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Article

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

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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.

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 (1):

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

where $\beta_{Glu/Phe}$ denotes the offset between the δ^{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) to characterize TDF':

$$TDF'_{Glu/Phe} = (\delta^{15}N_{sample,Glu} - \delta^{15}N_{sample,Phe}) - \beta_{Glu/Phe} \qquad (2)$$

where $\delta^{15}N_{sample}$ indicates the plant organ of interest, and

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

	Collection date (yy/mm/dd)	δ^{15}N (‰)[1]								$TP_{Glu/Phe}$[2]	TDF'[3]	Reference
		Alanine	Glycine	Valine	Leucine	Isoleucine	Proline	Glutamic acid	Phenyl alanine			
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 δ^{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.

$\beta_{Glu/Phe}$ 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. (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 $\delta^{15}N_{AAs}$ 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 $\delta^{15}N_{AAs}$ values in food webs.

2. Materials and Methods

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 (35°08′N, 139°07E) (Table 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. The bloom of flowers generally starts for about 2-3 weeks prior to the flush of leaves. 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 −20ºC until the isotope analysis.

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 δ^{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 δ^{15}N values of alanine, glycine, valine, leucine, isoleucine, proline, glutamic acid, and phenylalanine were determined for the sample leaves and flowers (Table 1), based on the S/N ratio of ≥20 with baseline separation on the chromatogram.

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 (1) and (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

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 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

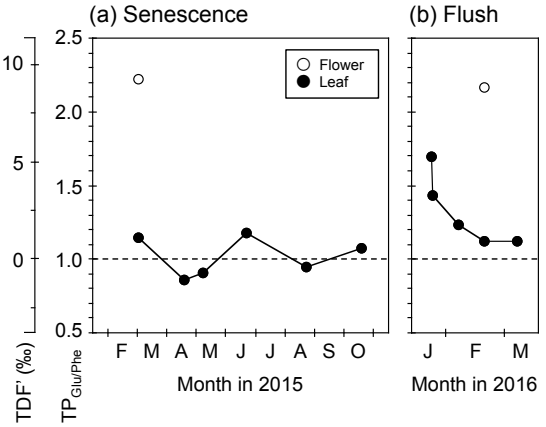

Fig. 1. $TP_{Glu/Phe}$ values and TDF' for leaf (a) senescence and (b) flush.

(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. 1b), which is very consistent with that for the same plant collected in 2015 (TDF'=9.3‰) (Fig. 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., 2017), allowing us approximately to employ this high TDF' (i.e., 8.9‰) as a signal of excess catabolism against photosynthesis in this plant.

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. 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 $TP_{Glu/Phe}$=1.3±0.2) for the flush leaves (Fig. 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. 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.

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 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) (Fig. 2b; from 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 the term of leaf flush even for the leaves collected from a single plant (Fig. 2a). 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. 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

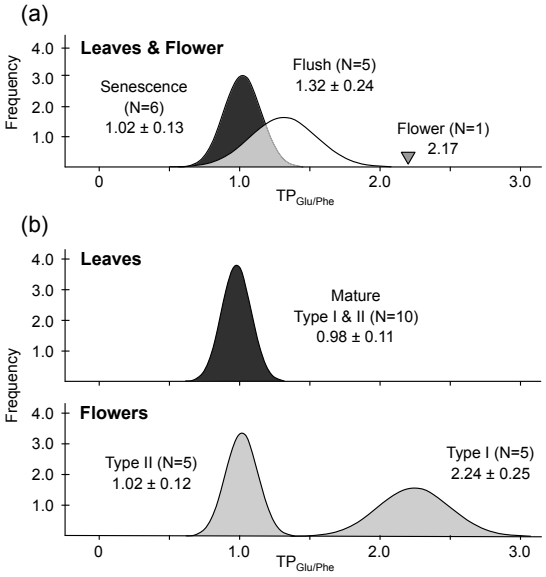

Fig. 2. Density distribution of (a) the $TP_{Glu/Phe}$ of senescence and flush leaves determined in the present study, together with (b) that of the leaves and flowers in deciduous plants reported by Takizawa et al. (2017). The $TP_{Glu/Phe}$ values of flowers in the present study is also shown (inverted triangle).

the 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

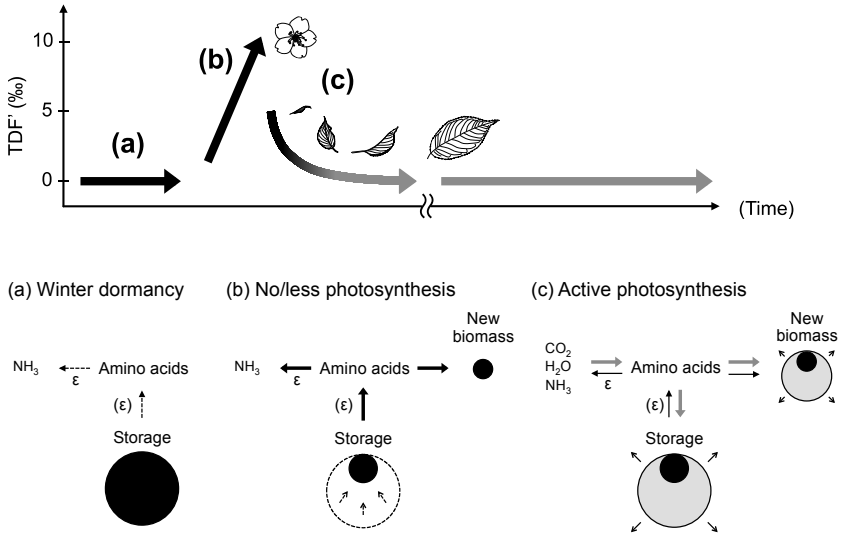

Fig. 3. Schematic illustration of the TDF' correlated to the metabolic flow with respect to the anabolism (i.e., production) and catabolism (i.e., consumption) of storage amino acids in plant phenology. (a): substantially zero TDF' of the storage amino acids (filled circle) is found in winter dormancy; (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.

always composed of large-sized storage compounds for adjusting the energy balance between supply and demand in phenology (Fig. 3), as similar to the requirement of huge-sized storage battery in the full cycle of solar-power (photovoltaic) generation system.

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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