Compound- and position-specific stable isotope methods for measuring the consumption of storage protein and lipids in plant physiology: implications for studies in ecology and geochemistry

（植物の貯蔵アミノ酸・脂質の消費に伴う分子レベル／分子内安定同位体分別：
生態学・地球化学研究への応用を目指して）

Graduate School of Environmental Science

Hokkaido University

Yuko Takizawa

北海道大学大学院環境科学院

滝沢　侑子

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ABSTRACT

Introduction

A large diversity in the stable isotopic composition (e.g., $^{13}C/^{12}C$, and $^{15}N/^{14}N$) of organic compounds is closely related to the isotopic fractionation on 'key' reactions in biosynthetic and metabolic pathways of organisms. The identification of 'key' reactions and comprehension of their fluxes in the pathways are indispensable to understand (1) 'universality vs. specificity' on the isotopic composition or fractionation found in organisms and (2) the accuracy and precision on the isotope evidence in biological, ecological, and geochemical application studies. However, conventional isotope methods and methodologies are inaccessible enough to identify and comprehend these issues, particularly for the metabolism of lipids in organisms.

Scientific objectives

To evaluate the biosynthetic/metabolic balance in organisms, we quantified the isotopic fractionation on organic compounds in plants with respect to the consumption and utilization of storage materials, based on the isotopic composition of nitrogen in amino acids for sprouting, flowering, and leafing with storage proteins (Chapter II), and of (1) the whole carbon in individual fatty acids and (2) the carboxyl carbon within a single fatty acid for sprouting with storage lipids (Chapter III).

Consumption of storage protein in the plant phenology (Chapter II)

1. The enrichment in ^{15}N slightly for mother sweet potato and considerably for its sprout growing under dark condition reveals a first data that storage protein will be metabolized to produce maintenance and growth energy for overwintering and sprouting.
2. A large enrichment in ^{15}N recorded in deciduous plant flowers that bloom before leafing reveals that the rational consumption and utilization of storage protein are universally found in plant phenology.
3. The enrichment in ^{15}N is rapidly decreased from significantly large for flush to substantially zero for senescence leaves, which reveals a gradual change in the energy flow from catabolically-released to photosynthetically-fixed during the short term of leafing.

Consumption of storage lipids in the plant phenology (Chapter III)

1. A small but significant enrichment in ^{13}C is found in sesame sprouts growing under dark condition, implying that the hydrolysis of triacylglycerol (i.e., consumption of storage lipids) is a key reaction responsible for the isotopic fractionation on the fatty acids during sprouting of seeds.
2. A position-specific isotope method established can access a huge enrichment in ^{13}C specifically on carboxyl carbon within the fatty acids of sesame seeds, which allows us to proof the utilization of storage lipids as major energy resource for the sprouting.

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CHAPTER I.

General Introduction

Molecular and stable isotopic compositions of organic compounds, particularly 'generally called' biomarkers that can characterize fundamental information in the biological sources of them and the function of biogeochemical processes, have long, widely been employed as potential powerful tools in the study (1) for tracing sources and delivery of organic compounds in geological and geographical samples and (2) for reconstructing /understanding environments and ecosystems over a wide range of geological timescales. This is proposed by that:

(1) organic compounds have a large diversity in the stable isotopic compositions (e.g., D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, etc.) in biological samples, which are always closely related to isotopic fractionation associated with specific processes in biosynthetic and metabolic pathways;

(2) the magnitude of isotopic fractionation for a process is apparently universal among species; and

(3) by mixing of multiple fractionation processes, the isotopic compositions of organic compounds are therefore numerous diversity specific to biological classes, species, and individuals, as well as growth condition including seasonality.

Isotopic composition of organic compound A ($\delta_{\mathrm{CompoundA}}$) is frequently given by a simplistic equation (eq. 1-1):

$$\delta_{Compound\ A} = \delta_{Source} + \varepsilon_{Biosynthesis} \qquad (eq.1-1)$$

where δ_{Source} and $\varepsilon_{\mathrm{Biosynthesis}}$ denote the isotopic composition of sources (e.g., CO_2 and organic substrates) and isotopic fractionation along biosynthesis for compound A, respectively. It has

been expected that a small or negligible variation in the isotopic compositions should be observed for the organic compound A in any organisms, if the compound A is produced from isotopically-homogenous sources accompanied by the isotopic fractionation associated with a single, common biosynthetic process even in different organisms. However, there is apparently a large variation in the isotopic fractionation during biosynthesis for a number of organic compounds, for example, among spatially- or temporally-different tissues even within a single organism (e.g., Sessions, 2006; Tipple et al., 2012; Sachse et al., 2015). Therefore, generally speaking, an identification of factors controlling this variation in the isotopic fractionation is thus indispensable for better understanding of 'universality vs. predictable fluctuation' on the isotopic compositions of organic compounds in organisms, as well as for crediting of the accuracy and precision on the isotope evidence in application studies.

To our knowledge of physiology, although CO_2, H_2O, and inorganic nitrogen (e.g., nitrate and ammonia) are original substrates of organic compounds, organisms can frequently produce organic compounds from isotopically heterogeneous organic substrate (e.g., storage proteins and lipids, diets, etc.) in natural environments, with respect to biogeochemical energy cycles. The production of photosynthate from inorganic chemicals 'with solar energy' during photosynthesis including the conversion of the photosynthate to various organic compounds (e.g., storage and organs) is generally called 'autotrophic production (AP)' (Fig. 1-1A). In the autotrophic production, it is explained by that the isotopic compositions of inorganic chemicals and the isotopic fractionation along biosynthesis (δ_{IC} and ε_{AP}, respectively) is recorded in the isotopic composition of organic compounds, like equation (1-1). Such organic compounds produced are employed as energy resources for heterotrophs,

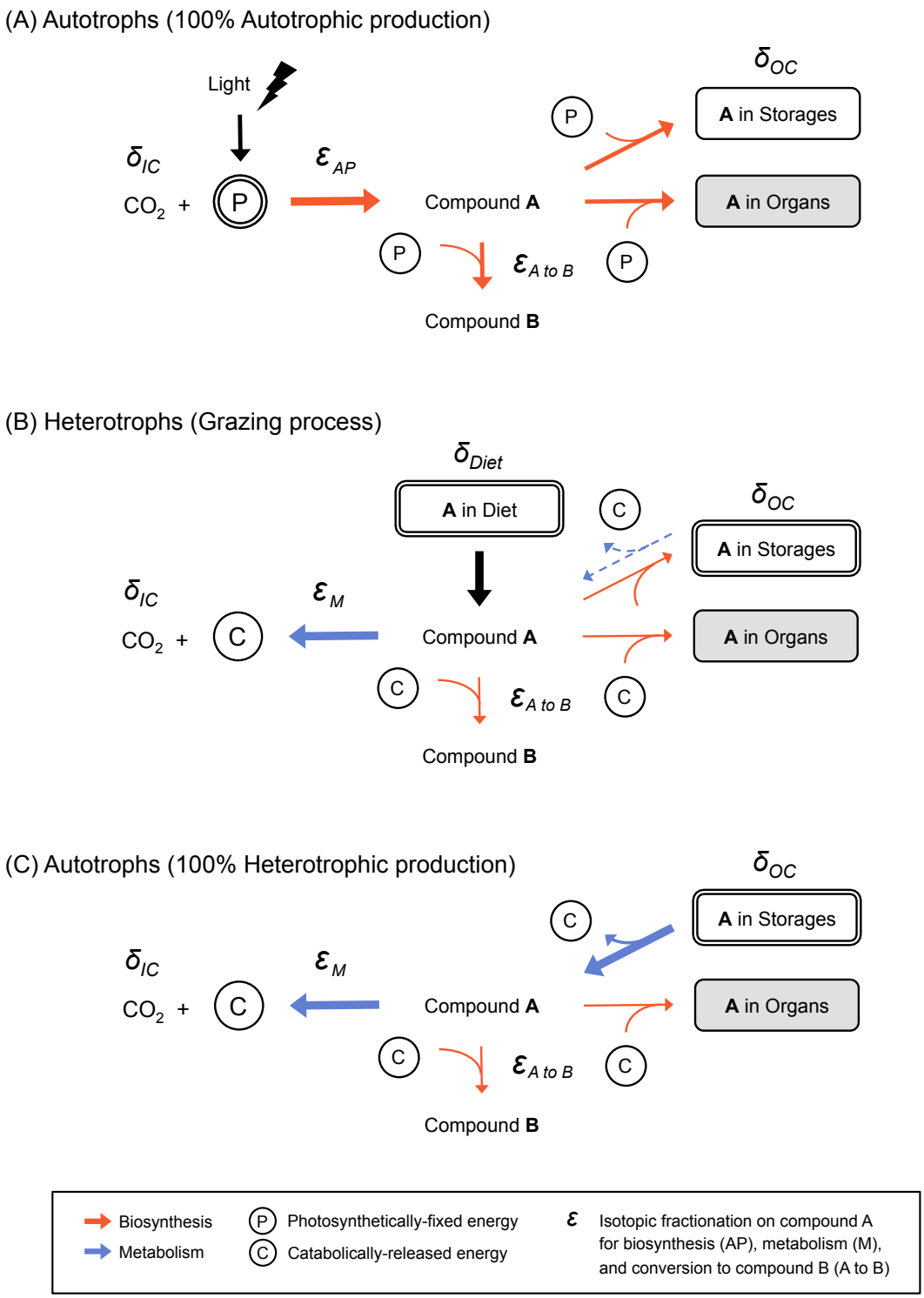

Fig. 1-1.

Schematic illustration of energy resources of biosynthesis and metabolism in autotrophs and heterotrophs: Photoautotrophs produce organic compounds by using photosynthetically-fixed energy during photosynthesis, and the fixed energy is released during metabolic breakdown in not only heterotrophs but also autotrophs; and these energies are used during biosynthesis to construct of their organ and storage biomass.

because heterotrophs cannot use solar energy to produce their own organic compounds. Instead of solar energy, heterotrophs obtain alternative energy from diet-derived organic compounds during the catabolism and produce their own organic compounds using the residual diet-derived organic compounds 'with catabolically-released energy' to keep the life of them (Fig. 1-1B).

Similar process is also found even in autotrophs, when autotrophs can obtain 'the alternative' energy from storage-derived organic compounds during the catabolism and produce other organic compounds 'with catabolically-released energy' under less/no photosynthetic activities (e.g., at night). Such the production of organic compounds 'with catabolically -released energy' is separately called 'heterotrophic production (HP)' (Fig. 1-1C), more specifically external HP in heterotrophs and internal HP in autotrophs.

Assuming these AP and HP with respect to biosynthesis and metabolism in organisms, the isotopic composition of organic compound A is given by:

$$\delta_{Compound\ A} = f(\delta_{\mathrm{IC}} + \varepsilon_{\mathrm{AP}}) + (1 - f)(\delta_{\mathrm{OC}} + \varepsilon_{\mathrm{HP}}) \qquad (eq. 1-2)$$

where δ_{IC} and δ_{OC} denote the isotopic composition of inorganic chemicals and organic compounds, respectively, $\varepsilon_{\mathrm{AP}}$ and $\varepsilon_{\mathrm{HP}}$ denote the isotopic fractionation associated with autotrophic and heterotrophic productions (sum of $\varepsilon_{\mathrm{M}} + \varepsilon_{\mathrm{AtoB}}$), respectively. f represents the fractional contribution of each. Thus, HP can contribute to the isotopic composition of organic compounds for not only heterotrophs but also autotrophs, which potentially allows us to see a large variation in the isotopic fractionation for organic compounds that described above. We predict that this equation (1) would be replaced by the equation (2) for better understanding

of the isotopic composition of organic compounds.

Indeed, the presence of such HP and its contribution to the isotopic compositions of organic compounds have been qualitatively or somewhat quantitatively discussed by using the isotopic compositions for plants and insects in a few number of studies, although the interpretation of data observed in these studies is indirect rather than direct enough to identify the presence and contribution of HP. To identify directly the presence and contribution of HP, it is thought that quantitative analysis for (i) *de novo* synthesize, (ii) degradation, (iii) re-synthesize, and (iv) re-organize of organic compounds in organisms may be required.

In this study, to evaluate the effect of HP on the isotopic composition of organic compound, we investigated the isotopic fractionation and its mechanisms for organic compounds in plants with respect to the consumption/utilization of storage materials, viewed from nitrogen isotopic composition of amino acids for sprouting, flowering, and leafing with storage proteins (Chapter II), and from (1) carbon and hydrogen isotopic compositions of the whole carbon and hydrogen in palmitic acid and (2) carbon isotopic composition of the carboxyl carbon within the palmitic acid for sprouting with storage lipids (Chapter III). We in this study used a standard compound-specific isotope method for measuring the isotopic compositions of amino acids and fatty acids, and developed a position-specific isotope method for measuring the isotopic composition of the carboxyl carbon in fatty acids.

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CHAPTER II.

The Consumption of Storage Protein in the Plant Phenology, based on Compound-specific Stable Isotope Analysis of Nitrogen within Amino Acids

Introduction

It is assumed that the protein (amino acids) is an essential resource to operate 'heterotrophic production (HP)' under no/less photosynthesis, particularly for sprouting, flowering, and leafing in specific terms of plant phenology, instead of the supply of energy and organic substrates under active photosynthesis with mature leaves. In this chapter, we applied compound-specific stable isotope analysis (CSIA) of nitrogen within amino acids for plant organs to identify and quantify the HP in plants.

During the last decade, the CSIA of nitrogen within amino acids has been employed as a potential powerful tool to estimate the resource utilization and trophic energy transfer among organisms in food chains and food webs (e.g., Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007). This is based on that difference in the isotopic composition between two types of amino acids ('trophic' amino acids represented by glutamic acids and 'source' amino acids represented by phenylalanine) in organisms, which can record the isotopic discrimination responsible for distinct 'key' processes in the amino acid metabolism of organisms (Fig 2-0-1) (e.g., Chikaraishi et al., 2007, 2009; Ohkouchi et al., 2015). For the trophic amino acids, significantly large isotopic discrimination (ca. 3-10‰) is found between a consumer and its diet (Chikariashi et al., 2007), reflecting large activity of deamination in the metabolism of consumers. The magnitude of discrimination is dependent of the metabolic flux what percentage of the amino acids is deaminated. In contrast, for the source amino acids, substantially no/little isotopic discrimination (ca. 0-1‰) is found between a consumer and its diet (Chikariashi et al., 2007), reflecting no/little activity of deamination in the amino acid metabolism of organisms.

Thus, we applied this traditional amino acid method for evaluating 'HP from stor-

age protein in plants', particularly for better understanding the utilization of storage amino acids for energy and newly-constituted protein in plant growth under no/less photosynthesis.

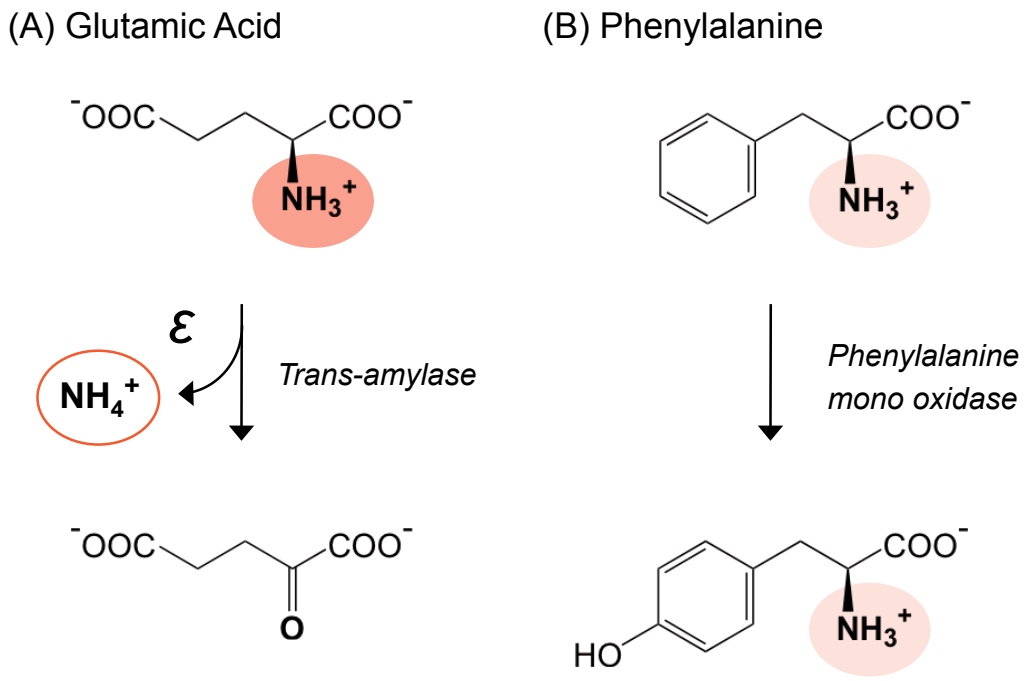

Fig. 2-0-1. The nitrogen isotopic discrimination of amino acid metabolism in organisms: (A) residual glutamic acid is much enriched in ^{15}N because of preferential ^{14}N elimination during the deamination (the first step of transamination); (B) residual phenylalanine is less enriched in ^{15}N because of non-preferential ^{14}N elimination during the hydroxylation of phenylalanine to produce tyrosine (After Chikaraishi et al., 2007).

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(The section 2-1 starts from next page.)

2-1. Are baby sprouts eating the proteins in the mother sweet potato?

Abstract

Sweet potatoes sometimes sprout the purple color of stems with several small leaves in the house pantry. In the present study, we investigated the trophic hierarchy between a mother sweet potato and its baby sprouts grown without any light in a dark house pantry, based on stable nitrogen isotopic composition (δ^{15}N, ‰ vs. AIR) of glutamic acid and phenylalanine. The isotope data reveal that glutamic acid has a significant ^{15}N-enrichment (by 6.9‰) from the mother sweet potato to its baby sprout while phenylalanine has a little ^{15}N-enrichment (by 0.6‰) between them. Interestingly, the isotopic heterogeneity found within the sweet potato is very similar to the isotopic discrimination generally found in the combination between plants and herbivores during grazing food webs (ca. 8.0‰ for glutamic acid and ca. 0.4‰ for phenylalanine). These results suggest that the proteins in the mother sweet potatoes are major resources for not only proteins in their baby sprouts but also growth energy in the sprouting, when they are grown heterotrophically without any light.

Key words:

plants, heterotrophy, nitrogen isotopic composition, amino acids, and trophic hierarchy

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1. Introduction

Sweet potatoes are stored without any light in a dark place to overwinter and ready to sprout in the spring. In spring, some small baby sprouts are found on the mother sweet potatoes, when it will be time to bed down the roots. However, these sprouts can grow for more than 5 months until the early autumn without any light in a dark place, and finally appear the purple colored long stems (>15 – 20 cm) with several small leaves on the mother sweet potatoes. For this phenomenon, we have a very simple question on the trophic hierarchy between mother sweet potatoes and their sprouts, whether the latters are ecologically one trophic level higher than the formers.

This question may seem to be no or little relationship with geochemistry, but it is very closely related to biogeochemical studies investigating the effects of catabolism in algal and vascular plants as well as heterotrophic organisms (e.g., Sessions, 2006; Zhang et al., 2009; Aoyagi et al, 2013). Plants can convert CO_2, H_2O, and light energy into organic materials (e.g., glucose) and O_2 during photosynthesis (i.e., anabolism), which is much larger than respiration (i.e., catabolism) that converts the organic materials and O_2 into CO_2, H_2O and life energy (or growth energy), when plants are growing with light. On the other hand, plants can produce organic materials even in catabolic stages without any light, which may appear totally different signals in the molecular and isotopic compositions from anabolic stages (e.g., Sessions, 2006). To clarify the effect of catabolism in plants is therefore an essential issue to reduce uncertainty on the molecular and isotope proxies used in organic geochemical studies. After harvest, the catabolism only occurs in sweet potatoes, because of no input of photosynthates from leaves. It is simply expected that sweet potatoes can employ their stocked organic materials (e.g., sugars, proteins, and/or lipids) as energy resources to survive and sprout in the

catabolic stage. Thus, to answer the simple question on the trophic hierarchy within sweet potatoes allows better understanding not only plant physiology to survive and sprout, but also its associated changes in the molecular and isotopic compositions of organic materials in catabolic stages.

Stable nitrogen isotopic composition (δ^{15}N, ‰ vs. AIR) of a couple of amino acids, glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), has recently been employed as a potential powerful tool to evaluate the trophic linkage and energy flow among plant and animal species in the network of ecosystems (e.g., Chikaraishi et al., 2007; McCarthy et al. 2007; Popp et al. 2007).

This tool has been constructed based on the isotopic discrimination associated with the amino acid metabolisms: glutamic acid shows significant ^{15}N-enrichment by 8.0 ± 1.1‰ from resource to consumer species in the grazing food web, whereas phenylalanine shows a little ^{15}N-enrichment by 0.4 ± 0.4‰ (Chikaraishi et al., 2010). Thus, a comparison of the δ^{15}N values between these two amino acids from a single organism corresponds to the trophic position of the organism in the ecological food web (e.g., Chikaraishi et al., 2009), which defined by the following equation (eq. 2-1-1):

$$\mathrm{TP} = \{(\delta^{15}\mathrm{N}_{\mathrm{Glu}} - \delta^{15}\mathrm{N}_{\mathrm{Phe}}) + \beta \,/\, 7.6\} + 1 \qquad (\mathrm{eq.}\, 2-1-1)$$

where the TP represents the estimated trophic position and the β represents a fix number related to the difference between δ^{15}NGlu and δ^{15}NPhe values in algal (− 3.4 ± 0.9‰) and vascular plants (+ 8.4 ± 1.6‰) (Chikaraishi et al., 2010). The standard deviation (1σ) of accuracy in the TP value was estimated to be 0.17 units for terrestrial organisms (Chikaraishi et al., 2011).

The nitrogen isotope analysis of amino acids is thus a potential approach to access the trophic hierarchy within sweet potatoes. In the present study, we (1) determined the isotopic composition of glutamic acid and phenylalanine in a mother sweet potato and its baby sprouts grown in a dark house pantry, (2) revealed the trophic hierarchy within a sweet potato, and (3) discussed its catabolism associated with the sprouting in sweet potatoes.

2. Materials and methods

A sweet potato (ca. 30 cm long, 10 cm maximum diameter) was collected from a farm in Yugawara (35º08'N, 139º07'E) in October 2013, washed down by tap water, and stored in a paper bag in a dark house pantry. A number of purple colored sprouts (ca. 10 – 20 cm) was found on the sweet potato in August 2014. A couple of pieces of the baby sprout stems (ca. 2.0 cm length from the middle of sprouts) and mother sweet potato (ca. 0.25 cm^3 from the middle of the potatoes) were collected at that moment and stored at −20°C until analysis.

These samples were prepared for stable nitrogen isotope analysis of glutamic acid and phenylalanine, after HCl hydrolysis and *N*-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedure in Chikaraishi et al. (2009). The isotopic composition was determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using a 6890N GC (Agilent Technologies) instrument coupled to a Delta^{plus}XP IRMS instrument through combustion (950ºC) and reduction (550ºC) furnaces via a GC-C/TC III interface (Thermo Fisher Scientific). The isotopic composition was expressed relative to atmospheric nitrogen (δ^{15}N, ‰ vs. AIR) on a scale normalized to the known δ^{15}N values of nine isotopic reference amino acids (from –25.9‰ to + 45.6‰, Indiana University and SI science co., Sato et al.,

2014). The accuracy and precision for the isotope measurements of the reference amino acids were 0.0‰ (mean of Δ) and 0.4‰ (mean of 1σ), respectively. The TP values were calculated using equation (1) with 8.4‰ for the β value.

3. Results and discussion

3.1. Stable nitrogen isotopic composition of amino acids and estimated trophic position

The $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values were −0.4‰ and + 5.2‰, respectively, for the mother sweet potato and + 6.5‰ and + 5.8‰, respectively, for its baby sprout (Fig. 2-1-1). Thus, a significant heterogeneity in the $\delta^{15}N_{Glu}$ value was found between mother sweet potato and its baby sprout within a single sweet potato, whereas a little heterogeneity was found in the $\delta^{15}N_{Phe}$ value between them. These isotope data lead to a significant difference in the TP value between the mother sweet potato (1.4) and its baby sprout (2.2), as the latter has 0.8 unit higher trophic position than the former.

3.2. Trophic hierarchy of mother sweet potatoes and their baby sprouts

In general, the grazing food web starts from primary producers such as algae and plants, and they are eaten by herbivores and omnivores. Then herbivores and omnivores are eaten by carnivores and finally by predators at the top of the food web pyramid. It has been designed that the TP values of organisms estimated based on the equation (1) with the observed $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values can correspond to the defined, actual trophic position of organisms within 0.17 units as an error (1σ) of accuracy for terrestrial food webs (Chikaraishi et al., 2011). Moreover it has been proved by several investigations using controlled feeding experiments and well-characterized wild species (e.g., Steffan et al., 2013; Bradley et al.,

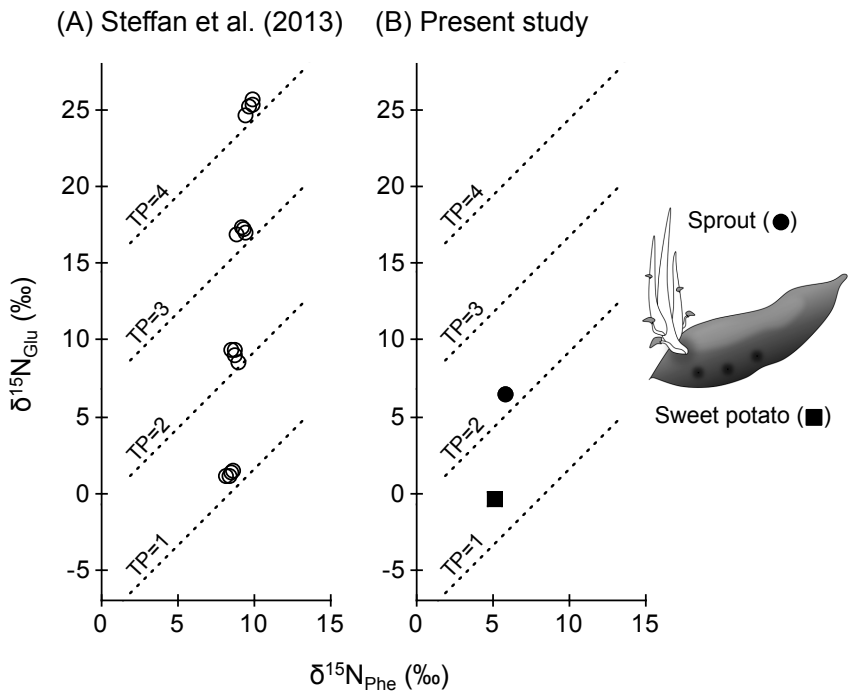

Fig. 2-1-1.
Cross-plots of the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values for (A) plants, caterpillars, and carnivorous insects in a controlled feeding experiment in Steffan et al., (2013) and (B) the mother sweet potato and its sprout in the present study. Dash lines indicate the trophic isocline for TPs 1-4 with slope of 1.0 created based on the equation (1).

2014; Downs et al., 2014). On cross-plots for the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values (Fig. 2-1-1), resource and consumer species should be arrayed in an almost vertical column within a narrow range on the $\delta^{15}N_{Phe}$ values of the resource species and with 8.0‰ interval of the $\delta^{15}N_{Glu}$ values for each integer-based number of the trophic position, if the consumers feed on only the resource species (Chikaraishi et al., 2014).

In the present study, the $\delta^{15}N_{Phe}$ value of the baby sprout (+ 5.8‰) is very close to that of the mother sweet potato (+ 5.2‰), while the $\delta^{15}N_{Glu}$ value of the sprout (+ 6.5‰) is more positive value than that of the sweet potato (− 0.4‰). Thus, both baby sprout and mother sweet potato are likely arrayed in a vertical column on the $\delta^{15}N_{Phe}$ value of the sweet

potato (Fig. 2-1-1B). Moreover, although the magnitude of ^{15}N-enrichment is slightly small for glutamic acid, the isotopic heterogeneity within the sweet potato (i.e., between the mother sweet potato and its baby sprout) is similar to the isotopic discrimination generally found in the combination between plants and herbivores during grazing food webs (8.0 ± 1.1‰ for glutamic acid and 0.4 ± 0.4‰ for phenylalanine, Chikaraishi et al., 2010). These results strongly suggest an interesting trophic hierarchy that the baby sprouts are approximately one trophic level higher than the mother sweet potatoes.

For the sprouting, proteins in mother sweet potatoes could be reconstructed to build proteins in baby sprouts. Simultaneously, their stocked organic materials (e.g., sugar, protein, and/or lipids) could be employed as energy resources for the reconstruction of proteins. In our knowledge, because sweet potatoes stock a large pool of starch (i.e., sugars), it may be assumed that the sugars stocked in mother sweet potatoes are available as major energy resources in sprouting, while the proteins stocked may be only converted into proteins in baby sprouts (but not into growth energy). If this assumption is correct, an absence of the change in the δ^{15}N value should be found for any amino acids between mother sweet potatoes and baby sprouts due to no isotopic deamination occurring on amino acids. However, much and less heterogeneities in the δ^{15}N value for glutamic acid and phenylalanine, respectively, within a single sweet potato (as shown in Fig. 2-1-1) suggest that the proteins in the mother sweet potatoes are major resources for not only proteins in their baby sprouts but also growth energy in the sprouting when they are grown heterotrophically without any light, as very similar to general phenomenon (i.e., catabolism) found in resource and consumer species during grazing food webs. Plants have both pathways for anabolism and catabolism, whereas heterotrophic animals have a single pathway only for catabolism. It is highly likely that the isotopic

discrimination process–isotopic fractionation factor and flux on the deamination of amino acids–in the catabolism is common or very similar in both plants and animals.

The TP values of plant materials such as leaves, nuts, and sap have always been reported to be 1.0 within 0.2 as 1σ so far (e.g., Chikaraishi et al., 2011, Steffan et al., 2013). However, the TP value of the sprout in the present study is 2.2, which implies that the TP values of plant materials are potentially not always found within 1.0 ± 0.2, particularly if the respiration (i.e., catabolism) is much larger than the photosynthetic production (i.e., anabolism), as the stages of sprouting and blooming. Moreover, the TP value of the mother sweet potato in the present study is 1.4, which borders on the upper TP value of plants within 2σ. Although we have only one data point in the present study, this relatively high TP value (i.e., 1.4) for the mother sweet potato may potentially suggest that sweet potatoes employ stocked proteins as energy resources to survive for a long time in winter or to sprout in spring season.

Base on the data in the present study, we found three tentative summaries as follows:

(1) the baby sprouts are approximately one trophic level higher than the mother sweet potatoes,

(2) this is probably because the proteins in the sweet potatoes are major resources for not only proteins in the baby sprouts but also growth energy in the sprouting, when they are grown without any light, and

(3) the TP values of plant materials may somewhat increase (i.e., not always within 1.0 ± 0.2) when the respiration is much larger than the photosynthetic production, as the stages of overwintering, sprouting, and/or blooming.

3.3. Implication in isotope ecology

The number of studies using the nitrogen isotopic composition of amino acids has increased significantly during the last 5 years. Indeed, it has been employed as a potential powerful tool to understand ecosystems and elucidate ecological issues accurately and precisely. However, the universality of the trophic isotopic discrimination of amino acids is still in the investigation stage for various taxa of organisms at this moment. In the present study, we demonstrated that magnitude of the isotopic discrimination during the catabolic stages of sweet potatoes is very similar to that generally found in heterotrophic animals (Fig. 2-1-1). If these results are commonly found in other plants, it is highly supportive of that magnitude of the trophic isotopic discrimination of amino acids is, in principal, common for the catabolic stages of both plants and animals.

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(The section 2-2 starts from next page.)

2-2. Intra-trophic isotopic discrimination of $^{15}N/^{14}N$ for amino acids in autotrophs: Implications for nitrogen dynamics in ecological studies

Abstract

The differential discrimination of nitrogen isotopes ($^{15}N/^{14}N$) within amino acids in consumers and their diets has been routinely used to estimate organismal tropic position (TP). Analogous isotopic discrimination can occur within plants, particularly in organs lacking chloroplasts. Such discrimination likely arises from the catabolic deamination of amino acids, resulting in a numerical elevation of estimated TP, within newly synthesized biomass. To investigate this phenomenon, we examined the $^{15}N/^{14}N$ of amino acids ($\delta^{15}N_{AA}$) in spring leaves and flowers from eight deciduous and two annual plants. These plants were classified on the basis of their time of bloom; plants that bloomed when their leaves were absent (Type I), versus plants that bloomed while leaves were already present (Type II). Based on the $\delta^{15}N_{AA}$ values from leaves, both plant types occupied comparable and ecologically realistic mean TPs (= 1.0 ± 0.1, mean ± 1σ). However, the estimated TPs of flowers varied significantly (Type I: 2.2 ± 0.2; Type II: 1.0 ± 0.1). We hypothesize that these results can be interpreted by the following sequence of events: (1) Type I floral biomass is synthesized in absence of active photosynthesis, (2) the catabolic deamination of amino acids in particular,

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leaves behind ^{15}N in the residual pool of amino acids, and (3) the incorporation of these ^{15}N-enriched amino acids within the biomass of Type I flowers results in the numerical elevation of the TPs. In contrast, the actively photosynthesizing Type II leaves energetically sustain the synthesis of Type II flower biomass, precluding any reliance on catabolic deamination of amino acids. Amino acids within Type II flowers are therefore isotopically comparable to the Type II leaves. These findings demonstrate the idiosyncratic nature of the $\delta^{15}N_{AA}$ values within autotrophic organs and have implications for interpreting trophic hierarchies using primary producers and their consumers.

Keywords:
$\delta^{15}N$, isotopic fractionation, food web, trophic position, plant phenology, and winter dormancy

1. Introduction

Amino acid metabolism within consumers causes predictable enrichment in their ^{15}N compared to their diets. By quantifying the magnitude of this isotopic discrimination among unique amino acids through compound-specific isotope analysis (CSIA), ecologists have achieved unprecedented insights into complex food webs (e.g., Gaebler et al., 1966; McClelland and Montoya 2002; Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007; Sherwood et al. 2011; Batista et al. 2014;Choy et al. 2015; Sackett et al. 2015; Steffan et al. 2015a; Naito et al. 2016; Dharampal and Findlay 2017). Chikaraishi et al. (2009) measured this 'inter-trophic' isotopic discrimination between consumers and their diets, and established the following equation (2-2-1) to estimate the trophic position ($TP_{Tr/Src}$) of organisms in food webs:

$$\mathrm{TP_{Tr/Src}} = \left(\frac{\delta^{15}\mathrm{N_{Tr}} - \delta^{15}\mathrm{N_{Src}} - \beta_{\mathrm{Tr/Src}}}{\mathrm{TDF_{Tr/Src}}} \right) + 1 \qquad (eq. 2-2-1)$$

where $\delta^{15}N_{Tr}$ and $\delta^{15}N_{Src}$ denote stable nitrogen isotopic composition of trophic (Tr, including alanine, valine, isoleucine, proline, and glutamic acid) and source (Src, including methionine and phenylalanine) amino acids in a single organism examined, respectively; $\beta_{Tr/Src}$ denotes the isotopic difference between Tr and Src amino acids in primary producers at the base of food webs; and $TDF_{Tr/Src}$ $(= \Delta\delta^{15}N_{Tr} - \Delta\delta^{15}N_{Src})$ stands for the net inter-trophic discrimination factor of Tr and Src amino acids between a consumer and its diet. By investigating several pairs of Tr and Src amino acids, Chikaraishi et al. (2009) identified glutamic acid and phenylalanine as the best combination to return the most accurate estimation of the trophic position of consumers ($TP_{Glu/Phe}$). Since then, several studies further suggested that using the average $\delta^{15}N$ values of Tr and Src amino acids of multiple amino acids may provide greater sta-

tistical power to TP calculations than a single pair of amino acids (e.g., Sherwood et al. 2011; Décima et al. 2013; Bradley et al. 2015; Nielsen et al 2015).

The unique metabolic pathway of individual amino acids can affect their isotopic behavior (whether Tr or Scr amino acids). Within heterotrophs, these differential enrichment (or depletion) patterns determine the amount of inter-trophic isotopic discrimination (e.g., Chikaraishi et al. 2007, 2009; Ohkouchi et al. 2015). For instance, it has been proposed that catabolic deamination (preceding transamination) of Tr amino acids causes the preferential cleavage of the ^{14}N amino group, resulting in an accumulation of ^{15}N (by up to ~3-8‰ per trophic level) in the Tr amino acids of consumer (Chikaraishi et al. 2007). In *in vitro* trials, Miura and Goto (2012) reported that the magnitude of isotopic discrimination of glutamic acid strongly correlates with its deamination flux (i.e., the deamination of a large pool generates greater isotopic discrimination compared to that from a smaller pool). However, the metabolism of Src amino acids does not involve the formation or cleaving of carbon-nitrogen bonds. Therefore there is negligible isotopic discrimination in Src AA between consumer and diet (Chikaraishi et al. 2007). The metabolic routing of amino acids may invoke alternative patterns of isotopic discrimination, particularly in the carbon isotopes of non-essential amino acids (McMahon et al. 2010). Although discrimination in nitrogen isotopes associated with metabolic routing has not been evidenced (Chikaraishi et al. 2007), the balance of amino acids, lipids, and carbohydrates as metabolic energy sources can potentially cause a significant variation in the isotopic discrimination of amino acids (Chikaraishi et al., 2015; McMahon et al., 2015; Blanke et al. 2017).

With the exception of photosynthesis, there are several metabolic parallels between plants and heterotrophs (Fig. 2-2-1, cf: Buchanan et al. 2000). For example, plants can store

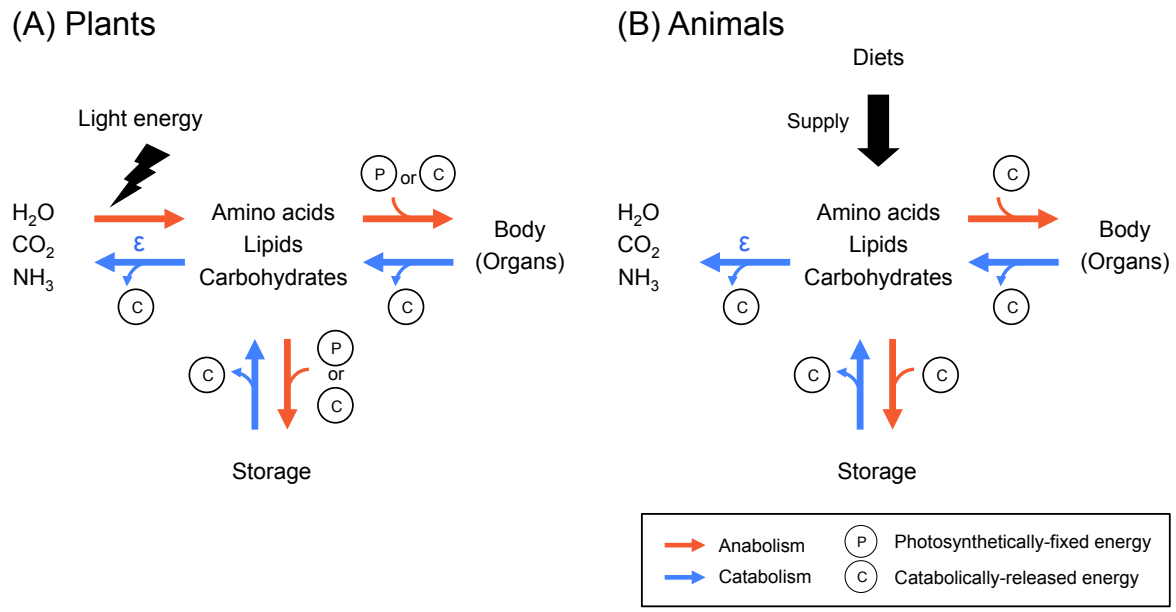

Fig. 2-2-1.
Schematic illustration of the catabolism and anabolism in plants and animals: The solar energy is fixed as organic molecules (e.g., amino acids, lipids, and carbohydrates) during photosynthesis in plants, and the fixed energy is released during metabolic breakdown of complex to simple molecules (i.e., catabolism) in both plants and animals; and these energies are used during anabolism to construct of their body and storage biomass (after Buchanan et al. 2000).

photosynthetically fixed energy in form of carbohydrates, lipids, and/or amino acids (Chapin et al., 1990; Millard, 1996; Buchanan et al. 2000; Kermode et al., 2011). During periods of lean photosynthesis, like heterotrophs, the catabolism of these storage compounds releases energy (i.e., ATP) that is subsequently used for the anabolism of new constituents (Buchanan et al. 2000), particularly in organs without chloroplasts (e.g., flower and root). If this catabolism within autotrophic biomass involves the deamination of amino acids, the resulting residual pool of amino acids (particularly for Tr amino acids) will be more enriched in ^{15}N than the original source pool. The mobilization and assimilation of these ^{15}N-enriched amino acids may generate isotopic differences between the source pool and newly synthesized biomass.

Unlike 'inter-trophic' isotopic discrimination that involves two separate organisms with unique trophic identities (e.g., Chikaraishi et al. 2007), 'intra-trophic' isotopic discrimination arises as a result of catabolic deamination of storage amino acids among different tissues within a single plant. In almost all cases, photosynthetic energy fixation in plants exceeds catabolic energy release, even during limited availability of sunlight (Reich et al. 1998). The intra-trophic isotopic discrimination in plants, primarily an outcome of amino acid deamination, is therefore hardly detectable during the growing season when metabolism is largely geared toward photosynthesis. The significant reduction or even absence of photosynthesis during winter dormancy however, temporarily severs the energy supply for the homeostasis (Damesin 2003). During this time plants (mostly deciduous), must meet the energetic demands for the maintenance of basic cellular function through the catabolism of organic storage compounds (Olofinboba 1969; Loescher et al. 1990; Arora et al., 1992; Gomez and Faurobert, 2002), which may include deamination of storage amino acids, that ultimately results in the intra-trophic isotopic discrimination within a plant tissue. Indeed, Takizawa and Chikaraishi (2014) first reported that sweet potato sprout grown in the absence of light has an unusually high $TP_{Glu/Phe}$ value of 2.2. Given that sprouting occurred in dark, and in absence of photosynthesis, sprout biomass likely recorded the ^{15}N-enrichment derived from the amino acid deamination during catabolism.

We hypothesize that plant organs lacking chloroplasts may undergo intra-trophic isotopic discrimination *via* the aforementioned mechanisms. This leads to an increased $\delta^{15}N_{Tr}$ values in these organs, and therefore, to an ecologically erroneous overestimation ($TP_{Glu/Phe} > 1.0$) for plant trophic position. The objective of our study was to investigate if indeed there was a measurable amount of intra-trophic isotopic discrimination between chloroplast

-bearing leaves and chloroplast-lacking flowers, and to assess its implication for trophic position calculation.

2. Materials and methods

2.1. Leaf and flowers samples

Flowers and mature leaves of eight deciduous trees and two annual plants were collected in their blooming season (February-May) from either a farm or a house-garden in Yugawara, Japan (35º08'N, 139º07E) (Table 2-2-1). These plants commonly begin to grow leaves in spring and completely lose their leaves in autumn. They were classified into Type I and Type II, with respect to the timing of their bloom relative to leaf emergence (Fig. 2-2-2). Type I plants included four stone fruit plants (*Amygdalus persica*, *Cerasus lannesiana*, *Cerasus pseudocerasus*, and *Prunus mume*) and one wisteria (*Wisteria floribunda*). These

Table 2-2-1. Plant leaves and flowers induced in the present study.

	Collection date (yyyy/mm/dd)	
Sample	Leaf	Flower
Type I		
Amygdalus persica	2015/4/24	2015/4/01
Cerasus lannesiana	2015/3/02	2015/3/02
Cerasus pseudocerasus	2015/4/17	2015/3/13
Prunus mume	2015/4/24	2015/2/24
Wisteria floribunda	2015/4/27	2015/4/27
Type II		
Akebia quinata	2015/4/17	2015/4/17
Benthamidia japonica	2015/5/07	2015/5/07
Cucumis sativus	2015/5/23	2015/5/23
Hydrangea macrophylla	2015/5/25	2015/5/25
Solanum melongena[*]	2015/5/23	2015/5/23

[*] *S. melongena* is a perennial, but it was commonly grown as an annual in the temperate region.

plants bloom for about 2 to 3 weeks prior to the emergence of their first leaves. Type II plants included three deciduous tree species (*Akebia quinata*, *Benthamidia japonica*, and *Hydrangea macrophylla*) and two annual plant species (*Cucumis sativus* and *Solanum melongena*). These plants bloom for 2 to 3 weeks (for deciduous plants), or continually for 1 to 2 months (for annual plants) only after their leaves have emerged. The flowers of the Type II plants were collected approximately halfway through the spring bloom. Both Type I and Type II plants were chosen as they are commonly found in agricultural area and/or house gardens in the temperate region of Japan. Approximately ten leaves and ten flowers were collected for each plant. The collected samples were cleaned with distilled water to remove surface contaminants, homogenized to a fine powder using a Tube-Mill (IKA, Staufen, Germany), freeze-dried, and then stored at −20°C.

2.2. Analysis of $\delta^{15}N_{AA}$ values

The samples were prepared for the $\delta^{15}N_{AA}$ analysis after HCl hydrolysis and *N*-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedure in Chikaraishi et al. (2009). In brief, the homogenized samples were hydrolyzed using 12M HCl at 110ºC overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. The derivatization was performed sequentially with thionyl chloride/2-propanol (1/4, v/v) at 110ºC for 2 hours, and pivaloylchloride/dichloromethane (1/4, v/v) at 110ºC for 2 hours. The $\delta^{15}N_{AA}$ values were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using a 6890N GC (Agilent Technologies, Palo Alto, USA) instrument coupled to a Delta^{plus}XP IRMS instrument through combustion (950ºC) and reduction (550ºC) furnaces, a countercurrent dryer (Permeable

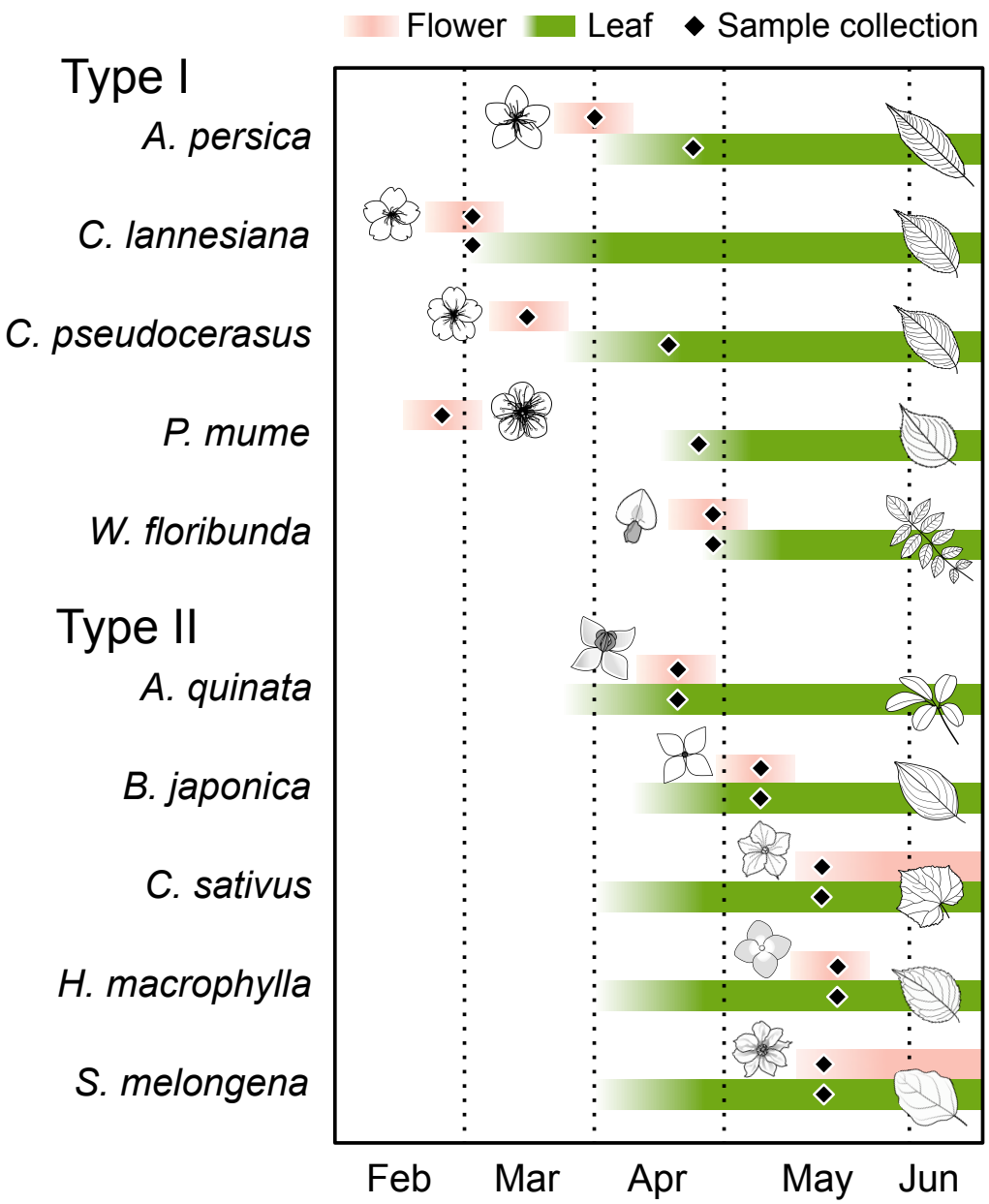

Fig. 2-2-2.

The periods of blooming and leafing for the plants induced in the present study. Flowers of the Type I plants (*Amygdalus persica*, *Cerasus lannesiana*, *Cerasus pseudo cerasus*, *Prunus mume, and Wisteria floribunda*) bloomed for 2 to 3 weeks in the leafless period or just prior to the leafing period in spring. Flowers of the Type II plants bloomed for 2 to 3 weeks (for deciduous tree plants; *Akebia quinata*, *Benthamidia japonica*, and *Hydrangea macrophylla*) or continually for 1 to 2 months (for annual plants; *Cucumis sativus* and *Solanum melongena*) during the mature-leaf period. Diamond symbols approximately indicate the collection dates (Table 2-2-1) of the flowers and leaves in the present study.

membrane, Nafion™), and a liquid nitrogen CO_2 trap *via* a GC-C/TC III interface (Thermo Fisher Scientific, Bremen, Germany). The Pv/iPr derivatives were injected using a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany) into an HP Ultra-2 capillary column (50 m; i.d. 0.32 mm; film thickness 0.52 μm; Agilent Technologies). The carrier gas (He) flow rate was maintained at 1.4 ml min^{-1}. To assess the reproducibility of the isotope measurement, a standard amino acid reference mixture (Indiana University, Bloomington, USA; SI science co., Sugito-machi, Japan) was analyzed after every five or six sample runs, with three pulses of reference N_2 gas discharged at the beginning and end of each run. The $\delta^{15}N_{AA}$ values were expressed relative to the isotopic composition of atmospheric nitrogen (AIR) on scales normalized to known $\delta^{15}N$ values of the reference amino acids. The accuracy and precision for the reference mixtures were 0.0‰ (mean of Δ) and 0.3-0.5‰ (mean of 1σ) for sample sizes of ≥0.5 nmol N, respectively. The $\delta^{15}N$ values of alanine, glycine, valine, leucine, isoleucine, proline, serine, glutamic acid, and phenylalanine were determined for sample leaves and flowers (Table 2-2-2), based on the S/N ratio of ≥20 with baseline separation on the chromatogram. All analyses were performed in triplicate, and the precision (1σ) for the $\delta^{15}N_{AA}$ values in the sample amino acids was 0.0 to 0.8‰ (mean: 0.3 ± 0.2‰).

2.3. Calculation of the $TP_{Glu/Phe}$ values

The $TP_{Glu/Phe}$ value was calculated from the observed $\delta^{15}N$ values of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), using eq. (1) with the $\beta_{Glu/Phe}$ and $TDF_{Glu/Phe}$ (inter $TDF_{Glu/Phe}$) being −8.4 ± 1.6‰ and +7.6 ± 1.2‰, respectively, values commonly applied for terrestrial samples from previous studies (Chikaraishi et al. 2010, 2011, 2014).

2.4. Statistical analysis

Independent samples Mann-Whitney U test was used to compare the $TP_{Glu/Phe}$ values of Type I leaves versus Type I flowers, and Type II leaves versus Type II flowers. Here, the null hypothesis was that $TP_{Glu/Phe}$ values of leaves and flowers from any particular plant type would be indistinguishable. Independent samples Mann-Whitney U test was used to compare the $TP_{Glu/Phe}$ values between Type I and Type II leaves, and independent samples t-test was used to compare the $TP_{Glu/Phe}$ values between Type I and Type II flowers. The null hypothesis assumed that the $TP_{Glu/Phe}$ values of both leaves type would be comparable, as would both flower types. One sample Wilcoxon signed rank test was used to compare the $TP_{Glu/Phe}$ values of Type I and Type II leaves, and one sample t-test was used to compare Type I and Type II flowers to a test value of 1.0. The null hypothesis underlying these tests was that any plant sample, whether leaves or flowers would have $TP_{Glu/Phe} = 1.0$.

3. Results and discussion

3.1. The $\delta^{15}N_{AA}$ and $TP_{Glu/Phe}$ values in leaves and flowers

Leaves and flowers fell within a similar but wide range in the $\delta^{15}N_{AA}$ value within Type I and Type II plants (Type I leaves = −2.5 ± 8.2‰; Type I flowers = −3.5 ± 5.9‰ and Type II leaves = −6.3 ± 9.2‰; Type II flowers = −5.9 ± 9.2‰; mean ± 1σ, Table 2-2-2). As expected, $TP_{Glu/Phe}$ value for both Type I and Type II leaves reported a mean of 1.0 ± 0.1 (Fig. 2-2-3), consistent with previously reported values (1.0 ± 0.2) of plant samples such as leaves, nuts, and sap (Chikaraishi et al. 2011; Steffan et al. 2013; Chikaraishi et al. 2014). However, the $TP_{Glu/Phe}$ value of Type I flowers (2.2 ± 0.2) was significantly higher than that of Type II flowers (1.0 ± 0.1) ($t_8 = 10.63$, $p < 0.001$). Additionally, the $TP_{Glu/Phe}$ value of Type I flowers

Table 2-2-2 Nitrogen isotopic composition of amino acids in plant leaves and flowers.

Sample	δ^{15}N (‰)[1]																		$TP_{Glu/Phe}$[2]		$TDF'_{Glu/Phe}$[3]
	Alanine		Glycine		Valine		Leucine		Isoleucine		Proline		Serine		Glutamic acid		Phenylalanine				
	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	
Type I																					
Leaf																					
Amygdalus persica	-13.1	0.1	-16.7	0.2	-14.2	0.2	-13.1	0.1							-13.0	0.1	-5.1	0.1	1.1	0.02	
Cerasus lannesiana	-3.1	0.2	-13.0	0.4	-0.1	0.4	-4.2	0.2	-5.7	0.5	7.3	0.1			-0.7	0.4	6.9	0.3	1.1	0.09	
Cerasus pseudocerasus	-4.5	0.4	-17.4	0.5	-3.5	0.5	-5.7	0.4	-4.4	0.2	-0.2	0.2	-14.2	0.1	-2.1	0.3	6.3	0.3	1.0	0.03	
Prunus mume	3.5	0.4	-12.8	0.2	7.6	0.2	0.6	0.6	1.3	0.3	4.8	0.3			2.8	0.2	11.0	0.4	1.0	0.08	
Wisteria floribunda	2.1	0.6	1.1	0.8	3.8	0.3									5.1	0.3	13.3	0.6	1.0	0.12	
Flower																					
Amygdalus persica	-6.6	0.6	-15.2	0.3	-6.3	0.1							-10.0	0.4	-5.8	0.4	-4.0	0.1	1.9	0.08	6.5
Cerasus lannesiana	-1.7	0.1	-14.4	0.5	-0.3	0.2	-7.0	0.3	-2.8	0.3					1.4	0.4	0.6	0.4	2.2	0.00	9.2
Cerasus pseudocerasus	-3.3	0.3	-7.0	0.4	2.7	0.1	-1.2	0.4	-0.4	0.4	2.0	0.2			5.2	0.3	1.8	0.3	2.6	0.03	11.8
Prunus mume	-2.0	0.7	-8.2	0.7	0.5	0.6									7.3	0.3	5.8	0.3	2.3	0.06	9.9
Wisteria floribunda	-3.4	0.3	-14.3	0.5	-4.0	0.4	-9.1	0.7	-3.3	0.2			-15.3	0.3	0.2	0.6	-0.5	0.3	2.2	0.06	9.2
Type II																					
Leaf																					
Akebia quinata	-16.4	0.3	-25.6	0.2	-20.2	0.3	-17.5	0.2							-18.7	0.2	-10.0	0.1	1.0	0.02	
Benthamidia japonica	-12.1	0.2	-22.9	0.3	-10.4	0.4	-10.0	0.4							-12.4	0.7	-4.1	0.4	1.0	0.07	
Cucumis sativus	-1.3	0.5	-15.7	0.4	-2.1	0.2	-2.3	0.1	-2.7	0.4					0.3	0.4	8.9	0.3	1.0	0.09	
Hydrangea macrophylla	-3.7	0.4	-12.6	0.4	-2.1	0.2	-0.7	0.6	-1.7	0.0	-0.9	0.6	-11.0	0.0	-0.9	0.2	7.3	0.2	1.0	0.05	
Solanum melongena	-0.2	0.4	-18.4	0.4	0.5	0.5	0.6	0.2	2.5	0.2					2.4	0.6	11.8	0.3	0.9	0.11	
Flower																					
Akebia quinata			-24.9	0.3			-16.8	0.2							-16.6	0.5	-7.2	0.6	0.9	0.12	-0.9
Benthamidia japonica	-14.9	0.7	-30.8	0.5	-9.7	0.2									-13.2	0.2	-4.9	0.1	1.0	0.02	0.1
Cucumis sativus	-1.1	0.4	-16.6	0.1	-0.9	0.2	-1.2	0.1	-1.6	0.1					0.5	0.1	8.6	0.2	1.0	0.01	0.3
Hydrangea macrophylla	-3.1	0.5	-11.3	0.6	-1.4	0.3	-1.0	0.1	-0.5	0.4	1.0	0.3			0.1	0.3	8.3	0.3	1.0	0.07	0.2
Solanum melongena	-2.2	0.2	-14.9	0.0	-2.2	0.3	-4.5	0.1	-7.1	0.2					-1.0	0.2	7.0	0.4	1.1	0.07	0.4

[1]The δ^{15}N value was determined by triplicate analysis for each sample.

[2]$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4) / 7.6] + 1$.

[3]$TDF'_{Glu/Phe} = (\delta^{15}N_{Flower, Glu} - \delta^{15}N_{Flower, Phe}) - \beta$

was significantly higher than the functional trophic position of autotrophs (TP ~1.0) in any ecosystem (t_4 = 11.05, p < 0.001). Conversely, the $TP_{Glu/Phe}$ value of Type II flowers was virtually identical to the expected trophic position values (Fig. 2-2-3). The $TP_{Glu/Phe}$ value of Type I flowers was significantly higher than that of Type I leaves ($TP_{Flower} - TP_{Leaf}$ =1.2 ± 0.3; U = 25.0, p = 0.008). However, there was no such difference between the $TP_{Glu/Phe}$ value of Type II flowers and leaves ($TP_{Flower} - TP_{Leaf}$ = 0.0 ± 0.1).

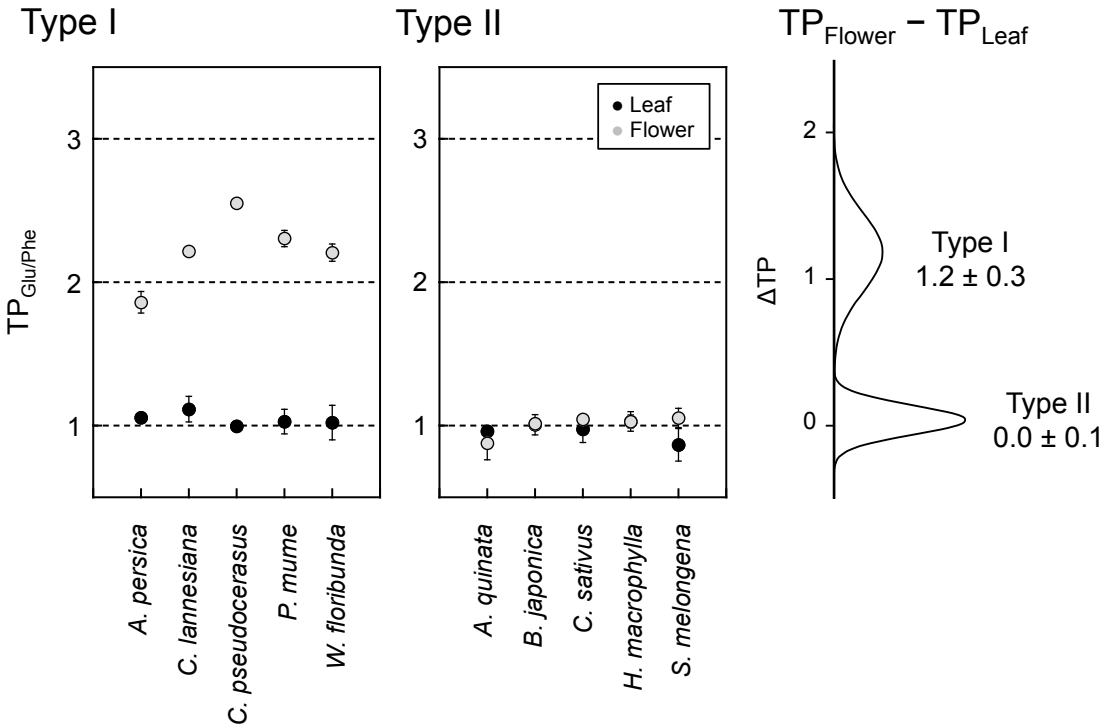

Fig. 2-2-3.
The $TP_{Glu/Phe}$ values of leaves and flowers from Type I and Type II plants, and the density distribution in the $TP_{Glu/Phe}$ value between leaves and flowers ($\Delta TP_{Flower-Leaf}$) for these plants.

3.2. Energy resources for blooming

As primary producers, plants occupy TP = 1.0 (Elton, 1927; Lindeman, 1942), a value that has been validated using the CSIA method (Chikaraishi et al., 2011; McCarthy et al., 2013; Steffan et al., 2013). However, whether this well-documented trophic identity ap-

plies to all organs within an individual plant, has not been fully investigated (Takizawa and Chikaraishi, 2014). Our results indicate that even within a single plant, the $\Delta\delta^{15}N_{Glu-Phe}$ value (and therefore the $TP_{Glu/Phe}$ value) of different organs can vary significantly (Figs. 2-2-3 and 2-2-4). We propose possible physiological scenarios that could contribute to this isotopic heterogeneity between leaves and flowers of Type I and Type II plants, potentially skewing their trophic identities.

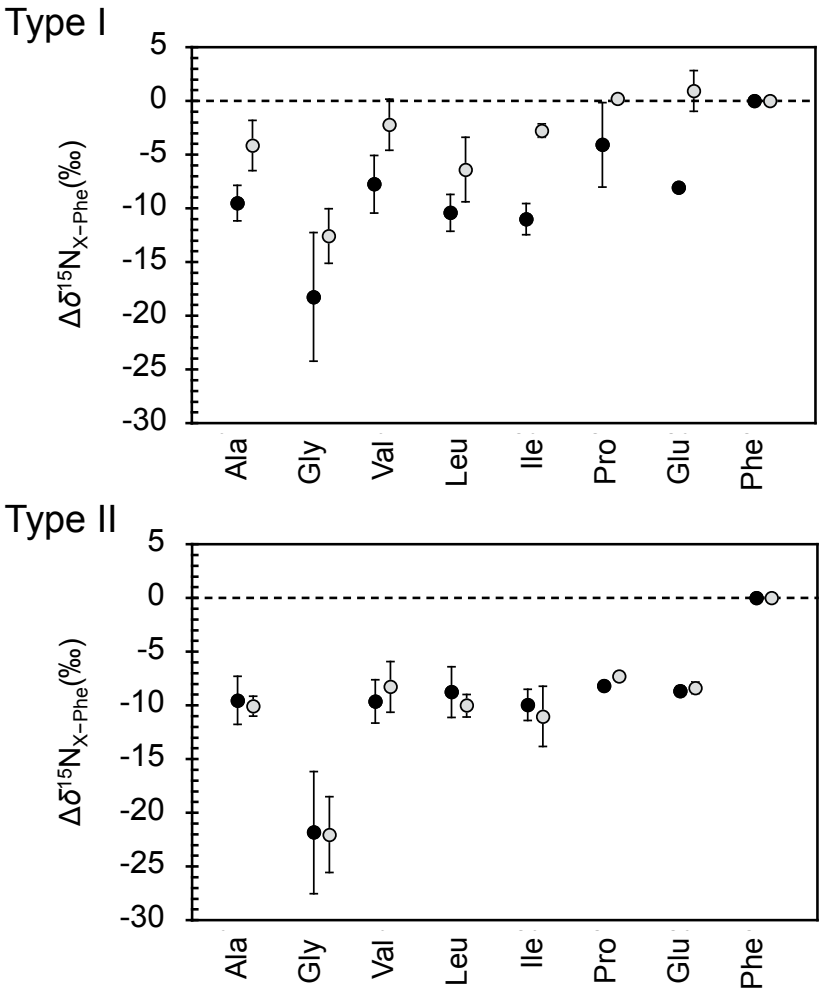

Fig. 2-2-4.
Difference in the $\delta^{15}N$ value between amino acids and phenylalanine ($\delta^{15}N_{X-Phe}$). Black and gray filled circles indicate the value for leaves and flowers, respectively. Bar indicates 1σ variation within the plant types.

Flowers have limited/no photosynthetic capacity, and must therefore rely on other sources of energy to support their bloom. The elevation in the $TP_{Glu/Phe}$ value (>1.0) in Type I flowers indicates that the Tr amino acids used to synthesize the flower biomass were enriched in ^{15}N. One possible mechanism to explain this enrichment is that in Type I plants, bloom occurs before the appearance of leaves (i.e., in the absence of photosynthetically-fixed energy). During this time, overwintered storage compounds are broken down to liberate metabolic energy (i.e., ATP) required to sustain Type I bloom. In case of stored proteins, the deamination of amino acids can preferentially eliminate the ^{14}N amino group as ammonia, leaving behind the enriched ^{15}N in the residual pool of Tr amino acids (Fig. 2-2-5). If the enriched end products of deamination are used to assimilate Type I flowers, it would explain the enrichment of ^{15}N in floral Tr amino acids. Previous studies have shown that antifreeze protein help deciduous trees to survive through winter dormancy (Arora et al., 1992; Hon et al., 1995). Because these proteins are not required during spring, they may be subsequently deaminated (Arora et al., 1992). The residual pool of ^{15}N-enriched amino acids generates 'intra-trophic' discrimination, especially in Tr amino acids (e.g., glutamic acid), and when incorporated in newly synthesized Type I floral tissue, inflates their $TP_{Glu/Phe}$ value (Fig. 2-2-5).

Type II plants represent a different phenology where bloom occurs in the presence of actively photosynthesizing leaves. Since energy fixed by active photosynthesis is sufficient to support bloom, this may preclude the necessity of deamination of amino acids (Fig. 2-2-5), which can explain the negligible 'intra-trophic' isotopic discrimination of amino acids in Type II plants. Therefore the $TP_{Glu/Phe}$ value of Type II leaves and Type II flowers remain comparable with each other, and to the expected value of 1.0.

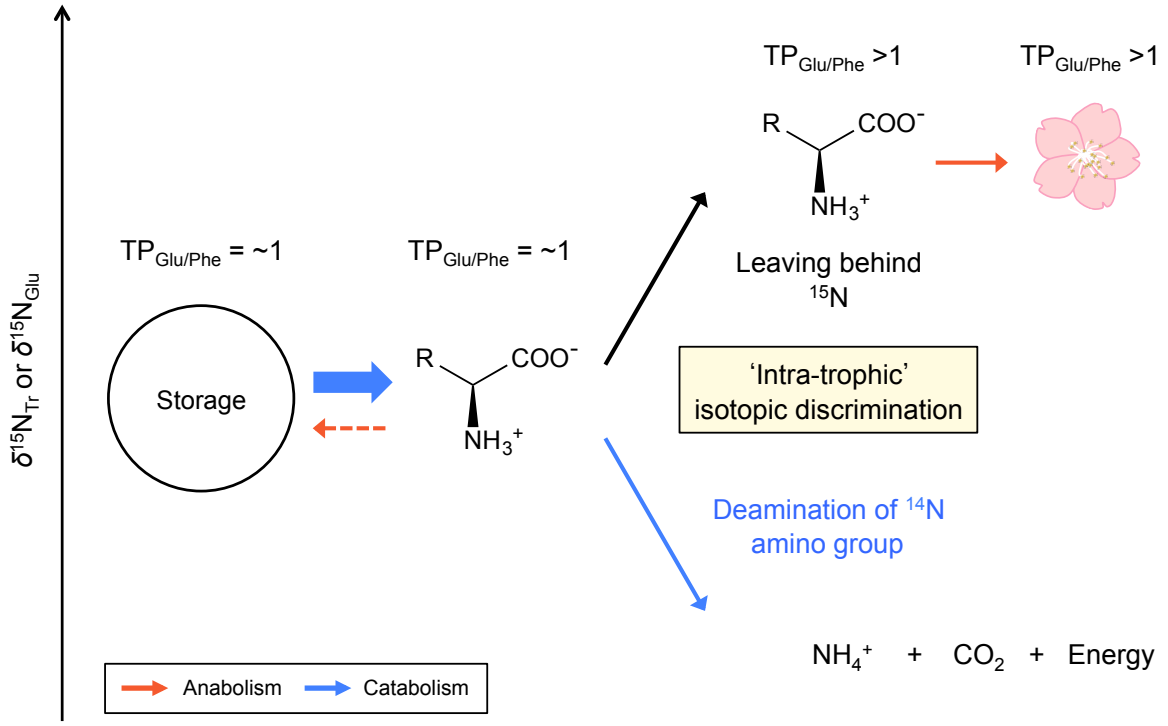

Fig. 2-2-5.
Possible metabolic states for flowering of the Type I plants, which includes deamintion of amino acids and therefore alternative isotopic discrimination leading to significant elevation in the $TP_{Glu/Phe}$ value of amino acids in flowers.

3.3. Intratrophic isotopic discrimination

We suggest the following equation (2-2-2) to illustrate the intra-TDF of glutamic acid and phenylalanine ($TDF'_{Glu/Phe}$) in plant organs (e.g., flowers):

$$TDF'_{Glu/Phe} = \left(\delta^{15}N_{Organ,Glu} - \delta^{15}N_{Organ,Phe}\right) - \beta \qquad (eq. 2-2-2)$$

where the subscript *Organ* indicates the plant organ of interest. The β should be derived from the $\delta^{15}N$ value offset between glutamic acid and phenylalanine that has never undergone the deamination *via* plant catabolism. Since our data show no substantial deamination in both Type I and Type II leaves, the standard β value (−8.4 ± 1.6 ‰, Chikaraishi et al. 2010) re-

ported for primary producers was incorporated in equation (2).

Our data show that the $TDF'_{Glu/Phe}$ for Type I flowers was 9.3 ± 1.9‰, elevating $TP_{Glu/Phe}$ values of flowers correspondingly by 1.2 ± 0.3 trophic units than expected (Fig. 2-2-3). This high $TP_{Glu/Phe}$ value can be explained by a large $TDF'_{Glu/Phe}$, probably as an outcome of the assimilation of deaminated amino acids. Type II flowers were most likely sustained by foliar photosynthesis during bloom, resulting in a low $TDF'_{Glu/Phe}$ (0.0 ± 0.5‰), and a $TP_{Glu/Phe}$ value ~1.0, as expected. Moreover, when equation (2) is applied to the previously reported data for sprout and sweet potato ($TP_{Glu/Phe}$ = 2.2 and 1.4, respectively) (Takizawa and Chikaraishi 2014), the $TDF'_{Glu/Phe}$ is 9.1‰ for the sprout, and 2.8‰ for the sweet potato, indicative of strong reliance on deamination of amino acids for sprouting under dark conditions.

Interestingly, three of the five Type I plants (*A. persica*, *C. lannesiana*, and *W. floribunda*) included in the present study started sprouting leaves from middle to end of the blooming period (Fig. 2-2-2). It is plausible that these newly sprouted leaves are initially catabolically supported, and later sustain themselves as photosynthesis ramps up. If so, the first few leaves to sprout could also present with higher TDF', elevating their $TP_{Glu/Phe}$ value temporarily, before returning to ecologically realistic values as the season progresses. Although we currently do not have sufficient data to support this assumption, further research is required to investigate intra-trophic discrimination in leaves sprouting early in the season.

The $TDF'_{Glu/Phe}$ will be a useful parameter to assess the magnitude of deamination of amino acids, with respect to the energy consumption in specific phenology, within plants. Several factors could result in variation of the $TDF'_{Glu/Phe}$, including length of dormancy relative to growing season (short versus long), flower biomass relative to storage amino acids

(small versus large), and availability of storage lipids and/or carbohydrates as alternative catabolic energy sources (low versus high). With further research, the TDF' parameter could potentially be extended and used to investigate unusually high trophic positions of herbivores ($TP_{Glu/Phe} > 2.0$).

3.4. The $\delta^{15}N$ values of phenylalanine in leaves and flowers

There was a large variation in the $\delta^{15}N$ value of phenylalanine within Type I and Type II plants (Type I leaves = 6.5 ± 7.1‰; Type I flowers = 0.7 ± 3.6‰ and Type II leaves = 2.8 ± 9.3‰; Type II flowers = 2.3 ± 7.8‰), as well as a difference in phenylalanine value between leaves and flowers ($\Delta\delta^{15}N_{Phe} = \delta^{15}N_{Flower,\ Phe} - \delta^{15}N_{Leaf,\ Phe}$) within a plant (Type I $\Delta\delta^{15}N_{Phe} = -5.8 \pm 5.3$‰, and Type II $\Delta\delta^{15}N_{Phe} = -0.4 \pm 2.8$‰). Such large variability in the $\delta^{15}N_{Phe}$ and $\Delta\delta^{15}N_{Phe}$ values is uncharacteristic, given that it is a Src amino acid. However, this variation is consistent with data published in previous studies using leaves collected from the same farm (10.6 ± 3.8‰) (Chikaraishi et al. 2011, 2014). One likely source of such variability is the temporal and spatial heterogeneity in the abundance and $\delta^{15}N$ values of organic and inorganic nitrogen sources (NH_4^+, NO_3^-, and N_2) in soils. The timing of bloom was different among the plants examined (Fig. 2-2-2), and this could have introduced temporal variability in their $\delta^{15}N$ values of phenylalanine. Additionally, the rate of incorporation of this isotopically variable phenylalanine may vary between leaves and flowers within a plant. However, evaluating the difference in the $\delta^{15}N$ value between phenylalanine and other amino acids ($\Delta\delta^{15}N_{X-Phe} = \delta^{15}N_X - \delta^{15}N_{Phe}$) can control for this background heterogeneity. Our data show that, notwithstanding this background noise, there was a significantly large difference between leaves and flowers for all-examined amino acids in Type I plants ($\Delta\delta^{15}N_{X-Phe} = 9.0 \pm 2.1$‰), but was negligible in Type II plants ($\Delta\delta^{15}N_{X-Phe} = 0.3 \pm 0.8$‰) (Fig. 2-2-4). Therefore,

the background heterogeneity in the $\delta^{15}N$ value of phenylalanine does not sufficiently explain the high $\Delta\delta^{15}N_{Phe}$ value for Type I plants (Fig. 2-2-4) and likely did not affect the numeral inflation of the $TP_{Glu/Phe}$ values of Type I flowers (Fig. 2-2-3). While our data show a clear pattern of differential isotopic discrimination in amino acids among plant tissues, we recommend further investigation using additional representative organs from Type I and Type II plants to evaluate the co-variations in the $TP_{Glu/Phe}$ value and $TDF'_{Glu/Phe}$.

4. Implications

CSIA has expanded the ecologists' toolbox by allowing high-resolution insights into trophic interactions. However, little information is available about the factors controlling inter- and/or intra-trophic isotopic discrimination of amino acids in plants, animals, fungi, and bacteria (Gutiérrez-Rodríguez et al. 2014; Chikaraishi et al., 2015; McMahon et al., 2015; Steffan et al. 2015a). Our findings reveal unique isotopic heterogeneity among wild plant organs, which can confound trophic estimations of these plants and the consumers that they support. For example, will feeding on Type I pollen in early spring elevate the $TP_{Glu/Phe}$ of pollinators and nectarivores above that expected of herbivores (> 2.0)? How will the preferential feeding of herbivores on early spring flowers or new leaves imprint on the trophic positions of higher order consumers? What will be the trophic identity of the detritus derived from Type I flowers as they become a basal resource in the brown food web?

Ideally, the $TP_{Glu/Phe}$ (or $TP_{Tr/Src}$) value enables isotopic ecologists to deduce the ecological function (e.g., primary producer, herbivore, omnivore, and carnivore) of organisms (Chikaraishi et al. 2014; Bradley et al. 2015; Nielsen et al. 2015; Steffan et al. 2015b). However, our data indicate that the $TP_{Glu/Phe}$ value does not always reflect an organism's

functional trophic position in the food web. For instance, although Type I flowers returned a $TP_{Glu/Phe}$ value of 2.2, such value is typical of omnivores (TP > 2.0), and certainly does not represent the 'functional' trophic identity of plants and their organs. Therefore it appears that during sum of intra- and inter-trophic isotopic discriminations, organismal $TP_{Glu/Phe}$ values may represent 'energetic' tendencies rather than the organism's true functional trophic position in food webs. These differences between the energetic and functional trophic positions arising from intra-trophic isotopic discrimination can complicate food web studies. We therefore encourage continued investigations to re-evaluate how CSIA-derived trophic position correlates to the $\delta^{15}N_{AA}$ values of organisms in food webs.

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2-3. Change in the δ^{15}N value of plant amino acids on the phenology of leaf flush and senescence

Abstract

Elevation in the δ^{15}N value of amino acids ($\delta^{15}N_{AAs}$) from the diet to its consumer (i.e. 'inter'-trophic discrimination factor: TDF) has been widely used to illustrate the trophic hierarchy among organisms in ecological food webs. However, there is 'intra'-trophic discrimination factor (TDF') within a single organism, which is attributable to the catabolism of storage compounds for adjusting the energy balance between supply and demand, independent of the TDF between two separate organisms. The $\delta^{15}N_{AAs}$ values of the deciduous plant *Cerasus lannesiana* reveal that the TDF' is 0.1±1.0‰ (mean ± 1σ) for leaf senescence from spring to autumn, whereas that is gradually decreased from 5.3‰ to 0.9‰ for leaf flush in early spring. These results imply that plants can use sufficient photosynthetically-fixed energy for the leaf senescence, but use a large amount of catabolically-released energy (from deamination of storage amino acids) for the leaf flush under no/less photosynthetic activities. Thus, we predict that the metabolic energy fluxes can be considered in the isotope ecology, as such TDF' potentially propagates into the $\delta^{15}N_{AAs}$ values in consumers that particularly feed on buds and flush leaves.

This section was originally in press as; Takizawa Y. and Chikaraishi Y. (2017) Change in the δ^{15}N value of plant amino acids on the phenology of leaf flush and senescence. *Researches in Organic Geochemistry*.

1. Introduction

Stable nitrogen isotopic composition of amino acids ($\delta^{15}N_{AAs}$) has been recently employed as a potential powerful tool to illustrate high-resolution trophic hierarchies among organisms in ecological food webs (e.g., Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007; Steffan et al., 2015), which is based on the 'inter'-trophic discrimination factor (TDF) of nitrogen isotopes associated with the catabolic deamination of amino acids in consumers for the grazing process between consumers and their diets (e.g., Ohkouchi et al., 2015; McMahon and McCarthy, 2016). The position on the food web hierarchies (trophic position, TP) is generally estimated by the following equation (2-3-1):

$$\mathrm{TP_{Glu/Phe}} = \left(\frac{\delta^{15}\mathrm{N_{Glu}} - \delta^{15}\mathrm{N_{Phe}} - \beta_{\mathrm{Glu/Phe}}}{\mathrm{TDF_{Glu/Phe}}} \right) + 1 \qquad (eq.\ 2-3-1)$$

where $\beta_{Glu/Phe}$ denotes the offset between the $\delta^{15}N$ values of glutamic acid and phenylalanine ($\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$, respectively) in primary producers at the base of food webs, and $TDF_{Glu/Phe}$ ($= \Delta\delta^{15}N_{Glu} - \Delta\delta^{15}N_{Phe}$) stands for the net TDF of glutamic acid and phenylalanine between a consumer and its diet (Chikaraishi et al., 2009).

However, numerical isotopic discrimination was reported in amino acids from plant organs such as an overwintered sweet potato (Takizawa and Chikaraishi, 2014) and some deciduous plant flowers (Takizawa et al., 2017). This numerical discrimination has been recently explained by 'intra'-trophic discrimination factor (TDF'), which is attributable to the deamination of storage amino acids in plant catabolism. Because plants require the catabolically-released energy to adjust energy balance between supply and demand for the homeostasis in overwintering and the productivity in flowering under no/less photosynthetic activities. Like heterotrophic animals, the deamination preferentially releases ^{14}N as ammonia from the

storage amino acids, and simultaneously leaves behind ^{15}N in the residual pools of amino acids. The plants construct their organs with the later ^{15}N-enriched amino acids. As a result of this discrimination (i.e., TDF'), it is considered that the estimated TP is frequently more than 1.0 even in plant organs. Takizawa et al. (2017) previously suggested the following equation (2-3-2) to characterize TDF':

$$\mathrm{TDF'_{Glu/Phe}} = \left(\delta^{15}\mathrm{N_{sample,Glu}} - \delta^{15}\mathrm{N_{sample,Phe}}\right) - \beta_{\mathrm{Glu/Phe}} \qquad (eq.\ 2-3-2)$$

where $\delta^{15}\mathrm{N_{sample}}$ indicates the plant organ of interest, and $\beta_{\mathrm{Glu/Phe}}$ is derived from the same offset in the δ^{15}N value between glutamic acid and phenylalanine in primary producers to that used in the eq. (2-3-1).

As similar to the flowers, if the energy supply frequently leans from the photosynthesis to the catabolism of amino acids even in plant leaves, the TDF' can propagate through consumers in food webs. Takizawa et al. (2017) indeed speculated that the TDF' would be detectable in buds and flush leaves during plant phenology. Identifying specific factors, when/how the TDF' is substantial large in plant leaves, is thus required to improve accuracy of the TP estimation in the isotope ecology, particularly for studies of green food webs where plant leaves considerably contribute to basal resources.

In the present study, we determined the $\delta^{15}\mathrm{N_{AAs}}$ values in leaves of the deciduous plant *Cerasus lannesiana* for leaf senescence (March-October in 2015) and leaf flush (January-March in 2016) periods, to evaluate diversity and variation in the TDF' with respect to the phenology of plant leaves. Furthermore, we discuss the potential impact whether or not the TDF' in leaves propagates into the $\delta^{15}\mathrm{N_{AAs}}$ values in food webs.

2. Materials and Methods

2.1. Leaf and flower samples

We collected leaves of the deciduous plant *C. lannesiana* for leaf senescence (March-October in 2015) and flush (January-March in 2016) periods and flowers of the same plant for blooming periods (early spring in both 2015 and 2016), from a house-garden in Yugawara, Japan (35º08'N, 139º07E) (Table 2-3-1). This plant commonly thrives in the temperate region of Japan. The phenology of this plant is composed of growing seasons and winter dormancy, as flush leaves and flowers in spring, mature leaves in summer, turned leaves in autumn, and no leaves in winter. Flowers bloom for about 2-3 weeks prior to the leaf flush. For leaf-senescence and flowering, approximately ten leaves and ten flowers were collected, respectively, cleaned with distilled water to remove surface contaminants, homogenized to a fine powder using a Tube-Mill (IKA), and freeze-dried. On the other hand, for leaf flush, approximately five small leaves were collected, cleaned with distilled water, and cut into small pieces, and total ~4 mm x 8 mm area of each sample were used. These samples were stored at −20°C until the isotope analysis.

2.2. Analysis of the $\delta^{15}N_{AA}$ values

These samples were prepared for the $\delta^{15}N_{AAs}$ analysis after HCl hydrolysis and N-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedure in Chikaraishi et al. (2009). In brief, the samples were hydrolyzed using 12M HCl at 110ºC overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. The derivatization was performed sequentially with thionyl chloride/ 2-propanol (1/4, v/v) at 110ºC for 2 hours, and pivaloyl chloride/dichloromethane (1/4, v/v)

at 110ºC for 2 hours. The $\delta^{15}N_{AAs}$ values were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using a 6890N GC (Agilent Technologies) instrument coupled to a Delta^{plus}XP IRMS instrument through combustion (950ºC) and reduction (550ºC) furnaces, a countercurrent dryer (Permeable membrane, Nafion™), and a liquid nitrogen CO_2 trap via a GC-C/TC III interface (Thermo Fisher Scientific). The Pv/iPr derivatives were injected using a programmable temperature vaporizing (PTV) injector (Gerstel) into an HP Ultra-2 capillary column (50 m; i.d. 0.32 mm; film thickness 0.52 μm; Agilent Technologies). The carrier gas (He) flow rate was maintained at 1.4 ml min^{-1}. To assess the reproducibility of the isotope measurement, a standard amino acid reference mixture (Indiana University; SI science co.) was analyzed after every five or six sample runs, with three pulses of reference N_2 gas discharged at the beginning and end of each run. The $\delta^{15}N_{AAs}$ values were expressed relative to the isotopic composition of atmospheric nitrogen (AIR) on scales normalized to known $\delta^{15}N$ values of the reference amino acids. The accuracy and precision for the reference mixtures were 0.0‰ (mean of Δ) and 0.3-0.5‰ (mean of 1σ) for sample sizes of ≥0.5 nmol N, respectively. The $\delta^{15}N$ values of alanine, glycine, valine, leucine, isoleucine, proline, glutamic acid, and phenylalanine were determined for sample leaves and flowers (Table 2-3-1), based on the S/N ratio of ≥20 with baseline separation on the chromatogram.

2.3. Calculation of the $TP_{Glu/Phe}$ values and $TDF'_{Glu/Phe}$

The $TP_{Glu/Phe}$ value and $TDF'_{Glu/Phe}$ were calculated using the equations (2-3-1) and (2-3-2), respectively. We used the $\beta_{Glu/Phe}$ and $TDF_{Glu/Phe}$ to −8.4‰ and +7.6‰, respectively, which are commonly applied for terrestrial samples in previous studies (Chikaraishi et al. 2010, 2011, 2014)

3. Results and Discussion

3.1. The $\delta^{15}N_{AAs}$ value and TDF' in plants

Amino acids have the δ^{15}N values (1) between −12.6‰ for glycine and −1.2‰ for isoleucine, with −1.3‰ for glutamic acid and −1.8‰ for phenylalanine in the flower, (2) between −15.8‰ for glycine (in the April leaves) and 11.7‰ for phenylalanine (in the April leaves), with −1.2±3.5‰ (mean ± 1σ) for glutamic acid and 7.1±3.6‰ for phenylalanine as a mean of the six samples in the senescence leaves, and (3) between −7.2‰ for glycine and 9.9‰ for proline, with 2.3±0.4‰ for glutamic acid and 8.2±1.5‰ for phenylalanine as a mean of the five samples in the flush leaves (Table 2-3-1). One likely source of such variability is the temporal and spatial heterogeneity in the abundance and δ^{15}N value of organic and inorganic nitrogen sources (NH_4^+, NO_3^-, and N_2) in soils (Chikaraishi et al., 2014; Takizawa et al., 2017). The $\delta^{15}N_{Phe}$ values (from −1.8 to 11.7‰) of flowers and leaves in the present study indeed highly overlap the values (from 1.6 to 17.0‰) reported in Chikaraishi et al. (2014). The TDF' (and $TP_{Glu/Phe}$) is however independent of such variability (because of normalizing to the $\delta^{15}N_{Phe}$ values) and represents the trophic isotopic discrimination specific to a single phenological process (e.g., flowering) in plants (Takizawa et al., 2017).

The TDF' of flowers is highly positive (8.9 ‰ in the present study, corresponding to $TP_{Glu/Phe}$ = 2.2) for the *C. lannesiana* collected in 2016 (Fig. 2-3-1B), which is very consistent with that for the same plant collected in 2015 (TDF'=9.3‰) (Fig. 2-3-1A) as well as for the some other deciduous plants (TDF'=9.4±2.2) (Takizawa et al., 2017). Such highly positive TDF' of these flowers have been explained by the enrichment in ^{15}N associated with the catabolism (that starts from deamination) of storage amino acids for supplying blooming energy prior to starting photosynthetic energy fixation (i.e., no leaves) (Takizawa et al.,

Table 2-3-1. Nitrogen isotopic composition of amino acids in plant leaves and flowers, examined in this study.

	Collection date (yy/mm/dd)	$\delta^{15}N$ (‰)[1]								$TP_{Glu/Phe}$[2]	TDF'[3]	Reference
		Alanine	Glycine	Valine	Leucine	Isoleucine	Proline	Glutamic acid	Phenylalanine			
Flowers												
Flower	2015/3/2	-1.8	-14.1	-0.3	-7.0	-2.9		1.2	0.3	2.2	9.3	Takizawa et al. (2017)
Flower	2016/2/19	-5.6	-12.6	-5.6	-7.7	-1.2		-1.3	-1.8	2.2	8.9	
Senescence leaves												
Immature*	2015/3/2	-3.1	-12.8	-0.4	-4.2	-5.8	7.2	-0.6	6.7	1.1	1.1	Takizawa et al. (2017)
Mature*	2015/4/18	1.4	-15.8	-1.8	-2.0	2.1	1.0	2.3	11.7	0.9	-1.1	
Mature*	2015/5/7	-0.7	-13.4	-2.2	-3.7	-1.3	-1.9	-1.4	7.7	0.9	-0.7	
Mature*	2015/6/21	-1.2	-6.9	2.3	-4.0	0.1	-0.1	3.0	10.1	1.2	1.3	
Mature*	2015/8/23	-5.0	-13.6	-1.2	-6.4	-4.8		-4.3	4.5	0.9	-0.4	
Mature*	2015/10/18	-6.0	-15.4	-2.9	-7.6	-7.3		-5.9	1.9	1.1	0.6	
Flush leaves												
Immature*	2016/1/23	1.0	-2.4	2.0	-1.3	1.9	9.9	2.8	6.0	1.7	5.3	
Immature*	2016/1/24	-0.4	-3.0	0.9	-2.0	0.8	6.1	2.4	7.5	1.4	3.3	
Immature*	2016/2/6	-1.3	-4.8	0.6	-1.9	0.6	2.8	1.9	8.5	1.2	1.8	
Immature*	2016/2/19	-2.2	-7.2	2.3	-2.7	1.0	3.2	2.2	9.6	1.1	0.9	
Mature*	2016/3/7	-1.7	-6.2	0.7	-1.3	0.6	2.9	2.0	9.5	1.1	0.9	

[1]The $\delta^{15}N$ value was determined by single analysis for each sample.

[2]$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4) / 7.6] + 1$.

[3]$TDF'_{Glu/Phe} = (\delta^{15}N_{Sample, Glu} - \delta^{15}N_{Sample, Phe}) - \beta$

*Immature and Mature are defined by leaves that has leaf length less than and more than 5 cm, respectively.

2017), allowing us approximately to employ this high TDF' (i.e., 8.9‰) as a signal of excess catabolism against photosynthesis in this plant.

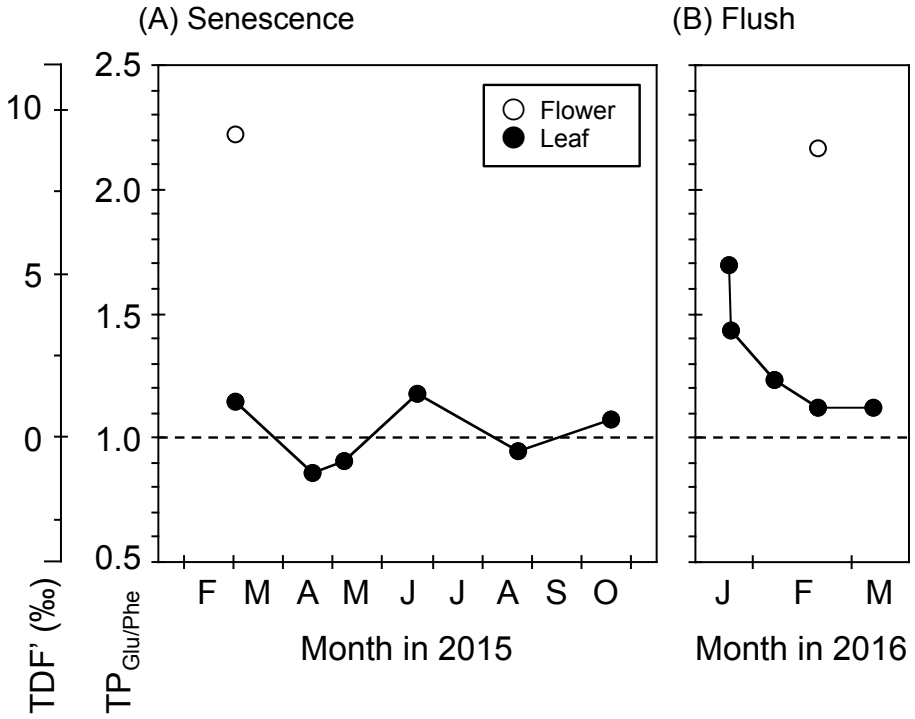

Fig. 2-3-1.
The TDF' and $TP_{Glu/Phe}$ for leaf (A) senescence and (B) flush.

The TDF' of senescence leaves are substantially close to zero (0.1±1.0, corresponding to $TP_{Glu/Phe}$=1.0±0.1) for the *C. lannesiana* (Fig. 2-3-1A), which is consistent with the functional trophic position of primary producers as well as the absence of TDF' for plant leaves reported in previous studies (e.g., Chikaraishi et al., 2010; Steffan et al., 2013; Takizawa et al., 2017). Such negligible small value of TDF' in these leaves has been explained by no catabolism of storage amino acids for supplying growth energy in the period of leaf senescence, allowing us to employ this zero TDF' as a signal of excess photosynthesis against catabolism in this plant.

In the amino acids from the flush leaves, the net TDF' has 2.4±1.8‰ (corresponding to TP=1.3±0.2) for the flush leaves (Fig. 2-3-1B). These substantially positive TDF'

in leaves have not been found yet in previous studies. Moreover, these δ^{15}N values reveal that the TDF' is large (5.3‰) for the first flush leaves (collected in January 23rd) and is gradually decreased by the asymptotic curve to 0.9‰ for March 7th (Fig. 2-3-1B). These results well demonstrate that, as expected in Takizawa et al. (2017), the positive TDF' (and TP>1) is certainly found in plant leaves for the early stage of leaf flush, and proof that plants can use catabolically-released energy derived from storage amino acids in the growth even for leaves. The asymptotic curve observed in the TDF' implies that positive TDF' of the amino acids (i.e., residual pool of storage amino acids and their reconstituents) in flush leaves is rapidly diluted with zero TDF' of the newly-produced amino acids along the activation of photosynthesis in leaf growth. On the simplistic assumption that the maximum and minimum TDF' are 8.9‰ (equal to the flowers collected in 2016) for excess catabolism and 0.1‰ (equal to the senescence leaves) for excess photosynthesis, respectively, the proportion of residual amino acids with positive TDF' in flush leaves is accounted to be 59% in the first sampling date, which is decreased to 36% after only 1 day and finally to 9 % after 44 days.

3.2. Contribution of TDF' to the TP estimate

The $TP_{Glu/Phe}$ values calculated are 1.02±0.13 and 1.32±0.24 for the senescence and flush leaves, respectively, in our study, which are illustrated in Fig. 2-3-2 together with TPs for the deciduous leaves and flowers in the previous study (Takizawa et al., 2017). The $TP_{Glu/Phe}$ close to 1.0 was reported for the leaves independent of the plant types in the previous study: plants that bloomed when their leaves were absent (Type I), versus plants that bloomed while leaves were already present (Type II) (Takizawa et al., 2017). However, the $TP_{Glu/Phe}$ of flush leaves is elevated to 1.32 as mean with a large variation ($1\sigma = 0.24$) during

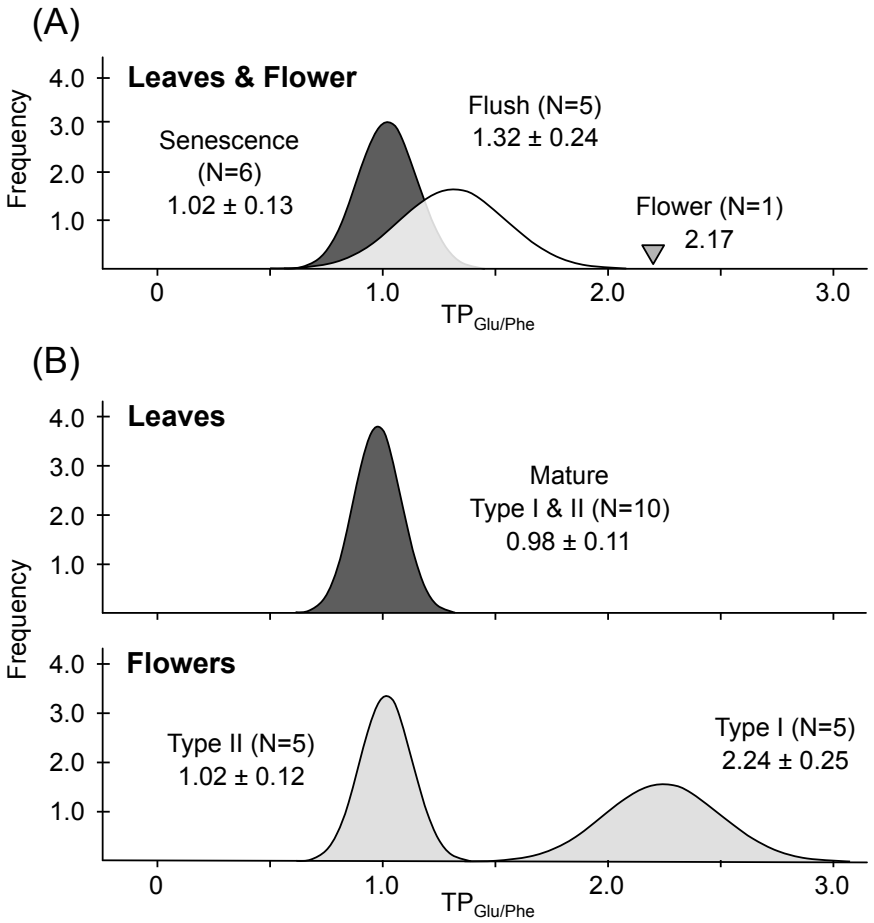

Fig. 2-3-2.

Density distribution of (A) the $TP_{Glu/Phe}$ of senescence and flush leaves determined in the present study, together with (B) that of the leaves and flowers in deciduous plants reported by Takizawa et al. (2017). The $TP_{Glu/Phe}$ values of flowers in the present study is also shown (inverted triangle).

the term of leaf flush even for the leaves collected from a single plant. The $TP_{Glu/Phe}$ of flush leaves thus inflates and falls between the $TP_{Glu/Phe}$ of senescence leaves and flowers in the *C. lannesiana* as well as between that of mature-leaves in both type plants and of flowers in the Type I plants (Fig. 2-3-2). These results reveal that the $TP_{Glu/Phe}$ of plants does not always close to 1.0 and certainly can elevate to substantial high values for specific organs in plants, at least 2.2 and 1.7 as maximum for flowers and flush leaves, respectively. This elevation can be explained by the common process of energy consumption for flowering in plant phenology

when plants supply catabolically-released energy from metabolism (i.e., resulting in TDF' associated with deamination of amino acids) for the growth of specific organs under no/less photosynthetic activities.

It is assumed that the impact of TDF' found in flowers to the green food web study is limited in the region where Type I plants are dominant, in the ephemeral term of early spring, and in several herbivores such as pollinators and nectarivores (Takizawa et al., 2017). However, the impact of TDF' found in flush leaves to the food web study would be extended farther than that in flowers, because the leaf flush is certainly observed in all deciduous plants. For instance, Naito et al. (2010, 2013) reported unusually high $TP_{Glu/Phe}$ (2.3-2.5) for bone collagen of deers (6000–4000 cal BP.) collected from Hokkaido (the subarctic zone), Japan. The elevation in the $TP_{Glu/Phe}$ by 0.3-0.5 for the deer can be interpreted by where the deer feed on flush leaves with high TDF'. The TDF' in flush leaves thus potentially increases uncertainty on the traditional 'functional' TP estimates in ecological studies. In the previous study, Takizawa et al. (2017) suggested a new concept that the $\delta^{15}N_{AAs}$ values reflect 'energetic' but not 'functional' trophic position to explain an uncertainty derived from TDF', because the $\delta^{15}N_{AAs}$ values mirror the sum of isotopic discrimination (TDF + TDF') associated with the catabolically energy release within organisms but never mirror a count of grazing process (i.e., primary producers, herbivores, omnivores, carnivores, etc.) in food webs. Indeed, the flowers and flush leaves from the *C. lannesiana* return the mean $TP_{Glu/Phe}$ value of 2.2 and 1.3, respectively, as the trophic position in the energetic hierarchy of food webs, although such values are typical of omnivores ($TP > 2.0$) in the functional hierarchy of food webs.

Based on the present results, we predict that catabolically-released energy from storage compounds and photosynthetically-fixed energy are both important as the energy

supply against temporal and functional diversity in the energy demand for plant phenology. In other words, our results imply that plants are always composed of large-sized storage compounds for adjusting the energy balance between supply and demand in phenology (Fig. 2-3-3), as similar to the requirement of huge-sized storage battery in the full cycle of solar-power (photovoltaic) generation system.

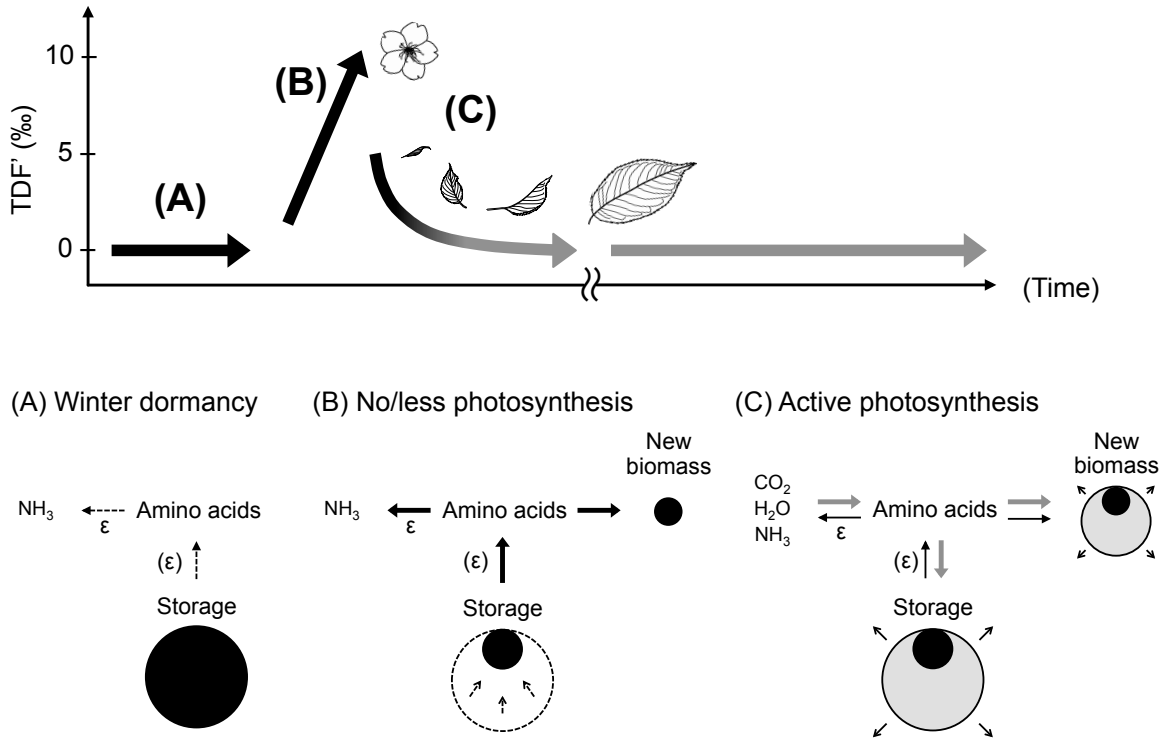

Fig. 2-3-3.

Schematic illustration of the TDF' correlated to the metabolic flow with respect to the anabolism (i.e., production) and catabolism (i.e., consumption) of storage amino acids in plant phenology. (A): substantially zero TDF' of the storage amino acids (filled circle) is found in winter dormancy; (B): the storages are catabolized to release growth energy, resulting in high TDF' on amino acids in floral and foliar organs in growing buds; (C): the high TDF' in foliar organs (filled circle) is diluted with zero TDF' of the newly-produced amino acids (gray circle) along the activation of photosynthesis in flush leaves.

4. Conclusion

We determined the $\delta^{15}N_{AAs}$ values in leaves for the deciduous plant *C. lannesiana*, and found that TDF' is 0.1±1.0‰ for leaf senescence from spring to autumn, whereas that is gradually decreased from 5.3‰ to 0.9‰ for leaf flush in early spring. This implies that plants can use sufficient photosynthetically-fixed energy for the leaf senescence, but use a large amount of catabolically-released energy (from deamination of storage amino acids) for the leaf flush. Based on these results, we predict that the energy balance can be considered in the isotope ecology: although the impact of TDF' in flowers to green food web studies may be limited, that in flush leaves to the food web studies would be extended farther than that in flowers. The investigation of amino acid isotopes in plants can trace both flux of energy and fate of organic compounds in plant phenology, and assess the functional importance of storage amino acids in plants.

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(The chapter III starts from next page.)

CHAPTER III.

The Consumption of Storage Lipids in the Plant Phenology, based on Compound- and Position-specific Stable Isotope Analysis of Carbon within Fatty Acids

Introduction

As the observation in the nitrogen isotopic composition of amino acids with respect to the HP on the utilization of storage protein (Chapter II), it is thought that other organic storages such as fat (i.e. lipids) and carbohydrates (i.e. sugars) are also employed as energy and organic substrate resources for the HP in plants under no/less photosynthetic activity. In such storage utilization, any biochemical elements (e.g. hydrogen, carbon, nitrogen, and oxygen) can be involved either forming or cleaving of chemical bonds in 'key process' responsible for the isotopic discrimination. Since lipids are major storages as important as amino acids in both plants and animals, knowing of metabolic flux of lipids on the HP is probably contributable for better understanding the whole cycle of energy and organic compounds in organisms' physiology. In this chapter, we have applied a standard compound-specific isotope method (e.g., Sessions 2006) for measuring the isotopic compositions of the whole carbon and hydrogen of palmitic acid to identify the isotopic discrimination with respect to HP from storage lipids in plants, and developed a position-specific isotope method for measuring the isotopic composition of the carboxyl carbon in fatty acids to ascertain the key process responsible for isotopic fractionation (i.e. hydrolysis of triacylglycerol) and to attempt quantifying the isotopic discrimination directly on the functional position of carbon atom in fatty acids.

The CSIA of carbon and hydrogen for lipids such as fatty acids and sterols has widely been employed in geochemistry to characterize sources and delivery of organic compounds and their elements in biogeochemical cycles, and to illustrate environments where, when, and/or how the lipids were produced (e.g., Naraoka and Ishiwatari, 1999; Huang et al., 2002; Chikaraishi and Naraoka, 2005). However, isotopic discrimination associated with the

utilization of storage lipids has poorly studied so far, mainly because there is no method, no methodology, and no evidence enough to discuss the isotopic discrimination in organisms due to the following two methodological concerns. Firstly, unlike the $\Delta\delta^{15}N_{key}$ values of amino acids, isotopic discrimination on the carbon (or hydrogen) atom ($\Delta\delta^{13}C_{key}$), that involved either forming or cleaving of chemical bonds in 'key process' responsible for the isotopic discrimination, within a single compound is generally much diluted with a number of other carbon (or hydrogen) atoms ($\Delta\delta^{13}C_{other}$) that has no isotopic discrimination in the process (equation 3-0-1).

$$n \times \Delta\delta^{13}C_m = 1 \times \Delta\delta^{13}C_{key} + (n-1) \times \Delta\delta^{13}C_{other} \qquad (eq.3-0-1)$$

where $\Delta\delta^{13}C_m$ indicates change in the measured $\Delta\delta^{13}C$ value of compounds between before and after reaction. If the $\delta^{13}C_{other}$ value is no change ($\Delta\delta^{13}C_{other} = 0$) in the key process, the equation (3-0-1) is given by:

$$\Delta\delta^{13}C_m = \frac{1}{n}\Delta\delta^{13}C_{key} \qquad (eq.3-0-2)$$

meaning that, for example, the $\Delta\delta^{13}C_m$ values are unfortunately considerably small, 0.5‰ for palmitic acid (n=16) and 0.3‰ for cholesterol (n=27), if the $\Delta\delta^{13}C_{key}$ values are even 8.0‰ (as large as the isotopic discrimination of glutamic acid) for both compounds. Indeed, it has long been thought that isotopic discrimination associated with the degradation of large carbon numbered organic compounds is negligible commonly in biological and geochemical samples. Secondly, there is no method for measuring the isotopic discrimination of a single carbon (or hydrogen) atom ($\Delta\delta^{13}C_{key}$), that involved either forming or cleaving of chemical bonds in 'key process' responsible for the isotopic discrimination, within a single compound. A standard CSIA can only access an average $\delta^{13}C$ values of whole carbon atoms (or δD values of

whole hydrogen atoms) in each compound, but have never been accessible the $\delta^{13}C$ value of single carbon atom (or δD values of single hydrogen atom) within a compound, particularly for large carbon numbered (Cn > 3).

Against these two methodological concerns, in this chapter, we have designed the following two experiments: (1) to find measurable $\Delta\delta^{13}C$ and $\Delta\delta D$ values of palmitic acid (even much diluted) during sesame seed sprouting where a large portion of storage lipids is consumed, as a evidence for the HP on the utilization of storage lipids; and (2) to develop a method for measuring the $\Delta\delta^{13}C_{key}$ value on a single carbon atom (i.e. carboxyl carbon) within a fatty acid during the seed sprouting. Based on these experiments, we would like to contribute to further refinement of our knowledge about change in the $\delta^{13}C$ and δD values of lipids during the HP in organisms as well as of lipid biomarkers during burial process into sediments.

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3-1. Enrichment in ^{13}C of fatty acids for sesame seed sprout

Abstract

Isotopic discrimination associated with fat (or lipid) metabolism within a single organism, between organisms, or environments (e.g., water column and sediments) has been poorly understood so far, even though fat is employed as one of major energy and substrate resources of organisms' growth. In the present study, we investigated the change in molecular abundance and carbon and hydrogen isotopic compositions of palmitic acid, a major lipid component, during the germination of sesame seeds under dark condition, to find numerous isotopic discrimination associated with storage fat consumption. The molecular abundance of palmitidc acid is decreased by 51%, which is accompanied by increasing considerably in the carbon isotopic composition from −34.0 to −31.1‰ but negligibly in the hydrogen isotopic composition −189 to −191‰, respectively. These results indicate that a reaction to a carbon atom within fatty acids is 'key' process of isotopic discrimination associated with storage fat consumption. As a possible key process, we suggest the enzymatic hydrolysis of triacylglycerol (TAG), which reacts preferentially ^{12}C carboxyl carbon of fatty acids (that esterified to glycerol), leaving behind the enriched ^{13}C in the residual pool of fatty acids (as TAG form) in storage fat of seeds. Carbon isotopic discrimination calculated is 48‰ for the carboxyl carbon of palmitic acid, if the enzymatic hydrolysis is responsible for the observed change in carbon isotopic composition of fatty acids during the germination.

1. Introduction

As illustrated by changes in the $\delta^{15}N_{AA}$ value of plant organs with respect to the HP on the utilization of storage protein (Chapter II), other organic storages such as fat (i.e. lipids) and carbohydrates (i.e. sugars) are potentially employed as energy and organic substrate resources for the HP in plants under no/less photosynthetic activity (e.g., Takizawa et al., 2017). Particularly it is thought that fat is usually found in many organisms not only plants but also animals, and that it is used to adapt the environmental fluctuation (e.g., low temperature, little resources) and its associated phenological events (e.g., dormancy). For example, triacylglycerol (TAG: TAG consist of three fatty acids esterified to a glycerol back-bone) is major component of storage fat, and have function as an efficient source of carbon and energy for germination of seeds of plants (e.g., Buchanan et al., 2000). During germination of seeds, a large proportion of the TAG stored in seeds is broken down to fatty acid and glycerol units and is finally incorporated into the central pathway to supply energy and substrate resources for the re-synthesis of other components such as carbohydrates and protein in plant growth (Fig. 3-1-1). In the utilization of TAG, the isotopic composition of carbon and/or hydrogen is potentially detectable if the elements is involved either forming or cleaving of chemical bonds in 'key process' responsible for the isotopic discrimination.

In this study, we designed laboratory pot experiments of sesame seed germination to find measurable $\Delta\delta^{13}C$ and $\Delta\delta D$ values of palmitic acid (even much diluted) in plants as a evidence for the HP on the utilization of storage fat, with the following three assumptions:

(1) the utilization of TAG results in carbon and/or hydrogen isotopic discrimination ($\Delta\delta^{13}C$

and/or $\Delta\delta$D, respectively) of fatty acids;

(2) the $\Delta\delta^{13}$C and/or $\Delta\delta$D values will be significant enough to measure even much diluted, when large proportion of TAG is utilized;

(3) seeds of the sesame *Sesamum indicum* contains considerable abundance of TAG, which is likely employed abundantly as almost solo energy and substrate resources for germination.

Based on the observed results, we discuss possible mechanism of the isotopic discrimination associated with the utilization of storage fat in plants.

2. Materials and Methods

Seeds of the sesame *Sesamum indicum* were germinated in a pot under dark condition for 5 days. Seeds (t=0) and the sprouts (t=5) were stored at −20°C until analysis. Palmitic acid was analyzed according to a modified procedure in Chikaraishi and Naraoka (2005). In brief, the frozen sample was saponified with KOH in CH_3OH/H_2O (95/5 w/w) to hydrolyze ester-bonds. Acidic lipids (including *n*-fatty acids) were then extracted with *n*-hexane/acetic acetate (4/1, v/v) after addition of 12M HCl (to pH<1). The acidic lipids were esterified with HCl in CH_3OH to form methylesters. Palmitic acid was identified by gas chromatography/mass spectrometry (GC/MS) using an 7890A GC instrument connected to an 5975C MS instrument (Agilent Technologies, Palo Alto, CA).

The carbon and hydrogen isotopic compositions of palmitic acid were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using an Agilent Tech-

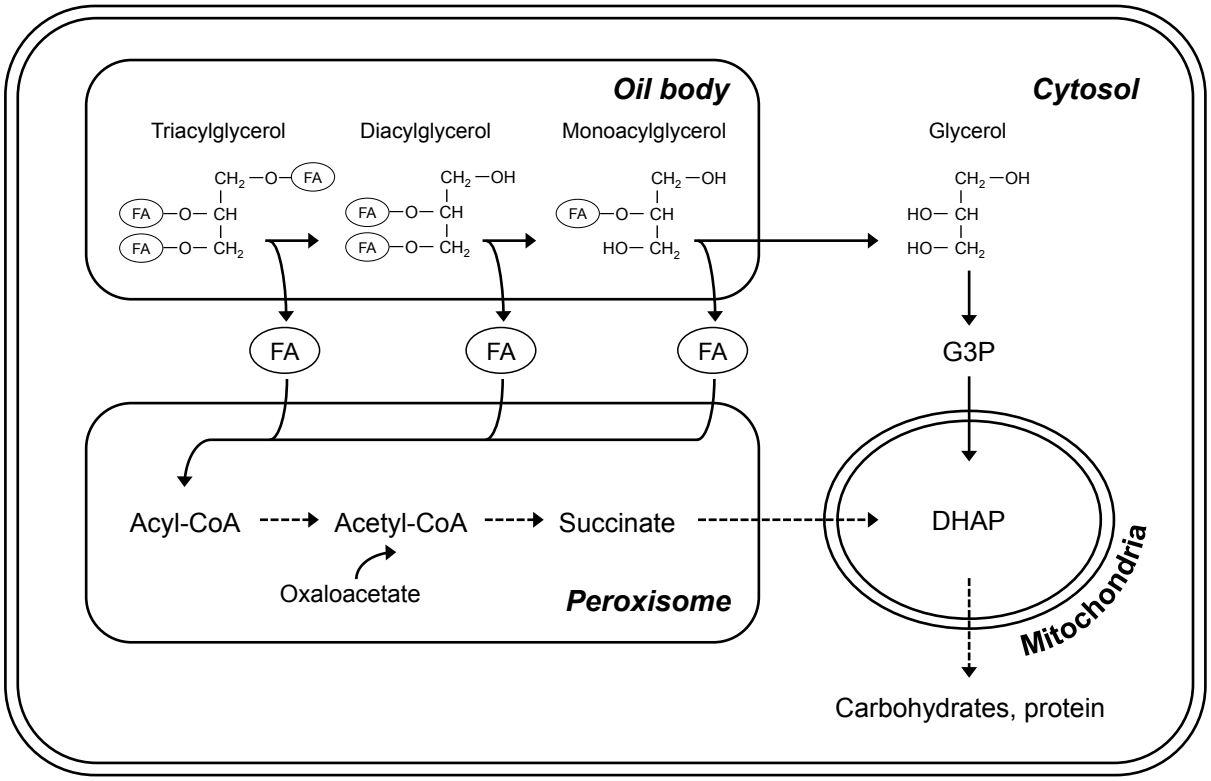

Fig. 3-1-1.

Triacylglycerol (TAG) stored in oil bodies is hydrolyzed sequentially with lipases to fatty acids (FAs) and glycerol. The glycerol is converted to dihydroxyacetone phosphate (DHAP) with glycerol kinase *via* Gly-3-phosphate dehydrogenase. The FAs are transported into the peroxisome, where they are activated into acyl-CoAs, and enter the *β*-oxidation spiral. Acetyl-CoA condenses with oxaloacetate to form succinate. The succinate and DHAP are converted to other components such as carbohydrates and protein (After Quttier and Eastmond, 2009; Barros et al., 2010).

nologies 7890N GC coupled to a Thermo Fisher Scientific Delta^{plus}XP IRMS with a GC-Isolink interface, with a combustion temperature of 1020°C for carbon and a pyrolysis temperature of 1450°C for hydrogen. The palmitic acid methylester was injected by using a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany) into an HP-5ms capillary column (30 m; i.d. 0.25 mm; film thickness 0.25 μm; Agilent Technologies). To assess the reproducibility of isotope measurements and to obtain the δ values in samples, reference mixtures of 14 *n*-alkanes (C_{18}–C_{36}) having known $\delta^{13}C$ and δD values were analyzed several times prior to sample runs. Two pulses of reference CO_2 or H_2 gas were discharged into the IRMS at the commencement and completion of each chromatography run, for both reference mixtures and samples. The isotopic compositions are expressed relative to Vienna Peedee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW), on scales normalized to the known δ values of the reference *n*-alkanes. The standard deviation of measurements for the reference *n*-alkanes was less than 1.2‰ for carbon and 3‰ for hydrogen.

3. Results and discussion

3.1. $\delta^{13}C$ and δD of palmitic acid in sesame seeds and sprouts

The molecular abundance of palmitic acid is decreased by 51% from seeds (t=0) to sprouts (t=5) for the germination (Fig. 3-1-2A). These results are consistent with a general knowledge that seeds indeed contain considerable amount of TAG, and that a large propor-

tion of the TAG in storage fat is utilized as major energy and organic substrate resources for the growth of sprouts (e.g., Buchanan et al., 2000).

The isotopic composition of palmitic acid is increased by considerably from −34.0 to −31.1‰ for carbon but negligibly from −189 to −191‰ for hydrogen in the germination (Fig. 3-1-2B, C). Thus, we can find measurable $\Delta\delta^{13}C$ values but immeasurable $\Delta\delta D$ values of fatty acids attributable to the HP on the utilization of storage fat, even though it is thought that changes in the $\delta^{13}C$ value on a carbon atom within organic compounds is much diluted with a number of other carbon atoms in the compounds (which have no change in the $\delta^{13}C$ values) in the key process responsible for the isotopic discrimination. In other words, for the

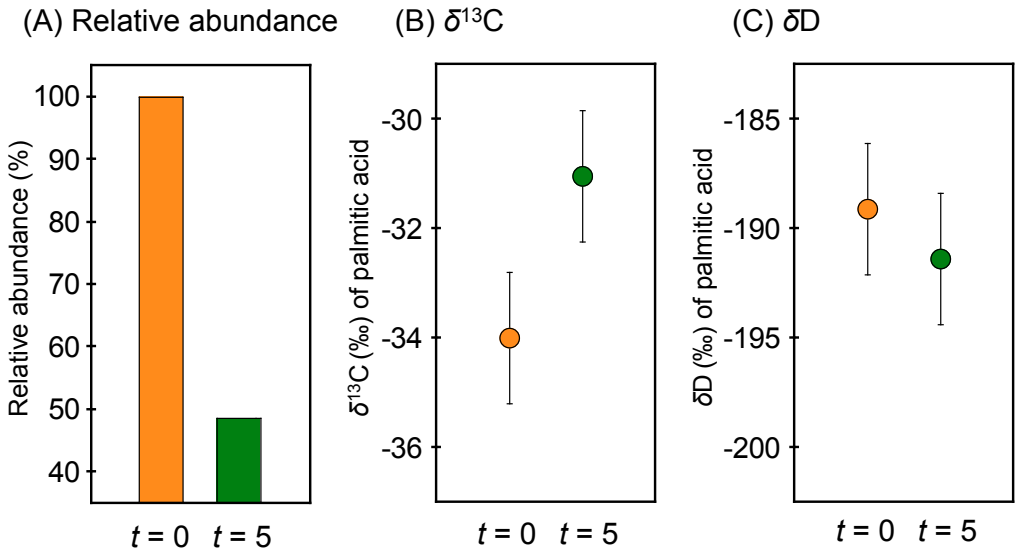

Fig. 3-1-2.
(A) Abundance, (B) the $\delta^{13}C$ values, and (C) the δD values of palmitic acid from seeds (t=0, indicated in orange color) and sprouts (t=5, indicated in green color). Error bars indicate the standard deviation (1σ) of the isotope measurements.

sesame germination, the isotopic discrimination of carbon isotopes on the single carbon atom is considerably large (e.g., > 10‰) compared to that in our general knowledge.

3.2. Possible mechanism for the enrichment in ^{13}C

TAG in storage fat is hydrolyzed sequentially with lipases to fatty acids (FAs) and glycerol (Fig. 3-1-1), where the first step of hydrolysis may forms a chemical bond between a functional carbon and/or hydrogen of TAG and lipase enzyme. Since the isotopic discrimination on the fatty acids is found for carbon but not hydrogen in this study, 'the connection of enzyme to a carbon atom within fatty acid components in TAG' is probably 'key' process of isotopic discrimination associated with storage fat consumption. Based on these results, as a possible key process, we suggest that the enzymatic hydrolysis of TAG reacts preferentially ^{12}C carboxyl carbon of fatty acid components, leaving behind the enriched ^{13}C in the residual pool of fatty acid components in storage fat of seeds. Because the depleted ^{13}C in hydrolysis products (i.e., fatty acid derivatives such as acyl-CoA) is subsequently transported into metabolic intermediates (e.g., acetyl-CoA and succinate), we can find the enrichment in ^{13}C on fatty acids in the saponified products of samples (i.e., t=5 sprouts). Carbon isotopic discrimination calculated by mass balance is 48 ± 27‰ for the carboxyl carbon of palmitic acid, if the enzymatic hydrolysis is responsible for the observed change in carbon isotopic composition of fatty acids during the germination.

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3-2. A position-specific isotope method for measuring the $\delta^{13}C$ values of carboxyl carbon in fatty acids

Abstract

Stable isotopic compositions of organic compounds have widely been employed as a potential powerful tool in the study for tracing sources and delivery of organic compounds in the earth surface and for reconstructing environments in the geological timescales. Organic compounds, however, continuously play in the physiological energy cycle including production and degradation of the compounds even during transfer and burial processes, which are potentially recorded in their isotopic compositions. In the present study, we have developed a position-specific method for measuring heterogeneity in the carbon isotopic composition within fatty acid components of triacylglycerol (TAG), to understand the isotopic discrimination associated with the utilization of storage fat (i.e., TAG) in plants, as well as of fatty acids found in environmental samples. The results indicate that the isotopic composition of carboxyl carbon in fatty acid components can be measured with a GC-IRMS coupled to a pyrolysis module (1100°C), and found that the carboxyl carbon is considerably enriched in ^{13}C by more than 60‰ if 50% of TAG is hydrolyzed for the germination of sesame seeds under dark condition. Thus, we succeeded to access the isotopic composition directly for carboxyl carbon atom and indirectly for alkyl-chain carbon atoms within a single fatty acid, which hopefully allows us to contribute better understanding the balance between production and degradation of fatty acids within a single organism as well as in environments.

1. Introduction

Degradation of organic products (e.g., protein, fat, and carbohydrates) is an essential process to obtain metabolic energy in all organisms during many biogeochemical processes (e.g., transfer, precipitation, and burial) in the earth surface. Moreover, knowing the balance between production (biosynthesis) and degradation (metabolism) is important to understand the physiological energy cycle within a single organism as well as in turn processes in the biogeochemical cycles. Indeed, we reported the detectable carbon isotopic discrimination by 2.9‰ for fatty acid components in triacylglycerol (TAG) that correlates to 50% of storage fat being utilized in sesame seeds (Section3-1). For this case, we suggested an isotopic discrimination mechanism that the enzymatic hydrolysis of TAG reacts preferentially ^{12}C carboxyl carbon of fatty acid components (Fig. 3-2-1A), leaving behind the enriched ^{13}C in the residual pool of the components in storage fat (Fig. 3-2-1B). The degradation ratio thus ideally correlates to difference in the isotopic composition between carboxyl and alkyl-chain carbon atoms ($\delta^{13}C_{COOH}$ and $\delta^{13}C_{alkyl}$, respectively) within a fatty acid ($\delta^{13}C_{FA}$) (Fig. 3-2-2). The mass balance for the fatty acid with *n* carbon number is given by:

$$n \times \delta^{13}C_{FA} = (n-1) \times \delta^{13}C_{alkyl} + \delta^{13}C_{COOH} \qquad (eq.\ 3-2-1)$$

Carbon isotopic discrimination calculated is 48 ± 27‰ for the carboxyl carbon of palmitic acid, if the enzymatic hydrolysis is responsible for the observed change in the $\delta^{13}C_{FA}$ value (by 2.9‰) during the germination of sesame seeds (Section 3-1). On the other hand, no substantial change in the $\delta^{13}C_{alkyl}$ value is likely found in the enzymatic hydrolysis, implying that

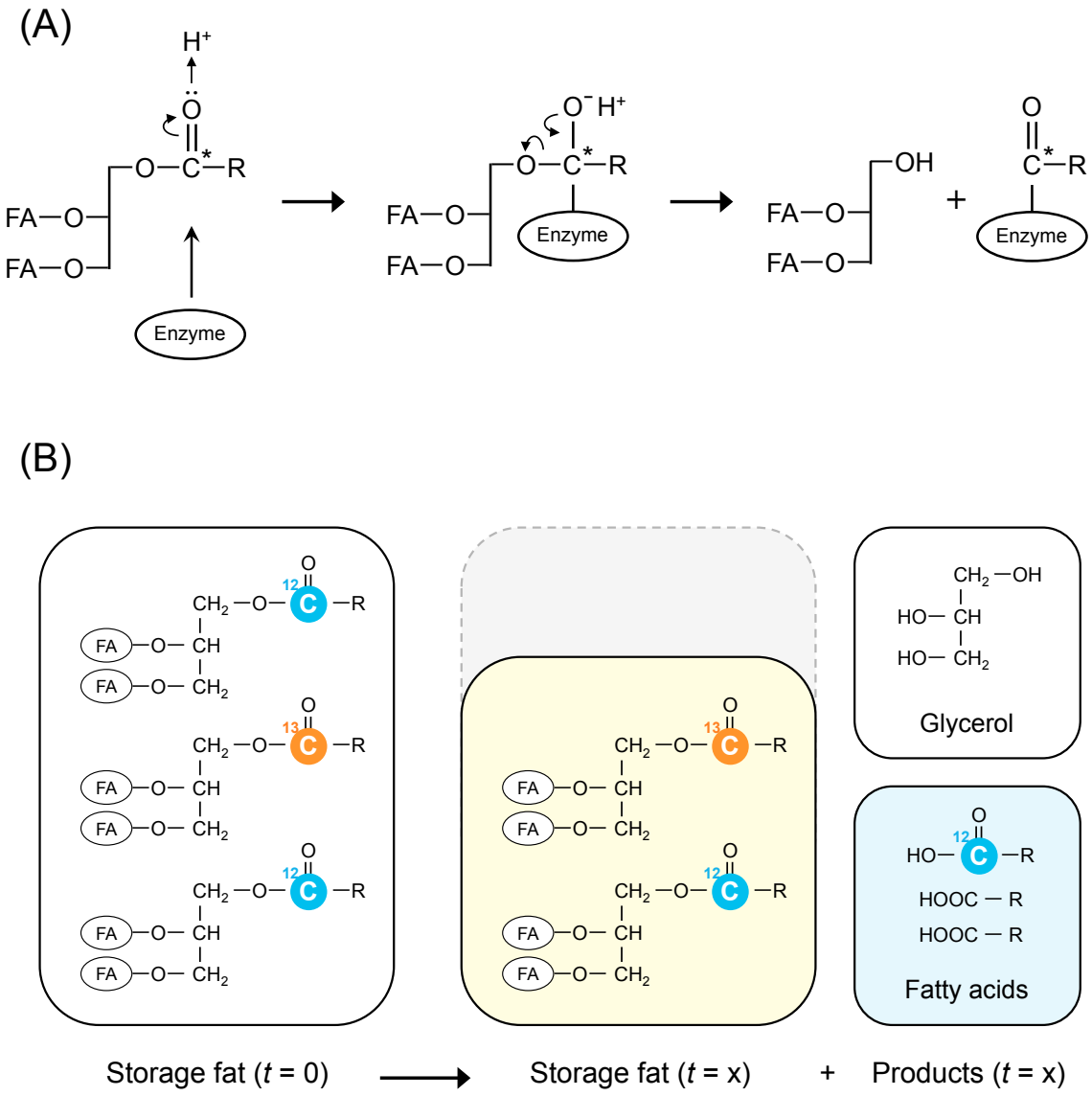

Fig. 3-2-1.

(A) Reaction mechanism of enzymatic hydrolysis of TAG. Asterisk indicate atom where isotopic discrimination occurs. (B) Schematic view of enzymatic hydrolysis of TAG with respect to the discrimination of $^{13}C/^{12}C$: ^{12}C carboxyl carbon of fatty acid components in storage fat (t=0) is preferentially reacted, resulting in increase in $^{13}C/^{12}C$ of carboxyl carbon in residual pool of storage fat (t=x).

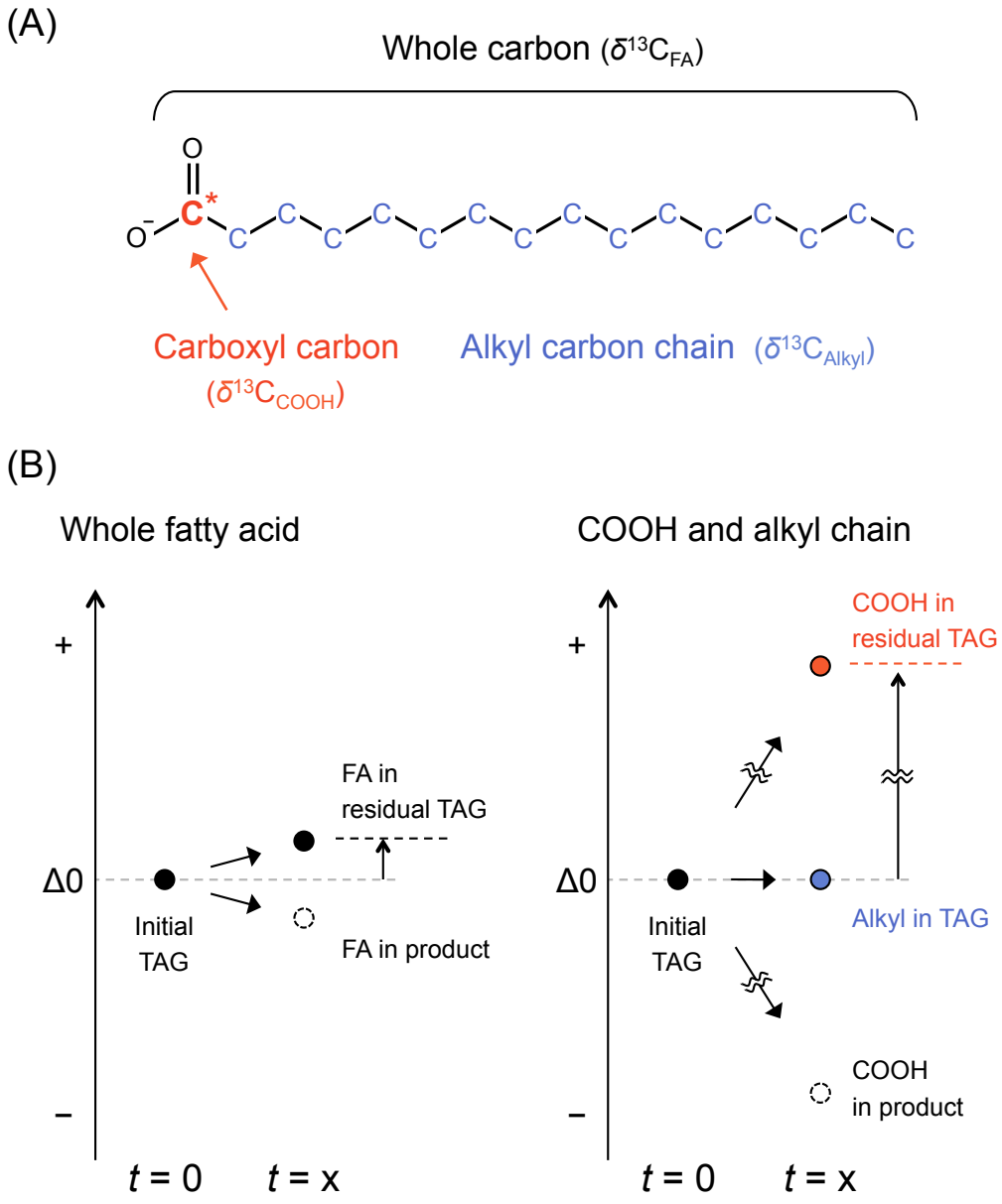

Fig. 3-2-2.

(A) Isotopic heterogeneity in the $\delta^{13}C$ value for carboxyl and alkyl chain carbons in a single fatty acid, which is caused by utilization of TAG: (B) the $\delta^{13}C$ values are increased slightly for an average of whole carbons, considerably for carboxyl carbon, and negligibly for alkyl-chain carbons of a fatty acid components in residual pool of TAG after the enzymatic hydrolysis.

the $\delta^{13}C_{alkyl}$ values have well preserved the environment where the fatty acid was produced. However, to our knowledge, no method is available for measuring the $\delta^{13}C_{COOH}$ and $\delta^{13}C_{alkyl}$ values within a single fatty acid.

In this study, we have developed a position-specific method for measuring the $\delta^{13}C_{COOH}$ values of fatty acids to proof the considerable enrichment in ^{13}C on carboxyl carbon of fatty acid components observed in storage fat during the germination of sesame seeds. For the development, we designed a GC-IRMS coupled to a pyrolysis module (Fig. 3-2-3), according to a modified GC-IRMS instrument for position-specific isotope analysis of acetic acid (Yamada et al., 2002) and a modified TCEA-IRMS instrument for the $\delta^{13}C_{COOH}$ analysis of aromatic carboxylic acids (Oba and Naraoka, 2006). The whole carbon atoms in fatty acids are oxidized to CO_2 in the traditional GC-IRMS through a micro ceramic combustion furnace with CuO, NiO, and Pt wires at 1000°C (Fig 3-2-3A). In contrast, the carboxyl carbon in fatty acids is decarboxylated and released as CO_2 in the designed GC-IRMS through a micro ceramic pyrolysis furnace without any reagents and catalysts at 1100°C (Fig 3-2-3B).

2. Materials and methods

2-1. Samples and authentic standard

Seeds of the sesame *Sesamum indicum* were germinated in a pot under dark condition for 5 days, and palmitic acid was extracted from the seeds (t=0) and the sprouts (t=5) (Section 3-1). The palmitic acid was esterified with HCl in CH_3OH to form methylesters. An

authentic standard of palmitic acid metylester was purchased from Wako Pure Chemical Industries Ltd.

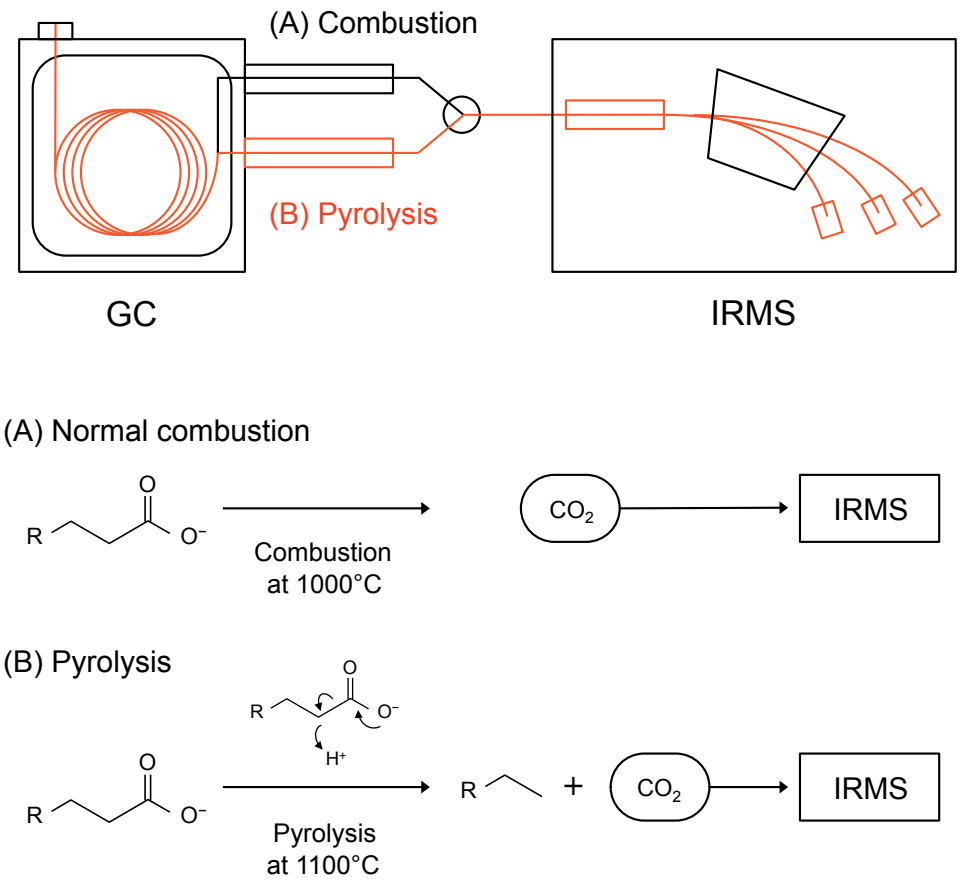

Fig. 3-2-3.

Schematic of GC-IRMS used in this study: (A) combustion at 1000°C converts whole carbon atoms of a fatty acid to CO_2, and (B) pyrolysis at 1100°C releases carboxyl carbon atom of a fatty acid to CO_2.

2-2. Position-specific carbon isotope analysis

The carbon isotopic composition of carboxyl carbon in palmitic acid was determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using an Agilent Technologies 6890N GC coupled to a Thermo Fisher Scientific Delta^{plus}XP IRMS with a Combustion III interface, with a pyrolysis temperature of 1100°C. The palmitic acid was injected by using a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany) into an HP Ultra-2 capillary column (50 m; i.d. 0.32 mm; film thickness 0.52 μm; Agilent Technologies). In this condition, the carboxyl carbon in palmitic acid was thermally decarboxylated, released as CO_2, introduced to the IRMS (Fig 3-2-4). The isotopic composition is reported relative to reference CO_2 gas but, at this moment, is not normalized to scale on multiple international reference materials.

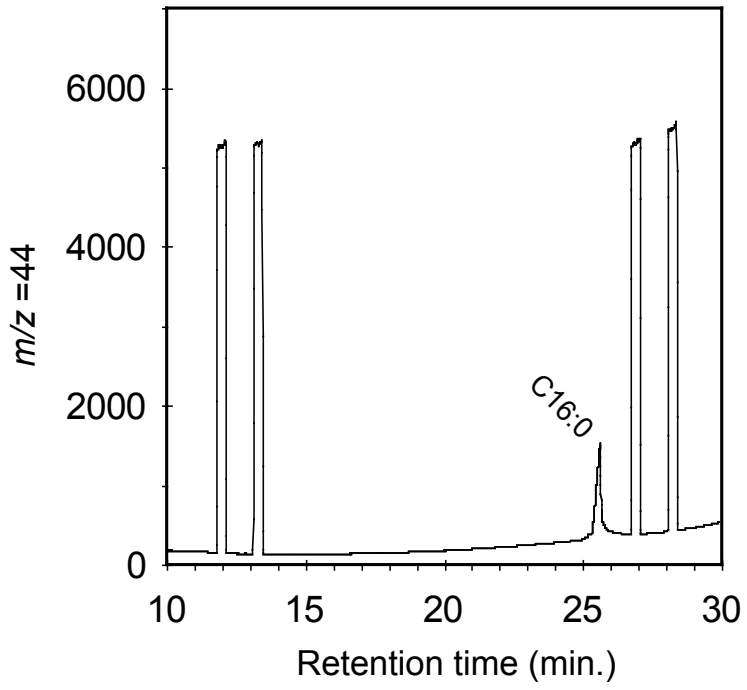

Fig. 3-2-4.
A representative *m/z* 44 chromatogram of the modified GC-IRMS. The CO_2 peak is derived from the carboxyl carbon of palmitic acid.

3. Results and discussion

3.1. Heterogeneity in the $\delta^{13}C$ value within palmitic acid

As mentioned above, carbon isotopic composition of carboxyl carbon was reported by ‰ relative to that of laboratory reference CO_2 gas, but not to that of international standard because no standard is available at this moment for the position-specific isotope analysis. Accordingly, in this study, we cannot use the eq. 3-2-1 to compare the observed $\delta^{13}C_{COOH}$ values with the $\delta^{13}C_{alkyl}$ and $\delta^{13}C_{FA}$ values.

By using the modified GC-IRMS, CO_2 derived from carboxyl carbon in palmitic acid is successfully found as a single peak on the chromatogram for both t=0 seeds (Fig. 3-2-5A) and t=5 sprouts (Fig. 3-2-5B) of the sesame. The isotopic composition ($\delta^{13}C$, ‰ relative to reference CO_2 gas) of carboxyl carbon in palmitic acid components is −52‰ for the t=0 seeds and +11‰ for the t=5 sprouts (Fig. 3-2-5C). Such enrichment in ^{13}C by 63‰ from seeds to sprouts is considerably large compared to the isotopic discrimination generally found in primary producers including plants (e.g., Farquhar et al., 1980, 1989), but is likely consistent with the enrichment in ^{13}C (isotopic discrimination = 48 ± 27‰) speculated in the isotope analysis for whole carbon atoms of palmitic acid from the same samples (Section 3-1). Thus, we succeeded to access the isotopic composition (even though ‰ relative to reference CO_2 gas) directly for carboxyl carbon atom within a single fatty acid, and proof a considerable isotopic discrimination on the carboxyl carbon associated with the enzymatic hydrolysis of TAG.

3.2. Possible contribution

Although we succeeded to access the isotopic composition directly for carboxyl carbon atom within a single fatty acid in this study, the analytical method used is still in very-early development stage. We need to optimize many factors including GC column, oven program, He flow rate, pyrolysis temperature, injection volume, derivatization, etc. to apply this method widely to biogeochemical issues. However, we expect that this position-specific isotope method will be a essential tool to quantify the degradation of fatty acids and their derivatives (e.g., fatty acid esters including TAG) in both physiological and biogeochemical cycles. The quantification of such isotopic heterogeneity within a single fatty acid will allow us to contribute better understanding the balance between production and degradation of fatty acids within a single organism as well as in environments and to extract the isotopic composition of initial products without any degradation even from high-variably degraded samples such as soil and sedimentary records.

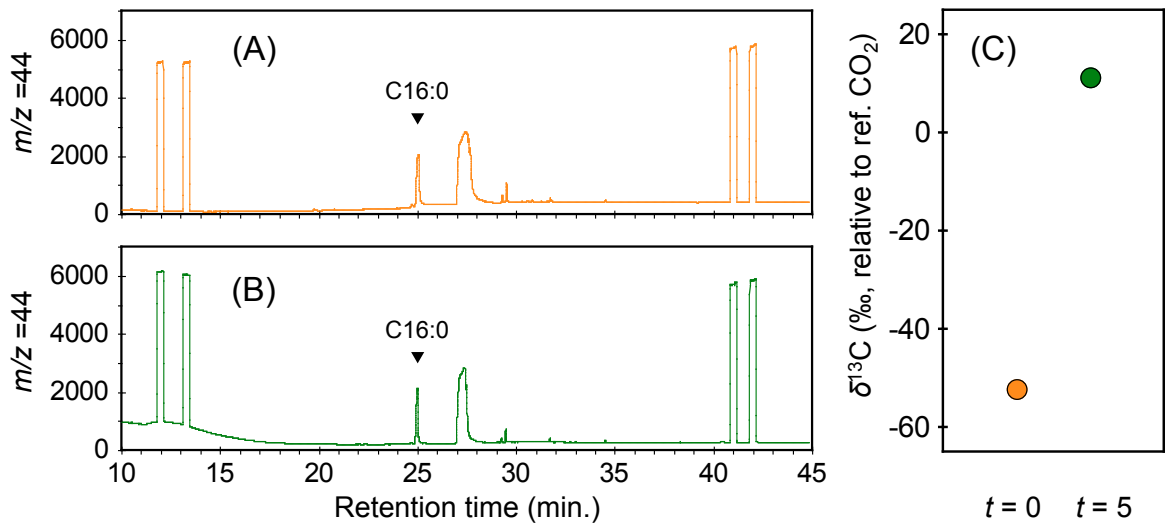

Fig. 3-2-5.

m/z 44 chromatograms of the modified GC-IRMS for fatty acid components of TAG: (A) t=0 seeds and (B) t=5 sprouts. (C) The carbon isotopic composition (‰ relative to reference CO_2 gas) of the seeds and sprouts, determined by the modified GC-IRMS.

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(The chapter IV starts from next page.)

CHAPTER IV.

General Conclusions

In this study, to evaluate the effect of heterotrophic production (HP) on the isotopic composition of organic compound, we investigated the isotopic fractionation and its mechanisms for organic compounds in plants with respect to consumptions/utilizations of storage materials such as protein and lipids. For the protein consumption, we illuminate that:

1. the enrichment in ^{15}N slightly for amino acids in mother sweet potato and considerably in its sprouts growing under dark condition reveals a first data that storage protein will be metabolized to produce maintenance and growth energy for overwintering and sprouting (Section 2-1);
2. a large enrichment in ^{15}N of amino acids recorded in deciduous plant flowers that bloom before leafing reveals that the rational consumption and utilization of storage protein are universally found in plant phenology (Section 2-2);
3. the enrichment in ^{15}N of amino acids is rapidly decreased from significantly large for flush to substantially zero for senescence leaves, which reveals a gradual change in the energy flow from catabolically-released to photosynthetically-fixed during the short term of leafing (Section 2-3).

For the lipid consumption, we first present that:

1. a small but significant enrichment in ^{13}C is found in fatty acids from sesame sprouts growing under dark condition, implying that the enzymatic hydrolysis of triacylglycerol (i.e., consumption of storage lipids) is a key reaction responsible for the isotopic fractionation on the fatty acids during germination of seeds (Section 3-1);
2. a position-specific isotope method established can access a huge enrichment in ^{13}C spe-

cifically on carboxyl carbon within the fatty acids of sesame seeds, which allows us to proof the utilization of storage lipids as major energy resource for the sprouting (Section 3-2).

Based on these results, we particularly proofed that, like heterotrophic animals, the HP on the utilization of storage materials such as protein and lipids is usually (or universally) found in plants if the plants need growth energy and substrates under no/less photosynthetic activity. Moreover, we further ascertain that the stable isotopic compositions of organic compounds record isotopic discrimination associated with 'key process' in not only biosynthesis (production) but also metabolism (degradation) regarding to the energy flux (i.e., input/output) in physiological cycles. In other word, we suggest that consumption of organic compounds (e.g., amino acids and fatty acids) is a essential factor correctly to interpret the isotopic composition of them in any natural samples including plants, animals, soils, sediments, etc. as mentioned by equation 1-2 in the general introduction. To quantify the consumption, a combination analysis of compound-specific isotope analysis of nitrogen within amino acids, which has been conventionally used in ecological studies, and position-specific isotope analysis of carboxyl carbon within a single fatty acid, which is newly established in this study (Section 3-2) will be highly useful.

APPENDIX

Introduction

In the main chapters, we demonstrate that isotopic discrimination associated with the consumption of storage materials is a factor to interpret possible variation in the isotopic composition of organic compounds found in environmental samples. For instance, nitrogen isotopic composition of amino acids in sprouts, flowers, and flush leaves record that plants can consume and utilize amino acids derived from storage protein as energy and substrate resources under no/less photosynthetic activities, and simultaneously that plants can re-organize fresh protein from residual pool of amino acids that enriched in ^{15}N (Chapter II). This phenomena is called 're-organization', a process of heterotrophic production, which is explained by the transport of a single amino acid from storage to the fresh protein. Thus, we discussed 'isotopic discrimination associated with re-organization' in Chapter II, as a solo factor controlling this possible variation in the isotopic composition of organic compounds found in environmental samples.

However, there is 're-synthesis' as the other factor potentially to contribute the possible variation in the isotopic composition of organic compounds (Table A-0-1), which is explained by transport of a compound (e.g., glucose) from storage (or substrate) to new products but as other compounds (e.g., alanine) *via* central biosynthetic and metabolic path-ways. For example, alanine enriched in ^{15}N is always found in re-organized protein (Chapter II), but may potentially not in re-synthesized protein.

The purpose of Appendix is to investigate the effect of re-synthesis on isotopic composition of amino acids in organisms. The results will be useful for better understanding of the balance between production and degradation at molecular level and the energy cycle within a single organism and among organisms in food webs.

Table A-0-1. A possible mechanism for isotopic discrimination during biosynthetic pathways with respect to the autotrophic and heterotrophic productions.

	Autotrophic	Heterotrophic	
Name	De-novo synthesis	Re-organization	Re-synthesis
Energy source	Light	Organic compounds	Organic compounds
Substrate source	Inorganic chemicals (δ_{IC})	A in Storage (δ_{AinS})	A and B in Storage (δ_{AinS}, δ_{BinS})
Metabolism (Degradation)	–	+	+
Isotopic discrimination (ε)	$\varepsilon_{Biosynthesis}$	ε_{MofA}	$\varepsilon_{MofA} + \varepsilon_{MofB} + \varepsilon_{BtoA}$
Pathway	CO_2 ↓ ↓ (directly) ↓ ↓ Organs	(CO_2) ↓ A in Storage ↓ A ↙ ↘ Organs CO_2	(CO_2) ↓ A in Storage ↓ A ↙ ↘ CO_2 Organs (CO_2) ↓ B in Storage ↓ B ← A ↙ ↘ Organs CO_2

A-1. A biosynthetic and metabolic perspective of the trophic discrimination of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ on amino acids in food webs

Abstract

Isotopic discrimination of carbon and nitrogen within amino acids between a consumer and its diet ($\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$, respectively) has recently been employed as a unified approach potentially to provide unprecedented accuracy and precision in our understanding of resource utilization and trophic connections among organisms in diverse ecosystems. However, a little is known the biosynthetic and metabolic factors responsible for the trophic isotopic discrimination (particularly for carbon) of amino acids in consumer species. In the present study, we determined the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values in four pairs of consumer–resource invertebrates: the sea slug *Hypselodoris festiva* – the sponge *Halichondria okadai*, and the ladybug beetle *Menochilus sexmaculatus* – the aphid *Aphidoidea* sp., collected from coastal and terrestrial environments, respectively; and the green lacewings *Chrysoperla rufilabris* – the fall armyworm *Spodoptera frugiperda*, and the green lacewings *C. rufilabris* – the green lacewings *C. rufilabris*, reared in laboratory controlled feeding experiments, to illustrate a biosynthetic and metabolic perspective of the trophic isotopic discrimination on amino acids. The $\Delta\delta^{15}N_{AA}$ values in the combinations are consistent with those in many other combinations reported in previous studies, further validating a standard scenario: the deamination reacts preferentially with ^{14}N amino group of diet-derived amino acids, leaving behind the enriched ^{15}N in the residual pool of amino acids in consumer biomass. The trophic isotopic discrimination of $^{15}N/^{14}N$ thus mirrors the metabolic activity of

amino acid deamination. On the other hand, the $\Delta\delta^{13}C_{AA}$ values have a trend far different from the $\Delta\delta^{15}N_{AA}$ values, which allows us to predict the following scenario: decarboxylation reacts preferentially with ^{12}C α-carbon (i.e., carbony-carbon) of pyruvate in the glycolysis and of α-ketoglutarate in the TCA cycle, leaving behind the enriched ^{13}C on these intermediates, and the enriched ^{13}C is propagated into amino acids via re-biosynthesis (or metabolic routing) in consumers. The trophic isotopic discrimination of $^{13}C/^{12}C$ thus can mirror the biosynthetic activity of amino acid production. The proposed bridged perspective between the trophic isotopic discrimination and its responsible process in the biosynthesis and metabolism of consumers will provide for further refinements of the observed change in the $\delta^{13}C$ and $\delta^{15}N$ values for bulk tissues as well as amino acids of wild organisms in ecological food webs.

1. Introduction

Organic compounds including amino acids have a large diversity in the stable isotopic compositions (e.g., $^{13}C/^{12}C$ and $^{15}N/^{14}N$), which is generally cased by isotopic discrimination associated with the isotope effect and branched flux on 'key' biochemical processes in biosynthetic and metabolic pathways of organisms (e.g., Hayes, 2001; Chikaraishi, 2014; Ohkouchi et al., 2015). An identification of the key process is thus indispensable for better understanding of 'universality vs. predictable fluctuation' on the isotopic composition and/or discrimination found in organisms, as well as for crediting of the accuracy and precision on the isotope evidence in application studies.

During food webs, it has very long been known that a large diversity in the isotopic discrimination of nitrogen within amino acids ($\Delta\delta^{15}N_{AAs}$) is observed in the trophic transfer from resource to consumer species (e.g., Gaebler et al., 1963, 1966; Hare et al., 1991; McClelland and Montoya, 2002; Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007). More recently, in late 2000's, these $\Delta\delta^{15}N_{AA}$ values have been interpreted by metabolic activities of the amino acid deamination in consumer species (Chikaraishi et al., 2007, 2015; Takizawa and Chikaraishi, 2014, 2017; Takizawa et al., 2017). For instance, dominant metabolism of certain amino acids (e.g., alanine, valine, isoleucine, proline and glutamic acid) starts deamination (preceding transamination), which includes the preferential cleavage of the ^{14}N amino group of diet-derived amino acids and prompts an accumulation of ^{15}N (by up to ~3-8‰ per trophic transfer) in the residual pool of amino acids in consumer biomass (Chikaraishi et al. 2007). On the other hand, deamination is a quite minor metabolism of other amino acids (e.g., methionine and phenylalanine), which is well consistent with negligible isotopic discrimination between consumer and resource species (Chikaraishi et al. 2007).

These two groups of amino acids are nowadays commonly called ‘trophic’ and ‘source’ amino acids, respectively (e.g., reviewed in Ohkouchi et al., 2017). With respect to this theory, the $\delta^{15}N$ analysis of amino acids has rapidly evolved into a unified approach potentially to provide unprecedented accuracy and precision in our understanding of resource utilization and trophic connections among organisms in diverse ecosystems: the $\delta^{15}N$ values of the source amino acids in organisms directly provide those in primary producers at the base of food webs, whereas difference in the $\Delta\delta^{15}N_{AA}$ value between trophic and source amino acids within a single organisms correlates with the trophic position of organisms in food webs (e.g., Chikaraishi et al., 2014; Choy et al., 2015; Steffan et al., 2015).

A large diversity in the carbon isotopic composition ($\delta^{13}C$) of amino acids within organisms has also very long been discussed in a number of studies (e.g., Abelson and Hoering 1961; Macko et al. 1987; Fantle et al. 1999; O’Brien et al., 2002; McCarthy et al., 2004; Larsen et al., 2009, 2013; McMahon et al., 2010, 2015). To our knowledge, it has been commonly reported that trophic isotopic discrimination of carbon within amino acids ($\Delta\delta^{13}C_{AA}$) is very small or negligible for aromatic (e.g., phenylalanine) and branched amino acids (e.g., valine, leucine, and isoleucine), but highly varies from −10‰ to +10‰ for other amino acids (e.g., glycine, alanine, aspartic acid, glutamic acid, and proline) (e.g., McMahon et al., 2010. 2015). Since these two types of amino acids are apparently consistent with a traditional ‘essential (or indispensable)’ and ‘non-essential (or dispensable)’ grouping on amino acids, respectively, these $\Delta\delta^{13}C_{AA}$ values have been simplistically understood to be that the negligibly small $\Delta\delta^{13}C_{AA}$ value for the former amino acids (i.e., essential ones) is attributable to no or little activity of *de novo* biosynthesis, whereas a large variation in the $\Delta\delta^{13}C_{AA}$ value for the latter amino acids (i.e., non-essential one) is attributable to great activity of *de novo*

biosynthesis and/or metabolic routing from other carbon pools from dietary protein, fat, and carbohydrates (PFC) in consumers (e.g., McMahon et al., 2010, 2015). Indeed, based on these findings, a principal component analysis of the essential amino acid $\delta^{13}C$ values has recently been employed as an unfungible powerful 'finger-printing' tool to trace carbon-flow from diverse resource end-members (e.g., phytoplankton, macroalgae, bacteria, and fungi) to consumer species through food webs (e.g., Larsen et al., 2009, 2013, 2015; McMahon et al., 2010, 2016). However, there remains unbridged knowledge between the observed $\Delta\delta^{13}C_{AA}$ values and their responsible 'key' processes in the biosynthetic and/or metabolic pathways, which strongly hinders the advance of application studies with the use of $\Delta\delta^{13}C_{AA}$ evidence.

Compound-specific isotope analysis (CSIA) of carbon and nitrogen within amino acids was rapidly distributed after commercial production of a gas chromatograph-isotope ratio mass spectrometer (GC-IRMS) in the 1990's, which has been carried out *via* derivatization to neutralizes polar carboxyl (-COOH), amino ($-NH_2$), and hydroxyl (-OH) groups in amino acids by less-polar moieties prior to the GC-IRMS analysis (e.g., Engel et al., 1990; Silfer et al., 1991; Demmelmair and Schmidt, 1993; Merritt and Hayes, 1994; Metges et al., 1996; Chikaraishi et al., 2010a). However, this method is still in the development stages particularly for the CSIA of carbon within amino acids (e.g., Metges and Daenzer, 2000; Docherty et al., 2001; Corr et al., 2007a, 2007b; Chikaraishi and Ohkouchi, 2010), because acylation (or acetylation) of the amino group of amino acids always accompanies with considerable degree of artificial isotopic fractionation on the carbonyl carbon of the acyl moiety in the derivatives (Silfer et al., 1991; Rieley, 1994; Chikaraishi and Ohkouchi, 2010). The magnitude of isotopic fractionation is indeed much variable depending on (1) the kinetic isotope effect specific to a given amino acid, (2) the molar balance between amino acids and derivat-

ization reagent in the derivatization, and (3) the contribution of matrix constituents (particularly as hydroxyl group content in there) in samples (e.g., Chikaraishi and Ohkouchi, 2010), which always leads to the grave risk of unreliable determination of the $\delta^{13}C_{AA}$ values with GC-IRMS analysis (e.g., Chikaraishi and Ohkouchi, 2010; Dunn et al., 2011). Although, the former two (i.e., isotope effect and molar balance) can be calculated and controlled (Corr et al., 2007a, 2007b), the control (or correction) of the isotopic fractionation derived from the third concern (i.e., contribution of matrix) is highly difficult in many cases (Chikaraishi and Ohkouchi, 2010). For example, there is a high dissimilarity in the PFC constituent between plant (i.e., cellulose-base) and herbivorous animal (i.e., protein-base) tissues, resulting in that the magnitude of the artificial isotopic fractionation is always considerably far different at the level of individual amino acids between these samples.

In the present study, we determined the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values in four pairs of consumer–resource invertebrates: sea slug–sponge and ladybug beetle–aphid collected from coastal marine (a stony shore) and terrestrial (a shrub) environments, respectively, and green lacewings–fall armyworm and green lacewings–green lacewings reared in laboratory controlled feeding experiments, to illustrate a biosynthetic and metabolic perspective on the trophic isotopic discrimination of amino acids during the grazing process in food webs. These pairs were designed as animal-animal combination within a single step of grazing food webs, because a predicted small difference in the molar balance of amino acid and matrix components within a pair can minimizes the difference in the artificial isotopic fractionation within a pair. In the other words, the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values of amino acids will be obtainable with minimum uncertainty in these samples, which will provide further refinements of the

trophic change in the $\delta^{13}C$ and $\delta^{15}N$ values for bulk tissues as well as amino acids of wild organisms in ecological food webs.

2. Materials and methods

The sea slug *Hypselodoris festiva* and the sponge *Halichondria okadai* were collected in May 2016 from a stony shore in Yugawara (35°080N, 139°070E), Japan. *H. festiva* is monophagous on the sponge *H. okadai* (e.g., Watanabe et al., 2009). Since *H. okadai* is found everywhere in this shore for all seasons, *H. festiva* could not starved of the diet throughout the year. This stony share was previously investigated the $\delta^{15}N$ values of amino acids from various free-roaming organisms, where a little variation in the baseline $\delta^{15}N$ value (i.e., 3.6-6.6‰ for phenylalanine) were observed (Chikaraishi et al., 2014). Larva of the ladybug beetle *Menochilus sexmaculatus* and the aphid *Aphidoidea* sp. were collected in September, 2014, from a shrub at a house-garden in Yugawara (35°080N, 139°070E), Japan. *M. sexmaculatus* is aphidophagous in both larvae and adult stages (e.g., Khan and Khan, 2002), and the larvae of M. *sexmaculatus* collected in the present study was able to feed only on a single species of the aphid *Aphidoidea* sp. within a small shrub habitat, where we found neither larvae and adult of parasitic wasps in and around aphids in the observation. Respective one individual of *H. festiva,* one portion (ca. 20 mm × 50 mm) of *H. okadai,* five individuals of M. *sexmaculatus*, and fifty individuals of *Aphidoidea* sp. were collected, dried, and homogenized for the analysis.

The controlled feeding experiments for the two combinations, the green lacewings *Chrysoperla rufilabris* – the fall armyworm *Spodoptera frugiperda*, and the green lacewings *C. rufilabris* – the green lacewings *C. rufilabris*, were conducted in Steffan et al. (2013).

Briefly, *S. rufilabris* larvae were incubated with a single homogeneous diet, and frozen to serve as prey for the *C. rufilabris* larvae. Then, the *C. rufilabris* larvae were frozen and used for prey for the *C. rufilabris* larvae. Respective four individuals were used for the analysis. The $\delta^{15}N$ values of amino acids were reported in Steffan et al. (2013).

These samples were prepared for the isotope analysis after HCl hydrolysis and *N*-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedure in Chikaraishi et al. (2009). In brief, the homogenized samples were hydrolyzed using 12M HCl at 110ºC overnight (>12 hours). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. The derivatization was performed sequentially with thionyl chloride/2-propanol (1/4, v/v) at 110ºC for 2 hours, and pivaloyl chloride/dichloromethane (1/4, v/v) at 110ºC for 2 hours. The $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using a 6890N GC (Agilent Technologies, Palo Alto, USA) instrument coupled to a Delta^{plus}XP IRMS instrument through combustion (950ºC) and reduction (550ºC) furnaces, a countercurrent dryer (Permeable membrane, Nafion™), and a liquid nitrogen CO_2 trap (only for the $\delta^{15}N_{AA}$ the analysis) *via* a GC-C/TC III interface (Thermo Fisher Scientific, Bremen, Germany). The Pv/iPr derivatives were injected using a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany) into an HP Ultra-2 capillary column (50 m; i.d. 0.32 mm; film thickness 0.52 μm; Agilent Technologies). The carrier gas (He) flow rate was maintained at 1.4 ml min^{-1}. To assess the reproducibility of the isotope measurement, a standard amino acid reference mixture (Indiana University, Bloomington, USA; SI science co., Sugito-machi, Japan) was analyzed after every five or six samples runs, with three pulses of reference CO_2 or N_2 gas discharged at the beginning and end, each run. The $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values were ex-

pressed relative to the isotopic composition of Vienna Peedee Belemnite (VPDB) and atmospheric nitrogen (AIR), respectively, on scales normalized to known δ values of the reference amino acids. The accuracy and precision for the reference mixtures were 0.0‰ (mean of Δ) and 0.7-1.4‰ (mean of 1σ) for the $\delta^{13}C_{AA}$ values, 0.0‰ (mean of Δ) and 0.3-0.6‰ (mean of 1σ) for the $\delta^{15}N_{AA}$ values, respectively.

For adjusting to the same size of artificial isotopic fractionation during derivatization within a single combination (i.e., to calculate the $\Delta\delta^{13}C_{AA}$ values), we prepared exactly the same weight (within 0.1-1.0 mg) of consumer and resource species for the derivatization, and confirmed that concentration and molar-balance of amino acids were varied by less than 10% within each combination (Fig. A-1-1). The mass balance for the $\delta^{13}C$ value of an amino acid (measured: $\delta^{13}C_m$ and original: $\delta^{13}C_o$) and the artificial isotopic fractionation (ε_a) is given by:

$$(n_i + n_d) \times \delta^{13}C_m = n_i \times \delta^{13}C_o + n_d \times \varepsilon_a \qquad (eq. A-1-1)$$

where n_i and n_d indicate the number of carbon atoms in an amino acid (e.g., 2 for glycine) and the derivative groups (8 for glycine Pv/iPr), respectively. Based on the negligible variation in the concentration and molar-balance of amino acids (Fig. A-1-1), the artificial isotopic fractionation (ε_a) is successfully cleansed in the $\Delta\delta^{13}C$ values between organisms A and B, given by:

$$\Delta\delta^{13}C_{A-B} = \left(\delta^{13}C_{o,A} - \delta^{13}C_{o,B}\right) = [(n_i + n_d)/ n_i] \times \left(\delta^{13}C_{m,A} - \delta^{13}C_{m,B}\right)$$

$$(eq. A-1-2)$$

The sea slug *H. festiva* – the sponge *H. okadai*, and the ladybug beetle *M. sexmaculatus* – the aphid *Aphidoidea* sp. were analyzed the $\delta^{13}C_{AA}$ and $\delta^{15}N_{AA}$ values by triplicate injections for

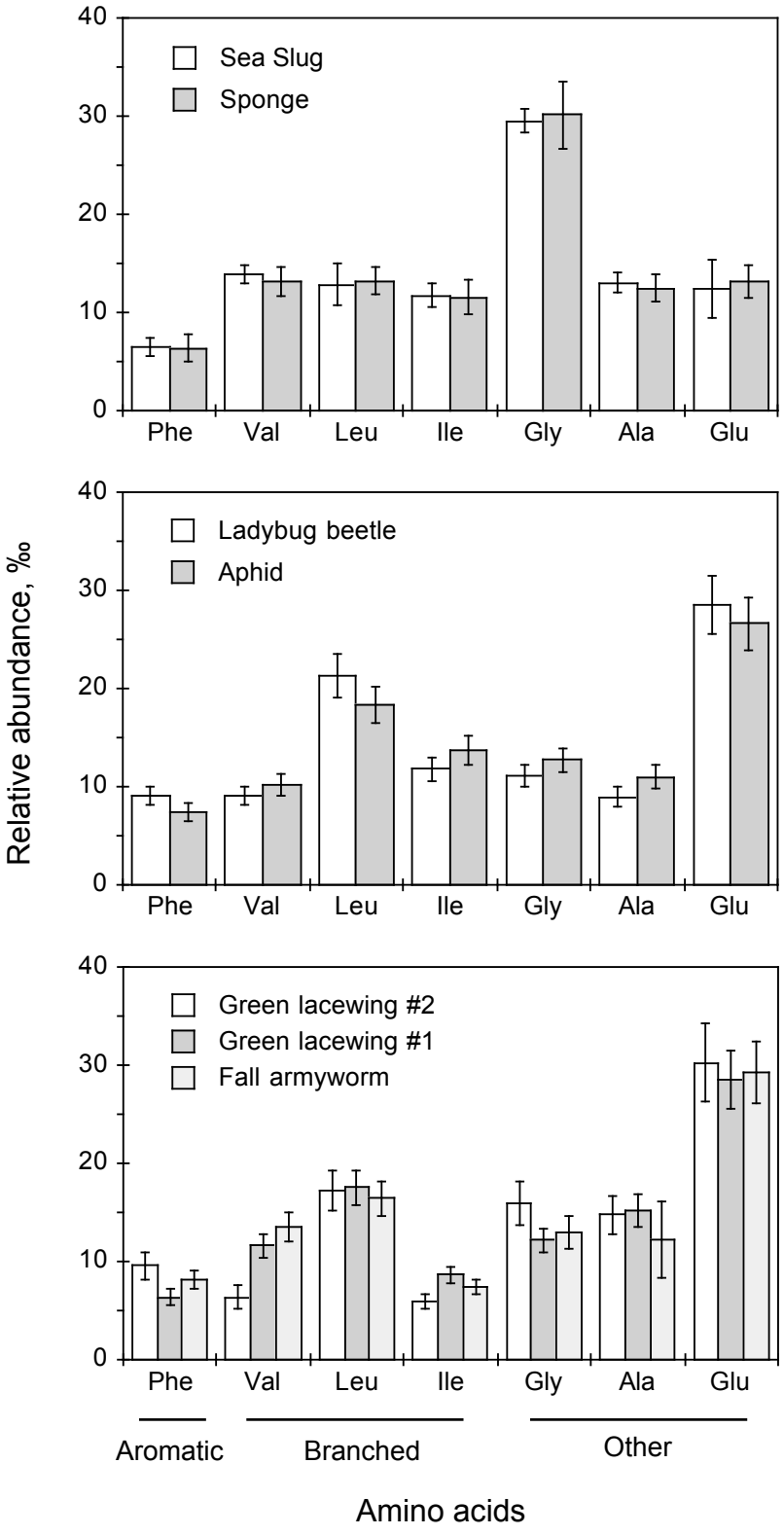


Fig. A-1-1.

Molar balance of amino acids for four combinations of consumer and its diet. Bars indicate standard deviation (1σ) of replicate measurements.

each single sample, and the green lacewings *C. rufilabris* – the fall armyworm *S. frugiperda*, and the green lacewings *C. rufilabris* – the green lacewings *C. rufilabris*, were analyzed these values by a single injection for each of four specimens. The $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values were thus obtained as the mean ± 1σ, in multiple injections or specimens. The Δ values were reported for one aromatic (i.e., phenylalanine), three branched (i.e., valine, leucine, and iso-leucine), and three other amino acids (i.e., glycine, alanine, and glutamic acid) in the present study.

3. Results and discussion

3.1. Isotopic discrimination of nitrogen

The $\Delta\delta^{15}N_{AA}$ values in these samples widely vary from 0.3 to 8.1‰, and have a great diversity in the mean value and its 1σ variation among amino acids. As general trend for four combination samples, there are a small mean (0.4‰) with a little variation (0.1‰) for phenylalanine, a large mean (from 4.0 to 7.9‰) with a little variation (form 0.2 to 1.0‰) for valine, leucine, isoleucine, glycine alanine, and glutamic acid, and a medium-sized mean (2.5‰) with a great variation (2.8‰) for glycine (Fig. A-1-2, Table A-1-1).

These results are well consistent with the $\Delta\delta^{15}N_{AA}$ values reported in a number of previous studies (e.g., Chikaraishi et al., 2009, 2010; Steffan et al., 2015). For example, the $\Delta\delta^{15}N$ values of glutamic acid and phenylalanine are 7.9±0.4‰ and 0.4±0.3‰ (mean±1σ), respectively, in the present study, which are very similar to these values (8.0±1.1‰ and 0.4±0.4‰, respectively) reported in Chikaraishi et al. (2010b). Thus, our data further validate the traditional interpretation that these small and larger $\Delta\delta^{15}N_{AA}$ values can be explained by metabolic activity of deamination for source (e.g., phenylalanine) and trophic (e.g., valine,

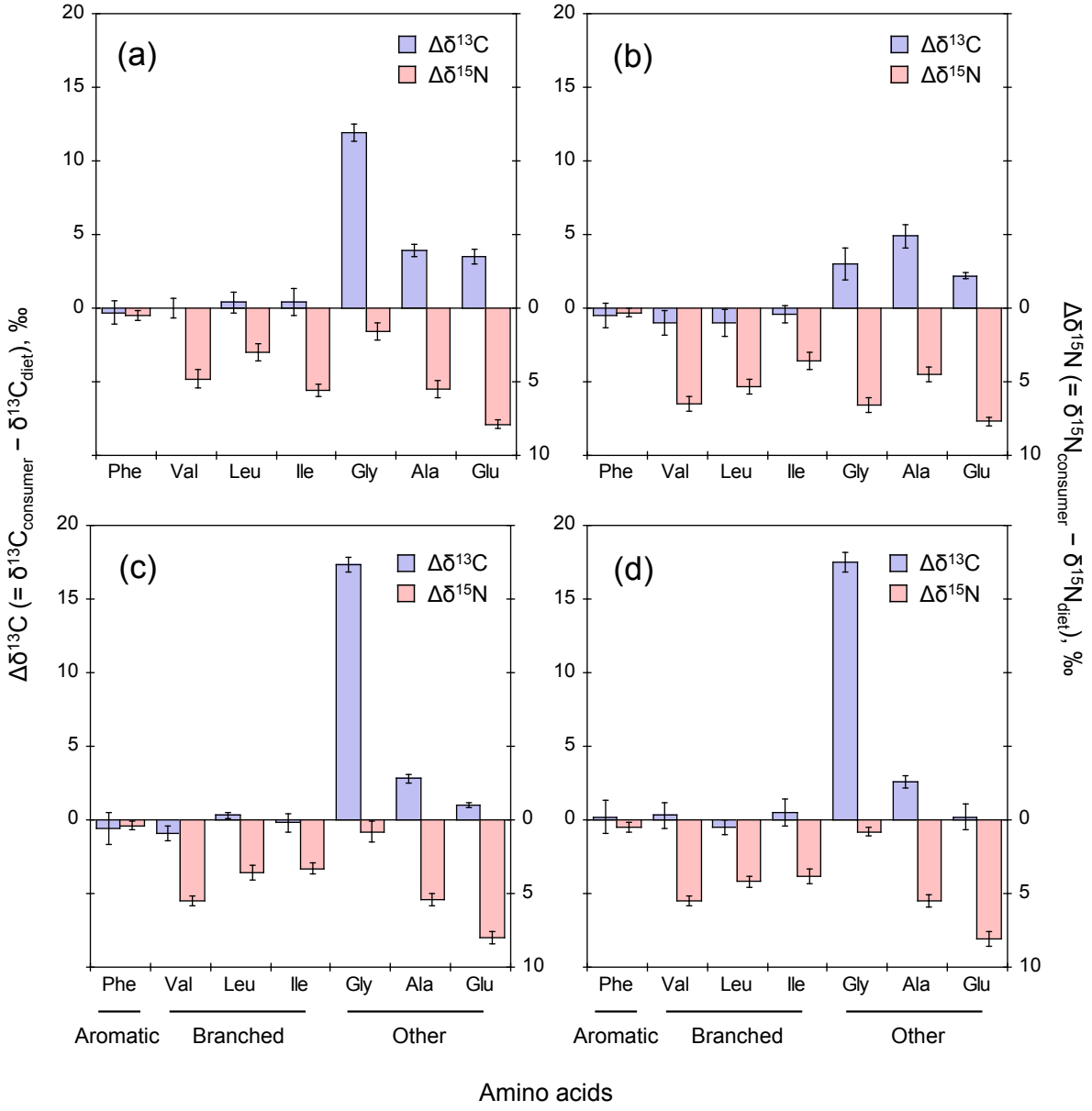


Fig. A-1-2.

$\Delta\delta^{13}$C and $\Delta\delta^{15}$N values of amino acids: (a) sea slug feeds on sponge, (b) lady beetle feeds on aphid, (c) green lacewing #1 feeds on fall armyworm, and (d) green lacewing #2 feeds on green lacewing #1. Bars indicate standard deviation (1σ) of replicate measurements.

leucine, isoleucine, galanine, and glutamic acid) amino acids, respectively (Fig. A-1-3a): for great activity on the trophic amino acids, the deamination reacts preferentially with ^{14}N amino group of diet-derived amino acids, leaving behind the considerably enriched ^{15}N in the residual pool of amino acids in consumer biomass; in contrast, little activity on the source amino acids results in the little enriched ^{15}N in phenylalanine (e.g., Chikaraishi et al., 2007; Takizawa et al., 2017).

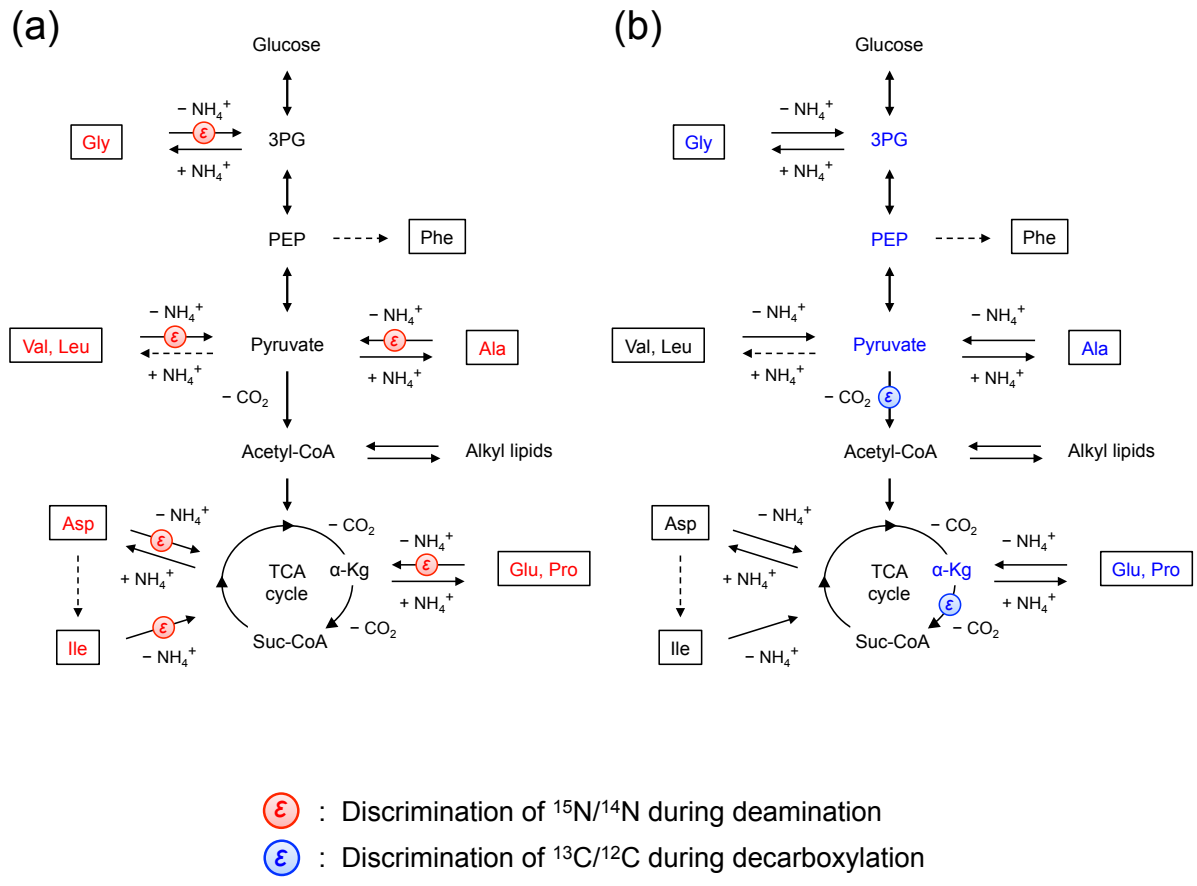


Fig. A-1-3.

Simplified map of biosynthetic and metabolic networks of amino acids with respect to isotopic discrimination of (a) $^{15}N/^{14}N$ associated with deamination of amino acids and (b) $^{13}C/^{12}C$ associated with decarboxylation of pyruvate and α-ketoglutarate.

3.2. Isotopic discrimination of carbon

The $\Delta\delta^{13}C_{AA}$ values in these samples much vary from −1.0 to 17.5‰, by approximately twice as wide as the variation in the $\Delta\delta^{15}N_{AA}$ value. Consistent with the previous studies (e.g., McMahon et al., 2010. 2015), there are a small mean (from −0.4 to 0.1‰) with a little variation (from 0.4 to 0.7‰) for aromatic (i.e., phenylalanine) and branched amino acids (i.e., valine, leucine, and isoleucine), and a large mean (from 1.7 to 12.4‰) with a large variation (form 1.1 to 6.8‰) for other amino acids (i.e., glycine, alanine, and glutamic acid). The largest mean (12.4‰) with the greatest variation (6.8‰) are found for glycine among the amino acids examined in the present study (Fig. A-1-2, Table A-1-1).

It is assumed that the isotopic discrimination of carbon within organic compounds is principally controlled by the isotope effect and relative flux at a branching point that either forming or cleaving carbon-carbon bond in biosynthetic and metabolic pathways (e.g., Hayes, 2001; Chikaraishi, 2014; Ohkouchi et al., 2015). Four decades ago, DeNiro and Epstein (1977) first demonstrated that metabolic products (i.e., lipids) are greatly depleted in ^{13}C (by 7‰–8‰) for *E. coli* cultured with glucose or pyruvate, but less depleted in ^{13}C (by 1‰ only) for that with acetate, and suggested that isotopic discrimination is closely related to the decarboxylation of pyruvate to form acetyl-CoA. More recently, it was proposed that the decarboxylation of pyruvate causes the depletion in ^{13}C at the position of the carbonyl-carbon on acetyl-CoA, maybe due to the preferential cleaving of the ^{12}C-^{12}C bond on the pyruvate in the decarboxylation (e.g., Monson and Hayes, 1980, 1982; Melzer and Schmidt, 1987). Based on this knowledge, we further propose the following processes (Fig. A-1-3b) to interpret the $\Delta\delta^{13}C_{AA}$ values observed in the present study:

(1) decarboxylation of pyruvate in glycolysis preferentially eliminates ^{12}C as acethyl-CoA,

leaving behind the enriched ^{13}C in the residual pool of pyruvate (a biosynthetic precursor of alanine and glycine);

(2) similarly, decarboxylation of α-ketoglutarate in the TCA cycle preferentially eliminate ^{12}C as succinyl-CoA, leaving behind the enriched ^{13}C in the residual pool of α-ketoglutarate (e.g., a biosynthetic precursor of glutamic acid) in there;

(3) the enriched ^{13}C in the residual pyruvate and α-ketoglutarate can be propagated into amino acids if the consumers produces the amino acids via re-biosynthesis (or metabolic routing).

In these process, the absence of isotopic discrimination (i.e., substantially zero $\Delta\delta^{13}C_{AA}$) for the aromatic (i.e., phenylalanine) and branched amino acids (i.e., valine, leucine, and isoleucine) is attributable to no or little activity of re-biosynthesis of these amino acids in consumers, because these amino acids need long process (i.e., multiple steps) for biosynthesis (which are therefore called 'essential' or 'indispensable' amino acids). Moreover, the high $\Delta\delta^{13}C$ values (and their large variations) for glycine, alanine, and glutamic acid are attributable to great activity (and its variability) of re-biosynthesis in consumers, because these amino acids need short processes for the biosynthesis (which are therefore called 'non-essential' or 'dispensable' amino acids). Thus, we predict that the decarboxylation in both glycolysis and TCA cycle elevates ^{13}C content on the biosynthetic precursors of amino acids, and that re-biosynthesis of amino acids incorporates the enriched in ^{13}C into the newly produced amino acids in consumers.

3.3. Coupling vs. decoupling between $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$ in food webs

There is the empirical observation that the isotopic discrimination of bulk organ-

isms is 0-1‰ for carbon ($\Delta\delta^{13}C_{bulk}$) and 3-4‰ for nitrogen ($\Delta\delta^{15}N_{bulk}$) with each step of trophic transfer in food webs (e.g., DeNiro and Epstein 1981; Minagawa and Wada 1984; and reviewed in Post, 2002; Fry 2006; Ohkouchi et al., 2015). Regarding to apparent positive correlation between $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$ values, it has long been discussed whether the trophic isotopic discrimination process for carbon is metabolically coupling or decoupling with that for nitrogen within a single organism as well as food chains (e.g., Aita et al., 2011; Wada et al., 2013). For instance, Aita et al. (2011) found significant liner correlations between $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$ values with similar size of slopes in diverse oceanic food chains, which inevitably allows us to suppose a hypothesis that the trophic isotopic discrimination of carbon is coupling with that of nitrogen in food webs.

Moreover, because the isotopic discrimination is generally cased by either forming or cleaving chemical bond in biosynthetic and metabolic key processes (e.g., Hayes, 2001; Chikaraishi, 2014; Ohkouchi et al., 2015), the following scenarios are assumed as a potential process responsible for the isotopic discrimination during deamination:

(1) forming of the C=N bond between amino acid and enzyme-pyridoxal phosphate (enzyme-PLP) (Fig. A-1-4a), or

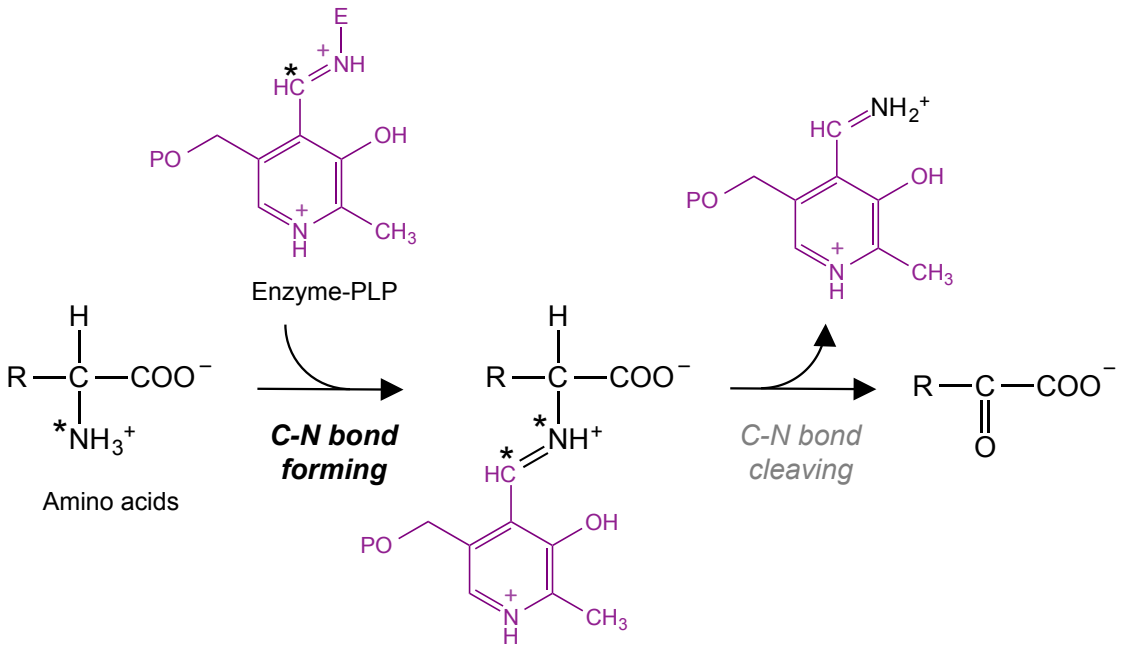
(2) cleaving of the C-N bond (α carbon – amino group nitrogen) within amino acids (Fig. A-1-4b),

and that during decarboxylation:

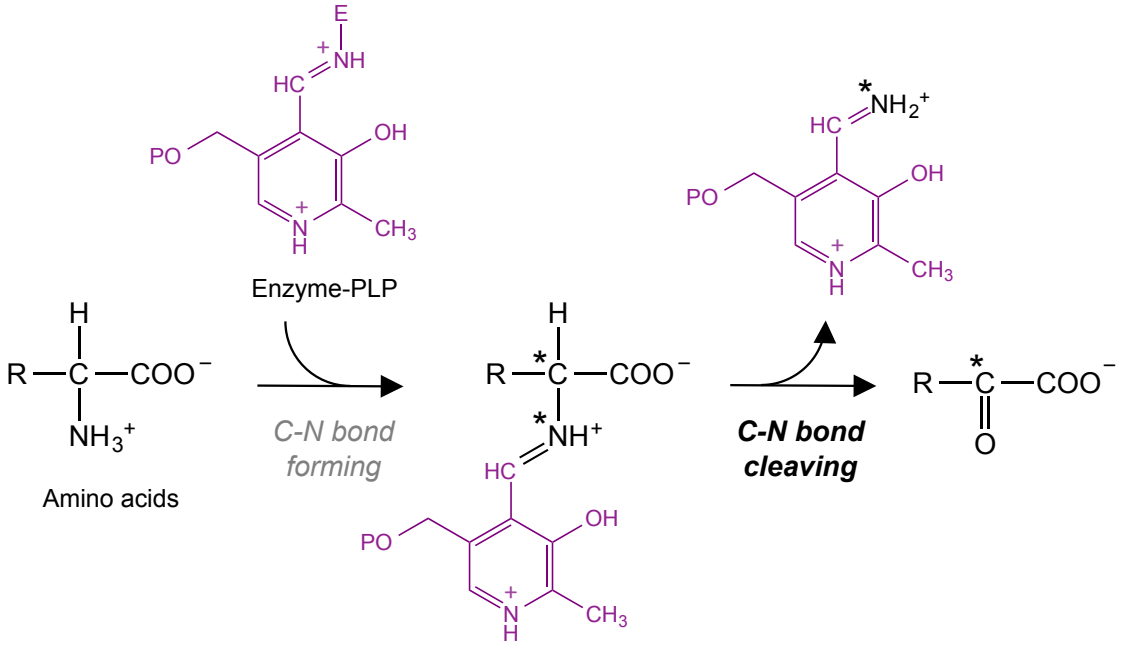
(3) forming of C-C bond between pyruvate (and α-ketoglutarate) and thiamine pyrophosphate (TPP) (Fig. A-1-5a), or

(4) cleaving of C-C bond (α carbon – carboxyl group carbon) within pyruvate (or α-ketoglutarate) (Fig. A-1-5b).

(a) Isotopic fractionation in C-N bond forming



(b) Isotopic fractionation in C-N bond cleaving

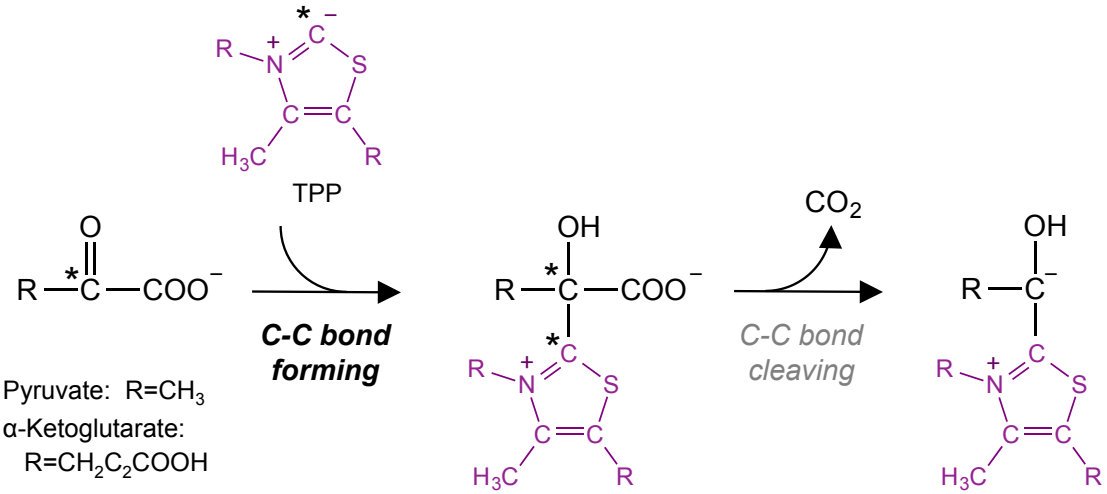


PLP (Pyridoxal phosphate)

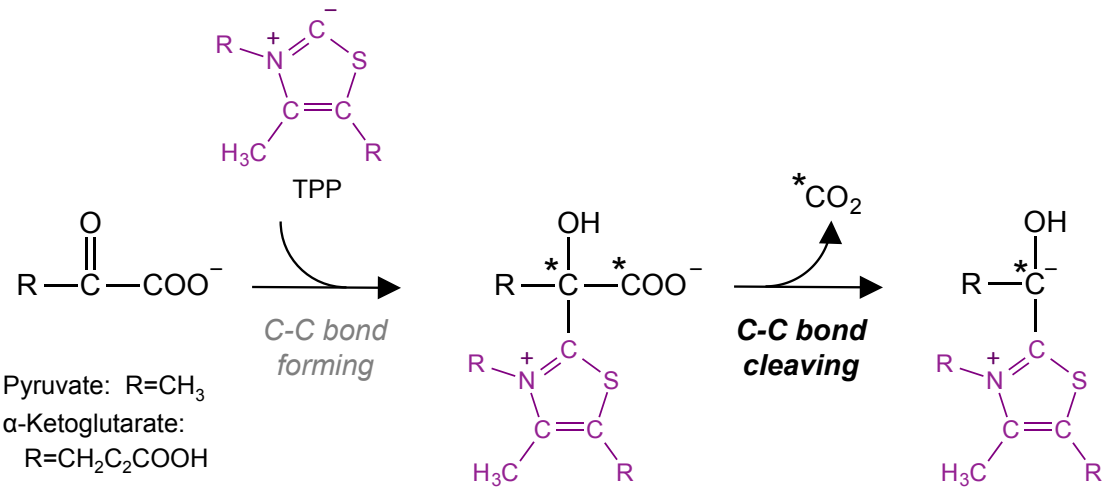
Fig. A-1-4.

Possible reaction mechanism of deamination of amino acids. Asterisk indicate atom where isotopic discrimination occurs.

(a) Isotopic fractionation in C-C bond forming



(b) Isotopic fractionation in C-C bond cleaving



TPP (thiamine pyrophosphate)

Fig. A-1-5.

Possible reaction mechanism of decarboxylation of pyruvate and α-ketoglutarate. Asterisk indicate atom where isotopic discrimination occurs.

The positive correlations between $\Delta\delta^{13}C_{bulk}$ and $\Delta\delta^{15}N_{bulk}$ values found in diverse ecosystems (e.g., Aita et al., 2011; Wada et al., 2013) are may be consistent with the above scenario (2), as the deamination preferentially cleaves ^{12}C α carbon – ^{14}N amino group nitrogen bound in the diet-derived amino acids, leaving behind the enriched both ^{13}C and ^{15}N in the residual pool of amino acids in consumer biomass.

The data observed in the present study, however, illustrate no positive correlation between $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values in any amino acids for the studied consumers (Fig. A-1-6). A marked absence of both re-biosynthesis and deamination activities of the aromatic amino acid in consumers result in the $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values very close to zero for phenylalanine (Fig. A-1-6a). An absence of re-biosynthesis but the presence of deamination activities of the branched amino acids in consumers result in substantially zero $\Delta\delta^{13}C$ and high $\Delta\delta^{15}N$ values for valine, leucine, and isoleucine (Fig. A-1-6b). Moreover, both re-biosynthesis and deamination activities of the other amino acids in consumers results in a large variation in both $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values for alanine, glycine, and glutamic acid, accompanied with the inverse correlation between the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values (Fig. A-1-6c). Moreover, the no positive correlation between $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values suggests that the forming chemical bond (i.e., above scenarios 1 and 3) are potential processes responsible for the isotopic discrimination during deamination of amino acids and decarboxylation of pyruvate (and α-ketoglutarate), respectively, as preferential forming of the C=N bond between ^{14}N amino group and enzyme-PLP during deamination is independent of preferential forming of C-C bond between ^{12}C carboxyl carbon and TPP during decarboxylation. Our results thus reveal that the trophic isotopic discrimination process for carbon is, at least in the amino acid metabolism, decoupling with that for nitrogen within a single organism as well as food chains.

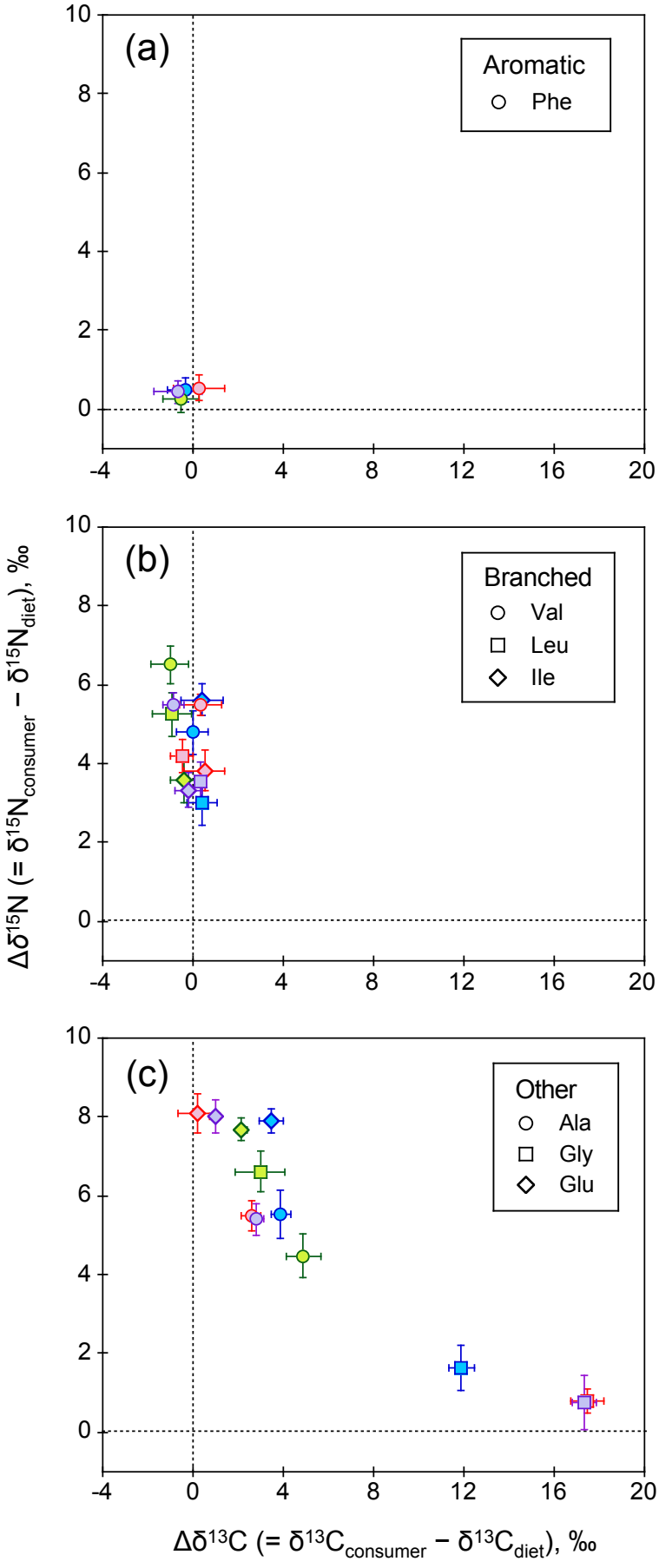


Fig. A-1-6.

Cross-plots for the $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ values for (a) aromatic, (b) branched, and (c) other amino acids.

Blue: sea slug feeds on sponge

Green: lady beetle feeds on aphid

Purple: green lacewing #1 feeds on fall armyworm

Red: green lacewing #2 feeds on green lacewing #1

3.4. $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ of glycine, alanine, and glutamic acid

For the isotopic discrimination of amino acids in food webs, a great variation in the $\Delta\delta^{15}N$ values have been frequently found in glycine in the number of previous studies (e.g., 4.0±3.5‰, Chikaraishi et al., 2010), even though glycine has been sometimes employed as a source amino acid in food web studies (e.g., Popp et al., 2007; Sherwood et al., 2011; Vander Zanden et al., 2013; Bradley et al., 2015). Moreover, the universality of the $\Delta\delta^{15}N$ values of glutamic acid has been questioned for several animals, particularly for high trophic level consumers that have urea/uric acid cycle, and for any trophic level consumers that feed on diet with unusual PFC balance (e.g., Chikaraishi et al., 2015; McMahon et al., 2015; McMahon and McCarthy 2016).

The data observed in the present study illustrate the inverse correlation between the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values for alanine, glycine, and glutamic acid (Fig. A-1-6c). We predict that this inverse correlation mirrors the balance between re-biosynthesis and deamination activities for these three amino acids in consumers, as the $\Delta\delta^{15}N$ values are inherently compressed if the re-biosynthesis is dominated against the deamination of these amino acids in consumers. It is the most likely cases when consumers can take a deficiency of glycine, alanine, and/or glutamic acid from their diets, because such cases allow the consumers to re-biosynthesize (but not to deaminate) these amino acids for their biomass constituents. Based on these results, we suggest that the balance between re-biosynthesis and deamination activities is a dominant factor for controlling the isotopic discrimination of carbon and nitrogen within bulk tissues as well as amino acids (particularly for glycine) in consumers. In other words, the combination analysis of the $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values of individual amino acids can be useful in the assessment of the central biosynthetic and metabolic fluxes regard-

ing to amino acids during a trophic transfer from diets to consumers, as well as in turn in food webs.

4. Conclusions

In the present study, we determined the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values in four pairs of consumer–resource invertebrates to illustrate a bridged perspective between the trophic isotopic discrimination (in both carbon and nitrogen) and its responsible process in the biosynthesis and metabolism. Our results provides the following refinements of diversity in the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values among amino acids:

(1) as a fundamental role, the $\Delta\delta^{13}C_{AA}$ values mirror the metabolic activity of decarboxylation and subsequent re-biosynthesis, whereas the $\Delta\delta^{15}N$ values mirror that of deamination in consumer species;

(2) aromatic amino acid (i.e., phenylalanine) has the $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values very close to zero, due to an absence of both re-biosynthesis and deamination activities.

(3) branched amino acids (e.g., valine, leucine, and isoleucine) have substantially zero $\Delta\delta^{13}C$ and high $\Delta\delta^{15}N$ values, due to an absence of re-biosynthesis but the presence of deamination activities;

(4) other amino acids (e.g., glycine, alanine, and glutamic acid) have an inverse correlation between the $\Delta\delta^{13}C_{AA}$ and $\Delta\delta^{15}N_{AA}$ values, due to diversity in the metabolic balance between re-biosynthesis and deamination activities

These results imply that the combination analysis of the $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values of individual amino acids can be useful for better understanding the central biosynthetic and metabolic fluxes with respect to the observed change in the $\delta^{13}C$ and $\delta^{15}N$ values (i.e., trophic discrimi-

Table A-1. Nitrogen and carbon isotopic discriminations on individual amino acids in the four combination samples.

	Alanine		Glycine		Valine		Leucine		Isoleucine		Glutamic acid		Phenylalanine	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
$\Delta\delta^{15}$N (‰)														
Sea sulg	5.5	0.6	1.6	0.6	4.8	0.6	3.0	0.6	5.6	0.4	7.9	0.3	0.5	0.3
lady beetle	4.5	0.5	6.6	0.5	6.5	0.5	5.3	0.5	3.6	0.6	7.7	0.3	0.3	0.3
green lacewing #1	5.4	0.4	0.8	0.7	5.5	0.3	3.6	0.5	3.3	0.4	8.0	0.4	0.4	0.3
green lacewing #2	5.5	0.4	0.8	0.3	5.5	0.3	4.2	0.4	3.8	0.5	8.1	0.5	0.5	0.3
$\Delta\delta^{13}$C_corrected (‰)														
Sea sulg	3.9	0.4	11.9	0.6	0.0	0.7	0.4	0.7	0.4	0.9	3.5	0.5	-0.3	0.8
lady beetle	4.9	0.8	3.0	1.1	-1.0	0.8	-1.0	0.9	-0.4	0.6	2.2	0.2	-0.5	0.8
green lacewing #1	2.8	0.3	17.3	0.5	-0.9	0.5	0.3	0.2	-0.2	0.6	1.0	0.2	-0.6	1.1
green lacewing #2	2.6	0.4	17.5	0.7	0.3	0.9	-0.5	0.5	0.5	0.9	0.2	0.9	0.2	1.1

'Sea sulg': Sea sulg feed on sponge, 'lady beetle': lady beetle feeds on aphid, 'green lacewing #1': green lacewing #1 feeds on fall armuworm, and 'green lacewing #2': green lacewing #2 feeds on green lacewing #1.

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