Leaf Transition from Heterotrophy to Autotrophy is Recorded in the Intraleaf C, H and O Isotope Patterns of Leaf Organic Matter

Zhenyu ZHU^a, Xijie YIN^b, Xin SONG^c, Bo WANG^a, Ran MA^a, Yu ZHAO^a, Andleeb RANI^a, Ying WANG^a, Qiulin YAN^a, Jing SU^b, Arthur GESSLER^d, *Youping ZHOU^a

^aIsotopomics in Chemical Biology & Shaanxi Key Laboratory of Chemical Additives for Industry, School of Chemistry & Chemical Engineering, Shaanxi University of Science & Technology, Weiyang University Park, Xi'an 710021, China

^bLaboratory of Marine & Coastal Geology, MNR Third Institute of Oceanology, 178 Daxue Rd, Xiamen 361005, China

^cSchool of Life and Marine Sciences, Shenzhen University, 3688 Nanhai Rd, Shenzhen 518060, China

^dSwiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf 8903, Switzerland

*Author for correspondence

Youping ZHOU, PhD

Isotopomics in Chemical Biology

School of Chemistry & Chemical Engineering

Shaanxi University of Science & Technology

Weiyang University Park, Xi'an 710021, China

Email: youping.zhou@sust.edu.cn

Tel: (86) 29 86168973

This document is the accepted manuscript version of the following article:
Zhu, Z., Yin, X., Song, X., Wang, B., Ma, R., Zhao, Y., ... Zhou, Y. (2020).
Leaf transition from heterotrophy to autotrophy is recorded in the intraleaf
C, H and O isotope patterns of leaf organic matter. *Rapid Communications in
Mass Spectrometry*, 34(19), e8840 (22 pp.). <https://doi.org/10.1002/rcm.8840>

ABSTRACT

RATIONALE: Quantitatively relating $^{13}\text{C}/^{12}\text{C}$, $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of plant α -cellulose and $^2\text{H}/^1\text{H}$ of *n*-alkanes to environmental conditions and metabolic status should ideally be based on the leaf, the plant organ most sensitive to environmental change. The fact that leaf organic matter is composed of isotopically different heterotrophic and autotrophic components means that it is imperative that we are able to disentangle the relative heterotrophic and autotrophic contributions to leaf organic matter.

METHODS: We tackled this issue by 2D sampling of leaf water and α -cellulose and specific *n*-alkanes from greenhouse-grown immature and mature and field-grown mature banana leaves, taking advantage of their large areas and thick waxy layers. Leaf water, α -cellulose and *n*-alkanes isotope ratios were then characterized by EA/IRMS or GC/IRMS. A three-member (heterotrophy, autotrophy and photoheterotrophy) conceptual linear mixing model was then proposed to disentangle the relative contribution of the three trophic modes.

RESULTS: We discovered distinct spatial leaf water, α -cellulose and *n*-alkanes isotope ratio patterns that varied with leaf developmental stages. We infer with the conceptual model that averaged over the leaf blade only 20% of α -cellulose in banana leaf is autotrophically laid down in both greenhouse-grown and field-grown banana leaves, while approximately 60% and 100% of *n*-alkanes are produced autotrophically in greenhouse-grown and field-grown banana leaves, respectively. There exists distinct lateral (edge to midrib) gradients in autotrophic contributions of α -cellulose and *n*-alkanes.

CONCLUSIONS: Efforts to establish quantitative isotope-environment relationships should take into account the fact that the evaporative leaf water ^{18}O and ^2H enrichment signal recorded in autotrophically laid-down α -cellulose is significantly diluted by the heterotrophically formed α -cellulose. The $\delta^2\text{H}$ of field-grown mature banana leaf *n*-alkanes is much more sensitive than α -cellulose as a recorder of the growth environment. Quantitative isotope-environment relationship based on greenhouse-grown *n*-alkanes $\delta^2\text{H}$ may not be reliable.

Keywords: autotrophy, heterotrophy, wax, α -cellulose, leaf blade isotope pattern, banana

1. INTRODUCTION

The molecular and positional level of the C, H and O isotopic ratios ($^{13}\text{C}/^{12}\text{C}$, $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$) of higher plant leaf wax (*n*-alkanes) and carbohydrates (α -cellulose) have proven to be valuable quantitative recorders of the plant growth environment (and by extension, the prevailing climate¹⁻³) and the concomitant metabolic status of the leaf⁴⁻⁹. Efforts to seek a quantitative relationship between isotope ratios (or ratio change) and environmental and metabolic parameters have traditionally been focused on wax and α -cellulose extracted from (pooled) whole leaves (in addition to stem wood). Leaf is arguably the plant organ, whose isotopic compositions are most sensitive to environmental change as its major activity, photosynthesis, is dependent on an array of abiotic environmental factors, including light, temperature, humidity, nutrients and the prevailing CO_2/O_2 ratio. It is the only organ of higher plants that experiences a transition from full heterotrophy to full autotrophy in its life cycle. The early phase of leaf growth and development (e.g., laying down primary cell walls) is dependent on imported photosynthate (heterotrophy) from other leaves or plant carbon stores and thus the isotopic ratios of C, H and O will be a reflection of past environmental conditions (days to months) rather than the currently prevailing conditions. As the leaf matures and gains photosynthetic competence ((photo)autotrophy and photoheterotrophy) the C, H and O isotopic ratios of newly synthesized α -cellulose (secondary cell walls) and wax will directly reflect the prevailing environmental conditions. These different growth phases should be observable in the C, H and O isotopic ratios of the α -cellulose and wax laid down during the different trophic states, as the isotopic signatures of the immediate C, H and O sources (sucrose and local cellular water) will also vary with the trophic status of the leaf^{4,10,11}. If we are to achieve a more reliable understanding of how these isotope signatures relate to the environmental conditions of the growing leaf, it is imperative that we be able to tease apart the relative contribution of heterotrophy from autotrophy in the accumulated organic material of the leaf. A simple analysis of whole leaf isotopic signatures, even a series of analyses, following the different developmental stages^{3,5,12-14} is unlikely to distinguish materials laid down in each growth phase. This is because two dimensional (2D: base to tip, and midrib to edge) translocation of sucrose¹⁵ and the venation-pattern dictated leaf water isotopic gradient (both theoretically predicted^{16,17} and experimentally confirmed^{18,19}, and growth environment-dependent) is known to exist in photosynthesizing mature leaves, confounding the ability of a whole leaf analysis to distinguish heterotrophic and autotrophic growth.

The growth and development of the banana (*Musa basjoo*, a C_3 perennial monocotyledon²⁰) leaf with its large size and waxy cuticle represents an opportunity to

directly compare the heterotrophic and autotrophic phases of growth. The banana leaf grows as a “candle” in which one half of the leaf wraps the other half. This candle then naturally unfurls when the leaf is approximately 90% full size²¹ (**Fig1 A**). The internally wrapped half of the leaf is, except for the tip, largely unexposed to light and its growth to that point is therefore (very likely exclusively) heterotrophic with photosynthate imported to support its growth. The leaf’s subsequent growth to maturity in the unfurled state then occurs autotrophically, with the leaf sourcing its own photosynthate. In this paper, we describe and compare the 2D isotopic maps of leaf water, *n*-alkanes and α -cellulose in banana leaves. We looked at firstly, the immature, non-photosynthetically competent half of a greenhouse-grown “candle” (heterotrophic growth, **Fig 1B**) and compared it to the mature (fully autotrophic) greenhouse-grown (**Fig 1C**) and field-grown mature (fully autotrophic) leaves (**Fig 1D**). From these isotopic maps, we propose a conceptual model to quantitatively disentangle the relative contributions of autotrophy and heterotrophy to the growth of a banana leaf.

2. MATERIALS and METHODS

2.1 Leaf wax, α -cellulose and water sampling and extraction, and gas exchange measurement

Sampling and extraction. Glasshouse-grown healthy banana leaves were sampled on Jan 11, 2018 in the Fengdong Horticultural Expo (Xi’an, China). The day/night temperature and relative humidity inside the greenhouse were maintained at 30($\pm$ 1)/20($\pm$ 1) °C and 60($\pm$ 5)/80($\pm$ 5)% humidity. The plants were watered with tank water with stable $\delta^2\text{H}$ (-112.0 ± 3.6 mUr) and $\delta^{18}\text{O}$ (-11.5 ± 1.7 mUr) while the force-circulated air (CO_2) inside the greenhouse has a stable $\delta^{13}\text{C}$ (-9.2 ± 0.2 mUr) for CO_2 over the entire period of leaf development. The leaf blade of an immature leaf (**Fig 1B**) was harvested while still rolled in a “candle”. A mature fully unfurled leaf (**Fig 1C**) with exposure to solar radiation was also harvested. Although ideally the study of the leaf heterotrophy to autotrophy transition should use leaves from the same plant, the immature and mature leaves used in this study were sampled from two different plants as sampling from the same plant would have significantly decreased the horticultural value of the plant in the Horticultural Expo. The field-grown healthy leaf (**Fig 1D**) was sampled on a sunny day (Feb 28, 2017) from a banana plant grown on a hillslope on the campus of the Chinese Academy of Tropical Agricultural Sciences (Hainan, China). All leaves were cut off from the petiole and immediately subdivided lengthwise into 11 (for immature leaf) and 20 (for mature leaves) segments and each longitudinal segment was then further sectioned laterally into 4-5 sub-segments (**Fig 1**; segments numbered

from base to tip. Each segment subdivided and alphabetically labelled from midrib to edge). For the immature leaf (**Fig 1B**), only the photosynthetic incompetent half was sub-segmented and leaf water and organic matter extraction were attempted on the same subsegments in sequence; for the mature leaves (**Fig 1C and 1D**), both halves were used, with one half for organic matter extraction and the other water for water extraction at symmetrical positions. Leaf subsegments for water extraction were immediately sealed in pre-labelled and pre-cooled plastic tubes and kept frozen with dry ice in the field and in fridge when in laboratory before water extraction while those subsegments for organic matter extraction were placed in pre-labelled paper bags and dried naturally (to prevent fungal growth) before being transferred back to the laboratory. Once in the laboratory, leaf sub-segments (including those from the immature leaf following water extraction) were rinsed with high purity water (Youpu Ltd, China) water, dried in an oven at 55 °C and then powdered before wax and α -cellulose extraction. The midrib of the glasshouse grown mature leaf (**Fig 1C**) was also divided lengthwise to 20 segments for water extraction. To compare the spatial patterns of α -cellulose content in leaves of various developmental stages, a mature leaf (on which gas exchange measurements were conducted, see below the subsection **Gas exchange measurement** for details) and an immature leaf sampled from a field-grown banana plant on Shenzhen University campus were segmented and subsegmented in a similar fashion to those leaves for leaf water, α -cellulose and *n*-alkanes extraction.

Leaf water extraction. Leaf waters were extracted on a purpose-built cryogenic extraction line. Vacuum at the initial and final stage of extraction of <1 Pa was routinely achieved, ensuring near complete recovery of leaf water and minimal isotopic fractionation associated with water recovery. Extracted water was then passed through charcoal and then a 0.22 μ m PTFE filter to remove any particles and organic matter that may interfere with the isotopic analysis.

Lipid extraction. Lipids were Soxhlet-extracted from dried power overnight at 75 °C with KOH-CH₃OH (v/v = 9/1) solution following the method described in Zhou et al.^{8,22}. Neutral lipid was then separated from the total extract on an activated silica gel column. The hydrocarbons, of which the alkanes are a component, were eluted with *n*-hexane. After being dried under a dry N₂-stream, the hydrocarbon fractions were kept frozen until GC/MS and GC/IRMS analysis (see details below). Control experiments showed no significant isotopic fractionations during the extraction and drying procedure.

α -Cellulose extraction. Leaf α -celluloses were extracted following the method of Zhou et al.²³. Briefly, dried and powdered leaf materials were first delipified with a

combination of CH₃OH and CH₂Cl₂, then treated with concentrated NaOH to hydrolytically remove hemicellulose and finally delignified with acidified NaClO₂. The α -celluloses obtained after washing with purified water were dried and stored up in a desiccator until isotope analysis.

Gas exchange measurement. Gas exchange measurement was conducted using a Licor 6400 Photosynthetic System (Licor, Lincoln, Nebraska) on a field grown mature leaf (on the campus of Shenzhen University) between 11am-3pm when the gas exchange behaviour was stabilized. The obtained C_i/C_a ratios were used for the modelling described in section 3.3.

2.2 Isotope analysis

α -Cellulose C, O and H isotope analysis. α -Celluloses were analysed for O and H isotope composition following the method described in Zhou et al.²⁴. For O and H isotope analysis, α -cellulose and isotope standards (benzoic acid, IAEA-601, $\delta^{18}\text{O}=23.3$ mUr (equivalent to ‰)) and certified reference materials (EMA P1: $\delta^{13}\text{C}=-28.10$ mUr; $\delta^{18}\text{O}=26.88$ mUr; $\delta^2\text{H}=-87.80$ mUr) were pyrolyzed in a glassy-carbon lined ceramic tube at 1380 °C where O and H in samples, standards and reference materials were quantitatively converted to CO and H₂ in a Flash HT (1112 series) EA (elemental analyzer) (Thermo Fisher GmbH, Bremen) coupled to a MAT 253 plus IRMS (Isotope Ratio Mass Spectrometry) via a ConFlo III (Thermo Fisher GmbH, Bremen). For C isotope analysis, all Cs were quantitatively converted to CO₂ and chromatographically purified online before being introduced to the IRMS for isotope ratio measurement. Corrections were made for the absorbed water due to the hygroscopic –OH groups of α -cellulose according to Zhou et al.²⁴ and Ma et al.⁶. The isotopic composition of carbon-bound H ($\delta^2\text{H}_{\text{C-H}}$ value) in the α -cellulose was calculated following the equation:

$$\delta^2\text{H}_{\text{C-H}} = \frac{[\delta_{\text{total}} - p(\alpha(\delta_{\text{moisture}} + 1) - 1)]}{(1-p)} \quad (\text{mUr}) \quad (\text{I})$$

where $\delta^2\text{H}_{\text{total}}$ value is the isotopic compositions of all hydrogen atoms in α -cellulose pre-equilibrated with air moisture of known isotopic composition ($\delta^2\text{H}_{\text{moisture}}$ value). A value of 0.28 rather than 0.3 (3 hydrogen atoms in the OH groups out of a total of 10 hydrogen atoms in the glucose unit of α -cellulose) was assigned to p as not all hydrogen atoms in –OH groups are exchangeable²⁵. A value of 1.22 was assigned to α (fractionation factor between moisture and the hydrogen atoms in the exchangeable positions at a given equilibrium temperature) according to Feng et al.²⁶.

GC/MS and CSIA-H analyses of n -alkanes. GC/MS analyses of n -alkanes were carried out using a Trace GC1310 gas chromatograph interfaced with a Thermo

Scientific DSQ single quadrupole mass spectrometry. The mass spectrometer was run in EI mode and scanned 40-400 amu. *n*-Alkanes were identified by comparison of their retention times and mass spectra to those of standards. CSIA-H and -C were performed on a Thermo Scientific MAT 253 plus mass spectrometer interfaced to a Thermo Scientific Trace 1310 gas chromatograph (Bremen, Germany). Indiana *n*-alkane mix (C₁₆-C₃₀) was used as H standard (see <http://pages.iu.edu/~aschimme/hc.html> for the details of the reference materials). The isotopic compositions of the waxes in this study fell within the isotopic range of the standard mix. $\delta^2\text{H}$ value for *n*-alkanes was reported either individually or as a weight-averaged.

Leaf water isotopic analysis. Leaf water H and O isotopic analyses conducted on a Thermo Scientific Delta V advantage coupled to a Flash 2000 elemental analysis. Samples and in-house water isotope standards (GBW-0440: $\delta^{18}\text{O}=0.32$ mUr; $\delta^2\text{H}=-0.4$ mUr; GBW-0443: $\delta^{18}\text{O}=-24.52$ mUr, $\delta^2\text{H}=-189.1$ mUr) for calibration were pyrolytically converted to H₂ and CO at 1380 °C and then chromatographically separated before being analysed by IRMS.

In all H isotope analysis, the H₃⁺ factor was monitored daily and the average value was always less than 6 ppm/nA. Isotopic compositions were reported in the standard δ -notation relative to V-SMOW (Vienna Standard Mean Ocean Water) for H and O and V-PDB (Vienna PeeDee Belemnite) for C:

$$\delta = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \text{ (mUr)}$$

where R_{sample} and R_{standard} are the isotopic ratios ($^2\text{H}/^1\text{H}$, $^{18}\text{O}/^{16}\text{O}$ or $^{13}\text{C}/^{12}\text{C}$) of sample and standard respectively.

All analyses were performed in duplicate or triplicate. Precisions for *n*-alkane H isotope analyse were better than 3 mUr, for α -cellulose C, H and O isotopes were better than 0.1 mUr, 2 mUr and 0.3 mUr, respectively, and for water H and O isotopes were better than 2 mUr and 0.3 mUr, respectively.

3. RESULTS and DISCUSSION

To quantitatively relate the spatially mapped organic isotope compositions to the trophic status of the banana leaf, we first present the 2D isotopic maps of leaf water (H and O), α -cellulose (C, H and O) and *n*-alkanes (H) (**Section 3.1**, see **Table S1** for data) for leaves at different developmental stages. This is followed by a discussion of banana leaf ontogeny in relation to wax and α -cellulose deposition (**Section 3.2**). On the observational (**Section 3.1**) and theoretical (**Section 3.2**) bases, we then develop a conceptual model linking the isotopic composition of leaf organic matter to the transition from heterotrophic to autotrophic growth (**Section 3.3**). We examine the quality of the fit between the model predicted and observed isotopic spatial trends in the leaf and finally discuss the implications of the model for the isotope-based environment (climate)-reconstruction (**Section 3.4**).

3.1 Spatial C, H and O isotopic patterns of leaf water, α -cellulose and *n*-alkanes of greenhouse- and field-grown banana leaves

Leaf and midrib water $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values. In the photosynthetically incompetent half of the “candle” (**Fig 1B**), both $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of leaf water display a distinct tipward increasing trend (**Fig 2A**). Close to the base, there was no discernable lateral trend of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values, in sharp contrast to a clear edgeward gradient (increase from midrib to edge) of 99 mUr for $\delta^2\text{H}$ value and 25.8 mUr for $\delta^{18}\text{O}$ value, respectively, to the tip. This is consistent with the fact that when the leaf was sampled, it was still in a “candle” (rolled up state), so that the inner part of the “candle” received no solar radiation while obviously the tip and edge did. In the greenhouse-grown mature leaf (**Fig 1C**), both $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of leaf water (**Fig 2B**) display a very strong edgeward increasing trend (segmental gradient ranging from 131.4 to 97.7 mUr with an average of 117.4 mUr for the $\delta^2\text{H}$ value, and 24.7 to 36.0 mUr with an average of 28.9 mUr for $\delta^{18}\text{O}$) and an overall moderate tipward increasing trend (segmental average ranging from -49.2 to -29.4 with a gradient of 19.8 mUr for the $\delta^2\text{H}$ value, and from 7.2 to 14.2 mUr with a gradient of 7.1 mUr for the $\delta^{18}\text{O}$ value). This is consistent with the fact that in a fully unfurled mature leaf, evaporation induces ^2H and ^{18}O enrichment and vasculature dictates the spatial enrichment pattern. Midrib sampled longitudinally showed a slight tipward increase in the $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values, and on average slight enrichment relative to that of the irrigation water (-112.0 mUr for the $\delta^2\text{H}$ value and -11.5 mUr for the $\delta^{18}\text{O}$ value) (**Fig 2B**). The field-grown mature leaf (**Fig 1D**) displayed similar edgeward trend (segmental gradient ranging from 63.8 to 106.4 mUr with an average of 90.0 mUr for the $\delta^2\text{H}$ value, and 16.2 to 25.2 mUr with an

average of 21.2 mUr for the $\delta^{18}\text{O}$ value) and tipward (segmental average ranging from 10.9 to 31.5 mUr with a gradient of 20.6 mUr for the $\delta^2\text{H}$ value, and from 13.3 to 19.2 mUr with a gradient of 5.9 mUr for the $\delta^{18}\text{O}$ value) increasing trends (**Fig 2C**), similar to those of the greenhouse grown mature leaf, again a reflection of the evaporation and vasculature-dictated enrichment patterns.

α -Cellulose $\delta^{18}\text{O}$ values. There was no discernable lateral trend in the heterotrophic part of the greenhouse-grown immature leaf (**Fig 1B**), but there was a clear edgeward gradient of 5.5 mUr at the (autotrophic) tip of the same leaf (**Fig 3A**). This edgeward gradient is roughly 1/5 (5.5/25.8) of the leaf water $\delta^{18}\text{O}$ value gradient (25.8 mUr, see **Fig 2A**) in the tip segment which was subjected to evaporative enrichment. In the greenhouse grown mature leaf (fully expanded and unfurled, **Fig 1C**), there was a clear tipward (segmental average ranging from 31.1 to 33.5 mUr with a gradient of 2.35 mUr) and edgeward enrichment in $\delta^{18}\text{O}$ values (segmental gradient ranging from 3.3 to 4.5 mUr with an average of 4.2 mUr for the $\delta^{18}\text{O}$ values) (**Fig 3B**). This edgeward trend is similar to that found in monocotyledonous grasses^{27,28}. The field-grown mature leaf α -cellulose displayed neither edgeward nor tipward trend in the $\delta^{18}\text{O}$ values across the whole blade (**Fig 3C**), in sharp contrast to the clear edgeward and tipward trend in the greenhouse- and field-grown mature leaf water (**Fig 2B** and **2C**) and α -cellulose (**Fig 3B**). The distinct edgeward and tipward increasing trends across the whole blade of the greenhouse-grown mature leaf and at the tip of immature leaf indicates that autotrophy initiates in the tip and edge and expands toward the base and the midrib. Note that on average the glasshouse grown mature leaf (**Fig 3B**) is more enriched in ^{18}O than that of the immature leaf (**Fig 3A**), consistent with the idea that secondary cell wall synthesis proceeds (expands) in more enriched water (under fully unfurled and irradiated state).

α -Cellulose $\delta^2\text{H}$ values. In the greenhouse-grown immature leaf (**Fig 1B**), there was a clear edgeward increasing, but no discernable longitudinal trend in $\delta^2\text{H}$ values across much of the (heterotrophic part of) leaf blade (with segmental gradient ranging from 13.0 to 22.6 and averaging 17.4 mUr) except that at the leaf tip (autotrophic part) there was an overall edgeward dip by 32.1 mUr (**Fig 4A**). In the greenhouse-grown mature leaf, there was a clear tipward decreasing trend (segmental average ranging from -76.8 mUr to -90.3 mUr, with a gradient of 13.5 mUr) and an overall (with fluctuations) edgeward increasing trend close to the base and an overall (with fluctuations) decreasing trend close to the tip, indicating increasing contribution of relative ^2H -depleted α -cellulose during autotrophic growth (**Fig 4B**). Again, no discernable trend existed in the field-grown mature leaf α -cellulose $\delta^2\text{H}$ values except close to the leaf tip, where the segmental average (-20.5 mUr) was slightly elevated relative to the rest of the leaf blade (-33.2 mUr) by 12.7 mUr (**Fig 4C**). The similarity of the acropetal

decreasing trend between greenhouse-grown mature and immature leaves indicates that autotrophy proceeds in the basipetal direction. Again, the shift from an overall edgeward increasing trend near the base to an overall edgeward decreasing trend close to the tip as seen in the greenhouse-grown mature leaf also implies that autotrophy proceeds from the leaf edge towards the midrib.

α -Cellulose $\delta^{13}\text{C}$ values. In the greenhouse-grown immature leaf, there was a discernable tipward (segmental average ranging from -27.9 to -27.5 mUr with a gradient of 0.4 mUr) and edgeward (segmental gradient ranging from 0.43 mUr to 0.50 mUr with an average of 0.45 mUr) increasing trend in $\delta^{13}\text{C}$ values across the blade except in the tip segment, there was clear edgeward decrease by 0.68 mUr in $\delta^{13}\text{C}$ values (**Fig 5A**). In the greenhouse-grown mature leaf, there was a clear edgeward (segmental average ranging from 0.67 to 1.74 with an average of 1.30 mUr) and tipward (-27.37 to -26.02, with a gradient of 1.35 mUr) decreasing trend in $\delta^{13}\text{C}$ values across the whole leaf blade. Notably there was a reversal of the edgeward decreasing trend in the segments close to the base: the $\delta^{13}\text{C}$ values for the subsegments at the edge were greater than their immediate neighbors, but such reversal was lost in the segments closer to the tip (**Fig 5B**). Again, no discernable edgeward trend was found in the field-grown mature leaf α -cellulose $\delta^{13}\text{C}$ values except that a decrease by 0.50 mUr was observed in the segmental average near the tip (-28.02 mUr) from the rest of the leaf blade (-27.53 mUr) (**Fig 5C**), contrary to the observed tipward increase in $\delta^2\text{H}$ values for the segmental average near the tip of the greenhouse-grown immature leaf (**Fig 4C**). The overall acropetal and edgeward decreasing trend in this greenhouse-grown mature leaf again implies that autotrophy expands both basipetally and laterally toward the midrib.

***n*-alkane $\delta^2\text{H}$ values.** In the greenhouse-grown immature leaf, there was no discernable lateral trend but a clear tipward decreasing trend (segmental average ranging from -179.0 to 193.1 mUr with a gradient of 14.1 mUr for the C_{27} -alkane and 171.3 to -192.7 mUr with a gradient of 21.4 mUr for the C_{29} -alkane) in $\delta^2\text{H}$ values across the heterotrophic part of the leaf blade except in the tip where there was a distinct edgeward increasing trend (ranging from -182.4 to -152.1 mUr with a gradient of 30.3 mUr for C_{27} -alkane, and -200.3 to -171.5 with a gradient of 28.8 mUr for C_{29} -alkane) (**Fig 6A**) in parallel to that of the leaf water (**Fig 2A**). Overall, the dip (trough) in the curve connecting segmental average values is close to the leaf tip. In the greenhouse-grown mature leaf, there was a clear edgeward increasing trend across the whole leaf blade with a segmental gradient ranging from 60.3 mUr (the tipmost segment excluded) to 29.4 mUr with an average of 39.4 mUr for the C_{27} -alkane and 37.2 mUr to 22.7 mUr with an average of 29.6 mUr for the C_{29} -alkane (**Fig 6B**). This edgeward trend is similar

to those found in monocotyledonous grasses²⁹⁻³¹. Similar to the immature leaf there was a distinct trough (dip) in the curve connecting segmental average $\delta^2\text{H}$ values for both the C_{27} and C_{29} -alkanes, but here the trough was closer to the leaf base. In the field-grown mature leaf, there was a clear edgeward increasing trend (segmental gradient ranging from 29.5 mUr to 52.4 mUr with an average of 38.4 mUr for odd-carbon numbered *n*-alkanes, 29.1 mUr to 61.0 mUr with an average of 46.1 mUr for even-carbon numbered *n*-alkanes; 29.9 mUr to 55.6 mUr with an average of 41.4 mUr and for the weighted average of all *n*-alkanes) across the whole leaf blade (**Fig 6C**), similar to that in the greenhouse-grown mature leaf. The dip (trough) in the curve connecting the segmental averages (ranging from the -182.9 mUr to -161.3 mUr, with a gradient of 21.6 mUr for weighted average of all *n*-alkanes) across the whole leaf blade was again even closer to the leaf base. The distinct edgeward increasing trend across the entire blade of the greenhouse-grown mature and near the tip of the immature leaf indicate that autotrophy proceeds laterally toward the midrib. The basipetal shift of the dip (trough) of the curves from immature to mature leaves also implies that autotrophy proceeds basipetally.

3.2 Banana leaf ontogeny in relation to wax and α -cellulose deposition

The growth of banana leaf starts with a primordium initiated within an apical meristem close to the point of insertion^{32,33}. Developmentally, cells close to the tip and edge are older while those close to the base and midrib are younger. Prior to emergence, lamina growth is largely due to, first, acropetal (longitudinal) and, later, lateral expansion of cells within a ‘sheltered’ environment created by the pseudosheath of older leaves²¹. The lamina develops with one-half wrapped within the trough of the midrib while the other half envelops both midrib and the first half, so that when the blade is still in the “candle”, the enveloping half is fully autotrophic while the concealed or wrapped half is autotrophic only at the tip (**Fig 1A and 1B**) where it is exposed to light. A new leaf starts growing only after the growth of the immediate neighboring leaf is completed³⁴ and unrolling only after it is completely out of the pseudosheath.

Synthesis of organic matter (including α -cellulose and wax) across the entire leaf blade is purely a heterotrophic process using imported sugars (mainly sucrose¹⁵) from neighboring leaves (**Fig 7A, upper panel**). Deposition of the primary cell wall (and veins of various orders) occurs when the cell is still expanding while deposition of the secondary cell wall (and veins of various orders) occurs after cell expansion has stopped³⁵, so it is inferable that the secondary cell wall most likely contributes to the cell wall thickness, and hence leaf width increase following cessation of longitudinal

growth as seen in the C₃ monocotyledonous grass *Festuca arundinacea* Shreb^{36,37}. As Liu et al.³⁸ showed that α -cellulose (a major component of cell wall) deposition stopped at (or shortly after) full leaf expansion in a perennial C₄ monocotyledonous grass *Cleistogenes squarrosa*, it is also inferrable that the hemicellulose is most likely the dominant component of the secondary wall.

Once emerged from the pseudosheath, structural and functional maturation of the low order veins proceeds in basipetal and lateral (toward the midrib) directions³⁹. This process is accelerated once the “candle” emergence ends and the blade starts unrolling. Cells near the edge and tip first gain photosynthetic competence and start depositing wax and α -cellulose using their own, locally assimilated, autotrophic carbon (**Fig 7B, upper panel**). Cells away from the edge and tip and which are not yet photosynthetically competent will still be heterotrophic, receiving photosynthate from both cells closer to the photosynthetic edge and imported via the midrib (**Fig 7B, upper panel**). The basipetal and lateral (toward the midrib) procession of autotrophy continues until all cells become photosynthetically competent and have transitioned from carbon sinks to carbon sources (**Fig 7C, upper panel**).

3.3 Conceptual isotope model linking autotrophy and heterotrophy

Model establishment. From Section 3.1 and from an understanding of the ontogeny and biosynthesis of α -cellulose and wax (Section 3.2), it is clear that in a mature leaf α -cellulose and *n*-alkanes have been deposited heterotrophically (photosynthates imported into the developing leaf) prior to leaf emergence, and both autotrophically (photosynthates produced locally) and also photoheterotrophically from photosynthates transported from the newly photosynthetically competent leaf edge (**Fig 7B**) after emergence in spatially opposing directions. Therefore, at any point across the blade of a mature leaf, the C, H and O isotope compositions of total α -cellulose ($\delta^A X_{cel}^{total}$) and wax can be modelled as the weighted average of the *n*-alkanes and α -celluloses produced in the three trophic modes. Mathematically, they can be expressed as (note that α -cellulose is synthesized and laid down locally and is non-recyclable):

$$\delta^2 H_{cel}^{total} \approx \delta^2 H_{cel}^{het} f_{cel}^{het} + f_{cel}^{auto} (\delta^2 H_{water}^{local} + {}^2\epsilon_{water2cel}^{auto} + \delta^2 H_{water}^{local} {}^2\epsilon_{water2cel}^{auto}) + f_{cel}^{photohet} (\delta^2 H_{water}^{local} + {}^2\epsilon_{water2cel}^{photohet} + {}^2\epsilon_{water2cel}^{photohet} \delta^2 H_{water}^{local}) \quad (II)$$

$$\delta^{18} O_{cel}^{total} \approx \delta^{18} O_{cel}^{het} f_{cel}^{het} + f_{cel}^{auto} (\delta^{18} O_{water}^{local} + {}^{18}\epsilon_{water2cel}^{auto} +$$

$$\delta^{18}O_{water}^{local} \delta^{18}\epsilon_{water2cel}^{auto} + f_{cel}^{photohet} (\delta^{18}O_{water}^{local} + \delta^{18}\epsilon_{water2cel}^{photohet} + \delta^{18}\epsilon_{water2cel}^{photohet} \delta^{18}O_{water}^{local}) \quad (III)$$

$$\delta^{13}C_{cel}^{total} \approx \delta^{13}C_{cel}^{het} f_{cel}^{het} + \delta^{13}C_{cel}^{auto} f_{cel}^{auto} + \delta^{13}C_{cel}^{photohet} f_{cel}^{photohet} \quad (IV)$$

where $\delta^A X_{cel}^{total}$, $\delta^A X_{cel}^{auto}$, $\delta^A X_{cel}^{het}$ and $\delta^A X_{cel}^{photohet}$ are the $^A X$ ($=^{13}C$, 2H , ^{18}O) isotope compositions of total, heterotrophically, autotrophically and photoheterotrophically synthesized α -cellulose, respectively; $\delta^A X_{water}^{local}$ is the isotopic composition of cellular water ($^A X=^2H$ or ^{18}O) at the site of α -cellulose and n -alkane synthesis; $\delta^A \epsilon_{water2cel}^{auto}$ refers to the enrichment of 2H ($^2\epsilon$) or ^{18}O ($^{18}\epsilon$) of α -cellulose synthesized autotrophically over that of the local leaf water ($\delta^2 H_{water}^{local}$ or $\delta^{18} O_{water}^{local}$), respectively ($\epsilon = \alpha - 1$, where α is the fractionation factor); $\delta^A \epsilon_{water2cel}^{photohet}$ refers to the enrichment of 2H ($^2\epsilon$) or ^{18}O ($^{18}\epsilon$) of α -cellulose synthesized photoheterotrophically over that of leaf water respectively; f_{cel}^{auto} , f_{cel}^{het} and $f_{cel}^{photohet}$ ($f_{cel}^{het} + f_{cel}^{auto} + f_{cel}^{photohet} = 1$) are the proportions of α -cellulose generated in heterotrophic, autotrophic, and photoheterotrophic modes, respectively. Theoretically the f_{cel}^{auto} , f_{cel}^{het} and $f_{cel}^{photohet}$ for a given site (in our case, a given subsegment) for different isotopes on the leaf blade should be the same, but may vary across the leaf blade.

Similarly, the $\delta^2 H$ values of n -alkanes at a given site on the leaf blade can be expressed as (note that n -alkanes are synthesized and laid down locally and are very likely non-recyclable):

$$\delta^2 H_{alkane}^{total} \approx \delta^2 H_{alkane}^{het} f_{alkane}^{het} + f_{alkane}^{auto} (\delta^2 H_{water}^{local} + \delta^2 \epsilon_{water2alkane}^{auto} + \delta^2 H_{water}^{local} \delta^2 \epsilon_{water2alkane}^{auto}) + f_{alkane}^{photohet} (\delta^2 H_{water}^{local} + \delta^2 \epsilon_{water2alkane}^{photohet} + \delta^2 \epsilon_{water2alkane}^{photohet} \delta^2 H_{water}^{local}) \quad (V)$$

where $\delta^2 H_{alkane}^{het}$ is the H isotopic composition of heterotrophically synthesized n -alkanes; f_{alkane}^{auto} , f_{alkane}^{het} and $f_{alkane}^{photohet}$ ($f_{alkane}^{het} + f_{alkane}^{auto} + f_{alkane}^{photohet} = 1$) are the proportions of n -alkanes generated in heterotrophic, autotrophic, and

photoheterotrophic modes, respectively. Other parameters are defined in a similar fashion to equations II to IV.

Model parameterization. To estimate $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$ from equations II and III, H and O isotope enrichments for the synthesis of α -cellulose in all three trophic modes must be known, in addition to the isotopic compositions of heterotrophically synthesized α -cellulose, and the isotopic compositions of water at the site of autotrophic/photoheterotrophic biosynthesis. In this study we adopt the $^{18}\epsilon$ values ($^{18}\epsilon_{\text{water2cel}}^{\text{auto}}$, $^{18}\epsilon_{\text{water2cel}}^{\text{het}}$ and $^{18}\epsilon_{\text{water2cel}}^{\text{photohet}}$) for α -cellulose synthesized in the three trophic modes reported by Sternberg and Deniro⁴⁰ and Yakir and Deniro¹⁰, and the $^2\epsilon$ values ($^2\epsilon_{\text{water2cel}}^{\text{auto}}$ and $^2\epsilon_{\text{water2cel}}^{\text{het}}$) reported by Cormier et al.⁴¹ as these are the only ϵ values relevant to our study here (Table 1).

To estimate $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$ from equation IV, $\delta^{13}\text{C}_{\text{cel}}^{\text{het}}$, $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}}$ and $\delta^{13}\text{C}_{\text{cel}}^{\text{photohet}}$ should be known. In this study we use the average $\delta^{13}\text{C}$ values of all equivalent heterotrophic subsegments as $\delta^{13}\text{C}_{\text{cel}}^{\text{het}}$ for the modelling purpose. To estimate the $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}}$ and $\delta^{13}\text{C}_{\text{cel}}^{\text{photohet}}$ for the subsegments, we use the relationship between $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}}$ and the C_i/C_a ratio (C_i and C_a are the leaf internal and external CO_2 concentrations): $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}} = \beta + \delta^{13}\text{C}_{\text{CO}_2} - \left[\mathbf{a} + (\mathbf{b} - \mathbf{a}) \frac{\text{C}_i}{\text{C}_a} \right]$, where $\mathbf{a}$ and $\mathbf{b}$ are the fractionations associated with CO_2 diffusion and carboxylation by RubisCO during photosynthetic CO_2 assimilation into bulk organic matter⁴². The slope $(\mathbf{b} - \mathbf{a})$ is set as 19.5 mUr according to the greenhouse growth experiments by Brugnoli et al.⁴³ for this study. The adjustable β is the isotopic offset between bulk organic matter and that of the α -cellulose. Its value was estimated by assuming the $\delta^{13}\text{C}$ value of the edge subsegment matches the $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}}$. On another field grown mature banana leaf, we measured the lateral gradient of C_i/C_a and obtained values between 0.38 to 0.8 (Fig 8). The same lateral C_i/C_a gradient was also applied to the photoheterotrophically synthesized α -cellulose, with an estimated offset value between $\delta^{13}\text{C}_{\text{cel}}^{\text{photohet}}$ and $\delta^{13}\text{C}_{\text{cel}}^{\text{auto}}$ of 0.3 mUr for the same subsegment. In doing so, it is assumed that $\mathbf{b}$ does not vary across leaf blade.

Model prediction. Although heterotrophy, autotrophy and photoheterotrophy proceed in both longitudinal and lateral directions, the leaf water isotopic gradient is, however,

mostly evident in the lateral direction. We therefore limit our modeling to only the lateral direction. It should be pointed out that modeling in the longitudinal direction can be carried out in a similar way which involves adding an adjustment factor (which can be derived by fitting the model to the data) to the equations II-V to reflect the (observed) longitudinal isotope gradients. The model predictions are visualized in **Fig 9**. Since $0 > {}^2\varepsilon_{\text{water2cel}}^{\text{het}} > {}^2\varepsilon_{\text{water2cel}}^{\text{photohet}} > {}^2\varepsilon_{\text{water2cel}}^{\text{auto}}$ ¹⁰, depending on the actual values of ${}^2\varepsilon_{\text{water2cel}}^{\text{photohet}}$, ${}^2\varepsilon_{\text{water2cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$, any heterotrophically formed α -cellulose $\delta^2\text{H}$ gradient may be lost or even reversed. In this study, the heterotrophically generated edgeward increasing trend of the α -cellulose $\delta^2\text{H}$ value (**Fig 4A**) is predicted to be gradually weakened/lost and eventually reversed as $f_{\text{cel}}^{\text{auto}}$ and $f_{\text{cel}}^{\text{photohet}}$ increase because $0 > {}^2\varepsilon_{\text{water2cel}}^{\text{photohet}} > {}^2\varepsilon_{\text{water2cel}}^{\text{auto}}$. This is predicted even though the procession of autotrophy should strengthen the edgeward increasing trend of the $\delta^2\text{H}$ value due to the presence of an edgeward leaf water ($\delta^2H_{\text{water}}^{\text{local}}$) increasing trend. However, for the $\delta^{18}\text{O}$ value, a gradient parallel to that of leaf water is always predictable since ${}^{18}\varepsilon_{\text{water2cel}}^{\text{het}} \approx {}^{18}\varepsilon_{\text{water2cel}}^{\text{photohet}} \approx {}^{18}\varepsilon_{\text{water2cel}}^{\text{auto}} \approx 27 \text{ mUr}$ ^{10,40}. In this case, the lack of a discernable $\delta^{18}\text{O}_{\text{cel}}^{\text{het}}$ trend (**Fig 3A**) will be replaced with an edgeward trend mimicking that of the leaf water $\delta^{18}\text{O}_{\text{water}}^{\text{local}}$. Since ${}^{13}\text{C}_{\text{cel}}^{\text{auto}} < \delta^{13}\text{C}_{\text{cel}}^{\text{photohet}} < \delta^{13}\text{C}_{\text{cel}}^{\text{het}} < 0$, depending on the actual values of as $f_{\text{cel}}^{\text{auto}}$ and $f_{\text{cel}}^{\text{photohet}}$, any heterotrophically formed trend may be lost or even reversed. In this study, the edgeward increasing trend in the heterotrophically produced $\delta^{13}\text{C}_{\text{cel}}^{\text{het}}$ (**Fig 5A**) is predicted to be first weakened/lost and eventually reversed as the procession of autotrophy will generate an edgeward decreasing trend.

For n -alkanes, since $0 > {}^2\varepsilon_{\text{water2alkane}}^{\text{het}} > {}^2\varepsilon_{\text{water2alkane}}^{\text{photohet}} > {}^2\varepsilon_{\text{water2alkane}}^{\text{auto}}$ ^{5,41} and approximately 50% of the H in n -alkanes is derived directly from cellular water⁴⁴ (the other 50% is from NADPH), any heterotrophically formed trend may also be lost or even reversed depending on $f_{\text{alkane}}^{\text{auto}}$ and $f_{\text{alkane}}^{\text{photohet}}$. In this case, the absence of a trend in the $\delta^2\text{H}$ value of the heterotrophically generated n -alkanes (**Fig 6A**) is predicted to be replaced with an edgeward (and tipward, although weak) increasing trend parallel to that of leaf water $\delta^2H_{\text{water}}^{\text{local}}$.

Estimating $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$. To disentangle the contribution of heterotrophy to

the (synthesis of) total α -cellulose, effort should be made to find a set of values for $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$ that give the best fit between model prediction and observation. These f values can be estimated from any two of the three equations **II**, **III** and **IV** provided that $\delta^A X_{\text{het}}$, ${}^A \varepsilon_{\text{water2cel}}^{\text{het}}$, ${}^A \varepsilon_{\text{water2cel}}^{\text{photohet}}$ and ${}^A \varepsilon_{\text{water2cel}}^{\text{auto}}$ ($A=^{18}\text{O}$, ^2H or ^{13}C) are known as $\delta^A X_{\text{cel}}^{\text{total}}$ and $\delta^A X_{\text{cel}}^{\text{het}}$ are measured. The values for ${}^{18} \varepsilon_{\text{water2cel}}^{\text{het}}$, ${}^{18} \varepsilon_{\text{water2cel}}^{\text{photohet}}$, ${}^{18} \varepsilon_{\text{water2cel}}^{\text{auto}}$, $\delta^{13} C_{\text{cel}}^{\text{auto}}$, $\delta^{13} C_{\text{cel}}^{\text{photohet}}$ and $\delta^{13} C_{\text{cel}}^{\text{het}}$ are known, but values for ${}^2 \varepsilon_{\text{water2cel}}^{\text{het}}$, ${}^2 \varepsilon_{\text{water2cel}}^{\text{photohet}}$ and ${}^2 \varepsilon_{\text{water2cel}}^{\text{auto}}$ are uncertain. We therefore chose equations **III** (for O isotopes) and **IV** (for C isotopes) to solve for $f_{\text{cel}}^{\text{auto}}$, $f_{\text{cel}}^{\text{het}}$ and $f_{\text{cel}}^{\text{photohet}}$. This is done by correlating the modelled $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values under varying $f_{\text{cel}}^{\text{het}}$ with the measured values (averaged over all equivalent subsegments). The correlations slopes, intercepts and R^2 are displayed in **Fig 10** (see also **Table S2** for data). The $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$ value that gives the best fits for both ^{13}C and ^{18}O is around 19% (**Fig 10**), in very close agreement with the measurement of α -cellulose from another set of field-grown mature (unfolded) and immature (folded) banana leaves sampled from the same plant (**Fig 11**): the averages of measured α -cellulose percentages of all equivalent sub-segments in both mature ($f_{\text{mature-mean}}$) and immature ($f_{\text{immature-mean}}$) leaves and the calculated $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$ all showed an edgeward increasing trend with minimal fluctuations.

To test whether or not constant $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$ (and $f_{\text{cel}}^{\text{het}}$) values might also be consistent with our data, the model was run at a constant $f_{\text{cel}}^{\text{het}}$ value of 81%, the resulting slopes of the regression lines at three different combinations of $f_{\text{cel}}^{\text{auto}}$ and $f_{\text{cel}}^{\text{photohet}}$ values (**Table 2**) were significantly different from the expected **1** (although the correlation coefficient R^2 s for ^{18}O and in particular ^{13}C are not significantly different from those obtained with variable f values). Also, at fixed $f_{\text{cel}}^{\text{het}}$ values across the leaf blade, the correlation for the $\delta^2\text{H}$ values was significantly worse in terms of slope, R^2 and intercept of the regression lines. We therefore reject a fixed $f_{\text{cel}}^{\text{het}}$. A lateral gradient in $f_{\text{cel}}^{\text{het}}$ is probably a more realistic reflection of the different roles the heterotrophically and autotrophically generated α -cellulose play: heterotrophic investment for α -cellulose close to the midrib is higher as stronger veins (and therefore more α -cellulose to build cell walls) are needed than close to the edge.

With the above estimated $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}} = 19\%$, and the published ${}^2\epsilon_{\text{water2cel}}^{\text{auto}}$ (-73 mUr), ${}^2\epsilon_{\text{water2cel}}^{\text{het}}$ (-9 mUr) (**Table 1**)⁴¹ we varied the ${}^2\epsilon_{\text{water2cel}}^{\text{auto}}$ and ${}^2\epsilon_{\text{water2cel}}^{\text{photohet}}$ while keeping a constant offset (20 mUr) between them to find the best set of ϵ value pairs (${}^2\epsilon_{\text{water2cel}}^{\text{auto}}$ and ${}^2\epsilon_{\text{water2cel}}^{\text{photohet}}$). The slopes and intercepts (of the regression lines) increases while R^2 decreases (**Fig 12**, see also **Table S3** for data) monotonically with these value pairs. Even at the lowest possible and realistic ${}^2\epsilon_{\text{water2cel}}^{\text{auto}} = -160$ mUr (estimated from Chikaraishi et al⁴⁵), and ${}^2\epsilon_{\text{water2cel}}^{\text{photohet}} = -140$ mUr, an edgeward increasing trend still exists, contrary to the observed edgeward decreasing trend. Only at even lower values for ${}^2\epsilon_{\text{water2cel}}^{\text{auto}} = -260$ mUr and ${}^2\epsilon_{\text{water2cel}}^{\text{photohet}} = -240$ mUr, the observed decreasing trend can be modelled (**Fig 13**). Such values are however unrealistic (note that they are lower than that of the *n*-alkanes, **Table 1**), implying that our understanding of the factors that control the autotrophic and photoheterotrophic hydrogen isotopic fractionation during α -cellulose biosynthesis is inadequate.

Estimating $f_{\text{alkane}}^{\text{auto}}$, $f_{\text{alkane}}^{\text{het}}$ and $f_{\text{alkane}}^{\text{photohet}}$. Similar to the procedure used to estimate the f values for α -cellulose the best correlation was obtained when $f_{\text{alkane}}^{\text{auto}} + f_{\text{alkane}}^{\text{photohet}} = 87\%$, i.e., over 87% of *n*-alkanes is derived from autotrophic and photoheterotrophic synthesis. Since about a half of the H in fatty lipids (to which *n*-alkanes belong) is derived directly from leaf (to be exact, cellular) water at the site of biosynthesis, the *n*-alkane lateral gradient should be 43.5% of that of the leaf water. This is consistent with the fact that in the greenhouse-grown leaf, *n*-alkane lateral $\delta^2\text{H}$ value gradient is about 44% of that of the leaf water (**Fig 14**).

3.4 Physiological and biochemical explanations for the consistency and inconsistency between model prediction and observation

Although in **section 3.3**, the predictive power of the model is demonstrated by the close fit of the predicted and measured patterns (in particular for α -cellulose C and O and *n*-alkanes H) in greenhouse-grown mature leaf, the model has not been established to predict the isotopic patterns formed in pure heterotrophic processes in the immature leaf, nor tested with field-grown leaves. In this section, we first provide physiological and biochemical explanations for the patterns (or a lack thereof) in the heterotrophic growth stage. We then highlight and provide explanations for inconsistencies between

prediction and measurement in the greenhouse-grown leaf. Finally, we compare the isotopic patterns between greenhouse- and field-grown mature leaves.

Physiological and biochemical explanations for isotopic patterns in the greenhouse-grown immature leaf.

In the immature leaf where transition from heterotrophy to autotrophy can be visually identified, the α -cellulose $\delta^2\text{H}$ values ($\delta^2H_{\text{cel}}^{\text{total}}$) show a distinct edgeward increasing trend across the heterotrophic part of the leaf blade (see **Fig 4A**). Such a trend in the $\delta^2\text{H}$ values cannot be explained by an edgeward leaf water isotopic gradient even in the heterotrophic part of the leaf as, firstly, leaf water sampled across the rolled-up leaf blade showed no such edgeward gradient in the heterotrophic part (**Fig 2A**). We must, however, acknowledge that this is only a snapshot, rather than a time/growth integrated signal. If such a leaf water gradient did exist during the dominant period of leaf heterotrophic expansion, it must be formed as a result of evaporation and this is unlikely in a rolled up (and still shielded) state. Secondly, if such a leaf water isotopic gradient did exist, it should also be reflected in the α -cellulose $\delta^{18}\text{O}$ value ($\delta^{18}O_{\text{cel}}^{\text{total}}$), but such a gradient wasn't found (**Fig 3A**). Neither can it be explained by variations of the isotopic composition of the imported sucrose as that would mean the sucrose feeding to the very first leaf of a banana plant should have similar variation, which again is highly unlikely. Therefore the $\delta^2H_{\text{cel}}^{\text{total}}$ trend must be mainly, if not exclusively, due to the isotopic effects (IEs) associated with utilization of the sucrose for the heterotrophic synthesis of α -cellulose. Similarly, since processes that may cause an edgeward and tipward C_i/C_a gradient (which may translate into a gradient of the $\delta^{13}\text{C}$ values in organic matter) do not exist in the heterotrophic part of the leaf, the edgeward and tipward gradient in α -cellulose $\delta^{13}\text{C}$ values in the heterotrophic part of the leaf (**Fig 5A**) must also be due to isotope effects (IEs) derived during the heterotrophic synthesis of α -cellulose. It is likely that these IEs occur in the cleavage of the C-3~C-4 bond in sucrose-derived hexose (phosphates) to produce triose phosphates during the (futile) triose cycle⁴⁶. This bond cleavage enriches ^{13}C in the residual hexoses as reflected in the observed tipward increase in the $\delta^{13}\text{C}$ value of α -cellulose.

The distinct edgeward dip in $\delta^2H_{\text{cel}}^{\text{total}}$ (**Fig 4A**) and $\delta^{13}\text{C}_{\text{cel}}^{\text{total}}$ (**Fig 5A**) and increase in $\delta^{18}\text{O}_{\text{cel}}^{\text{total}}$ (**Fig 3A**) in the tip segment can be explained by the addition of autotrophically and photoheterotrophically produced α -cellulose. As the leaf tip (and edge) is exposed to light first, autotrophy must proceed both longitudinally (basipetally) and laterally (toward midrib), resulting in a lateral (toward midrib) decreasing relative contribution of autotrophically and photoheterotrophically ($f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$) produced ^{13}C -depleted α -cellulose (as a result of RubisCO carboxylation⁴⁷). Although an

edgeward leaf water gradient of $\delta^2\text{H}$ values ($\delta^2H_{\text{water}}^{\text{local}}$) for the tip segment (**Fig 2A**) should result in a similar gradient in α -cellulose, such a gradient is more than cancelled out by the contribution of highly ^2H -depleted (transferable H of) NADPH generated in the chloroplast (which is mainly responsible for the highly negative $^2\varepsilon_{\text{water}2\text{cel}}^{\text{auto}}$) to the autotrophically synthesized α -cellulose. Autotrophically generated α -cellulose obviously faithfully recorded the edgeward water $\delta^{18}\text{O}$ value ($\delta^{18}O_{\text{water}}^{\text{local}}$) gradient.

In the already autotrophic tip of the immature leaf, there is a clear edgeward gradient of 30 mUr (averaged for the two *n*-alkanes, **Fig 6A**). This represents approximately 30% (=30/99) of the leaf water gradient (99 mUr, **Fig 2A**), far lower than the theoretical 50% of H directly derived from cellular water when *n*-alkanes are synthesized⁴⁴, implying that even at the leaf tip, autotrophic addition to and/or replacement of the heterotrophic wax was incomplete when the leaf was sampled.

Although there was no discernable lateral *n*-alkanes $\delta^2\text{H}$ ($\delta^2H_{\text{alkane}}^{\text{total}}$) gradient in the heterotrophic part of the leaf, a tipward decreasing trend was still clear (17.7 mUr averaged for both the C_{27} and C_{29} *n*-alkanes, **Fig 6A**). This longitudinal trend must be a result of decreasing replacement and/or addition ($f_{\text{alkane}}^{\text{auto}} + f_{\text{alkane}}^{\text{photohet}}$) of wax synthesized autotrophically and photoheterotrophically using newly assimilated carbon (note that unlike plants grown in the field, no compensatory growth of wax is expected as loss of wax due to abrasion or insect invasion is minimal in the glasshouse), and/or isotope effect during the heterotrophic synthesis of wax. The presence of such a decreasing $\delta^2H_{\text{alkane}}^{\text{total}}$ trend, in contrast to the lack of a similar trend in $\delta^2H_{\text{cel}}^{\text{total}}$ and $\delta^{18}O_{\text{cel}}^{\text{total}}$ indicates that wax synthesis may utilize a different source of sucrose, possibly similar to that proposed by Joy⁴⁸ but is more likely due to the fact that wax synthesized from sucrose must experience H isotopic exchange with water while the exchange of O with leaf water can be minimal if the majority of hexose from sucrose is incorporated into cellulose directly (i.e., without going through the futile triose phosphate cycle, **Fig 7**, see also supplementary information **Fig S1** for further details).

Consistencies and inconsistencies between model prediction and observation in the greenhouse-grown mature leaf.

In the glasshouse-grown mature leaf where autotrophy has been fully established, the 4.17 mUr (average over all segments) edgeward gradient of $\delta^{18}O_{\text{cel}}^{\text{total}}$ (**Fig 3B**) is only 14.4% (= 4.17/28.9) of that of the leaf water $\delta^{18}\text{O}$ values ($\delta^{18}O_{\text{water}}^{\text{local}}$, **Fig 2B**). Since the observed $\delta^{18}O_{\text{cel}}^{\text{total}}$ gradient might have included contributions from isotopic fractionations associated with leaf phloem loading which tends to enrich the $\delta^{18}\text{O}$ value of the remaining sucrose⁴⁹ as sucrose is exported laterally toward the midrib³² and

basipetally, the actual $\delta^{18}O_{\text{cel}}^{\text{total}}$ gradient should be smaller and significantly lower than that observed in the autotrophic tip segment of the immature leaf (21% = 5.5/25.8, see **Fig 3A** and **2A**). This is in close agreement with the model estimated average $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$ value of 19% and that this value drops gradually toward the midrib.

The gradient of δ^2H values for the heterotrophically generated α -cellulose ($\delta^2H_{\text{cel}}^{\text{het}}$) is gradually diluted out by the expansion (toward midrib and base) of autotrophy (**Fig 4B**): close to the edge and tip, leaf water is more enriched but at the same time, the contribution of autotrophically generated NADPH (highly depleted in $^2H^{50}$) to the total α -cellulose $f_{\text{cel}}^{\text{auto}}$ is higher; toward the midrib and base, leaf water is less enriched or unenriched in 2H but $f_{\text{cel}}^{\text{auto}}$ is lower. Although a gradually decreasing $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$ can explain the distinct tipward and overall edgeward decrease in δ^2H values, the model cannot predict the fluctuations observed in most of the segments. Since lateral gradients in f values are required to successfully reproduce the trends observed in $\delta^{13}C$ and $\delta^{18}O$ values of α -cellulose, the fluctuations mean that effective $^2\varepsilon_{\text{water}2\text{cel}}^{\text{auto}}$ values may not be constant across the leaf blade. This is not surprising since $^2\varepsilon_{\text{water}2\text{cel}}^{\text{auto}}$ is largely determined by the relative contributions of isotopically vastly different NADPH sources: the highly 2H depleted chloroplastic NADPH and the much 2H -less depleted cytosolic NADPH. Any variation in the relative contribution of NADPH from different sources will likely result in the $^2\varepsilon_{\text{water}2\text{cel}}^{\text{auto}}$ variation.

The clearly weakened lateral gradient in α -cellulose $\delta^{13}C$ values ($\delta^{13}C_{\text{cel}}^{\text{total}}$, **Fig 5B**) toward the base can also be easily explained by the gradually weakened addition (lower $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$) of α -cellulose synthesized using new assimilates depleted in ^{13}C . Nevertheless, it is surprising that toward the leaf base, the edge subsegment is less depleted in ^{13}C than its immediate lateral neighbor as the model predicts otherwise: the edge subsegment should be more depleted in ^{13}C than its neighbor. We currently do not have a reasonable explanation for such a reversal.

Again, the clear increasing edgeward trend of δ^2H values for the n -alkanes ($\delta^2H_{\text{alkane}}^{\text{total}}$, on average 39.4 mUr and 29.6 mUr for C_{27} and C_{29} n -alkane respectively, **Fig 6B**) reflects the leaf water gradient (on average 117.4 mUr, **Fig 2B**) across the whole leaf blade. Nevertheless, this leaf water signal ($\delta^2H_{\text{water}}^{\text{local}}$) is clearly diluted by the heterotrophically generated n -alkanes as the $\delta^2H_{\text{alkane}}^{\text{total}}$ gradient is only 29.4% ($= [(39.4 + 29.6)/2]/117.4$) of the leaf water ($\delta^2H_{\text{water}}^{\text{local}}$) gradient, and thus lower than the theoretical 50%. The dip in the segmental average indicates that basipetal replacement

of wax/addition was not completed yet when the leaf was sampled.

Physiological explanation for the isotopic pattern differences between field and greenhouse-grown mature leaves.

In the field-grown mature leaf, there was a distinct edgeward gradient (**Fig 6C**) in *n*-alkane δ^2H values ($\delta^2H_{\text{alkane}}^{\text{total}}$) but no such gradient is found in the $\delta^{18}O$, δ^2H and $\delta^{13}C$ values ($\delta^{18}O_{\text{cel}}^{\text{total}}$, $\delta^2H_{\text{cel}}^{\text{total}}$ and $\delta^{13}C_{\text{cel}}^{\text{total}}$) of α -cellulose (**Fig 3C, 4C and 5C**). This is in sharp contrast to the glasshouse-grown mature leaf where $\delta^{18}O$ and $\delta^{13}C$ values ($\delta^{18}O_{\text{cel}}^{\text{total}}$ and $\delta^{13}C_{\text{cel}}^{\text{total}}$) both show a clear edgeward gradient (**Fig 3B and 4B**). Since variations in precipitation isotopic compositions, relative humidity, temperature and solar radiation during the accumulation of wax are likely, even though the autotrophic expansion of banana leaf and deposition of wax is short (less than two weeks, see the **Materials and Methods** section for further information), the lack of pattern in precipitation isotopic composition and/or the asynchronicity with that of the autotrophic growth must have contributed to the absence of trend in the $\delta^{18}O$, δ^2H and $\delta^{13}C$ values ($\delta^{18}O_{\text{cel}}^{\text{total}}$, $\delta^2H_{\text{cel}}^{\text{total}}$ and $\delta^{13}C_{\text{cel}}^{\text{total}}$) observed in the field-grown banana α -cellulose. The clear lateral and an overall longitudinal (albeit weak, **Fig 6C**) increasing trend in *n*-alkanes δ^2H value ($\delta^2H_{\text{alkane}}^{\text{total}}$) which roughly mimics that of the leaf water pattern (**Fig 2C**) implies that the leaf water evaporative signal is sufficiently strong as not to be masked by the isotopic variability of precipitation. Even though there is no lateral gradient in either $\delta^2H_{\text{cel}}^{\text{total}}$ or $\delta^{18}O_{\text{cel}}^{\text{total}}$ or $\delta^{13}C_{\text{cel}}^{\text{total}}$, the distinct dip in $\delta^{13}C_{\text{cel}}^{\text{total}}$ and rise in $\delta^{18}O_{\text{cel}}^{\text{total}}$ and $\delta^2H_{\text{cel}}^{\text{total}}$ observed in the tip and penultimate segments clearly indicates a significant autotrophic contribution to the total α -cellulose. The ratio of the *n*-alkane δ^2H value ($\delta^2H_{\text{alkane}}^{\text{total}}$) gradient to that of the leaf water ($\delta^2H_{\text{water}}^{\text{local}}$) ($48.6\% = [(46.1+41.4)/2]/90.0$) is close to the theoretical 50%, higher than that observed in the glasshouse grown mature leaf (29.4%), indicating that the replacement of heterotrophically generated *n*-alkanes with autotrophically and photoheterotrophically generated *n*-alkanes is near completion in field-grown mature leaves but not in the glasshouse-grown leaves. This is not surprising as compensatory generation of wax using (enriched) leaf water during autotrophic and photoheterotrophic growth to counterbalance the wax loss due to wind driven abrasion and insect invasion in the field-grown leaves is expected to be higher than that in the glasshouse grown leaves.

4. CONCLUDING REMARKS

C, H and O isotope compositions of leaf organic matter (LOM) are a repository/integrator of metabolic, physiological and growth environmental (and by extension, climate) signal. The fact that the isotope compositions of the autotrophically

formed component of LOM is arguably a more sensitive recorder than its heterotrophically formed component, makes it necessary to disentangle their contributions if they are to be exploited for retrospective and prospective understanding of plant's metabolic status and responses to environmental (climate) change. This fundamental challenge is tackled here by comparing the isotope patterns leaf water, α -cellulose and *n*-alkanes from greenhouse and field grown banana leaves at various developmental stages and a three-member conceptual linear isotope mixing model linking heterotrophic, autotrophic and photoheterotrophic contributions to LOM. Some inferences and implications from the mode which may put the practice of reconstruction of environmental conditions from LOM isotope composition on a firmer basis include:

1. Only approximately 1/5 of the α -cellulose is produced during autotrophic growth of the banana leaf, implying that leaf water evaporative-enrichment signal is significantly diluted by the heterotrophically formed α -cellulose. Future effort to recover climatic signals such as relative humidity from leaf α -cellulose should take into account the effect of dilution of the signal by the heterotrophic contribution.
2. There exists a clear lateral (edgeward) gradient in ^{18}O exists in the heterotrophically laid-down α -cellulose in a stable environment, indicating that that environmental variation during leaf ontogeny is not needed to effect a cross-blade isotope gradient in α -cellulose $\delta^{18}\text{O}$ values, contrary to that implied by Helliker and Ehleringer²⁷. Environmental variation during leaf ontogeny may weaken or strengthen the gradient depending on the direction of variation.
3. Wax recovered in field-grown mature banana leaf is nearly 100% autotrophic in origin, implying that it is much more sensitive than α -cellulose as a recorder of the growth environment; Approximately 60% of the wax in the greenhouse-grown banana leaf is produced autotrophically, implying that quantitative isotope-environment relationship based on greenhouse-grown plants may not be reliable. McNerney et al.⁵¹ failed to observe an expected leaf water evaporative ^2H enrichment signal in grass leaf wax, in sharp contrast to a clearly recorded signal in the ^{18}O of α -cellulose in the same greenhouse-grown grass leaves and in wax ^2H of field-grown grass leaves. They attributed this to a temporal mismatch (asynchronicity) of leaf water enrichment and wax deposition. In the light of the above finding of vast differences in the contribution of autotrophically generated wax between field-grown and greenhouse-grown leaves, this could simply be a result of failure to include autotrophically (regardless if is originally produced or regenerated) synthesized wax for isotope analysis, i.e., leaves might have been sampled before autotrophy has been firmly established.

ACKNOWLEDGEMENTS

We thank the Fengdong Horticultural Expo (Xi'an, China) for generous permission to sample their greenhouse-grown banana leaves. YPZ was supported by Chinese NSF grants [41773032 and 41973072]. XS acknowledges the support from a Chinese NSF grant [31770435]. Assistance from J Gao, XY Kang, YP Xu and YD Xing was appreciated. Funding from Shaanxi Natural Science Basic Research Plan [2019JQ-232 to RM, -037 to ZZ and -776 to BW respectively] and China National Postdoctoral Fellowship [2019M650253 to RM] were also gratefully acknowledged. Prof. CH Hocart and Ms K Wang were gratefully acknowledged for improving the English in this manuscript. Dr Robert Turgeon was thanked for answering queries about leaf ontogeny. This is the contribution #x of the Isotopomics in Chemical Biology group.

REFERENCES

1. Helliker BR, Ehleringer JR. Establishing a grassland signature in veins: ^{18}O in the leaf water of C_3 and C_4 grasses. *Proc Natl Acad Sci USA*. 2000;97:7894-7898.
2. Song X, Barbour MM, Saurer M, Helliker BR. Examining the large-scale convergence of photosynthesis-weighted tree leaf temperatures through stable oxygen isotope analysis of multiple data sets. *New Phytologist*. 2011;192:912-924.
3. Tipple BJ, Berke MA, Doman CE, Khachatryan S, Ehleringer JR. Leaf-wax n -alkanes record the plant-water environment at leaf flush. *Proc Natl Acad Sci USA*. 2013;110:2659-2664.
4. Cormier M-A, Werner RA, Sauer PE, et al. ^2H -fractionations during the biosynthesis of carbohydrates and lipids imprint a metabolic signal on the $\delta^2\text{H}$ values of plant organic compounds. *New Phytol*. 2018;218:479-491.
5. Kimak A, Kern Z, Leuenberger M. Qualitative distinction of autotrophic and heterotrophic processes at the leaf level by means of triple stable isotope (C–O–H) patterns. *Front Plant Sci*. 2015;6:222-227.
6. Ma R, Zhu ZY, Wang B, et al. Novel position-specific $^{18}\text{O}/^{16}\text{O}$ measurement of carbohydrates. I. O-3 of glucose and confirmation of $^{18}\text{O}/^{16}\text{O}$ heterogeneity at natural abundance levels in glucose from starch in a C_4 plant. *Anal Chem*. 2018;90:10293-10301.
7. Yakir D. Variations in the natural abundance of oxygen-18 and deuterium in plant carbohydrates. *Plant, Cell Environ*. 1992;15:1005-1020.
8. Zhou YP, Grice K, Stuart-Williams H, Hocart CH, Gessler A, Farquhar GD. Hydrogen isotopic differences between C_3 and C_4 land plant lipids: consequences of compartmentation in C_4 photosynthetic chemistry and C_3 photorespiration. *Plant, Cell Environ*. 2016;39:2676-2690.
9. Zhou YP, Zhang BL, Stuart-Williams H, et al. On the contributions of photorespiration and compartmentation to the contrasting intramolecular ^2H profiles of C_3 and C_4 plant sugars. *Phytochemistry*. 2018;145:197-206.
10. Yakir D, Deniro MJ. Oxygen and hydrogen isotope fractionation during cellulose metabolism in *Lemna gibba* L. *Plant Physiol*. 1990;93:325-332.
11. Terwilliger VJ, Huang J. Heterotrophic whole plant tissues show more ^{13}C enrichment than their carbon sources. *Phytochemistry*. 1996;43(6):1183-1188.
12. Newberry SL, Kahmen A, Dennis P, Grant A. n -Alkane biosynthetic hydrogen isotope fractionation is not constant throughout the growing season in the riparian tree *Salix viminalis*. *Geochim Cosmochim Acta*. 2015;165:75-85.
13. Terwilliger VJ, Kitajima K, Le Roux-Swarthout DJ, Mulkey SJ. Intrinsic water-use efficiency and heterotrophic investment in tropical leaf growth of two Neotropical pioneer tree species as estimated from d^{13}C values. *New Phytol*. 2001;152(2):267-281.
14. Tipple BJ, Ehleringer JR. Distinctions in heterotrophic and autotrophic-based metabolism as recorded in the hydrogen and carbon isotope ratios of normal alkanes. *Oecologia*. 2018;187:1-23.
15. Liu DD, Chao WM, Turgeon R. Transport of sucrose, not hexose, in the phloem. *J Exp Bot*. 2012;63:4315-4320.

16. Farquhar GD, Gan KS. On the progressive enrichment of the oxygen isotopic composition of water along a leaf. *Plant, Cell Environ.* 2003;26:801-819.
17. Ogée J, Cuntz M, Peylin P, Bariac T. Non-steady-state, non-uniform transpiration rate and leaf anatomy effects on the progressive stable isotope enrichment of leaf water along monocot leaves. *Plant, Cell Environ.* 2007;30:367-387.
18. Gan KS, Wong SC, Yong JWH, Farquhar GD. Evaluation of models of leaf water ^{18}O enrichment using measurements of spatial patterns of vein xylem water, leaf water and dry matter in maize leaves. *Plant, Cell Environ.* 2003;26:1479-1495.
19. Gerlein-Safdi C, Gauthier PPG, Sinkler CJ, Caylor KK. Leaf water ^{18}O and ^2H maps show directional enrichment discrepancy in *Colocasia esculenta*. *Plant, Cell Environ.* 2017;40:2095-2108.
20. Skutch AF. Anatomy of leaf of banana, *Musa sapientum* L. var. Hort. Gros Michel. *Bot Gaz.* 1927;84:337-391.
21. Barker WG. Growth and development of the banana plant: gross leaf emergence. *Ann Bot.* 1969;33:523-535.
22. Zhou YP, Grice K, Chikaraishi Y, Stuart-Williams H, Farquhar GD, Ohkouchi N. Temperature effect on leaf water deuterium enrichment and isotopic fractionation during leaf lipid biosynthesis: results from controlled growth of C_3 and C_4 land plants. *Phytochemistry.* 2011;72:207-213.
23. Zhou YP, Stuart-Williams H, Farquhar GD, Hocart CH. The use of natural abundance stable isotopic ratios to indicate the presence of oxygen-containing chemical linkages between cellulose and lignin in plant cell walls. *Phytochemistry.* 2010;71:982-993.
24. Zhou YP, Yin X, Yang HB, et al. Stable isotopic evidence for the widespread presence of oxygen-containing chemical linkages between α -cellulose and lignin in Poaceae (Gramineae) grass leaves. *ACS Sust Chem Eng.* 2017;5:3250-3260.
25. Zhu ZY, Yin XJ, Lu FY, et al. The effect of processing medium on the $^2\text{H}/^1\text{H}$ of carbon-bound hydrogen in α -cellulose extracted from higher plants. *Rapid Commun Mass Spectrom.* 2020;34:e8641.
26. Feng XH, Krishnamurthy RV, Epstein S. Determination of D/H ratios of nonexchangeable hydrogen in cellulose: A method based on the cellulose-water exchange reaction. *Geochim Cosmochim Acta.* 1993;57:4249-4256.
27. Helliker BR, Ehleringer JR. Grass blades as tree rings: environmentally induced changes in the oxygen isotope ratio of cellulose along the length of grass blades. *New Phytol.* 2002;155:417-424.
28. Helliker BR, Ehleringer JR. Differential ^{18}O enrichment of leaf cellulose in C_3 versus C_4 grasses. *Funct Plant Biol.* 2002;29:435-442.
29. Sessions AL. Seasonal changes in D/H fractionation accompanying lipid biosynthesis in *Spartina alterniflora*. *Geochim Cosmochim Acta.* 2006;70:2153-2162.
30. Gao L, Guimond J, Thomas E, Huang YS. Major trends in leaf wax abundance, $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values along leaf venation in five species of C_3 plants: Physiological and geochemical implications. *Org Geochem.* 2015;78:144-152.
31. Gao L, Huang Y. Inverse gradients in leaf wax δD and $\delta^{13}\text{C}$ values along grass blades of *Miscanthus sinensis*: implications for leaf wax reproduction and plant physiology. *Oecologia.* 2013;172:347-357.

32. Richardson A, Franke R, Kerstiens G, Jarvis M, Schreiber L, Fricke W. Cuticular wax deposition in growing barley (*Hordeum vulgare*) leaves commences in relation to the point of emergence of epidermal cells from the sheaths of older leaves. *Planta*. 2005;222:472-483.
33. Van Volkenburg E. Leaf expansion – an integrating plant behaviour. *Plant, Cell Environ*. 1999;22:1463–1473.
34. Maxwell W. The rate and mode of growth of banana leaves. *Bot Gaz*. 1896;21:365-370.
35. Harris PJ. Primary and secondary plant cell walls: A comparative overview. *N Z J Forestry Sci*. 2006;36:36-53.
36. Brégard A, Allard G. Grass leaf blade development: modification of the source. *Can J Plant Sci*. 1999;79:361-363.
37. MacAdam JW, Nelson CJ. Secondary cell wall deposition causes radial growth of fibre cells in the maturation zone of elongating tall fescue leaf blades. *Ann Bot*. 2002;89:89-96.
38. Liu HT, Yang F, Gong XY, Schäufele R, Schnyder H. An oxygen isotope chronometer for cellulose deposition: the successive leaves formed by tillers of a C₄ perennial grass. *Plant, Cell Environ*. 2017;40:2121-2132.
39. Turgeon R. The sink-source transition in leaves. *Ann Rev Plant Physiol Plant Mol Biol*. 1989;40:119-138.
40. Sternberg L, Deniro DG. Biogeochemical implications of the isotopic equilibrium fractionation factor between the oxygen atoms of acetone and water. *Geochim Cosmochim Acta*. 1983;47:2271-2274.
41. Cormier M-A, Werner RA, Leuenberger MC, Kahmen A. ²H-enrichment of cellulose and *n*-alkanes in heterotrophic plants. *Oecologia*. 2019;189(2):365-373.
42. Farquhar GD, O’Leary MH, Berry JA. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust J Plant Physiol*. 1982;9:121-137.
43. Brugnoli E, Hubick KT, Von Caemmerer S, Wong SC, Farquhar GD. Correlation between the carbon isotope discrimination in leaf starch and sugars of C₃ plants and the ratio of intercellular and atmospheric partial pressures of carbon dioxide. *Plant Physiol*. 1988;88(4):1418-1424.
44. Zhou YP, Grice K, Stuart-Williams H, et al. Biosynthetic origin of the saw-toothed profile in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of *n*-alkanes and systematic isotopic differences between *n*-, *iso*- and *anteiso*-alkanes in leaf waxes of land plants. *Phytochemistry*. 2010;71:388-403.
45. Chikaraishi Y, Naraoka H, Poulson SR. Hydrogen and carbon isotopic fractionations of lipid biosynthesis among terrestrial (C₃, C₄ and CAM) and aquatic plants. *Phytochemistry*. 2004;65(10):1369-1381.
46. Hill SA, Waterhouse JS, Field EM, Switzer VR, Ap Rees T. Rapid recycling of triose phosphates in oak stem tissue. *Plant, Cell Environ*. 1995;18:931-936.
47. Francey RJ, Gifford RM, Sharkey TD, Weir B. Physiological influences on carbon isotope discrimination in huon pine (*Lagarostrobos franklinii*). *Oecologia*. 1985;66:211-218.
48. Joy KW. Carbon and nitrogen sources for protein synthesis and growth of sugar-beet leaves. *J Exp Bot*. 1967;18:140-150.

49. Gessler A, Brandes E, Keitel C, et al. The oxygen isotope enrichment of leaf-exported assimilates--does it always reflect lamina leaf water enrichment? *New Phytol.* 2013;200:144-157.
50. Luo YH, Sternberg L. Spatial D/H heterogeneity of leaf water. *Plant Physiol.* 1992;99:348-350.
51. McInerney FA, Helliker BR, Freeman KH. Hydrogen isotope ratios of leaf wax *n*-alkanes in grasses are insensitive to transpiration. *Geochim Cosmochim Acta.* 2011;75:541-554.

Table 1. The parameters used for the modelling of α -cellulose and n -alkanes spatial patterns in this study. For the convenience of modelling, an ε offset between autotrophic and photoheterotrophic syntheses of α -cellulose and n -alkanes was assumed. As the contribution of photoheterotrophy relative to that of autotrophy is small, such an assumption will not significantly change the model prediction.

Trophic Status	α -cellulose (mUr)			n -alkanes (mUr)
	$^2\varepsilon$	$^{18}\varepsilon$	$\delta^{13}\text{C}$	$^2\varepsilon$
heterotrophy	-9 [♦]	26 [♣]	-27.6 [♣]	-166 [♦]
photoheterotrophy	-60 [*]	27 [♣]	-28.0 [*]	-185 [*]
autotrophy	-73 [♦]	27 [♣]	-28.3 [♣]	-199 [♦]

♦: from Cormier et al.⁴¹; ♣: from Yakir and Deniro¹⁰; ▲: from Brugnoli et al.⁴³; *: assumed in this study.

Table 2. The slopes, intercepts and R^2 of the regression lines between measured and modelled $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of α -cellulose when modeled with a fixed value of 19% for $f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$. Note that the f values in the table are scaled by 100.

$f_{\text{cel}}^{\text{photohet}}$	$f_{\text{cel}}^{\text{auto}}$	$f_{\text{cel}}^{\text{auto}} + f_{\text{cel}}^{\text{photohet}}$	$f_{\text{cel}}^{\text{het}}$	$\delta^{18}\text{O}$			$\delta^{13}\text{C}$			$\delta^3\text{H}$		
				slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2
1.0	18.0	19.0	81.0	0.8410	4.1452	0.9901	0.6019	-10.5400	0.8003	1.9284	52.7670	0.4962
2.0	17.0	19.0	81.0	0.8410	4.1452	0.9901	0.6019	-10.5400	0.8003	1.9284	52.7670	0.4962
3.0	16.0	19.0	81.0	0.8410	4.1452	0.9901	0.6019	-10.5400	0.8003	1.9284	52.7670	0.4962