Metabolic exchange between pathways for isoprenoid synthesis and implications for biosynthetic hydrogen isotope fractionation

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Summary

- Hydrogen isotope ratios of plant lipids are used for paleoclimate reconstruction, but are influenced by both source water and biosynthetic processes. Measuring δ2H : δ1H ratios of multiple compounds produced by different pathways could allow these effects to be separated, but hydrogen isotope fractionations during isoprenoid biosynthesis remain poorly constrained.
- To investigate how hydrogen isotope fractionation during isoprenoid biosynthesis is influenced by molecular exchange between the cytosolic and plastidial production pathways, we paired position-specific 13C-pyruvate labeling with hydrogen isotope measurements of lipids in Pachira aquatica saplings.
- We find that acetogenic compounds primarily incorporated carbon from 13C2-pyruvate, whereas isoprenoids incorporated 13C1- and 13C2-pyruvate equally. This indicates that cytosolic pyruvate is primarily introduced into plastidial isoprenoids via glyceraldehyde 3-phosphate and that plastidial isoprenoid intermediates are incorporated into cytosolic isoprenoids. Probably as a result of the large differences in hydrogen isotope fractionation between plastidial and cytosolic isoprenoid pathways, sterols from P. aquatica are at least 50‰ less 2H-enriched relative to phytol than sterols in other plants.
- These results provide the first experimental evidence that incorporation of plastidial intermediates reduces δ2H : δ1H ratios of sterols. This suggests that relative offsets between the δ2H : δ1H ratios of sterols and phytol can trace exchange between the two isoprenoid synthesis pathways.

Introduction

Paleoclimate proxies are used to understand the Earth’s climate system and inform predictions of future change. One such proxy is based on the hydrogen isotope composition (δ3H = (δH/δH)Sample/(δH/δH)VSMOW − 1) of leaf wax n-alkanes and n-alkanoic acids, which are strongly correlated with δ3H values of mean annual precipitation on a global scale (Sachse et al., 2012; McFarlin et al., 2019; Ladd et al., 2021). Reconstructing δ3H values of precipitation is useful for learning about past changes in temperature, moisture transit pathways, and precipitation intensity, depending on location (Bowen et al., 2019), and sedimentary leaf wax δ3H values are increasingly used for this purpose (e.g. Tierney et al., 2017; Bhattacharya et al., 2018; Guillemet et al., 2019; Hinojosa et al., 2019). Although used as a proxy for precipitation δ3H values, leaf wax δ3H values are also influenced by δ2H enrichment of leaf water, as well as biosynthetic hydrogen isotope fractionation relative to leaf water (Sachse et al., 2012). Overall, controls on leaf water δ3H values and their influence on leaf wax δ3H values are relatively well understood and supported by robust data–model comparisons (Kahmen et al., 2013; Tipple et al., 2015). Compared with our understanding of leaf water δ2H enrichment, much less is known about how and why biosynthetic hydrogen isotope fractionation between leaf water and waxes varies among and within species (Chikaraishi et al., 2004a; Liu & Yang, 2008; Zhou et al., 2011; Gao et al., 2014; Cormier et al., 2018). This leads to uncertainty in the reliability of leaf wax δ3H values as recorders of precipitation δ3H values over time, because biosynthetic hydrogen isotope fractionation between leaf water and waxes can vary with environmental conditions such as aridity and salinity (Douglas et al., 2012; Ladd & Sachs, 2015).

Measuring δ3H values of multiple plant compounds could help to deconvolve changes in leaf water δ3H values that are driven by changes in leaf water δ3H values from those caused by changes in biosynthetic fractionation. Specifically, changes in leaf water δ3H values should affect δ3H values of all compounds, while changes in biosynthetic fractionation could occur independently for compounds synthesized by different pathways. Isoprenoid lipids could be particularly helpful in this regard, as there are two independent biosynthetic pathways for their production, the
mevalonic acid (MVA) and the methylerythritol phosphate (MEP) pathways (Lichtenthaler et al., 1997; Hemmerlin et al., 2012). Although most organisms are limited to either the MEP or the MVA pathway, some algae and all higher plants produce isoprenoids by both (Lichtenthaler et al., 1997; Hemmerlin et al., 2012; Vranova et al., 2013). Although specific compounds are typically produced by only one of the two pathways, there is potential for metabolic crosstalk to occur, during which precursors common to all isoprenoids, such as isopentenyl pyrophosphate (IPP), are exchanged between the MEP and MVA pathways (Hemmerlin et al., 2012). Metabolic crosstalk may allow plants to cope more flexibly with changing stressors and limiting resources (Arigoni et al., 1997; Hemmerlin et al., 2003; 2012; Laule et al., 2003). However, it remains unclear to what extent such crosstalk happens and, if it does, which molecular precursors are most significantly involved (Rasulov et al., 2018; Souza et al., 2018).

If metabolic crosstalk between the MVA and MEP pathways for isoprenoid biosynthesis is an important process in plants, it should be reflected in the relative offset in δ²H values between compounds primarily produced by each pathway. Sterols (primarily produced by the MVA pathway) are typically greatly ²H-enriched relative to compounds mainly produced by the MEP pathway, such as phytol (Sessions et al., 1999; Chikaraishi et al., 2004a; Sessions, 2006; Zhou et al., 2011). Differences in the source of IPP are thought to explain some of the ²H-enrichment of MVA-derived isoprenoids relative to those from the MEP pathway (Sessions et al., 1999; Sachs et al., 2016, 2017). Isopentenyl pyrophosphate produced by the MEP pathway in the plastid should incorporate relatively more H from ²H-depleted photosynthetic NADPH than MVA-derived IPP, which should have more H from ²H-enriched NAD(P)H from the oxidative pentose phosphate pathway (Schmidt et al., 2003; Sachs et al., 2017). Among isoprenoids, sterol ²H values frequently vary with environmental gradients such as nutrient availability, light availability, and salinity, which has been attributed to changes in the relative proportion of IPP derived from the MEP pathway via molecular crosstalk (Maloney et al., 2016; Sachs et al., 2016, 2017; Ladd et al., 2018). However, direct experimental evidence of a significant contribution of IPP from the MEP pathway to sterol synthesis is lacking.

One way to address this gap is through position-specific ²¹³C glucose or pyruvate labels. This approach makes use of the fact that there are multiple ways by which these common lipid precursors can be incorporated into product molecules, and that only some pathways will conserve carbon from all positions, while other pathways will only incorporate carbon atoms from certain parts of the precursor (Ghirado et al., 2011; Jardine et al., 2014). When alternate possibilities exist to synthesize a molecule, the relative amount of ²¹³C from different positions found in product molecules can be used to determine the relative importance of each possible synthesis pathway. Position-specific ²¹³C labeling has been successfully employed to track the flow of glucose and pyruvate into smaller isoprenoids, including isoprene, monoterpenes, sesquiterpenes and diterpenes, illuminating their fluxes and production pathways (Ghirado et al., 2014; Jardine et al., 2014; Fasbender et al., 2018; Yáñez-Serrano et al., 2018, 2019; Werner et al., 2020). Although it has similar promise to resolve the amount of interchange of molecular precursors between the MVA and MEP pathways for larger isoprenoids such as sterols, position-specific ²¹³C labeling has not yet been used for that purpose.

To investigate the degree of molecular interchange between the MVA and MEP pathways and its potential impact on hydrogen isotope fractionation associated with phytol and phytosterol synthesis, we paired position-specific ²¹³C-pyruvate labeling with natural abundance hydrogen isotope measurements of lipids from the leaves of Pachira aquatica Aubl., grown in control and mild drought treatments. P. aquatica is a tropical plant with demonstrated ability to adjust the source of pyruvate used to produce the volatile isoprenoid compound isoprene in response to stress (Yáñez-Serrano et al., 2019). We show that interchangeable precursors between the MVA and MEP pathways are more prevalent than is typically assumed, even for unstressed plants, and that this exchange probably influences the δ²H values of sterols produced by P. aquatica.

Materials and Methods

Plant growth conditions and mild drought treatment

Twenty P. aquatica saplings were planted in 3 l pots containing a 2 : 1 ratio of soil multiplication substrate (Floragard, Oldenburg, Germany) and sand. In January 2019, they were moved from a glasshouse to a climate-controlled growth chamber (ThermoTec Weilburg, Weilburg, Germany) with a photon flux density of 800 μmol m⁻² s⁻¹ and a 12 h : 12 h, day : night cycle. Relative humidity was maintained at 60%, daytime (08:00–20:00 h) temperatures at 25°C and nighttime temperatures at 20°C.

From 24 January to 13 March, all plants were watered three times a week to keep the soil moisture between 25% and 27%. The amount of water added was equal to the mass the plants had transpired since the last watering event (determined by change in mass), ± 50 ml of water per 3% deviation from desired soil moisture. Mean soil moisture content was measured with a soil moisture sensor (theta Probe ML3; delta T, Cambridge, UK) from three locations per pot.

On 13 March plants were divided into a control group (kept under conditions described earlier) and a drought group, which was dried by reducing water additions by half. After 22 March, relative soil moisture in the drought group was maintained at 10–12% through to the conclusion of the experiment on 5 April.

Plant water potential

Predawn (07:00–07:45 h) and midday (12:30–13:30 h) leaf water potential was measured on 3 April. Freshly cut leaves were placed in a Scholander type pressure chamber (model 3015G4; Soilmoisture, Santa Barbara, CA, USA) (Scholander et al., 1965). Pressure inside the chamber was increased by adding N₂ gas until xylem water was visible at the cut end of the leaf.
Plant material and position-specific $^{13}$C-pyruvate labeling

Plant sampling and position-specific labeling took place from 26 March to 5 April 2019. Unlabeled leaves were collected from the same plant when the leaf designated for labeling was placed in the cuvette (Fig. 1). The mid-vein from one leaflet was carefully removed, and each half of the remaining leaf tissue was placed in separate exetainers (Labco, Lampeter, UK). One-half was used for leaf water analyses, and the other for lipid analyses (Fig. 1). Samples were stored at $-80^\circ$C until extraction.

Leaves selected for position-specific $^{13}$C-pyruvate labeling were placed in a cuvette lined with fluorinated ethylene propylene foil the day before labeling and allowed to acclimate overnight. The petiole of the leaf selected for $^{13}$C-pyruvate labeling was cut, placed in demineralized water and cut again $c.$ 1 cm from the end to prevent xylem embolism (Fasbender et al., 2018) (Fig. 1). The petiole was then transferred under water into an Eppendorf tube and allowed 5 min to recover from the wounding. Afterwards the Eppendorf tube was replaced by a new tube containing 2 ml of 10 mM pyruvate solution, labeled with $^{13}$C (99.9 atom %) in either the C1 or C2 position (Cambridge Isotope Laboratories, Andover, MA, USA). The petiole remained in the solution for 95 min (Werner et al., 2009; Fasbender et al., 2018). The leaf was then removed from the cuvette and one leaflet was stored in two exetainers as for unlabeled leaves (Fig. 1).

Gas fluxes

Leaf cuvettes were attached to an online measuring system (described by Fasbender et al., 2018). Cuvettes were connected to instruments via perfluoro alkoxyl alkane tubing (1/4 in OD, 1/8 in ID). Cuvettes (volume $c.$ 7 l) were supplied with volatile organic compound free air with defined CO$_2$ concentration ($c.$ 480 ppm) under constant flow (0.6 l min$^{-1}$ ± 2.5%), maintained by mass flow controllers (Omega Engineering, Stamford, CT, USA). The CO$_2$ concentration of air entering the cuvette was determined before and after each measurement by analyzing the air exiting an empty cuvette attached to the system. CO$_2$ concentrations and $\delta^{13}$CCO$_2$ values were measured by isotope ratio infrared spectrometry (IRIS, Delta Ray with Universal Reference Interface; Thermo Fisher Scientific, Bremen, Germany). H$_2$O content in the air was recorded using an infrared gas analyzer (LI-7000 CO$_2$/H$_2$O Analyzer; Li-Cor, Lincoln, NE, USA). The Li-Cor was calibrated with a Dew Point Generator (LI-610; Li-Cor). The transpiration rate $E$ (mmol m$^{-2}$ s$^{-1}$) was calculated from the concentration of water vapor from the empty cuvette $w_e$ and the concentration of water vapor from the leaf cuvette $w_0$ (ppt):

$$E = \frac{u(w_0 - w_e)}{s(1 - w_0)}$$

Eqn 1

where $u$ is molar flow at the cuvette entrance (mmol s$^{-1}$), and $s$ is leaf area (m$^2$) (von Caemmerer & Farquhar, 1981).

The IRIS was calibrated before and after each plant measurement with two internal isotopic reference gases (Thermo Fisher Scientific) (Fasbender et al., 2018). Standards were diluted with CO$_2$ free synthetic air (Synthetic Luft 5.0 KW-frei; Messer Gase, Bad Soden, Germany) to match the concentration of sample gas. The assimilation rate $A$ (μmol m$^{-2}$ s$^{-1}$) was calculated as in von Caemmerer & Farquhar (1981) using the difference in CO$_2$ concentration exiting the leaf cuvette ($c_e$) relative to the empty cuvette ($c_0$):

$$A = \frac{u}{s} \left(c_e - c_0\right) \frac{(1 - w_e)}{(1 - w_0)} - E c_e$$

Eqn 2

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**Fig. 1** Schematic representation of the $^{13}$C labeling procedure and sample collection. Full details of the gas supply and flux measurements are provided in Fasbender et al. (2018).
Instantaneous water-use efficiency was calculated in μmol CO₂ mmol⁻¹ H₂O as the ratio A : E.

Leaf water hydrogen isotope analyses
Water isotope analyses were limited to unlabeled leaves. Leaf water was cryogenically extracted from half of the leaf for 2 h as in Newberry et al. (2017). The δ²H value of the extracted water was subsequently measured with a thermal conversion/elemental analyzer (TC-EA) coupled to a Delta Plus V isotope ratio MS (IRMS) via a ConFlo IV interface (Thermo Fisher) as in Newberry et al. (2017). Raw measurements were normalized to the VSMOW scale using calibrated laboratory standards (δ²H values of −77.7‰ and +45.4‰), which were analyzed repeatedly throughout each sample sequence. A quality control standard (δ²H = +1.0‰) was analyzed as an unknown alongside each set of standard analyses. Measured values of the quality control standard were δ²H = +0.9 ± 0.2‰ (n = 5).

Leaf lipid extraction, purification, and derivatization
A recovery standard containing 5α-androstane, 3-ecosanone, n-C₁₉-alkanol, and n-C₁₉-alkanoic acid was quantitatively added to freeze-dried, homogenized leaves before extraction in 30 ml of 9 : 1 dichloromethane/methanol (DCM/MeOH) in a SolvPro Microwave Reaction System (Anton Parr, Graz, Austria) following the extraction scheme of Ladd et al. (2018). Total lipid extracts were base saponified and separated into neutral and acidic fractions, and the neutral fraction was dissolved in hexane and purified by Si gel column chromatography as in Ladd et al. (2018). Alkanes eluted in 4 ml of hexane (Hex), squalene in 4 ml of 2 : 1 Hex/DCM, and phytol and phytosterols in 4 ml of 19 : 1 DCM/MeOH. The alcohol fractions were acetylated by adding 200 μl of pyridine and 25 μl of acetic anhydride of known isotopic composition (Ladd et al., 2018) and heating at 70°C for 30 min.

Fatty acids were methylated to produce fatty acid methyl esters (FAMEs) using 4 ml of 5% HCl in MeOH overnight at 70°C. Ten milliliters of solvent-extracted ultrapure water was added to each sample, and FAMEs were extracted with Hex. Saturated n-alkanoic FAMEs were isolated from unsaturated FAMEs with Si columns (500 mg/6 ml; Biotage, Uppsala, Sweden). Saturated n-alkanoic FAMEs eluted in 4 ml of 2 : 1 Hex/DCM, while unsaturated FAMEs eluted in 4 ml of 19 : 1 DCM/MeOH.

Leaf lipid quantification and identification
All neutral and acid fractions resulting from column chromatography were analyzed by gas chromatography/flame ionization detection (GC-FID) using previously described instrumentation and chromatographic conditions (Ladd et al., 2018). Peak areas were normalized to areas of recovery standards in order to account for any loss during sample handling. Alkanes and FAMEs were identified by comparing retention times with external standards. A subset of samples was analyzed by GC-MS under identical chromatographic conditions in order to identify sterols and confirm compound identities (instrumentation as in Ladd et al., 2018) (Supporting Information Fig. S1). The resulting mass spectra were compared with published mass spectra and the NIST14 library to identify compounds.

Leaf lipid isotope analyses
Carbon isotopes ratios (δ¹³C values) were measured from all samples. Hydrogen isotope analyses (δ²H values) were limited to unlabeled samples to avoid the influence of H from the pyruvate solution. Leaf lipid stable isotope ratios were analyzed by GC-IRMS, using instrumentation (Thermo Fisher) and GC conditions as in Nelson et al. (2018). Samples were dissolved in an appropriate amount of heptane so that the signals for targeted analytes were within the linear range for each isotope (CO₂ between 15 and 100 Vs for δ¹³C analyses, and H₂ between 20 and 115 Vs for δ²H analyses). For δ¹³C analyses, column effluent was converted to CO₂ in a combustion reactor at 100°C. For δ²H analyses, column effluent was converted to H₂ and a pyrolysis reactor at 1420°C. All samples were measured in duplicate, and all analyses were completed between September 2019 and March 2020.

Measured values were normalized to VPDB (δ¹³C values) or VSMOW (δ²H values) scales using a mix of n-alkanes of known isotopic compositions (Mix A7 or A6, plus n-C₃₂ #1 and n-C₃₅ #1; Arndt Schimmelmann, Indiana University), injected in sets of three different concentrations throughout each sequence after every seven samples. Analytical accuracy and precision were assessed through routine analyses of a quality control sample of n-C₂₉ alkane, which was co-injected with the same n-C₃₂ and n-C₃₅ alkanes used in the standard injections (in this case analyzed as unknowns). The δ¹³C values for the repeat analyses of these compounds were −37.58 ± 0.21‰ (n = 71), −29.84 ± 0.29‰ (n = 42; known value = −29.47‰), −29.51 ± 0.24‰ (n = 42; known value = −29.84‰), for the n-C₂₉, n-C₃₂, and n-C₃₅ compounds, respectively. For the δ²H analyses these values were −142.2 ± 5.5‰ (n = 37), −215.0 ± 4.2‰ (n = 36; known value = −212.4‰) and −198.9 ± 3.2‰ (n = 34; known value = −194.8‰). During δH isotope analyses, the H₃⁺ factor was measured at the beginning of each sequence (Sessions et al., 2001) to ensure it was within the manufacturer-specified range.

Stable isotope calculations
Changes in the δ¹³C values of CO₂ as a result of carbon assimilation were calculated as $ε^{13}C_{CO2} = R_{olut}/R_{olu} - 1$, where $R_{olut} = ^{13}C/^{12}C$ in CO₂ leaving the leaf cuvette and $R_{olu} = ^{13}C/^{12}C$ in CO₂ leaving the blank chamber directly before and after the labeling event. All ε-values are expressed in ‰. Pre-cut $ε^{13}C_{CO2}$ values are mean values from 15 min before cutting the petiole and maximum $ε^{13}C_{CO2}$ values are mean values from minutes 85–88 in the $^{13}C$-pyruvate label solution.

Incorporation of $^{13}$C from each type of pyruvate label (C1 or C2 carbon) into specific compounds was calculated as $ε^{13}C_{Lipid} = (R_{labeled}/R_{unlabeled} - 1)$, where $R_{labeled} = ^{13}C/^{12}C$ in
leaves exposed to the pyruvate label and $R_{\text{unlabeled}} = \frac{13C}{12C}$ in unlabeled leaves from the same plant. Incorporation of $^{13}C$ from $^{13}C_1$-pyruvate and $^{13}C_2$-pyruvate labels was also calculated in terms of the relative amount of each compound that was replaced by carbon from the added pyruvate, using an isotopic mass balance approach. The concentration of compound produced with the label, $[G]$, was calculated as $[G] = \left[ C \right] \times (F_t - F_0)/(F_t - F_i)$, where $[C]$ is the total concentration in the labeled leaf, $F_t$ is the fraction of $^{13}C$ in the compound from the labeled leaf ($F_t = (^{13}C/^{12}C)/(^{13}C/^{12}C + 1)$), $F_i$ is the fraction of $^{13}C$ in the same compound from the unlabeled leaf, and $F_0$ is the fraction of $^{13}C$ in pyruvate added from the label (0.99). As a relatively small amount of $^{13}C$-pyruvate was added to avoid interfering with the leaf's metabolism (Werner et al., 2009; Fasbender et al., 2018), most of the compounds synthesized during the labeling event were probably produced using natural pyruvate, amounts of which are not possible to quantify as they are constantly in flux. Therefore, $C_t$ represents a minimum turnover rate for each compound. If c. 10% of newly produced compounds incorporate $^{13}C$ from the labeled pyruvate, turnover rates for fatty acids and n-alkanes would be well within the range of previous studies using other label substrates (Gao et al., 2012; Gamarra & Kahmen, 2017).

Biosynthetic hydrogen isotope fractionation for each compound was calculated as $\varepsilon_{2H_{\text{bio}}} = ((^{2H}/^{1H})_{\text{lipid}} - 1). ^2H$ enrichment of sitosterol relative to phytol for $P. aquatica$ and for other plants compiled from published studies (Sessions et al., 1999; Chikaraishi et al., 2004a,b; Chikaraishi & Naroaka, 2006; Zhou et al., 2011, 2016) were calculated as $\varepsilon_{2H_{\text{sitosterol-phytol}}} = ((^{2H}/^{1H})_{\text{sitosterol}} - 1).$

Statistics
All statistical tests were carried out in GraphPad PRISM v.8.3. Differences among groups were assessed using unpaired parametric two-tailed t-tests. Differences are reported as statistically significant when $P < 0.05$. Box plots are plotted using Tukey's method to define whiskers and outliers.

Results
Leaf water potential, carbon assimilation, transpiration and water-use efficiency

$Pachira aquatica$ saplings showed limited responses to reduced soil moisture content. Pre-dawn and midday leaf water potential,

Fig. 2 Leaf-level measurements of transpiration (a), carbon assimilation (b), leaf water potential (c), and instantaneous water-use efficiency (WUE) (d) in $Pachira aquatica$ saplings. Control plants are shown in blue and drought plants are in pink. Boxes represent the median (center line) and 25–75% range of eight to 20 replicates. Outliers and whiskers are calculated using Tukey’s method. Statistical differences are marked with asterisks: *, $P < 0.05$; **, $P < 0.01$. 

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and carbon assimilation rate did not differ significantly between the drought and control treatments (Fig. 2b,c). Drought effects were only visible in significantly reduced transpiration rates ($P < 0.01$) (Fig. 2a), corresponding to higher instantaneous water-use efficiency values ($P < 0.05$) (Fig. 2d).

**Leaf water $\delta^2$H values**

Irrigation water had a $\delta^2$H value of $-63.8 \pm 0.2 \%$. Leaf water from unlabeled plants was significantly $^2$H-enriched relative to irrigation water and ranged from $-4.0 \%$ to $8.1 \%$ (Fig. S2). Although the mean values were higher for plants in the drought treatment relative to the control plants ($2.1 \%$ vs $0.0 \%$) these values were not significantly different from each other (Fig. S2).

**Leaf lipid contents**

Lipid contents relative to dry leaf mass were calculated for several compounds, including fatty acids, $n$-alkanes, phytol, squalene and sitosterol (Table S1). There were no significant differences in lipid contents between the drought and control treatments for any analyzed compound (Table S1). In all leaves, the most abundant fatty acids were $n$-$C_{16}$-alkanoic acid and unsaturated $n$-$C_{18}$ acids (Table S1). The most abundant alcohol was typically the sterol sitosterol (Table S1), which was well resolved at concentrations appropriate for quantification and $\delta^{13}$C analyses, but sometimes coeluted with the pentacyclic triterpenoid $\beta$-amyrin at the increased concentrations needed for $\delta^2$H measurements. Phytol was also abundant in the alcohol fractions (Table S1). The most abundant $n$-alkanes were $n$-$C_{29}$ and $n$-$C_{31}$, with lesser amounts of $n$-$C_{33}$ (Table S1).

Incorporation of $^{13}$C label into different compounds

$^{13}$C from the pyruvate label was incorporated into several compounds representing three biosynthetic pathways (acetogenic, MVA, and MEP). The greatest $^{13}$C-enrichment relative to the compound in unlabeled leaves was observed for $n$-$C_{16}$-alkanoic acid labeled with $^{13}$C2-pyruvate (Fig. 3). The only compounds that were not significantly $^{13}$C-enriched relative to those from unlabeled leaves from the same plant were $n$-$C_{16}$-alkanoic acid, $n$-$C_{29}$- and $n$-$C_{31}$-alkanes from control plants labeled with $^{13}$C1-pyruvate, and sitosterol from both treatments with both labels (Fig. 3).

There were clear differences in the incorporation of $^{13}$C from the two pyruvate labels for the acetogenic compounds, all of which were significantly more $^{13}$C-enriched in both treatments when labeled with $^{13}$C2-pyruvate rather than $^{13}$C1-pyruvate ($P < 0.0001$ for $n$-$C_{16}$-alkanoic acid and $n$-$C_{29}$-alkane in both treatments, and $< 0.001$ for $n$-$C_{31}$-alkane in both treatments; Fig. 3). In the case of the isoprenoid lipids (phytol, squalene, and sitosterol), $^{13}$C-incorporation was similar regardless of which carbon position of pyruvate was labeled (Fig. 3). CO$_2$ leaving the leaf cuvette became enriched in $^{13}$C relative to initial values.

![Fig. 3 $^{13}$C enrichment of diverse compounds from *Pachira aquatica* leaves following position-specific $^{13}$C-pyruvate labeling relative to unlabeled leaves from the same plant. Measurements are grouped by the type of label used ($^{13}$C1- or $^{13}$C2-pyruvate) and grouped by treatment (drought or control). Boxes represent the median (center line) and 25–75\% range of 7–10 replicates. Whiskers and outliers are calculated using Tukey’s method.](image-url)
during the labeling event \((P < 0.0001)\), with maximum values that were more enriched for the \(^{13}\)C1-pyruvate than for \(^{13}\)C2-pyruvate \((P < 0.001)\; \text{Fig. S3).}"

Normalizing to the pool size did not alter any of the trends for \(^{13}\)C incorporation into different lipids (Fig. 4). Overall, a very small portion of the lipid pool was replaced with molecules derived from the pyruvate added during the 95 min labeling event (Fig. 4). The highest concentration replaced with labeled material was 276 ± 99 ppm, which was observed for \(n\)-C\(^{16}\)-alkanoic acid from control plants labeled with \(^{13}\)C2-pyruvate (Fig. 4).

In most cases, there were not significant differences in \(^{13}\)C incorporation between plants in the control and the drought treatments for either lipids or \(\text{CO_2}\) (Figs 3, S3). The only exceptions were for \(n\)-C\(^{29}\)- and \(n\)-C\(^{31}\)-alkanes labeled with \(^{13}\)C1-pyruvate, both of which were slightly more \(^{13}\)C-enriched in the drought treatments relative to the control treatments \((P < 0.01\) and < 0.05, respectively; Figs 3, 4).

**Lipid \(\delta^2\)H values and \(\epsilon^2\)H\(_{\text{bio}}\) values**

Lipid \(\delta^2\)H values spanned a range > 200‰, with \(n\)-C\(^{16}\)-alkanoic acid having the most \(2^H\)-enriched values and pyroglutamyl having the most \(2^H\)-depleted (Table S2). The long-chain \(n\)-alkanes were slightly more \(2^H\)-depleted than \(n\)-C\(^{16}\)-alkanoic acid (Table S2). Squalene and sitosterol had \(\delta^2\)H values intermediate between the acetogenic compounds and pyroglutamyl (Table S2). As leaf water \(\delta^2\)H values were close to zero (Fig. S2), biosynthetic fractionation factors, expressed as \(\epsilon^2\)H\(_{\text{bio}}\) were nearly identical to the \(\delta^2\)H values of each compound. \(\epsilon^2\)H\(_{\text{bio}}\) values averaged −126 ± 11‰ for \(n\)-C\(^{16}\)-alkanoic acid, −148 ± 10‰ for \(n\)-C\(^{29}\)-alkane, −147 ± 8‰ for \(n\)-C\(^{31}\)-alkane, −251 ± 10‰ for squalene, −245 ± 7‰ for sitosterol, and −306 ± 7‰ for pyroglutamyl (Fig. 5a).

There were no significant differences in \(\delta^2\)H values or in \(\epsilon^2\)H\(_{\text{bio}}\) values between the drought and control treatments, with the exception of sitosterol, which was slightly more enriched in the drought treatment \((P < 0.05)\; \text{Fig. 5a).}"

However, sitosterol was only well resolved enough to measure its \(\delta^2\)H values for two of the control plants.

The MVA-derived isoprenoids from \(P\). \textit{aquatica} were highly \(2^H\)-enriched relative to pyroglutamyl \((\epsilon^2\)H\(_{\text{sitosterol}}\)-pyroglutamyl = 84 ± 15‰, \(n = 9\) \(\text{Fig. 5b).}\)

However, the magnitude of this \(2^H\)-enrichment was significantly \((P < 0.0001)\) less than values calculated from all previous studies that report \(\delta^2\)H values of both sitosterol and pyroglutamyl from plant leaves \((\epsilon^2\)H\(_{\text{sitosterol}}\)-pyroglutamyl = 135 ± 36‰, \(n = 85\); data compiled from Sessions \textit{et al}, 1999; Chikaraishi \textit{et al}, 2004a,b; Chikaraishi & Naroaka, 2006; Zhou \textit{et al}, 2011, 2016 \(\text{Fig. 5b).}\)

### Discussion

Incorporation of \(^{13}\)C from each pyruvate label matched expectations for the production of \(\text{CO}_2\) (more enrichment from \(^{13}\)C1-pyruvate) and acetogenic compounds (more enrichment from \(^{13}\)C2-pyruvate) (Figs 3, S2). By contrast, \(^{13}\)C was incorporated in isoprenoids at equivalent rates from \(^{13}\)C1- and \(^{13}\)C2-pyruvate (Fig. 4), demonstrating that compounds derived from pyruvate that maintain all three initial C atoms must be incorporated into compounds synthesized by both the MEP and MVA pathways. As we demonstrate in the following discussion, this suggests a high degree of metabolic interaction between the two pathways, with important implications for the overall hydrogen isotope fractionation associated with isoprenoids.

\(^{13}\)C-incorporation into acetogenic lipids consistent with current biochemical models

\(^{13}\)C from the \(^{13}\)C2-pyruvate is readily incorporated into acetogenic compounds through the decarboxylation of pyruvate to form acetyl-CoA. This process removes C1 but retains C2 and C3 (Fig. 6). Acetyl-CoA is the base of all acetogenic compounds (Fig. 6). The primary compound produced by the acetogenic pathway within the plastid is \(n\)-C\(^{16}\)-alkanoic acid, which is typically abundant within plant leaves and also the precursor for the longer-chain-length \(n\)-C\(^{29}\)- and \(n\)-C\(^{31}\)-alkanes (Samuels \textit{et al}, 2008). In order to form these compounds, \(n\)-C\(^{16}\)-alkanoic acid is elongated by sequential addition of acetyl-CoA in the endoplasmic reticulum and forms \(n\)-alkanes following the loss of the carbonyl group (Samuels \textit{et al}, 2008) (Fig. 6).

There was also small, but significant, \(^{13}\)C enrichment for acetogenic compounds labeled with \(^{13}\)C1-pyruvate (Figs 3, 4). This is probably a result of refixation of respired \(^{13}\)C from the pyruvate label (Fig. 6). The \(\delta^{13}\)C values of \(\text{CO}_2\) in the cuvettes increased dramatically during pyruvate labeling. This change was faster and more pronounced for \(^{13}\)C1-pyruvate than for \(^{13}\)C2-pyruvate (Fig. S3), consistent with previous results (Fasbender...
Research using Tukey’s method. Sitosterol in P. aquatica and in compiled literature values. Boxes represent the median (center line) and 25–75% range of seven to 10 replicates. Whiskers are calculated using Tukey’s method. Sitosterol in P. aquatica is significantly less enriched relative to phytol than in previous observations of other species (****, P < 0.0001).

et al., 2018; Werner et al., 2020). C1 is more likely to be respired than C2 during daylight as there are numerous processes that decarboxylate pyruvate (e.g. to form acetyl-CoA) and release C1 carbon as CO2 (Fig. 6), while the only direct mechanism to respire carbon from C2 is through the complete breakdown of pyruvate in the tricarboxylic acid (TCA) cycle, which mainly occurs at night (Werner et al., 2009; Sweetlove et al., 2010; Tcherkez et al., 2012). For acetygenic compounds, synthesis from primary photosynthate, which is enriched in 13C as a result of refxation of respired 13CO2 from 13C1-pyruvate, occurred more slowly than the initial incorporation of 13C from the 13C2-pyruvate (Figs 3, 4). The difference in 13C incorporation from each carbon position of the pyruvate molecule is especially striking for n-C16-alkanoic acid, which has a high turnover rate in plant leaves (Gao et al., 2012). The maximum amount of 13C incorporation from 13C1-pyruvate is only 6% of that from 13C2-pyruvate (Fig. 3), implying that refxation is not the main process for adding 13C to compounds synthesized on the short timescale of pyruvate labeling. Likewise, new long-chain n-alkanes in P. aquatica were primarily derived from cytosolic pyruvate rather than from carbon fixed during the labeling event, as indicated by greater incorporation of 13C from 13C2-pyruvate than from 13C1-pyruvate, but at an overall lower rate than that at which n-C16-alkanoic acid was synthesized (Figs 3, 4).

GA-3-P is an important intermediate between pyruvate label and MEP-derived isoprenoids

In contrast to acetygenic lipids, 13C was incorporated into phytol at a similar rate from both the 13C1- and 13C2-pyruvate (Fig. 4). This result is surprising, as the 5C compound 1-deoxy-D-xylulose-5-phosphate (DOXP), a precursor for all MEP-derived compounds such as phytol, is also produced in the plastid with decarboxylated pyruvate, resulting in a loss of C1 (Fig. 6). This should result in relatively more incorporation of 13C from 13C2-pyruvate (Fig. 6). The relatively high rate of 13C incorporation from 13C1-pyruvate cannot be explained by refxation of respired 13CO2, as this would have also resulted in roughly equal 13C uptake from both label positions for the acetygenic lipids, which did not occur (Figs 3, 4). Rather, equivalent incorporation of 13C from the 13C1- and 13C2-pyruvate suggests that the primary way in which the label was added to phytol was via glyceraldehyde-3-phosphate (GA-3-P). GA-3-P is a three-C molecule and, in the first part of the MEP pathway, it combines with decarboxylated pyruvate to form the five-C compound 1-deoxy-D-xylulose-5-phosphate (DOXP) (Fig. 6). DOXP is converted to IPP and dimethylallyl pyrophosphate (DMAPP), which form the building blocks of all MEP-derived isoprenoids (Fig. 6). Because GA-3-P retains all three carbons when it is produced from pyruvate via PEP, routing the pyruvate label through this intermediate would be an efective way to incorporate 13C from the 13C1-pyruvate into MEP-derived compounds (Fig. 6) (Werner et al., 2020). We suggest that this may be the primary way in which 13C from both 13C1- and 13C2-pyruvate was added to newly synthesized phytol in P. aquatica, as signifcant incorporation via decarboxylated pyruvate would have resulted in relatively higher 13C uptake in leaves labeled with the 13C2-pyruvate. This result contrasts with previous position-specifc 15C-labeled pyruvate experiments, where some 13C from 13C1-pyruvate was also incorporated into MEP-derived monoterpenes, but at a lower rate than from 13C2-pyruvate (Fasbender et al., 2018; Werner et al., 2020).

Signficant role of plastidial precursors in synthesis of MVA-derived isoprenoids

Equal rates of 13C incorporation from the 13C1- and 13C2-pyruvate into squalene (Fig. 4) were also unexpected, as MVA-derived isoprenoids from other plants also tend to be more strongly enriched by 13C2-pyruvate than by 13C1-pyruvate.
As is the case for acetogenic compounds, acetyl-CoA is an important building block for the MVA pathway, but will only allow $^{13}$C from the $^{13}$C$_2$-pyruvate to be incorporated into these compounds in the absence of widespread refixation of respired $^{13}$CO$_2$ (Fig. 6). There is no known way for $^{13}$C from the $^{13}$C$_1$-pyruvate to be incorporated into MVA-derived compounds while staying exclusively in cytosol (Fig. 6). However, it would be possible to incorporate $^{13}$C from $^{13}$C$_1$-pyruvate by exchange of $^{13}$C-enriched (via GA-3-P as described above) IPP across the plastid membrane. Isopentenyl pyrophosphate crosstalk between the MEP and MVA pathways has long been suggested (Arigoni et al., 1997; Hemmerlin et al., 2003; Hemmerlin et al., 2012), but remains controversial as no transporter has been identified and clear evidence of IPP exchange is lacking from several recent studies (Rasulov et al., 2018; Souza et al., 2018). In addition to IPP, there are other common precursors of squalene and phytol that are produced by both the MEP and MVA pathways, specifically geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) (Fig. 6). Transport of both of these isoprenoid intermediates from the plastid to the cytosol has also been observed (Bick & Lange, 2003; Gutensohn et al., 2013), and could also result in $^{13}$C-enriched squalene from $^{13}$C$_1$-pyruvate (Fig. 6).

As $^{13}$C is incorporated into squalene at equal rates from $^{13}$C$_1$- and $^{13}$C$_2$-pyruvate, our data suggest that squalene is primarily produced from MEP-derived isoprenoid intermediates in P. aquatica leaves. Flexibility between the two isoprenoid-producing pathways has been proposed as a strategy to help plants overcome environmental stresses (Hemmerlin et al., 2012) and high rates of crosstalk by P. aquatica may contribute to the robustness of its lipid metabolism in response to reduced water availability. However, the prevalence of metabolic crosstalk for the production of
squalene in the well-watered control plants is notable (Fig. 4), and suggests that this process may be more widespread than is commonly assumed.

The use of plastidial intermediates during squalene synthesis implies that they are also incorporated into phytosterols. Phytosterols, such as sitosterol, are all produced from squalene (Fig. 6) (Killops & Killops, 2004), and should incorporate $^{13}$C from each label similarly to squalene. Although sitosterol from $^{13}$C-pyruvate labeled leaves were, on average, enriched in $^{13}$C relative to sitosterol from unlabeled leaves in the same plant, these differences were small and not significant (Figs 3, 4). The most probable explanation for this discrepancy is that sitosterol is a long-lasting compound that accumulates within the leaf, while squalene rapidly turns over into downstream products. It is likely that we would have observed $^{13}$C-enriched sitosterol if we had allowed the pyruvate labeling to continue for a longer time period.

**Low $^{2}$H$_{sitosterol-phytol}$ values in *P. aquatica***

Phytosterols are highly enriched in $^2$H relative to phytol, with a mean enrichment from published values of $135\%$ (Sessions et al., 1999; Chikaraishi et al., 2004a,b; Chikaraishi & Naraoka, 2006; Sessions, 2006; Zhou et al., 2011) (Fig. 5b). This $^2$H-enrichment is typically attributed to incorporation of relatively more $^2$H-depleted photosynthetic NADPH into MEP-derived intermediates in comparison to those derived from the MVA pathway, which should have more $^2$H-enriched NAD(P)H from sugar metabolisms such as the oxidative pentose phosphate pathway (Sessions et al., 1999; Schmidt et al., 2003; Sachs et al., 2017) (Fig. 6). If the biosynthetic pathway producing IPP and other isoprenoid intermediates were the only cause of $^2$H-offsets between sterols and phytol, these compounds would have nearly identical $^2$H values in *P. aquatica*, where position-specific $^{13}$C-pyruvate labeling indicates that IPP used for squalene synthesis is all MEP-derived. However, squalene and its product sitosterol are still $^2$H-enriched relative to phytol, albeit with reduced $^{2}$H$_{sitosterol-phytol}$ values (85\%, Fig. 5). Additional $^2$H-depletion of phytol relative to other MEP-derived isoprenoids is almost certainly a result of strong fractionation that occurs during hydrogenation of geranylgeraniol and other intermediate compounds (Chikaraishi et al., 2009).

Directly comparing $^{2}$H$_{sitosterol-phytol}$ values allows the effect of precursor exchange between the MEP and MVA to be assessed independently of source water $^2$H values or leaf water $^2$H enrichment, as all leaf biomass should be synthesized from the same water, provided the compounds are produced at similar times. In the case of *P. aquatica*, $^{2}$H$_{sitosterol-phytol}$ values are c. 50\% lower than is typically observed in other plants (Sessions et al., 1999; Chikaraishi et al., 2004a,b; Chikaraishi & Naraoka, 2006; Sessions, 2006; Zhou et al., 2011, 2016) (Fig. 5b). This is consistent with our expectations that incorporation of MEP-derived precursors will result in relatively $^2$H-depleted sterols. This indicates that > 50\% of variability in sedimentary sterol $^2$H values may be a result of shifting precursor sources, complicating their use as a proxy for water $^2$H values.

Although the drought effect in this study was not strong enough to induce changes in the relative amount of crosstalk or in the $^2$H values of MVA-derived compounds (Figs 4, 5), our results suggest that pairing position-specific $^{13}$C labeling with natural abundance isoprenoid $^2$H measurements under more extreme stress conditions, or in comparisons among species, would permit the hypothesis that crosstalk drives variability in sterol $^3$H$_{itauro}$ values to be tested more directly than ever before, and could lead to the use of natural $^{2}$H$_{sitosterol-phytol}$ values as an indicator of the relative amount of exchange between the MEP and MVA pathways taking place within a plant’s cells.

**Conclusions**

Metabolic exchange between the MVA and MEP pathways has long been suspected to influence isoprenoid $^2$H values in plants, but supporting experimental evidence has been lacking. Through position-specific $^{13}$C-pyruvate labeling in *P. aquatica* saplings, we demonstrated the important role played by exchange of precursor molecules (GA-3-P, IPP, GPP, FPP) between pathways for isoprenoid synthesis under nonstressed conditions. Additionally, the low degree of $^2$H enrichment of sitosterol relative to phytol in comparison to literature values from other plant species is consistent with the hypothesis that incorporation of MEP-derived precursor molecules into sterols will result in lower sterol $^2$H values, and we demonstrate for the first time that this effect can exceed 50\%. Sensitive information about isoprenoid production and the degree of metabolic crosstalk between the MVA and MEP pathways can thus be determined by measuring changes in the relative natural abundance of $^2$H among different compound classes without the addition of isotopic labels.

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**Author contributions**

NL and CW designed the research. NL, DN, IB and ED performed the research and collected data. NL, DN, JK, AK and CW analyzed and interpreted the data. NL wrote the manuscript with input from all co-authors.

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Data availability

All data are available in Tables S1–S3.

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data availability

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Gas chromatogram of neutral alcohol fraction from Pachira aquatica leaves.

Fig. S2 Hydrogen isotopic composition of leaf water extracted from unlabeled Pachira aquatica leaves, grouped by treatment.

Fig. S3 \( ^{13} \text{C} \) enrichment of \( \text{CO}_2 \) exiting cuvettes relative to \( \text{CO}_2 \) supplied to cuvettes.

Table S1 Lipid contents (\( \mu \text{g compound g}^{-1} \text{ dry leaf} \)) in Pachira aquatica leaves.

Table S2 Compound-specific \( ^{2} \text{H} \) values of various lipids from Pachira aquatica leaves.

Table S3 Stable isotope \( (^{2} \text{H}, ^{18} \text{O}, ^{13} \text{C}) \) values of leaf water and lipids from individual Pachira aquatica leaves.

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