NOTE

Organic hydrogen-carbon isotope signatures of terrestrial higher plants during biosynthesis for distinctive photosynthetic pathways

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(Received March 15, 2001; Accepted November 24, 2001)

Stable isotopic compositions of organic hydrogen (δD) have positive correlations ($r^2 > 0.95$) with those of carbon ($\delta^{13}C$) among several compound fractions of terrestrial plant leaves possessing distinctive photosynthetic pathways (C_3 , C_4 and CAM). The $\delta D/\delta^{13}C$ slopes of C_3 plants vary from ~25 to 42, which are larger than those of C_4 plants (11 to 12). CAM plants have intermediate $\delta D/\delta^{13}C$ slopes (~17 to 20) between C_3 and C_4 plants. Using a δD - $\delta^{13}C$ diagram, photosynthetic metabolisms are clearly discriminated, even though they sometimes cannot be distinguished from each other only by carbon isotopes. Relative to bulk organic matter, hydrogen and carbon of lipid fraction are more depleted in ^{13}C than those of pigment fraction, respectively. Furthermore, δD values of lipid and pigment fractions relative to bulk organic hydrogen have negative correlations with $\delta^{13}C$ values of corresponding fractions among the three photosynthetic pathways. This isotopic covariance among each fraction may be attributable to kinetically-controlled molecular biosyntheses using similar enzymes but with different isotope fractionations. Or the intermediate molecules for the biosyntheses have isotopically different pools in hydrogen and carbon.

INTRODUCTION

It is well known that terrestrial plants use three photosynthetic pathways (C_3 , C_4 and CAM) during carbon fixation. While C_3 plants use a Calvin-Benson cycle characterized by the use of ribulose biphosphate carboxylase oxydase (RUBISCO), C_4 plants use a Hatch-Slack cycle by phosphoenolpyruvate carboxylase (PEPC). The third metabolism known as crassulacean acid metabolism (CAM) has both C_3 - and C_4 -like characteristics depending on growth environment such as light intensity (Lerman and Queiroz, 1974). These different photosynthetic metabolisms are important to understanding plant physiology (O'Leary, 1981; Farquhar *et al.*, 1982) and ecology (Cerling *et al.*, 1993; Morgan *et al.*, 1994) as well as having ap-

plications to paleoenvironmental studies (Cerling *et al.*, 1998; Pagani *et al.*, 1999). A different carbon isotope fractionation during carbon fixation is expected with respect to the different photosynthetic mechanisms. The RUBISCO of C_3 higher plants has the largest kinetic isotope effect (~29‰) between bulk organic matter and CO_2 , while the isotope effect of C_4 plants (~6‰) is much smaller (O'Leary, 1993; Lajtha and Marshall, 1994). CAM plants usually have an intermediate isotope effect between C_3 and C_4 plants (Deines, 1980). It is also known that lipid carbon is depleted in ^{13}C relative to bulk carbon (Degens, 1969; Collister *et al.*, 1994). This is due to the kinetic isotope fractionation of intermediate compounds such as acetyl-CoA during lipid synthesis (DeNiro and Epstein, 1977). The fractionation mechanisms of

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organic carbon during photosynthesis have been extensively studied experimentally and theoretically (e.g., Farquhar *et al.*, 1989).

In contrast to carbon isotope, several studies have shown that total organic hydrogen is depleted in D relative to ambient water hydrogen, and that lipid hydrogen is depleted in D relative to total organic hydrogen (Smith and Epstein, 1970; Ziegler *et al.*, 1976; Estep and Hoering, 1980). Epstein *et al.* (1976) reported the importance of δD study for non-exchangeable hydrogen of cellulose nitrate, because non-carbon bound hydrogens such as in OH may be subjected to H-D exchange with ambient water. Using cellulose nitrate of various plant materials, the δD and $\delta^{13}C$ values have been determined to discriminate among C_3 , C_4 and CAM plants (Sternberg *et al.*, 1984a, b; Ting *et al.*, 1985). Also, hydrogen isotopic compositions of cellulose nitrate of tree rings were used for paleoclimate studies (Northfelt *et al.*, 1981). Due to the relatively large variability on hydrogen isotope fractionations during cellulose synthesis, δD of plant lipids (Sternberg, 1988) as well as individual organic compounds by recent analytical development (Xie *et al.*, 2000) may provide more significant information to study palaeoclimate with respect to environmental waters. However, hydrogen isotopic distributions are not clarified with respect to various types of organic matters within a species, or among different types of photosynthetic metabolism. In this study, we examined stable isotopic compositions of organic hydrogen and carbon on several compound fractions consisting of terrestrial plant leaves which have distinctive photosynthetic pathways (C_3 , C_4 and CAM).

SAMPLES AND EXPERIMENTAL METHODS

Eleven species of terrestrial plant leaves (including five C_3 , four C_4 and two CAM) were examined (Table 1). These samples were collected from suburbs of Tokyo, Japan (7 species), and from farms in Thailand (4 species). As for the Japanese samples, the leaf samples were cleaned up to remove surface contaminants and stored at

Fig. 1. A procedural scheme to obtain several compound fractions from terrestrial plant leaves for organic hydrogen and carbon isotope analyses.

$-20^{\circ}C$ immediately after the collection. The samples were freeze-dried and powdered before analysis. A separation procedure to obtain several compound fractions is shown in Fig. 1. The powdered bulk samples were extracted with hexane (lipid fraction, LF), a part of which was further developed by silica gel column chromatography to obtain the hydrocarbon fraction (HF). Based on gas chromatography-mass spectrometry analysis, the lipid fraction was composed mainly of *n*-alkanols, *n*-alkanoic acids and sterols, being the hydrocarbon fraction composed of *n*-alkanes and terpenoid hydrocarbons. A part of the hexane-insoluble fraction (HIF) was extracted with acetone (pigment fraction, PF) until the residue (acetone-insoluble fraction, AIF) became colorless. The PF was composed mainly of chlorophyll-*a*, based on the UV-visual absorbed spectra. Each extract was carefully subjected to evaporation by N_2 -flow to remove the organic solvent completely. In this study,

Table 1. Organic hydrogen and carbon isotopic compositions of several compound fractions of terrestrial plant leaves (C_3 , C_4 and CAM)

No.	Plants	Country*	Bulk**	Concentrations (mg/g)				δD (‰, relative to SMOW)				$\delta^{13}C$ (‰, relative to PDB)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
				H/C	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF			HF			Bulk	LF					

*J and T denote the samples from Japan and Thailand, respectively.

**Bulk H/C ratio is given as a molar ratio, determined using a CHN elemental analyzer.

nitrate-derivatization had not been done for AIF fraction, which is composed mainly of cellulose (see below).

Bulk and 5 sub-fractions (LF, HF, PF, HIF and AIF) were analyzed for δD and $\delta^{13}C$. All organic solvents used here are non-protic, and hence no hydrogen-exchange reaction was expected during the procedures. Each fraction was combusted in an evacuated and sealed quartz tube at *ca.* 800°C for 3h in the presence of CuO. Evolved CO₂ and H₂O were separated cryogenically using a vacuum line. H₂O was reduced to H₂ in a sealed quartz tube at 750°C for 1h in the presence of sandy Zn (WakoTM). The sandy Zn was preheated just prior to use to remove adsorbed gases using a burner under the vacuum. The CO₂ and H₂ are analyzed for isotope ratios using a dual inlet mass spectrometer (Finnigan delta S). δD and $\delta^{13}C$ values are given in per mil (‰) relative to SMOW and PDB, respectively. The δD values are calibrated using three international isotopic standards (SMOW, GISP and SLAP). Standard deviations of hydrogen and carbon isotope measurements are better than 3‰ and 0.2‰, respectively. Isotopic precision was tested using NIST Reference Material 8540 (Polyethylene Foil 1, PEF1; Gerstenberger and Herrmann, 1983), and gave accurate values within the analytical standard deviations.

RESULTS AND DISCUSSION

C₃ and C₄ plants have bulk organic carbon $\delta^{13}C$ values of $-28.7(\pm 2.2)$ and $-12.6(\pm 0.7)$ ‰, respectively (Table 1). Bulk CAM plants have a wide variation in $\delta^{13}C$ (-21.9 and -13.6 ‰), with intermediate isotopic compositions between C₃ and C₄ plants. The carbon isotope distribution is consistent with many previous studies (e.g., Deines, 1980). In comparison, bulk isotopic compositions of organic hydrogen range from -80 to -45 ‰, showing no distinctive isotopic trend in spite of the different photosynthetic metabolisms as well as different sample origins. The δD values of precipitation in Tokyo area range from -75 to -10 ‰ with an annual mean of -42 ‰. The δD values of

precipitation in Thailand also range from -80 to -10 ‰ (IAEA/WMO, 1999). For C₃ and C₄ plants, the δD values of bulk organic hydrogen are similar or slightly lower relative to that of meteoric water hydrogen, consistent with a previous study (Ziegler *et al.*, 1976; White, 1989).

On the other hand, the bulk δD value of CAM plants (~ -70 to -50 ‰) in this study is lower than those in the previous study (~ -40 to -20 ‰; Ziegler *et al.*, 1976). In addition, the δD value of cellulose nitrate from CAM plants is much higher (~ 0 to $+70$ ‰; Sternberg *et al.*, 1984a) than the bulk δD value of this study. The δD difference is probably due to environmental factors such as humidity or δD value of underground water, because the δD value of CAM plants is highly variable depending on natural conditions (Ziegler *et al.*, 1976). Another possibility for the δD difference is H-D isotope exchange between the organic hydrogen and ambient water during analytical procedures, because such isotopic exchange are suggested by the previous investigators (Epstein *et al.*, 1976; DeNiro, 1981). However, the samples of this study were refrigerated at -20 °C immediately after the collection, followed by freeze-drying. In addition, non-protic organic solvents such as hexane and acetone were used for the extraction. Such analytical procedures could minimize the H-D isotope exchange. In the present study, environmental factors should be more important for the D-depleted bulk organic matter of CAM plants.

The lipid and pigment fractions (LF and PF) are minor components of leaves (10–60 mg/g and 5–40 mg/g respectively, Table 1), and are depleted in ^{13}C relative to bulk organic matter. The carbon isotopic compositions of C₄ plant lipids are depleted in ^{13}C by 7 to 9‰ relative to bulk carbon (LF-bulk), those of C₃ plants are only depleted by 2.5 to 5.5‰. CAM plants have an intermediate degree of lipid-bulk carbon fractionation (~ 7 ‰). Such a larger fractionation in C₄ plants was reported in the case of total wax and total tissue (Collister *et al.*, 1994). The similar isotope trend is observed between pigment fraction and bulk carbon (PF-bulk, 3.1 to 4.8‰ for C₃ plants; 5.1 to

6.1‰ for C_4 plants). Isotopic fractionation degree of PF-bulk is smaller than that of LF-bulk up to 2.9‰ except *Chamaecyparis*. The δD values of LF range from ~ -230 to -150 ‰, being more depleted in D by ~ 50 to 150 ‰ relative to bulk hydrogen (Table 1). Such a δD range of lipid fraction is also consistent with the reported range (Smith and Epstein, 1970; Sternberg *et al.*, 1984c; Sternberg, 1988). However, this study clearly clarified that hydrogen isotope composition of C_4 plants relative to bulk organic hydrogen (~ -95 ‰) is much higher than that of C_3 plants (~ -160 ‰, except for *Chamaecyparis*). The hydrogen isotopic trend is opposite to the carbon isotopic trend between C_3 and C_4 plants. The similar hydrogen-carbon isotopic trend is also observed in pigment-bulk isotopic fractionation. As a result, δD variations of LF and PF relative to bulk organic hydrogen have negative correlations with the $\delta^{13}C$ values of LF and PF relative to bulk carbon (see below).

Both HIF and AIF have only slightly higher δD and $\delta^{13}C$ values relative to the isotopic compositions of bulk organic matter (Table 1). This is because bulk organic matter is composed mainly of HIF and AIF, the major components of which is probably high-molecular-weight cellulose (Epstein *et al.*, 1976), and is consistent with plant leaves being composed mainly of carbohydrates (Hunt, 1995). The bulk and the 5 sub-fractions have a good positive correlation ($r^2 > 0.95$) between δD and $\delta^{13}C$ (Fig. 2) within each sample. $\delta D/\delta^{13}C$ slopes are clearly different (25 to 42 for C_3 , 11 to 13 for C_4 , and 17 to 21 for CAM plants) using the δD - $\delta^{13}C$ diagram. In C_3 plants (Fig. 2a), annual deciduous species (filled symbols) have higher $\delta D/\delta^{13}C$ slopes (35 to 42) than the other species (open symbols, 25 to 28). All types of photosynthesis can be discriminated with this diagram, in contrast to the use of carbon isotopes only, which cannot easily distinguish CAM plants from C_3 or C_4 plants (for example, *Ananas* from C_4 plants). Since CAM plants use two photosynthetic pathways (i.e., C_3 and C_4) depending on environmental conditions such as light changes (Lerman and Queiroz, 1974), this isotopic rela-

Fig. 2. Organic δD - $\delta^{13}C$ diagrams of several compound fractions consisting of terrestrial plant leaves. a) C_3 plants, filled circle: *Quercus* (slope: 35, $r^2 = 0.95$); open circle: *Camellia* (slope: 25, $r^2 = 0.99$); filled triangle: *Pinus* (slope: 42, $r^2 = 1.00$); open triangle: *Chamaecyparis* (slope: 28, $r^2 = 0.98$); filled square: *Manihot.* (slope: 36, $r^2 = 0.96$). b) CAM plants, open triangle: *Ananas* (slope: 21, $r^2 = 1.00$); filled triangle: *Hymenocallis* (slope: 20, $r^2 = 0.98$). c) C_4 plants, filled circle: *Saccharum* (slope: 13, $r^2 = 0.98$); open circle: *Sorghum* (slope: 12, $r^2 = 0.99$); filled square: *Tripsacum* (slope: 12, $r^2 = 0.99$); open square: *Zoysia* (slope: 11, $r^2 = 1.00$).

Fig. 3. Organic hydrogen and carbon isotopic compositions of lipid (LF) and pigment fractions (PF) relative to those of bulk hydrogen and carbon. Circle, triangle and square symbols represent C_3 , CAM and C_4 plants, respectively. Filled and open symbols, showing straight and broken lines for regression calculations (except *Chamaecyparis*), represent samples from Japan and Thailand, respectively.

tionship of hydrogen and carbon may a useful means to evaluate the relative importance of C_3 vs. C_4 metabolism for these plants.

Relative to both bulk δD and $\delta^{13}C$ values (probably representative values of carbohydrate), PF is generally less depleted in both D and ^{13}C than LF (Fig. 3). This may indicate two distinctive hydrogen and carbon pools for each lipid and pigment biosynthesis. The porphyrin framework of chlorophyll (the dominant component of PF) is synthesized from succinyl-CoA and glycine, while lipid components are synthesized from acetyl-CoA (Conn *et al.*, 1987). The different starting compounds may have isotopically different compositions, because amino acids are usually enriched in ^{13}C relative to bulk organic carbon (Degens, 1969). Or both hydrogen and carbon have the isotopically same pools, but different fractionation factors during lipid or pigment syntheses.

Isotopic fractionation during lipid and pigment biosynthesis is a kinetic process (DeNiro and Epstein, 1977). If the kinetic isotope effect causes preferential ^{12}C -condensation during C-C combination, it can be applicable to H-condensation during C-H combination. In such case, the slope of the $\Delta D/\Delta^{13}C$ ($\delta D_{LF-Bulk}/\delta^{13}C_{LF-Bulk}$ or $\delta D_{PF-Bulk}/$

$\delta^{13}C_{PF-Bulk}$) must be positive. However, $\Delta D/\Delta^{13}C$ of this study are negative for both lipid and pigment syntheses. The negative $\Delta D/\Delta^{13}C$ may infer a partial dehydrogenation which causes the residual hydrogen to be enriched in D during the C-H cleavage reaction. Terpenoid and porphyrin are synthesized from intermediate molecules such as geranyl pyrophosphate and porphobilinogen, respectively (Conn *et al.*, 1987). Hydrogen atoms are dehydrogenated or dehydrated from the intermediate molecules in contrast to carbon coupling through each reaction pathway. Dehydrogenation involving kinetically preferential H elimination during lipid and chlorophyll synthesis may be probable to control the isotope distributions.

Figure 3 also shows that the δD and $\delta^{13}C$ values of LF and PF relative to bulk organic matter are systematically variable regardless of any photosynthetic metabolisms (except for *Chamaecyparis*). These isotopic distributions suggest that terrestrial plants with different photosynthetic metabolisms utilize similar enzymes but different isotope effects for lipid and pigment biosyntheses. The isotopic discrimination lines differ slightly between Japan and Thailand samples (Fig. 3). Such a difference is probably attributable to physiological response to different environment such as temperature and/or humidity. One exceptional isotopic composition for the lipid component (*Chamaecyparis*) may suggest a different pathway during the lipid synthesis. Compound-specific hydrogen and carbon isotope analyses will be promising to clarify the biosynthesis mechanism in detail.

CONCLUSIONS

There is a good correlation between δD and $\delta^{13}C$ values of several compound fractions of terrestrial plant leaves. The slope values of $\delta D/\delta^{13}C$ clearly discriminate the photosynthetic pathways (C_3 , C_4 and CAM). The δD values of lipid and pigment fractions relative to bulk organic matter are negatively correlated with the $\delta^{13}C$ values of corresponding fractions regardless of the three photosynthetic metabolisms. In addition to the

previous classification of C₃, C₄ and CAM plants using $\delta\text{D}-\delta^{13}\text{C}$ values of cellulose nitrate by Sternberg *et al.* (1984), this study also indicates that $\delta\text{D}-\delta^{13}\text{C}$ relationships of several organic fractions provide important information on classification of photosynthesis as well as lipid and pigment biosyntheses. The organic $\delta\text{D}-\delta^{13}\text{C}$ relationship will also be a novel means to study the sources as well as geochemical cycles of organic compounds.

Acknowledgments—Thailand leaf samples are kindly donated by S. Ando of Japan International Research Center for Agricultural Sciences and Dr. T. Yoneyama of National Agricultural Research Center. The authors are grateful to Profs. S. R. Poulson, O. Matsubaya, R. Ishiwatari and T. Yoneyama for invaluable comments. This work was supported by the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (Y.C.) and the Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture (H.N.).

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