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Biosynthetic and environmental effects on the stable carbon isotopic compositions of *anteiso*- (3-methyl) and *iso*- (2-methyl) alkanes in tobacco leaves

Kliti Grice [a,*], Hong Lu [a,b], Youping Zhou [c,e], Hilary Stuart-Williams [d], Graham D. Farquhar [d]

[a] *WA Organic and Isotope Geochemistry Centre, Institute for Geoscience Research, Curtin University of Technology, Perth, Australia*
[b] *Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou, China*
[c] *Research School of Earth Sciences, Australian National University, Canberra, ACT 0200, Australia*
[d] *Environmental Biology Group, Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia*
[e] *Institute of Earth Environment, Chinese Academy of Sciences, Xi'an, China*

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ABSTRACT

Nicotiana tabacum is the only plant known to synthesise large quantities of *anteiso*- (3-methyl) alkanes and *iso*- (2-methyl) alkanes. We investigated the carbon isotope ratios of individual long-chain *n*-alkanes, *anteiso*- and *iso*-alkanes (in the C_{29}–C_{33} carbon number range) extracted from tobacco grown in chambers under controlled conditions to confirm the pathway used by the tobacco plant to synthesise these particular lipids and to examine whether environmental data are recorded in these compounds. Tobacco was grown under differing temperatures, water availabilities and light intensities in order to control its stable carbon isotope ratios and evaluate isotopic fractionations associated with the synthesis of these particular lipids. The *anteiso*-alkanes were found to have a predominant even-carbon number distribution (maximising at C_{32}), whereas the *iso*-alkanes exhibit an odd-carbon number distribution (maximising at C_{31}). *Iso*-alkanes were relatively more abundant than the *anteiso*-alkanes and only two *anteiso*-alkanes (C_{30} and C_{32}) were observed.

The *anteiso*-alkanes and *iso*-alkanes were found to be enriched in ^{13}C by 2.8–4.3‰ and 0–1.8‰ compared to the *n*-alkanes, respectively, consistent with different biosynthetic precursors. The assumed precursor for the odd-carbon-numbered *iso*-alkanes is *iso*-butyryl-CoA (a C_4 unit derived from valine) followed by subsequent elongation of C_2 units and then decarboxylation. The assumed precursor for even-carbon-numbered *anteiso*-alkanes is α-methylbutyryl-CoA (a C_5 unit derived from isoleucine) and subsequent elongation by C_2 units followed by decarboxylation. The ratio of carbon atoms derived from α-methylbutyryl-CoA and subsequent C_2 units (from malonyl-CoA) is 1:5 for the biosynthesis of a C_{30} *anteiso*-alkane. The ratio of carbon atoms derived from *iso*-butyryl-CoA and subsequent C_2 units (from malonyl-CoA) is 4:25 for the synthesis of a C_{29} *iso*-alkane. An order of ^{13}C depletion *n*-alkanes > *iso*-alkanes > *anteiso*-alkanes is evident from compound specific isotope data. This trend can probably be attributed to the ratio of the two different sources of carbon atoms in the final wax components.

Higher water availability generally results in more depleted stable carbon isotope ratios due to maximised discrimination during carboxylation, associated with less diffusional limitation. This was confirmed in the present study by compound specific isotope analyses of *iso*-alkanes, *anteiso*-alkanes and *n*-alkane lipids extracted from the tobacco leaves. Likewise, light intensity has been shown to influence plant bulk $\delta^{13}C$ in previous studies. The carbon isotope ratios of *n*-alkanes in tobacco grown under low-light conditions were about 2‰ more depleted in ^{13}C than those of lipids extracted from tobacco grown under elevated light conditions. A similar order of difference is observed for the *iso*-alkanes and *anteiso*-alkanes (1.8‰ and 1.9‰, respectively). A negligible depletion in carbon isotope ratios was observed for the *iso*-alkanes and *anteiso*-alkanes extracted from tobacco grown under elevated temperatures. These results are consistent with the work of Farquhar [Farquhar, G.D., 1980. Carbon isotope discrimination by plants: effects of carbon dioxide concentration and temperature via the ratio of intercellular and atmospheric CO_2 concentrations. In: Pearman, G.I. (Ed.), Carbon Dioxide and Climate: Australian Research. Springer, Berlin, pp. 105–110] where temperature appears to have only a minor effect on plant bulk $\delta^{13}C$.

1. Introduction

Odd carbon-numbered long-chain *n*-alkanes in the C_{25}–C_{35} range are characteristic components of the epicuticular leaf waxes

* Corresponding author. Tel.: +61 8 9266 2474; fax: +61 8 9266 2300.
E-mail address: K.Grice@curtin.edu.au (K. Grice).

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of higher plants (Eglinton and Hamilton, 1967; Reddy et al., 2000). The tobacco plant (*Nicotiana tabacum*) is the only plant known to synthesise large quantities of *anteiso*-alkanes and *iso*-alkanes (close to 68% of total alkanes of the plants) (Heemann et al., 1983; Rogge et al., 1994) although these compounds may be present to a lesser extent (~1.5%) in other plants' leaf waxes (Reddy et al., 2000). Environmental studies have depended upon the abundance of *anteiso*- and *iso*-alkanes in tobacco as molecular indicators of cigarette smoke pollution in indoor and urban aerosols (Kavouras et al., 1998; Rogge et al., 1994; Bi et al., 2005).

In higher plants, alkanes are the decarboxylative products of fatty acids. *n*-Fatty acids are biosynthesized by chain elongation of precursor acetyl-CoA with malonyl-ACP (Fig. 1a, Heldt, 2005; Kunst and Samuels, 2003; Kunst et al., 2005; Lea and Leegood, 1999) while branched long-chain fatty acids are synthesized by the successive elongation of CoA thiolesters of short branched-chain acids derived from branched-chain amino acids (BCAA) such as valine, leucine and isoleucine (Fig. 1b and c; Singh and Shaner, 1995; Kaneda, 1991). Branched-chain fatty acids can be classified according to their respective terminal precursors: *iso*butyryl-CoA, *iso*valeryl-CoA, and α-methylbutyryl-CoA are the terminal precursors for *iso*-fatty acids having an even number of carbon atoms, *iso*-fatty acids having an even-number of carbon atoms and *anteiso*-fatty acids having an odd-number of carbon atoms, respectively. The terminal precursors for the branched-chain fatty acids can be formed by the oxidative decarboxylation of the α-keto acids corresponding to valine, leucine and isoleucine (e.g. Fig. 1). No data are available on the carbon isotope ratios of corresponding *anteiso*-alkanes (having an even number of carbon atoms) and *iso*-alkanes (having an odd-number of carbon atoms) in tobacco plants. In the present study we have investigated the carbon isotope ratios of individual long-chain *n*-alkanes, *anteiso*- (3-methyl) and *iso*- (2-methyl) alkanes extracted from tobacco grown under controlled conditions to confirm the pathways used by the tobacco plant to synthesise specific lipids. Tobacco plants were also grown under differing temperatures, water availabilities and light intensities in order to obtain their carbon isotope ratios to evaluate isotope fractionation effects.

2. Materials and methods

For all the growth experiments, seeds of *Nicotiana tabacum* were first germinated in plastic pots (10 L) filled with potting mix and fertilized with Osmocote (Osmocote, PlusScott, Sierra Horticultural Products, The Netherlands) in a temperature-controlled glasshouse in the Research School of Biological Sciences (ANU) and watered with Canberra tap water. When seedlings were about 3–4 cm high (7–10 days after sowing), the plants were thinned to 2–3 plants per pot and 3 pots of seedlings of similar height were chosen and transferred to environment-controlled growth cabinets. The photoperiod was maintained at 12 h (8 a.m. to 8 p.m.). Purely on the basis of mass balance, the contribution of the inherited carbon from the seedlings to the final organic matter at harvest was negligible. Seven days after being transferred into cabinets, the plants were further thinned to 1 plant per pot. Every 3 days the positions of the pots were rotated to minimise the differences due to spatial heterogeneity of light inside the cabinets, the circulating system and the humidifying system.

2.1. Temperature, humidity and light intensity control

The temperature inside the cabinets was continuously monitored by a sensor and controlled by heating with a radiator or cooling with a fan to dissipate the heat to the outside. The humidity was also continuously monitored and maintained by injecting misted water into the cabinets when it fell below the set value or by condensing the moisture when the humidity rose above the set value. Mixing of air inside the cabinets was achieved by forced circulation. Measurement of the air temperature at different positions inside the cabinet showed that it was laterally uniform across the cabinet with a variation of ±0.5 °C but relative humidity measurements near different leaves did show deviations of ±5% from the set value and from each other. The precision of humidity control was affected by the temperature and the light intensity inside the cabinet. Light (halide lamp) was ramped to full set value in the first hour of the day (8 a.m.–9 a.m.) and kept constant at full set value before being ramped down to zero in the last hour (8 p.m.–9 p.m.) of the day.

2.2. Watering

Plants were watered twice a day. The amount of water added was adjusted with the growth stage so that the plants were always well-watered except for the growth experiment testing the effect of drought. Water-stress was imposed by watering the plants once every 3 days instead of twice a day. The stressed plants had noticeably shorter stems plus darker, narrower and shorter leaf blades and curly or folding leaves while well-watered plants showed all signs of healthy growth (Zhou, 2005).

2.3. Temperature experiment

Plants (3 pots) were grown in one cabinet with growth conditions set as 40% for relative humidity, 400 ppm for [CO_2], 780 μmol photons $m^{-2}\,s^{-1}$ for photosynthetically active radiation (PAR). Day/night-time temperatures for the relatively high temperature and 'moderate' temperature cabinets were set at 30 °C/20 °C and 18 °C/15 °C, respectively.

2.4. Light intensity experiment

Plants (3 pots) were grown in one cabinet with growth conditions set as 40% for relative humidity, 400 ppm for [CO_2] and 30 °C/20 °C for day/night-time temperatures. Canopy top PAR for the high-light level and low-light level cabinets were set at 1200 μmol photons $m^{-2}\,s^{-1}$ at the top of the leaf canopy and 780 μmol photons $m^{-2}\,s^{-1}$, respectively. As the effective intensity impinging on the plant canopy changes with the distance between the plant canopy and the lights, the light level was adjusted by either changing the maximum intensity of the lamps or changing the distance between the lamp and plant canopies such that the effective intensity was relatively constant at least for the top leaves throughout this growth experiment. Some heating of the upper leaves by the lamps was inevitable but this effect was minimised as much as possible by circulating the air in the cabinet rapidly.

2.5. Extraction and separation of lipids

Leaves were sampled when plants were 42–45 days old and well beyond the exponential biomass accumulation period (26–35 days). The dried and powdered leaf material (60–200 g) was extracted with distilled dichloromethane: methanol (9:1) in a Soxhlet apparatus for 48 h to obtain both aliphatic and polar lipids. Polar lipids are not the focus of the present study but were utilised in another research project. Extracts were dried under a nitrogen purge and weighed. Aliquots of the extracts (100 mg) were then separated using two column volumes of *n*-hexane (15 cm × 0.5 cm i.d.) by silica gel chromatography (150 °C, overnight) in columns which had been pre-eluted with *n*-hexane yielding a hydrocarbon fraction. All the hydrocarbons for this study were collected in the *n*-hexane fraction. The solvent was carefully evaporated by heating in a sand bath (80 °C).

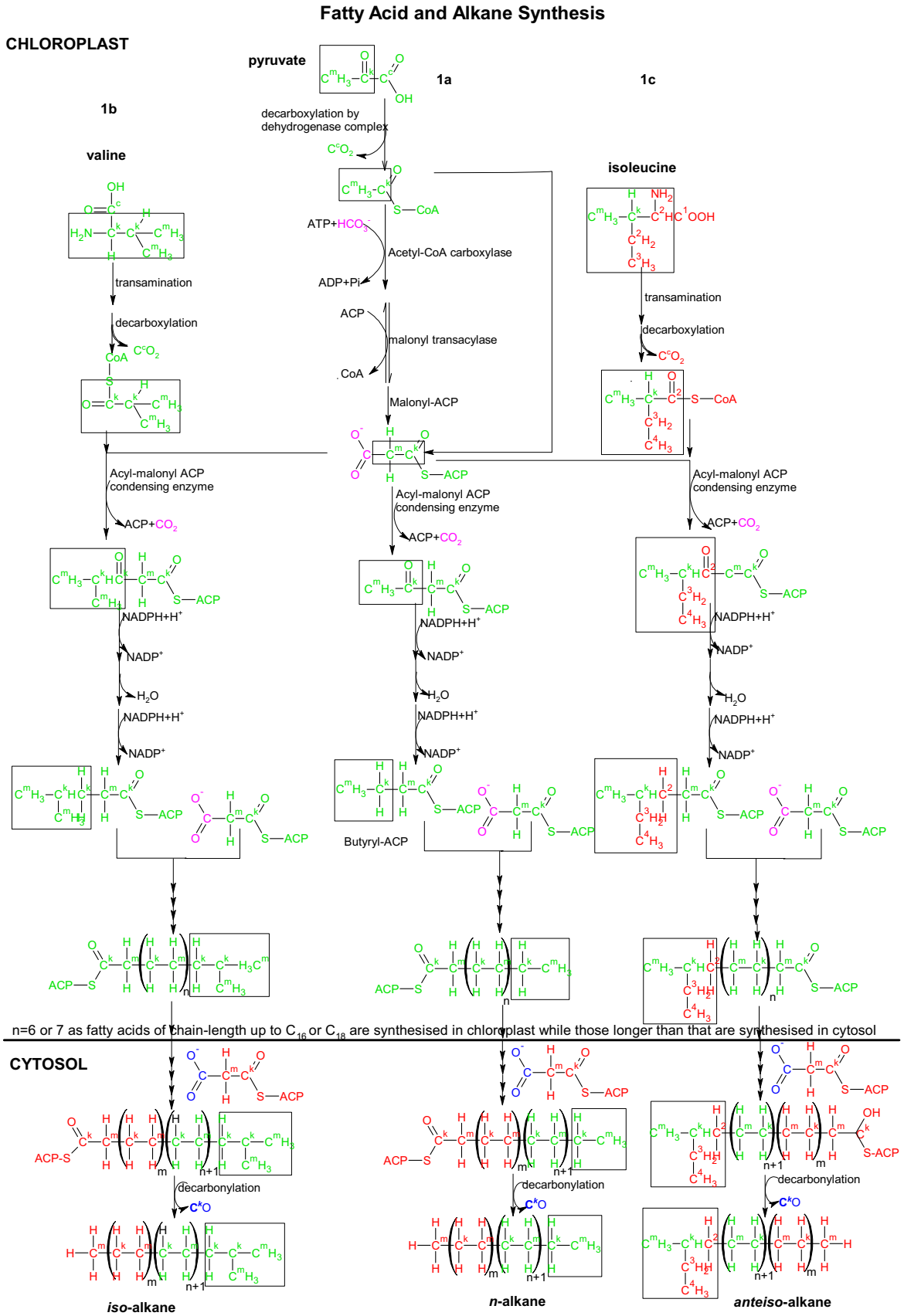

Fig. 1. Fatty acids and alkane syntheses in higher plants. *n*-Fatty acids synthesis starts with synthesis of chain-extender molecule malonyl-CoA from precursor pyruvate in chloroplast. Subsequent extension of acetyl-CoA (derived from chloroplastic pyruvate) by repetitive addition of C_2 unit from malonlyl-CoA yields fatty acids of chain-length up to C_{16} or C_{18} in chloroplast (a). In cytosol, elongation of fatty acids involves also repetitive addition of C_2 unit from malonlyl-CoA derived from cytosolic pyruvate. *Iso*-alkanes (b) and *anteiso*-alkanes (c) have similar biosynthetic pathways to that of *n*-alkanes but with different precursor molecules: the former being valine and latter being isoleucine. Hydrocarbons are the direct product of fatty acids decarbonylation. An even (odd)-numbered fatty acid will give an odd (even)-numbered alkane.

2.6. GC/MS and GC-irMS analyses

Qualitative analyses of the hydrocarbons were carried out using an HP 6890 gas chromatograph interfaced to a mass-selective detector. The GC was equipped with a temperature-programmable, septum-less injector system and a 60 m × 0.25 mm i.d. column containing a DB-1 phase (0.25 μm phase thickness). The samples were injected in a split/splitless mode while the oven was held constant at 40 °C and the injector programmed to 320 °C over 2 min. The oven then followed a temperature program of 10 °C min^{-1} to 120 °C and 4 °C min^{-1} to 320 °C where it was held constant for 50 min. Helium was used as a carrier gas at constant flow (1 mL/min).

Compound specific isotope analyses were performed on a Micromass IsoPrime isotope ratio gas chromatography-mass spectrometer (ir-GCMS). The IsoPrime consists of a gas chromatograph, combustion unit and a dual reference gas injection system, all online with an isotope ratio mass spectrometer fitted with an electromagnet. The samples were dissolved in *n*-hexane and analysed in helium carrier gas using an HP 6890 gas chromatograph equipped with an autosampler and a split/splitless injector. A 60 m × 0.25 mm i.d. column containing a DB-1 phase (0.25 μm phase thickness) was used and the sample was injected using pulsed splitless mode (injection holding for 30 s at 15 psi above the head pressure of the column and the purge time of 35 s). Helium was used as a carrier gas at constant flow (1 mL/min) The GC oven was programmed from 40 °C (1 min) to 300 °C at 3 °C min^{-1} and held isothermally at 300 °C for 30 min. The isotopic compositions were calculated by integration of the mass 44, 45 and 46 ion currents of the peaks (including the Craig correction) produced by combustion (CuO packed quartz-glass tube at 850 °C) of the gas chromatographically separated compounds. The compositions are reported relative to those of CO_2 reference gas pulses calibrated with a mixture of compounds with known $\delta^{13}C$ previously calibrated against Vienna Peedee belemnite (VPDB). Average values of at least two runs were determined. The stable carbon isotopic compositions are reported in the δ-notation.

3. Results and discussion

3.1. Distributions and $\delta^{13}C$ of waxy hydrocarbons in Nicotiana tabacum

The GC–MS chromatogram of one of the waxy hydrocarbon fractions from a tobacco plant is shown in Fig. 2. The waxy hydrocarbon distributions of all tobacco leaf samples grown under varying chamber conditions were similar (results not shown here). There are three homolog series: *n*-alkanes, *iso*-alkanes and *anteiso*-alkanes with *n*-alkanes being dominant. The *anteiso*- (3-methyl) and *iso*- (2-methyl) alkanes also have similar distributions, which elute in the same region of the chromatogram as the *n*-alkanes (C_{29}–C_{33}). Examining mass spectra and published data allowed us to identify a homologous series of *n*-alkanes, 2-methyl (*iso*-alkane) and 3-methyl (*anteiso*-alkane). $(M-15)^+$. and $(M-43)^+$. ions are characteristic fragment ions for *iso*-alkanes and $(M-29)^+$. is a characteristic fragment ion for *anteiso*-alkanes. The *anteiso*-alkanes have a predominant even-carbon number distribution (maximising at C_{32}), whereas the *iso*-alkanes exhibit an odd-carbon number distribution (maximising at C_{31}). *Iso*-alkanes were relatively more abundant than the *anteiso*-alkanes and only two *anteiso*-alkanes (C_{30} and C_{32}) were observed. Comparable patterns have been reported before and probably reflect the biosynthetic pathways of these components (Kolattukudy, 1969). The *anteiso*-alkanes are believed to originate from an isoleucine-derived α-methylbutyryl-CoA (C_5 unit), which is then elongated by C_2 units and further decarboxylated, resulting in an even-numbered dominance. The *iso*-alkanes, however, have an odd-numbered dominance as they are mainly biosynthesized from an *iso*-butyryl-CoA (C_4 unit) precursor derived from valine, which is elongated by C_2 units and then decarboxylated.

The stable carbon isotopic compositions of waxy hydrocarbons from the tobacco samples are listed in Tables 1–3 and are also shown in Fig. 3. Fatty acids are biosynthesized by chain elongation of acetyl-CoA. *Anteiso*-alkanes are enriched in ^{13}C by 2.8–4.3‰ compared to *n*-alkanes of the same carbon number, consistent with a set of different precursors and biosynthetic pathway (Fig. 1c). The stable carbon isotopic composition of the *iso*-alkanes is also slightly enriched in ^{13}C by 0–1.8‰ compared to the *n*-alkanes, also possibly supporting a different set of precursors and biosynthetic pathway (Fig. 1b). A similar but slightly smaller difference (1.5‰) has been recognised by Hayes (1993). Studies on the bacterium *Escherichia coli* (Monson and Hayes, 1982) and some sedimentary biomarker data led to a theoretical prediction of some carbon isotopic consequences of the different biosynthetic pathways. One such prediction was related to the difference between the stable carbon isotopic compositions of polyisoprenoid versus *n*-alkyl lipid compounds in the same organism. Hayes (1993) showed that isoprenoids biosynthesised by the "classical" mevalonate pathway are ca. 1.5‰ enriched in ^{13}C compared to fatty acids biosynthesised by chain elongation of acetyl-CoA. This difference was attributed to

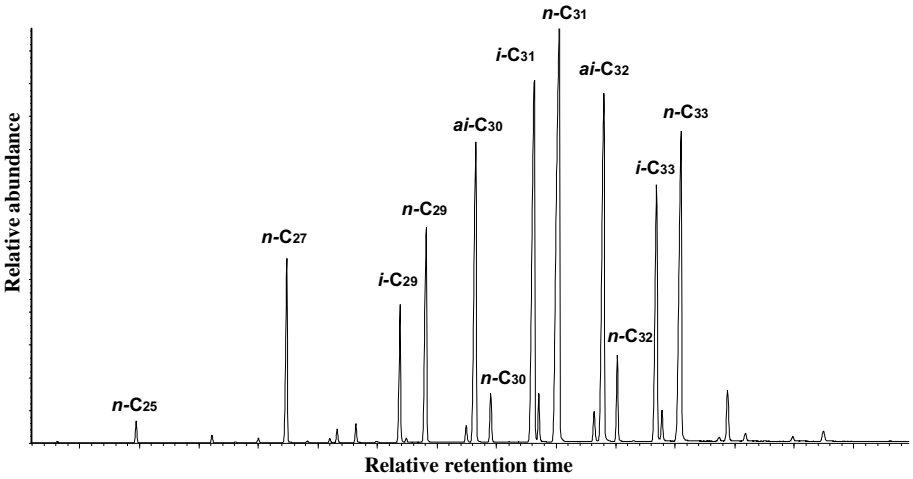

Fig. 2. GC/MS chromatograph of waxy hydrocarbons (*n*-alkanes, *iso*-alkanes and *anteiso*-alkanes) extracted from tobacco plant leaf grown under low temperature treatment as described in the materials and methods.

Table 1
The stable carbon isotopic compositions (‰) of *n: normal* alkanes, *ai: anteiso*-alkanes and *iso*: *iso*-alkanes in tobacco samples grown under different conditions

Treatments	*n*-C_{25}	*n*-C_{27}	*n*-C_{29}	*n*-C_{30}	*n*-C_{31}	*n*-C_{32}	*n*-C_{33}
Well-watered	−43.9(0.6)	−43.6(0.2)	−44.6(0.2)	−45.8(0.4)	−44.8(0.1)	−45.5(0.3)	−44.5(0.1)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−44.7(0.3)	−42.7(0.2)	−43.9(0.1)	−42.3(0.1)	−44.2(0.1)
Water-stressed		−41.8(0.1)	−42.7(0.2)	−44.0(0.5)	−42.8(0.1)	−43.9(0.5)	−42.3(0.0)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−41.9(0.2)	−40.4(0.5)	−41.2(0.2)	−39.8(0.2)	−41.2(0.2)
n-Alkanes $\Delta\delta^{13}C_{\text{stress-well-watered}}$		1.9	1.9	1.8	2	1.6	2.2
iso-Alkanes $\Delta\delta^{13}C_{\text{stress-well-watered}}$			2.7		2.7		3
Anteiso-alkanes $\Delta\delta^{13}C_{\text{stress-well-watered}}$			2.3		2.5		
Low-light	−42.6(0.6)	−42.5(0.2)	−44.3(0.1)	−45.4(0.4)	−45.2(0.2)	−46.(0.2)	−45.3(0.1)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−43.4(0.2)	−42.0(0.3)	−43.4(0.1)	−42.0(0.4)	−43.9(0.2)
High light	−40.5(0.4)	−40.6(0.1)	−42.0(0.2)	−42.9(0.2)	−42.8(0.1)	−44.4(0.2)	−42.9(0.0)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−41.7(0.3)	−40.1(0.2)	−41.6(0.0)	−40.1(0.3)	−41.9(0.1)
n-Alkanes $\Delta\delta^{13}C_{\text{high-low-light}}$		2.1	1.9	2.3	2.4	2.1	2.4
iso-Alkanes $\Delta\delta^{13}C_{\text{high-low-light}}$		1.7		1.8		2	
Anteiso-alkanes $\Delta\delta^{13}C_{\text{high-low-light}}$			1.8		1.8		
Moderate temperature 18 °C/15 °C	−41.8(0.7)	−41.5(0.1)	−42.3(0.4)	−43.7(0.5)	−42.0(0.1)	−43.0(0.1)	−42.0(0.1)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−41.6(0.2)	−39.7(0.1)	−41.9(0.2)	−39.5(0.0)	−42.7(0.5)
High temperature 30 °C/20 °C		−42.1(0.2)	−42.4(0.2)	−44.3(0.4)	−42.6(0.1)	−43.4(0.3)	−42.5(0.1)
			i-C_{29}	*ai*-C_{30}	*i*-C_{31}	*ai*-C_{32}	*i*-C_{33}
			−42.1(0.1)	−40.8(0.2)	−41.7(0.0)	−40.5(0.0)	−42.3(0.2)
n-Alkanes $\Delta\delta^{13}C_{\text{high-moderate temp}}$		−0.6	−0.1	−0.6	−0.7	−0.5	−0.6
iso-Alkanes $\Delta\delta^{13}C_{\text{high-moderate temp}}$			−0.5		−0.2		−0.4
anteiso-alkanes $\Delta\delta^{13}C_{\text{high-moderate temp}}$			−1		−1		

Numbers in brackets are standard error from two or three analyses. Note that the average bulk lipid leaf stable carbon isotopic composition across all treatments was −35.1‰.

Table 2
$\delta^{13}C$ (‰) differences between odd-numbered *n*-alkanes and *iso*-alkanes, expressed as $\delta_{n\text{-alkanes}} - \delta_{iso\text{-alkanes}}$

Experiment	$\delta^{13}C$‰ differences between odd *n*-alkanes and *iso*-alkanes			
	Carbon number	C_{29}	C_{31}	C_{33}
Water-stressed		−0.1	−0.9	−0.3
Well-watered		−0.8	−1.6	−1.1
Low-light		−0.9	−1.8	−1.4
High-light		−0.3	−1.2	−1
Moderate-temperature 18 °C/15 °C		−0.7	−0.1	0.7
High-temperature 30 °C/20 °C		−0.3	−0.9	−0.2

Table 3
$\delta^{13}C$ (‰) differences between even-numbered *n*-alkanes & *anteiso*-alkanes expressed as $\delta_{n\text{-alkanes}} - \delta_{iso\text{-alkanes}}$

Experiment	$\delta^{13}C$‰ differences between even *n*-alkanes and *anteiso*-alkanes		
	Carbon number	C_{30}	C_{32}
Water-stressed		−3.1	−3.2
Well-watered		−3.6	−4.1
Low-light		−3.4	−4
High-light		−2.8	−4.3
Moderate-temperature 18 °C/15 °C		−4	−3.5
High-Temperature 30 °C/20 °C		−3.5	−2.9

the biosynthetic step which forms acetyl-CoA from the decarboxylation of pyruvate (DeNiro and Epstein, 1977; Monson and Hayes, 1982). The carboxyl carbon of acetyl-CoA (keto-carbon (C^k) of pyruvate in Fig. 1) is depleted in ^{13}C compared to the methyl group. The ratio of carboxyl-derived (C^k) to methyl-derived (C^m) carbon in fatty acids is 1:1, and in polyisoprenoid components it is 2:3 where the building group is *iso*pentenyl diphosphate (IPP). Consequently, biological and sedimentary isoprenoidal compounds (i.e. acyclic isoprenoids such as phytol and its derivatives like steroids) of organisms using this pathway are generally enriched in ^{13}C compared to the fatty acids and their derivatives (e.g. *n*-alkanes) from the same organism (Schouten et al., 1998).

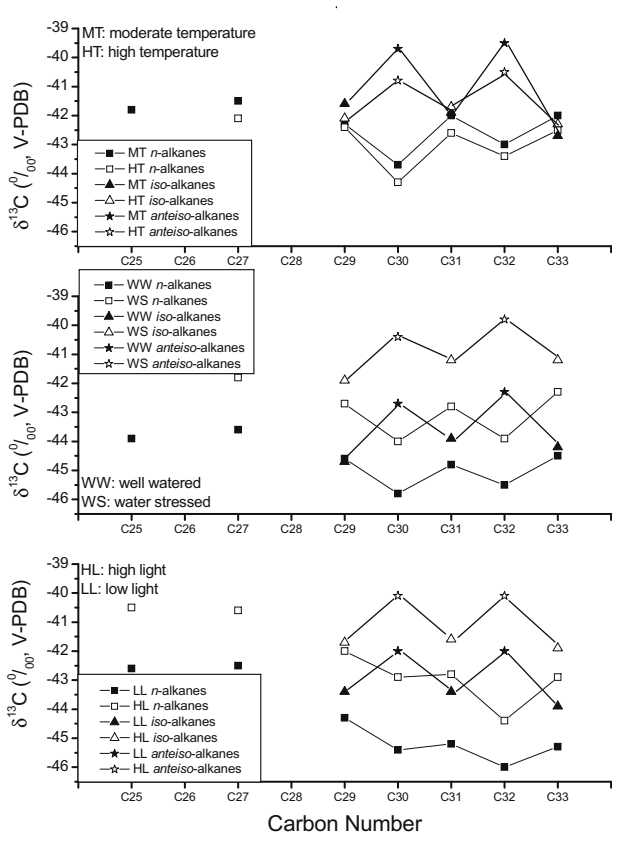

Fig. 3. $\delta^{13}C$ of individual *n*-alkanes, *iso*-alkanes and *anteiso*-alkanes extracted from tobacco leaf grown under contrasting growth conditions.

In the present study we propose that the precursor for even-carbon-numbered *anteiso*-alkanes is α-methylbutyryl-CoA derived from isoleucine and subsequent elongation by C_2 units followed by decarboxylation (Fig. 1c). In higher plants, the synthesis of isoleucine occurs in the chloroplast (Fig. 4). The precursor for isoleucine synthesis is oxaloacetate imported from the cytosol. Oxaloacetate can be synthesised in the cytosol by carboxylating PEP (phosphoenolpyruvate from glycolysis in cytosol) using PEP carboxylase. As bicarbonate (HCO_3^-) is enriched in ^{13}C, its signal is passed onto the C_4 of oxaloacetate which in turn passes the signal to C_4 of aspartate (Fig. 4). During synthesis of isoleucine from aspartate, the signal is retained in the isoleucine and ultimately in odd-numbered *anteiso*-fatty acids and even-numbered *anteiso*-alkanes. Oxaloacetate can also be synthesised from pyruvate in the tricarboxylic acid (TCA) cycle in mitochondria. Cyclic decarboxylation from isocitrate may produce isotopically heavier oxaloacetate for export to the chloroplast for isoleucine synthesis as well (Fig. 4).

We also propose that the precursor for the odd-carbon-numbered *iso*-alkanes is *iso*-butyryl-CoA derived from valine and subsequent elongation by C_2 units followed by decarboxylation (Fig. 1b). As the syntheses of *n*-alkanes, *anteiso*-alkanes and *iso*-alkanes all share similar pathways, the $\delta^{13}C$ differences amongst these three alkane types must lie in $\delta^{13}C$ of precursors. *Anteiso*-alkanes are the most enriched as they derive three carbons from ^{13}C enriched oxaloacetate from the cytosol. As valine derives its carbon exclusively from the chloroplastic pyruvate pool (Fig. 4) with C^m:C^k ratio of 1:1, the same ratio for C^m and C^k in pyruvate for *n*-fatty acids (*n*-alkanes) synthesis, the relative ^{13}C enrichment of *iso*-alkane against *n*-alkane is surprising and may be caused by a selective-utilisation of heavier pyruvate-derived acetyl-CoA in the chloroplast by the enzymes responsible for the synthesis of valine. The ratio of carbon atoms derived from α-methylbutyryl-CoA and subsequent C_2 units is 1:5, for example for the biosynthesis of a C_{30} *anteiso*-alkane. The ratio of carbon atoms derived from *iso*-butyryl-CoA and subsequent C_2 units is 4:25 for the biosynthesis of a

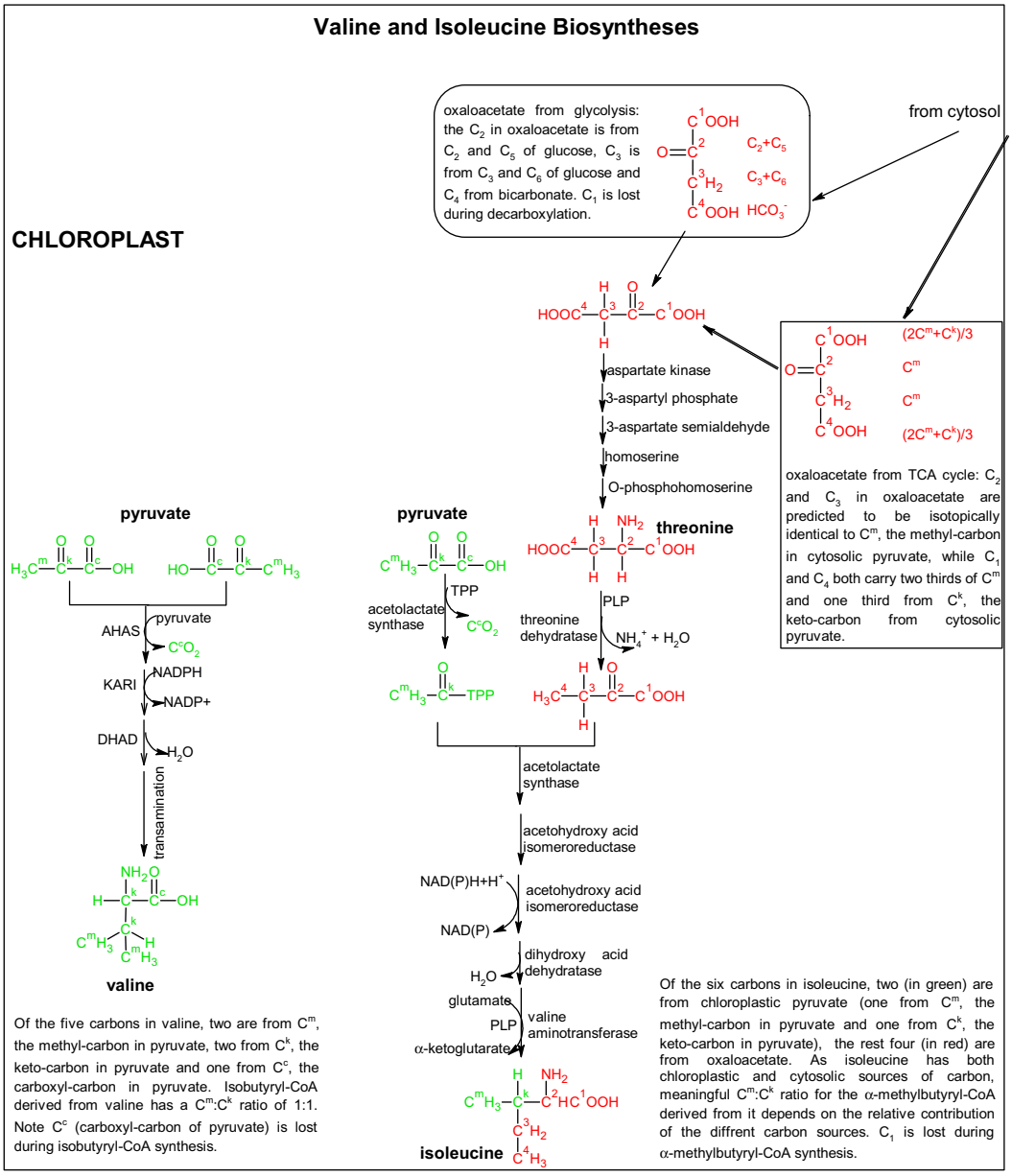

Fig. 4. Biosynthetic pathways of valine, the precursor molecule for *iso*-fatty acids synthesis and isoleucine, the precursor molecule of *anteiso*-fatty acids synthesis.

C_{29} *iso*-alkane. An order of ^{13}C depletion is evident, *n*-alkanes > *iso*-alkanes > *anteiso*-alkanes.

3.2. Stable carbon isotopic compositions of n-alkanes, anteiso-alkanes and iso-alkanes in Nicotiana tabacum from contrasting conditions

The stable carbon isotopic compositions of alkanes from the tobacco samples grown under well-watered and water-stressed conditions are listed in Table 1 and are also shown in Fig. 3. *n*-Alkane ^{13}C ($\delta^{13}C$ −44.9‰ on average) from plants grown under well-watered conditions are ca. 1.8‰ more depleted than those lipids extracted from tobacco grown under water-stressed conditions ($\delta^{13}C$ −43.1‰ on average). A similar order of difference, although larger, is observed for the *iso*-alkanes and *anteiso*-alkanes (2.8‰ and 2.4‰, respectively), the tobacco grown under well-watered conditions being depleted in ^{13}C.

Higher water availability generally results in more negative $\delta^{13}C$ bulk values, and this is confirmed in the present study by compound specific isotopic analyses of *iso*-alkanes, *anteiso*-alkanes and *n*-alkanes lipids from tobacco. Our results are consistent with previous studies based on the carbon isotope ratios of bulk biomass of natural plant communities where plants exposed to elevated levels of soil moisture and/or elevated air humidity are typically more ^{13}C-depleted than plants grown under conditions of low water availability and/or low humidity (e.g. Farquhar, 1980; Farquhar and Richards, 1984; Downton et al., 1985; Martin and Thorstenson, 1988; Madhaven et al., 1991; Garten and Taylor, 1992; Handley et al., 1994; Kohorn et al., 1994; Stewart et al., 1995). Similar orders of difference have been also been reported for Cannabis grown indoors and outdoors (Denton et al., 2001). Indoor-grown Cannabis plants typically had more negative $\delta^{13}C$ than outdoor-grown plants. The ^{13}C depletion of indoor-grown plants was attributed to high relative humidity, poor ventilation and recycling of ^{13}C-depleted respired CO_2 (Denton et al., 2001) but we note that it could also be caused by low-light intensity, with effects as discussed below.

Light intensity has been shown to influence plant bulk $\delta^{13}C$ as lower stomatal conductance, proportionally still lower photosynthetic rate, and the higher ratio of intercellular and atmospheric CO_2 concentrations (C_i/C_a) under low-light conditions result in larger ^{13}C depletion compared with leaves from high light environments (Farquhar, 1980; Farquhar et al., 1982; Denton et al., 2001; Ehleringer et al., 1986, 1987; Zimmerman and Ehleringer, 1990; Gutierrez and Meinzer, 1994; Medina et al., 1994). The stable carbon isotopic compositions of *n*-alkanes in tobacco grown under low-light conditions are ca. 2.2‰ more depleted in ^{13}C ($\delta^{13}C$ −44.5‰ on average) than those lipids extracted from tobacco grown under elevated light conditions ($\delta^{13}C$ −42.3‰ on average). A similar order of difference is observed for the *iso*-alkanes and *anteiso*-alkanes (1.8‰ and 1.9‰, respectively), the tobacco grown under low-light conditions being depleted in ^{13}C.

The stable carbon isotopic compositions of *n*-alkanes in tobacco grown under a higher temperature (30 °C/20 °C) are only ca. 0.5‰ more depleted in ^{13}C ($\delta^{13}C$ −42.9‰ on average) than those lipids extracted from tobacco grown under lower temperature conditions ($\delta^{13}C$ −42.4‰ on average at 18 °C/15 °C). A negligible difference is observed for the *iso*-alkanes and *anteiso*-alkanes, the tobacco grown under elevated temperature being depleted in ^{13}C. These results are consistent with Farquhar (1980) where temperature appears to have only a minor effect in general on plant bulk $\delta^{13}C$.

We have provided a tentative and qualitative phytochemical explanation of the observed $\delta^{13}C$ differences among *n*-alkane, *iso*-alkane and *anteiso*-alkane extracted in tobacco leaf and a physiological explanation for the observed $\delta^{13}C$ shift when growth environments changes. However, we also noticed a saw-toothed pattern for $\delta^{13}C$ (of these alkanes, in particular *n*-alkanes) with odd-number *n*-alkanes being enriched in ^{13}C compared to even-numbered *n*-alkanes in the C_{27}–C_{33} range. A similar but opposite pattern is observed for δD of *n*-alkanes (unpublished). This may be related to intracellular compartmentation of acetyl-CoA used for syntheses of these alkanes and will be described in a future paper.

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