Abstract for Ph.D thesis

博士学位論文 要約

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

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CONTENTS OF ORIGINAL Ph.D THESIS

要約

生体分子中有機化合物の安定同位体比（δ，例えば，D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$ など）は，一般的に，生合成時における「基質の δ 」と，その物理化学・生化学的プロセスにおける「同位体分別 (ε)」，特に生体試料の δ に影響を与えうる ε を持った‘Key プロセス’を記録すると考えられている．これらの組み合わせの普遍性・一般性によってもたらされる生体試料の δ に基づいて，環境試料から得られた δ は「生合成時の環境因子」や地球表層における「物質の起源や移動」を理解するためのツールとして，幅広い分野で用いられている．その一方で，生物は自身を成長させるために，あるいはエネルギーを獲得するために，比較的短い期間で有機化合物の合成と分解を繰り返すため，その δ には「生合成（生産）の ε 」のみならず「代謝 （分解）の ε」も潜在的に含まれると考えられる．にもかかわらず，この「代謝」すなわち「有機物分解」の過程に，生体試料の δ に影響を与える‘Key プロセス’が存在するかどうか，その影響の見積もりに関しては，これまで定量的な議論や証拠が不十分であった．それは「有機物分解に由来する ε を観察することの出来る生体試料」を手に入れる困難さと，有機物分解を定量的に評価するための手法・方法論が，現状で 1 つ（アミノ酸安定窒素同位体比 ($\delta^{15}N_{AA}$) を用いたアミノ酸異化（脱アミノ反応）の評価）に限られており，どの基質（糖，タンパク質，脂質）が分解・利用された時に，どの有機分子の，どの元素に記録されるかを，多面的・複合的に議論することができないためである．

そこで本論文では，植物試料を対象に，有機物分解が植物の δ を変えうるファクターになりえるかどうかを，(1) 既存の解析手法である $\delta^{15}N_{AA}$ から確認（Chapter II）するとともに，脂質を対象とした有機物生産／分解のバランスを評価するための新規手法の確立を目的として (2) 脂質分解に伴う ε の有無をパルミチン酸安定炭素・水素同位体比（$\delta^{13}C_{16FA}$，δD_{16FA}）を用いて検討（Chapter III, Section 3-1）し，(3) その Key プロセスと反応部位を明らかにするために脂肪酸分子内安定炭素（カルボキシル基の炭素）同位体比（$\delta^{13}C_{FA\text{-}COOH}$）測定法の開発（Chapter III, Section 3-2）をおこなった．「有機物分解の寄与が大きい δ を持つ生体試料」として，Chapter II では，「暗室で保管したサツマイモとそこから出た芽 （Section 2-1)」，「葉が芽吹く前に咲く花 （Section 2-2)」，「落葉樹の芽吹き始めの葉 （Section 2-3)」の，光合成が無い，もしくは弱い条件下で育った 3 つの植物試料を対象に，Chapter III では「暗室下で発芽させたゴマの種子」を対象とした．

一般的に，光合成ができる「葉」などの組織が持つアミノ酸の $\delta^{15}N$ 値は，グルタミン酸とフェニルアラニンの差にして −8.4‰, そこから算出されるエネルギー的栄養段階 ($TP_{Glu/Phe}$) は 1.0 であるのに対して, Section 2-1 では, イモは−5.6‰ ($TP_{Glu/Phe}$=1.4), 芽は 0.7‰ ($TP_{Glu/Phe}$=2.2) という高い値を示した．これらの結果は，植物試料から有機物分解（アミノ酸の異化）のシグナルを検出できた初めての例である．また Section 2-2 では，葉が芽吹く‘前’に咲く花における ^{15}N の濃縮（$TP_{Glu/Phe}$ の上昇）は，葉が芽吹いた‘後’に咲く花（$TP_{Glu/Phe}$=1.0±0.1）に比べ

て，顕著に大きくなる（$TP_{Glu/Phe}$=2.2±0.3）ことが明らかとなった．これらの事実は，光合成が弱い状況において，植物はエネルギーや基質を得るために，生体内に存在するアミノ酸を分解・利用すること，そしてそのアミノ酸の分解（脱アミノ反応）とそれに伴う ε は，植物の δ を変えうる Key プロセスとして機能することを証明している. そして Section 2-3 では，このようなアミノ酸の分解と利用は, 落葉樹の葉が芽吹くプロセスにおいても共通しており，そのシグナルは芽吹きの初期において顕著になり（$TP_{Glu/Phe}$=1.7），その後，葉の成長に伴って減少（$TP_{Glu/Phe}$=1.1）することを明らかにした．これらの結果から，植物の δ には，確かに有機物分解のシグナルが保存される可能性があり，そこにはアミノ酸の生産／分解のバランスの情報が記録されることが明らかとなった.

Section 3-1 では「脂質の分解過程において，検出可能な ε が得られるかどうか」を検証するために，暗室下で「種子（貯蔵性脂質であるトリアシルグリセロール：TAG を多く含む）」を発芽させる実験をおこない，発芽前と後との試料を対象に，相対存在量，安定炭素・水素同位体比（それぞれ $\delta^{13}C_{16FA}$ 値，δD_{16FA} 値）を測定した．その結果，発芽前（種子）に比べて，発芽後（芽）では，試料中のパルミチン酸成分の相対存在量は 49%まで減少し，$\delta^{13}C_{16FA}$ 値のみにおいて有意な差（+2.9±1.7‰）を得た（δD_{16FA} 値は測定誤差範囲内）．これらの「相対存在量の減少「と「^{13}C の濃縮」という結果は (1) 暗室下で発芽する種子は，確かに TAG を消費すること，そして(2)「脂質の分解過程に‘炭素’を反応部位とした ε が存在する」ことを示唆した. Key プロセスの候補としては，脂質の異化・利用の初期過程である「酵素による貯蔵性脂質の加水分解（ TAG →グリセロール + 脂肪酸）」が考えられ，もしこれが正しければ，その時の反応部位は「貯蔵性脂質中の脂肪酸成分のカルボキシル基の炭素」となり，$\delta^{13}C_{FA\text{-}COOH}$ 値はマスバランスにより 48±27‰と算出される．これを検証するために，Section 3-2 では貯蔵性の脂肪酸（C16, C18 など）を対象として，分子内安定炭素（カルボキシル基の炭素）同位体比測定法を開発した. 分析系の構築は，ガスクロマトグラフ-同位体比質量分析計（GC-IRMS）に，カルボキシル基の炭素の脱炭酸をおこなう熱分解炉を設置することで達成した. これを用いて $\delta^{13}C_{FA\text{-}COOH}$ 値を測定した結果，発芽後の試料に含まれる脂肪酸成分のカルボキシル基の炭素に，顕著に高い $\delta^{13}C_{FA\text{-}COOH}$ 値（63‰の上昇）を検出した．以上の事実から，貯蔵性脂質（すなわち TAG）が，発芽のプロセスにおいて主要なエネルギー・基質の源になっていること，そして貯蔵性脂質の「酵素による加水分解」が，炭素同位体に関して大きな ε を生み出す Key プロセスであることを証明した．本研究で得られた成果は，今後，生体試料や環境試料を対象とした「生合成と代謝（生産と分解）を区別した」評価，特に「有機物分解の定量的評価」をおこなう上での有力なツールとなる可能性を持っており，物質循環・環境変遷の解析ツールとして役立てられることが期待される.

GENERAL BACKGROUNDS

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):

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

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)'. 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, 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.

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)', 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, ε_{AP} and ε_{HP} denote the isotopic fractionation associated with autotrophic and heterotrophic productions (sum of $\varepsilon_M + \varepsilon_{AtoB}$), 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.

References:

Sachse D., Dawson T. E., and Kahmen A. (2015) Seasonal variation of leaf wax n-alkane pro duction and δ2H values from the evergreen oak tree, *Quercus agrifolia*, *Iso. Environ. Heal. Stud.* 51:1, 124-142.

Sessions A. L. (2006) Seasonal changes in D/H fractionation accompanying lipid biosynthesis in *Spartina alterniflora*. *Geochim. Cosmochim. Acta*. 70, 2153–2162.

Tipple B. J., Berke M. A., Doman C. E., Khachaturyan S. and Ehleringer J. R. (2012) Leaf-wax *n*-alkanes record the plant–water environment at leaf flush. *Proc. Natl. Acad. Sci.* doi:10.1073/pnas.1213875110.

SUMMARY

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. This section was originally published as; Takizawa Y. and Chikaraishi Y. (2014) Are baby sprouts eating the proteins in the mother sweet potato? *Researches in Organic Geochemistry*. 30, 29-32.
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. This section was originally published as; Takizawa Y. and Dharampal P. S. Steffan S. A. Takano Y. Ohkoushi N. and Chikaraishi Y. (2017) Intra-trophic isotopic discrimination of $^{15}N/^{14}N$ for amino acids in autotrophs: Implications for nitrogen dynamics in ecological studies. *Ecology and Evolution*. 7, 2916-2924.
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. This section was originally in press as; Takizawa Y. and Chikaraishi Y. (2017) Change in the $\delta^{15}N$ value of plant amino acids on the phenology of leaf flush and senescence. *Researches in Organic Geochemistry*.

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

Because this chapter is still in the writing stage for a scientific journal, I briefly summarize our results:

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.