Hydrogen isotopic differences between C 3 and C 4 land plant lipids: consequences of compartmentation in C 4...

Article in Plant Cell and Environment · August 2016
DOI: 10.1111/pce.12821

CITATIONS
2

READS
134

6 authors, including:

Youping Zhou
Baker IDI Heart and Diabetes Institute
13 PUBLICATIONS  415 CITATIONS
SEE PROFILE

Charles H Hocart
Australian National University
85 PUBLICATIONS  2,219 CITATIONS
SEE PROFILE

Arthur Gessler
Swiss Federal Institute for Forest, Snow and…
214 PUBLICATIONS  6,270 CITATIONS
SEE PROFILE

Graham D Farquhar
Australian National University
392 PUBLICATIONS  59,930 CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:

- Permian-Triassic boundary View project
- Bridging in Biodiversity Science (BIBS) - Novel ecosystems View project

All content following this page was uploaded by Youping Zhou on 04 October 2016.
The user has requested enhancement of the downloaded file. All in-text references underlined in blue are added to the original document and are linked to publications on ResearchGate, letting you access and read them immediately.
Hydrogen isotopic differences between C₃ and C₄ land plant lipids: consequences of compartmentation in C₄ photosynthetic chemistry and C₃ photorespiration

Youping Zhou¹²₄₆, Kliti Grice², Hilary Stuart-Williams³, Charles H. Hocart³, Arthur Gessler⁴₅ & Graham D. Farquhar³

¹School of Chemistry and Chemical Engineering, Shaanxi University of Science and Technology, Xi’an 710021, China, ²WA-Organic and Isotope Geochemistry Centre, The Institute for Geoscience Research, Curtin University, Perth 6845, Australia, ³Research School of Biology, Australian National University, Canberra 2601, Australia, ⁴Institute for Landscape Biogeochemistry, ZALF, Müncheberg 15374, Germany, ⁵Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf 8903, Switzerland and ⁶Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

ABSTRACT

The $\delta^{2}H/\delta^{1}H$ ratio of carbon-bound H in biolipids holds potential for probing plant lipid biosynthesis and metabolism. The biochemical mechanism underlying the isotopic differences between lipids from C₃ and C₄ plants is still poorly understood. GC-pyrolysis-IRMS (gas chromatography-pyrolysis-isotope ratio mass spectrometry) measurement of the $\delta^{2}H/\delta^{1}H$ ratio of leaf lipids from controlled and field grown plants indicates that the biochemical isotopic fractionation ($\epsilon^{2}H_{lipid, biochem}$) differed between C₃ and C₄ plants in a pathway-dependent manner: $\epsilon^{2}H_{C₄} > \epsilon^{2}H_{C₃}$ for the acetogenic pathway, $\epsilon^{2}H_{C₄} < \epsilon^{2}H_{C₃}$ for the mevalonic acid pathway and the 1-deoxy-D-xylulose 5-phosphate pathway across all species examined. It is proposed that compartmentation of photosynthetic CO₂ fixation into C₄ mesophyll (M) and bundle sheath (BS) cells and suppression of photorespiration in C₄ M and BS cells both result in C₄ M chloroplastic pyruvate – the precursor for acetogenic pathway – being more depleted in $\delta^{2}H$ relative to pyruvate in C₃ cells. In addition, compartmentation in C₄ plants also results in (i) the transferable H of NADPH being enriched in $\delta^{2}H$ in C₄ M chloroplasts compared with that in C₃ chloroplasts for the 1-deoxy-D-xylulose 5-phosphate pathway pathway and (ii) pyruvate relatively $\delta^{2}H$-enriched being used for the mevalonic acid pathway in the cytosol of BS cells in comparison with that in C₃ cells.

Key-words: C₃ photorespiration; C₄ compartmentation; isotope effect; lipid biosynthesis; natural abundance hydrogen isotope composition.

INTRODUCTION

A clear understanding of the factors that contribute to the natural hydrogen isotopic makeup ($\delta^{2}H/\delta^{1}H$ ratio) of carbon-bound hydrogen of lipids from land plants is important as a tool in diagnostic and predictive studies of plant physiology (Yakir 1992). This importance extends to metabolism and biosynthesis (Zhang et al. 2009a, 2009b; Zhou et al. 2010, 2015), agriculture and food (Billault et al. 2001; Zhang et al. 2002) and environment and climate (Schefuß et al. 2005; Farquhar et al. 2007; Sachse et al. 2012). Plant photosynthates incorporate H from water, the $\delta^{2}H/\delta^{1}H$ ratio of which is determined by climatic conditions such as temperature and humidity (Dansgaard 1964) and geographical conditions, such as topography and soil physical and chemical properties. More elaborate molecules that are synthesized post-photosynthetically, including lipids, are imprinted with both photosynthetic and biosynthetic pathway-specific isotopic modifications (Chikaraishi et al. 2004; Sachse et al. 2012).

While the physical and biophysical processes governing water isotopic modification in soils (absorption, diffusion and evaporation) and leaves (evapotranspiration) are relatively well understood (Barnes & Allison 1983; Farquhar & Gan 2003; Gan et al. 2003), the biochemical reactions that incorporate leaf water H and later modify the metabolites isotopically remain relatively poorly understood (Hayes 2001; Wang et al. 2013). For example, the fundamental reason for lipids being depleted relative to the source water, despite evaporative enrichment of $\delta^{2}H$ in leaf water, is thought to be the incorporation of $\delta^{2}H$-depleted transferable H atoms from NADPH (Sachse et al. 2012). The isotopic composition of this hydrogen is unknown, although it is believed to be derived from the highly depleted free-proton pool in the chloroplast stroma (Luo et al. 1991; Hayes 2001; Schmidt et al. 2003). In studying natural abundance metabolite $\delta^{2}H/\delta^{1}H$ ratios, efforts have been made to devise in vitro experiments to understand isotopic fractionation through the steps leading to lipid biosynthesis (Robins et al. 2003; Baillif et al. 2009).

Unfortunately, attempts to relate lipid isotopic composition to such in vitro biochemical reactions are fraught with uncertainty as the in vivo isotope effects are influenced by the relative rates of competing metabolic processes (Hayes 2001; Schmidt et al. 2003). One way to tackle this problem is to take a comparative biochemical approach, that is to compare isotopic compositions of lipids synthesized via different pathways in both C₃ and C₄ plants (Chikaraishi et al. 2004;
et al. 2001; Sachse et al. 2012). This is because the three independent lipid pathways, namely the aliphatic lipid-synthesizing acetogenic (ACT) pathway, the sterol-synthesizing mevalonic acid (MVA) pathway and the phytol-synthesizing 1-deoxy-D-xylulose 5-phosphate (DXP) pathway are present in both C₃ and C₄ plants (see the representative structures of the lipids synthesized via the three pathways in Fig. 1, also the compounds 27a–30a in Fig. 2 and 27b–30b in Fig. 3A). For the convention of numbering these molecules, see the legend in Fig. 2) (Eisenreich et al. 2001; Sachse et al. 2012). The lipids all share common precursors, and all occur in distinct cellular compartments.

In C₃ plants, photosynthetic biochemistry occurs exclusively in mesophyll (M) cells (Fig. 2), while in C₄ plants, it is compartmented into anatomically distinct M cells and bundle sheath (BS) cells (Fig. 3A, NADP-ME type, and Fig. 3B, NAD-ME type). Thus, it might be expected that leaf water hydrogen isotopic enrichment and that of the transferable hydrogen atoms of the NADPH pool, which are two of the three contributors to the hydrogen in lipid molecules (the third being the biosynthetic precursor) (Sessions et al. 1999; Zhou et al. 2010), would be different in the C₄ BS compared with the C₃ M, with the isotopic composition of BS cellular water close to that of soil water and M cellular water closer to that at the sites of evaporation (Smith et al. 1991). In a C₄ BS cell, photorespiration is largely but not totally inhibited (see Berry & Farquhar 1978). The common precursor for ACT and DXP pathways, pyruvate (3e in Fig. 3A) in the M chloroplast in C₄ plants, is derived predominantly from the BS chloroplastic phosphoglyceric acid (PGA; 2g in Fig. 3A) pool generated by carboxylation of ribulose-1,5-bisphosphate (1b). As oxidation of ribulose-1,5-bisphosphate during photorespiration in a C₃ cell also influences the isotopic composition of the C₃ chloroplastic PGA (2 in Fig. 2) pool, it is possible that photorespiration also manifests itself in the ²H/¹H ratio of lipids.

To understand the fundamental biochemical basis of the differences in lipid hydrogen isotope ratios between C₄ and C₃ plants, controlled growth experiments were first conducted wherein different combinations of environmental parameters [temperature, humidity, (CO₂), photosynthetically active radiation and water supply] were used to influence the isotopic compositions of leaf water and metabolites in C₃ and C₄ species. Such growth experiments ensure that environmental effects that may exaggerate or mask the lipid isotopic differences between C₃ and C₄ plants can be averaged out, thus facilitating the formulation of a general biochemical explanation for the isotopic differences observed. Our results from the controlled growth experiments show that the biochemical isotopic fractionation (ε²Hₐ⁻l lipid/biochem lipid versus available leaf water) differences can be tied to photorespiration in C₃ cells and compartmentation of photosynthetic chemistry in C₄ cells. In the course of publishing these results, the recent work of Gao et al. (2014) and Liu et al. (2016) came to our attention (see also Liu et al. 2006; Liu & Yang 2008); the apparent fractionation (ε²Hₐ⁻alkane apparent) between leaf wax n-alkanes and source water (xylem water or averaged soil water) is tied to phylogeny, that is on average, the ε²Hₐ⁻alkane apparent of eudicots is smaller than that of monocots and the effect of photosynthetic mode is subordinate to lineage. To resolve this issue, we complemented our controlled growth experiments with a sampling campaign to collect field grown monocotyledonous C₃ and C₄ grass leaves from tropical Hainan Island (China). Results from the complementary measurements further strengthened our argument that C₃ photorespiration and C₄ compartmentation are the major, rather than subordinate, causes of C₃-versus-C₄ isotopic differences in lipids.

**MATERIALS AND METHODS**

**Growth experiments**

Controlled growth experiments with three species of plants with C₃ photo-assimilation (Nicotiana tabacum, Gossypium hirsutum and Ricinus communis) and three species of plants with C₄ photo-assimilation (Sorghum bicolor, Zea mays and Panicum coloratum) were conducted in environment-controlled growth chambers located at The Australian National University, Canberra (Australia). The growth conditions and leaf water are presented in Table 1. Details of the experimental design and environmental controls are reported in full in Zhou (2005) and in part in Grice et al. (2008) and Zhou et al. (2011).

**Field grown monocotyledonous grasses**

Whole leaves of monocotyledonous grasses representing 10 C₃ and 14 C₄ (including nine NADP-ME, one NAD-ME, three PCK and one uncertain C₄ subtype) were sampled from tropical Hainan Island. Detailed site conditions are listed in Table 2. All species sampled were perennials except Saccharum officinarum, which is an annual NADP-ME. Sampling was conducted between 1100–1500 h on sunny days in late June 2015 to ensure that leaf water had reached steady state under field evaporative conditions (Cernusak et al. 2002). Leaves were cut off from randomly chosen plants with scissors. After immediate removal of the main veins, the leaves were swiftly sealed in pre-chilled Falcon centrifuge tubes (CNWTC, Jiangsu, China) with Parafilm (Bemis, NA) and stored in dry ice before being transported to a laboratory refrigerator for further storage before water extraction. Although stem/culm...
Figure 2. C₃ photosynthetic biochemistry highlighting the histories of H from RuBP [ribulose-1,5-bisphosphate (1a)] for the three lipid classes (represented by alkanes, sterols and phytol) and glucose. Colour-coding is used to distinguish different cellular compartments: white: stroma of chloroplast; grey: cytosol; red: mitochondrion; yellow: peroxisome; H₂O: (chloroplastic) stromal water. H₂O: cytosolic water. For clarity, only steps where incorporation of H from either cellular water H (H₂O or H₂O) or NADPH [nicotinamide adenine (diphosphate)] occurs are illustrated. The Calvin–Benson–Bassham cycle (CBB) is enclosed in the bright yellow dotted polygon. Oxidative pentose phosphate pathway (OPPP) is enclosed in a red dashed polygon (only the NADPH-generating steps are shown); photorespiration, which spans chloroplastic stroma, cytosol, peroxisome and mitochondrion, is enclosed in the bright blue dotted polygon; the movement of H⁺ from the stroma to the lumen across the thylakoid membrane is via a redox reaction between plastoquinone oxidized (PQ) and plastoquinone reduced (PQH₂). Abbreviations are as follows: GAPDH: glyceraldehyde phosphate dehydrogenase; PGA: phosphoglyceric acid; RubisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; DHAP: dihydroxyacetone phosphate; TPI: triose phosphate isomerase; GAP: glyceraldehyde 3-phosphate; F-1,6-βP: fructose-1,6-bisphosphate; PGI: phosphoglucose isomerase; PS I(II): photosynthesis system I(II); TCA: tricarboxylic acid. THF-C₁H₁βH: tetrahydrofolate-C₁H₁βH; GGT: glutamate-glyoxylate transaminase; HPR: hydroxypyruvate reductase; GDC: glycine decarboxylase complex; SHMT: serine hydroxymethyltransferase; GGT: glyoxylate glycine aminotransferase. Chloroplastic NADPH is the sole source of reducing equivalent for DXP and ACT pathways up to the synthesis of 18-carbon fatty lipid chain length. The MVA pathway mainly utilizes NADPH generated from the oxidative pentose phosphate pathway, not shown (OPPP). Compounds are numbered approximately sequentially in relation to their metabolism from RuBP (1). Pools of compounds that are distinct, for example within a defined compartment or from a different metabolic origin, are additionally lettered. C-atoms in RuBP (1a) are numbered following the organic chemistry convention. Thus, PGA is compound 2, with 2a being the PGA derived from the positions C1 and C2 of RuBP plus CO₂, and 2b being the PGA derived from the C3, C4 and CS of RuBP (1a). Similarly, 2d is PGA derived from the glyoxylate cycle, for which the relationship of the C atoms to RuBP (1a) differs from that in 2a and 2b. The relationship of the C atoms to RuBP (1a) is marked for each position. For example, in GAP (5a), C₁₅ means that this C has equal contributions from C1 and C5 of RuBP (1a). The relationship of the C atoms to RuBP (1a) is marked for each position. For example, H₁ refers to one of the two H-atoms attached to C1 of RuBP (1a). Hence, they can be traced back to RuBP (1a). For example, the H connected to C₂₄ (written as H₁₂8) means that it has a contribution (but not necessarily an equal one) from H₁ and H₂ of the original RuBP, and β refers to the pro-R and pro-S positions in all compounds except in serine (25), hydroxypropionate (26) and PGA (2d) where H₁² occupies the pro-R position (Hanson 1984). Again, this notation relates the H atoms back to those in RuBP (1a). The conversion of glycine (22) to serine (25) involves first the decarboxylation of glycine to give NH₃, CO₂, NADH and a –CH₂– unit temporarily captured by tetrahydrofolate (THF) under the catalysis of GDC: glycine + NAD⁺ + THF →N⁺₂N⁺₃-methylene-THF + NADH + H⁺ + C₂O and subsequent N⁺₂N⁺₃-methylene-THF reaction with another molecule of glycine (22) to give serine (25): N⁺₂N⁺₃-methylene-THF + glycine → serine + THF under the catalysis of the GDC/SHMT complex.

© 2016 John Wiley & Sons Ltd, Plant, Cell and Environment.
Figure 3. (a) Compartmentation of C₄ photosynthesis into M and BS cells in a typical C₄ NADP-ME plant metabolism. In the NADP-ME C₄ type (represented by Z. mays and S. bicolor in this study), the malate (32a)-pyruvate (3e) shuttle is the dominant shuttle between the M and BS with a high flux indicated by a thick arrow. The PGA (2g) and DHAP (6d) in the M cell form an integral part of the Calvin–Benson–Bassham cycle (CBB) in the BS cell. For each complete shuttle, one of the three H-atoms in the M chloroplastic pyruvate (3e), the precursor for the synthesis of C₁₈ fatty acids in the M chloroplast, is replaced with BS chloroplastic H₂O (Hb). Eventually, nearly all H-atoms in pyruvate will be replaced with BS chloroplastic H₂O. NADPH produced in M chloroplasts (NADPHₘ) is shuttled to a BS chloroplast to meet the high demand for reducing power in the BS chloroplast because of the absence of PS II. The consequence of this NADPH shuttle is that the residual NADPH in an M cell chloroplast is enriched relative to its C₃ plant counterpart as a result of a high KIE associated with NADPH-ME (NADP malic enzyme) activity (Hermes et al. 1982; Grissom & Cleland 1985). Pyruvate (3f) in the BS cytosol, the precursor for sterol (30a) synthesis via the MVA pathway, is derived from the relatively enriched H₁,H₆-proR and H₆-proS in the glucose (Schleucher et al. 1999; Zhang et al. 2002). Although the majority of the DHAP (6d) and GAP (5e) in BS cells is shuttled from M cells, some of it is presumably generated from within BS by reducing PGA (2g) with a mixture of BS NADPHₘ and NADPHₜ, as comparative proteomics does show that GAPDH (the activity of which is indicated by the broken arrow between PGA (2g) and GAP (5e)), TPI and PGI (Majeran et al. 2005) are present. Therefore, GAP (5e) and DHAP (6d) and compounds downstream of them carry H atoms from NADPHₘ and NADPHₜ. The movement of H⁺ from stroma to lumen is omitted to improve the readability of the drawing. Abbreviations are as follows: NADP-ME: NADP malic enzyme; OAA: oxaloacetate; PEPC: phosphoenolpyruvate carboxylase; NADP-MDH: NADP malate dehydrogenase; TPI: triose phosphate isomerase; NDH: NADPH dehydrogenase; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde phosphate; F₆P: fructose-6-phosphate; G₆P: glucose-6-phosphate; F₁,6-bisP: fructose-1,6-bisphosphate. The numbering and lettering of the C and H-atoms follow the rules described in Fig. 2. (b) Compartmentation of C₄ photosynthesis into M and BS cells in a typical C₄ NAD-ME plant, represented by P. coloratum in this study (Edwards & Walker 1983; Edwards & Voznesenskaya 2011). In this C₄ type, the aspartate (33a)-alanine (34a) shuttle is the dominant shuttle between the M and BS; the PS IIIs in the chloroplasts of both cell types are competent to generate NADPH, and therefore, no NADPH is transported across the two cell types; no suberin lamella exists between M and BS cells that may restrict the flow of water between them. Reduction of OAA (31b) to malate (32b) occurs in BS mitochondria using NADH rather than NADPH. Subcellular locations of lipid biosynthesis in this subtype are currently unclear but are thought to be the same as those in the C₃ cells. It is also believed that both cell types are capable of synthesizing all three classes of lipids, without clear dominance of one subtype over the other. Abbreviations are as follows: NAD-ME: NAD malic enzyme; NAD-MDH: NAD malate dehydrogenase. Other abbreviations are as in Fig. 3A. The numbering and lettering of the C and H-atoms follow the rules described in Fig. 2.
water is the immediate source for leaves, the fact that few grass species have easily identifiable stem/culm and that the grass stem/culm can also be subjected to evaporative enrichment (McInerney et al. 2011) makes it a less than ideal candidate for source water extraction. Consequently, we used a hand-operated soil corer to collect depth-integrated soil samples (after removing the plant debris on the soil surface) near the plants to a depth of 30 cm and took this soil water as source water. The retrieved soils were sealed in pre-chilled Falcon centrifuge tubes with Parafilm and stored in dry ice in the field before being transported back to lab for further storage before water extraction. As grasses normally have a shallow rooting system, 30 cm is enough to capture the average water used by plants (Grieu et al. 2001). To remove spatial heterogeneity of leaf water, wax and soil water isotopic compositions, three sets of soil and leaf samples were taken from three sites roughly 50–100 m apart. After cleaning, drying and powdering, leaf wax together with leaf water and soil water was extracted following the procedure outlined in the next section (Isotopic Sampling); the respective extracts were then mixed to make a

Figure 3. (Continued).
Table 1. Environmental parameters for the controlled growth experiments and $\delta^{2}H$ & $\varepsilon^{2}H$ of $n$-alkanes, phytol and sterols for the three species of C$_3$ plant and three species of C$_4$ plant

| Species and photosynthetic modes | PAR $\mu$mol photons m$^{-2}$ s$^{-1}$ | [CO$_2$] ppm | Leaf temperature (°C) | Relative humidity (%) | Irrigation | Leaf $\delta^{2}H$ (%) of water | $\delta^{2}H$ $n$-alkane (ACT) | $\varepsilon^{2}H$ $n$-alkane (ACT) | $\delta^{2}H$ sterols (MVA) | $\varepsilon^{2}H$ sterols (MVA) | phytol (DXP) | $\delta^{2}H$ DXP- phytol (DXP) |
|---------------------------------|---------------------------------------|--------------|------------------------|----------------------|------------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------|------------------------------|
| Sorghum bicolor (C$_4$, NADP-ME) | 500 400                              | 32.9         | 65                     | -28                  | 12         | -119                           | -141                         | -152                         | -124                         | 188                         | -186           | -188                         |
| Zea mays (C$_4$, NADP-ME)       | 450 400                              | 33.0         | 64                     | -28                  | 8          | -94                            | -111                         | -107                         | 125                         | 146                         | -138           | -138                         |
| Panicum coloratum (C$_4$, NAD-ME) | 500 420                              | 32.1         | 60                     | -34                  | 13         | -118                           | -126                         | -122                         | 154                         | 146                         | -138           | -138                         |
| NADP-ME                        |                                        |              |                        |                      |            | 152±23                         | 198±12                       | 277±66                       | 152±23                     | 198±12                      | 277±66         | 152±23                      |
| NAD-ME                         |                                        |              |                        |                      |            | 153±3                           | 204±6                        | 240±86                       | 153±3                     | 204±6                       | 240±86         | 153±3                      |
| C$_4$ average                  |                                        |              |                        |                      |            | 152±23                         | 198±12                       | 277±66                       | 152±23                     | 198±12                      | 277±66         | 152±23                      |
| C$_3$ average                  |                                        |              |                        |                      |            | 153±3                           | 204±6                        | 240±86                       | 153±3                     | 204±6                       | 240±86         | 153±3                      |

[a]: average and standard deviation of $\delta^{2}H$ and $\varepsilon^{2}H$; C$_{28}$Δ$^5$: campesterol (24-methylcholest-5-en-3β-ol); C$_{29}$Δ$^5,22$: sigmasterol (24-ethylcholesta-5,22-dien-3β-ol); Refer to Fig. 1 for the representative structures.

C$_{29}$Δ$^5$: β-sitosterol (24-ethylcholesta-5-en-3β-ol). PAR: photosynthetically active radiation. ‘-’ means no isotopic value has been obtained due to insufficient materials for analysis.

All leaf water and lipids $\delta^{2}H$ data are the averages of $\delta^{2}H$ for 3–5 randomly chosen individual plants of each species. 1 σ of duplicated or triplicated analysis is smaller than 3‰ for all species and all treatments. The calculation of weighted average $\delta^{2}H$, which is used for the calculation of weighed average $\varepsilon^{2}H$ for alkanes, follows the same mathematical equation as outlined in Table 2.
| Species                                | Photosynthetic modes | δ^2H_{soil water} | δ^2H_{leaf water} | δ^2H | n-Alkanes (ACT) | Phytol (DXP) | Site names and geographical coordinates |
|----------------------------------------|----------------------|-------------------|-------------------|------|----------------|--------------|----------------------------------------|
| Saccharum officinarum                  | NADP-ME              | -58               | 2                 | 26   | -145 -177 -177 -182 -171 -166 -147 -174 | 241 -286 -456 | Toyang 19°17.11.81" 110°30.26.55" 17 |
| Emmeropsis atroiret                     | PCK                  | -47               | -4                | 23   | -166 -176 -180 -190 -182 -164 -183 | 252 -240 -346 | Lukiou 19°17.31.89" 109°32.12.54" 4   |
| Zoysia matrella                         | NADP-ME              | -55               | -9                | 12   | -137 -154 -161 -176 -188 -179 -180 | 235 -251 -351 | Shime 18°40.07.07" 110°16.03.90" 18  |
| Sporobolus virginicus                   | PCK                  | -55               | -9                | 17   | -1 -171 -173 -175 -166 -185           | 248 -252 -360 | Shime 18°40.07.07" 110°16.03.90" 18  |
| Digitaria violascos                     | NADP-ME              | -58               | -11               | 1    | -153 -170 -189 -196 -177 -177 -191 | 237 -259 -351 | Yinggeling 19°01.56.48" 109°32.08.53" 904 |
| Aplada mutica                          | NADP-ME              | -53               | -5                | 6    | 18.6 -252 -174 -166 -177 -180 -175   | 225 -231 -308 | Meilan 19°56.57.90" 110°52.20.03" 16 |
| Pennisetum alopecuroides               | NADP-ME              | -49               | -10               | 0    | 21.1 -168 -204 -207 -204 -195 -192 -180 | 249 -241 -318 | Meilan 19°57.09.70" 110°21.03.8" 16  |
| Bambusa chungii                        | NADP-ME              | -57               | -15               | -5   | 20.2 -178 -244 -256 -234 -210 -192   | -234 -264 -353 | Yinggeling 19°01.56.48" 109°32.08.53" 904 |
| Themeda villosa                        | NADP-ME              | -64               | -7                | 11   | 23.4 -160 -169 -185 -188 -191 -17 -184 | 238 -265 -375 | Yinggeling 19°01.56.48" 109°32.08.53" 904 |
| Carex nomenclostachys                   | ?                    | -56               | -17               | -3   | 26.2 -160 -196 -201 -201 -186 -195   | 238 -262 -350 | Diaolo 18°47.36.79" 109°32.34.31" 251 |
| Imperata flava                         | NADP-ME              | -59               | -21               | -4   | 30.4 -185 -183 -198 -198 -192 -173 -155 -219 | 233 -262 -350 | Maoyang 18°56.16.07" 109°30.19.99" 255 |
| Neyraudia reynaudiana                  | PCK                  | -59               | -18               | 0    | 30.4 -154 -169 -187 -197 -203 -203 -178 -219 | 251 -263 -357 | Maoyang 18°56.16.07" 109°30.19.99" 255 |
| Digitaria heterantha                   | NADP-ME              | -53               | -7                | 4    | 19.4 -222 -193 -190 -187 -192 -187 -179 -208 | 237 -262 -361 | Meilan 19°56.57.90" 110°52.03.04" 16  |
| Scleria parviflora                     | -44                  | 5                 | 17                | 19.7 | -208 -221 -220 -208 -205 -196 -215 | 296 -272 -398 | Meilan 19°56.57.90" 110°52.03.04" 16  |
| C4 average                             |                      | 55                | 9                 | 7    | 26 (±7) -138 -148 -183 -190 -199 -192 -192 | 249 (±22°) -259 (±16°) -360 (±35°) | Shime 18°40.07.07" 110°46.03.90" 18  |
| Phyllostachys heteroclada               | C3                   | -55               | -6                | -3   | 7.3 -138 -148 -183 -190 -199 -192 -192 | 235 -293 -410 | Shime 18°40.07.07" 110°46.03.90" 18  |
| Oplolenus compositus                   | C3                   | -56               | -9                | -5   | 7.9 -149 -166 -175 -179 -184 -175 -160 -176 | 207 -336 -500 | Diaolo 18°43.42.28" 109°52.02.05" 251 |
| Scleria levis                          | C3                   | -38               | -3                | 0    | 6.9 -140 -147 -173 -175 -163 -141 -92 -169 | 204 -292 -413 | Sanjiang 18°52.10.75" 110°27.43.54" 11 |
| Lophatherum gracile                    | C3                   | -53               | -21               | -17  | 11.3 -136 -187 -187 -196 -182 -179 -177 -183 | 203 -297 -397 | Diaolo 18°47.36.79" 109°52.34.31" 251 |
| Arundo donax                           | C3                   | -53               | -13               | -11  | 6.6 -156 -178 -194 -205 -202 -200    | 237 -276 -367 | Meilan 19°56.57.90" 110°52.03.04" 16  |
| Oryza rafipogon                        | C3                   | -35               | -15               | -14  | 7.1 -164 -183 -201 -220 -198 -171 -203 | 237 -354 -527 | Meilan 19°56.57.90" 110°52.03.04" 16  |
| Carex longipetolata                    | C3                   | -56               | -9                | -5   | 9.2 -149 -151 -166 -180 -182 -167    | 206 -281 -384 | Diaolo 18°43.36.68" 109°52.03.04" 251 |
| Sphaerocharum malacense                | C3                   | -56               | -10               | -7   | 7.1 -140 -145 -186 -169 -175 -164    | 189 -304 -427 | Diaolo 18°43.36.68" 109°52.03.04" 251 |
| Cyrtococcum oxypilum                   | C3                   | -56               | -13               | -10  | 8.2 -160 -159 -165 -180 -195 -189    | 216 -347 -451 | Diaolo 18°43.42.28" 109°52.02.05" 251 |
| C3                                    |                      | -70               | -16               | -12  | 6.8 -165 -159 -168 -182 -179 -171   | 195 -288 -389 | Diaolo 18°43.42.28" 109°52.02.05" 251 |

(Data continues)
Table 2. (Continued)

| Species | C25 | C27 | C29 | C31 | C33 | C35 | C37 | C25 C27 C29 C31 C33 C35 C37
|---------|-----|-----|-----|-----|-----|-----|-----| C25 C27 C29 C31 C33 C35 C37
| α-Alkanes (ACT) | δHleaf water | δHsource | δHmeasured | δHcorrected |
| Phytol (DXP) | δHleaf water | δHsource | δHmeasured | δHcorrected |
| Site names and geographical coordinates | Name | Lat. (E) | Longit. (N) | Alt. (m, asl) |

| Centotheca lappacea | 2Hαn | 2HACT | δε | 2HDXP | 2HDXP | δε | 2HDXP | 2HDXP |
|---------------------|------|------|-----|-------|-------|-----|-------|-------|
| Hsoil-photosynthetic modes | Measured | Corrected | Φ | δε | 2HACT | δε | 2HDXP | δε |

| Φ | δε | 2HACT | δε | 2HDXP | δε |
| (±10)* | (±5)* | (±5)* | (±5)* | (±5)* |

- Average and standard deviation of δHcorrected for leafwater, soil water and lipids is better than 3‰ for all species analyzed.

- The isotopic composition of M water is calculated according to the following equation:

\[
\delta H_{corrected} = \frac{\delta H_{measured} - \Phi \delta H_{source}}{1 - \Phi}
\]

where \(\delta H_{corrected}\), \(\delta H_{measured}\), \(\delta H_{source}\) and \(\Phi\) are the true and measured isotopic compositions of water directly available for metabolism in M tissue, the isotopic composition of source water and the volume proportion of the BS, respectively.

**Isotopic sampling**

Leaf water was cryogenically extracted following the removal of the main veins (Zhou et al. 2010). Extraction and isolation of leaf lipids was carried out as described by Grice et al. (2008). Briefly, 42- to 45-day-old, randomly chosen leaves of controlled grown plants were harvested (with main veins removed), cleaned with distilled water, dried and powdered before being extracted with CH\(_2\)Cl\(_2\)/CH\(_3\)OH (9/1, v/v) in a Soxhlet extractor for 48h. As plants were grown from seeds, the contribution of inherited H from seeds to lipids was negligible. Leaf water sampled at different growth stages showed insignificant variation over the entire growth period, ensuring that there was no asynchrony between leaf water composition and lipid accumulation (Sessions 2006). Sampling whole leaves removed the effects of leaf water spatial heterogeneity (Santrucek et al. 2007), and leaves of all ages were randomly sampled and mixed to avoid age- and growth-stage related differences (Zhang et al. 2009a; Sachse et al. 2010; Gessler et al. 2013; Kahmen et al. 2013a, 2013b; Tipple et al. 2013). Target compounds were recovered from extracts by separation on silica gel column chromatography according to Grice et al. (2008). Control experiments confirmed that no significant isotopic fractionation occurred during the extraction and drying procedures.
Chemical and isotopic instrumentation

Separation and identification of lipids were conducted on an Agilent GC/MS. Isotope ratio monitoring of controlled grown wax lipids was conducted on a Micromass GC/IRMS at the Western Australia Organic and Isotopic Geochemistry Centre of Curtin University following the method of Sessions et al. (1999). Analysis of lipids from field grown plant waxes was conducted on a Thermo Delta V Advantage IRMS interfaced to a Trace GC Ultra via a GC Isolink at the National Key Laboratory of Organic Geochemistry in the Chinese Academy of Sciences’ Guangzhou Institute of Geochemistry. Hydrogen isotopic compositions are reported using the standard δ-notation relative to Vienna Standard Mean Ocean Water (V-SMOW; 0‰) using

$$\delta^2H_{\text{sample}} = \frac{R_{\text{sample}} - R_{V-SMOW}}{R_{V-SMOW}} \times 1000 \text{‰},$$

where $R$ is the abundance ratio $^2H/\text{H}$. Lipid isotopic fractionation $\varepsilon^2H$ values are calculated using $\delta^2H_{\text{lipid_biochem}} = \frac{18}{12} \times \frac{1 - R_{\text{lipid_biochem}}}{1 - R_{\text{V-SMOW}}} - 1 \times 1000 \text{‰}$. This is the same form as used by plant physiologists for the fractionation of carbon isotopes during photosynthesis and which gives an effective rate constant for $^{12}\text{C}$ versus $^{13}\text{C}$ (Farquhar et al. 1989).

For analysis, phytol and sterols were acetylated [with acetic anhydride ($\text{Ac}_2\text{O}$) in pyridine] to remove exchangeable H in their $\text{–OH}$ groups prior to GC-MS and GC-pyrolysis-IRMS analyses, according to the method of Chikaraishi et al. (2004) and Zhou et al. (2011). Corrections were made for the introduced H-atoms (from $\text{Ac}_2\text{O}$). The overall analytical error was 7% (3σ average). Leaf water isotopic composition was determined according to the method of Stuart-Williams et al. (2008) and had an overall analytical precision of better than 1‰.

RESULTS

The data for measured leaf water and lipid isotopic compositions ($\delta$) and associated calculated biochemical isotopic fractionations ($\varepsilon^2H_{\text{lipid_biochem}}$; see the definition in the experimental section), together with the corrected leaf water isotopic compositions for the controlled growth experiments and for the field grown plants are given in Tables 1 and 2, respectively. The controlled growth trial lacked sufficient replication of individual growth conditions to be able to assess the effects of some parameters, for example temperature and (CO$_2$) on $\varepsilon^2H_{\text{lipid_biochem}}$ for lipids. Because assessing the effects of individual parameters on $\varepsilon^2H_{\text{lipid_biochem}}$ is not the focus of this work, we omitted such effects from this paper.

Averaged over all species and all growth conditions were examined in the controlled growth experiments; the isotopic fractionation occurring during production of lipids (n-alkanes, see 29b in Fig. 3A) synthesized via the ACT pathway in C$_4$ plants is consistently greater than in C$_3$ plants: $152 \pm 20$‰ ($1\sigma$, $n=6$) and $121 \pm 8$‰ ($1\sigma$, $n=8$) for C$_4$ and C$_3$ plants, respectively. If the C$_4$ subtypes are considered, then the values are $152 \pm 23$‰ ($1\sigma$, $n=6$) for NADP-ME and $152 \pm 3$‰ ($1\sigma$, $n=2$) for NAD-ME, respectively. This observed ACT C$_4$ versus C$_3$ metabolism difference of $\Delta \varepsilon^2H=31$‰ for n-alkanes is consistent with Chikaraishi et al. (2004) and more recent findings of Gao et al. (2014) (see Fig. 5 in this reference where eudicots represented mainly by trees exhibit a smaller fractionation than that of monocots represented mainly by grasses from both chamber-grown and field collected plants). However, direct comparison with other relevant results (Smith & Freeman 2006; Eley et al. 2014) is difficult as the fractionation calculations in those studies contained either uncertain estimated soil water isotopic compositions or failed to account for the contributions of unenriched BS cellular water in leaves of C$_4$ plants.

For the field grown grasses, the average $\varepsilon^2H$ (249 ± 21‰, $1\sigma$, $n=16$) for C$_4$ n-alkanes is greater than that of C$_3$ grasses (213 ± 18‰, $1\sigma$, $n=10$), while the average $\varepsilon^2H$ (360 ± 34‰, $1\sigma$, $n=16$) for C$_4$ n-alkanes is smaller than that of C$_3$ grasses (426 ± 52‰, $1\sigma$, $n=10$), entirely consistent with those observed with controlled grown plants, although the field-grown plants exhibit larger fractionations in both ACT and DXP pathways than those of the controlled grown plants. We did not measure the isotopic compositions of sterols (MVA pathway) as we did not invoke the M-versus-BS cellular water isotopic difference as an explanation for the sterol isotopic difference between C$_3$ and C$_4$ plants.

In C$_4$ plants, MVA pathway-derived lipids [\(\varepsilon^2H \approx 200 \pm 10\)‰ ($1\sigma$, $n=9$), represented by sterols, 30b in Fig. 3A] and DXP pathway-derived lipids [\(\varepsilon^2H \approx 264 \pm 70\)‰ ($1\sigma$, $n=9$), represented by phytol, 28b in Fig. 3A] show consistently less fractionation than their counterparts in C$_3$ plants [\(\varepsilon^2H = 250 \pm 20\)‰ ($1\sigma$, $n=8$) for MVA and 460 ± 84‰ ($1\sigma$, $n=8$) for DXP pathways, respectively]. Thus, an inter-photosynthetic mode fractionation difference (\(\Delta \varepsilon^2H_{\text{MVA, DXP}}\)) of −50‰ for the MVA pathway and −196‰ for the DXP pathway is found. If the C$_4$ subtypes are considered, the $\varepsilon^2H$ values are 198 ± 12‰ ($1\sigma$, $n=6$) for MVA and 277 ± 66‰ ($1\sigma$, $n=6$) for DXP for the NADP-ME type species and 205 ± 6‰ ($1\sigma$, $n=3$) for MVA and 240 ± 86‰ ($1\sigma$, $n=3$) for DXP for NAD-ME type species.

DISCUSSION

Because, in this paper, we take a comparative approach to understanding the factors that determine the H isotopic difference between C$_3$ and C$_4$ land plant lipids, we focus on the anatomical and biochemical features of C$_4$ plants that may have an isotopic consequence: the suberin lamella that may restrict the flow (and mixing) of water between BS and M, the shuttle between BS and M and the transport of reductive power NADPH between BS and M cells and within their respective cellular compartments. Of the two subtypes (NADP-ME and NAD-ME) of C$_4$ plants investigated here, only NADP-ME has all the features that may maximize the isotopic difference between C$_4$ and C$_3$ plant lipids: viz., (i) there exists a clear and well-defined suberin lamella between BS and M cells; (ii) photosynthetic chemistry is clearly shared between the BS and M, resulting in the need for BS–M shuttling of metabolites; and (iii) photosynthetic machineries located in

© 2016 John Wiley & Sons Ltd, Plant, Cell and Environment
BS and M are cooperative, resulting in the need for M-to-BS transport of NADPH. Consequently, in the following discussion, the comparative approach is first applied to understanding the C_{3} and C_{4} NADP-ME lipid isotopic differences. To complement such an approach, we also discuss the pathway-specific lipid isotopic difference between NADP-ME and NAD-ME subtypes in terms of their anatomical and biochemical feature differences. Because field and controlled grown plants exhibit similar patterns of isotopic fractionation across C_{3} and C_{4} plants and field measurement is obviously more prone to error because of natural variability of growth environments, we based our discussions in the succeeding texts on the results from the controlled growth experiments.

**Isotopic differences in n-alkanes (ACT pathway) between C_{3} and C_{4} plants**

In both C_{3} and C_{4} plants, the precursor for the ACT pathway is pyruvate, from which fatty acids are built by repeatedly extending the chain with acetyl-CoA units (which are also derived from pyruvate on decarboxylation and thioesterification), and n-alkanes (Fig. 1) are derived from fatty acids by decarboxylation. Compartment-specific proteomic analysis of (Majeran et al. 2005) has shown that synthesis of fatty acids occurs predominantly in the M chloroplast (Bao et al. 2000). Indeed, in C_{3} plants, the synthesis of all three classes of lipids occurs within the M cells (Fig. 2). The ACT fatty acid pathway is active first in the chloroplast with chain elongation up to 18 carbons and then continues in the cytosol (endoplasmic reticulum) where there is elongation up to a chain length of 40 carbons (Zhou et al. 2010). In C_{4} NADP-ME plants, the malate (23a)–pyruvate (3e) shuttle between the BS and M chloroplast results in a rapid exchange of the H-atoms of the methyl group of pyruvate (3e) with BS chloroplastic water. Eventually, nearly all H-atoms in pyruvate (3e) are replaced with H from the BS chloroplastic water (as the H^{2} and H^{3} derive from the H^{2}O, i.e. from the BS, Fig. 3A). The BS cell is adjacent to the vascular bundle, where water is subjected to only slight enrichment (because of the presence of the suberin lamella between M and BS cells, which vastly reduces the back-diffusion of evaporatively enriched water from the leaf M, see Fig. 3A), as opposed to M cells where evapotranspiration does cause enrichment (Smith et al. 1991). As a consequence, M cell pyruvate (3e) in C_{4} plants (which is directly shuttled in from BS cells) should be depleted in 2H relative to its C_{3} counterpart. This may explain, at least in part, why fatty lipids of C_{4} plants are more depleted in 3H than those of C_{3} plants. If this mechanism is indeed responsible for part of the differences in C_{3} versus C_{4} plants, then variations in 3H of n-alkanes could be a valuable indicator of the degree of leakiness of the suberized lamella allowing diffusion of evaporatively enriched M water to the BS. This clearly will have important consequences on the use of n-alkanes as biogeochemical markers (Sachse et al. 2012).

Theoretically, only 25% of H in fatty lipids comes directly from precursor pyruvate (3e in Fig. 3A and 3a–e in Fig. 2) (another 25% from cellular water (Zhou et al. 2010), in the case of C_{4} plants, M cellular water) and the remaining 50% from NADPH (and in the case of C_{4} plants, M NADPH). However, Sedgewick & Cornforth (1977) and Baillif et al. (2009) reported variable degrees of hydrogen exchange between malonyl-CoA (structure not shown here for clarity reasons) [when pyruvate (3e in Fig. 3A and 3a–e in Fig. 2) is converted to malonyl-CoA on the pathway to fatty lipid biosynthesis] and cellular water, which may increase the percentage of H in fatty acids derived from water from the theoretical 25% by an amount proportional to the degree of exchange. The upper limit percentage of H in fatty acids coming from water cannot be higher than 50%, even allowing for this post-malonyl-CoA exchange. n-alkanes (29b in Fig. 3A) in C_{4} plants are synthesized in M cells where cellular water is slightly enriched relative to the C_{3} M, and also NADPH is enriched compared with its C_{3} counterpart [see the section in the succeeding texts on phytol (28a in Fig. 2 and 28b in Fig. 3A) isotopic difference between C_{3} and C_{4} plants]. To account fully for the observed 3H difference of 31% between C_{4} and C_{3} plants, a minimum isotopic difference of 62‰ (when the percentage of H in fatty acid is 50%) and 124‰ (when the percentage of H in fatty acid is 25%) between BS and M cellular waters would be required. The bulk composition of lamina water in C_{4} plants is a mixture of enriched M cellular water and unenriched BS cellular water (equivalent to irrigation water). Using an average volume percentage of 26% for C_{4} BS cellular water (Hattersley 1984; Gan et al. 2003, Table 2) and using the δ of 11 ± 7‰ of lamina water in C_{4} plants (Table 1), the isotopic difference between BS and M cellular waters is calculated to be 58‰. This value is close to the required minimum isotopic difference of 62‰, when the percentage of H in fatty acid is 50%, but falls well short of the required 124‰ when the percentage of H in fatty acid coming from water is 25%, even though there is uncertainty in the estimation of the volume of BS cellular water. Given that it is unlikely that the percentage of H in fatty acids coming from water in vivo can reach the upper limit of 50%, another factor likely contributes to the observed isotopic difference between n-alkanes (29a in Fig. 2 and 29b in Fig. 3A) from C_{3} and C_{4} metabolic contexts. It is suggested that such a factor could be photorespiration.

In C_{3} plants, pyruvate (3a in Fig. 2) gives rise by decarboxylation to the activated form of acetate, acetyl-CoA (18c in Fig. 2), the immediate precursor for ACT pathway. This pyruvate (3a in Fig. 2) is synthesized from PGA (2 in Fig. 2), which has a number of potential origins, of which the primary is glycolytic cleavage of photo-assimilate in the chloroplast. Another important origin is photorespiration in the peroxisome, giving PGA (2c and 2d) derived from serine (25 in Fig. 2) via the glyoxylate cycle. In PGA (2d), two of the three carbon-bound H-atoms (H and H^{NAD}) are most likely to be enriched in 3H relative to the corresponding H-atoms in PGA (2a and 2b) generated by carboxylation because (i) the H is from cellular (peroxisomal) H$_{2}$O and (ii) the H$^{NAD}$ is the H from NADH, which is enriched by transhydrogenase when converted to NADPH (Keister et al. 1960; Bizouarn et al. 1995; Jackson et al. 1999; Zhang et al. 2009b). Note that in higher plant mitochondria, the predominant reducing equivalent is NADH (Heldt 2005) while, in microorganisms, it is NADPH.
(Zhang et al. 2009b). Conversion of the $^{2}$H$_{pro}$-R of NADH to $^{2}$H$_{pro}$-S of NADPH is predicted to have a kinetic isotope effect (KIE) similar to that of the reverse reaction in microorganisms where the $^{2}$H$_{pro}$-S of NADPH is converted to the $^{2}$H$_{pro}$-R of NADH with a KIE of 1.8 to 4.5 (Zhang et al. 2009b). The third hydrogen ($^{1}$H$_{fl}$) of PGA (2d) is, however, unchanged as the peroxisomal conversion of glycolate (20) to glyoxylate (21) is a stereospecific process wherein $^{14}$H is selectively removed (Hanson 1984). Such enrichment of $^{2}$H in PGA (2d) because of photorespiration could, at least qualitatively, account for the relative hydrogen isotopic depletion of $n$-alkanes in C$_{4}$ plants where photorespiration is largely inhibited.

Nevertheless, while it is possible to argue that the relative $^{2}$H enrichment of H and $^{2}$H$_{NAP}$ in PGA (2d) relative to the equivalent H-atoms in PGAs (2a and 2b) can be an important factor that contributes to the observed isotopic difference in $n$-alkanes between metabolism in C$_{4}$ and C$_{3}$ land plants, this is an oversimplification of the situation. It must be borne in mind that NADH in peroxisomes derives its transferable H from the malate/oxaloacetate shuttle operating across the chloroplast, mitochondrion, cytosol and peroxisome (not shown in Fig. 2 for reasons of clarity). KIEs associated with generating malate by reducing oxaloacetate (OAA) with NADPH (in the chloroplast) and NADH (in the mitochondrion), and the transfer of H from malate to hydroxypyruvate (26) to generate PGA (2d) may attenuate or even mask the previously mentioned $^{2}$H enrichment in $^{2}$H$_{NAD}$ caused by transhydrogenase. Furthermore, some peroxisomal water molecules derive their H atoms from the $^{14}$H of glycolate (20) during the glycolate oxidase reaction, a process wherein a large KIE is likely followed by the subsequent decomposition by catalase of hydrogen peroxide to water. Therefore, peroxisomal water (H) may not be fully representative of cellular water.

Isotopic differences in phytol (DXP pathway) between C$_{3}$ and C$_{4}$ plants

The DXP pathway occurs exclusively in the chloroplast. For the same reason that BS water is isotopically close to source water and less enriched relative to M water, PGA (2g in Fig. 3A) in the BS chloroplasts should be depleted in $^{2}$H relative to that in M cells of C$_{3}$ plants. When BS PGA (2g in Fig. 3A) is shunted to an M chloroplast, and reduced to GAP (glyceraldehyde 3-phosphate) (5f in Fig. 3A) by $^{2}$H-depleted chloroplastic NADPH, the depletion signal is retained in the GAP (5f in Fig. 3A). Therefore, the two precursors, pyruvate (3e in Fig. 3A) and GAP (5f in Fig. 3A) used for the synthesis of phytol (28b in Fig. 3A) in C$_{4}$ M cells via the DXP pathway are both depleted relative to pyruvate (3a in Fig. 2) and GAP (5a in Fig. 2) in M cells of C$_{3}$ plants. Phytol is, however, found to be more depleted in $^{2}$H in the C$_{3}$ plants than in the C$_{4}$ plants (Table 1). Because there is no difference in phytol biosynthetic pathways between C$_{3}$ (28a in Fig. 2) and C$_{4}$ (28b in Fig. 3A) plants, in C$_{4}$ plants, either M chloroplastic water or M chloroplastic NADPH, or both, must be enriched in $^{2}$H relative to their counterparts in C$_{3}$ plants. As M water of C$_{4}$ plants leaves, calculated previously, is approximately 11‰ ($\approx$ 18.6–7.9, which is also close to the estimated 16‰ for field grown plants listed in Table 2) enriched relative to that in C$_{3}$ plant leaves and the direct contribution of H of cellular water to the H in phytol is only 18%, less than 2% of the observed 196‰ difference (Table 1) can be accounted for by the (lamina) cellular water difference between plants exploiting the C$_{4}$ or C$_{3}$ assimilatory pathways. Therefore, it can reasonably be suggested that NADPH in C$_{4}$ plant M cell chloroplasts is relatively less depleted and thus could be responsible for the lower molecular level isotopic fractionation in phytol of C$_{4}$ ($^{2}$H = 264‰) than C$_{3}$ plants ($^{2}$H = 460‰).

There are at least two mechanisms that are likely to contribute to the $^{2}$H content of NADPH in C$_{4}$ plant M chloroplasts. The first of these relates to the NADPH-generating capacity of the cell types. The C$_{4}$ plant M chloroplast is equivalent to the C$_{3}$ plant chloroplast in terms of NADPH generating ability; it has exactly the same PS II and PS I systems essential for effective electron transfer and NADPH and ATP generation (Munekage et al. 2004). However, because of the lack of PS II in BS chloroplasts (at least in the NADP-ME subtype), the NADPH generating ability of BS chloroplasts is greatly damped. Consequently, NADPH generated in the C$_{4}$ plant M chloroplast is exported with malate (32a in Fig. 3A) at a high flux (with a molar ratio of 1:1 for malate:NADPH) to the BS cells to meet the active catabolic demand for reducing equivalent in the Calvin–Benson–Bassham cycle. As reducing OAA (31a in Fig. 3A) to malate (32a in Fig. 3A) with NADPH inside the M chloroplast involves a high KIE of 5.5–5.7 (Hermes et al. 1982; Grissom & Cleland 1985), the transferable H atom of NADPH remaining inside the M chloroplast (the residual NADPH) is consequently enriched in $^{2}$H.

The second of these relates to the involvement of the NADPH dehydrogenase complex (NDH), involved in the alternative route of electrons in PS I cyclic electron transport. In the chloroplast, NADPH is oxidized by NDH on the stromal side of PS I, enriching the transferable hydrogen atom of the NADPH remaining in the chloroplast. As M chloroplasts in C$_{4}$ plants have proportionally more NDH than chloroplasts in C$_{3}$ plants (Moller 2001; Majeran et al. 2005), they might therefore oxidize more NADPH, resulting in a relatively $^{2}$H-enriched NADPH in the stroma.

Isotopic differences in sterols (MVA pathway) between C$_{3}$ and C$_{4}$ plants

The MVA pathway occurs only in the cytosol. Hence, sterols in both C$_{4}$ (30b in Fig. 3A) and C$_{3}$ (30a in Fig. 2) plants are synthesized via the MVA pathway with cytosolic pyruvate (3b and 3e in Fig. 2 and 3f in Fig. 3A) as the common precursor. In C$_{3}$ plants, the glycolytic metabolism of glucose (9b in Fig. 2; the 6-phosphorylated form, G6P) occurs in the M cytosol, while, in C$_{4}$ plants, the glycolytic process, which generates pyruvate (3f in Fig. 3A), is located predominantly in the BS cytosol (Majeran et al. 2005). During glycolysis, only $^{1}$H, $^{15}$N$^{–}$pro–R and $^{15}$N$^{–}$pro–S in glucose are retained in pyruvate (3f in Fig. 3A; Zhang et al. 2002; Robins et al. 2003, 2008), and more than 2/3 of the hydrogen
atoms in the pyruvate (3f in Fig. 3A) are from cytosolic water (compare 9b and 3b and 3e in Fig. 2). Subsequent synthesis of sterols (30b in Fig. 3A) from pyruvate (3f in Fig. 3A) incorporates H from BS cytosolic water and from NADPH (both of which sources are depleted in 2H relative to their C3 plant counterparts). Thus, the smaller εH of sterols (30b in Fig. 3A) in C4 relative to C3 plants must be due to comparatively enriched H1, H6-proR and H8-proS in C4 glucose (9c, 9d and starch in Fig. 3A), relative to those of C3 glucose (9a, 9b, starch and sucrose in Fig. 2), an inference consistent with measurements of the position specific distribution of 2H in glucose (from sucrose) by 2H NMR (Schleucher et al. 1999; Zhang et al. 2002; Ehlers et al. 2015).

In arguing for a role of the relatively enriched H1, H6-proR and H8-proS in C4 NADP-ME glucose to the relative enrichment of C4 sterols (relative to C3 sterols), we made the implicit assumption that the pyruvate (3f in Fig. 3A) used for sterol (30b in Fig. 3A) synthesis comes from glycolysis in BS cells. We believe such an assumption is justified. Although the BS cytosolic pool of pyruvate can have contribution from that involved in the malate (32a in Fig. 3A)-pyruvate (3e in Fig. 3A) shuttle, as the glycolysis-generated pyruvate (3f in Fig. 3A) can theoretically mix with pyruvate (3e), such a contribution is most likely to be small. This is because (i) given that it is imperative to maintain a high flux of pyruvate (3e) across BS and M in NADP-ME, it is reasonable to assume that the vast majority of pyruvate (3e) is engaged in the malate (32a in Fig. 3A)-pyruvate (3e in Fig. 3A) shuttle, rather than being mixed with glycolytically generated pyruvate (3f in Fig. 3A); and (ii) anatomically, the channels responsible for exporting pyruvate (3e in Fig. 3A) generated during exposure to light may be physically too far away for effective mixing with the pyruvate (3f in Fig. 3A) generated by glycolysis to occur. Note that in the NADP-ME subtype, the BS chloroplast is centrifugally oriented (Edwards & Voznesenskaya 2011), that is the chloroplast is oriented in a way so that metabolites coming out from the Calvin–Benson–Bassham cycle can be channelled immediately to the BS M-plasmodesmata for export without being mixed with the pyruvate generated in glycolysis. We are not sure, however, if this export-without-mixing scenario also applies to the NAD-ME subtype. Nevertheless, our prediction is that this might not be the case as the following: (i) anatomically, a typical NAD-ME type plant has its chloroplasts oriented centripetally (therefore away from the plasmodesmata between BS and M cells) while abundant mitochondria may be oriented toward the plasmodesmata between BS and M cells (Edwards & Voznesenskaya 2011); (ii) aspartate-alanine rather than malate-pyruvate is the shuttled species in NAD-ME type plants; and (iii) the fractional contribution of the two cell types to the production of glucose in NAD-ME type plants is currently unknown.

Isotopic differences between NADP-ME and NAD-ME subtypes

The typical NAD-ME subtype plant (Fig. 3B) is both anatomically and biochemically different from its NADP-ME counterpart (Fig. 3A) in that (i) it has no suberin lamella; (ii) the shuttles between M and BS cells are aspartate (33 in Fig. 3B) (instead of malate, 32a in Fig. 3A and 32b in Fig. 3B) and alanine (34 in Fig. 3B) (instead of pyruvate, 3e in Fig. 3A and 3g in Fig. 3B); and (iii) there is no M-to-BS transport of reducing power (Edwards & Walker 1983; Edwards & Voznesenskaya 2011). In NADP-ME type C4 plants, the M-to-BS transport of NADPH and the BS-M cellular water isotopic difference are suggested to be responsible for the observed isotopic offset from that of C3 plant lipids. Thus, in the NAD-ME type C4 species, which lack a suberin lamella in BS cell walls (affecting water transport), and lack the shuttle of NADPH from M to BS, one might predict the following: compared with the NADP-ME type, in the NAD-ME species, phytol would have a higher εH, while n-alkanes and sterols a lower εH, because the NADPH in M chloroplasts of NAD-ME plants should not be as enriched as NADPH as in NADP-ME plants (as there is no transport-driven enrichment of the transferable H). Moreover, the ACT and MVA precursor pyruvate (3g in Fig. 3B) would be expected to be less depleted in the NAD-ME type (because there is no depletion because of exchange with unenriched BS water). However, this prediction is not supported by the results in that the average εHs for ACT [152 ± 23‰ (1σ, n = 6) for NADP-ME versus 152 ± 3‰ (1σ, n = 2) for NAD-ME] and MVA [198 ± 12‰ (1σ, n = 6) for NADP-ME versus 205 ± 6‰ (1σ, n = 3) for NAD-ME] pathways (see Table 1) are not significantly different, while for the DXP pathway, the average εH is in fact lower [240 ± 86‰ (1σ, n = 3)] than that of the NAD-ME [277 ± 66‰ (1σ, n = 6)]. While interpretation may be limited by sample size (note that there is only one NAD-ME species and two or three treatments are used to calculate the average εH), the NAD-ME species may have unique anatomical and biochemical features, which account for the isotopic results. It should be borne in mind that (i) unlike NADP-ME plants where the cellular locations of biosynthesis of lipids are clear and specific, the synthesis of the three classes of lipids in NAD-ME plants remains to be elucidated; (ii) as typical NAD-ME plants are known to have equally competent/functional chloroplasts in both M and BS cells (unlike in NADP-ME where the BS is deficient in PSII crucial for NADPH generation), it is reasonable to assume that the lipids are synthesized in both cell types without clear dominance of one type over the other; and (iii) the reducing power NADH (not NADPH) used for the conversion of OAA (31b) to malate (32b in Fig. 3B) is produced by the abundant mitochondria in BS cells of NAD-ME plants. The isotopic composition of (the transferable H of) NADH is likely to be relatively enriched compared with the NADPH produced photochemically in the BS chloroplast. The final isotopic composition of NAD-ME lipids therefore will be the weighted average isotopic compositions of the lipids produced by the two cell types. Lack of knowledge of the relative contribution of the two cell types to each of the three classes of lipids, and the difference in the isotopic composition between the transferable Hs of the mitochondrial NADH and the chloroplastic NADPH in NAD-ME type species, limits the interpretation.
of isotopic differences (or the lack there of) between NADP-ME and NAD-ME lipids.

When growth conditions vary, phytol (28b in Fig. 3A) \(^2\text{H}\) has a much larger range within C4 plants (158%) than in C3 (28a in Fig. 2) plants (49% if the only outlier of 258% is excluded, Table 1). We consider that the \(^2\text{H}\) enrichment of (the transferable H) NADPH because of the M-to-BS export of NADPH in the NADP-ME type is the main cause of the difference between C4 NADP-ME and C3 phytols. Thus, an intrinsic difference in the percentage of NADPH export from M to BS (Ziegler 1988) or perhaps a higher sensitivity of the (transferable H of) C4 NADPH isotopic composition to environmental changes are probably the causes of the \(^2\text{H}\) range difference.

**Inter-pathway isotopic differences**

The magnitudes of inter-pathway isotopic fractionation differences, \(\Delta \epsilon^2\text{H}\), are also found to be photosynthetic-mode dependent; the values for C3 plants (129% for \(\Delta \epsilon^2\text{H}_{\text{MVA-ACT}}\) and 210% for \(\Delta \epsilon^2\text{H}_{\text{DXP-MVA}}\)) are greater than those for C4 plants (48% for \(\Delta \epsilon^2\text{H}_{\text{MVA-ACT}}\) and 64% for \(\Delta \epsilon^2\text{H}_{\text{DXP-MVA}}\)). With the current state of knowledge, it is hard to put forward a quantitative explanation for the relationship of the absolute inter-pathway isotopic fractionation differences between photosynthetic modes. Nevertheless, the previously inferred relative enrichment of M cell chloroplastic NADPH in C4 plants relative to C3 plants is likely to be the most important amongst the three factors put forward as the determinants of these differences.

**CONCLUSIONS**

C4 land-plant lipids synthesized via the ACT pathway are found to be more depleted in \(^2\text{H}\) than the equivalent in C3 land-plants, while those synthesized via the MVA and DXP pathways in C4 plants are found to be less depleted in \(^2\text{H}\) than their counterparts in C3 plants. We surmise that these differences are the consequences of compartmentation of photosynthetic metabolism in C4 plants into anatomically and functionally different M and BS cells. Specifically, we suggest that this may result in the following:

1. pyruvate used in the ACT pathway in the M chloroplast of C4 plants being more depleted in \(^2\text{H}\) as a result of shuttling of pyruvate from the unenriched/slightly enriched BS cellular water and the possible contribution of enriched PGA from photosynthesis to the chloroplastic PGA pool in C3 plants;
2. the transferable H of NADPH used for the DXP pathway in the M chloroplast of C4 plants being less depleted than in C3 cells, because of the export of NADPH in the form of malate/NADPH from the M cells to the BS cells; and
3. a more enriched pyruvate used for the MVA pathway in the BS cytosol of C4 plants in comparison with C3 plants as a result of the suppression of photosuppression in C4 plants.

In proposing the three-part biochemical explanation for the observed C3 versus C4 plant lipid isotopic fractionation between different classes of lipids and leaf water, we have made the assumption that there is no cross-talk between chloroplastic and cytosolic pyruvate pools. Recent work, however, has showed that such cross-talk does occur (Schuur et al. 2003). Such cross-talk may diminish/enhance the isotopic differences between the three classes of lipids depending on the dominant direction of the cross-talk flux, thus further complicating the interpretation of the C3 versus C4 plant isotopic depletion differences. Nevertheless, it does not invalidate the arguments for the role of photorespiration in C3 plants and compartmentation in C4 plants in influencing the pathway-specific isotopic differences found in lipid from land plants exploiting different photosynthetic metabolisms.

**ACKNOWLEDGEMENTS**

This work was supported by discovery grants (DP130100577 and DP1096729) from ARC to KG and GF; a PhD scholarship from the Australian National University and a visiting fellowship (SKLQG0916) and a Talent 100 Fellowship from the Chinese Academy of Sciences to YZ. GF and AG acknowledge support from the Alexander-von-Humboldt Foundation.

**REFERENCES**

Baillif V., Robins R.J., Le Feunteun S., Lesot P. & Billault I. (2009) Investigation of fatty acid elongation and desaturation steps in Fusarium lateritium by quantitative two-dimensional deuterium NMR spectroscopy in chiral oriented media. Journal of Biological Chemistry 284, 10783–10792.

Bao X., Focke M., Pollard M. & Ohlrogge J. (2000) Understanding precursor supply for fatty acid synthesis in leaf tissue. The Plant Journal 2, 39–50.

Barnes C.J. & Allison G.B. (1983) The distribution of deuterium and \(^18\text{O}\) in dry soils: I. Theory. Journal of Hydrology 60, 141–156.

Berry J. & Farquhar G.D. (1978) The CO2 concentrating function of C4 photosynthesis. A biochemical model. In: Proc. of the 4th International Congress on Photosynthesis, Reading, England, 1977 (eds. Hall D., Coombs J., Goodwin T.), pp. 119–131. The Biochemical Society, London.

Billault I., Guiet S., Mabo F. & Robin R.J. (2001) Natural deuterium distribution in long-chain fatty acids is non-statistical: a site-specific study by quantitative \(^1\text{H}\) NMR spectroscopy. Chemical and Biochemical Engineering Quarterly 2, 425–431.

Bizouarn T., Grimley R.L., Cotton N.P.J., Stilwell S.N., Hutton M. & Baz J.J. (1995) The involvement of NADP(H) binding and release in energy transduction by proton-translocating nicotinamide nucleotide transhydrogenase from Escherichia coli. Biochimica et Biophysica Acta 1229, 49–58.

Cernusak L.A., Pate J.S. & Farquhar G.D. (2002) Diurnal variation in the stable isotope composition of water and dry matter in fruiting Lupinus angustifolius under field conditions. Plant Cell & Environment 25, 983–907.

Chikaraishi Y., Naraoka H. & Poulson S.R. (2004) Hydrogen and carbon isotopic fractionations of lipid biosynthesis among terrestrial (C3, C4 and CAM) and aquatic plants. Phytochemistry 65, 1369–1381.

Dansgaard W. (1964) Stable isotopes in precipitation. Tellus 16, 191–198.

Farquhar G.D., Ehleringer J.R. & Hubick K.T. (1989) Carbon isotope discrimination and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 40, 503–537.

© 2016 John Wiley & Sons Ltd, Plant, Cell and Environment
Zhang X.N., Gillespie A. & Sessions A.L. (2009a) Large D/H variations in bacterial lipids reflect central metabolic pathways. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 12580–12586.

Zhang Z.H., Sachs J. & Marchetti A. (2009b) Hydrogen isotope fractionation in freshwater algae: II. Temperature and nitrogen limited growth rate effects. *Organic Geochemistry* **40**, 428–439.

Zhou Y.P. (2005) Carbon, oxygen and hydrogen isotope compositions of plant material: Insights from controlled growth experiments and modelling. PhD thesis, Australian National University, Canberra, Australia.

Zhou Y.P., Grice K., Stuart-Williams H., Farquhar G.D., Hocart C.H., Lu H. & Liu W.G. (2010) Biosynthetic origin of the saw-toothed profile in $\delta^{13}$C and $\delta^2$H of n-alkanes and systematic isotopic differences between n-, iso- and anteiso-alkanes in leaf waxes of land plants. *Phytochemistry* **71**, 388–403.

Zhou Y.P., Grice K., Chikaraishi Y., Stuart-Williams H., Farquhar G.D. & Ohkouchi N. (2011) Temperature effect on leaf water deuterium enrichment and isotopic fractionation during leaf lipid biosynthesis: results from controlled growth of C3 and C4 land plants. *Phytochemistry* **72**, 207–213.

Zhou Y.P., Stuart-Williams H., Grice K., Kayle Z.E., Zavadlav S., Gessler A. & Farquhar G.D. (2015) Allocate carbon for a reason: priorities are reflected in the $^{13}$C/$^{12}$C ratios of plant lipids synthesized via three independent biosynthetic pathways. *Phytochemistry* **111**, 14–20.

Ziegler H. (1988) Hydrogen isotope fractionation in plant tissues. In *Stable Isotopes in Ecological Research* (eds Rundel P., Eheringer J.R. & Nagy K.A.), pp. 195–123. Springer-Verlag, New York.

Received 18 January 2016; accepted for publication 17 August 2016