA variants on carotenoid production. IspA and its variants were co-expressed with (a) 4,4'-diaponeurosporene biosynthetic pathway or (b) lycopene biosynthetic pathway. The transformant harboring indicated plasmids were grown, harvested, and evaluated the level of carotenoids accumulated.

production of the strain harboring IspA_{S80F, L112W} was 30% higher than those expressing IspA_{S80F}. Here again, IspA_{S80F, L112W} was shown to be effective in GPP accumulation, probably by transiently converting *ispA* to GPP synthesizer, rather than its consumer.

Conclusions

Carotenoid biosynthesis is “secondary” to various non-photosynthetic organisms, while their precursor isoprenyl pyrophosphates are “primary”. Especially, FPP is indispensable for all living organism, and its intermediate GPP is difficult to accumulate within the cell. This has been hampering the high-titer production of monoterpenoids [6] for years, and probably for the establishment of novel smaller carotenoids such as C₂₀ carotenoids. As of today, such pathways are yet to be found, both in nature and in the lab. However, there are known examples of the carotenoid synthase-like enzymes that can accept dimethylallyl pyrophosphate (DMAPP, C₅PP) [23] and geranyl pyrophosphate (GPP, C₁₀PP) [24] in nature. Thus, it is biochemically obvious that laboratory evolution would expand the scope of carotenoid kingdom to the mini (C₂₀) carotenoids. The GPP accumulating strain described herein should be useful precursor feeder of these novel carotenoids.

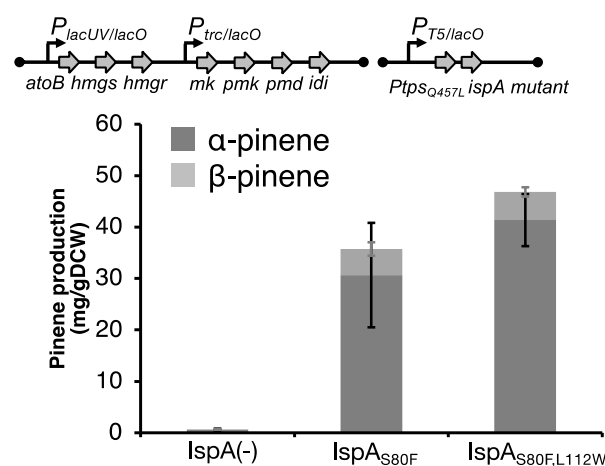


Figure 5. Effect of expressing IspA variants on pinene biosynthesis. The results are shown as the average of six biological replicates; error bars represent the standard deviation from the mean.

Acknowledgments

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Synthesis and Biological Evaluation of Heteroarotinoids with a 2*H*-Chromene Ring as Retinoid X Receptor-Selective Agonists

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Abstract: Retinoic acid analogs, in which the cyclohexene ring and adjacent double bond were replaced by a 2*H*-chromene ring, were synthesized by a palladium-catalyzed coupling reaction of 6-iod-2*H*-chromenes and trienylstannyl ester, and their biological activities were evaluated. None of the analogs exhibited transcriptional activities for the retinoic acid responsive element gene, however, 2- and 4-alkyl-2*H*-chromene analogs showed higher transcriptional activities for the retinoid X receptor responsive element gene than 9-*Z*-retinoic acid.

1. Introduction

β -Carotene is a good precursor for vitamin A because central degradation by an enzyme affords two molecules of retinal, which is an important metabolite of vitamin A and a chromophore for visual pigments [1, 2]. Other important vitamin A metabolites are retinoic acids, which are ligand molecules for nuclear receptors. All-*E*-retinoic acid (ATRA; **1**) and 9-*Z*-retinoic acid (9CRA; **2**) are natural ligands for retinoic acid receptors (RARs) α , β , and γ and retinoid X receptors (RXRs) α , β , and γ , respectively [3]. RARs and RXRs are members of the nuclear receptor superfamily and are involved in fundamental biological functions, including cell differentiation, cell proliferation, embryonic development, and apoptosis [4, 5].

RXRs form homodimers or heterodimers with other partners, such as RARs, liver X receptors, peroxisome proliferator-activated receptors, farnesoid X receptors, thyroid receptors, and vitamin D receptors. Over the past two decades, much research has focused on preparing receptor-selective ligand molecules to define the receptor functions and to develop therapeutic agents [6-8].

For our study on the structure-activity relationship of retinoic acid analogs [9-16], we prepared retinoic acid analogs in which the cyclohexene and adjacent

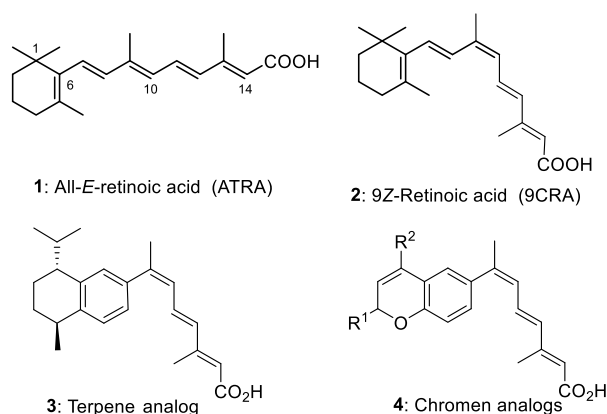


Figure 1. Structures of retinoic acid and its analogs.

double bond were replaced with a cyclic terpenoid moiety [17, 18]. The analog with the menthane skeleton (**3**) exhibited higher activity than 9CRA. Retinoic acid analogs are highly lipophilic, which causes undesirable side effects for clinical applications [6]. Although **3** has not yet been tested *in vivo*, it is expected that **3** will also have undesirable side effects due to high lipophilicity because it contains many carbon atoms. In this work, we prepared 9CRA analogs containing 2*H*-chromene skeleton **4** to decrease the lipophilicity by replacing carbon atoms with heteroatoms, and we evaluated their biological activity (**Fig. 1**).

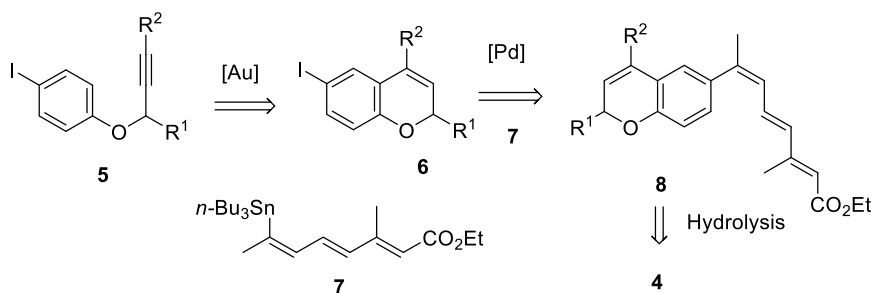


Figure 2. Synthetic pathway for chromene analogs.

2. Chemistry

The synthetic pathway of 2*H*-chromene analogs (**4**) is shown in **Fig. 2**. The key compounds are 6-iodochromenes (**6**), which were obtained by hydroarylation of 4-iodophenyl alkynyl ethers (**5**), the palladium-catalyzed coupling reaction with trienylstannylester (**7**) to give esters (**8**), and then basic hydrolysis of **8** to produce desired analogs (**4**).

4-Iodophenyl alkynyl ethers (**5**) were prepared by three methods (**Fig. 3**). Method I was a Mitsunobu reaction [19, 20] of 4-iodophenol (**9**) with a commercially available alcohol. Method II was a Sonogashira coupling reaction [21, 22] of 4-iodophenyl propargyl ether with an aryl iodide (**10**).

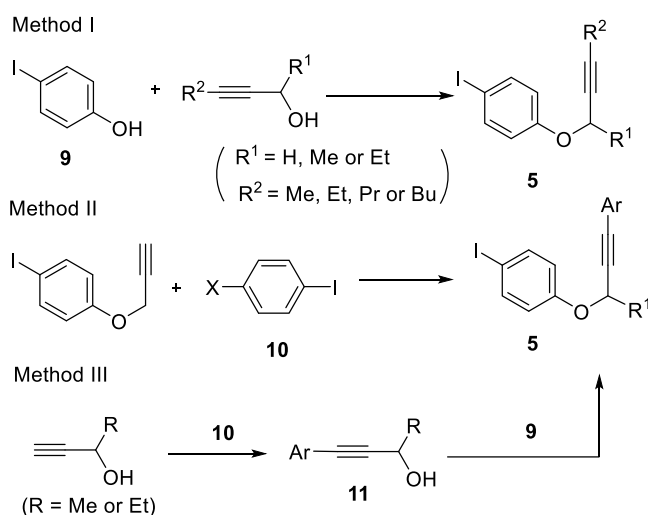


Figure 3. Preparation of iodophenyl propargyl ethers.

A large excess of the aryl iodide (5–10-fold excess) was used to reduce the dimeric product of 4-iodophenyl propargyl ether. Method III was a Mitsunobu reaction of 4-iodophenol with an (aryl)alkynyl alcohol (**11**) derived from the

corresponding alcohol by a Sonogashira coupling reaction. All reactions proceeded smoothly to afford the desired products in moderate to excellent yields (**Table 1**).

Hydroarylation of **5** using a gold catalyst [23, 24] gave 2*H*-chromene derivatives (**6**) in good to excellent yield. When R^1 was hydrogen and R^2 was an alkyl substituent in **5** (runs 2–4), isomeric products containing a double bond (**6'**) were obtained according to the bulkiness of the substituent (**Fig. 4**). Thus, the ratio of **6'** gradually increased with the number of carbon atoms in the alkyl chain. Because it was difficult to separate these products, the structures of cyclized products **6** and **6'** were confirmed after the next reaction. In contrast, when R^1 was Me or Et and R^2 was an alkyl substituent (runs 11, 12, and 18), 2*H*-chromene (**6**) was obtained as the sole product (**Table 1**).

A palladium-catalyzed coupling reaction of **6** with trienylstannyl ester (**7**) was easily accomplished by our procedure using CsF [14], giving the coupling products in moderate yields. The coupled products were finally hydrolyzed under basic aqueous conditions to afford desired 2*H*-chromene analogs (**4**) in good yields. The reaction yields are summarized in **Table 1**.

The results of the hydroarylation of **5** with the gold catalyst led us to propose the following plausible mechanism for the formation of **6** and **6'** (**Fig. 5**). The Au catalyst coordinates with the acetylenic bond and activates an electrophilic attack of the aromatic ring to generate an organoaurated intermediate [**A**]. Aromatization of [**A**] by deprotonation gives intermediate [**B**], and subsequent protonolysis of the

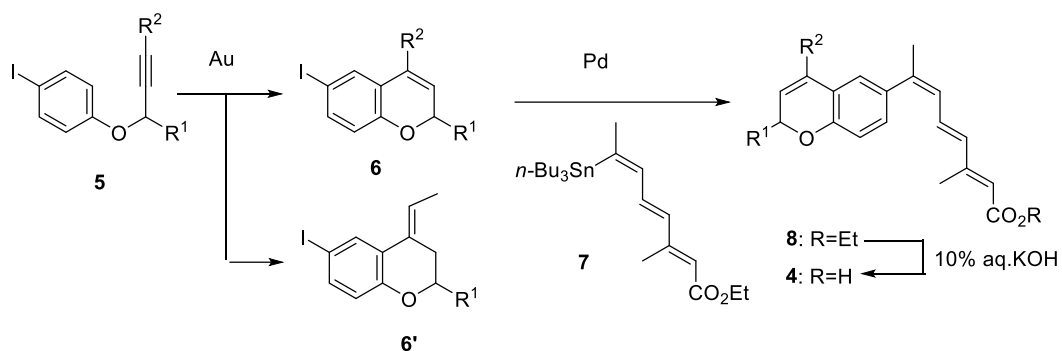


Figure 4. Preparation of retinoic acid analogs.

Table 1. Yields of reactions for the synthesis of retinoic acid analogs.

Run	R ¹	R ²	Yield of 5 ^a (method)	Yield of 6	Yield of 8	Yield of 4
1 (a)	H	Me	86 (I)	88 ^c	30	78
2 (b)	H	Et	91 (I)	92 ^d	44	82
3 (c)	H	Pr	87 (I)	93 ^e	48	78
4 (d)	H	Bu	92 (I)	93 ^f	57	72
5 (e)	H	Ph	70 ^b (II)	85	23	83
6 (f)	H	4-MeC ₆ H ₄	58 ^b (II)	74	38	88
7 (g)	H	4-MeOC ₆ H ₄	68 ^b (II)	84	33	89
8 (h)	H	4-MeCOC ₆ H ₄	67 ^b (II)	85	28	90
9 (i)	H	4-MeOCOC ₆ H ₄	75 ^b (II)	87	38	79 ^g
10 (j)	Me	H	90 (I)	86	69	87
11 (k)	Me	Me	82 (I)	94	59	92
12 (l)	Me	Et	89 (I)	50	57	94
13 (m)	Me	Ph	60 (III)	76	58	93
14 (n)	Me	4-MeC ₆ H ₄	64 (III)	76	46	80
15 (o)	Me	4-MeOC ₆ H ₄	41 (III)	74	68	76
16 (p)	Me	4-NO ₂ C ₆ H ₄	Quant. (I)	85	80	67
17 (q)	Et	H	49 (I)	41	47	76
18 (r)	Et	Me	83 (I)	81	68	89

^a Mitsunobu reaction yield. ^b Sonogashira reaction yield.

^c 6a:6'a = 100:0. ^d 6b:6'b = 94:6. ^e 6c:6'c = 87:13. ^f 6d:6'd = 67:33. ^g R² = 4-COOHC₆H₄.

carbon–gold bond affords expected product **6**. However, when R^2 is an alkyl substituent, 1,3-hydrogen shifts in **[B]** from the two allylic positions give organoaurated intermediates **[C]** and **[D]**, and the protic cleavage of the carbon–gold bond provides the geometric isomers **6'** and **6''**, respectively. Product **6''** is easily isomerized to **6** because the double bond conjugated with the phenyl group is more stable than that in the non-conjugated system.

When R^2 was methyl (runs 1, 11, and 18), no isomeric product **6'** was obtained because product **6'** ($R^2 = H$) with a disubstituted *exo*-conjugated double bond

bond was less stable than product **6** with a trisubstituted *endo*-conjugated double bond. When R^1 was methyl and R^2 was ethyl (run 12), expected product **6** was exclusively obtained because of the stability of the cationic species obtained from the 1,3-hydrogen shift intermediate. Thus, intermediate **[E]** was a tertiary cationic species and the cationic carbon was still stabilized by the adjacent oxygen atom, and therefore a 1,3-hydrogen shift from **[B]** to **[D]** occurred preferentially to give product **6''**, which was spontaneously isomerized to afford **6** as the sole product.

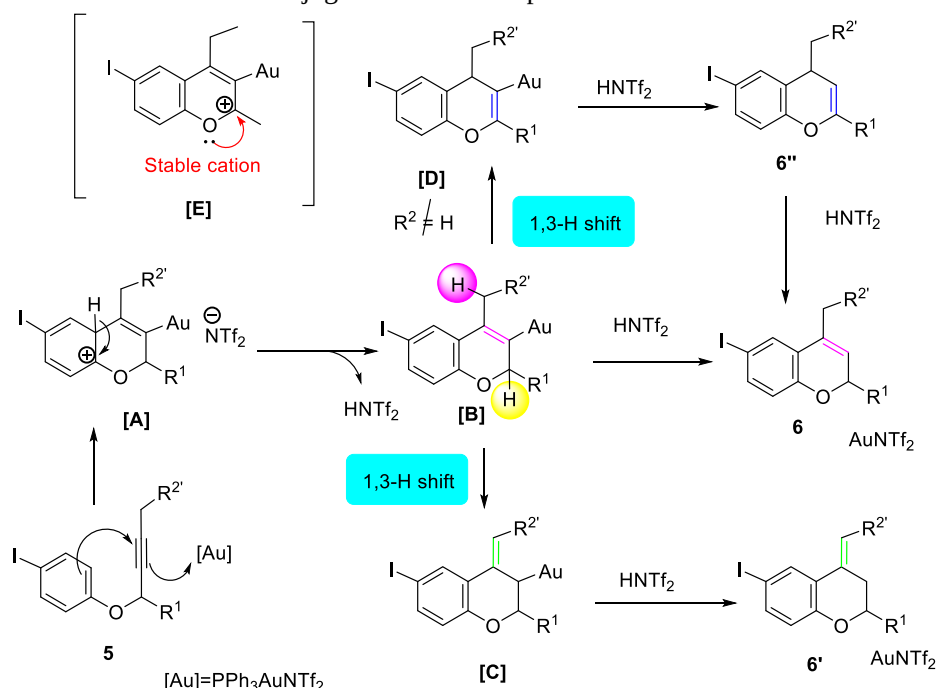


Figure 5. A plausible mechanism for hydroarylation.

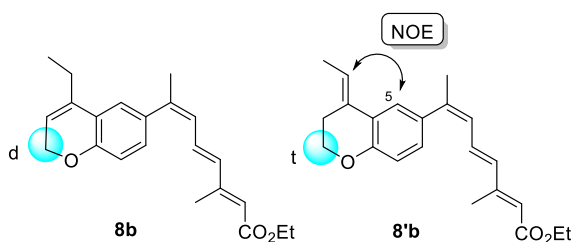


Figure 6. NOE correlation in the NMR spectra.

The structures of **6** and **6'** were determined from coupling products **8** and **8'** (Fig. 6). The olefin geometry of **8'** was determined as *E* based on an ^1H -NMR study. Thus, for **8'b**, an NOE correlation was observed between the *exo*-olefinic proton and the aromatic proton at position 5 of the *2H*-chromene ring.

3. Biological Activity

The retinoic acid analogs were evaluated in three transcriptional assays (Fig. 7). In the transcriptional assays for a human $\text{RAR}\beta$ gene, retinoic acid responsive element (*RARE*), none of the analogs exhibited higher activity than those of ATRA (**1**) and 9CRA (**2**) (Fig. 7A and B). The results of the transcriptional activity toward a rat CRABPII-retinoid X receptor responsive element gene (*RXRE*) luciferase reporter gene are shown in Fig. 7C and D. Among the H series of analogs ($R^1 = H$), all alkyl and some phenyl-substituted analogs (**4b–f**) showed higher activity than 9CRA (Fig. 7C). In the Me series ($R^1 = \text{Me}$) and Et series ($R^1 = \text{Et}$), the H and Me analogs (**4j**,

4k, **4q**, and **4r**) exhibited higher activity than 9CRA (**Fig. 7D**). To clarify whether RXRE transcriptional activity was reflected by the binding affinity of RXR, the analogs were tested in the RXR α binding assay using a RAR α -GAL4 hybrid luciferase assay (**Fig. 7E**

and F). In this assay, a trend almost the same as that in the RXRE assay was observed. In addition, the Me series of analogs (**4j**, **k**) were more active than the Et series of analogs (**4q**, **r**).

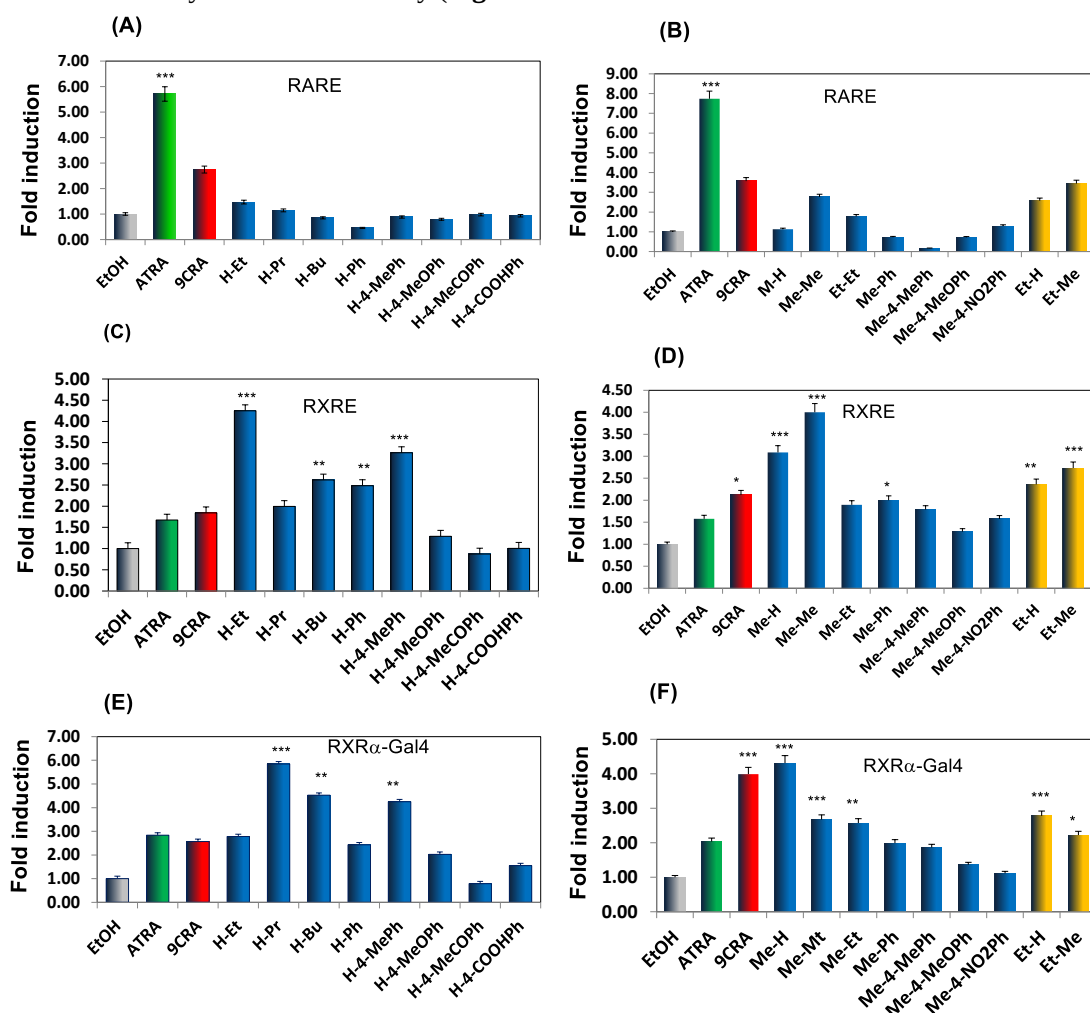


Figure 7. Transcriptional assay of analogs. (A), (C) (E) H series. (B), (D) (F) Me and Et series. Significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs EtOH), Dunnett's test.

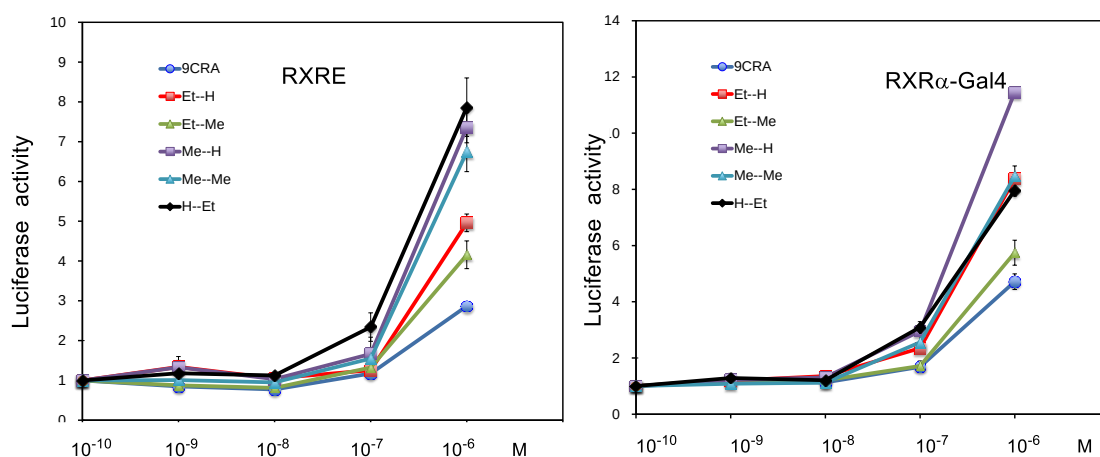


Figure 8. Dose-dependent transcriptional activities for RXRE (left) and RXR α -Gal4 (right).

Next, we performed a dose-dependent assay for RXRE and RXR α -GAL4 with selected analogs (**Fig. 8**). All selected analogs showed higher activity than that of 9CRA, the native ligand of RXR. In particular, in the RXRE assay, a characteristic feature was that the activity decreased as the number of carbon atoms in the R¹ and R² substituents increased (H-Et > Me-H > Me-Me > Et-H > Et-Me). In the RXR α -GAL4 assay, there were no remarkable characteristic features, and analog **4j** (Me-H) exhibited the highest activity among the selected analogs.

4. Conclusion

We developed a method for synthesizing heteroarotinoids in which the cyclohexene and adjacent double bond of retinoic acid were replaced with a 2H-chromene ring by using a palladium-catalyzed coupling reaction of 6-iodo-2H-chromens and trienylstannyl ester in the presence of CsF. In the structure-activity relationship study, the 2- and 4-alkyl-substituted analogs showed higher RXR transcriptional activities than 9CRA. Our findings provide useful information for the design of further analogs. We are currently conducting docking studies of these analogs and determining their isotype selectivity and efficacy in treating metabolic disease.

5. Experimental section

IR spectra were recorded on a Perkin-Elmer FT-IR Paragon 1000 spectrometer in CHCl₃. ¹H NMR spectra were obtained on a Varian Gemini-300 or a Varian VXR-500 superconducting FT-NMR spectrometer with tetramethylsilane as an internal standard in CDCl₃. ¹³C-NMR spectra were obtained on a Varian Gemini-300 (75 MHz) or a Varian VXR-500 (125 MHz) superconducting FT-NMR spectrometer in CDCl₃. Mass spectra were determined on a Hitachi M-4100 instrument. Column chromatography was performed using Kanto Silica Gel 60 N (spherical, neutral). All reactions were carried out under an argon atmosphere. Materials obtained from commercial suppliers were used without further purification. Standard workup means that the organic layers were finally washed with brine, dried over anhydrous sodium sulfate (Na₂SO₄), filtered, and concentrated in *vacuo* below 30 °C using a rotary evaporator.

5.1. General procedure for the Mitunobu reaction (GP1)

To a solution of 4-iodophenol (**11**, 1.0 eq.), 3-substituted propargyl alcohol (**6**, 1.1 eq.), and Ph₃P (1.0 eq.) in dry THF (6mL/mmol) was added diethylazodicarboxylate (DIAD, 1.0 eq.) at 0 °C. The solution was stirred at room temperature overnight then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to afford the propargyl ether (**5**).

5.2. General procedure for the gold-catalyzed hydroarylation (GP2)

To a solution of 4-iodophenyl 3-substituted propargyl ether (**5**, 1 mmol) in dry CH₂Cl₂ (5 mL) was added Ph₃PAuNTf₂ (Au-catalyst) at room temperature. The resulting solution was stirred for an additional 18 to 24 hours and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to afford the desired cyclic compound (**6**).

5.3. General procedure for the coupling reaction (GP3)

A mixture of the iodide (**6**, 1.3 eq.) and the stannanyl ester (**7**, 1.0 eq.) was dissolved in dry DMF (0.1 M), then Pd₂(dba)₃·CHCl₃, AsPh₃ (10 mol%) and CsF (2.0 eq.) were added. The flask was evacuated and refilled with argon five times. The mixture was stirred at 40-45 °C for 12-15 h, cooled to room temperature, and diluted with CH₂Cl₂ and water. After vigorous stirring, the mixture was filtered through Celite with CH₂Cl₂/AcOEt (1:1). The organic layer was separated, followed by standard workup. The residue was purified by column chromatography using neutralized SiO₂/powdered KF (9:1) to give coupling product (**8**). The data of isomeric products **8** and **8'** were shown.

5-3-1. Ethyl (2E,4E,6Z)-3-methyl-7-(4-ethyl-2H-chromen-6-yl)-2,4,6-octatrienoate (**8b**) and Ethyl (2E,4E,6Z)-3-methyl-7-(4-ethylidene-chroman-6-yl)-2,4,6-octatrienoate (**8'b**)

According to GP3, esters were prepared from **6b** (150 mg, 0.524 mmol), **7** (224 mg, 0.4 mmol), Pd₂(dba)₃·CHCl₃ (20.0 mg, 19.1 μmol), AsPPh₃ (23.2 mg, 76.4 μmol) and CsF (145 mg, 0.953 mmol) in 45%

yield (78.6 mg) as a pale red oil. HPLC separation afforded the pure esters **8b** (69 mg, 39%) and **8'b** (10 mg, 6%).

8b: ^1H NMR (600 MHz) δ : 7.07 (1H, d, J = 1.8 Hz), 7.03 (1H, dd, J = 8.4, 1.8 Hz), 6.81 (1H, d, J = 8.4 Hz), 6.77 (1H, dd, J = 15.6, 11.4 Hz), 6.25 (1H, d, J = 15.6 Hz), 6.21 (1H, d, J = 11.4 Hz), 5.74 (1H, s), 5.60 (1H, tt, J = 3.6, 1.8 Hz), 4.80 (2H, dt, J = 3.6, 1.8 Hz), 4.15 (2H, q, J = 7.2 Hz), 2.39 (2H, qq, J = 7.8, 1.8 Hz), 2.18 (3H, s), 2.16 (3H, s), 1.23 (3H, t, J = 7.2 Hz), 1.19 (3H, t, J = 7.8 Hz); ^{13}C NMR (150 MHz) δ : 167.21, 153.86, 152.92, 142.76, 135.35, 134.43, 133.72, 132.51, 128.62, 126.64, 123.25, 123.20, 118.35, 116.88, 115.76, 65.66, 59.62, 25.71, .23.93, 14.36, 13.86, 12.20; HR-EIMS calcd for $\text{C}_{22}\text{H}_{27}\text{O}_3$ ($\text{M}+\text{H}^+$): 339.1955. Found: 339.1957.

8'b: ^1H NMR (600 MHz) δ : 7.41 (1H, d, J = 1.8 Hz), 7.01 (1H, dd, J = 8.4, 1.8 Hz), 6.83 (1H, d, J = 8.4 Hz), 6.77 (1H, dd, J = 15.6, 10.8 Hz), 6.25 (1H, d, J = 15.6 Hz), 6.21 (1H, d, J = 10.8 Hz), 6.11 (1H, qt, J = 7.2, 1.8 Hz), 5.74 (1H, s), 4.23 (2H, t, J = 5.4 Hz), 4.15 (2H, q, J = 7.2 Hz), 2.65 (2H, t, J = 5.4 Hz), 2.18 (3H, s), 2.16 (3H, s), 1.79 (3H, d, J = 7.2 Hz), 1.28 (3H, t, J = 7.8 Hz); ^{13}C NMR (150 MHz) δ : 167.24, 153.56, 153.05, 142.92, 134.24, 133.32, 132.27, 128.14, 127.08, 126.58, 123.51, 122.74, 118.74, 118.26, 117.02, 66.34, 59.65, 25.69, .25.51, 14.36, 13.74, 13.27; HR-EIMS calcd for $\text{C}_{22}\text{H}_{27}\text{O}_3$ ($\text{M}+\text{H}^+$): 339.1955. Found: 339.1958.

5-3-2. Ethyl (2E,4E,6Z)-3-methyl-7-(4-propyl-2H-chromen-6-yl)-2,4,6-octatrienoate (8c) and Ethyl (2E,4E,6Z)-3-methyl-7-(4-propyldene-chroman-6-yl)-2,4,6-octatrienoate (8'c)

According to GP3, esters were prepared from **6c** (200 mg, 0.666 mmol), **7** (284 mg, 0.606 mmol), $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (25.1 mg, 24.3 μmol), AsPPh_3 (29.3 mg, 95.8 μmol) and CsF (184 mg, 1.21 mmol) in 48% yield (112 mg) as a pale red oil. HPLC separation afforded the pure esters **8c** (90.8 mg, 39%) and **8'c** (20.8 mg, 9%).

8c: ^1H NMR (600 MHz) δ : 7.05 (1H, d, J = 2.4 Hz), 7.02 (1H, dd, J = 8.4, 2.4 Hz), 6.81 (1H, d, J = 8.4 Hz), 6.76 (1H, dd, J = 15.6, 11.4 Hz), 6.25 (1H, d, J = 15.6 Hz), 6.21 (1H, d, J = 11.4 Hz), 5.74 (1H, s), 5.60 (1H, tt, J = 3.6, 1.2 Hz), 4.79 (2H, dt, J = 3.6, 1.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 2.34 (2H, tq, J = 7.8, 1.2 Hz), 2.18 (3H, s), 2.17 (3H, s), 1.57 (2H, sext, J = 7.8 Hz), 1.23 (3H, t, J = 7.2 Hz), 0.97 (3H, t, J = 7.8 Hz);

^{13}C NMR (150 MHz) δ : 167.19, 153.96, 152.91, 142.75, 134.47, 133.78, 133.70, 132.46, 128.57, 126.65, 123.37, 123.20, 118.33, 118.02, 115.79, 65.56, 59.60, 33.31, 25.73, .20.98, 14.35, 13.91, 13.83; HR-EIMS calcd for $\text{C}_{23}\text{H}_{29}\text{O}_3$ ($\text{M}+\text{H}^+$): 353.2111. Found: 353.2113.

8'c: ^1H NMR (600 MHz) δ : 7.43 (1H, d, J = 2.4 Hz), 7.17 (1H, dd, J = 8.4, 2.4 Hz), 6.83 (1H, d, J = 8.4 Hz), 6.78 (1H, dd, J = 15.6, 10.8 Hz), 6.25 (1H, d, J = 15.6 Hz), 6.22 (1H, d, J = 10.8 Hz), 6.02 (1H, tt, J = 7.2, 1.2 Hz), 5.74 (1H, s), 4.23 (2H, t, J = 6.0 Hz), 4.15 (2H, q, J = 7.2 Hz), 2.67 (2H, t, J = 6.0 Hz), 2.19 (2H, quint, J = 7.2 Hz), 2.18 (3H, s), 2.17 (3H, s), 1.28 (2H, t, J = 7.2 Hz), 1.05 (3H, t, J = 7.2 Hz); ^{13}C NMR (150 MHz) δ : 167.23, 153.71, 153.02, 142.88, 134.27, 133.27, 132.71, 128.18, 127.41, 126.59, 124.66, 123.55, 122.68, 118.17, 117.17, 66.45, 59.60, 25.69, 25.65, 20.98, 14.36, 14.08, 13.85; HR-EIMS calcd for $\text{C}_{23}\text{H}_{29}\text{O}_3$ ($\text{M}+\text{H}^+$): 353.2111. Found: 353.2116.

5-3-3. Ethyl (2E,4E,6Z)-3-methyl-7-(4-butyl-2H-chromen-6-yl)-2,4,6-octatrienoate (8d) and Ethyl (2E,4E,6Z)-3-methyl-7-(4-butyldene-chroman-6-yl)-2,4,6-octatrienoate (8'd)

According to GP3, esters were prepared from **6d** (300 mg, 0.955 mmol), **7** (408 mg, 0.869 mmol), $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (36.2 mg, 35.0 μmol), AsPPh_3 (43.0 mg, 140 μmol) and CsF (264 mg, 1.74 mmol) in 56% yield (112 mg) as a pale red oil. HPLC separation afforded the pure esters **8d** (81.0 mg, 40%) and **8'd** (20.8 mg, 16%).

8d: ^1H NMR (600 MHz) δ : 7.05 (1H, d, J = 1.8 Hz), 7.02 (1H, dd, J = 8.4, 1.8 Hz), 6.81 (1H, d, J = 8.4 Hz), 6.76 (1H, dd, J = 15.6, 11.4 Hz), 6.25 (1H, d, J = 15.6 Hz), 6.21 (1H, d, J = 11.4 Hz), 5.74 (1H, s), 5.60 (1H, tt, J = 4.2, 1.2 Hz), 4.79 (2H, dt, J = 4.2, 1.2 Hz), 4.15 (2H, q, J = 7.2 Hz), 2.36 (2H, tq, J = 7.8, 1.2 Hz), 2.18 (3H, s), 2.16 (3H, s), 1.55-1.49 (2H, m), 1.39 (2H, sext, J = 7.2 Hz), 1.23 (3H, t, J = 7.2 Hz), 0.92 (3H, t, J = 7.2 Hz); ^{13}C NMR (150 MHz) δ : 167.20, 153.95, 152.91, 142.76, 134.47, 134.03, 133.72, 132.47, 128.60, 126.66, 123.33, 123.26, 118.33, 117.86, 115.79, 65.58, 59.60, 30.86, 29.94, 25.74, .22.48, 14.36, 13.89 (2C); HR-EIMS calcd for $\text{C}_{24}\text{H}_{31}\text{O}_3$ ($\text{M}+\text{H}^+$): 367.2268. Found: 353.2273.

8'd: ^1H NMR (600 MHz) δ : 7.43 (1H, d, J = 1.8

Hz), 7.20 (1H, dd, $J = 8.4, 1.8$ Hz), 6.83 (1H, d, $J = 8.4$ Hz), 6.79 (1H, dd, $J = 15.6, 11.4$ Hz), 6.25 (1H, d, $J = 15.6$ Hz), 6.22 (1H, d, $J = 10.8$ Hz), 6.03 (1H, tt, $J = 7.8, 1.2$ Hz), 5.74 (1H, s), 4.23 (2H, t, $J = 6.0$ Hz), 4.15 (2H, q, $J = 7.2$ Hz), 2.67 (2H, t, $J = 6.0$ Hz), 2.19 (3H, s), 2.17 (3H, s), 2.16 (2H, qt, $J = 7.2$ Hz), 1.47 (2H, sext, $J = 7.2$ Hz), 1.28 (2H, t, $J = 7.2$ Hz), 0.94 (3H, t, $J = 7.2$ Hz); ^{13}C -NMR (150 MHz) δ : 167.23, 153.67, 153.024, 142.87, 134.29, 133.24, 132.70, 128.17, 128.09, 126.59, 123.61, 123.01, 122.71, 118.27, 117.18, 66.50, 59.59, 29.73, 25.79, 25.68, 22.77, 14.36, 13.86, 13.84; HR-EIMS calcd for $\text{C}_{24}\text{H}_{31}\text{O}_3$ ($\text{M}+\text{H}^+$): 367.2268. Found: 353.2273.

5.4. General procedure for the preparation of 9Z-retinoic acid analogs

A mixture of the ester (**8**, 1.0 equiv.) and 10% KOH (0.133 M) in ethanol (0.08 M) was heated at 50 °C for 3-6 hours. After cooling, the reaction mixture was made acidic with 5% HCl, and the organic compounds were extracted with ethyl acetate followed by standard workup. The residue was purified by flash column chromatography on silica gel to give the acid (**4**). Representative spectral data of analogs (Me-series and Et-series) were listed below.

5-4-1. (2E,4E,6Z)-3-Methyl-7-(2-methyl-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-H)

IR (CHCl_3) ν_{max} cm^{-1} : 2977, 1677, 1595, 1491; ^1H -NMR (300MHz, CDCl_3) δ : 7.00 (1H, dd, $J = 2.1, 8.4$), 6.86 (1H, brs), 6.82 (1H, d, $J = 11.1$), 6.77 (1H, dd, $J = 8.4, 4.5$), 6.37 (1H, dd, $J = 1.5, 9.75$), 6.27 (1H, d, $J = 15.3$), 6.21 (1H, d, $J = 11.1$), 5.76 (1H, s), 5.69 (1H, dd, $J = 3, 9.6$), 5.03~5.07 (1H, m), 2.19 (3H, s), 2.15 (3H, s), 1.47 (3H, d, $J = 6.6$); ^{13}C -NMR (75 MHz, CDCl_3) δ : 172.0, 155.6, 153.1, 143.3, 134.1, 133.6, 133.5, 129.2, 127.3, 126.5, 126.2, 123.5, 121.5, 117.3, 115.7, 71.7, 25.7, 21.5, 14.0; HR-ESIMS calcd for $\text{C}_{19}\text{H}_{21}\text{O}_3$ [$\text{M}+\text{H}$]: 297.1485. Found: 297.1483.

5-4-2. (2E,4E,6Z)-3-Methyl-7-(2,4-dimethyl-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-Me)

IR (CHCl_3) ν_{max} cm^{-1} : 3031, 1679, 1597, 1488; ^1H -NMR (300MHz, CDCl_3) δ : 7.02~7.06 (2H, m), 6.83 (1H, dd, $J = 11.4, 15.5$), 6.82 (1H, s), 6.28 (1H, d, $J = 15.9$), 6.23 (1H, d, $J = 12.9$), 5.76 (1H, s), 5.4 (1H, brs), 2.19 (3H, s), 2.18 (3H, s), 2.00 (3H, brs), 1.44

(3H, d, $J = 6.6$); ^{13}C -NMR (75 MHz, CDCl_3) δ : 172.0, 155.5, 153.2, 143.6, 134.0, 133.6, 133.3, 129.1, 128.9, 126.4, 124.1, 123.4, 123.2, 117.3, 115.7, 71.5, 25.7, 21.5, 18.0, 14.0; HR-ESIMS calcd for $\text{C}_{20}\text{H}_{22}\text{O}_3$ [$\text{M}+\text{H}$]: 311.1642. Found: 311.1640.

5-4-3. (2E,4E,6Z)-3-Methyl-7-(4-ethyl-2-methyl-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-Et)

IR (CHCl_3) ν_{max} cm^{-1} : 3525, 3008, 2973, 2931, 2468, 1680, 1594, 1492; ^1H -NMR (300MHz, CDCl_3) δ : 7.06 (1H, d, $J = 2.1$), 7.03 (1H, dd, $J = 1.8, 8.1$), 6.82 (1H, $J = 8.1$), 6.81 (1H, dd, $J = 11.1, 15.3$), 6.28 (1H, d, $J = 15.3$), 6.22 (1H, d, $J = 11.7$), 5.76 (1H, s), 5.48 (1H, brs), 4.99~5.00 (1H, m), 2.38 (2H, q, $J = 7.2$), 2.18 (3H, s), 2.17 (3H, s), 1.45 (3H, d, $J = 6.6$), 1.16 (3H, t, $J = 7.5$); ^{13}C -NMR (75 MHz, CDCl_3) δ : 171.8, 155.6, 153.4, 143.7, 134.6, 134.1, 133.6, 133.3, 128.6, 126.5, 123.1, 122.7, 122.1, 117.3, 115.9, 71.5, 25.8, 23.9, 21.5, 14.0, 12.2; HR-ESIMS calcd for $\text{C}_{21}\text{H}_{24}\text{IO}_3$ [$\text{M}+\text{H}$]: 325.1798. Found: 325.1801.

5-4-4. (2E,4E,6Z)-3-Methyl-7-(4-phenyl-2-methyl-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-4-Ph)

IR (CHCl_3) ν_{max} cm^{-1} : 3011, 2978, 1678, 1595, 1488; ^1H -NMR (300MHz, CDCl_3) δ : 7.35 (5H, m), 7.07 (1H, dd, $J = 2.1, 8.1$), 6.92 (1H, s), 6.89 (1H, brs), 6.74 (1H, dd, $J = 11.4, 15.3$), 6.22 (1H, d, $J = 1.5, 6.14$ (1H, d, $J = 11.1$), 5.75 (1H, s), 6.18 (1H, brs), 5.05~5.13 (1H, m), 2.19 (3H, s), 2.08 (3H, s), 1.55 (3H, d, $J = 6.6$); ^{13}C -NMR (75 MHz, CDCl_3) δ : 172.4, 155.5, 153.7, 143.4, 138.0, 136.1, 134.1, 133.5, 133.4, 129.2, 128.5, 128.4, 127.9, 126.5, 125.7, 125.6, 122.7, 117.3, 116.3, 71.5, 25.7, 21.1, 14.0; HR-ESIMS calcd for $\text{C}_{25}\text{H}_{24}\text{O}_3$ [$\text{M}+\text{H}$]: 373.1798. Found: 373.1792.

5-4-5. (2E,4E,6Z)-3-Methyl-7-(2-methyl-4-(4-methylphenyl)-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-4-MePh)

IR (CHCl_3) ν_{max} cm^{-1} : 3014, 2978, 2926, 1679, 1597, 1488; ^1H -NMR (300MHz, CDCl_3) δ : 7.16~7.26 (4H, m), 7.07 (1H, dd, $J = 2.4, 4.2$), 6.92 (1H, d, $J = 3.3$), 6.89 (1H, s), 6.75 (1H, dd, $J = 10.8, 15.2$), 6.25 (1H, s), 6.18 (1H, d, $J = 9.3$), 6.15 (1H, d, $J = 9.3$), 5.66 (1H, d, $J = 3.0$), 5.06~5.10 (1H, m), 2.38 (3H, s), 2.20 (3H, s), 2.09 (3H, s), 1.52 (3H, s); ^{13}C -NMR (75 MHz, CDCl_3) δ :

155.6, 153.8, 143.5, 137.7, 136.0, 135.0, 134.1, 133.5, 129.1, 128.4, 126.5, 125.6, 125.3, 122.8, 117.0, 116.2, 71.5, 25.7, 21.2, 21.1, 14.0; HR-ESIMS calcd for $C_{26}H_{26}O_3$ [M+H]: 387.1955. Found: 387.1955.

5-4-6. (2E,4E,6Z)-3-Methyl-7-(4-(4-methoxy-phenyl)-2-methyl-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-4-MeOPh)

IR (CHCl₃) $\nu_{\max}$ cm⁻¹: 3010, 1678, 1596, 1511, 1488; ¹H-NMR (300MHz, CDCl₃) δ : 7.24~7.27 (2H, m), 7.06 (1H, dd, J = 2.1, 8.1), 6.90 (2H, J = 8.7), 6.89 (1H, s), 6.77 (1H, dd, J = 11.1, 15.3), 6.22 (1H, d, J = 15), 6.15 (1H, J = 10.8), 5.75 (1H, s), 5.64 (1H, J = 3.3), 5.05~5.08 (1H, m), 2.19 (3H, brs), 2.08 (3H, s), 1.53 (3H, d, J = 6.6); ¹³C-NMR (75 MHz, CDCl₃) δ : 172.2, 159.3, 155.6, 153.8, 143.5, 135.7, 134.1, 133.4, 130.3, 129.6, 129.1, 126.5, 125.6, 125.0, 123.0, 117.2, 116.2, 113.8, 71.5, 55.3, 25.7, 21.1, 14.1; HR-ESIMS calcd for $C_{26}H_{27}IO_4$ [M+H]: 403.1904. Found: 403.1901.

5-4-7. (2E,4E,6Z)-3-Methyl-7-(2-methyl-4-(4-nitrophenyl)-2H-chromen-6-yl)octa-2,4,6-trienoic acid (Me-4-NO₂Ph)

IR (CHCl₃) $\nu_{\max}$ cm⁻¹: 3027, 1683, 1599, 1520, 1489, 1350, 1232; ¹H-NMR (300MHz, CDCl₃) δ : 8.25 (2H, d, J = 8.7), 7.51 (2H, d, J = 8.7), 7.11 (1H, dd, J = 2.1, 8.1), 6.94 (1H, d, J = 8.4), 6.77 (1H, d, J = 2.1), 6.69 (1H, dd, J = 11.1, 15.3), 6.23 (1H, d, J = 15.6), 6.17 (1H, d, J = 11.1), 5.78 (1H, d, J = 3.3), 5.76 (1H, s), 5.09~5.12 (1H, m), 2.18 (3H, s), 2.08 (3H, s), 1.57 (3H, d, J = 6.6); ¹³C-NMR (75 MHz, CDCl₃) δ : 171.7, 155.1, 153.6, 147.5, 144.8, 142.9, 134.8, 134.4, 133.9, 132.9, 130.0, 129.4, 127.5, 126.9, 124.8, 123.8, 121.8, 117.5, 116.6, 71.3, 25.7, 20.8, 14.1; HR-ESIMS calcd for $C_{25}H_{24}NO_5$ [M+H]: 418.1649. Found: 418.1649.

5-4-8. (2E,4E,6Z)-7-(2-Ethyl-2H-chromen-6-yl)-3-methylocta-2,4,6-trienoic acid (Et-H)

IR (CHCl₃) $\nu_{\max}$ cm⁻¹: 2973, 2932, 1680, 1594, 1492, 1257, 1236, 1192; ¹H-NMR (300MHz, CDCl₃) δ : 7.00 (1H, dd, J = 2.1, 8.4), 6.85 (1H, d, J = 2.4), 6.79 (1H, s), 6.78 (1H, dd, J = 3.6, 11.7), 6.39 (1H, d, J = 10.2), 6.26 (1H, d, J = 15), 6.21 (1H, d, J = 11.4), 5.76 (1H, s), 5.71 (1H, dd, J = 3, 9.9), 4.82~4.90 (1H, m), 2.19 (3H, s), 2.15 (3H, s), 1.76~1.84 (2H, m), 1.05

(3H, t, J = 7.5); ¹³C-NMR (75 MHz, CDCl₃) δ : 171.2, 155.6, 153.3, 143.4, 134.0, 133.6, 133.5, 129.2, 126.5, 126.2, 126.1, 123.9, 121.6, 117.1, 115.6, 68.2, 28.6, 25.7, 14.0, 9.2; HR-ESIMS calcd for $C_{20}H_{22}IO_3$ [M+H]: 311.1642. Found: 311.1642.

5-4-9. (2E,4E,6Z)-7-(2-Ethyl-4-methyl-2H-chromen-6-yl)-3-methylocta-2,4,6-trienoic acid (Et-Me)

IR (CHCl₃) $\nu_{\max}$ cm⁻¹: 3694, 3532, 2977, 2937, 2602, 1680, 1597, 1490; ¹H-NMR (300MHz, CDCl₃) δ : 7.02~7.05 (2H, m), 6.80~6.88 (2H, m), 6.28 (1H, d, J = 15.3), 6.23 (1H, d, J = 13.5), 5.76 (1H, s), 5.51~5.53 (1H, m), 4.75~4.78 (1H, m), 2.20 (3H, s), 2.18 (3H, s), 2.01 (3H, s), 1.72~1.81 (2H, m), 1.04 (3H, J = 7.2); ¹³C-NMR (75 MHz, CDCl₃) δ : 172.1, 155.6, 153.4, 143.6, 134.0, 133.6, 133.2, 129.3, 128.8, 126.4, 123.4, 123.4, 122.8, 117.3, 115.6, 28.6, 25.7, 18.1, 14.1, 9.3; HR-ESIMS calcd for $C_{21}H_{24}IO_3$ [M+H]: 325.1798. Found: 325.1803.

5.5. Biological assay

5-5-1. Transfection and luciferase activity assay (RARE)

Human osteosarcoma MG-63 cells, which are positive for RXR gene expression, were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 1% penicillin, 1% streptomycin, and 10% dextran-coated charcoal-treated FCS (Gibco BRL). The day before transfection, cells were seeded on six-well culture plates at a density of 2×10^5 cells per well, so that they were confluent on the day of transfection. The retinoid-responsive luciferase reporter construct, human RAR β -RARE3-SV40-Luc, was generated by cloning three copies of the RARE from the RAR β promoter (59/33: GGGTAAAGTTCACCGAAAGTTCACTCG). The pRL-CMV vector was used as an internal control. After transfection using the Tfx-50 reagent, the cells were incubated with retinoid (10^{-6} M) for 2 days. Luciferase activity of the cell lysates was measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation determined from the luciferase activity was standardized with the luciferase activity of the same cells measured with the Sea Pansy luciferase assay system (Toyo Ink Co., Ltd.) as a

control. Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as mean $\pm$ S.E.

5-5-2. Transfection and luciferase activity assay (RXRE)

Human osteosarcoma MG-63 cells, which are positive for RXR gene expression, were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 1% penicillin, 1% streptomycin, and 10% dextran-coated charcoal-treated FCS (Gibco BRL). The day before transfection, cells were seeded on six-well culture plates at a density of 2×10^5 cells per well so that they were confluent on the day of transfection. The retinoid-responsive luciferase reporter construct, rat CRBP-II-RXRE-SV40-Luc, was generated by cloning three copies of the RXRE from the rat CRBP-II promoter (639/605: GCTGTCACAGGTCACAGGTCACAGGTCACAGTTCA) in the pGL3 vector. The pRL-CMV vector was used as an internal control. After transfection using the Tfx-50 reagent, the cells were incubated with retinoids (10^{-6} M) for 2 days. Luciferase activity of the cell lysates was measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation determined from the luciferase activity was standardized with the luciferase activity of the same cells measured with the Sea Pansy luciferase assay system (Toyo Ink Co., Ltd.) as a control. Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as mean $\pm$ S.E.

5-5-3. Transfection and luciferase activity assay (RXR α -GAL4)

Human osteosarcoma MG-63 cells, which are positive for RXR gene expression, were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 1% penicillin, 1% streptomycin and 10% dextran-coated charcoal-treated FCS (Gibco BRL). Cells (2×10^5) were suspended in 2 mL of medium and transfected with 1.0 μ g of a one-hybrid plasmid (pM vector, Promega Corp., Madison, WI, USA) containing a human RXR α cDNA linked with a yeast GAL4 DNA-binding domain cDNA (GAL-DBD), 0.5 μ g of luciferase reporter plasmid (pGVP2 vector, Toyo Ink Co., Ltd.) containing GAL4 binding

site (GAL-BS) and pRL-CMV vector as an internal control, using the Tfx-50 reagent (Promega Corp.). The cells were incubated with retinoids (10^{-6} M) for 2 days. The luciferase activities of the cell lysates were measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation measured by luciferase activity was standardized with the luciferase activity of the same cells determined by the Sea Pansy luciferase assay system (Toyo Ink Co. Ltd.) as a control. Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as mean $\pm$ S.E.

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Conflict of Interest

The authors declare no conflict of interest.

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Unveiling the Role of Carotenoid Species in Photoprotection: Triplet-to-Triplet Energy Dissipation in Spheroidene-Reconstituted LH1 Complex from *Rhodospirillum rubrum*

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Abstract: Purple photosynthetic bacteria utilize carotenoids as important light harvesters and photoprotectors in the process of capturing solar energy. The carotenoids play a crucial role in transferring excitation energy to bacteriochlorophyll *a* and dissipating excess energy delivered to the reaction center. In this study, we investigated the effect of carotenoid species on photoprotection processes using sub-nanosecond time-resolved absorption spectroscopy. By reconstituting spheroidene, extracted from *Rhodobacter sphaeroides* strain 2.4.1, into the carotenoidless light-harvesting complex from *Rhodospirillum* (*Rsp.*) *rubrum* strain G9+, we discovered that the energy dissipation process was predominantly triplet to triplet type. This finding contrasts with the previous understanding of the dominant singlet to triplet energy dissipation in the native spirilloxanthin-bound LH1 complex from *Rsp. rubrum* strain S1. Our results provide valuable insights into the photoprotection mechanism and may contribute to the design of artificial light-harvesting systems.

1. Introduction

Purple photosynthetic bacteria mainly have two different types of light-harvesting (LH) complexes, called LH1 and LH2, which have ring-like structures. The LH2 complex surrounds the LH1 complex, which in turn surrounds the RC complex where charge separation takes place. The LH1 complex is composed of 15 to 16 subunits, consisting of two bacteriochlorophyll *a* (Bchl *a*) and one carotenoid (Car), as well as a pair of polypeptides (refer to **Fig. 1**) [1].

In the photosynthetic process, solar light energy in the region around 500 nm is captured by Car and transferred to Bchl *a*, which is bound to the LH pigment-protein complexes (light-harvesting process). This efficiency of excitation energy-transfer (EET) depends on the number of conjugated C=C double bonds of the carotenoid and is typically 36-78% in nature [1, 2].

On the other hand, in situations where an excess amount of light is irradiated, the excess energy on the Bchl *a* flows back to Car in order to prevent the emergence of harmful singlet oxygen derived from triplet excited Bchl *a* (photoprotective process). The photoprotective process of the LH2 complex from *Rhodobacter* (*Rba.*) *sphaeroides* strain 2.4.1 has already been reported and concluded that the process was accomplished with 100% triplet-triplet EET transfer from Bchl *a* to Car [3]. Conversely, in the LH1 complex from *Rhodospirillum* (*Rsp.*) *rubrum* strain S1, 80% singlet-triplet and 20% triplet-triplet EET transfer was observed [4]. Although the reason for this difference is thought to be due to the size of LH rings or the number of Bchl *a* molecules, the details have not yet been revealed. Elucidating how the photoprotective pathway depends on the carotenoid may provide us with a new outlook on photosynthetic mechanisms.

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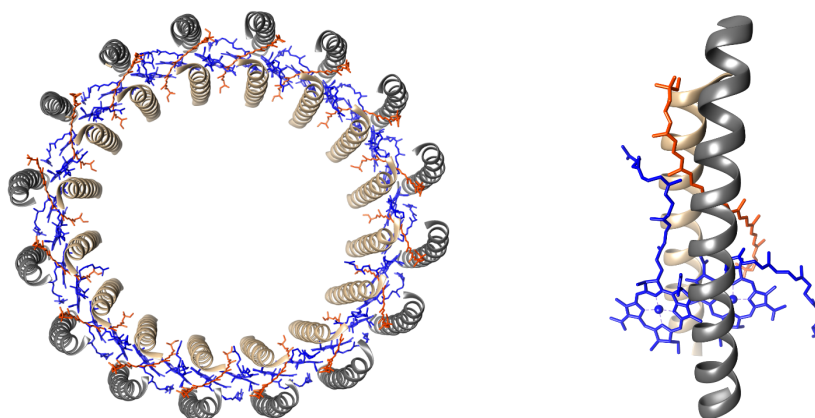


Fig. 1. The structure of LH1-RC complex (left, PDB: 7OY8) and LH1 heterodimer subunit (right) from *Rsp. rubrum* strain S1.

Purple photosynthetic bacterium *Rsp. rubrum* strain S1 predominantly uses spirilloxanthin (13 conjugated double bonds) as their main carotenoid. However, this strain only achieves a 27% efficiency of singlet-singlet excitation energy transfer (EET) from Car to Bchl *a* [5,6]. Since the primary processes of photosynthesis occur on a time scale ranging from femtoseconds to sub-milliseconds, time-resolved absorption spectroscopy has been employed to observe and study these processes.

2. Experimental

2.1 Cell growth and isolation of the chromatophores

Cells of *Rsp. rubrum* strain G9+ were grown photosynthetically under anaerobic conditions using C-succinate medium at room temperature. The cells were harvested by centrifugation ($18,800 \times g$, 20 min at 4 °C) in 20 mM MES buffer (pH 7.0) and stored in a freezer (−30 °C) until use. To disrupt the cell walls, the cells of *Rsp. rubrum* were subjected to a French pressure cell. Chromatophores were then prepared by successive centrifugation ($27,000 \times g$, 30 min at 4 °C) and ultracentrifugation ($258,000 \times g$, 90 min at 4 °C).

2.2 Isolation and purification of B820 heterodimers of the LH1 complex of *Rsp. rubrum* strain G9+

The fresh chromatophores were solubilized in 1.2% octyl- β -glucopyranoside (β -OG) in 50 mM PBS buffer to obtain the B820 heterodimer complex. This complex was then purified using a sucrose density gradient (0.6 M to 0.8 M linear gradient) via

ultracentrifugation ($165,000 \times g$, 16 hrs. at 4 °C) and stored in a freezer (−30 °C) until use [3].

2.3 Extraction of spheroidene from *Rba. sphaeroides* strain 2.4.1

The harvested cells of *Rba. sphaeroides* strain 2.4.1 (following Experimental 2.1) were stirred for 10 minutes in a mixed solution of acetone and methanol in a ratio of 7/2 (v/v), under a flow of N_2 gas. Subsequently, the solution was centrifuged ($4,000 \times g$, 20 min at 4 °C) to obtain the supernatant containing spheroidene. The obtained supernatant was evaporated, and the solvent-free substances were dissolved in a small amount of *n*-hexane. Finally, the purification of spheroidene were performed using silica gel column chromatography with 10% diethyl ether in *n*-hexane.

2.4 Reconstitution of spheroidene into the LH1 complex

Under a vigorous flow of N_2 gas, the acetone solution of spheroidene was slowly added to a 50 mM PBS buffer solution of B820 ($OD_{820} = 0.7$) containing 1.2% β -OG at 4 °C. The solution was then diluted with an equal volume of 50 mM PBS buffer for the reassembly of the B820 to form the LH1 ring structure mediated by Cars [7, 8]. Next, the surfactant β -OG was removed by ultrafiltration, and β -DDM was added to the solution at a concentration of 0.03%. The reconstituted LH1 was purified using an anion-exchange column chromatography (Whatman, DE-52)

with 0.03% β -DDM and NaCl (concentration: 0 M, 500 mM, and 1 M stepwise) in a 20 mM Tris-HCl buffer.

2.5 Steady-state spectroscopic measurements

The absorption spectra of the samples were recorded on a JASCO V-730 UV-vis spectrophotometer at room temperature.

2.6 Sub-nanosecond time-resolved absorption measurements

Details of sub-nanosecond spectroscopic measurements are described in references [6, 9]. The excitation pulse at 885 nm used to excite the $S_0 - S_2$ absorption band of Bchl *a* was generated by an optical parametric amplifier (Spectra Physics, OPA-800 CF). The excitation pulse condition was as follows: intensity: 50 nJ/pulse, pulse width: 100 fs, the beam diameter: 200 μ m. The probe pulses cover spectral region of 400 to 900 nm and was detected by a sub-nanosecond transient absorption spectrometer (EOS Vis-NIR, Ultrafast System).

Sodium *L*-ascorbate was added to the sample to a concentration of 20 mM prior to measurement. The sample was then placed in a static quartz cuvette with a 2.0 mm optical path length. The optical density of the sample solution was adjusted to 0.3 at 880 nm. All time-resolved measurements were performed at room temperature.

3. Results and Discussion

3.1 Steady-state absorption spectra

The steady-state absorption spectra before and after the reconstitution are shown in **Fig. 2**. The spectrum after the reconstitution contains the spectral property of Car (0-0: 504 nm, 0-1: 471 nm, 0-2: 444 nm) and the red-shifted spectral property of Bchl *a*. The red-shift is due to the conformational change of the LH1 complex. Bchl *a* in the heterodimers show the spectral peak at 822 nm, while those in ring-like structure show the spectral peak at 880 nm.

3.2 Sub-nanosecond time resolved absorption spectral measurements

Fig. 3 shows a set of transient absorption spectra recorded at selected delay times after photo-excitation.

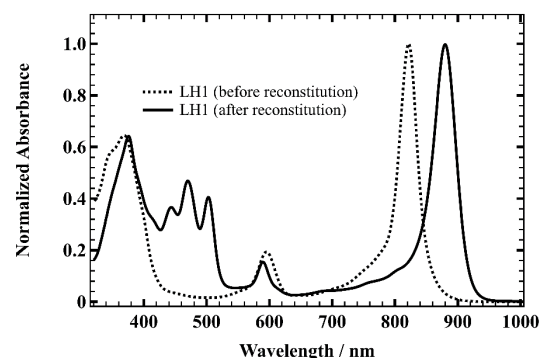


Fig. 2. Steady-state absorption spectra of LH1 complexes of *Rsp. rubrum* G9+ before and after the reconstitution of spheroidene.

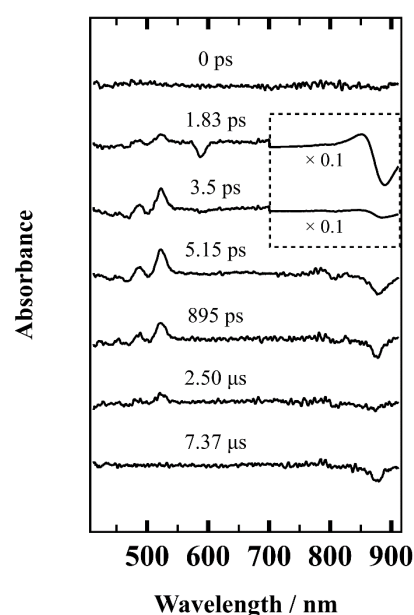


Fig. 3. Transient absorption spectra at selected delay times after photo-excitation of the spheroidene-reconstituted LH1 complex.

In the first phase (1.83 ps), temporal bleaching signals of Bchl *a* Q_y (850 nm) and Bchl *a* Q_x (600 nm) were observed, and the absorption property of Car T_1 around 510 nm emerged in competition with the Bchl *a* signals. After the bleaching signals of Bchl *a* disappeared (3.5 ps), a long-lived Bchl *a* cation signal appeared in the same spectral region [10].

The results of the global analysis of the time-resolved absorption spectra of the spheroidene-reconstituted LH1 complex are shown in **Figure 4(a)**, and the analytic scheme used for the analysis is presented in **Figure 4(b)**. The Glotaran program was used for the global analysis [11]. The analysis revealed that the evolution associated difference spectrum (EADS) of Bchl *a* S_1 decays within 400 ps, followed by

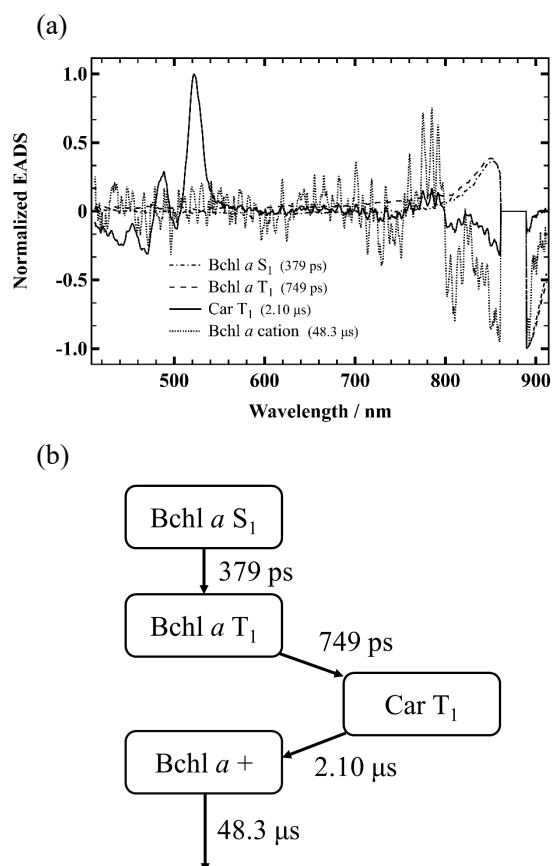


Fig. 4. Evolution associated difference spectrum (EADS) from the global analysis against transient absorption spectrum of the spheroidene-reconstituted LH1 (a) and its analytic scheme (b).

the decay of EADS of Bchl *a* T₁ within 750 ps. The energy on Bchl *a* T₁ then flowed to Car T₁ by triplet-triplet (TT) type dissipation. The target analysis with singlet-triplet (ST) type dissipation did not perform well, indicating that photoprotection was only proceeded with TT-type dissipation. The energy was finally received by Bchl *a* cation, which is an unfavorable energy receiver. These processes, the TT-type dissipation and the emergence of Bchl *a* cation, are different from the native LH1 complex from *Rsp. rubrum* strain S1, indicating a unique photoprotection process in the spheroidene-reconstituted LH1. The reason why the photoprotection process in the spheroidene-reconstituted LH1 does not contain fast (ST-type) dissipation is not yet revealed, but this result may provide a new perspective on the spheroidene-synthesizing biological pathway, which is not observed in the native LH1 from *Rsp. rubrum* strain S1.

4. Conclusion

Our findings indicate that photoprotection in the LH1 complex, when using spheroidene, is solely achieved through triplet-triplet (TT) type dissipation, resulting in the generation of toxic Bchl *a* cation. This discovery challenges the conventional understanding of LH1 complexes and sheds light on a previously unobserved cation formation pathway in the spheroidene-synthesizing LH1 system in *Rba. sphaeroides* strain 2.4.1.

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Structural optimization of the isomers of β -apo-8'-carotenal using semi-empirical molecular orbital calculations and evaluation of their photophysical properties

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Abstract: In this study, the most stable structure was determined for each isomer of β -apo-8'-carotenal using WinMOPAC 3.9, and $\Delta\mu$ (change in static dipole-moment associated with optical transition) was calculated for each isomer. For each isomer, a structure without aldehyde C=O conjugation was also prepared by rotating the aldehyde group 180° with respect to the polyene plane. Similarly, $\Delta\mu$ was calculated for each isomer. The photophysical properties of *cis-trans* β -apo-8'-carotenal were investigated by comparing these calculations to experimental data.

1. Introduction

Photosynthesis is a process that utilizes sunlight to produce biological energy from water and carbon dioxide. Conjugated polyene molecules, such as carotenoids and chlorophylls, have been selected to carry out this function. The unique structures of these pigment molecules are closely related to their light-harvesting function, which plays a crucial role in the primary process of photosynthesis [1]. Therefore, understanding the structure-function relationship of pigment molecules is vital to elucidate the mechanisms of photosynthetic light-harvesting [2]. To achieve this goal, it is important to determine the most stable structure of the pigment molecule using computational chemistry and to predict $\Delta\mu$, which represents the change in static dipole moment associated with optical transition [3]. $\Delta\mu$ indicates the direction and magnitude of charge transfer associated with the photoexcitation of the pigment molecule. In this study, we focused on the *cis-trans* isomers of β -apo-8'-carotenal, whose $\Delta\mu$ has been experimentally determined [4]. β -apo-8'-carotenal (shown in **Figures 1 and 2**) is a carotenoid with nine conjugated double bonds and a terminal carbonyl group. It is interesting to compare the $\Delta\mu$ of the all-*trans* isomer, which consists of a one-dimensional polyene backbone, with those of

the *cis* isomers, where the polyene skeletons extend two-dimensionally.

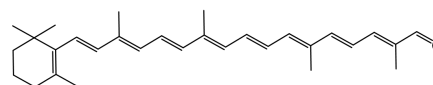


Fig. 1. Chemical structure of all-*trans* β -apo-8'-carotenal

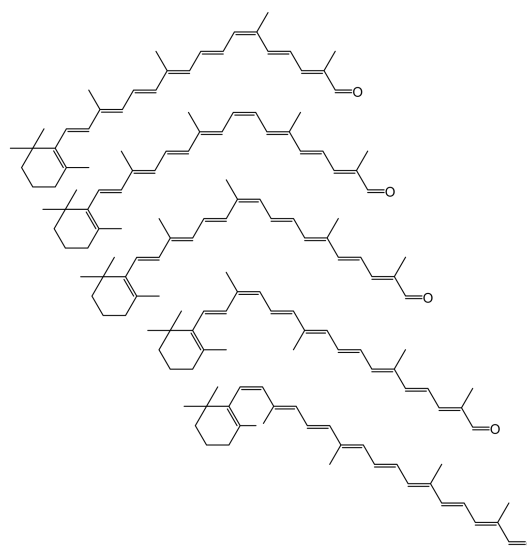


Fig. 2. chemical structures of the *cis* isomers of β -apo-8'-carotenal. From top to bottom: 13'-*cis*, 15-*cis*, 13-*cis*, 9-*cis*, 7-*cis* isomer

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2. Experimental

We determined the most stable structure of each isomer of β -apo-8'-carotenal using the WinMOPAC 3.9 program (Fujitsu Ltd.), a semi-empirical molecular orbital calculation software, and calculated $\Delta\mu$ for each isomer.

2.1 Structural Optimization

The molecular skeleton of all-*trans* β -apo-8'-carotenal was generated using Chem3D software (Cambridge Software Inc.) and structurally optimized by the AM1 and MNDO methods using WinMOPAC 3.9. Using these optimized structures as reference parameters, the bond angles of the β -ionone ring to the polyene segment were fixed at 10° intervals from -180° to +180°, and the two lowest energy angles were determined using minimum energy-path calculation. Since the AM1 method provided more detailed results than the MNDO method, we performed structural optimization using the AM1 method for all degrees of freedom (bond length, bond angle, and dihedral angle) for the structures with the obtained angles, respectively, to obtain more stable structures.

Next, five *cis* conformations were generated by arbitrary C=C bending of the previously obtained all-*trans* conformation, and the structures were again optimized using the AM1 method. For each isomer, the aldehyde group was rotated 180° with respect to the polyene plane, creating a structure in which the aldehyde C=O conjugation was absent.

2.2 Calculation of $\Delta\mu$

$\Delta\mu$ was calculated for the optimized structure using the MOS-F program included in WinMOPAC 3.9, with the INDO/S, CNDO/S2, CNDO/S3, CNDO/2, and PM5 functions. By comparing with the experimental values obtained in previous studies, it was determined that the CNDO/2 method was suitable for the calculation. The vector components of $\Delta\mu$ obtained by the CNDO/2 method were plotted in a two-dimensional XY plane using Wolfram Mathematica 12.

2.3 Calculation of $\theta_{\Delta\mu}$

The angle between the transition dipole moment $\mathbf{M}$ and $\Delta\mu$ is denoted as $\theta_{\Delta\mu}$. However, the transition dipole moment $\mathbf{M}$ cannot be determined on its own. Light

absorption in carotenoids is caused by transitions of the π -electrons that constitute the conjugated polyene. The transition dipole moment has the direction of the conjugated polyene. In the all-*trans* isomer, the conjugated polyene is on the linear chain, and the transition dipole moment is mainly in that direction. However, in the *cis* isomer, where the conjugated polyene is folded, the transition dipole moment appears in two directions; along the long and short axes of the molecule. Therefore, we determined the angle between the long axis (C6-C8') and $\Delta\mu$. To calculate $\theta_{\Delta\mu}$, we took the inner product of the vector component of the long axis and that of $\Delta\mu$.

3. Results and Discussions

Fig. 3 displays the results of the minimum energy path calculations for the β -ionone ring and the polyene segment. Table 1 summarizes the values of θ (torsion angle around C6-C7 bond) and the heat of formation after the structural optimization of the structures with C6-C7 rotation angles of 50° and -50°. In the most stable structure of the all-*trans* isomer, θ are found to be -50.80° and 48.49°, respectively, as determined by the minimum energy path calculation using the AM1 method.

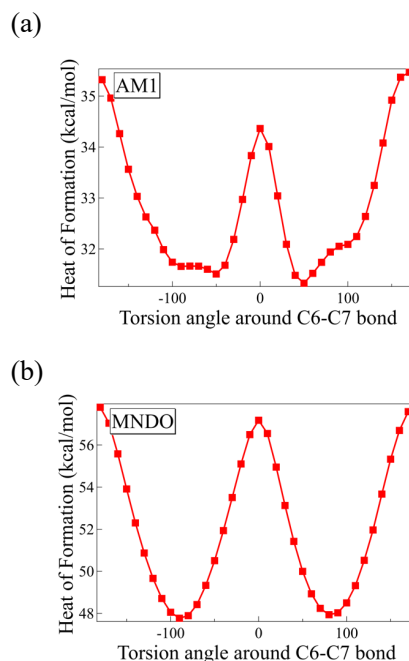


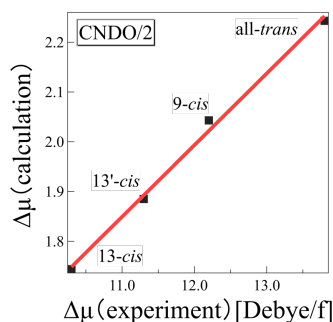
Fig. 3. Minimum energy path calculations for the rotation of the β -ionone ring relative to the polyene segment of all-*trans* β -apo-8'-carotenal using (a) the AM1 method and (b) the MNDO method

Table 1. The rotation angles of C6-C7 and heat of formation after structural optimization.

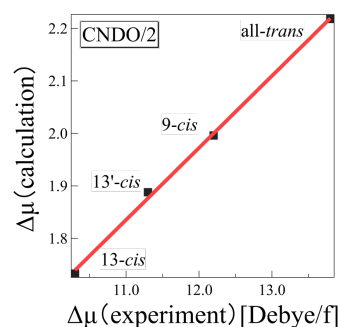
θ (setting)	θ (result of calculation)	heat of formation
-50	-50.80	31.50
50	48.49	31.32

Table 2 summarizes the results of previous experimental measurements of $|\Delta\mu|$ for the all-*trans*, 9-*cis*, 13-*cis*, and 13'-*cis* isomers [4, 5]. We compared the experimental and calculated $|\Delta\mu|$ values for the structures of the all-*trans*, 9-*cis*, 13-*cis*, and 13'-*cis* isomers with conjugation. The result of this comparison is shown in **Figure 4**. It reveals that the CNDO/2 method provided the best agreement between experimental and calculated $|\Delta\mu|$ values, thus confirming its optimality.

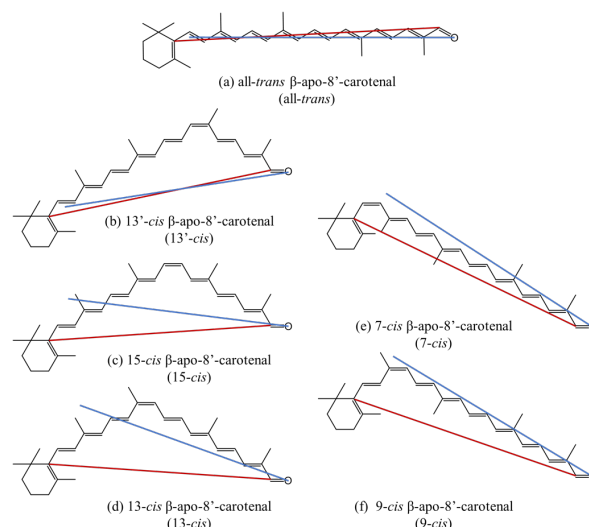
(a)



(b)

**Fig. 4.** Comparison of experimental and calculated values for $|\Delta\mu|$ (a) with and (b) without conjugation of carbonyl group.

Next, we determined the direction of $\Delta\mu$ ($\theta_{\Delta\mu}$) with respect to the long axis, which might be parallel to the direction of transition dipole moment of each isomer. To this end, we used the values of $|\Delta\mu|$ calculated by the CNDO/2 method and the coordinate system of $\Delta\mu$ output in the calculation process. **Table 3** summarizes the values of $\theta_{\Delta\mu}$ obtained from the results of our calculations for all-*trans* and all the *cis* isomers. **Figure 5** depicts a diagram illustrating the long axis of the transition moment and $\Delta\mu$, based on the obtained values of $\theta_{\Delta\mu}$.

**Fig. 5.** Long axis of the transition moment and the direction of $\Delta\mu$ overlaid on the chemical structure of β -apo-8'-carotenal (red line: direction of transition moment, blue line: direction of $\Delta\mu$).

Intriguingly, a comparison of the $\Delta\mu$ directions between the isomers revealed a remarkable trend. For the all-*trans*, 7-*cis*, and 9-*cis* isomers, $\Delta\mu$ was present along the polyene backbone conjugated to C=O. For the remaining *cis* isomers, the direction of $\Delta\mu$ was along the long axis and changed as the central *cis* position approached, with the order being 13-*cis*, 15-*cis*, and 13'-*cis* isomers. While previous studies have clarified the absolute value of $\Delta\mu$, little investigation has been conducted on the angle $\theta_{\Delta\mu}$. More detailed investigations

Table 2. $|\Delta\mu|$ of electronic transitions from the S_0 state to the S_2 /ICT excited state determined experimentally [4,5].

geometrical isomer	Experiment	$ \Delta\mu /\text{Debye} \cdot \text{f}^{-1}$	
		calculation	
		with conjugation	without conjugation
all- <i>trans</i>	13.8 ± 0.3	2.24	2.22
9- <i>cis</i>	12.2 ± 0.3	2.14	2.00
13- <i>cis</i>	10.3 ± 0.3	1.74	1.73
13'- <i>cis</i>	11.3 ± 0.3	1.89	1.89

Table 3. Comparison of the calculated $\theta_{\Delta\mu}$ values of the isomers of β -apo-8'-carotenal.

	with conjugation		without conjugation	
	calculation ($\theta=50$)	calculation ($\theta=-50$)	calculation ($\theta=50$)	calculation ($\theta=-50$)
all- <i>trans</i>	3.71	3.91	4.68	4.68
13'- <i>cis</i>	3.49	3.44	4.69	4.57
15- <i>cis</i>	11.67	11.44	12.82	12.76
13- <i>cis</i>	16.06	14.88	17.61	18.90
9- <i>cis</i>	12.91	11.75	14.30	13.33
7- <i>cis</i>	7.22	6.59	8.52	7.75

are needed in the future to gain a deeper understanding of $\theta_{\Delta\mu}$. The results of this study have the potential to provide interesting insights into the unique functional properties of *cis*-isomers in photosynthesis.

4. Conclusion

This study delved into the structures and properties of the isomers of β -apo-8'-carotenal using cutting-edge quantum chemical calculations. Our findings highlighted that the CNDO/2 method outperformed other approaches in determining the agreement between experimental and calculated $|\Delta\mu|$ values, while also revealing intriguing insights into the direction of $\Delta\mu$ ($\theta_{\Delta\mu}$) for all-*trans* and all the mono-*cis* isomers. Specifically, we discovered a remarkable trend where the direction of $\Delta\mu$ was aligned along the polyene backbone conjugated to C=O for the all-*trans*, 7-*cis*, and 9-*cis* isomers, while those of the central *cis* isomers were along the long axis and changing as the central *cis* position approached for the remaining *cis* isomers, with the order being 13-*cis*, 15-*cis*, and 13'-*cis* isomers. Although more extensive investigation is warranted to gain a deeper understanding of $\theta_{\Delta\mu}$, our results have the potential to provide exciting and novel insights into the unique functional properties of *cis*-isomers of carbonyl containing carotenoids in photosynthesis.

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Assessment of the photoprotective function of the light-harvesting systems containing bacteriochlorophyll *b* in *Blastochloris viridis*

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Abstract: The photoprotective function of the light-harvesting 1 and reaction center (LH1-RC) core complex containing bacteriochlorophyll *b* from the purple photosynthetic bacterium *Blastochloris* (*Blc.*) *viridis* has not been fully elucidated to date. The predominant carotenoids within the LH1-RC core complex of *Blc. viridis* are primarily composed of 1,2-dihydroneurosporene and 1,2-dihydrolycopene. In this study, we conducted steady-state and ultrafast time-resolved optical spectroscopy measurements on the LH1-RC core complex of *Blc. viridis* at room temperature, utilizing Q_y excitation (1010 nm excitation) conditions. Our analysis of the transient absorption spectra revealed the excitation energy-transfer pathways. These findings unexpectedly lead to the suggestion that 1,2-dihydroneurosporene and 1,2-dihydrolycopene, the primary carotenoids present in the LH1-RC core complex from *Blc. viridis*, do not demonstrate a photoprotective function.

1. Introduction

Photosynthetic bacteria have served as invaluable research models for gaining insights into the fundamental processes of photosynthesis. This is due to their relatively uncomplicated photosynthetic systems and the ease with which biochemical samples can be prepared. Despite the wealth of research on light-harvesting (LH) systems containing bacteriochlorophyll (Bchl) *a*, there has been a notable scarcity of studies that have directed their attention towards LH systems incorporating Bchl *b* [1]. The photosynthetic system of the purple photosynthetic bacterium *Blastochloris* (*Blc.*) *viridis* consists solely of the LH1-RC complex, with an LH1 pigment-protein complex surrounding the reaction center (RC). The LH1 complex consists of two carotenoid pigments, namely 1,2-dihydro-neurosporene and 1,2-dihydrolycopene, as well as Bchl *b* dimers [1]. Notably,

this complex exhibits an extended Q_y absorption band in the far-NIR region (1010 nm) compared to the LH1 complex containing Bchl *a*. The recent discovery of the

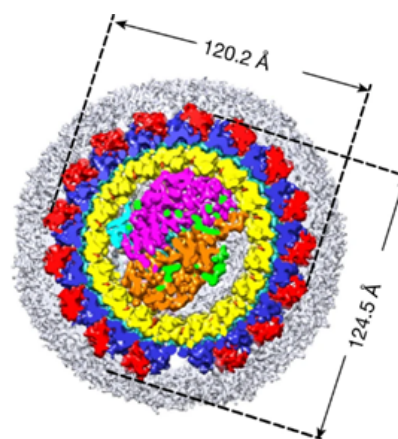


Fig. 1. Cryo-EM structure of the LH1-RC core complex from *Blc. viridis*., displayed with permission from ref. [2].

cryo-EM structure of the *Blc. viridis* LH1-RC complex is depicted in **Fig. 1** [2].

Purple photosynthetic bacteria indeed produce reactive oxygen species that can be detrimental to living organisms when exposed to excessive light energy. However, the LH1-RC complex serves as a critical photoprotective mechanism, effectively dissipating excess light energy into the surrounding environment as heat. This function prevents cell death and ensures the organism's survival when exposed to high-intensity light.

In this study, we isolated the LH1-RC complex from *Blc. viridis* and conducted sub-nanosecond time-resolved absorption spectroscopy measurements on the excitation within the Q_y absorption band (at 1010 nm) of the isolated LH1-RC complex. The primary objective was to investigate the process of triplet-triplet excitation energy transfer from Bchl *b* to carotenoids within this complex. Additionally, we assessed the photoprotective function of the isolated LH1-RC complex. These findings hold promise in contributing to the development of robust artificial photosynthetic systems, enabling the efficient utilization of light energy, particularly in the far-NIR spectral region.

2. Experimental

2.1 Sample Preparation [1,3,4].

The cells of *Blc. viridis* strain DSM 133 were cultivated photosynthetically using C-succinate media under anaerobic conditions at approximately 27°C for a duration ranging from 5 days to 1 week. Subsequently, the cells were harvested through centrifugation and then resuspended in 20 mM Tris-HCl buffer (THB) with a pH of 8.0. A small amount of DNase and $MgCl_2$ were introduced into the sample, and the cells were disrupted using a French Press. After disruption, the mixture was subjected to centrifugation at $12,000 \times g$ for 10 minutes at 4°C. The resulting supernatant was further processed through ultracentrifugation at $210,000 \times g$ for 2 hours at 4°C, ultimately yielding photosynthetic membranes.

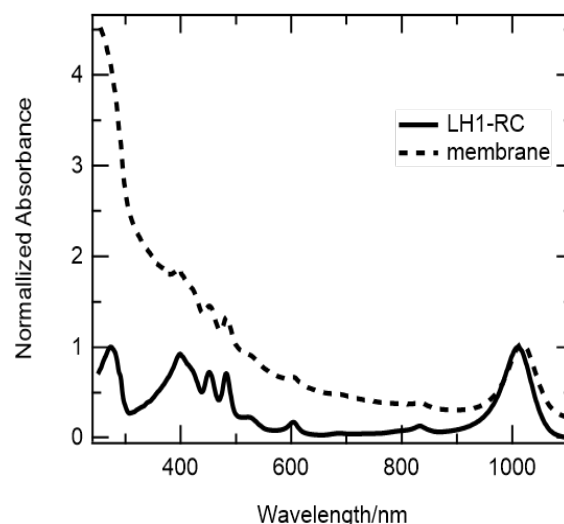


Fig. 2. Steady-state absorption spectra of the LH1-RC complex (solid line) and the photosynthetic membrane (broken line) from *Blc. viridis* at room temperature normalized at 1010 nm and 1008 nm, respectively.

The pelleted membrane was resuspended in 20 mM Tris-HCl buffer (pH 8.0) to an optical density (OD_{1010}) of 100 at 1010 nm. Subsequently, 30% (v/v) lauryl-dimethylamine *N*-oxide (LDAO) was added to achieve a final concentration of 0.3% (v/v) and incubated for approximately 5 minutes at room temperature, followed by another round of ultracentrifugation at $260,000 \times g$. The resulting supernatant was discarded, and the pellet was resuspended in 20 mM THB (pH 8.0) to an OD_{1010} of 100. To this suspension, dodecyl- β -*D*-maltoside (DDM) was added to a final concentration of 5%, and the pellet was gently stirred for 2 hours at room temperature in the absence of light. The supernatant was subsequently subjected to another round of ultracentrifugation at $260,000 \times g$ for 1 hour at 4°C.

Approximately 4 mL of the resulting supernatant was carefully layered onto a pre-prepared sucrose density gradient, which consisted of layers with sucrose concentrations of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 M. The entire gradient was then subjected to ultracentrifugation at $210,000 \times g$ for 16 hours at 4°C.

After centrifugation, two distinct bands were observed. The lower band, representing the LH1-RC core complex, was meticulously collected and subsequently purified by removing sucrose using a DE52 column that had been equilibrated with THB (pH 8.0) containing 0.02% DDM. Finally, the resulting samples were stored in a -30°C freezer for further analysis and experimentation.

2.2 Spectroscopic Methods

Steady-state absorption spectra at room temperature were recorded using a JASCO V730 spectrophotometer.

The specifics of the time-resolved absorption spectroscopic measurements have been previously documented and can be referenced elsewhere [8]. To provide a brief overview, 1010 nm excitation laser pulses with a pulse duration of 100 fs and an energy of 80 nJ per pulse were generated using an optical parametric amplifier (Spectra Physics, OPA-800C), which was excited by the output from a femtosecond regenerative amplifier (Spectra Physics, Hurricane-X).

The probe pulse utilized for the transient absorption (TA) measurements was a white-light continuum, spanning the spectral range from approximately 400 to 1000 nm, with a pulse duration of approximately 500 ps. The optical density of the sample solution was adjusted to be approximately 3.0 at the absorption maxima. This sample solution was placed within a quartz cuvette with a 2.0 mm optical path-length. To maintain the integrity of the samples during the measurements, the sample solution was continually stirred using a magnetic micro-stirrer bar.

Measurements were conducted in two conditions: with the presence of the electron mediator sodium ascorbate in the sample (referred to as the "opened state") and without it (referred to as the "closed state"). The TA data obtained from the experiments were subjected to analysis using a global analysis approach,

specifically utilizing the Glotaran Program and employing a sequential model.

3. Results and Discussion

3.1. Steady-state absorption spectra

The steady-state absorption spectra of the LH1-RC complex and the photosynthetic membrane from *Blc. viridis* at room temperature are depicted in **Fig. 2**. These spectra exhibit multiple bands that correspond to various components, primarily Bchl *b* bound to the LH1 antenna. Notable features include the Soret band at approximately 400 nm, the Q_x transition at around 600 nm, and the Q_y transition at approximately 1015 nm.

Moreover, the band observed at approximately 830 nm is assigned to the Q_y transition of monomeric accessory Bchl *b*, and the peak at 790 nm is attributed to bacteriopheophytins. Both of these components are bound within the reaction center [1,5,6]. It is worth noting that the lower exciton state, formed by the splitting of the Q_y band of Bchl *b* that functions as the primary donor in the reaction center, is apparent at approximately 960 nm [1,5,7]. However, this feature is somewhat concealed by the Q_y band originating from LH1-bound Bchls.

Additionally, the absorption spectrum showcases three distinctive peaks at 453, 484, and 525 nm, which are predominantly associated with the carotenoids found within the LH1 complex [1].

3.2 Sub-nanosecond time-resolved absorption spectra

Fig. 3 displays the transient absorption (TA) spectra captured at various delay times subsequent to excitation at 1010 nm. These TA spectra provide insight into both the open and closed states of the RC, denoted as (A) and (B), respectively.

At a delay time of 1.9 ns, the transient signal is attributed to Bchl S₁ in the open state. Conversely, at

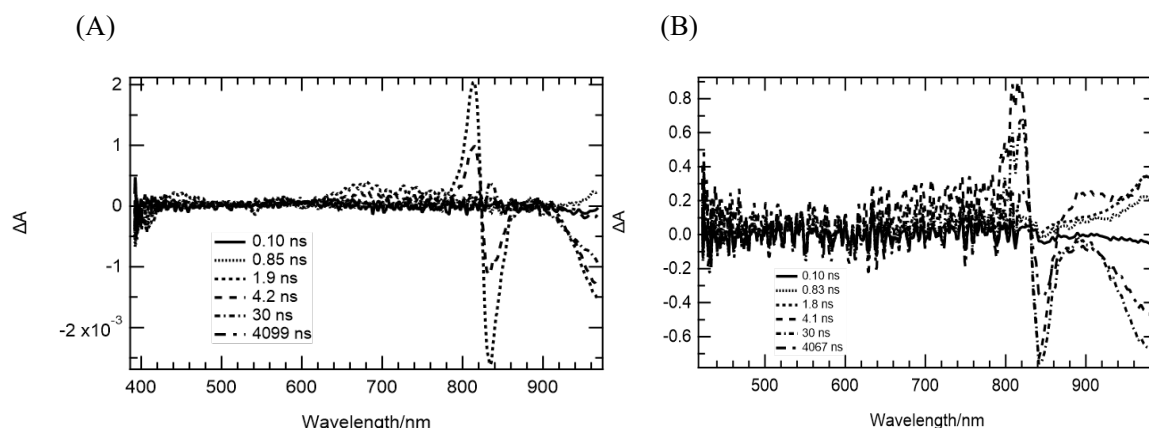


Fig. 3. Transient absorption spectra of the LH1-RC from *B. viridis* (A) with and (B) without sodium ascorbate at room temperature. The excitation wavelength was set to be 1010 nm.

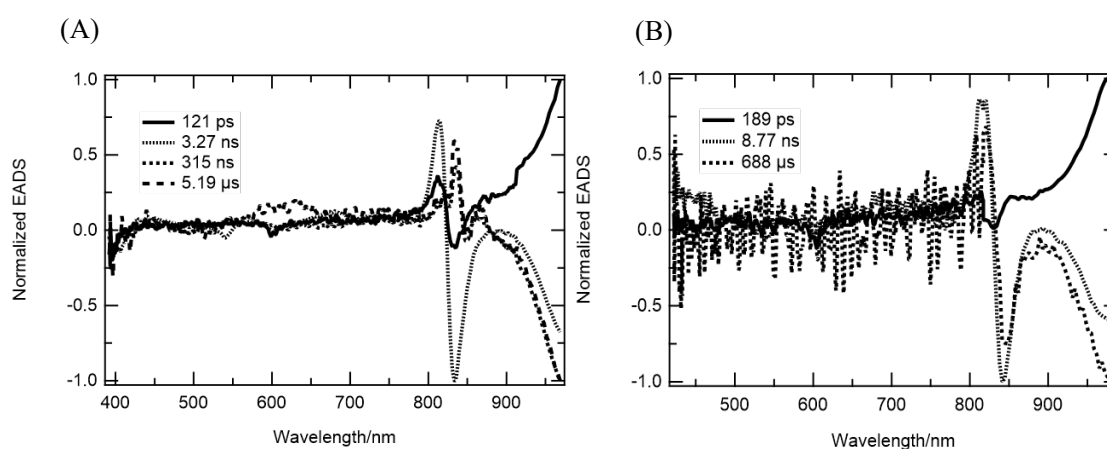


Fig. 4. Evolution associated difference spectra (EADS) obtained from global fitting of the TA data at 1010 nm excitation recorded (A) with and (B) without sodium ascorbate at room temperature.

4.1 ns, the same signal is evident in the closed state. Notably, lifetimes were observed to be generally longer in the open RC state compared to the closed RC state. Additionally, it is worth mentioning that carotenoid signals were not discernible at around 600 nm. A peak originating from the reaction center was observed at 810 nm in the closed RC state as well as in the open RC state.

In **Fig. 4**, we observe Evolution Associated Difference Spectra (EADS) that have been derived from the global fitting of the transient absorption (TA) data. In the "opened state" (A), this fitting yielded four distinct components:

1. The first EADS, with a relatively short lifetime of 121 ps, represents the signal occurring before the relaxation of the S_1 state of Bchl.
2. The second EADS, with a longer lifetime of 3.27 ns, is associated with the S_1 state of Bchl.
3. The third EADS, featuring a considerably extended lifetime of 315 ns, signifies the Bchl S_1 signal regenerated by the energy transfer from the RC (EET from RC).
4. The final EADS, with an even longer lifetime of 5.19 μ s, corresponds to the T_1 state of Bchl.

In contrast, in the "closed state" (B), the global fitting resulted in three components:

1. The first EADS, with a lifetime of 189 ps, represents the signal preceding the relaxation of the S_1 state of Bchl.

2. The second EADS, with a lifetime of 8.77 ns, can be attributed to the S_1 state of Bchl.

3. The third EADS, characterized by a significantly extended lifetime of 688 μ s, represents the T_1 state of Bchl.

These findings shed light on the temporal dynamics and transitions within the system in both the open and closed states of RC [9].

The analysis conducted on the LH1-RC complex of *Blc. viridis* revealed an absence of signals associated with the carotenoid triplet state when the Bchl b Q_y state was excited. This observation strongly suggests that the two carotenoids present in LH1, namely 1,2-dihydroneurosporene and 1,2-dihydrolycopene, do not serve a photoprotective function in this context.

Conclusion

Sub-nanosecond time-resolved spectroscopic measurements were conducted to explore the photoprotective mechanisms within purple photosynthetic bacterium *Blc. viridis*. The global analysis of the obtained transient absorption (TA) data revealed the following key findings:

1. The lifetimes of the S_1 state of Bchl b in the open and closed RC states were determined to be 3.27 ns and 8.77 ns, respectively.

2. The lifetimes of the T_1 state of Bchl b in the open and closed RC states were determined to be 5.19 μ s and 688 μ s, respectively.

3. Overall, the lifetimes of the transient species were observed to be longer in the open RC state compared to the closed RC state.

4. In the closed RC state, a distinct peak at 810 nm originating from the RC bound accessory Bchl b was evident.

5. Notably, no signal indicative of the triplet state

of carotenoids was observed in the LH1-RC complex of *Blc. viridis* when exciting the Bchl b Q_y state. This suggests that carotenoids within LH1 do not fulfill a photoprotective function, while minor carotenoids within the RC may possess such a function.

As part of future research, investigations will aim to ascertain whether *cis*-carotenoids present within the RC exhibit photoprotective properties. These findings advance our understanding of the photoprotective mechanisms employed by *Blc. viridis* and have implications for broader applications in the field of photosynthesis research.

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Total NMR Assignment of Mytiloxanthinone, A Characteristic Carotenoid of Sea Squirt

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Abstract: Mytiloxanthinone, 3,8'-dihydroxy-7,8-didehydro- β , κ -carotene-3',6'-dione, is a characteristic carotenoid in the sea squirt *Halocynthia roretzi*. However, detailed ^1H and ^{13}C -NMR assignments of mytiloxanthinone have not been reported yet. In this experimental report, total ^1H and ^{13}C -NMR assignments of mytiloxanthinone are reported.

Keywords: Mytiloxanthinone, Sea squirt, ^1H and ^{13}C -NMR assignments

1. Introduction

The sea squirt *Halocynthia roretzi* is an edible tunicate that is classified in the family Pyuridae in Protochordata, and has been consumed as sashimi, vinegared food, and salted food in East Asia, especially in South Korea and the Northeast region of Japan. *H. roretzi* can show bright red colors due to containing various carotenoids such as (3*S*,3'*S*)-astaxanthin, diatoxanthin, alloxanthin, mytiloxanthin, mytiloxanthinone, halocynthiaxanthin etc [1-4].

Among them, mytiloxanthinone is a characteristic carotenoid in the sea squirt *H. roretzi*. In 1982~1984, Matsuno et al., isolated this carotenoid from *H. roretzi* and determined its structure to be 3,8'-dihydroxy-7,8-didehydro- β , κ -carotene-3',6'-dione by chemical and UV-VIS, IR and 80 MHz ^1H -NMR spectral data [1,2].

Mytiloxanthinone only distributes in sea squirts belonging to Plaurogona and is a final metabolite of fucoxanthin in these sea squirts as shown in Fig. 1 [3].

However, detailed ^1H and ^{13}C -NMR assignments of mytiloxanthinone have not been reported yet [4]. In this experimental report, total ^1H and ^{13}C -NMR assignments of mytiloxanthinone are reported.

2. Experimentals

The sea squirt *H. roretzi*, cultivated at Yamada Bay in Iwate Prefecture, was purchased at local fish market in Kyoto in October. The tunic (4 kg) of the sea squirt *H. roretzi* was extracted with acetone (Me_2CO) at room temperature. The Me_2CO extract was partitioned with hexane/ Et_2O (1:1) and water. The hexane/ Et_2O layer was evaporated to dryness and chromatographed on silica gel using increasing Et_2O in hexane. The fraction eluted with hexane/ Et_2O (2:8) was subjected to HPLC on silica gel with Me_2CO /hexane (3:7), RT at 12.2 min to yield (3*S*,3'*S*)-astaxanthin (3.5 mg), RT at 14.1 min, roretziaxanthin (1.5 mg), RT at 15.0 min, mytiloxanthinone (6.5 mg). The fraction eluted with Et_2O / Me_2CO (1:1) was subjected to HPLC on silica gel

with Me_2CO /hexane (3:7), RT at 15.0 min, zeaxanthin (3 mg), RT at 16.2 min, diatoxanthin (9 mg), RT at 18.0 min, alloxanthin (33 mg). The fraction eluted with Me_2CO was subjected to HPLC on silica gel with Me_2CO /hexane (3:7), RT at 19.0 min, diadinoxanthin (2.5 mg), RT at 21.2 min, mytiloxanthin (3.5 mg), RT at 23.0 min, halocynthiaxanthin (3 mg), and RT at 25.0 min, fucoxanthinol (2 mg). Identification of these carotenoids were described in previous paper [2].

The ^1H NMR (500 MHz) spectrum was measured with a Varian UNITY INOVA 500 spectrometer in CDCl_3 with TMS as an internal standard. Mytiloxanthinone; ESI TOF MS $\text{M}+\text{H}^+$ m/z 597.3924, $\text{MH}-\text{H}_2\text{O}$ m/z 579.3826.

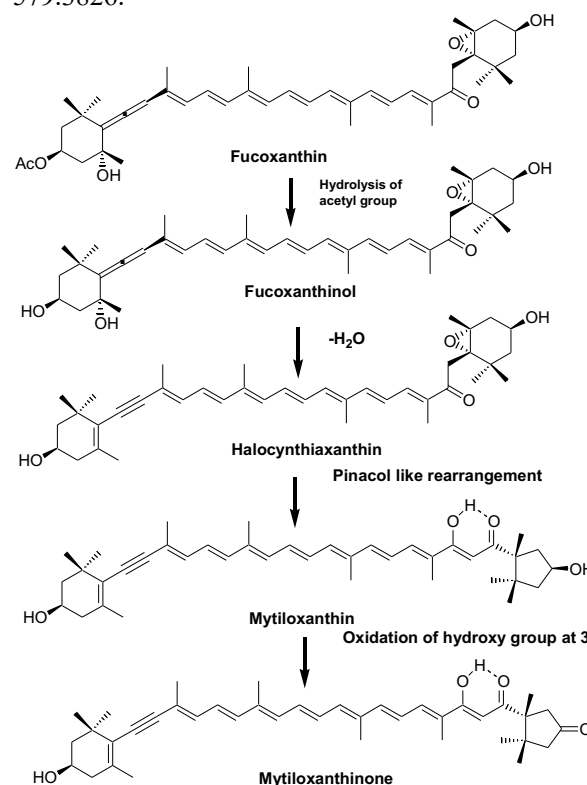


Fig. 1. Metabolic pathway from fucoxanthin to mytiloxanthinone in sea squirts.

3. Results and Discussion

Total ^1H - and ^{13}C - NMR assignments of mytiloxanthinone have been done detailed analysis of DEPT, COSY, NOESY, HSQC and HMBC experiments. Methylene protons signals at C2' and C4' in five-

membered ring (κ -end group) were assigned by NOE correlation between CH_3 16', 17', and 18' and referred with ^1H -NMR data of capsanthone [5]. These results are compiled in **Table 1**.

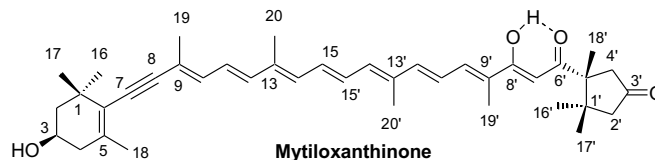


Table 1. total ^1H (500 MHz) and ^{13}C -NMR (125 MHz) assignments of mytiloxanthinone in CDCl_3 .

Positoin	^{13}C -NMR	^1H -NMR	Positoin	^{13}C -NMR	^1H -NMR
	δ	δ		δ	δ
		mult. J (Hz)			mult. J (Hz)
1	36.60		1'	41.49	
2	46.70	ax1.46 dd (12.0, 12.0)	2'	52.38	α 2.40 d (18.0)
		eq1.84 ddd (12.0, 3.5, 2.0)			β 2.23 d (18.0)
3	64.87	3.99 m	3'	216.53	
4	41.61	ax2.07 dd (17.5, 9.5)	4'	48.30	α 2.99 d (18.0)
		eq2.43 ddd (17.5,5.5, 2.0)			β 2.15 dd (18.0)
5	137.46		5'	53.12	
6	124.91		6'	200.34	
7	89.32		7'	94.10	5.86 S
8	98.56		8'	182.72	
9	119.64		9'	136.60	
10	135.04	6.46 d (11.5)	10'	135.82	7.23 d (9.5)
11	124.22	5.54 dd (14.5, 11.5)	11'	126.41	6.62 d (15, 9.5)
12	137.89	6.36 d (14.5)	12'	144.00	6.65 d (15.0)
13	136.50		13'	137.81	
14	133.11	6.28 d (10.5)	14'	135.96	6.38 d (10.5)
15	130.00	6.70 dd (14.0, 10.5)	15'	132.10	6.65 dd (14.0, 10.5)
16	28.77	1.15 s	16'	25.91	1.04 S
17	30.50	1.20 s	17'	20.50	1.23 S
18	22.49	1.93 s	18'	22.21	1.34 S
19	18.09	2.01 s	19'	12.91	1.98 S
20	12.83	1.98 s	20'	12.83	1.99 S
			8'-OH		16.26 s

Multiplicity: s: singlet, d: doublet, q: quartet, m: multiplet

Concerning the carotenoids having a 3-keto- κ -eng group, capsanthone and bastaxanthin C [6] have been reported. Because of presence of enolic group at 8' and 9' position in polyene chain, ^1H and ^{13}C chemical shift values of the 3-keto- κ -eng group in mytiloxanthinone are slightly different from those of capsanthone and bastaxanthin C. Therefore, total ^1H and ^{13}C -NMR assignments of mytiloxanthinone are conducted in the present report.

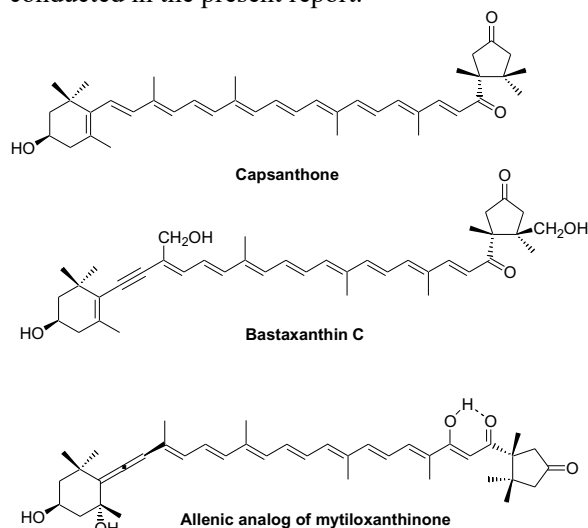


Fig. 2. Structures of capsanthone, bastaxanthin C, and allenic analog of mytiloxanthinone (unknown carotenoid).

Sea squirt contains various carotenoids. Still, new structure of carotenoid, roretziaxanthin has been isolated [7]. Therefore, another new structure carotenoids, such as allenic analog of mytiloxanthinone (**Fig. 2**) might be found in sea squirt. So, NMR data of mytiloxanthinone may be useful screening and structural elucidation of these unknown carotenoids.

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Solvent dependences on optical properties of a marine algal carotenoid, siphonaxanthin: effect of the non-conjugated functional group

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Siphonous algae, a group of marine macroalgae, that inhabit the coastal seafloor, are exposed to a variety of light, ranging from weak blue-green light to strong white light, depending on tidal change in water depth. Siphonous algae have thrived invasively in the various coasts around the world¹. Therefore, they need to have light-harvesting systems that are highly adapted to conditions of alternating extreme irradiation. Siphonous algae have a major light-harvesting complex (LHCII) containing a unique carbonyl carotenoid, siphonaxanthin (Sx). Sx contains an asymmetric carbonyl group attached to the conjugated chain (Fig. 1), which induces the intramolecular charge transfer (ICT) characters strongly coupled to the singlet excited states. In the LHCII, the ICT-state strongly coupled to the S_1 state of Sx may be essential for utilization of green light and efficient energy transfer²). In addition to the carbonyl group, Sx contains a hydroxy group attached to the 19-carbon adjacent to the conjugated carbonyl group, but the biological significance of this non-conjugated hydroxy group has not yet been clarified. Recently, we discovered a 19-deoxy Sx (dS) that is biosynthetic precursor of Sx and accumulates only when the green algae are exposed to intense illumination³). Also, the binding of dS to LHCII instead of Sx suggests that this carotenoid interconversion may be a novel light-adaptation mechanism. However, since dS and Sx have the same conjugated structure, their optical properties are also thought to be the same, and the underlying molecular mechanism of the adaptation is highly unclear. In this research, we compared the optical properties of dS and Sx in various solvents to clarify their differences and discussed in relation to the effect of the non-conjugated OH group in Sx.

First, we performed sub-pico second time-resolved absorption spectroscopy (TRAbs) of dS and Sx in non-polar and polar solvents. Fig. 2 shows TRAbs spectra of 2 ps after excitation at 490 nm. The spectral shape of dS resembles that of Sx

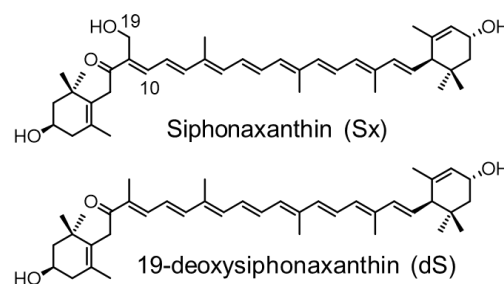


Fig. 1. The chemical structure of siphonaxanthin and 19-deoxy siphonaxanthin

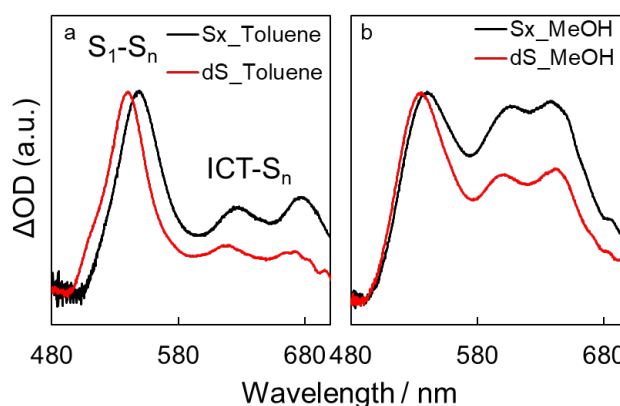


Fig. 2. Time-resolved Absorption spectrum of dS and Sx in toluene (a), acetonitrile (b), methanol (c) 2ps.

reported previously⁴⁾: The two peaks on the longer wavelength side of the sharp $S_1 \rightarrow S_n$ signal (S_1-S_n) were clearly enhanced in polar solvents and can be attributed to the $S_1/ICT \rightarrow S_n$ signal ($ICT-S_n$). However, the magnitude of $ICT-S_n$ in dS was smaller than that of Sx in both solvents. This strongly suggests that dS exhibits less ICT character than Sx. These results imply that when LHCII binds dS, the lowering ICT property reduces the energy transfer efficiency, thus favoring protection from intense light.

In the presentation, we will present steady-state absorption and 1H -NMR chemical shifts of dS and Sx in various solvents and will discuss the influence of the non-conjugated functional group on the asymmetric carbonyl conjugated system.

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Biosynthesis of capsanthin, capsorubin and cucurbitaxanthin A in *E. coli* cells

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Capsanthin and capsorubin are biosynthesized by capsanthin/capsorubin synthase (CCS) from antheraxanthin and violaxanthin. These carotenoids, especially capsorubin, show higher scavenging effect for singlet oxygen among carotenoids. In order to produce such profitable compounds effectively, we have tried pathway-engineering of *E. coli* using the *Capsicum annuum* CCS (*CaCCS*) gene and upstream carotenogenic genes.

Recently, Furubayashi *et al.* succeeded in producing capsanthin in *E. coli* by combining high level expression of *CaCCS* with violaxanthin/antheraxanthin biosynthetic pathway that consists of zeaxanthin epoxidase (*CaZEP*) and zeaxanthin biosynthesis genes (Furubayashi *et al.* 2021 [1]). However, capsorubin production remained at a low level. We speculated that the reason was owing to dual activity of *CaCCS* that functions as capsanthin/capsorubin synthase and as lycopene β -cyclase. In the latter case, *CaCCS* receives lycopene instead of antheraxanthin and violaxanthin as the substrate, which should lead to reduced CCS activity. Therefore, we thought to promote interaction between *CaZEP* and *CaCCS* to enhance the CCS reaction (**Fig. 1**).

S-tag and S-protein are derived from RNase A cleaved by subtilisin. The former consisting of N-terminal 20 amino acids and the latter composed of about 100 amino acid residues are associated tightly by non-covalent interactions. If *CaZEP* and *CaCCS* are fused to S-tag respectively, each of them could bind to S-protein through S-tag. Notably, S-proteins were reported to oligomerize by themselves with swapping their C-terminal 9 amino acids. Hence, we expected that the expression of S-tag-*CaZEP*, S-tag-*CaCCS* and S-protein in *E. coli* would make S-tag-*CaZEP* and S-tag-*CaCCS* align on the scaffold of S-protein oligomer and let *CaZEP* and *CaCCS* interact easily. Thus, we introduced such constructs into *E. coli*, cultured the obtained cells, and analyzed their carotenoids. Consequently, capsorubin was detected for the first time in addition to capsanthin. Surprisingly, cucurbitaxanthin A, whose synthetic route had been unknown, was found to be produced there, which means that this pigment is also synthesized by *CaCCS* from antheraxanthin.

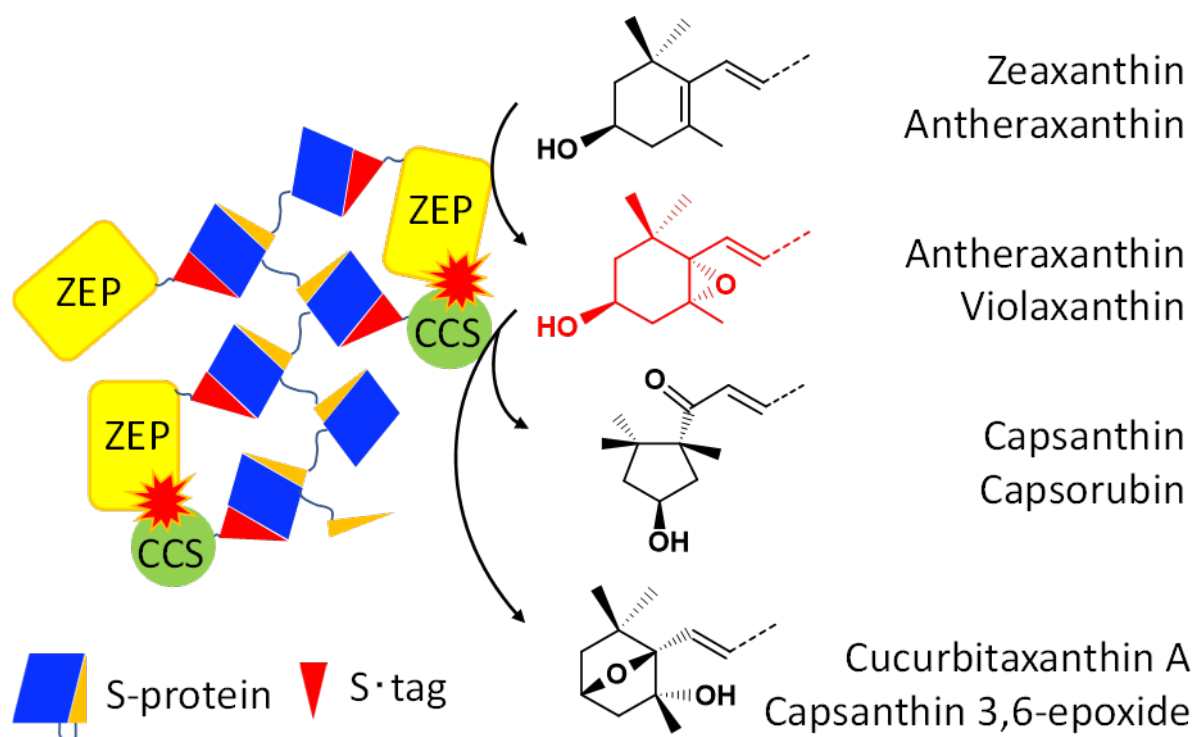


Fig. 1. A schematic diagram of S·tag-CaZEP and S·tag-CaCCS interaction on the S-protein scaffold. The interaction make it enhanced to exchange the substrate antheraxanthin and violaxanthin from CaZEP to CaCCS. These substrates are converted to capsanthin, capsorubin and cucurbitaxanthin A on the CaCCS catalytic center.

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Modification of Chloroplast Pigment Composition by Overexpression of Carotenoid Synthethis Genes

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In green leaves of plants, carotenoids have antioxidant properties and are important in alleviating excessive light stress. Lutein, which is the most abundant carotenoid in green leaves, has attracted attention as a functional ingredient because of its preventive effects against cataracts and age-related macular degeneration in humans, and is widely marketed as an eye supplement. In this study, we attempted to modify the carotenoid composition of leaves by overexpression of carotenoid synthase genes in order to grow crops that accumulate high levels of lutein.

In the model plant *Arabidopsis thaliana* (*Brassicaceae*), lycopene β -cyclase (*LcyB*), lycopene ϵ -cyclase (*LcyE*), CYP97A3 (*Lut5*) and CYP97C1 (*Lut1*) have been identified as enzyme genes involved in lutein biosynthesis from lycopene. To identify the most important enzymes for increasing lutein accumulation, we integrated *LcyB*, *LcyE*, *Lut5*, and *Lut1* into the pBYR2HS plasmid, a transient high-expression vector, and introduced each gene alone or in combination into the green leaves of *Nicotiana benthamiana* plants by Agroinfiltration.

We found that leaves overexpressing the genes alone did not show a significant increase in lutein content, while leaves overexpressing *LcyE* and *Lut5* simultaneously increased lutein content per chlorophyll by more than 20% over the control. This indicates that expression of an appropriate combination of multiple genes is effective in increasing lutein content.

In addition, phytoene synthase (*PSY*), which regulates total carotenoid content, and *ORANGE*, a protein known to interact with *PSY*, were transiently overexpressed in tobacco leaves alone or in combination with *LcyE* and *Lut5*. The results showed that *PSY* and *ORANGE* did not increase lutein content either alone or in combination with *LcyE* and *Lut5*. Currently, we are trying to further increase lutein content by reviewing light intensity and number of days of culture, and by spraying ascorbic acid, which has an inhibitory effect on plant cell death.

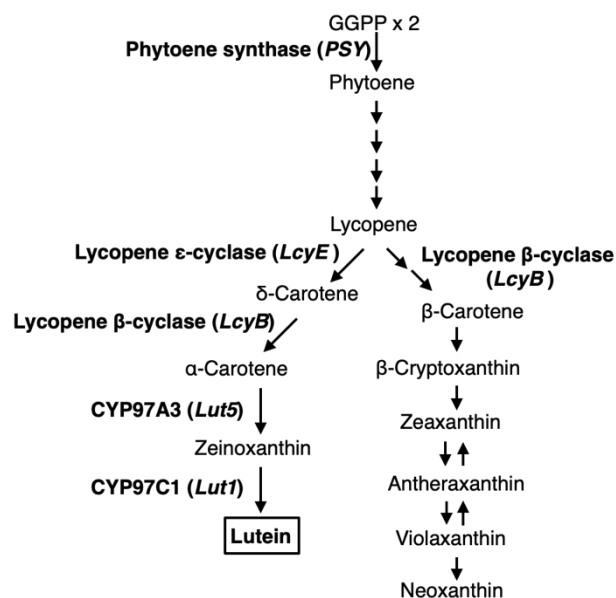


Fig. 1. Carotenoid biosynthesis pathway in *Arabidopsis*

Spectroscopic analyses on the radical cation species of carotenoids produced by chemical and electrochemical oxidation

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Carotenoids are natural pigments biosynthesized by photosynthetic organisms and play an important role of light-harvesting. On the other hand, they also have the function of photoprotection against singlet oxygen and reactive oxygen species generated by the photosensitization reaction. Radical cations generated by the oxidation of carotenoids are involved in the electron transfer process of the photosystem II (PSII) reaction center. Under the condition that $P680^{+}$ can accumulate excess light energy, *i.e.* when the PSII reaction center is photoactivated, β -carotene protects the reaction center by forming its radical cations [1]. In this way, carotenoid radical cations perform the photoprotective function in the PSII and are useful for the survival of photosynthetic organisms. Therefore, the study on the electro-optical properties of carotenoids is important to understand the different oxidation states of carotenoids and their ground and excited states properties [2]. In this study, radical cations of various carotenoids were generated in the laboratory and their photophysical properties were investigated.

We have used β -carotene, canthaxanthin, astaxanthin, and β -apo-8'-carotenal in this study. Carotenoid radical cations were generated using chemical and electrochemical oxidation. The chemical oxidation was performed using iron chloride. In the electrochemical oxidation, a carrier (electrolyte) was added to the sample solution to allow the oxidation-reduction reaction to proceed between the electrodes. We measured the absorption and resonance Raman spectra of these carotenoid radical cations.

The absorption spectra showed characteristic absorption bands ascribable to carotenoid radical cations in the near infrared spectral region, which guarantees that radical cations were truly produced in the laboratory. The effect of substituents was observed depending on the species of carotenoid by the absorption and resonance Raman measurements.

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Structural and functional analysis of the far-red light harvesting Chl *a/b* binding protein purified from the Antarctic alga

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Prasiola crista, an aerial green alga, forms layered colonies under the severe terrestrial conditions of Antarctica, which include severe cold, drought, and strong sunlight. Cells of deeper layer escape from photodamage at the sacrifice of photosynthetically active radiation except infrared. At deeper layers, *P. crista* achieves effective photosynthesis by low energy far-red light for photosystem II (PSII) excitation[1]. However, the molecular basis of the PSII excitation by far-red light remains elusive. Here, we identified a far-red light-harvesting complex of photosystem II in *P. crista*, Pc-frLHC (*Prasiola crista* far-red light-harvesting Chl-binding protein complex), and proposed a molecular mechanism of uphill excitation energy transfer based on its cryogenic electron microscopy structure. Pc-frLHC forms a ring-shaped homo-undecamer, and each monomer-monomer interface contains five chlorophyll *a* molecules that seem to contribute to long-wavelength absorption.

This is an abstract of the 34th Annual Meeting on Carotenoid Research, on 17-18 September 2022 at Kwansei Gakuin University, Nishinomiya, Japan. This will be published as a research article.

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Elucidation of astaxanthin photoisomerization properties and its utilization in material processing

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1. Introduction

Astaxanthin is universally found in algae, shrimp, or crabs, and is a kind of carotenoid with red color ^[1,2]. It has been reported that astaxanthin exhibits high antioxidant activity which is reported to be about 100-fold of α -tocopherol, and 10-fold of other carotenoids ^[3,4]. Due to these properties, it is attractive to utilize astaxanthin in functional food, food pigment, and cosmetics. Astaxanthin can be divided into all-*E* (*trans*)-isomer and *Z* (*cis*)-isomers, which exists mainly in plants and living animals, respectively. All-*E*-isomer is the main type in manufacturing product but it has high crystallinity and poor solubility in various organic solvents. These properties of all-*E*-isomer limit the efficiency of its extraction or emulsification and its bioavailability in human body. On the other hand, it is reported that *Z*-isomers of astaxanthin exhibit several hundred higher solubilities as well as higher biological activities (e.g., anti-inflammatory and anticancer activities) than those of the all-*E*-isomer. This work aimed at developing a photoisomerization method of astaxanthin, which is considered to have a low energy consumption than conventional thermal method. Moreover, the effects of wavelength, light-irradiation time, and types of organic solvents on the photoisomerization and physical properties of astaxanthin were investigated.

2. Methods

Astaxanthins with different isomer ratio (total *Z*-isomer ratio = 4% and 91%) were dissolved in three different organic solvents (ethanol, acetone, ethyl acetate; purity 99.5%), and its concentrations were adjusted at 2 $\mu\text{g/mL}$ ^[5]. As shown in **Fig. 1**, the wavelength of LED lamp was set at 365 nm, 405 nm, 470 nm, and 631 nm, and the light irradiation time was set in range of 30 ~ 120 min (when ethanol was used, it was set to 300 min). There was no light irradiation for the control group. When ethyl acetate was used as solvent, to inhibit the decomposition of astaxanthin, α -tocopherol was added and the concentration was adjusted at 1 mg/mL. The isomer ratio and remaining ratio of astaxanthin were determined by normal-phase HPLC, and the *Lab*-value were determined by UV-vis.

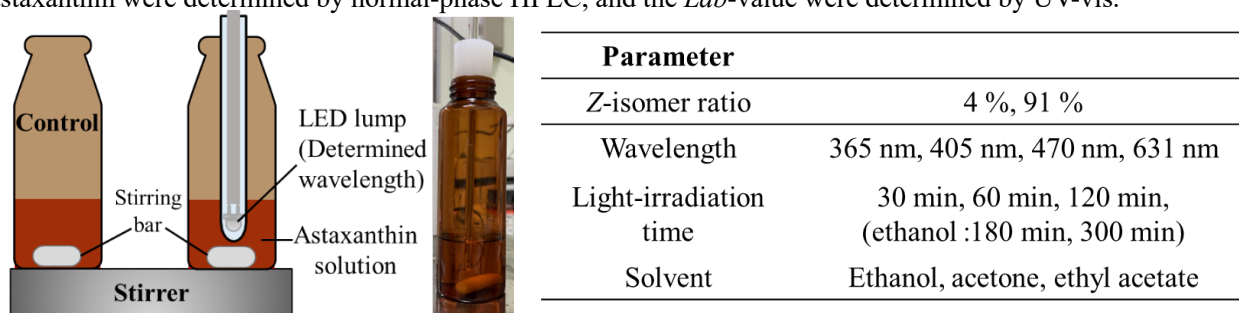


Fig. 1. Images of experiment and experimental parameters.

3. Results

The photoisomerization of astaxanthin was successfully achieved through light-irradiation with certain wavelength. We discovered that the photoisomerization of the *Z*-isomers to the all-*E*-isomer was easily achieved, but the photoisomerization of the all-*E*-isomer to the *Z*-isomers was difficult (**Fig.2**). Additionally, we found that the redness (*a* value) of astaxanthin solution got stronger as the ratio of all-*E*-isomer increased. When ethanol and acetone was used as dissolving solvent, the decomposition ratio of astaxanthin were not high, but it was high when

ethyl acetate was used. Notably, when LED lamp with 405 nm was used in ethyl acetate solution, all the astaxanthin decomposed after 5-minute light irradiation.

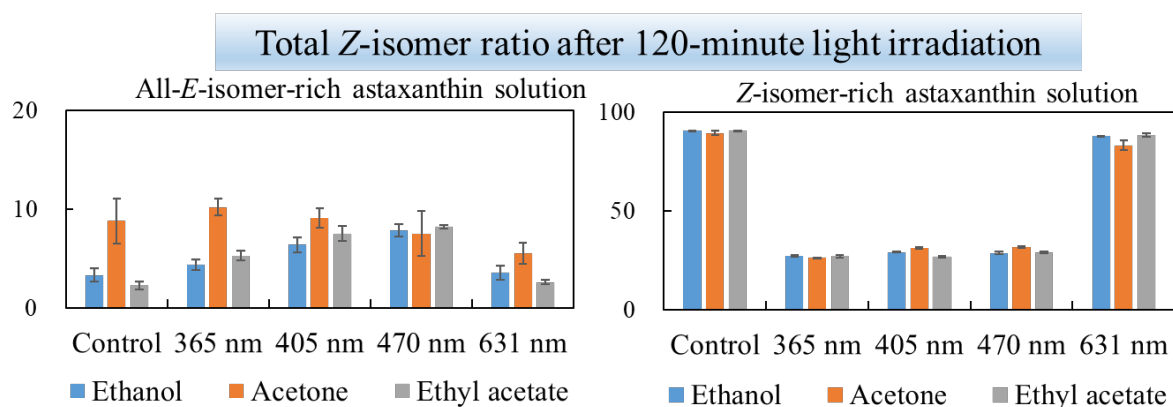


Fig. 2. Total Z-isomer ratio after 120-minute light irradiation under 365 nm, 405 nm, 470 nm, and 631 nm.

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The relationship between the structures and the excited states in *cis* isomers of β -apo-8'-carotenal

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Carotenoids absorb photons in visible light where (bacterio)chlorophylls have scant absorption, and are play an important roles which transfer to (bacterio)chlorophylls in the primary process of photosynthesis. In one-photon optical transition of carotenoids, they are excited up to the second excited singlet (S_2) state from the ground (S_0) state, while the optical transition to the first excited singlet state (S_1) state is forbidden. The carotenoids have some unclarified excited singlet states. One of them is the intramolecular charge transfer (ICT) excited state. It is suggested that the ICT excited state is very important to improve the efficiency of excitation energy-transfer from carotenoid with carbonyl group to (bacterio)chlorophyll. The number of the spectroscopic researches about the ICT excited state have been carried out, such as the dependence of the solvent and the conjugation length [1-2], however, the properties of the origin of the ICT excited state remain to be completely elucidated. In this study, we focused on the *cis* isomers of carotenoid whose structural symmetry is broken than that of all-*trans* isomers. The research on *cis* isomers of carbonyl carotenoids is very few because *cis* isomers is structurally unstable, which is difficult to obtain them with high purity. Therefore, we investigated the excited energy state dynamics of β -apo-8'-carotenal which is assigned 8 *cis* isomers [3], and evaluated the relationship between the chemical structure and the excited states.

We obtained the 8 *cis* isomers of β -apo-8'-carotenal (13'-, 15-, 13-, 9-, 7-, 9,13'-, 9,13-, 13,13'-*cis*) by means of the photoisomerization and the isolation using the high performance liquid chromatography (HPLC). The femtosecond time-resolved transient absorption spectroscopy in visible and near infrared regime was applied to each isomer dissolved in *n*-hexane, acetone, and methanol.

In polar solvents, acetone and methanol, the lifetimes of the S_1 /ICT excited state are shorter than in nonpolar solvents, *n*-hexane, due to the stabilization of the ICT excited state. The amount of changes in the S_1 /ICT lifetimes between *n*-hexane and methanol; 7-*cis* (20.8 ps) > 9-*cis* (18.4 ps) > all-*trans* (17.3 ps) > 13'-*cis* (15.2 ps) > 13-*cis* (12.7 ps) > 15-*cis* (11.3 ps). In this results, the values in terminal-*cis* isomer, 7- and 9-*cis* isomers, are higher than that of all-*trans* isomer, while the values in central-*cis* isomer, 13'-, 13-, and 15-*cis* isomers, are lower. Therefore, it is suggested that the terminal-*cis* isomers are sensitivity to the influence of the ICT excited state in highly polar solvents.

Table 1. The S_1 /ICT lifetimes of β -apo-8'-carotenal

<i>n</i> -hexane	acetone	methanol
13- <i>cis</i> (18.2 ps)	13- <i>cis</i> (10.9 ps)	13- <i>cis</i> (5.5 ps)
15- <i>cis</i> (18.8 ps)	15- <i>cis</i> (12.9 ps)	9- <i>cis</i> (5.9 ps)
13'- <i>cis</i> (22.7 ps)	9- <i>cis</i> (14.5 ps)	all- <i>trans</i> (6.5 ps)
all- <i>trans</i> (23.8 ps)	all- <i>trans</i> (15.8 ps)	15- <i>cis</i> (7.5 ps)
9- <i>cis</i> (24.3 ps)	13'- <i>cis</i> (17.2 ps)	13'- <i>cis</i> (7.5 ps)
7- <i>cis</i> (30.6 ps)	7- <i>cis</i> (25.8 ps)	7- <i>cis</i> (9.8 ps)

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Biosynthesis of asymmetric carotenoid backbone for the realization of “rare” polyene

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Introduction

More than 750 carotenoids are known in nature. Most of their molecular diversity is at the level of modification, with surprisingly little diversity in the pigment clusters (polyene structures) that determine their properties as pigments. For example, there are very few pigments with an even number of conjugated double bonds (cdb), and carotenoids with cdb = 4, 6, or 8 are especially rare. Phytofluene with cdb=5 is detected only slightly as a intermediate of ζ -carotene, and the pathway by which it is selectively synthesized is not known. All natural carotenoids are derived from phytoene (condensation product of two molecules of geranylgeranyl pyrophosphates; C₂₀PP), or from 4,4'-diapophytoene (condensation product of two molecules of farnesyl pyrophosphates; C₁₅PP). Because they are symmetric, desaturation and modification reaction also occur with respect to both ends. This results in “missing” numbers such as 4,5,6,8 etc. in the carotenoid polyenes cdb. We previously reported that CrtM, which C₃₀ phytoene synthase from *Staphylococcus aureus*, can synthesize C₃₅ phytoene by heteromolecular condensation of C₂₀PP and C₁₅PP. Cyclization of C₃₅ phytoene with cyclase CrtY derived from C₄₀ carotenoid synthetic pathway resulted in preferential cyclization of C₂₀ end. As a result, carotenoid with the acyclic C₁₅ end and the cyclic C₂₀ end were selectively synthesized. Thus, it is expected that “Break of symmetry” introduced at the backbone can regulate the polyene size of biosynthesized pigments with a higher degree of flexibility.

Result

There is a large reaction pocket in CrtM where two substrate (C_xPP) bind. We redesigned the reaction pocket by substituting various amino acid residues by site-directed mutagenesis to facilitate the introduction of hetero-size substrates. Next, we generated a variety of *E. coli* strains that efficiently accumulate substrates of different sizes (C_xPP, x=5~35). Expression of the above CrtM mutant in various CrtM mutant in these *E. coli* strains enabled selective biosynthesis of asymmetric phytoene of various sizes (C₃₅₊₂₅, C₃₅₊₂₀, C₃₅₊₁₅, C₃₀₊₂₅, C₃₀₊₂₀, C₃₀₊₁₅, C₂₅₊₂₀, C₂₅₊₁₅).

By desaturation these asymmetric phytoenes with various desaturase, we synthesized a variety of new carotenoids. In particular, the co-expression of CrtM_{F22A,F26A,W38A,E180G,F233A}/HexPS/FDS_{Y81M}/CrtI/CrtE2/CrtYg/CrtYh has synthesized CDB=6 carotenoid, and CrtM_{F22A,F26A,W38A,E180G,F233A}/HexPS/FDS_{Y81A,V157A}/CrtI_{N304P}/CrtE2/CrtYg/CrtYh has synthesized CDB=8 carotenoid respectively.

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Formation of Triplet-Excited State of Carotenoid in the Photosynthetic Reaction Center Complex of Green Sulfur Bacteria

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Green Sulfur Bacteria (Gsb) are obligatory anaerobic photoautotrophic bacteria, grown by anoxygenic photosynthesis oxidizing reduced sulfur compounds, such as sulfide, as electron sources. Their photosynthetic reaction center (RC) complex contains three 4Fe-4S clusters serving as electron acceptors for a long-lived charge separated state; it is thus classified into PS1-type (type-1), which is a functional homologue of photosystem I of oxygenic photosynthesis. Although its structure and function are not fully elucidated yet, an atomic resolution 3D structure of the Gsb-RC complex of *Chlorobaculum tepidum* was reported recently ^[1]. There are four carotenoids in the core-protein; the two of which is Chlorobactene (CB) and the other two is its glycosylated derivative, OH-chlorobactene-glucoside laurate (CB-G-FA). Since the core-protein has a homodimeric structure, the four carotenoids are bound in a C₂-symmetrical fashion. However, CB and CB-G-FA are arranged in different orientations; CB is laid almost in a horizontal direction relative to the membrane surface, while CB-G-FA is in a vertical. Function of these carotenoids in the Gsb-RC complex is still little known and has never been targeted for any intensive study. In the past meeting held in 2019, our research group reported that these carotenoids serve as effective light-harvesting antennae in the Gsb-RC complex ^[2].

In the present study, we further explored processes including any photochemical reaction of carotenoids in the Gsb-RC complex of *C. tepidum* by ultra-fast transient absorption spectroscopy. As a result, we successfully observed formation of a triplet-excited state of carotenoid (Car-T₁). Since the Car-T₁ formation was depending on excitation of Bacteriochlorophyll *a* (BChl *a*), it seemed be mediated by triplet-to-triplet excitation energy transfer (T-T EET) from some BChl *a* to the carotenoid. However, the observed kinetics had a lifetime faster than 1 ns, which is much faster than the rate for intersystem crossing between the lowest energy singlet and triplet states of a free BChl *a*. Therefore, the Car-T₁ formation in the Gsb-RC complex could be driven by an unknown mechanism rather than a simple T-T EET. We will discuss the reaction mechanism and the responsible carotenoid molecule of the Gsb-RC complex.

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In-vitro reconstitution of the photosynthetic antenna of marine green algae toward regulation of binding carotenoid

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Light-harvesting complex II (LHCII) is a pigment-protein complex ^[1] that absorbs solar energy for photosynthesis. The LHCII of the marine green alga (*Codium fragile*), called SCP (siphonaxanthin chlorophyll *a/b* binding protein), absorbs green light more efficiently than LHCII of higher plants. This green light absorption is attributable to the polar carotenoid siphonaxanthin interacting with the protein of SCP. Greenlight is known to have the highest intensity in the solar spectrum, therefore, elucidating the mechanism of efficient light absorption in this wavelength region is extremely important for the efficient use of solar energy.

"In-vitro reconstitution" has been established for LHCII of higher plants. This method artificially recreates LHCII from pigments extracted from natural sources and proteins expressed in *Escherichia coli*. It can also modify pigments and proteins and is widely used for LHCII in plants and green algae. However, reconstitution in systems containing highly polar carotenoids such as siphonaxanthin has not yet been reported.

Therefore, we attempted to reconstitute SCP using the conditions established for the LHC of higher plants ^[2]. As a result, a pigment-protein complex was successfully formed, with a small green absorption band characteristic of SCP. However, the green band was much smaller than that of native SCP (Figure 1), and the number of binding-siphonaxanthin relative to the protein was also smaller than that of native SCP. In this study, the composition of additive pigments was systematically varied and the correlation between the pigment compositions of the additive pigments and the reconstituted SCP was evaluated. Based on these results, we will determine the composition of additive pigments that will reduce differences in the binding efficiency of carotenoids with widely different physical properties and establish a technique for reconstituting SCP.

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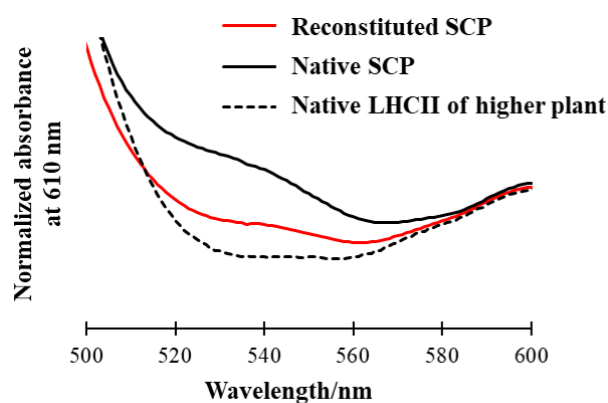


Figure. 1 Absorption spectrum in green region

Identification and tissue distribution of fucoxanthin-derived novel metabolites

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1. Introduction

Marine carotenoid fucoxanthin (Fx), which is expected to have various nutraceutical functions such as preventing non-alcoholic steatohepatitis [1], is converted to fucoxanthinol (FxOH) in intestinal tract and then absorbed in body. FxOH is further metabolized to amarouciaxanthin A (AmxA) and paracentrone [2]. In recent years, identification of carotenoid fatty acid esters in mammals has attracted attention to various metabolisms of carotenoids. However, there is no information on the conversion of Fx-derived metabolites to fatty acid esters. In this study, we aimed to identify novel ester-type metabolites and clarify their distribution in tissues of Fx-fed mice.

2. Materials and Methods

5-week-old C57BL/6J male mice (n=7) were fed AIN-93G diet containing 0.2% Fx for 1 week. After extraction of total lipids from the tissues and serum by Folch method, Fx metabolites were quantified by HPLC. Hydrolysis of total lipids extracted from liver and adipose tissue was performed using cholesterol esterase. Carotenoid fatty acid esters were prepared by esterification reaction of FxOH or AmxA with fatty acid chlorides.

3. Results and Discussion

When total lipids extracted from liver and adipose tissue were subjected to HPLC, several unidentified peaks were observed in addition to known Fx-derived metabolites such as FxOH and AmxA. These peaks were purified by HPLC and subjected to enzymatic hydrolysis. As a result, the unidentified peaks disappeared and new peaks with retention times corresponding to FxOH and AmxA appeared, suggesting that the unidentified peaks may be esterified derivatives of FxOH and AmxA. Next, carotenoid fatty acid esters were synthesized from free-form FxOH or AmxA and various fatty acid chlorides to use as standards. Comparison of HPLC chromatograms with those standards and total lipids revealed that the unidentified peaks were carotenoid fatty acid monoesters in which fatty acids such as palmitic acid and oleic acid were bound to FxOH and AmxA. Interestingly, the monoesters were abundantly accumulated in liver and adipose tissue, whereas the contents of those esters in small intestine, kidney, skeletal muscle, and serum were low. Furthermore, the composition of accumulated FxOH and AmxA fatty acid esters differed between liver and adipose tissue. Therefore, it was suggested that FxOH and AmxA are mainly transported as free forms to each tissue via the bloodstream and then actively converted to fatty acid esters, especially in the liver and adipose tissue.

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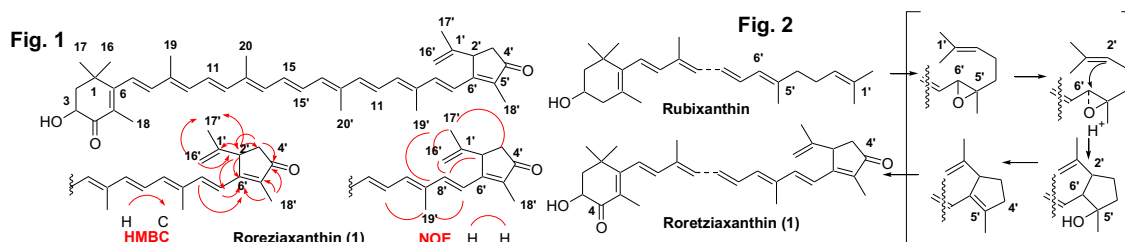
A novel carotenoid roretziaxanthin, having a unique 2,6-cyclo- Ψ -end group, from the sea squirt *Halocynthia roretzi*

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Tunicates are marine invertebrate belonging to Protochordate show bright red color due to contain various carotenoids. The sea squirt *Halocynthia roretzi* is an edible tunicate that is classified into the family Pyuridae in Protochordate and has been consumed as sashimi, as vinigated food, as salted food etc. in East Asia, especially in South Korea and in the Northeast region of Japan. A novel carotenoid, having a unique 2,6-cyclo- Ψ -end group, named roretziaxanthin (**1**) was isolated from the sea squirt *Halocynthia roretzi* as minor carotenoid along with astaxanthin, halocynthiaxanthin, mytiloxanthinone etc. This structure was determined to be 3-hydroxy-1',16'-didehydro-1',2'-dihydro-2',6'-cyclo- β . Ψ -carotene-4,4'-dione by uv-vis, MS, and NMR data. Structure of new end group was elucidated by detailed analysis of HMBC and NOE correlations as shown in **Fig. 1**.



This is the first report the isolation of carotenoid having a 2,6-cyclo- Ψ -end group in marine animals. The formation mechanism roretziaxanthin (**1**) was proposed in **Fig. 2**. Namely, epoxidation of double bond at C5, C6 in carotenoid with Ψ -end group formed carotenoid with 5,6-epoxy- Ψ -end group. However, carotenoid with 5,6-epoxy- Ψ -end group was very unstable and soon converted carotenoids with 2,6-cyclo- Ψ -end group through cleavage of epoxy ring and cyclization between C2 and C6. Oxidation at C4, C4' formed roretziaxanthin (**1**). It is reported that some carotenoids in marine invertebrate are produced by symbionts. Therefore, roretziaxanthin (**1**) might be produced from rubixanthin by symbionts in sea squirt.

Spectroscopic study on the LH1-RC light-harvesting antenna pigment-protein complex from the purple photosynthetic bacterium *Blastochloris viridis*

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Photosynthetic bacteria have been actively used as research targets to understand the primary process of photosynthesis because of their relatively simple structure of photosystems and easiness of biochemical sample preparation. Although there have already been performed many studies on the light-harvesting (LH) systems containing bacteriochlorophyll (Bchl) *a*, there are limited examples on the studies of LH systems containing Bchl *b* [1,2]. The photosystem from the purple photosynthetic bacterium *Blastochloris (Blc.) viridis* is solely composed of the LH1-RC complex, in which the LH1 pigment-protein complex surrounds the reaction center (RC). The LH1 complex is composed of two species of carotenoid pigments (1,2-dihydroneurosporene and 1,2-dihydrolycopene) and Bchl *b* dimers [1], which shows a Q_y absorption band in the far near-infrared region (1010 nm), the longer wavelength region than the Bchl *a*-containing LH1 complex. Purple photosynthetic bacteria produce reactive oxygen species that can destroy living organisms when they are exposed by excess amount of light energy. However, the LH1-RC complex prevents cell death with the photoprotection mechanism, by which dissipates the excess light energy as heat to the surroundings. In this study, we isolated the LH1-RC complex from *Blc. viridis* and evaluated its photoprotective function, which is expected to contribute to the development of robust artificial photosynthetic systems, especially for the purpose of effective usage of the light energy in the far near-infrared spectral region.

Photosynthetically grown cells of *Blc. viridis* were harvested and were subjected to French press to obtain photosynthetic membranes after ultra-centrifugation. The photosynthetic membranes were solubilized, and the LH1-RC complex was isolated by sucrose density gradient ultra-centrifugation. Sub-nanosecond time-resolved absorption spectroscopic measurement on the isolated LH1-RC complex was performed with Bchl *b* excitation in the Q_y absorption band (1010 nm) to investigate the excitation energy transfer processes from Bchl *b* to carotenoids.

Sub-nanosecond time-resolved absorption spectroscopy of the isolated LH1-RC complex did not afford the meaningful signals that are ascribable for the generation of the triplet carotenoids. This outstanding finding suggests that the photoprotective function of carotenoids that are bound to the LH1-RC complex from *Blc. viridis* may not be very effective.

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Metabolism of Siphonaxanthin in Human Hepatocyte Model

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Siphonaxanthin is a carotenoid of several specific green algae (*e.g.*, *Codium fragile* and *Caulerpa lentillifera*). We have previously shown that siphonaxanthin has potent anti-inflammatory effects [1] and that its effects are increased by metabolic conversion to oxidative metabolites (Fig. 1). These metabolites were detected in mice ingesting siphonaxanthin [2]; however, it remains unclear whether they are also produced in humans. In this study, we analyze siphonaxanthin metabolism in HepaRG cells, a model for studies of human liver metabolism of xenobiotics. HepaRG cells generated 3 major metabolites during the 24-h incubation in siphonaxanthin-containing medium: one with much higher polarity than siphonaxanthin (metabolite A), one with similar polarity (metabolite B), and one with lower polarity (metabolite C). Since metabolite A is much more polar than siphonaxanthin and its maximum absorption is almost the same, it was predicted to be a conjugate of siphonaxanthin. Metabolite B was identified as didehydro-metabolite of siphonaxanthin (DDM, Fig. 1) based on comparison with the standard. Based on HPLC retention times, metabolite C was considered to be the fatty acid ester form of siphonaxanthin or DDM. Although the amount was very small, didehydro-siphonaxanthin (DDS, Fig. 1) was also detected. No peak was detected that could be considered as tetradehydro-metabolite of siphonaxanthin (TDM, Fig. 1). Comparing HepaRG cells before and after differentiation induction, DDS and metabolite C were produced more in cells before differentiation, while metabolites A and DDM were produced more in cells after differentiation. In conclusion, siphonaxanthin would undergo oxidative metabolic conversion to DDS and DDM and fatty acid esterification reactions in human hepatocytes. It is also suggested that the efficiency of these metabolic transformations is affected by the differentiation state of hepatocytes.

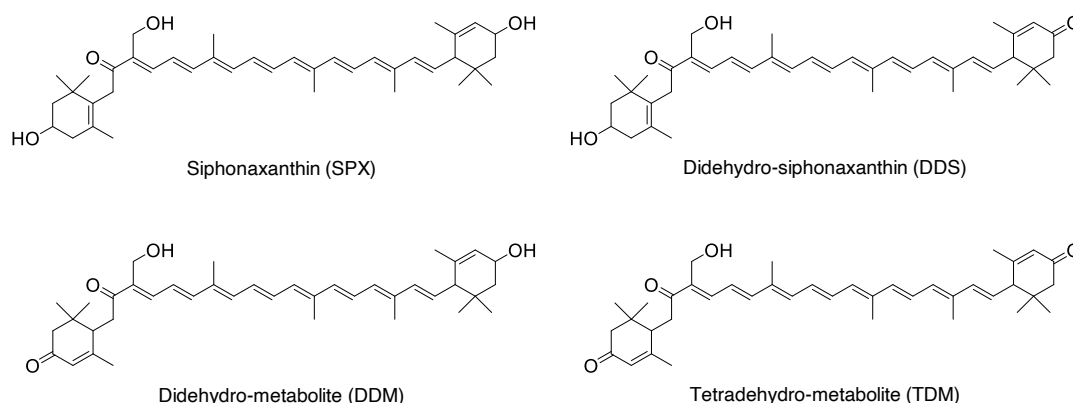


Fig. 1. Chemical structures of siphonaxanthin and its oxidative metabolites

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Structural optimization of *cis-trans* β -apo-8'-carotenal using molecular orbital calculations and evaluation of its photophysical properties

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Introduction

Photosynthesis is a reaction that uses sunlight to generate biological energy from water and carbon dioxide. Conjugated polyene molecules such as carotenoids and chlorophylls have been selected to carry out this function. It is known that the unique structures of these pigment molecules are closely related to the light-harvesting function, which plays an important role in the primary process of photosynthesis [1]. The detailed understanding of the structure-function relationship of the pigment molecules is important to clarify the mechanisms of the photosynthetic light-harvesting [2]. To this end, it is important to determine the most stable structure of the pigment molecule using computational chemistry and to predict $\Delta\mu$ (change in static dipole moment associated with optical transition). $\Delta\mu$ represents the direction and magnitude of charge transfer associated with photoexcitation of the pigment molecule. In this study, we focused on the *cis-trans* isomers of β -apo-8'-carotenal, whose $\Delta\mu$ has been determined experimentally [3]. It is interesting to compare the $\Delta\mu$ of the all-*trans* isomer, which consists of a one-dimensional polyene backbone, with those of the *cis* isomers, in which the polyene skeletons extend two-dimensionally. We have determined the most stable structure of each isomer of β -apo-8'-carotenal using WinMOPAC 3.9 program (Fujitsu Ltd.), a semi-empirical molecular orbital calculation software, and calculated $\Delta\mu$ for each isomer.

Experimental

Minimum energy path calculations with regard to the rotation around the C6-C7 bond, which corresponds to the rotation of β -ionone ring against the polyene backbone, were performed to find the most stable structures of the all-*trans* isomer. Using thus determined dihedral angle of the C6-C7 bond as the initial parameter, the most stable structure of each *cis* isomer was determined. For the most stable structures of each isomer, $\Delta\mu$ was calculated using the MOS-F program in WinMOPAC 3.9. The magnitude of $\Delta\mu$ predicted by the theoretical calculations were in good agreement with the experimental observation [3]. Furthermore, directions of $\Delta\mu$ were compared among the isomers. While the direction of $\Delta\mu$ of the all-*trans* isomer is almost parallel to its polyene backbone, those of the *cis* isomers were away from the all-*trans* fragment in the order, all-*trans* < 7-*cis* < 9-*cis* < 13-*cis* < 15-*cis* < 13'-*cis*. This observation suggests that the intra-molecular charge-transfer may take place along the long-axis of the molecule beside the two-dimensionality of the *cis*-isomers.

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Excitation wavelength dependence of excited state dynamics of β -carotene in PSII from *Thermosynechococcus vulcanus*

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Natural photosynthetic organisms capture sunlight energy and efficiently transfer it to reaction centers (RC) where charge separation takes place to gain electro-chemical potentials. Photosystem II (PS II) is the photosynthetic pigment-protein complex consisting of CP43/47 antenna and RC, and has the capability to oxidize H₂O to O₂ [1]. The high-resolution crystal structure revealed that the PS II-dimer from cyanobacterium *Thermosynechococcus* (*T.*) *vulcanus* contains photosynthetic chromophores of 35 Chlorophyll (Chl) *a* molecules and 11 β -carotene (β -car) molecules per PSII-monomer unit [2]. β -car in PSII absorbs sunlight in the 400~500 nm region of the spectrum and transfer to nearby Chl *a*. The energy transfer from β -car to Chl *a* has been reported

to be ~30% since the energy transfer from β -car S₁ state is inefficient (0%) [3]. The two Bcr absorption bands at 489 and 507 nm were observed in PSII at 77 K [4] and linear dichroism experiments showed that these peaks correspond to distinct β -car molecules, with different orientations relative to the membrane plane [5]. Yoneda *et al.*, have reported that the efficient charge separation in PSII takes place given by excitation to the red β -car absorption band at 510 nm [6]. However, it has been still a matter of controversial what the origin of the 507 nm absorption band and why the charge separation efficiently occurs after excitation to red β -car absorption band at 510 nm. In the present study, we performed fs pump-probe spectroscopies on the isolated PSII-dimer from a thermophilic cyanobacterium, *T. vulcanus* excited by the different wavelength at 500 and 520 nm to clarify the detailed energy transfer dynamics of β -car to Chl *a*.

Fig.1 shows the steady-state absorption spectrum of PSII-dimer from *T. vulcanus*. The absorption bands around 440 and 680 nm are attributed respectively to the Soret and Q_y transition of Chl *a* (contain partly pheophytin: Pheo). The S₂ absorption band of β -car appears around 500 nm. **Fig.2** (A) shows the photo-induced absorption spectra of PSII-dimer recorded at 1.0 ps after excitation into the β -car S₂ absorption band at 500 and 520 nm. The negative absorbance changes below 520 nm are bleaching signals of β -car due to the depletion of the ground state. The positive absorbance changes, assigned to the S₁ transient absorption of β -car [7], appeared around 570 nm. These signals originated from β -car depend strongly on the excitation wavelength. The signals excited at 520 nm shift to longer-wavelength compared with those excited at 500nm. The results demonstrate the selective excitation to β -cars having the different absorption wavelengths. The Chl *a* bleaching bands were observed around 680 nm. **Fig.2** (B) represents the kinetic traces of the S₁ transient absorption excited at 500 and 520 nm. The thin solid lines are the best fit curves using exponential decay/rise components convoluted with the instrument response function. The rise of the S₁ transient absorption of β -car corresponds to the S₂ decay and it depends not on the excitation wavelength.

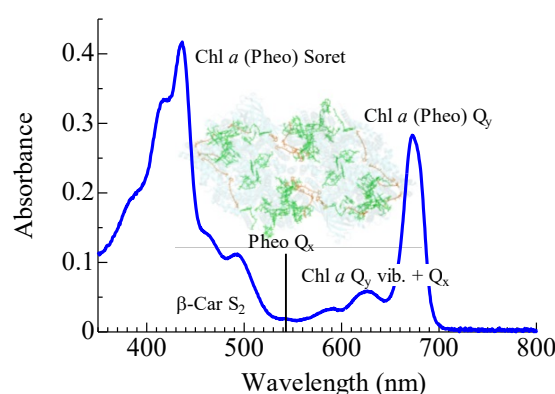


Fig.1: Steady-state absorption spectrum of the PSII dimer. An inset represents the PSII dimer.

On the other hand, the S_1 decay exhibits the pronounced excitation wavelength dependence, i.e. the faster decay of S_1 excited at 520 nm. It has been reported that the energy transfer from β -car to Chl *a* via the S_1 state was inefficient (0%) [3]. Thus, it is likely that the observed S_1 dynamics excited at 500 nm is the intrinsic S_1 decay of β -car in PSII-dimer. On the other hand, the faster S_1 decay of β -car excited at 520 nm implies the possibility of the energy transfer from β -car to Chl *a* via the S_1 state. Indeed, the Chl *a* bleaching excited at 520 nm enhanced and its peak is located at 677 nm slightly shorter-wavelength than that at 683 nm excited at 500 nm.

In this study, we performed fs pump-probe spectroscopies on the isolated PSII-dimer from a thermophilic cyanobacterium, *T. vulcanus* excited by the different wavelength at 500 and 520 nm to clarify the detailed energy transfer dynamics of β -car to Chl *a*. The excitation wavelength dependence of the excited state dynamics of β -car was observed in PSII. The excitation to the red β -car absorption band at 520 nm exhibits the faster decay of the S_1 transient absorption, implying the energy transfer from β -car to Chl *a* via the S_1 state.

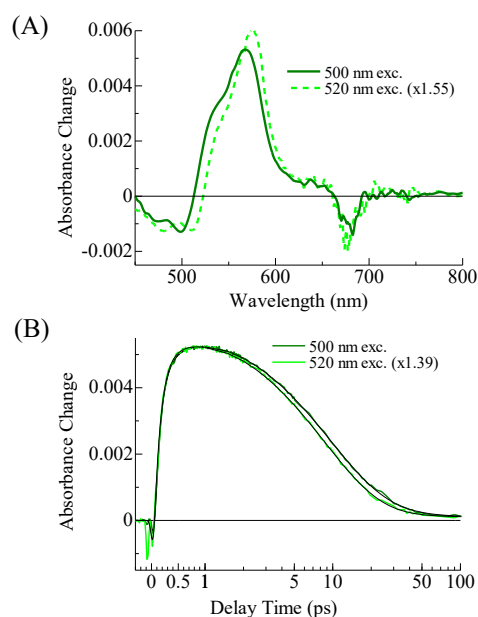


Fig.2: (A) Photo-induced absorption spectra at 1.0 ps and (B) kinetic traces at 570 nm of PSII-dimer excited at 500 and 520 nm.

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Creation of novel and rare carotenoids by new combination of known C40 carotenoid biosynthesis genes and investigation of their biological activities

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Purpose

Carotenoids are naturally widespread pigments, and many of them have been shown to have various pharmacological activities, including antioxidant activity. Since carotenoids show different biological activities depending on their chemical structures, we have been working to create rare or new antioxidant carotenoids using recombinant *E. coli* carrying plasmids containing previously discovered biosynthetic enzyme genes in new combinations.

In this study, we cultured recombinant *E. coli* carrying the biosynthesis gene of α -carotene (*HpIDI*, *crtE*, *crtB*, *crtI*, *MpLCYb*, *MpLCYe*), a ketolase gene which oxidize C-4 in β -ionone ring (*crtW*), and a hydroxylase gene which introduce OH at C-3 in ϵ -ring (*MpCYP97C*) as shown in **Fig. 1(a)** and succeeded to produce the rare carotenoid 3'-hydroxy- β , ϵ -echinenone shown in **Fig. 1(b)**.

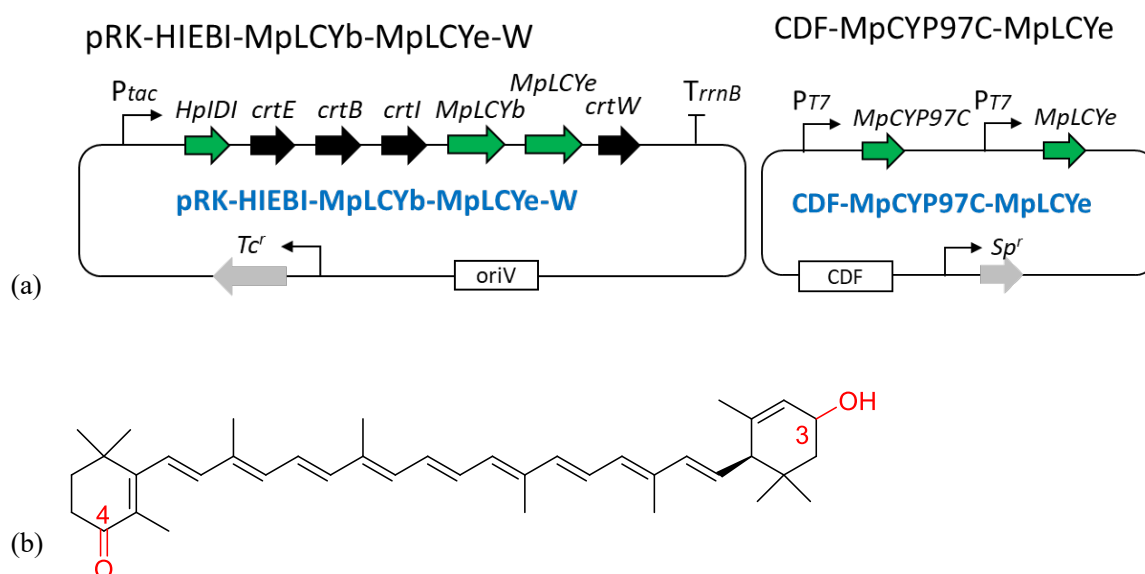


Fig. 1 Plasmid composition (a) and predicted chemical structure (b)

Method

We cultured the recombinant *E. coli* in 2YT medium (100 mL) at 27°C for 48-72 hours. The cells were collected by centrifugation and the produced carotenoids were extracted with CH_2Cl_2 -MeOH (1:1). DAD HPLC analysis of the extract revealed three carotenoids (1-3) were produced by the culture.

The recombinant *E. coli* was cultured in a large volume (2 L), and the cells were collected by centrifugation. The produced carotenoids were extracted by sonication in a CH_2Cl_2 -MeOH (1:1). The extract was partitioned with n-hexane/90% MeOH, and n-hexane layer containing carotenoids were concentrated to dryness in vacuo. The

orange syrup containing carotenoids were further separated by preparative ODS HPLC to afford pure **1** (8.8 mg), **2** (9.0 mg), and **3** (2.0 mg).

The structures of compounds **1-3** were analyzed by HRESI-MS and 1D (^1H and ^{13}C) and 2D (^1H - ^1H DQF COSY, HSQC, HMBC, and NOESY) NMR analyses. In addition, singlet oxygen scavenging activity of **1-3** was conducted.

Results and Conclusions

Structural analysis of **1-3** identified each compound as 3'-hydroxy- β , ϵ -echinenone (**1**) (a rare carotenoid in crustacea and mollusca), canthaxanthin (**2**) (a carotenoid in bacteria, algae, and some fungi), and β , ϵ -caroten-3'-ol (**3**) (a carotenoid in algae and plants), respectively (**Fig. 2**). All compounds possessed the structures that could be produced using the biosynthetic genes used in this study. This is the first study reporting the production of these rare carotenoids in recombinant *E. coli*.

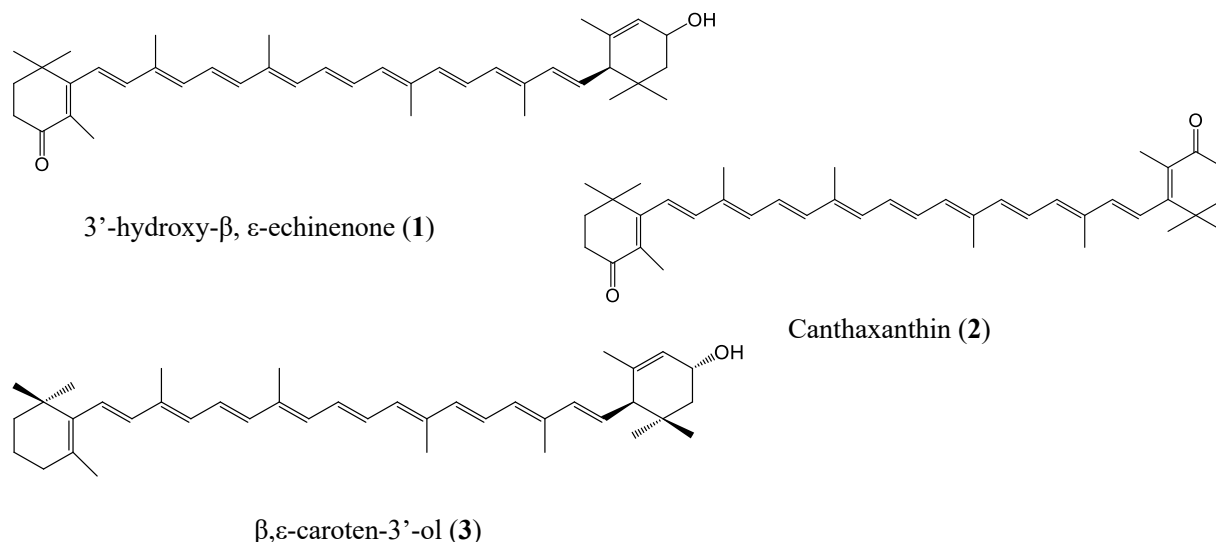


Fig. 2. The structures of produced carotenoids

The singlet oxygen scavenging activity of **1-3** were evaluated. The IC_{50} of compounds **1-3** were 4.6 μM (**1**), 2.3 μM (**2**), and 16.9 μM (**3**), respectively. (Astaxanthin IC_{50} 4.3 μM)

Development of a solid catalyst for carotenoid isomerization

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1. Introduction

Carotenoids are expected to be effective in preventing many diseases, but their low bioavailability is an important issue to be overcome. In order to improve the bioavailability of carotenoids, their “Z-isomerization” have recently been gaining traction [1,2]. For example, our recent study using rats have shown that the oral supplementation of a Z-isomer-rich astaxanthin diet (total Z-isomer ratio = 80.8%) resulted in a 36.8-fold increase in astaxanthin concentration in plasma and in a >10-fold increase in the concentrations of astaxanthin in specific tissues (e.g., adrenal, lung, and prostate) compared to the values obtained after the supplementation of an all-*E*-isomer-rich diet (total Z-isomer ratio = 0.5%) [2]. We have investigated various isomerization methods, such as heating, light irradiation, and microwave irradiation [3]. Moreover, we have discovered several plant-derived green catalysts (isothiocyanates and polysulfides) that can isomerize carotenoids efficiently [4]. However, these catalysts have a strong aroma and are homogeneous catalysts that are difficult to remove from the final product. Therefore, the objective of this study was to develop a heterogeneous (solid) catalyst that can be easily removed by filtering or centrifugation. Furthermore, we fabricated a column packed with solid catalyst and verified whether continuous isomerization is possible.

2. Methods

Isothiocyanate-functionalized silica gel (Si-NCS) was prepared and contacted with (all-*E*)-carotenoid (lycopene, β -carotene, astaxanthin, lutein, and fucoxanthin) solutions. *E/Z*-Isomer ratios were determined using high-performance liquid chromatography (HPLC). The carotenoid solutions were passed through a column packed with Si-NCS catalyst (**Fig. 1A**) and isomerization was performed continuously.

3. Results and Discussion

We found that the Si-NCS catalyst could enhance the Z-isomerization of (all-*E*)-carotenoids under mild conditions (<50°C), and little degradation of carotenoids occurred during the isomerization process. Furthermore, (all-*E*)-carotenoids were successfully Z-isomerized continuously by using a Si-NCS catalyst packed column unit (**Fig. 1B**).

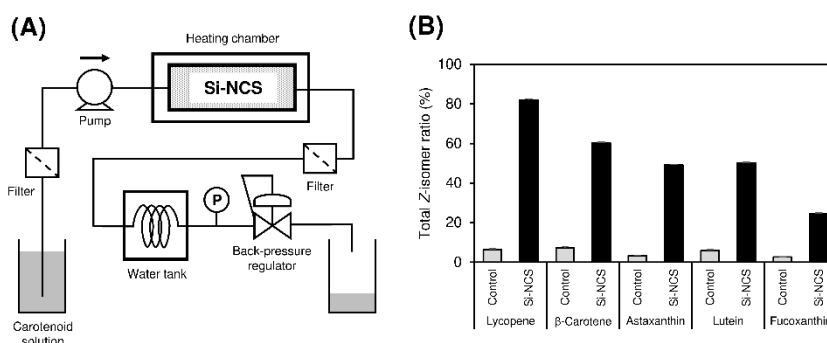


Fig. 1 Schematic diagram of the continuous-flow reactor for carotenoid isomerization using the Si-NCS catalyst (**A**) and total Z-isomer ratio of carotenoids after the isomerization treatment (**B**).

Among the carotenoids, lycopene was the most efficiently isomerized, i.e., when the lycopene solution was passed through the isomerization unit with Si-NCS catalyst, >80% of the carotenoid were isomerized to the *Z*-isomers. To the best of our knowledge, there have been no reports of such a high isomerization efficiency. The introduction of this isomerization unit immediately after the carotenoid extraction process, such as organic solvent and supercritical CO₂ extraction processes, is thought to produce an carotenoid extract with its high *Z*-isomer ratio.

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Development of high fucoxanthin production technology using Haptophyta *Pavlova* sp. based on metabolome knowledges

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Fucoxanthin has a wide range of applications such as an additive for functional foods, pharmaceuticals, and cosmetics. Haptophyta *Pavlova* sp. OPMS 30543 is a promising fucoxanthin producer due to its high ability to photosynthetically produce biomass from CO₂ and high fucoxanthin accumulation. A previous study suggested NaNO₃ as the suitable nitrogen source for OPMS 30543 fucoxanthin production. However, the mechanism by which nitrogen source affects the fucoxanthin production has not been elucidated. In order to develop a nitrogen supplementation strategy for fucoxanthin production, this study analyzed effect of nitrogen source on OPMS 30543 together with the underlying metabolic phenomena. When supplemented with either NaNO₃ or NH₄Cl as a sole nitrogen source, fucoxanthin content of OPMS 30543 cells was higher in the NaNO₃ medium. Metabolome analysis revealed that accumulation of phosphoenolpyruvate (PEP), pyruvate, and 1-deoxy-d-xylulose-5-phosphate (DXP) were not significantly different between the NaNO₃ and NH₄Cl media. On the other hand, accumulation of β -carotene was higher in the NaNO₃ medium, suggesting that the downstream process after DXP metabolism was affected by nitrogen source. When supplemented with different concentration of NaNO₃, fucoxanthin content of OPMS 30543 was almost same between these conditions at the initial stage, and turned to decrease in order from the lower concentration. Metabolome analysis revealed that DXP turned to decrease after nitrogen depletion. Thus, nitrogen source and its concentration were found to differently affect on the fucoxanthin biosynthesis process of OPMS 30543. The nitrogen supplementation strategy derived from these findings, in which OPMS 30543 is cultured with abundant NaNO₃, would increase feasibility of commercial production of fucoxanthin by the microalga.

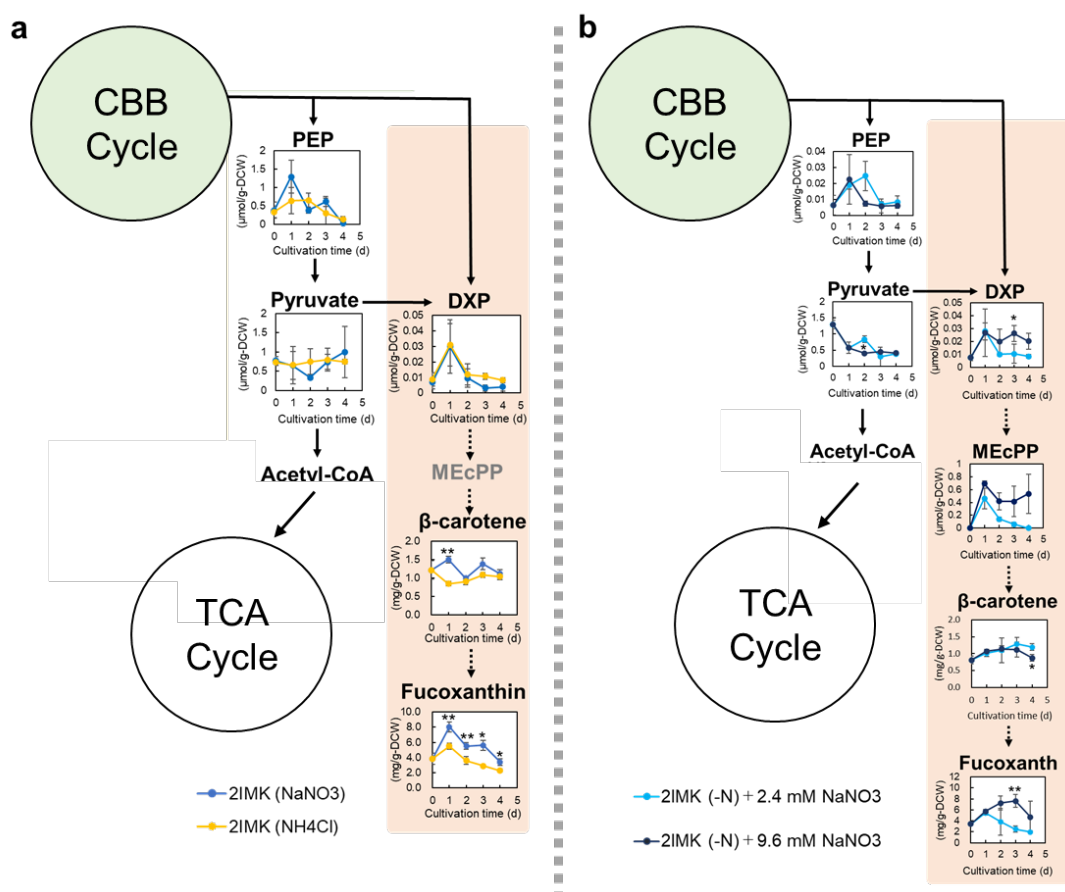


Fig. 1 Time-course changes of intracellular metabolite pool size. Changes in metabolites at different nitrogen sources (a). Changes in metabolites at different initial NaNO₃ concentrations (b). Statistical significance was determined using Welch's *t*-test (* <0.05, ** <0.01). Values are average ± SD from three replicated experiments. CBB cycle: Calvin-Benson-Bassham cycle, TCA cycle: Tricarboxylic acid cycle, PEP: Phosphoenolpyruvate, DXP: 1-Deoxy-d-xylulose 5-phosphate, MEcPP: 2-C-Methyl-d-erythritol-2,4-cyclopyrophosphate.

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Excitation energy transfer process in the LH2 light-harvesting complex from the purple photosynthetic bacterium, *Rhodoblastus acidophilus*

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Carotenoids bound to the light-harvesting complexes play significant roles, such as capturing sun-light and transferring the energy to bacteriochlorophyll *a* (Bchl *a*) in the primary process of photosynthesis. On the other hand, excess light exposure to the photosynthetic system promotes the production of triplet Bchl *a*, which results in the generation of singlet oxygen species. This is indeed harmful for photosynthetic organisms. Carotenoids can prevent the production of this singlet oxygen species by quenching the triplet Bchl *a* via triplet-triplet energy transfer from Bchl *a* to carotenoid. Thus, carotenoids in the light-harvesting complexes can also act as a photoprotector. The recent study with time-resolved spectroscopic measurements has clarified the excited state dynamics of the carotenoids following excitation of the B850 Bchl *a* in the LH2 complex from the purple bacterium *Rhodobacter (Rba.) sphaeroides* strain 2.4.1 [1]. The similar study has also been applied to the LH1 complex from *Rhodospirillum rubrum* strain S1. The relaxation dynamics following excitation of B880 Bchl *a* was very different from that of the LH2 from *Rba. sphaeroides* strain 2.4.1. The faster photoprotection mechanism mediated by the singlet-singlet fission process has newly been found [2]. The differences of these LH2 and LH1 complexes are (1) the ring size, (2) presence or absence of B800 Bchl *a*, and (3) the species of bound carotenoid. In this study, we have tackled to the issue (2) to clarify the difference found in LH2 and LH1. Here, we present the influence of the presence or absence of B800 Bchl *a* on the photoprotection process in the LH2 complex from *Rhodoblastus (Rbl.) acidophilus* strain DSM 145 [3].

The native LH2 complex was isolated from *Rbl. acidophilus* strain DSM 145. The LH2 complex in which the B800 Bchl *a* was depleted (B800-free LH2) was prepared from the native LH2 under acidic conditions [3]. Sub-nanosecond time-resolved absorption spectroscopies were applied to these LH2 complexes.

Transient absorption data recorded following excitation of the B850 absorption band showed unexpected difference at the beginning of the triplet carotenoid formation between the native and B800-free LH2. The detailed analysis was carried out by applying global and target analyses to the observed entire datasets of the LH2 complexes with or without B800 Bchl *a*.

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Preventive effects of carotenoids on UVA-induced damage in dermal fibroblasts

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Ultraviolet radiation is the major environmental stress on human skin. The UV light is classified into UVA, UVB and UVC by wavelength. UVC rays are mostly absorbed in the ozone layer. While UVB induces inflammation mainly in epidermal layer of the skin, UVA reaches dermal layer and promotes production of reactive oxygen species (ROS) that attack biomolecules. UVA also accelerates degradation of extracellular matrix (ECM), which maintains the strength and elasticity of the skin. Chronic exposure to UV causes “photoaging”, which has a significant influence on changes in appearance such as wrinkles and sagging. Although protection from UV is generally achieved by topical application of sunscreens to the skin surface, oral intake of functional substances which can transfer and accumulate in the skin, especially in the dermis, may be more effective.

We have previously found that ingested astaxanthin [1] and siphonaxanthin can accumulate in mouse skin and inhibit UV-induced photoaging. However, there is little information on the activity relationship of carotenoids with different structures in photoaging. In this study, we evaluated the preventive effects of various carotenoids in food against UV damage, focusing on UV-induced cell damage and ROS production in human dermal fibroblasts.

Twelve carotenoids were tested for their protective effects against UVA-induced cell damage and ROS production using normal human skin fibroblast cell line NB1RGB cells. Among them, canthaxanthin and fucoxanthin significantly suppressed the UVA-induced cell damage. Fucoxanthin also significantly inhibited UVA-induced ROS production whereas canthaxanthin had no effect on it. Therefore, we focused on these two carotenoids and tried to elucidate their underlying mechanisms. Canthaxanthin significantly suppressed the expression of several mRNAs associated with UVA-induced inflammation and degradation of ECM, but fucoxanthin did not. In mitogen-activated protein kinases (MAPK) signaling pathway, which regulates the expression of these mRNAs, canthaxanthin did not suppress p38 MAPK phosphorylation itself but downregulated the phosphorylation level or expression level of its downstream factors. In addition, we also evaluated the cellular lipidome changes by using liquid chromatography-mass spectrometry (LC-MS) analysis. UVA irradiation caused a decrease of sphingomyelin and an increase in ceramide, which were suppressed by canthaxanthin. Since the degradation of sphingomyelin to ceramide mediated by p38-induced sphingomyelinase activation has been reported, canthaxanthin might inhibit UVA-induced production of ceramide via suppression of p38 MAPK pathway. Our results suggest that fucoxanthin protects cells from UVA damage through its antioxidant effect, and canthaxanthin inhibits UV damage by acting on MAPK signaling pathway.

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Studies on the biosynthetic intermediate on C₃₀ carotenoid methyl 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate produced by *Planococcus maritimus* iso-3

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Purpose

A yellow marine bacterium *Planococcus maritimus* strain iso-3 was isolated from an intertidal sediment that looked industrially polluted, from the Clyde estuary in the UK. This bacterium was found to produce a yellow acyclic carotenoid with a basic carbon 30 (C₃₀) structure, which was determined to be methyl 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate (**5**) [1]. Much research has been conducted on the biosynthetic pathways and genes of C₄₀ carotenoids, while the biosynthesis of C₃₀ carotenoids produced by bacteria remains to be clarified. In this study, we investigated genes involved in the production of 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate from *P. maritimus* strain iso-3, to identify the functions of the isolated genes using *Escherichia coli*, and to obtain new biosynthetic intermediate C₃₀-carotenoids by combinations of the obtained genes.

In our previous study, five biosynthesis genes *crtM*, *crtN*, *cruF*, *crtNb* and *GT* genes have already been isolated and identified, and the structures of their biosynthetic intermediate carotenoids have been determined (blue framed part in **Fig. 1**) [2].

In the present study, we isolated and identified *crtP* and *aldH* genes, and determined the structures of their biosynthetic intermediate carotenoids (red framed part in **Fig. 1**). We also evaluated the singlet oxygen-quenching activities of them and compared their activities with methyl 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate. In addition, the biosynthetic pathway was presumed from the production titer of glucosyl and non-glucosyl carotenoids.

Methods

We searched *crtP* and *aldH* using homology to previously reported ones in other members of the genus *Planococcus* and isolated them from *Planococcus maritimus* iso-3. We constructed the following plasmids A to D and introduced them into *E. coli*.

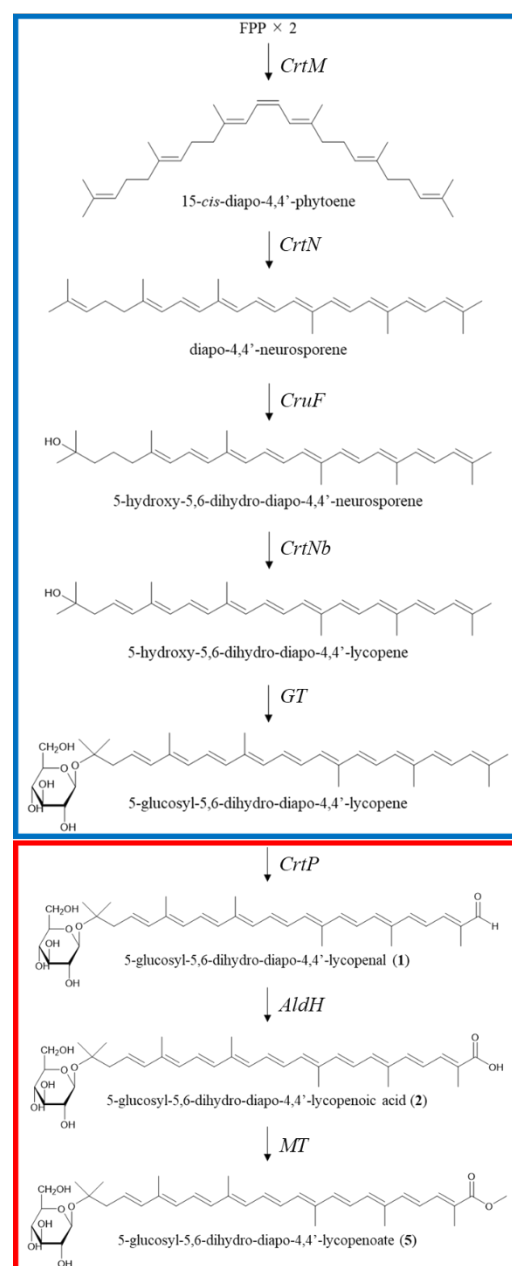


Fig. 1
Carotenoid biosynthetic pathway in
Planococcus maritimus strain iso-3

A *crtM*, *crtN*, *cruF*, *crtNb*, *GT* and *crtP*B *crtM*, *crtN*, *cruF*, *crtNb*, *GT*, *crtP* and *aldH*C *crtM*, *crtN*, *cruF*, *crtNb* and *crtP*D *crtM*, *crtN*, *cruF*, *crtNb*, *crtP* and *aldH*

To isolate the produced biosynthetic intermediate carotenoids, each transformed *E. coli* was grown in 2YT medium (100 mL medium in 500 mL Sakaguchi flask) at 20°C for 48-72 hours and the cells were collected by centrifugation and extracted with CH₂Cl₂-MeOH (1:1) with sonication. The extract was concentrated to dryness and partitioned between EtOAc/H₂O. The EtOAc layer containing carotenoids was concentrated to dryness and applied to preparative ODS HPLC to afford pure carotenoids. The structures of purified carotenoids were analyzed by HRESI-MS and 1D (¹H and ¹³C) and 2D (¹H-¹H DQF COSY, HSQC, HMBC, and NOESY) NMR analyses.

We also investigated ¹O₂-quenching activities of the produced biosynthetic intermediate carotenoids, and determined their IC₅₀ values, which represents the concentration at which 50% inhibition.

Results

The structures of biosynthetic mediate carotenoid produced by *E. coli* carrying A, B, C, D plasmid were determined to be 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenal (**1**, yield 2.0 μmol/L), 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoic acid (**2**, yield 3.1 μmol/L), 5-hydroxy-5,6-dihydro-diapo-4,4'-lycopenal (**3**, yield 0.75 μmol/L), and be 5-hydroxy-5,6-dihydro-diapo-4,4'-lycopenoic acid (**4**, yield 1.2 μmol/L), respectively. Carotenoids **1** - **4** were all new compounds according to the CAS database.

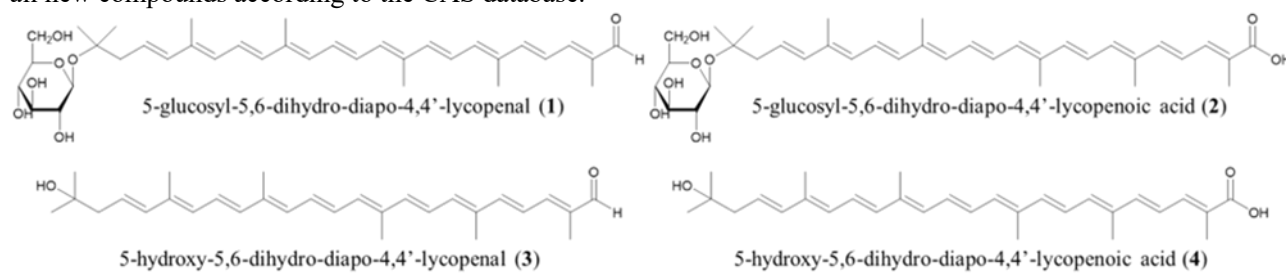


Fig. 2

The structures of new carotenoids (1–4)

The ¹O₂-quenching activities of carotenoids **1–4** was examined, and the results are shown in Table 1. Compounds **1–4** all showed potent ¹O₂-quenching activities, and biosynthetic intermediates at the end of biosynthetic showed more potent activities. This observation means that *P. maritimus* strain iso-3 may produce methyl 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate to prevent singlet oxygen damage.

Carotenoid	Singlet oxygen quenching IC ₅₀ (μM)
5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenal (1)	8.3
5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoic acid (2)	12
5-hydroxy-5,6-dihydro-diapo-4,4'-lycopenal (3)	9.3
5-hydroxy-5,6-dihydro-diapo-4,4'-lycopenoic acid (4)	7.8
methyl 5-glucosyl-5,6-dihydro-diapo-4,4'-lycopenoate (5)	5.1

Table 1

Singlet oxygen quenching activity of the intermediate carotenoids

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Ultrafast energy transfer dynamics in photosynthetic antenna acpPC from dinoflagellate *Symbiodinium*

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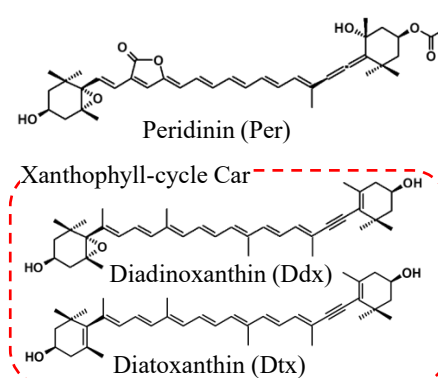
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Photosynthetic antenna apparatus efficiently capture sunlight and transfer the energy to reaction centers. Dinoflagellates contain two types of light-harvesting pigment-protein complexes. One is the peridinin-chlorophyll (Chl) protein (PCP) complex, which is water soluble protein and peripheral antenna of photosystem (PS) II¹. Another is Chl *a/c*-peridinin protein complex (acpPC), which is an integral membrane complex² and peripheral antenna of PSI³. The acpPC complex contains major carotenoid peridinin (per) and minor carotenoid diadinoxanthin (ddx)/diatoxanthin (dtx) (Scheme). It has been reported that acpPC shares high similarity to fucoxanthin Chl *a/c* protein (FCP) complex from diatom^{4,5}. Per and fucoxanthin exhibit spectral characteristics attributed to an intramolecular charge transfer (ICT) state located nearby the S₁ state that arises in polar environments due to the presence of the carbonyl group in its polyene backbone, leading to the generation of a strongly coupled S₁/ICT state⁶. Consequently, per and fucoxanthin efficiently transfer the absorbed energy to nearby Chl *a* via S₁/ICT state⁷. On the other hand, ddx/dtx is one of the xanthophylls cycle carotenoids that play a photoprotective function by dissipating excitation energy as heat. Thus, it has not been elucidated how ddx/dtx plays roles in light-harvesting function in acpPC. In this study, we isolated the two different fractions of functional acpPC from dinoflagellate *Symbiodinium* sp.strain SGCH-03⁸ and performed femtosecond pump-probe measurements on two different fractions of acpPC to clarify the light-harvesting functions of carotenoids.

Figure (A) presents the steady-state absorption spectra of the acpPC fractions (fr.1 and fr.2). The absorption bands around 440 and 460 nm are assigned to the Soret bands of Chl *a* and Chl *c*, respectively. The Q_y absorption band of Chl *a* is observed around 670 nm. The absorption spectra show the different carotenoid composition of fr.1 and fr.2 *i.e.*, fr.1 contains much per molecules while fr.2 contains much ddx/dtx. Femtosecond pump-probe spectroscopic measurements were performed on the two acpPC fractions to clarify energy transfer dynamic from carotenoids to Chl *a*. **Figure (B)** shows the photo-induced



Scheme: The chemical structures of carotenoids bound to acpPC.

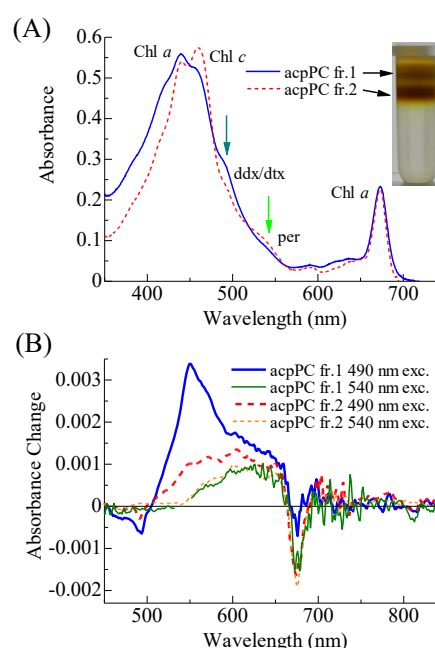


Figure: (A) Steady-state absorption spectra and (B) photo-induced absorption spectra of the different acpPC fractions.

absorption spectra of fr.1 and fr.2 taken at 1.0 ps after selective excitation to ddx/dtx at 490 and per at 540 nm. The positive absorbance changes due to the carotenoid transient absorption and the negative signals of the Chl *a* bleaching at 670 nm appears in the all spectra. In the spectrum of fr.1 excited at 490 nm, a sharp transient absorption band was observed around 550 nm. This sharp transient absorption band is assignable to ddx/dtx, while the Chl *a* bleaching was suppressed, suggesting an inefficient energy transfer from ddx/dtx to Chl *a*. In other cases, larger Chl *a* bleaching signals were observed, suggesting an efficient per→Chl *a* energy transfer.

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Biosynthetic pathways for non-standard apocarotenoids for shifting action spectrum of bacteriorhodopsin

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Introduction

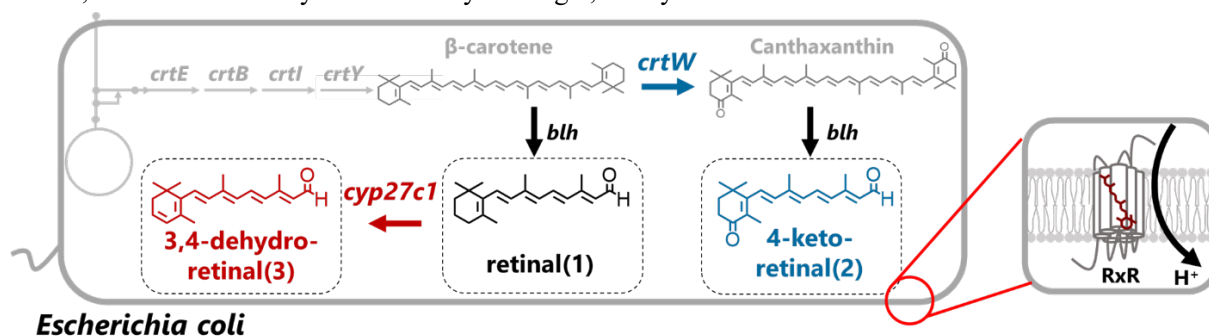
Retinal, an oxidative cleavage product of β -carotene, is an important apocarotenoid that binds to various proteins such as bacteriorhodopsins (BRs). Retinal binding confers BRs the proton-pumping activity, thereby making them the smallest stand-alone photosynthetic device. Unfortunately, each BRs is limited in the light it can absorb, and efforts have been made to shift/modify the absorption wavelength range, mainly by mutagenesis. In this report, we attempted to construct biosynthetic pathways for atypical and/or non-natural retinoids with different pigment properties to modify action spectrum of BRs.

Experiments and Discussion

By adding genes for β -carotene oxygenase to biosynthetic pathway of β -carotene, *Escherichia coli* strain produce, accumulate, and excrete retinal (**1**). Additional expression of keto-transferase (CrtW), this strain, we observe the accumulation of 4-keto-retinal (**2**) together with the retinal.

We also tested the additional expression retinal dehydrogenase (CYP27C1) from *Danio rerio* in the strain harboring standard retinal biosynthetic pathway. By co-expression of FDX1/FDXR, a mitochondria-type electron transferase, we could achieve near perfect conversion of retinal to 3,4-dehydro-retinal (**3**) in the cell.

The two biosynthetic pathways established above were co-expressed in *E. coli* with bacteriorhodopsin (RxR) from *Rubrobacter xylanophilus*. RxR fed with 4-keto-retinal exhibited a blue-shifted excitation spectrum compared to RxR fed with natural-type retinal, while RxR fed with 3,4-dehydroretinal exhibited a red-shifted excitation spectrum. Also, the action spectrum of RxR (as the light-driven proton pump) was blue-shifted by feeding 4-keto-retinal, while it was clearly red-shifted by feeding 3,4-dehydro-retinal.



Femtosecond Emission Spectroscopy of ICT State in Fucoxanthin

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Light-harvesting complexes (LH complexes) containing carotenoids (Car) and bacteriochlorophyll (BChl) play important roles in photosynthetic bacteria. In particular, the light-harvesting function of Car attracts attention. Fucoxanthin (Fx) is characterized by its intra-molecular charge transfer (ICT) state in polar solvents. In the energy transfer from Car to BChl, a highly efficient energy transfer is considered to occur from ICT because S_1 is an optically forbidden state, but ICT is an optically allowed state [1,2].

We have measured the dynamics of the ICT state of Fx in methanol by multi-excitation transient absorption spectroscopy [3]. The S_1 /ICT state has a relaxation with time constants of subpicoseconds and 5 ps. However, it is difficult to distinguish between the S_1 and ICT states by the transient absorption measurements. A stimulated emission from the ICT state is observed around 900 nm, but the dynamics of the ICT state could not be separately obtained because of the strong S_2 transient absorption signal in the same wavelength.

In this study, the dynamics of the ICT state of Fx was investigated in detail by femtosecond time-resolved measurements of ICT emission. Because the emission from the ICT state is weak and in the near-infrared region, we have detected ICT emission by the highly sensitive Upconversion method using sum frequency generation.

Figure 1 shows the time-resolved Fx emission signal at 970 nm induced by the 500 nm excitation light. Two rise components with a time constant below the time resolution (<0.3 ps) and 0.6 ps are observed. The component below the time resolution indicates that the relaxation from the initially excited S_2 state to the ICT state is ultrafast. The 0.6 ps component suggests that S_1 /ICT has some intermediate state before reaching equilibrium. A similar component has been observed in transient absorption spectroscopy, but it is difficult to determine whether this intermediate state is the S_1 state or the vibrationally excited state of the S_1 /ICT state. We will clarify the details of the intermediate state by measuring emission signals of broader wavelength range.

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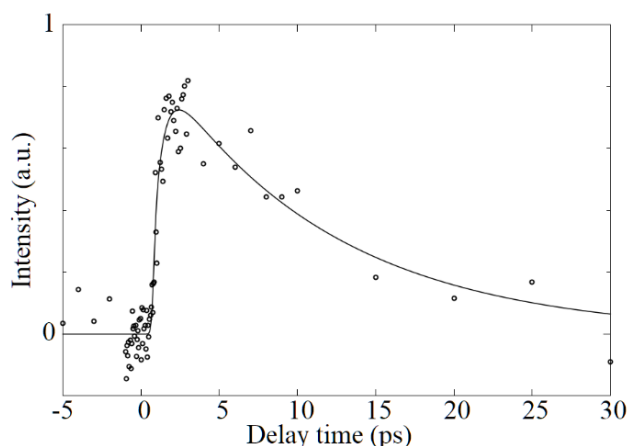


Fig. 1. Femtosecond time-resolved emission signal of fucoxanthin in methanol.

Important Role of Aphids on Arthropodal Food Chain, which was Told from Functional Analysis of the Carotenoid Biosynthesis Genes

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Carotenoids are pigments that protect organisms from photooxidative damage. Aphids and spider mites have been shown to possess genes of fungal origin for carotenoid biosynthesis since 2010, whereas a little knowledge was available about the functions of the genes and the biosynthetic pathway. The whole carotenoid biosynthetic pathway of the pea aphid (*Acyrtosiphon pisum*) was elucidated at the gene level, through comprehensive functional analysis of its carotenogenic genes, using the functional expression system in recombinant *Escherichia coli* [1]. Four genes were needed to synthesize all carotenoids accumulated in aphids from geranylgeranyl diphosphate (GGPP). We revealed that a gene designated *ApCrtYB3*, which was considered to have functionally evolved in aphids, can convert lycopene into uncommon carotenoids with the γ -ring such as (6'S)- β,γ -carotene and γ,γ -carotene. No organisms that can biosynthesize these atypical carotenoids have been reported so far, except for aphids, while a fungus *Caloscypha fulgens* was reported to possess β,γ -carotene [2].

We considered that the uncommon γ -carotenoids, which were here ascertained to be synthesized de novo in aphids, can work as ecological indicators for estimating the food chain from aphids to predatory arthropods, and showed that aphids contributed with significant levels to the food chain on predatory arthropods, *i.e.*, the red dragonfly (*Sympetrum frequens*; adults) and other dragonflies, seven-spotted ladybird (*Coccinella septempunctata*), two spiders, *Oxyopes sertatus* and *Nephila clavata*, and water sliders, *Acquarius elongatus* and *A. paludum* [1,3,4]. The content rate of all the γ -carotenoids that exist in seven carotenogenic aphids average 40.2%. Content rate and contribution rate (content rate per 40.2) of these γ -carotenoids in the individual arthropods are calculated as follows (shown as percentage, respectively): the immature adults of the red dragonfly (*S. frequens*) (5%, 12.4%), the mature adults of the dragonfly (17.8%, 44.3%), the larvae of the seven-spot ladybird beetle (*C. septempunctata*) (24.1%, 60%), the adults of the beetle (24.7%, 61.4%), sasa-spider (*O. sertatus*) (11%, 27.4%), joro spider (*N. clavata*) (1.9%, 4.7%), and water sliders (*A. elongatus* and *A. paludum*) (5.8%, 14.4% and 16.9%, 42%).

These results indicated that aphids made significant contributions to the food chain of widespread predatory arthropods. Aphids are likely to be positioned as an important “phytochemicals” source for some predatory insects and arachnids, which are often active under bright sunlight.

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Identification of carotenoids in photosynthetic protein complexes based on structural analysis

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Photosynthetic organisms, such as plants and algae, absorb solar energy and convert it into chemical energy by utilizing numerous pigments found in the photosynthetic membrane protein complexes (photosystems I and II; PSI and PSII) and then undergo electron transfer reactions. The carotenoids present in these protein complexes not only transfer energy to chlorophyll (Chl), but also possess photoprotective functions, whereby excitation energy is converted into heat and excess excitation energy is dissipated. The structure of these protein complexes has been actively analyzed in various species to elucidate the arrangement and functions of the pigments in the photosynthetic proteins. During this symposium, I aim to introduce and discuss the composition and arrangement of carotenoids in PSI and PSII of different species through structures analyzed using X-ray crystallography and cryo-electron microscopy (cryo-EM).

The PSII of *Thermosynechococcus vulcanus*, a thermophilic cyanobacterium, mainly contains β -carotene (β -Car). In 2011, we identified one β -cryptoxanthin (β -Crx) at the periphery of PSII by X-ray crystallography¹. In contrast, based on the structure obtained at a resolution of 2.5 Å, reported by Jordan et al., the PSI of *Thermosynechococcus elongatus* was found to contain only β -Car². However, our research group recently performed a cryo-EM analysis of the *T. vulcanus* PSI at a resolution of 2.1 Å. We obtained a difference map ($F_o - F_c$) between the structure factor (F_o) calculated using the cryo-EM map and structure factor (F_c) calculated using the structural model. The difference map suggested that a carotenoid with hydroxyl group is contained in the *T. vulcanus* PSI.

Additionally, *Acaryochloris marina*, a cyanobacterium, uses Chl *d* to absorb near-infrared light for photochemical reactions, whereas many oxygen-evolving photosynthetic organisms utilize Chl *a* for their photochemical reactions. Recently, we analyzed the structure of *A. marina* PSI at 2.6 Å resolution using cryo-EM and discovered a unique electron transfer mechanism involving Chl *d* and pheophytin *a* (Pheo *a*)³. The *A. marina* PSI contained a large number of carotenoids, and their arrangement was similar to the PSI of *T. vulcanus*; however, fine rearrangements were observed due to alteration in the surrounding amino acid residues. Furthermore, pigment analysis revealed that unlike β -Cars, α -carotenes (α -Cars) were identified in the *A. marina* PSI. Moreover, one zeaxanthin (Zea) was found in the *A. marina* PSI.

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Reconstitution of spheroidene into the LH1 complex from a carotenoidless purple photosynthetic bacterium, *Rhodospirillum rubrum* strain G9+

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In the primary process of purple photosynthetic bacteria, solar energy that is captured by the light-harvesting (LH) complex is transferred to the reaction center (RC), where charge separation takes place [1]. Both LH complex and RC have carotenoids that play important roles as the light harvesters (to boost the light-harvesting capacity) and the photoprotectors (to dissipate the excess energy delivered to bacteriochlorophyll *a* (Bchl *a*)) [1]. In the light-harvesting process, carotenoid (Car) is photoexcited up to the S₂ singlet excited-state and then relaxes to another optically forbidden S₁ singlet excited-state. Excitation energy is transferred from both the S₂ and S₁ states of Car to Bchl *a* (Figure 1(a)). On the other hand, in the photoprotection process, Car can dissipate the excess energy that has been delivered to Bchl *a* through singlet to triplet (ST) and/or triplet to triplet (TT) energy transfer pathways (Figure 1(b)). The ratio of ST/TT changes depending on the type of LH complexes [2, 3]. Only the TT-type energy dissipation is found in the LH2 complex from *Rhodobacter. (Rba.) sphaeroides* strain 2.4.1, which has spheroidene as a major Car [2], while the ST-type energy dissipation is dominant in the LH1 complex from *Rhodospirillum (Rsp.) rubrum* strain S1, which has spirilloxanthin as a major Car [3].

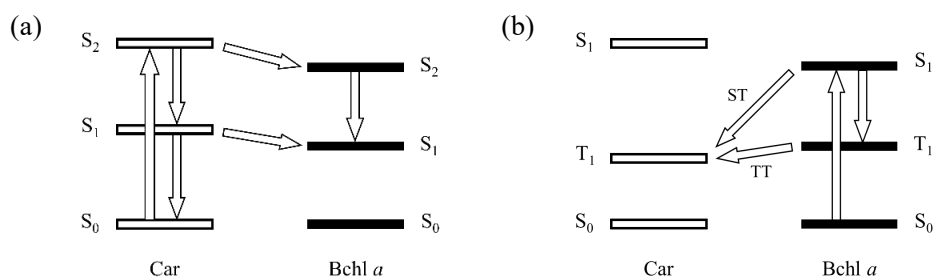


Figure 1. Schematic illustration of (a) light-harvesting and (b) photoprotection

In this study, we have extracted and purified spheroidene from *Rba. sphaeroides* strain 2.4.1 and reconstituted it to the carotenoidless LH1 complex from *Rsp. rubrum* strain G9+. We have investigated how the photoprotection processes would be modified depending on the species of Car bound to the LH1 complex using sub-millisecond time-resolved absorption spectroscopy. We found that TT-type energy dissipation process would be dominant in the spheroidene-reconstituted LH1 complex. This finding is quite different from the native spirilloxanthin-bound LH1 complex from *Rsp. rubrum* strain S1.

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Major Carotenoids of *Meiothermus ruber* Are Deinoxanthin Glucoside Esters, Not Meiothermoxanthin Glucoside Esters

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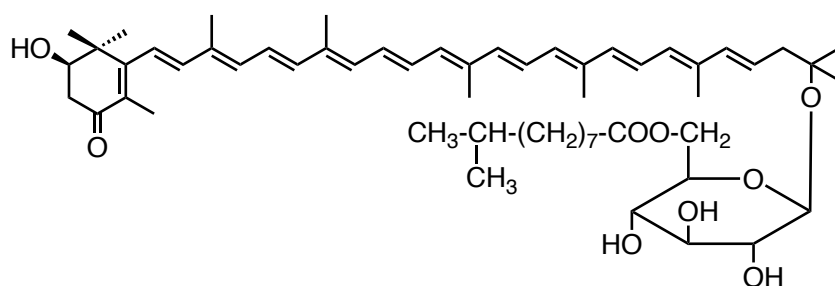
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Meiothermus ruber DSMZ 1279^T was isolated from a hot spring in Kamchatka and was red in color. The major carotenoid present was reported to be 1'-(β-D-glucopyranosyloxy)-3,4,3',4'-tetrahydro-1',2'-dihydro-β,ψ-caroten-2-one (meiothermoxanthin glucoside) after saponification (Burgess et al. 1999). In this study, we purified the major carotenoids in this species without saponification. We then reidentified the major carotenoids present using spectroscopic data, including ¹H NMR, ROESY, ¹³C NMR, HSQC, HMBC, MS, ECD, and enzymatic hydrolysis of fatty acid moieties, and found deinoxanthin glucoside iso fatty acid esters. The bound fatty acids present included four iso types, and their composition differed from cellular lipids. Moreover, the previously identified carotenoid glucoside (meiothermoxanthin glucoside) was a saponification artifact of deinoxanthin glucoside esters. Ketomyxocoxanthin glucoside esters and 1'-hydroxytorulene glucoside esters were also present. Based on the identification of carotenoids and the whole genome sequence of *M. ruber*, we propose a carotenoid biosynthetic pathway and note the corresponding genes.



Burgess et al. *J. Nat. Prod.* **1999**, 62, 859-863

This is an abstract of the 34th Annual Meeting on Carotenoid Research, on 17-18 September 2022 at Kwansei Gakuin University, Nishinomiya, Japan. This will be published as a research article.

Ultrafast excitation relaxation in keto-carotenoids

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Introduction

Contrary to polyene-type carotenoids having only C=C bonds in their π conjugation systems, keto-carotenoids, which possess a C=O bond in the conjugation systems, exhibit characteristic electronically excited states, such as the $^1(n, \pi^*)$ state(s) [1] and the charge-transfer state(s) [2]. Three types of keto-carotenoids, which have eight C=C bonds and one C=O bond in the conjugation systems, are found in oxygenic photosynthetic organisms (**Fig. 1**): siphonaxanthin in the siphonaxanthin–chlorophyll *a/b*-binding protein (SCP) of a green alga *Codium fragile*, fucoxanthin in the fucoxanthin–chlorophyll *a/c*-binding protein (FCP) of a diatom *Chaetoceros gracilis*, and peridinin in the peridinin chlorophyll *a* or *a/c*-binding protein (sPCP or iPCP, respectively) of a dinoflagellate *Symbiodinium* sp. In the light-harvesting pigment–protein complexes, these keto-carotenoids exhibit the function to capture the light energy especially in green region (500–550 nm) (**Fig. 2**), due to the unique conjugation systems.

We have examined the excitation relaxation processes of siphonaxanthin, fucoxanthin, and peridinin in solutions, and the excitation energy transfer processes from these keto-carotenoids to chlorophylls in the pigment–protein complexes [3–5], by means of time-resolved fluorescence spectroscopies. Besides these results, our recent progress for other types of keto-carotenoids will be presented in this talk.

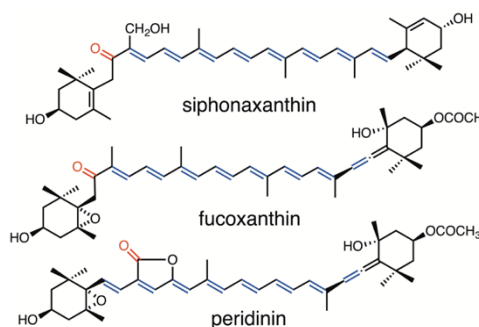


Fig. 1 Molecular structure of keto-carotenoids: siphonaxanthin, fucoxanthin, and peridinin from the top to the bottom.

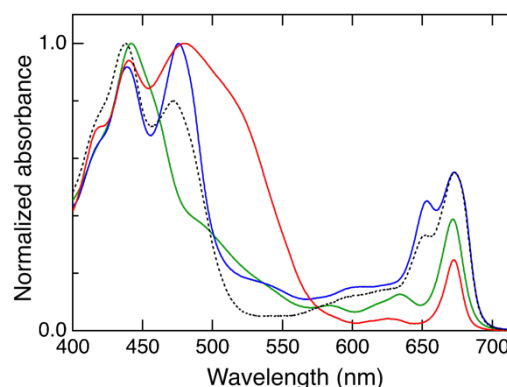


Fig. 2 Absorption spectra of SCP (blue), FCP (green), and iPCP (red). That of light-harvesting chlorophyll *a/b*-binding proteins isolated from the land plant *Arabidopsis thaliana*, which possesses no keto-carotenoid, is also shown by a black dotted line.

Measurements and analyses

To examine relaxation processes involving highly electronically excited states of carotenoids, fluorescence rise and decay curves were observed by the fluorescence upconversion system, in which the fundamental and the second harmonic of a Ti:sapphire laser were provided as gate and excitation pulses, respectively. The rise and decay curves ($I_F(t, \lambda)$) of carotenoids in solutions were analyzed by sums of three exponential functions with lifetimes (τ_i) independent of observed wavelengths (λ).

$$I_F(t, \lambda) = \sum_{i=1}^3 A_i(\lambda) \exp\left(-\frac{t}{\tau_i}\right)$$

Fluorescence decay-associated spectra (FDAS) were obtained by plotting of $A_i(\lambda)$ against λ .

Results and discussion

For all the keto-carotenoids examined here, all the values of $A_i(\lambda)$ were analyzed to be positive, indicating that only the decays of fluorescence were observed. This is in contrast to the fluorescence kinetics of the polyene-type carotenoids, in which negative values were analyzed at several wavelengths, showing the rises of fluorescence. The lifetime values were 30–80 fs for τ_1 and 180–230 fs for τ_2 (**Fig. 3**). Because the A_2 values were much smaller than the A_1 values, the first and second components were assigned due to the 1B_u state and the $^1(n, \pi^*)$ state, respectively. The third component exhibited further smaller A_3 with τ_3 longer than a few ps.

The lifetime values of the highly electronically excited states in the pigment–protein complexes are almost the same as those in solutions, suggesting that the efficiencies of energy transfer from the highly excited states of keto-carotenoids to chlorophylls are low [4, 5]. The fluorescence anisotropy decay of chlorophyll *a* in FCP exhibited a decay of 300 fs [4], whereas a rise component of 1.5 ps was resolved in the fluorescence kinetics of chlorophyll *a* in PCP [5]. These results indicate that energy transfers efficiently occur from the lowest excited states of keto-carotenoids.

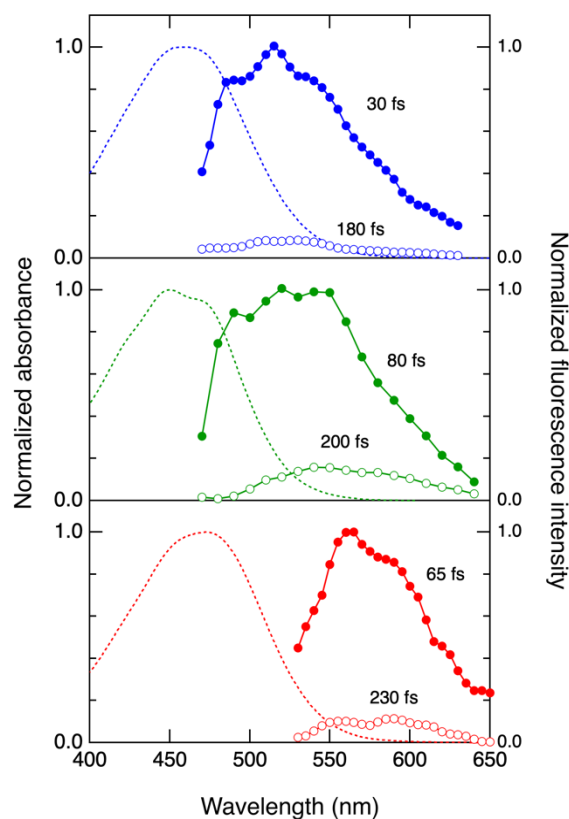


Fig. 3 Absorption spectra (dotted lines) and FDAS (open and closed circles) of the keto-carotenoids in alcohol solutions: siphonaxanthin (blue), fucoxanthin (green), and peridinin (red).

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Carotenoid production using *Escherichia coli*

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Several carotenoids have been extracted from plants and used as natural colorants, supplements and cosmetics, for example, lycopene from tomato fruits and lutein from marigold flowers. In contrast, rare carotenoids are difficult to be purified in large quantities. Consequently, their biological functions have not been elucidated.

As one solution of this problem, we have been developing the method to produce a great variety of carotenoids in recombinant *E. coli* cells. At the present time, we can produce not only C40 carotenoids but also C30 carotenoids in *E. coli* (**Fig. 1**) [1].

E. coli cannot synthesize carotenoids but FPP (Farnesyl pyrophosphate) which is a precursor of carotenoids. By introducing the genes for carotenoid biosynthesis following FPP, *E. coli* can produce carotenoids. For example, zeaxanthin is produced by using the *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes which are derived from the carotenoid producing bacteria *Pantoea ananatis*. These genes are functional in *E. coli* and therefore have been frequently used for carotenoid metabolic engineering research. On the other hand, since another xanthophylls rather than zeaxanthin such as lutein and violaxanthin are not produced by the bacteria, we have to use plant or algal genes to produce them. These eukaryotic genes sometimes do not work in *E. coli*.

Here, we introduce the methods to produce carotenoids using *E. coli* as a heterologous platform. We also discuss the problems and solutions.

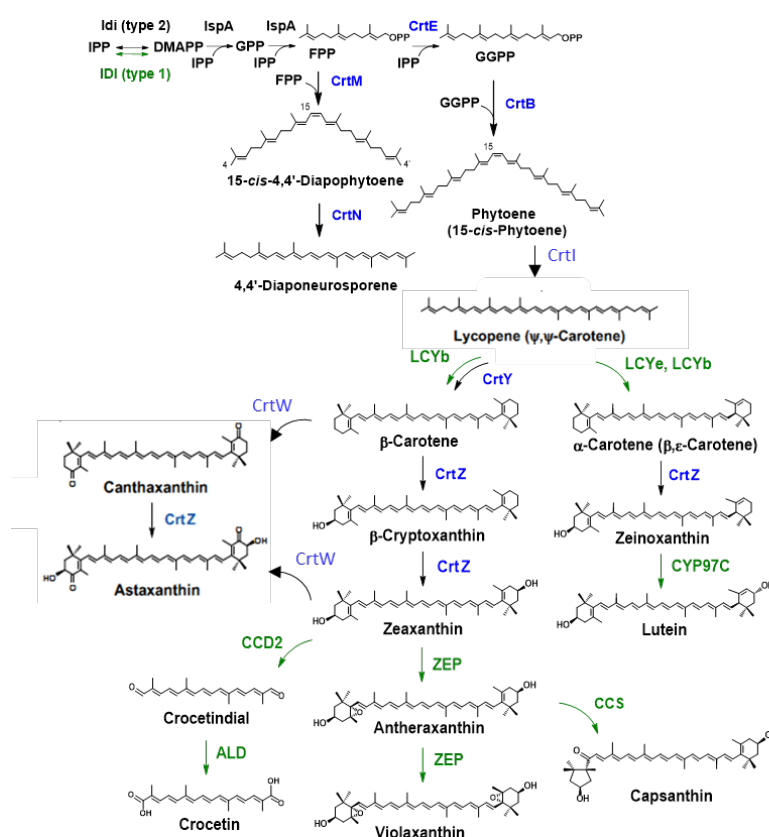


Fig. 1 Biosynthetic pathway of C30 and C40 carotenoid in recombinant *Escherichia coli*, and the enzymes required for biosynthesis in *E. coli*.

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The properties of paprika carotenoids

-Reaction mechanism with ROS, distribution into human blood, health benefits for humans-

Azusa Nishino

Carotenoids are the naturally occurring compounds including C₄₀ polyene chain as a common basic structure. There are over 750 known carotenoids based on the various structures of their end group. These compounds can be divided into two classes, which carotenes (hydrocarbons without oxygen) and xanthophylls (containing oxygen at their end group). The former is highly hydrophobic while the latter is amphipathic.

Dietary carotenoids which humans intake from mainly vegetables and fruits are only α -carotene, β -carotene and lycopene as carotenes and lutein, zeaxanthin and β -cryptoxanthin as xanthophylls. These 6 kinds of carotenoids can be absorbed from human small intestine and detected in human blood with their metabolites. It is known that xanthophylls preferentially are localized in erythrocytes while carotenes preferentially are localized in plasma due to the difference of molecular polarity. Recently, carotenoids have received increasing attention as phytochemicals for human health and they are applied to various supplements and functional foods. In particular, there are many studies on the health effects of astaxanthin and lutein classified as xanthophylls. It is considered that these effects are based on strong anti-oxidative activities for reactive oxygen species (ROS) of xanthophylls.

In our studies, we focused on paprika carotenoids as a good source of xanthophylls. It is well known that capsanthin, a characteristic xanthophyll in red paprika, has strong anti-oxidative activities against ROS, especially singlet oxygen. We investigated the relationship between anti-oxidative activities and chemical reaction of carotenoids with ROS. As a result, we found that their structure of end group might decide chemical reactivities with ROS and they might contribute to their scavenging effects against ROS. We also revealed that the structure of capsanthin might be beneficial in terms of chemical reactivities and scavenging effect with ROS.

On the other hand, we demonstrated that orally administrated paprika carotenoids including capsanthin could be distributed to both of plasma and erythrocytes and increases of carotenoid level are more effective in erythrocytes than in plasma. Erythrocytes are always exposed to risk of oxidative damage by ROS because they are responsible for transporting oxygen to the tissue and organs. Therefore, we expected that accumulation of capsanthin in erythrocytes might be effective to prevent oxidation of erythrocytes by ROS and improve oxygen delivery capacity of erythrocytes. During aerobic exercise, a plentiful oxygen supply to active muscle is needed and enormous ROS are occurred in body. A randomized clinical trial was conducted to evaluate the effect of supplementation of paprika carotenoids on respiratory metabolism during endurance exercise, which is closely related to erythrocytes function. As a result, we demonstrated oxygen uptake and heart rate during endurance exercise were significantly decreased and improved by supplementation of paprika carotenoids. It suggests that supplementation of paprika carotenoids is useful for improvement of athletic ability.

I would like to introduce research for paprika carotenoids mainly mentioned above.

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CAROTENOID SCIENCE
===INSTRUCTIONS TO AUTHORS===

Aims and scope

The purpose of Carotenoid Science is to provide a means of communication between scientists in different disciplines who share a common interest in the field of carotenoid research.

Submission of papers

Electronic manuscripts (Microsoft Word document and/or PDF) should be sent to the following e-mail address as attachments:

Prof. Dr. Hideki Hashimoto
Editor Carotenoid Science

Department of Applied Chemistry for
Environment, Graduate School of Science and
Technology, Kwansei Gakuin University, 1,
Gakuen-Uegahara, Sanda-shi, Hyogo, 669-
1330, Japan

e-mail: hideki-hassy@kwansei.ac.jp

Original material: Submission of a manuscript implies that it is not being simultaneously considered for publication elsewhere and that the authors have obtained the necessary authority for publication.

Types of contributions

Original research papers, letters to the editor, and review papers are welcomed. All of them should contain an Abstract (of up to 200 words) and a Conclusions section, which, particularly in the case of theoretical papers, translates the results into terms readily accessible to most readers. All the submitted manuscripts are subjected to critical reading by the reviewers who are authorized in the relevant field of the work. It is to be noted that the authors(s) cannot insist the priority of the work published in Carotenoid Science.

Instructions for the preparation of camera-ready manuscripts

1. All manuscripts should be written in proper English.
2. Manuscripts should be typed on A4 size (21.0 cm × 29.7 cm) papers.
3. Times or Times New Roman font should be used for the text of the manuscripts.
4. In principle, there is no limitation for the length of manuscripts. However, it is recommended to obey the following page limit: up to 10 pages for the research papers, up to 5 pages for the letters to the editor, and up to 20 pages for the review papers.
4. Camera-ready manuscripts are required, which should be typed according to the following instructions.

Margins

Leave 2.0 cm space for margins on both sides and on top, and 2.5 cm margin on bottom.

Mathematical symbols

All mathematical symbols representing physical quantities should be typed in italic.

Title and Authors(s)

- (i) The title of the manuscript should be typed in upper and lower case letters, and it should be centered.
- (ii) Leave one line space and then type author(s) name(s) in upper and lower case letters in centered position.
- (iii) Corresponding author should be marked with asterisk at the right-hand side up corner of the name. It is encouraged to write the e-mail address of the corresponding author as a footnote.
- (iv) Author(s)' affiliation should be typed in the next line italic and in centered position.

Abstract

- (i) Every manuscript must have an abstract, up to 200 words in lengths and typed in 10.5 pt font size.
- (ii) Abstract should have margin space of 3.0 cm on both sides.

Text

- (i) General text should be typed in 10.5 pt font size. It is preferable to be written in two columns.
- (ii) All section titles should be left justified, numbered in sequence and typed in bold face.
- (iii) All subsection titles should be left justified, numbered and written in bold and italic face.
- (iv) One line space should separate the end of a previous section or subsection with the next subsection or section's title.
- (v) Sections and subsections should not normally begin on a new page.
- (vi) The first line of each paragraph should be indented by 4 characters.
- (vii) References in the text should be cited with square brackets ([1], [2]). Two references in one bracket should be cited with a comma in between ([1, 2]), and more than two references should be cited with a hyphen in between ([1–3]).
- (viii) Figures and Tables should be numbered and placed in the text with captions, typed underneath, not at the end of the manuscript.
- (ix) References should be listed at the end of the text with their serial numbers in square brackets as cited within the text. Please refer to the recent issue of Carotenoid Science as an example.

After acceptance

You will be notified by the Editor of the journal of the acceptance of your article.