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The ‘photosynthetic C₁ pathway’ links carbon assimilation and growth in California poplar

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Although primarily studied in relation to photorespiration, serine metabolism in chloroplasts may play a key role in plant CO₂ fertilization responses by linking CO₂ assimilation with growth. Here, we show that the phosphorylated serine pathway is part of a ‘photosynthetic C₁ pathway’ and demonstrate its high activity in foliage of a C₃ tree where it rapidly integrates photosynthesis and C₁ metabolism contributing to new biomass via methyl transfer reactions, imparting a large natural ¹³C-depleted signature. Using ¹³CO₂-labelling, we show that leaf serine, the S-methyl group of leaf methionine, pectin methyl esters, and the associated methanol released during cell wall expansion during growth, are directly produced from photosynthetically-linked C₁ metabolism, within minutes of light exposure. We speculate that the photosynthetic C₁ pathway is highly conserved across the photosynthetic tree of life, is responsible for synthesis of the greenhouse gas methane, and may have evolved with oxygenic photosynthesis by providing a mechanism of directly linking carbon and ammonia assimilation with growth. Although the rise in atmospheric CO₂ inhibits major metabolic pathways like photorespiration, our results suggest that the photosynthetic C₁ pathway may accelerate and represents a missing link between enhanced photosynthesis and plant growth rates during CO₂ fertilization under a changing climate.

Terrestrial and marine ecosystems assimilate atmospheric CO₂ into biomass through photosynthesis¹ serving as a foundation for Earth’s carbon cycle. Since pre-industrial times (i.e. before 1850), global terrestrial gross primary production (GPP) is estimated to have increased by 31 ± 5%, largely driven by an increase in atmospheric CO₂ concentrations^{2,3}. However, while the mechanisms involving enhanced plant growth under elevated CO₂ have primarily focused on increased carboxylation and decreased oxygenation rates of ribulose biphosphate carboxylase/oxygenase (RuBisCO) and enhanced water use efficiency related to decreased stomatal conductance, these processes alone cannot fully explain the dynamic changes in plant growth to elevated CO₂⁴.

Plants assimilate CO₂ via gross photosynthesis (carboxylation) but also produce CO₂ via photorespiration and respiration. Photorespiration

involves a series of reactions linked to C₁ metabolism during the conversion of glycine to serine active during abiotic stress responses⁵. However, although the majority of research on serine metabolism in plants has focused on the role of photorespiration, the presence of a phosphorylated serine pathway has been established in cyanobacteria⁶ and higher plants⁷ and appears to be well conserved across prokaryotic and eukaryotic species⁸. High expression levels of key enzymes of the phosphorylated serine pathway have been documented in light-grown shoots⁹ with the phosphorylated serine pathway directly linked to nitrogen assimilation by providing 2-oxoglutarate for ammonia fixation¹⁰.

Disruption of the phosphorylated serine pathway leads to a reduction in nitrogen and sulfur contents in shoots and a general transcriptional response to nutrient deficiency¹¹. While the *in vivo* activity of the

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phosphorylated serine pathway, including its potential links with photosynthesis remains poorly characterized, genetic and molecular evidence revealed a critical role of the phosphorylated serine pathway for proper embryo and pollen development and root growth¹². In the model plant *Arabidopsis thaliana*, mutagenesis studies demonstrated its essential role in light and sugar-dependent growth promotion; A downregulation of the phosphorylated serine pathway led to a severe inhibition of shoot and root growth⁸ and mutants of the key enzymes phosphoglycerate dehydrogenase (PGDH) and phosphoserine phosphatase (PSP) resulted in an embryo-lethal phenotype^{10,13,14}. In addition, the phosphorylated serine pathway was shown to be critical for pollen development and metabolomics studies suggested that it directly integrates central carbon and energy metabolism with growth and development by affecting ammonia assimilation, glycolysis, the tricarboxylic acid cycle, and the biosynthesis of amino acids such as tryptophan^{10,13}. While most of what is known about leaf serine derives from studies on photorespiration⁷, the importance of the phosphorylated serine pathway as a source of serine was found to be particularly important under elevated ambient CO₂ concentrations associated with enhanced growth rates. Wild-type *A. thaliana* plants grown under elevated CO₂ concentrations showed enhanced leaf growth rates together with increased expression of PGDH1. In contrast, leaf serine content and growth rates of mature PGDH1-silenced plants were severely impaired while increased ammonia and some amino acid concentrations were observed¹⁰. These observations suggest that the phosphorylated serine pathway is particularly important under high CO₂ conditions that promote photosynthesis while suppressing photorespiration. Consistent with this view, a recent analysis of cell proliferation and elongation in *A. thaliana* revealed that the phosphorylated serine pathway is indispensable for plant growth and its loss cannot be compensated by photorespiratory serine biosynthesis¹⁵.

Although the potential direct connection between the phosphorylated serine pathway and the Calvin-Benson cycle for C₃ substrate linked with photosynthesis has not been investigated, a major role in plant growth and development and C₁ metabolism has been discussed, with serine involved in methyl group transfer reactions by supplying tetrahydrofolate (THF) metabolism C₁ units^{16,17}. Thus, while the mechanisms behind this critical and indispensable role of the phosphorylated serine pathway for growth and development remain under investigation, C₁ methyl transfer reactions are likely involved. In *A. thaliana*, chloroplasts were shown to be autonomous for the de novo synthesis of methionine, with the phosphorylated serine pathway the likely source of the serine used in the methylation of homocysteine to methionine¹⁸. Mediated by a chloroplast serine hydroxymethyltransferase (SHMT), serine from the phosphorylated serine pathway may be a major source of activated one-carbon units for plant C₁ metabolism¹⁹, especially under conditions like elevated CO₂ which suppresses photorespiration while enhancing photosynthesis.

Following the export of methionine from chloroplasts to the cytosol and its activation to the universal methyl group donor S-adenosylmethionine (AdoMet or SAM), AdoMet is subsequently imported into numerous organelles where it is utilized by diverse methyltransferase enzymes during biosynthetic and regulatory processes including the methylation of proteins, carbohydrates, nucleic acids, and metabolites²⁰. A large sustained turnover of methionine in plant tissues may be possible with AdoMet formation and regeneration, without net consumption of methionine²¹. AdoMet metabolism may be intimately involved in plant growth by sustaining nitrogen assimilation¹⁰ and a large, but poorly characterized flux of C₁ methyl transfers during new biopolymer and metabolite synthesis including regulatory components of plant growth such as nucleic acids³⁰, the growth hormones auxins¹⁰ and ethylene² and methylated pectin²². While previously considered a byproduct of growth, the release of methanol from methylated pectin is now recognized to dramatically alter the elasticity of the primary cell wall, a critical parameter controlling initiation and propagation of tissue morphogenesis and growth^{22–24} (see Supplementary Note 1: ‘pectin demethylation, methanol emission, and growth’).

In this study, we hypothesize that light-dependent methionine synthesis and metabolism are tightly linked with growth by providing a potentially large flux of C₁ transfers during biopolymer and metabolite synthesis and regulation via methylation. Moreover, synthesis of methylated pectin influences tissue morphogenesis and growth via subsequent demethylation on the primary cell wall, leading to changes in mechanical properties like elasticity and the associated release of methanol into the apoplast. We hypothesize that carbon for the activated methyl donor synthesis of AdoMet is directly produced by a ‘photosynthetic C₁ pathway’ in chloroplasts initiated by the carboxylation reaction catalyzed by RuBisCO which generates 3-phosphoglyceric acid (PGA) then utilized by the plastidic phosphorylated serine pathway. Serine then transfers C₁ units to THF which subsequently transfers C₁ units to homocysteine during methionine synthesis. Followed by the export of methionine to the cytosol and its activation to AdoMet, the universal methyl group donor²⁰, a high flux of methyl transfer reaction might be sustained through an AdoMet recycling pathway in the cytosol involving the regeneration of homocysteine, independently of photorespiration. To test these hypotheses, we employed dynamic ¹³C₂-labeling of California poplar (*Populus trichocarpa*) leaves under controlled environmental conditions and whole branches under day/night light cycles together with analysis of stable carbon isotope ratios (¹³C/¹²C, δ¹³C values) of leaf phosphoglyceric acid (PGA), serine, and methionine, cell wall methyl esters (OCH₃), and leaf methanol (CH₃OH), acetaldehyde (CH₃CHO), and isoprene (C₅H₈) emissions (that is, C₁, C₂, C₃ and C₅ metabolism products). We compared ¹³CO₂-branch labeling patterns under a 21% and 1% O₂ atmosphere to evaluate the potential roles of C₁-photosynthesis versus photorespiration in generating new cell wall methyl esters and the release of methanol during growth and development. We also evaluated *P. trichocarpa* leaf and branch gas exchange patterns in a field experiment to gain insight into potential temperature and hydraulic controls over diurnal growth and methanol emissions during the 2023 growing season in Berkeley, CA, USA. Finally, using comparative genomics, we examine the co-occurrence of proteins in the photosynthetic C₁ pathway and trace the evolutionary origin of methionine synthase through cyanobacteria and algae to land plants.

The dynamic labeling of ¹³CO₂ in California poplar demonstrated a strong connection between photosynthetic carbon assimilation and methanol emissions. Methanol emissions were highly responsive to light, with ¹³C-labeled pectin methyl esters (PMEs) observed within 5 min of light exposure and branch methanol emissions reaching 36% after two diurnal cycles. Methionine and PMEs were found to be primary recipients of ¹³C-labeled carbon from the Calvin cycle, confirming the involvement of the photosynthetic C₁ pathway in methyl group transfer and cell wall expansion during plant growth. This study reveals that the photosynthetic C₁ pathway plays a critical role in linking carbon assimilation with growth processes in California poplar by driving light-dependent methionine synthesis and methyl group transfer reactions. The observed diurnal fluctuations in methanol emissions and their correlation with photosynthesis underscore the importance of this pathway in regulating cell wall dynamics and overall plant growth. These findings provide new insights into the mechanisms by which elevated CO₂ and photosynthesis enhance plant growth, potentially representing a critical component of plant responses to changing environmental conditions.

Results

¹³C abundance in methanol, isoprene and acetaldehyde emitted by branches depends on light and % O₂ during diurnal ¹³CO₂ labeling

When a gas-exchange enclosure with elevated ¹³CO₂ (900 μmol mol⁻¹) was installed on an intact poplar branch at night in the dark under a 21% O₂ atmosphere, methanol emissions displayed a typical nocturnal pattern of initially decreasing in the evening, reaching a minimum around midnight, followed by an increase throughout the night (Fig. 1a). Despite continued branch methanol emissions in the dark, ¹³C-labeling was not observed, with a ¹³C/¹²C isotope ratio of 1.0–2.5%, similar to natural abundance (1.1%).

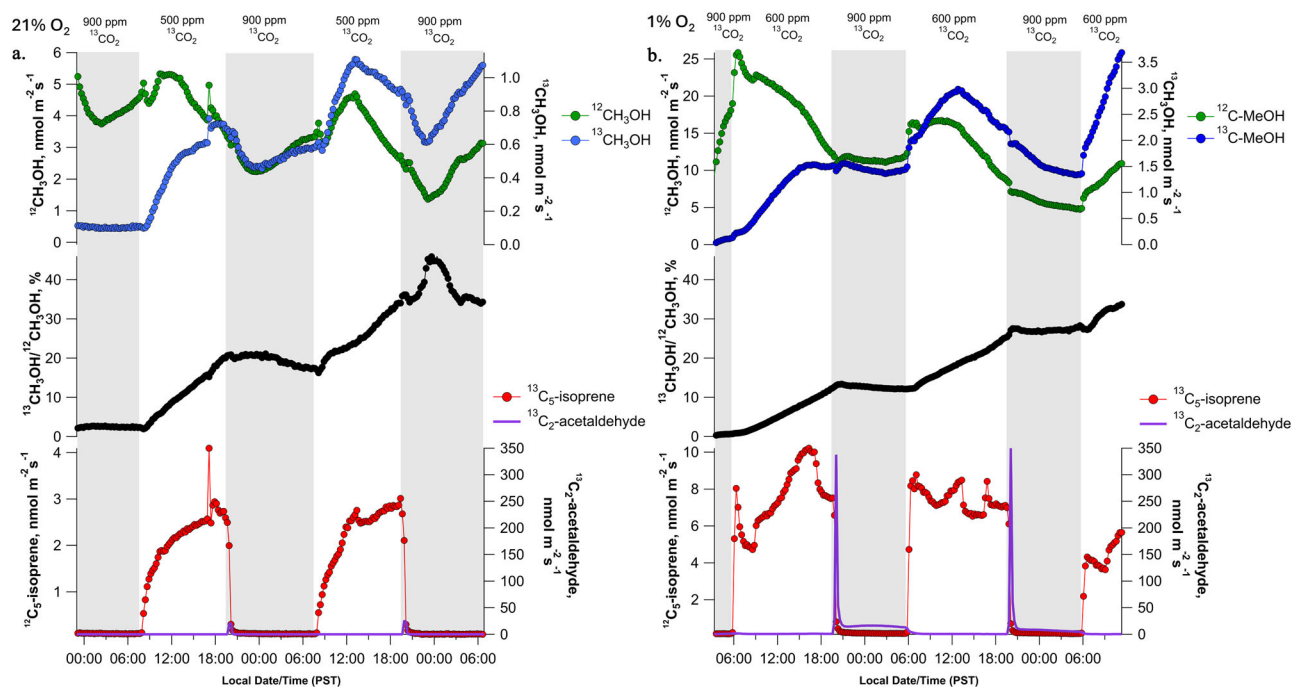


Fig. 1 | Diurnal ^{13}C -labeling dynamics of branch methanol emissions during light-dark cycles under an elevated $^{13}\text{CO}_2$ atmosphere. a Example 2-day time series in a 21% and (b) 1% O_2 atmosphere showing dynamic branch labeling of methanol

(CH_3OH) emissions via the photosynthetic- C_1 pathway under elevated $^{13}\text{CO}_2$. Night periods are shaded in gray. Also shown are branch emissions of $^{13}\text{C}_5$ -isoprene during the day and $^{13}\text{C}_2$ -acetaldehyde bursts at the beginning of the dark period.

Moreover, emissions of isoprene in the dark could not be detected. However, upon switching to the light in the morning, emissions of $^{13}\text{C}_5$ -isoprene (i.e., fully ^{13}C -labeled isotopologue) was immediately apparent and dominated other isoprene isotopologues ($^{13}\text{C}_5$ -isoprene emissions accounting for >95% of total isoprene emissions). In the light, the $^{13}\text{C}/^{12}\text{C}$ isotope ratio in methanol continuously increased, reaching 36% after two diurnal cycles (Fig. 1a). In contrast, at night, the isotope ratio in methanol showed some variation but did not increase. Interestingly, branch emissions of both ^{12}C - and ^{13}C -methanol started to increase at midnight, i.e. when leaf water potential recovered (-0.2 ± 0.1 MPa) from midday values (-1.0 ± 0.1 MPa). Replicate 2-day experiments showed similar diurnal patterns and maximum isotope ratio in methanol after 2 light periods (Supplementary Figs. S1–S4). A single long-term $^{13}\text{CO}_2$ branch labeling experiment (5 days) demonstrated that the pattern of light-dependent ^{13}C -methanol labeling continued to increase, with an isotope ratio in methanol reaching 80% by the end of day 5 (Supplementary Fig. S5).

Under a 1% O_2 atmosphere used to suppress photorespiration (Fig. 1b), branch $^{13}\text{CO}_2$ assimilation and O_2 production continued in the light (Supplementary Fig. S6) and the isotope ratio in methanol increased to 28% after two light periods. This value was slightly lower than that under 21% O_2 (36%). The branch also emitted $^{13}\text{C}_5$ -isoprene. Compared to 21% O_2 , there was a strong increase in fermentation-type metabolism in 1% O_2 . In fact, the burst of acetaldehyde emission at the light-to-dark transition increased considerably (nearly 20-fold) under 1% O_2 (purple line, Fig. 1a, b). Moreover, acetaldehyde emission lasted longer (2–3 h) and was followed by a period of elevated, but gradually decreasing $^{13}\text{C}_2$ -acetaldehyde emissions for the entire night (Fig. 1b). Collectively, it shows that C_1 metabolism producing methanol was mostly fed by photosynthetic carbon (and only marginally by photorespiration), with a relatively slow turn-over (compared to, e.g., isoprene).

Instantaneous leaf ^{13}C -methanol emission in the light correlates with cumulative $^{13}\text{CO}_2$ assimilation

A labeling experiment was carried out with a leaf placed in the chamber in the light under elevated $^{13}\text{CO}_2$ ($750 \mu\text{mol mol}^{-1}$). As expected, there was a progressive rise in assimilation rate (from 13 to $24 \text{ mol m}^{-2} \text{ s}^{-1}$, Fig. 2).

Light-dependent isoprene emissions were dominated by the $^{13}\text{C}_5$ -isotopologue (>95% as determined by PTR-MS) and slowly increased with time, while the monoisotopic species ($^{12}\text{C}_5$ -isoprene) decreased concurrently. As a result, after 3 h in the light, isoprene was more than 95% ^{13}C -labeled. Similarly, ^{13}C -labeling in methanol increased, reaching 8% ^{13}C after 3.5 h (Fig. 2). ^{13}C -labeling in methanol was verified using thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) (Supplementary Fig. S7). When individual leaves were illuminated for 1–5 h under elevated $^{13}\text{CO}_2$ (at 21% O_2), we found a strict linear correlation ($R^2 > 0.96$) between cumulative $^{13}\text{CO}_2$ assimilation in the light and instantaneous ^{13}C in methanol emissions (Supplementary Fig. S8). Also, with increasing labeling time from 1 to 2, 3, 4, or 5 h, the maximum $^{13}\text{C}/^{12}\text{C}$ ratio in methanol increased proportionally to 2.4%, 2.8%, 5%, 9%, and 11%, respectively. Interestingly, the slope of linear regressions, representing increase in $^{13}\text{C}/^{12}\text{C}$ ratio per μmol of assimilated ^{13}C per m^2 , varied from leaf to leaf with a mean of $2.7 \times 10^{-5} \pm 1.7 \times 10^{-5}$ (± 1 SD). Overall, it suggests that the carbon source used to synthesize methanol was fed directly by photosynthetic assimilation.

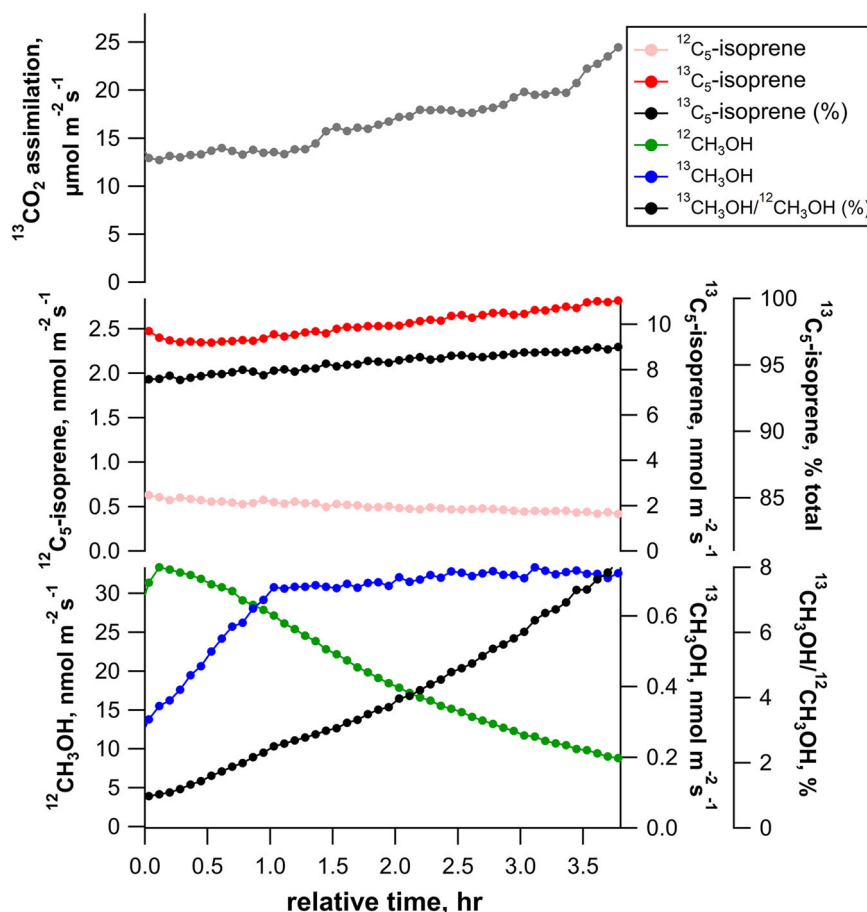
Leaf ^{13}C -methanol emission strongly depends on temperature upon $^{13}\text{CO}_2$ labeling

Selected branches from the 2-day $^{13}\text{CO}_2$ diurnal experiment (above) were detached from the tree with the stem recut under water, and one mature leaf placed in a gas exchange system under a $^{12}\text{CO}_2$ atmosphere ($400 \mu\text{mol mol}^{-1}$). There was a strong increase in both ^{13}C -methanol and ^{12}C -methanol emission with temperature, despite a rather constant $^{13}\text{C}/^{12}\text{C}$ isotope ratio, near 20% (Supplementary Fig. S9). Thus, temperature led to an increase in the rate of, but no change in the carbon pool used for methanol production.

^{13}C -pectin methyl esters are synthesized from assimilated $^{13}\text{CO}_2$ even without photorespiration

While measurement of a small % changes in $^{13}\text{C}/^{12}\text{C}$ isotope ratio in leaf-emitted methanol required 1 h or more, we used more sensitive measurements of changes of the $^{13}\text{C}/^{12}\text{C}$ ratio of pectin methyl esters (PMEs) using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and present results as $\delta^{13}\text{C}$ values (‰, see Methods). PMEs were

Fig. 2 | Example time series showing the ^{13}C -labeling dynamics of leaf isoprene and methanol in the light under constant environmental conditions including optimal conditions for photosynthesis ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 32°C leaf temperature, and $900\text{--}1000 \text{ ppm}$ reference $^{13}\text{CO}_2$). Note the rapid and near complete ^{13}C -labeling of isoprene emissions, and the comparably slower and incomplete labeling of ^{13}C -methanol emissions.



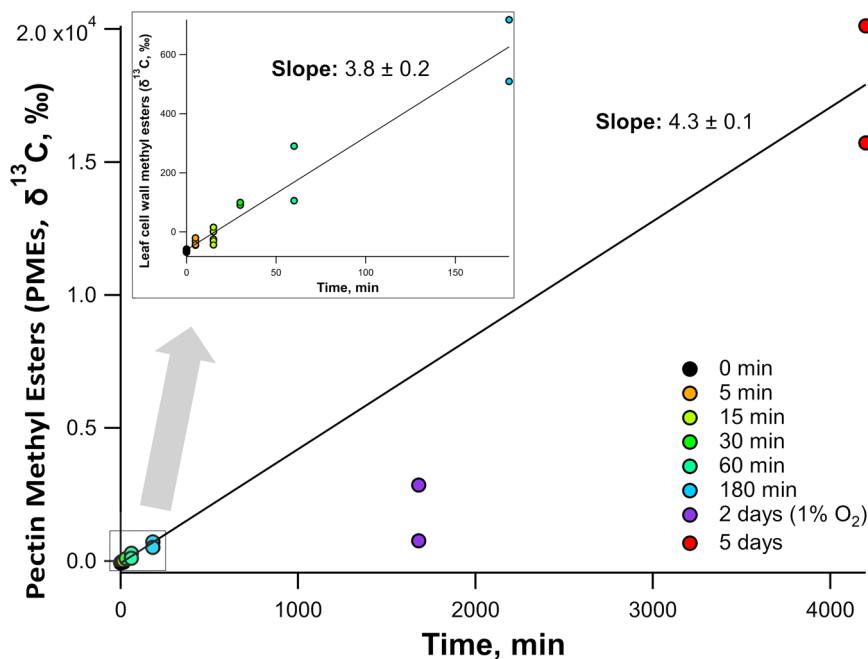
naturally ^{13}C -depleted and thus typical of C_1 carbon units of a C_3 plant. In effect, values obtained from control experiments confirmed the large natural ^{13}C -anomaly of plant C_1 carbon (i.e. down to -60% , Fig. 3). Upon $^{13}\text{CO}_2$ labeling in the light ($1000 \mu\text{mol mol}^{-1} \text{ }^{13}\text{CO}_2$, $21\% \text{ O}_2$), the ^{13}C -enrichment in leaf PMEs could be detected after the shortest possible time experimentally (5 min) to place a leaf in the chamber and reach steady state gas exchange (-35%) and after 180 min, reached $+614\%$ (i.e. about 1.8% ^{13}C). The $\delta^{13}\text{C}$ value increased after 5 d, reaching ca. $+18000\%$ (i.e. about 17.6% ^{13}C), thus showing a consistent rate of increase throughout ($3.8\text{--}4.3\%$ min^{-1}). Interestingly, $^{13}\text{CO}_2$ labeling for 2 days under $1\% \text{ O}_2$ led to an average ^{13}C -enrichment in PMEs of $+2860\%$ (purple circles, Fig. 3). Although slightly lower than expected compared to the situation under $21\% \text{ O}_2$, it shows that the suppression of photorespiration did not stop the synthesis of ^{13}C -labeled PMEs. Collectively, PMEs synthesis appeared to be consistent with methanol emission, that is, methyl groups of PMEs came, at least partly, from non-photorespiratory, recently assimilated photosynthetic carbon.

^{13}C -labeling in metabolites suggests the involvement of the Calvin–Benson cycle and P-serine pathway

Following $^{13}\text{CO}_2$ -labeling period under a $21\% \text{ O}_2$ atmosphere, liquid chromatography mass spectrometry (LC-MS/MS) analysis of extracted bulk leaf phosphoglyceric acid (3-PGA and/or 2-PGA), serine, and methionine showed significant ^{13}C -incorporation relative to control leaves exposed to $^{12}\text{CO}_2$ (Fig. 4a). PGA, an intermediate of the Calvin–Benson cycle²⁵, and serine, a product of the plastidic phosphorylated serine pathway⁹, showed near complete labeling on all three carbon atoms (total relative abundance $^{13}\text{C}_3/(\text{}^{13}\text{C}_3 + \text{}^{12}\text{C}_3)$ of $95.8 \pm 1.5\%$ and $91.2 \pm 1.0\%$, respectively). In addition, in leaves exposed to $^{13}\text{CO}_2$, the $^{13}\text{C}/^{12}\text{C}$ -methionine abundance ratio of singly ^{13}C -labeled methionine to unlabeled methionine was highly elevated

($57.2 \pm 2.8\%$). In contrast, the $^{13}\text{C}/^{12}\text{C}$ methionine abundance ratio from control leaves exposed to $^{12}\text{CO}_2$ was $4.55 \pm 0.17\%$, consistent with the value expected from natural ^{13}C -abundance (5.13% as $^{13}\text{C}_1/(\text{}^{13}\text{C}_1 + \text{}^{12}\text{C}_0)$). MS/MS analysis of methionine extracted from leaf samples identified 10 fragments observed in the natural abundance (^{12}C -methyl)-methionine and (^{13}C -methyl)-methionine standards (Supplementary Fig. S10), also previously reported in the literature²⁶. The molecular ion and all four fragments containing the sulfur-bonded methyl group ($\text{CH}_3\text{-S}$) showed ^{13}C -incorporation (m/z 61.0108, 87.0262, 104.0527, 133.0317). In contrast, all 6 fragments without the sulfur-bonded methyl group were not ^{13}C -labeled (Fig. 4b and Supplementary Fig. S10), also previously reported in the literature²⁶. The molecular ion and all four fragments containing the sulfur-bonded methyl group ($\text{CH}_3\text{-S}$) showed ^{13}C -incorporation (m/z 61.0108, 87.0262, 104.0527, 133.0317). In contrast, all 6 fragments without the sulfur-bonded methyl group were not ^{13}C -labeled. For example, the methionine $[\text{CH}_3\text{SCH}_2]^+$ fragment containing the sulfur-bonded methyl group showed ^{13}C -incorporation (m/z 61.0106 \rightarrow 62.0105) whereas the $[\text{C}_4\text{H}_8\text{NO}_2]^+$ fragment, which does not contain the sulfur-bonded methyl group, did not (m/z 102.0549) (Fig. 4b and Supplementary Fig. S10). These observations reveal that the ^{13}C -incorporation in methionine during photoassimilation of $^{13}\text{CO}_2$ occurred in the sulfur bonded methyl group, (^{13}C -methyl)-methionine. All serine fragments showed expected shifts in the fragmentation spectrum of the $^{13}\text{CO}_2$ labeled leaves based on the predicted fragment structures and ^{13}C -labeling of all 3 carbon atoms (Fig. 4c), while no shifts were observed in leaves exposed to $^{12}\text{CO}_2$ (Supplementary Fig. S10). 2-phosphoglyceric acid is known to elute on our methods at a similar retention time as 3-phosphoglyceric acid, and produce similar fragmentation spectra, thus we could not differentiate them in these samples, and no fragmentation spectra were collected for PGA due to the low abundance in the samples. However, mediated by a plastidic phosphoglycerate mutase,

Fig. 3 | $\delta^{13}\text{C}$ values of bulk leaf cell wall methyl esters by GC-C-IRMS plotted versus leaf $^{13}\text{CO}_2$ labeling period. Note the strong natural ^{13}C -depletion of control (0 min) leaf samples ($\delta^{13}\text{C}$ of -60‰). All leaves were under a 21% O_2 atmosphere except the 2-day samples which were under a 1% O_2 atmosphere. Trendlines only include data under a 21% O_2 atmosphere. Short labeling durations 0–180 min shown in inset.



photosynthetically generated 3-PGA is known to be converted to 2-PGA in the light²⁷.

The molecular ion and all four fragments containing the sulfur-bonded methyl group ($\text{CH}_3\text{-S}$) showed strong ^{13}C -incorporation (m/z 61.0108, 87.0262, 104.0527, 133.0317). In contrast, all 6 fragments without the sulfur-bonded methyl group were not ^{13}C -labeled (Fig. 4b and Supplementary Fig. S10). For example, the methionine $[\text{CH}_3\text{SCH}_2]^+$ fragment containing the sulfur-bonded methyl group showed strong ^{13}C -incorporation (m/z 61.0106 \rightarrow 62.0105) whereas the $[\text{C}_4\text{H}_8\text{NO}_2]^+$ fragment, which does not contain the sulfur-bonded methyl group, did not (m/z 102.0549) (Fig. 4b). These observations reveal that the majority of ^{13}C -incorporation in methionine during photoassimilation of $^{13}\text{CO}_2$ occurred in the sulfur-bonded methyl group, (^{13}C -methyl)-methionine.

In situ methanol emission rate in the field is mostly linked to air temperature

Diurnal branch gas exchange on *P. trichocarpa* individuals in the field showed a tight temporal relationship between air temperature and sap velocity, as well as branch gas exchange rates including net photosynthesis, transpiration, and methanol emissions during the early, mid, and late-growth season (April, May, and June 2023, Supplementary Figs. S11–S13, respectively). In April, in early growth season, night temperature was relatively low (i.e. 10 °C) and little to no methanol emission was observed. However, significant night-time methanol emission could be observed in May and June, although night temperatures kept relatively low (i.e. 3.0 $\text{nmol m}^{-2} \text{s}^{-1}$ at 12 °C). Methanol emissions tended to peak around midday (i.e. 13:00) whereas net photosynthesis, transpiration, and sap flow continued at elevated rates until later in the day (i.e. showing a relatively broad peak centered at 16:00). Therefore, during late afternoons, methanol emissions were low while photosynthesis remained elevated (along with sap velocity and air temperature). When put together, the dynamic branch gas exchange datasets showed that methanol emission increased exponentially with air temperature (Supplementary Fig. S14). Similar results were obtained when diurnal leaf gas exchange time series under controlled environmental conditions were compiled during April and June as bar plots (Supplementary Figs. S15 and S16). It was visible that methanol emission increased with temperature (and thus transpiration) throughout the mornings, peaked and declined in the late afternoon while temperature and transpiration remained high.

Discussion

To date, *in vivo* studies on plant serine and C_1 metabolism have focused on mitochondrial glycine-to-serine conversion during photorespiration, associated with THF-mediated methyl transfer^{28,29}. For example, in a previous study on photorespiration and sulfur metabolism using $^{13}\text{CO}_2$ labeling of sunflower (*Helianthus annuus*) leaves in the light³⁰, high-resolution ^{33}S and ^{13}C tracing with nuclear magnetic resonance (NMR) spectroscopy and LC-MS demonstrated a direct and rapid (2 h) ^{13}C -incorporation mostly in the methyl group (C-5 atom) of methionine; using various CO_2/O_2 ratios, it was shown that methionine C-5 labeling was directly dependent on RuBisCO-catalyzed oxygenation²⁹. However, although the methyl group of methionine can be redistributed to other compounds and thus participate in cellular C_1 metabolism, products of C_1 metabolism were not followed in that study. These results are consistent with studies using metabolic flux analysis, modeling, and gas exchange measurements that suggest a fraction of photorespiratory carbon can be exported as serine under photorespiratory conditions^{31–33}. Moreover, recent findings show that the photorespiratory pathway could contribute to C_1 metabolism; Disruption of mitochondrial SHMT1 boosted glycine and C_1 carbon (in the form of 5,10-methylene-THF) flux out of the photorespiratory cycle¹¹. Thus, it is important to note that photorespiratory C_1 carbon flux could be a substantial source of C_1 units, especially during abiotic stress conditions like heat waves and droughts that stimulate photorespiration while suppressing photosynthesis.

Here, we used $^{13}\text{CO}_2$ -labeling to monitor the isotope enrichment not only in leaf methionine, but also in PGA, serine, PMEs, and methanol, acetaldehyde, and isoprene emissions under conditions that promoted high rates of photosynthesis while suppressing photorespiration (e.g. moderate light, optimal leaf temperatures, elevated $^{13}\text{CO}_2$, and 1% O_2). Our results show that PMEs and methanol are ^{13}C -labeled even in the absence of photorespiration, suggesting that the plastidic phosphorylated serine pathway^{7,9} rather than photorespiration, plays the role of a light-dependent ‘photosynthetic C_1 ’ pathway initiated by RuBisCO-catalyzed carboxylation. That is, CO_2 fixation in the light can be a direct carbon source for cellular methyl transfers mediated by the methyl group of methionine. We nevertheless recognize that despite the strong ^{13}C -labeling in both PMEs and methanol in the light under non-photorespiratory conditions (1% O_2), it was slightly lower than that obtained under 21% O_2 (i.e. 28% compared with 32–42% after 2 diurnal cycles, Fig. 1). This effect was likely caused by (i) a slight inhibition of photosynthetic $^{13}\text{CO}_2$ fixation by O_2 mole fraction below

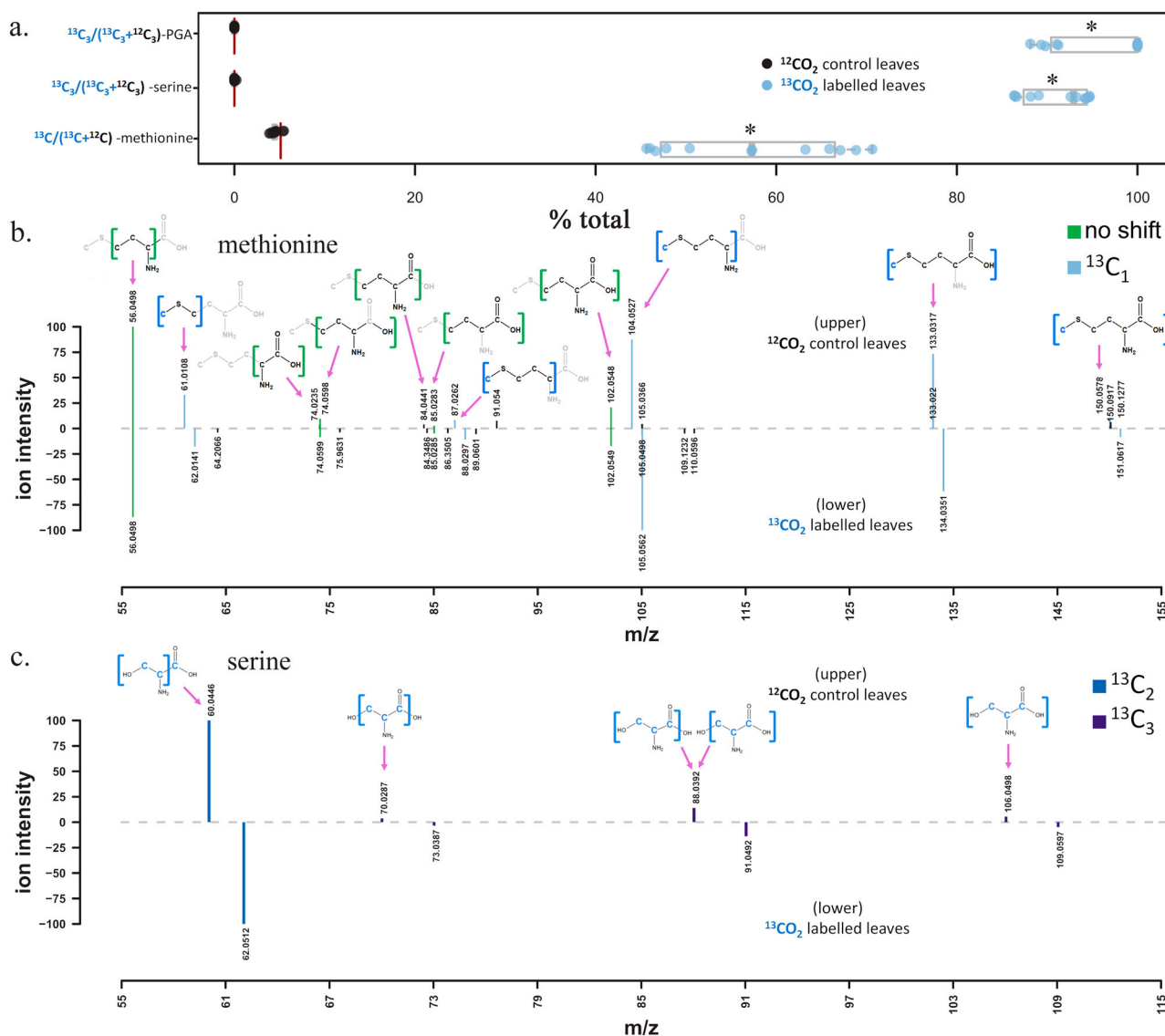


Fig. 4 | LC-MS/MS ¹³C-labeling analysis of phosphoglyceric acid (PGA), serine, and methionine extracted from *P. trichocarpa* leaves following 2-day gas exchange studies under a ¹³CO₂ and 21% O₂ atmosphere. a % total abundance ratio plot of [¹³C₃/(¹³C₃ + ¹²C₃)]-PGA, [¹³C₃/(¹³C₃ + ¹²C₃)]-serine, and [¹³C/¹²C]-methionine from control leaves exposed to a natural abundance ¹²CO₂ atmosphere (*n* = 10), and leaves exposed to ¹³CO₂ over two diurnal cycles (*n* = 12). The red vertical lines are the theoretical natural abundance ratios and * indicates significant difference by one tailed *t* test at *P* < 0.001. PGA was confirmed by RT, and *m/z*

comparison with pure reference standard, while serine and methionine were confirmed with RT, *m/z* and MSMS. Also shown are the mirror plots comparing fragmentation patterns of leaf methionine (b) and serine (c) from ¹²CO₂ and ¹³CO₂ exposed leaves. Serine and methionine carbon atoms within each fragment are shown as previously determined²⁶. Green fragments indicate matching ions within 15 ppm and blue and purple fragments indicate that the lower ion is within 15ppm of the expected ¹³C-labeled fragment isotopologue (see legend).

2%³⁴, (ii) an acceleration of fermentative metabolism at night (here, elevated nocturnal ¹³C₂-acetaldehyde emissions)³⁵, and (iii) reduced methanol dynamics (oscillations) at night-time, perhaps suggesting reduced growth rates (Fig. 1b).

The results demonstrate that the synthesis of PME via the light-dependent ‘photosynthetic C₁ pathway’ is independent of the release of methanol from PME during temperature-stimulated (day) as well as hydraulically-driven (night) growth processes. Thus, methanol emissions require light-dependent processes of C₁ photosynthesis leading to synthesis of new PME on the primary cell wall, and light-independent growth processes involving cell wall expansion, pectin demethylation, and methanol production. This implies that PME synthesis and export and incorporation into the primary cell wall occurs only during the day while PME demethylation and methanol production occurs during both the day and night, impacted by temperature and plant hydraulics.

Metabolic route from photosynthetic carbon to methanol

Methionine ¹³C-labeling was strong only in the C-5 atom position (methyl group, Fig. 4a, b and Supplementary Fig. S10), demonstrating the importance of THF-mediated C₁ metabolism. There was a dominance of ¹³C₃ isotopologues in both PGA and serine (more than 90% of total, Fig. 4a, c), consistent with the conversion of 3-PGA from the Calvin-Benson cycle to serine as the source of the methyl group (C-5 atom) of methionine^{1,9}. The very high ¹³C-enrichment in PGA and its direct products was also suggested by isoprene ¹³C-enrichment. As observed in other studies^{36,37}, more than 95% of isoprene emitted was fully ¹³C-labeled (¹³C₅ isotopologue) within minutes of placing the leaf into a ¹³CO₂ atmosphere in the light under elevated ¹³CO₂ (Fig. 2). Isoprene is not stored in plants and is produced via the isoprenoid pathway in chloroplasts which requires C₃ carbon skeletons synthesized by the Calvin-Benson cycle (i.e. 3-PGA)³⁶. A rapid conversion of 3-PGA to 2-PGA via plastidic phosphoglycerate mutase in the light²⁷

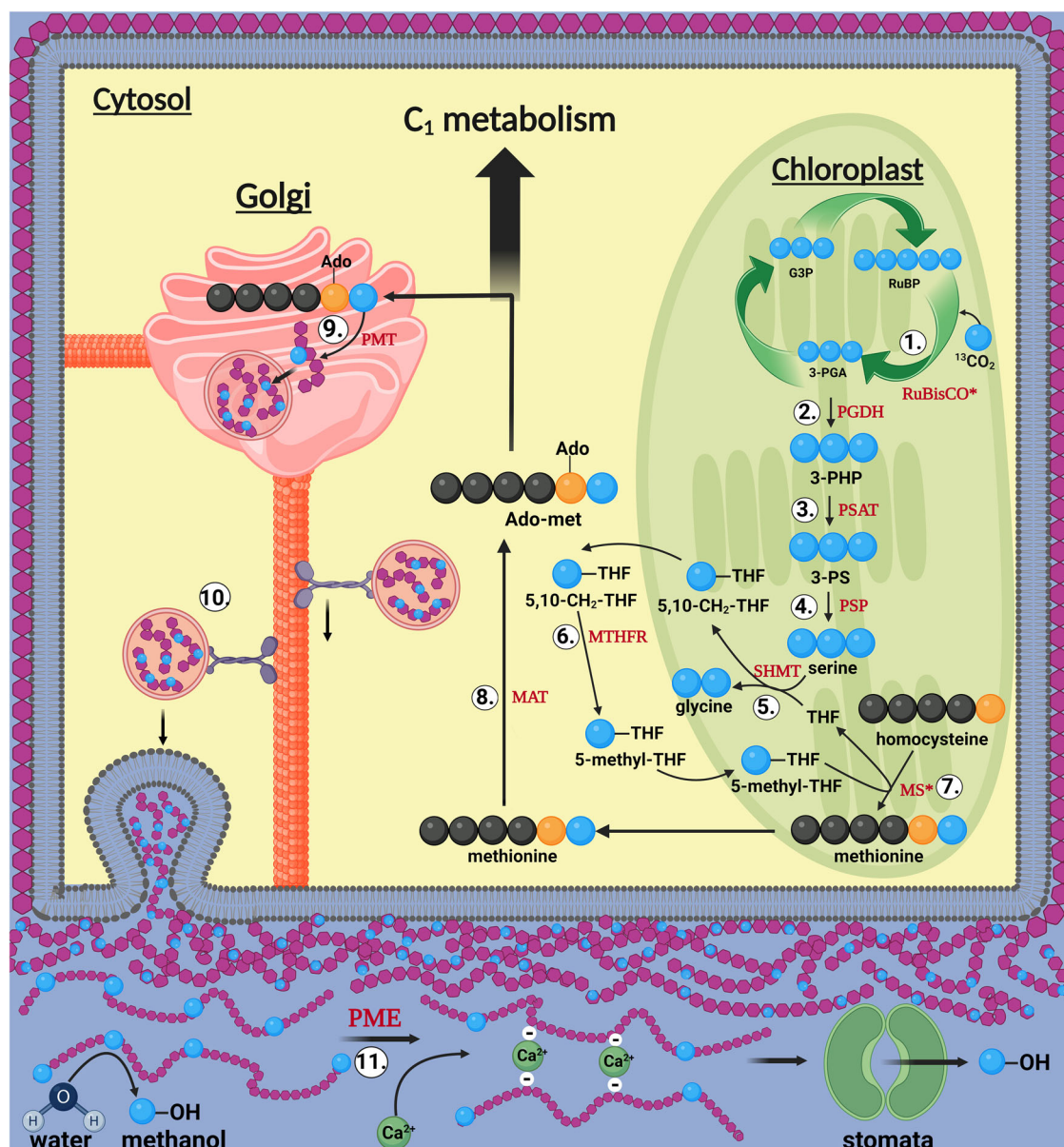
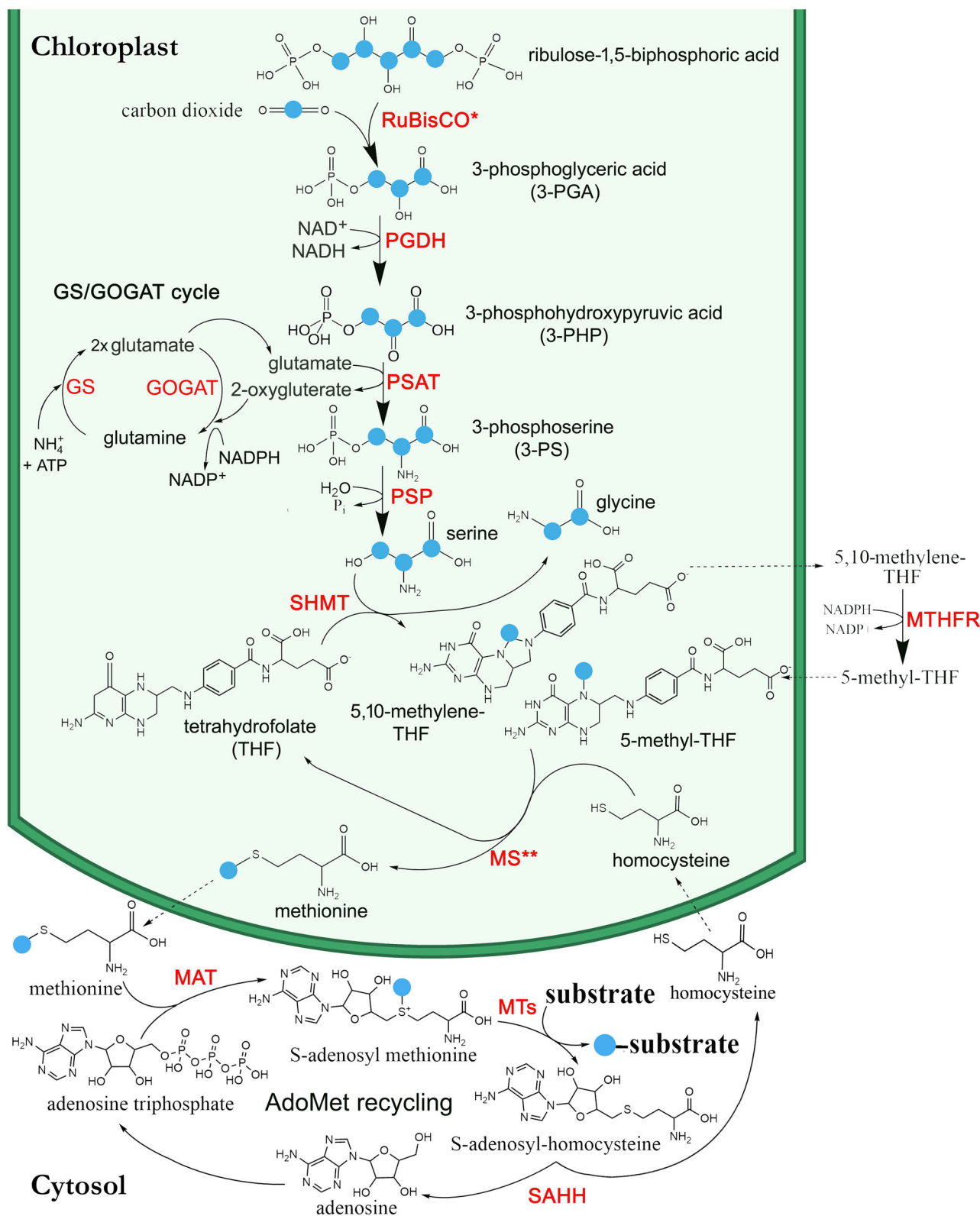


Fig. 5 | Simplistic model of the ‘Photosynthetic C_1 ’ pathway and methanol emission during growth. The ‘Photosynthetic C_1 ’ pathway (steps 1–7) results in light-dependent methionine synthesis followed by the activation of methionine to AdoMet in the cytosol (step 8) and its utilization as a major source of cellular methyl transfer reactions including in the Golgi during the methylation of newly synthesized pectin polysaccharides (step 9). Following the export and incorporation of newly synthesized, highly methylated pectin into the primary cell wall (step 10), cell wall expansion during growth processes is regulated by pectin demethylation (step 11). Pectin methyl ester hydrolysis results in the change in cell wall elasticity via formation of calcium pectinate “egg-box”⁹⁹ and release of methanol into the

apoplast. Methanol partitions between the plant aqueous and gas phases and rapidly escapes the plant via stomatal emission to the atmosphere. Blue balls represent carbon atoms recently assimilated by the Calvin–Benson cycle during photosynthesis as CO_2 ; Black balls represent stored or non-recently assimilated carbon atoms; Orange balls represent the sulfur atom of homocysteine, methionine and AdoMet. For abbreviations of metabolites and enzymes, see Fig. 6 for a more detailed schematic of the ‘Photosynthetic C_1 ’ pathway as well as Note 2 in the supplementary information document. Created in BioRender. K. Jardine (2023) BioRender.com/p03i521.

supports substrate flow to pyruvate and acetyl-CoA during light-dependent isoprenoid³⁸ and fatty acid synthesis³⁹, respectively. Moreover, the observation of strong near complete $^{13}C_2$ -labeling of acetaldehyde emissions during light-dark transitions supports the previous suggestions of a pyruvate overflow mechanism where photosynthetically generated pyruvate in the light is decarboxylated to acetaldehyde via aerobic fermentation upon sudden darkening, mediated by pyruvate decarboxylase^{40–42}. Under the 1% O_2 , this process was amplified resulting in nearly 20-fold increase relative to 21% O_2 with highly elevated $^{13}C_2$ -acetaldehyde persisting the entire night. Acetaldehyde oxidation to acetate is considered an effective alternative route to acetyl-CoA synthesis used in respiration as a part of a so called pyruvate

dehydrogenase bypass pathway⁴³, and O_2 concentrations of 2% or lower negatively impact aerobic respiration, with typical symptoms of hypoxia^{44,45} such as an upregulation of fermentation metabolism⁴¹. Also, the ^{13}C -enrichment in PME (including under 1% O_2) is consistent with the involvement of the phosphorylated serine pathway since the methyl group of methionine (and thus AdoMet) and the oxygen-bonded methyl group of PME are directly linked via a series of reactions (Fig. 5). A proposed biochemical model of the ‘Photosynthetic C_1 ’ pathway involves seven distinct enzymatic steps followed by AdoMet regeneration in the cytosol as a major source of cellular methyl transfer reactions in the light (see Fig. 6 and Supplementary Note 2: Biochemical steps of the ‘Photosynthetic C_1



pathway’). Here, chloroplast-localized methionine synthase¹⁸ (step 7) is crucial to transfer a C₁ unit from 5-methyl-THF to methionine. After activation to AdoMet (step 8), methyl esterification of new pectin monomers can occur via pectin methyltransferase⁴⁶ (step 9). Further utilization (transport, export, and incorporation to new PMEs in growing primary cell walls⁴⁷, step 10) and methanol production during pectin demethylation

(step 11)⁴⁶ can then take place. Our results effectively show that it was possible to trace photosynthetic carbon (¹³C) utilization through C₁ metabolism including methanol liberation (Fig. 1).

Interestingly, there was a considerable difference in ¹³C between metabolic end products examined here, namely PGA, serine, isoprene, acetaldehyde, PMEs and methanol. This effect was simply due to isotopic

Fig. 6 | Proposed biochemical model of the ‘Photosynthetic C₁ pathway’ in California poplar. Photosynthetic C₁ pathway initiated in chloroplasts by the Calvin-Benson cycle linked to NH₄⁺ assimilation via the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOAGT) cycle¹¹. Enzymes abbreviations shown in red include (1) ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), (2) 3-phosphoglycerate dehydrogenase (PGDH), (3) 3-phosphoserine aminotransferase (PSAT), (4) 3-phosphoserine phosphatase (PSP), (5) Serine hydroxymethyltransferase (SHMT), (6) methylenetetrahydrofolate reductase (MTHFR), and (7) methionine synthase (MS). PGDH, PSAT and PSP are enzymes of the phosphorylated serine pathway⁹. All enzymes of the photosynthetic C₁ pathway and GS/GOAGT cycle are chloroplast localized, except MTHFR which is

thought to be in the cytosol. Also shown is the AdoMet recycling pathway in the cytosol as a source of cellular methylation reactions with enzymes including methionine adenosyltransferase (MAT), methyltransferases (MT), and S-adenosyl-homocysteine hydrolase (SAHH). The blue balls represent carbon atoms recently assimilated by RuBisCO via the Calvin-Benson cycle during photosynthesis as CO₂. Note, the regeneration of THF and homocysteine intermediates, and the lack of ATP and NADPH requirements for methionine synthesis in the chloroplast. However, the GS/GOAGT cycle for NH₄⁺ assimilation requires both ATP and NADPH. * Large kinetic isotope effect leading a natural ¹³C-depletion of C₃ biomass relative to CO₂. ** Large kinetic isotope effect leading to natural ¹³C-depletion of C₁ carbon pools relative to C₃ biomass.

dilution (and pool size) along metabolic pathways. In particular, PGA, serine, isoprene and acetaldehyde were highly labeled while methanol and PME showed some isotopic dilution. The methyl group of methionine itself was at about 60% ¹³C under our conditions (Fig. 4a and Supplementary Fig. S10) and preexisting methyl groups in PMEs also participated in methanol production (Fig. 1). Although six of seven enzymes of the ‘photosynthetic C₁ pathway’ are chloroplast localized, 5-methyl-THF synthesis catalyzed by MTHFR is thought to occur in the cytosol, with the export of 5,10-methylene-THF out of chloroplasts together with 5-methyl-THF import into chloroplasts likely mediated by one or more chloroplastic folate transporters⁴⁸. Thus, export of strongly ¹³C-labeled 5,10-methylene-THF may be diluted by pre-existing 5,10-methylene-THF in the cytosol, resulting in a decrease in ¹³C-labeling of the methyl group of methionine (~60%) relative to serine (>90%). Methanol emission could continue at night in the absence of photosynthesis due to the large reservoir represented by PMEs in the primary cell wall⁴⁹ and continued growth processes at night. Thus, ¹³CO₂ photosynthetic fixation during the day allowed for the ¹³C-label to rapidly enter the PME pool (within 5 min), detectable in methanol emissions within 1 h (the initial, naturally ¹³C-depleted signature of PMEs is discussed further in Supplementary Note 3: Large, natural, and universal ¹³C depletion of plant C₁ pools). While the cause of the natural ¹³C-isotopic anomaly in the leaf C₁ pool remains unclear, we propose that the sequential events of natural discrimination against ¹³C during CO₂ diffusion and carboxylation by RuBisCO followed by methionine synthesis⁵⁰ as the last step of the photosynthetic C₁ pathway imparts an extreme ¹³C-depleted carbon isotope signature on the S-methyl group of methionine, and consequently all substrates that receive C₁ carbon during their synthesis or regulation via methyl transfer from AdoMet (see Fig. 6 and Note 3). Thus, the photosynthetic C₁ pathway may be responsible for the large carbon isotope anomaly observed in major leaf C₁ carbon pools⁵¹. However, due to the large pool size of preexisting ¹²C-PMEs at the beginning of the labeling experiments, ¹³C-labeling was still incomplete after 5 d with ¹³C/¹²C-methanol emission ratios reaching up to 80% (¹³C-methanol emissions representing 45% of total methanol emissions). This is in strong contrast to isoprene emissions, a known photosynthetic product that is not stored in plants³⁶. Within minutes with elevated ¹³CO₂, leaf isoprene emissions were dominated by the fully labeled (¹³C₅-isotopologue) isoprene molecular species (Fig. 2).

Relationship between PMEs, methanol production and eco-physiological parameters

We found a high resilience of the ¹³C/¹²C isotope ratio in emitted methanol with respect to time (i.e. rather stable ratio at night) (Fig. 1 and Supplementary Figs. S1–5) and temperature (Supplementary Fig. S9), suggesting that the metabolic pool feeding methanol production (i.e. PMEs on the primary cell wall) was large enough to dampen variations due to new PME synthesis in the light. In fact, after the first day of ¹³CO₂ labeling, methanol emission rates at night were not associated with a strong decline in the isotope ratio in methanol during the night (Fig. 1a and Supplementary Figs. S1–5), as would be expected if methanol emission represented a significant fraction of total active PMEs pool. Moreover, in leaves previously exposed to ¹³CO₂ and placed in a ¹²CO₂ atmosphere, the isotope ratio in methanol remained elevated and stable despite the considerable increase in individual ¹³C- and ¹²C-methanol emission rate with temperature

(Supplementary Fig. S9). The dependence of methanol emission rate on temperature (Supplementary Figs. S9 and S14) is consistent with previous observations of high temperature-sensitivity of foliar methanol emissions in many plant species⁵² as well as direct observations of the temperature sensitivity of PMEs hydrolysis and methanol release in hydrated leaf cell wall extracts⁴⁹. Our results also show that methanol emission occurs at night, likely possible due to high night-time stomatal conductance in *P. trichocarpa* leaves, and enhanced methanol emission starting at around midnight was associated with a recovery in leaf water potential; this was followed by an increase until midday and then reduction of afternoon methanol emission associated with increased leaf water stress (Fig. 1a, Supplementary Figs. S1–S5, S11–S13, and S15, S16).

Importantly, our results suggest that methanol emission is connected to photosynthesis metabolically (via 3-PGA and the phosphorylated serine pathway, see above) but also numerically, with a linear response of ¹³C-content in emitted methanol to cumulated fixed ¹³CO₂ (Fig. S8). This is in contrast with previous studies that suggested little direct connection between photosynthesis and methanol emission, considering the large reservoir of methylated pectin potentially independent of photosynthesis, and continued methanol emissions at night⁴⁹. Here, ¹³CO₂ labeling showed a rapid (within 5 min) metabolic connection between PMEs and CO₂ assimilation (Fig. 3).

Methanol emissions observed in the present study in poplar leaves are reminiscent of another C₁ volatile molecule produced during photosynthesis, methane. Light-dependent methane production and emission from ¹³C fixed photosynthetically have recently been observed in cyanobacteria⁵³. In that study, stable isotope techniques were used to demonstrate methane production during oxygenic photosynthesis in the light directly from ¹³CO₂. In land plants, while metabolic mechanisms are still under investigation, previous studies demonstrated that methane production and emission can be linked to the metabolism of the methyl group of methionine^{54–56}. Given that cyanobacteria also possess the phosphorylated serine pathway⁶, one possibility is that in both land plants and cyanobacteria, methane production is linked to photosynthesis via the ‘photosynthetic C₁’ pathway and light-dependent methionine biosynthesis. Since methane is not readily soluble and thus expected to be liberated, methane emission should be dependent on photosynthetic carbon fixation in the light. Accordingly, a recent study of methane emission by shoots of Scotts pine trees showed a pronounced diurnal cycle that closely followed incident photosynthetically active radiation⁵⁷.

Potential implications of the “photosynthetic C₁” pathway

Since C₁ metabolism and methanol emission was mostly independent of photorespiration and reflected photosynthetic carbon fixation as well as primary cell wall expansion, one may hypothesize that methanol production and emission can be used as an indirect indicator of growth. After midnight, branch emissions of both ¹²C-methanol and ¹³C-methanol under a 21% O₂ atmosphere increased steadily throughout the night (Fig. 1a and Supplementary Figs. S1–5), suggesting an important link to tissue water status (see graphical representation in Supplementary Fig. S17). This is consistent with PME hydrolysis liberating methanol linked to changes in primary cell wall elasticity and growth, which may be downregulated during tissue water stress, possibly via enhanced activity of pectin methyl esterase

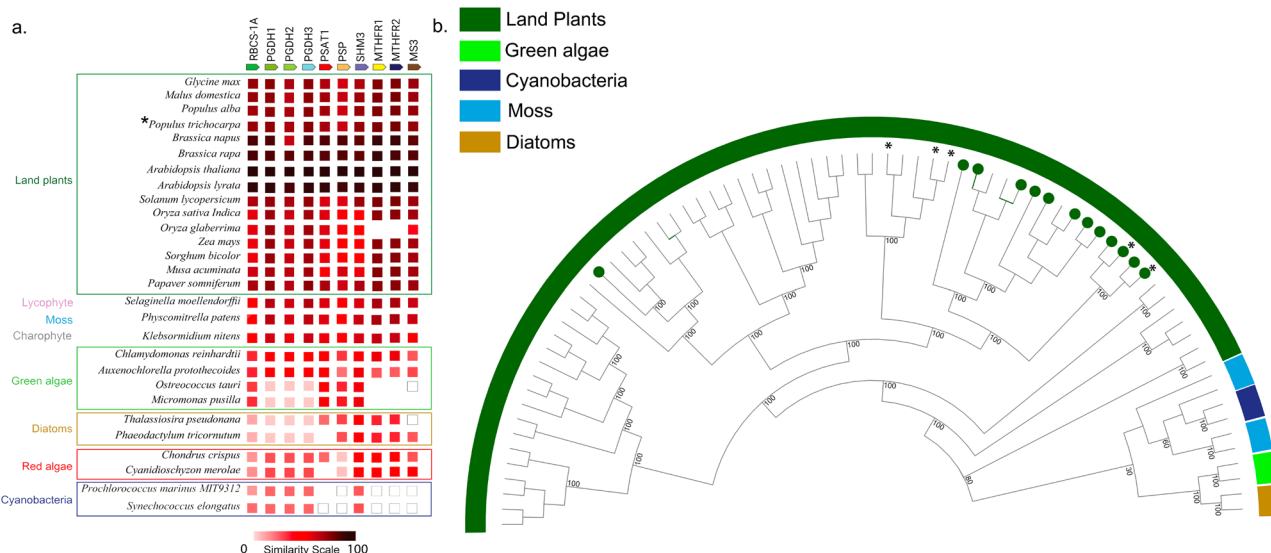


Fig. 7 | Photosynthetic C₁ pathway protein phylogeny and evolution across cyanobacteria and algae to land plants. a A co-occurrence plot for the presence of orthologs for the seven proteins of the C₁ pathway from representative species (see Fig. 6). Similarity between orthologous proteins is represented by color with a color scale ranging from white (low similarity) to black (high similarity) (see scale bar). A white box indicates that the sequence was detected or reported in literature but did not belong to the same orthogroup whereas a lack of a box indicates that the ortholog was not detected. Asterisk highlights *P. trichocarpa*. **b** Phylogenetic tree of

methionine synthase (MS) sequences from representative species. This tree is based on 76 amino acid sequences from 22 species and constructed using the maximum likelihood method (see Supplementary Data 2 for species and sequences). Sequences with predicted chloroplast localization based on transit peptide prediction have a green circle at the edge of the nodes. Asterisk indicates nodes of *P. trichocarpa* MS sequences. Bootstrap values (percentage of 1000 bootstraps) for selected nodes are indicated at the branch point.

inhibitors⁵⁸. In other words, it highlights the potential for methanol emission as a non-invasive chemical tracer of plant water status and growth processes across temporal and spatial scales (this is further discussed in Supplementary Note S4: Methanol emission: a metabolic biomarker of plant physiological status?).

Considering the central role of the ‘photosynthetic C₁’ pathway in synthesis of the methyl donor group of AdoMet, our results raise the question as to whether enzymes of this pathway are well-represented in the green lineage. A comparative genomics approach was used to explore the molecular evolution of the seven genes comprising the ‘photosynthetic C₁’ pathway leading to light-dependent methionine synthesis across photosynthetic lineages. Representative orthogroups for enzymes of the model plant *A. thaliana* C₁ pathway were identified using the STRING-DB⁵⁹. The co-occurrence analysis reveals that, within land plants, proteins in the C₁ pathway are present with high similarity (~60–100%) (Fig. 7a). Beyond land plants, photosynthetic C₁ proteins were present throughout photosynthetic organisms including in a lycophyte, a moss, a charophyte, green algae, diatoms, red algae, and cyanobacteria. Availability of more genomes will improve the understanding of this pathway, especially in lycophytes, mosses and charophytes where only a single species was represented in the database. Intriguingly, some green algae, which are at the base of Viridiplantae (the green lineage) and ancestral to land plants, do not have *methylene tetrahydrofolate reductase* (*MTHFR*) nor *methionine synthase* (*MS*) detected in their genomes, and we confirmed these absences manually (see *Methods*). It suggests that in the green lineage, enzymes of chloroplastic C₁ metabolism may have a shared common ancestor that was lost in the early diverging green algae (e.g. *Ostreococcus*) but was retained in the later diverging chlorophytes (e.g. *Chlamydomonas*). Surprisingly, we also observed a gene fusion event of *serine hydroxymethyltransferase* (*SHMT*) and *MS* sequences in the green alga *Chlorella sorokiniana* suggesting that the two sequences are translated into a single bifunctional polypeptide and act in the same pathway. Many cyanobacterial C₁ proteins had to be extracted from Cyanobase (white boxes) because they did not belong to the same orthogroup as algae and land plants suggesting that the domain was not

conserved. The absence of a protein may be due to improper genome annotation or an incomplete genome (e.g. *MTHFRs* in *Oryza glaberrima*).

Because MS is the key enzyme responsible for generating methionine for the ‘photosynthetic C₁’ pathway (with a large kinetic isotope effect, contributing to explain the natural ¹³C depletion⁵⁰, see Supplementary Notes S3), we performed a phylogenetic analysis of MS from representative species across photosynthetic lineages. MS of land plants form a distinct clade that branch from the basal clade which includes diatoms, green algae, and cyanobacteria (Fig. 7b). Land plants have multiple copies of *MS* and more than half (8 of 15 species) had a copy with predicted chloroplast localization. Since the ‘photosynthetic C₁’ pathway is predicted to occur in the chloroplast, the phylogenetic clustering of most chloroplastic MS sequences may suggest a common, cyanobacterial origin of the ‘photosynthetic C₁’ pathway in land plants. In *A. thaliana*, all proteins of the pathway except *MTHFR* have been found in the chloroplast proteome^{60,61}. Altogether, these results suggest that most enzymes of the ‘photosynthetic C₁’ pathway are found across photosynthetic lineages and that those within Angiosperms have high protein similarity, suggesting a conserved function in generating light-dependent synthesis of methionine.

Conclusions and perspectives

Our results show that C₁ metabolism at the origin of PMEs and methanol depends on RuBisCO-catalyzed carboxylation via the phosphorylated serine pathway. As such, methanol is linked to photosynthesis and partly independent of photorespiration. Considering the value of C₁ metabolism in support of biopolymer and metabolite synthesis and regulation, we presume that the carbon flux through the ‘photosynthetic C₁’ pathway may be significant relative to the rate of carboxylation. Nevertheless, future studies should focus on characterizing this photosynthetic C₁ flux in relation to carboxylation and how it varies with instantaneous environmental variables that impact photosynthesis (i.e. CO₂, light, temperature, soil moisture, etc.) and biological variables such as the maximum carboxylation velocities and growth rates as they vary across plant development and early and late successional species, as well as relationships with gene expression patterns.

Although limited information is available on the expression patterns of the seven genes of the photosynthetic C_1 pathway in leaves, a study of leaf gene expression in natural *P. trichocarpa* trees in the field during the growing season showed that most genes of the photosynthetic C_1 pathway have a ~4X increase during the growth phase (May)⁶². Although we used conditions that promote high rates of photosynthesis while suppressing photorespiration, we were not able to discriminate serine produced from photorespiration versus the phosphorylated serine pathway. Assuming that serine can move freely between organelles, one important area of research is the allocation of serine from photorespiration versus the phosphorylated serine pathway to C_1 metabolism and other major sinks like protein synthesis¹⁵. Given that the SHMT reactions operate in opposite directions in photorespiration (glycine and 5,10-methylene-THF converted to serine) versus C_1 photosynthesis (serine converted to glycine and 5,10-methylene-THF), exchange of substrates and products between these pathways should be investigated. This includes CO_2 and NH_4^+ generated from photorespiration which may be re-assimilated by C_1 photosynthesis linked to the GS/GOGAT cycle in chloroplasts (Fig. 6). Even though photorespiration generates high amounts of serine in plants, serine derived from the phosphorylated serine pathway appears to be more important for plant growth and its deficiency triggers the induction of nitrogen assimilation as an amino acid starvation response¹⁵. This may be because the photosynthetic C_1 pathway is a critical biosynthetic pathway required for the synthesis of numerous biopolymers and metabolites leading to the gain of carbon and nitrogen, whereas photorespiration is largely a recycling pathway for Calvin-Benson cycle intermediates important for abiotic stress signaling (via H_2O_2) associated with the loss of carbon (CO_2) and nitrogen (NH_4^+). It is important to point out that the production of methanol and other C_1 pools via the ‘photosynthetic C_1 ’ pathway proposed here is distinct from the previously described oxidative C_1 pathway^{17,63}. The latter is initiated by methanol oxidation to formaldehyde and formic acid which can then integrate into photorespiration via 5,10-methylene-THF formation⁶⁴. Moreover, formic acid can be oxidized to CO_2 which can be refixed and thus support photosynthesis⁶⁵. It is possible that the synthesis of AdoMet via the ‘photosynthetic C_1 ’ pathway, ammonia assimilation via the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle¹⁵, photorespiration, the release of methanol during cell wall expansion, and methanol recycling mechanisms via the oxidative C_1 pathway, operate concurrently during the day (involving photosynthesis and photorespiration) and/or separately during the night (methanol emissions at night).

Another important question that warrants more research work is the way the ‘photosynthetic C_1 ’ pathway changes (and acclimates) with rising CO_2 mole fraction. Depending on stomatal conductance and photosynthetic capacity, elevated CO_2 tends to enhance photosynthesis and decrease photorespiration. Therefore, the provision of C_1 units including for sulfur assimilation to methionine should decline and perhaps, the ‘photosynthetic C_1 ’ pathway plays an increasingly important role in both carbon and nitrogen assimilation¹⁵. Potentially mediated by methionine phloem loading and transport, we also note that it may have consequences for whole plant growth responses as well as microbial methanotrophy and methylotrophy above ground in the phyllosphere⁶⁶ and below ground in the rhizosphere⁶⁷. It is generally understood that elevated CO_2 tends to suppress biosynthetic pathways like the isoprenoids synthesis in chloroplasts that are unable to compete with the Calvin-Benson cycle for ATP and NADPH^{68,69}. Importantly, the ‘photosynthetic C_1 ’ pathway from RuBisCO-catalyzed carboxylation to methionine synthesis by MS has no requirement for ATP and generates one NADH. Although the MTHFR reaction in the cytosol consumes NADPH, the lack of ATP and NADPH requirements in the chloroplast means that in principle, the flux of methionine synthesis could increase together with the Calvin-Benson cycle as atmospheric CO_2 mole fraction increases. However, it is important to note that ammonia assimilation in chloroplasts via the GO/GOGAT cycle, which directly links with C_1 photosynthesis by providing glutamate for the formation of 3-phosphoserine (3-PSP) while consuming 2-oxyglutarate, requires ATP and NADPH (catalyzed by PSAT, see Fig. 6). Our observations suggest that

the ‘photosynthetic C_1 ’ pathway, which appears to be highly conserved in land plants (Fig. 7) may play a critical, yet poorly understood role in enhancing net primary productivity of C_3 plants as atmospheric CO_2 continues to rise, and therefore might represent a missing link between photosynthesis and growth of natural and managed forests under a changing climate (see Supplementary Video 1, The global photosynthetic C_1 Pathway and plant growth).

It should be kept in mind that while the total flux associated with carbon utilization from 3-PGA to overall C_1 metabolism may be large under elevated CO_2 , it remains uncharacterized. Future research should quantify the carbon flux through the photosynthetic C_1 pathway in relation to rates of RuBisCO carboxylation, carbohydrate, isoprenoid, and fatty acid synthesis, and its impact on 3-PGA and RuBP regeneration. Efforts to improve photosynthesis have shown that overexpression of a plastidic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which dedicates triphosphates to glycolysis, limits the regeneration of RuBP, but slightly increases photosynthesis rates under elevated CO_2 in rice⁷⁰. RuBisCO overexpression has also been considered as method to improve photosynthesis rates, but has generally been shown to not lead to substantial photosynthesis improvement, possibly due to over-accumulation of 3-PGA⁷¹. However, simultaneous overexpression of RuBisCO and the glycolytic enzymes GAPDH and triosephosphate isomerase (TPI) only slightly improved photosynthesis at elevated CO_2 . It was concluded that these modifications did not alleviate over-accumulation of 3-PGA⁷¹. Given its role in both CO_2 assimilation and utilization of 3-PGA, future work could evaluate if the overexpression of the seven enzymes of the ‘photosynthetic C_1 pathway’ under elevated CO_2 leads to more substantial improvements in photosynthesis by more effectively alleviating 3-PGA over-accumulation. Indeed, recent studies have shown that in most eukaryotic algae, RuBisCO is located in a microcompartment known as the pyrenoid in association with CO_2 -concentrations mechanisms that promote photosynthesis over photorespiration⁷². Moreover, the second enzyme of the photosynthetic C_1 pathway, PGDH which catalyzes the commitment step of serine synthesis, is located to puncta directly adjacent to the pyrenoid, likely acting as metabolic channel to enhance serine biosynthesis by capturing 3-PGA exiting the pyrenoid⁷³.

Given that the demonstrated role of the methyl group of methionine in methane production and emission in plants, the photosynthetic C_1 pathway provides a biochemical mechanism for light-dependent production and emission of methane observed from cyanobacteria⁵³ and trees⁵⁷ and may be expanded with additional studies to other C_1 volatiles like methanethiol and dimethyl sulfide⁷⁴. Thus, this resolves the controversy for many years that plants produce methane^{75–77} versus the widespread view that vascular plants do not possess a biochemical pathway to produce methane⁷⁸, but rather only act as a conduit for microbially produced methane in soils to the atmosphere⁷⁹. Thus, the presence of a ‘photosynthetic C_1 pathway’ across the photosynthetic tree of life may have major implications for not only understanding the evolution of oxygenic photosynthesis⁸⁰, but also represents the basis for the development of predictive Earth system models that simulate natural greenhouse gas emissions from marine and terrestrial ecosystems to the atmosphere and their feedback with the climate system.

Methods

Greenhouse plants

We used 15 potted California poplar (*Populus trichocarpa*) saplings (average height of 2 meter) obtained from Plants of the Wild (Washington State, USA) and maintained for three years in the South Greenhouse at the Oxford Tract Experimental Facility in Berkeley, CA, USA, where they were regularly watered and subject to standard pest control practices. Water was delivered to each individual using an automated watering system in 15 gal pots containing Supersoil planting media (Scotts Co., Marysville, Ohio, USA). Ambient natural light was supplemented with LED lighting using an Argus Titan environmental control system with photocell (Argus Controls, British Columbia, Canada). The controller was programmed to turn LED lights off when detecting exterior light levels above $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the 16-h photoperiod (6:00 to 22:00).

Methanol, acetaldehyde, and isoprene ¹³C-labeling analysis by PTR-MS

For all leaf and branch gas exchange experiments, a high sensitivity quadrupole proton transfer reaction- mass spectrometer (PTR-MS with QM422 quadrupole, Balzers, Switzerland) was utilized for real-time flux and stable carbon isotope analysis of methanol, acetaldehyde and isoprene emissions as previously described^{36,42,81}. The PTR-MS was operated with a drift tube voltage of 440 V and pressure of 1.8 mbar. For each measurement cycle lasting 24 s, the following mass to charge (*m/z*) ratios were monitored including *m/z* 21 (H₃¹⁸O⁺), 32 (O₂⁺), *m/z* 37 (H₃O⁺-H₂O), *m/z* 33 (H⁺-¹²C-methanol), *m/z* 34 (H⁺-¹³C-methanol), *m/z* 45 (¹²C₂-acetaldehyde), *m/z* 47 (¹³C₂-acetaldehyde), *m/z* 69 (H⁺-¹²C₅-isoprene), *m/z* 70 (H⁺-¹³C₁-isoprene), *m/z* 71 (H⁺-¹³C₂-isoprene), *m/z* 72 (H⁺-¹³C₃-isoprene), *m/z* 73 (H⁺-¹³C₄-isoprene), and *m/z* 74 (H⁺-¹³C₅-isoprene). Quantification of the methanol, acetaldehyde, and isoprene concentrations was based on dynamic dilution of a primary gas standard (1,000 ppb methanol and isoprene in nitrogen, Restek Corporation). Calibration curves were generated for *m/z* 33 (methanol), *m/z* 45 (acetaldehyde), and *m/z* 69 (isoprene) for 0–45 ppb generated by dynamic dilution of the primary standard (1000 ppb, Restek, Inc.) with hydrocarbon-free air. ¹³C/¹²C-methanol emission ratios were calculated as the ratio of ¹³C-methanol/¹²C-methanol and expressed as a % whereas ¹³C₅-isoprene emissions were expressed as a % of total isoprene emissions (¹²C₅-isoprene + ¹³C₅-isoprene) emissions.

¹³CO₂ leaf labeling under optimal environmental conditions for photosynthesis

In order to study ¹³CO₂ labeling of the putative photosynthetic-C₁ pathway in poplar leaves under constant environmental conditions and short time periods, a portable photosynthesis system (Li6800, Li-COR Biosciences) with a 36 cm² leaf chamber and light source (6800-03, Li-COR Biosciences) was coupled to a high precision cavity ringdown spectrometer (CRDS) for ¹³CO₂ (G2131-i, Picarro Inc.) and quadrupole PTR-MS inside the greenhouse laboratory (see configuration diagram in Supplementary Fig. S18a). Leaves were maintained under constant environmental conditions of 1200 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR), 32 °C leaf temperature, and an air flow rate entering the leaf chamber of 538 ml min⁻¹ maintained with humidity between 4 and 10 mmol mmol⁻¹. A small fraction of air exiting the chamber was diverted to a CRDS for determination of ¹³CO₂ concentrations (25 ml min⁻¹) and a PTR-MS (75 ml min⁻¹) for determination of ¹²C-methanol and ¹³C-methanol leaf emissions. CO₂-free zero air (ultra-zero air, CAS No: 132259-10-0, Linde Gas) was delivered to the Li6800 system after passing through a catalytic converter system (ZA30 catalyst, Aadc instruments, USA) to oxidize any trace volatile organic compounds present. A ¹³CO₂ cylinder (99% ¹³CO₂, CAS No: 1111-72-4, Sigma-Aldrich Inc., USA) was used to supply the internal CO₂ injector using the CO₂ tank adapter kit (part number 9968-109, Li-COR-Biosciences). An elevated ¹³CO₂ concentration in the leaf chamber was maintained between 900 and 1100 ppm ¹³CO₂ (determined by CRDS) throughout each leaf labeling experiment in the light. Net fluxes of ¹³CO₂ photoassimilation (μmol ¹³CO₂ m⁻² s⁻¹) were calculated as a function of time in the leaf chamber as $A(t)$ based on Eq. (1) where μ is the constant air flow rate entering the leaf chamber (400 mol air s⁻¹), $\Delta^{13}\text{CO}_2(t)$ is the difference in ¹³CO₂ mole fraction between leaf chamber and reference air (μmol mol⁻¹) as a function of time, and S is leaf surface area (0.0036 m²) placed inside the chamber.

$$A(t) = \mu \times \Delta^{13}\text{CO}_2(t) \times S^{-1} \quad (1)$$

Leaf ¹³CO₂ labeling studies lasting 1 h ($n = 5$), 2 h ($n = 2$), 3 h ($n = 1$), 4 h ($n = 2$), and 5 h ($n = 2$) were conducted together with ¹³C/¹²C-methanol emission ratios analysis. On the morning of the experiment, poplar branches were detached from one of the 15 potted California poplar trees in the greenhouse in the morning (9:00–11:00) and immediately immersed and recut under water and transferred to the nearby greenhouse laboratory. A selected leaf from the branch was placed inside the leaf chamber. To

minimize branch water loss through transpiration and maintain hydrated leaves, a mylar sheet was placed over the branch outside of the leaf chamber and moist paper towels were placed inside around the branch. This was found to be important for minimizing leaf water stress where high leaf transpiration rates occurred over long periods of time (e.g. up to 5 h). For each leaf ¹³CO₂ labeling experiment, ¹³C/¹²C-methanol emission ratios were plotted versus cumulative ¹³CO₂ photoassimilation (μmol ¹³CO₂ m⁻²). For a single leaf sample (5 h), ¹³C-methanol labeling was verified using TD-GC-MS (see supplementary methods, ‘TD-GC-MS verification of leaf methanol ¹³C-labeling during ¹³CO₂ photosynthesis’, for experimental details).

Diurnal ¹³CO₂ branch labeling of intact potted saplings

Diurnal branch ¹³CO₂ labeling occurred over a 2–5-day period in the plant growth area in the real-time volatile metabolomics laboratory at the Oxford greenhouse facilities in Berkeley, CA (see configuration diagram in Supplementary Fig. S18b). Potted *P. trichocarpa* trees were transported to the nearby laboratory from the greenhouse and placed under a full spectrum grow light (Bestva Diammble Pro 4000 W, USA) approximately 1 m above the top branches of the tree. The light intensity was maintained constant (700–900 μmol m⁻² s⁻¹ of photosynthetically active radiation, depending on the tree) during the light period (6:00–20:00). Tap water was automatically added to the soil every 1 h for 20 s (200 ml) using an automated watering system (smart watering device, Model: Sw-CO3-KA, sPlant Technology Co., Ltd.). A transparent branch chamber (Restek, 10 L ALTEF gas sampling bag, Catalog#: 22962) was continuously supplied with 2.75 l min⁻¹ of CO₂-free zero air (ultra-zero air, CAS No: 132259-10-0, Linde Gas) which first passed through a catalytic converter system (ZA30 catalyst, Aadc instruments, USA) to oxidize any trace volatile organic compounds present in the high purity air. A small flow (3.0 ml min⁻¹) of 99% ¹³CO₂ (Sigma-Aldrich Inc., CAS No:1111-72-4, USA) was mixed into the 2.75 l min⁻¹ dilution flow of zero air just prior to the catalytic converter to generate an expected ¹³CO₂ concentration in the empty branch chamber of 1090 ppm. CO₂ as well as isoprene and methanol concentrations and ¹³C/¹²C ratios were monitored in real-time inside the branch chamber by CRDS and PTR-MS, respectively. While the majority of the air flow entering the chamber passed through the enclosure (2653 ml min⁻¹), a small fraction was diverted through a 1/8” O.D. Teflon PFA tubing to the CRDS (25 ml min⁻¹) and PTR-MS (75 ml min⁻¹). Following the initiation of the ¹³CO₂ flow, background CRDS and PTR-MS data were collected for several hours without a branch inside the enclosure. A branch near the top of the tree canopy was subsequently placed carefully inside the chamber during the night period, and branch gas exchange measurements were initiated. In the light, ¹³CO₂ was drawn down inside the branch chamber between 700 and 900 ppm, depending on the branch studied. The experiment was repeated on five different potted poplar trees.

For all but one diurnal branch ¹³CO₂ labeling experiment, a 21% O₂ atmosphere was used and occurred over 2 days (5 different trees, 1 branch per tree) and 5 days (1 tree, 1 branch). For a single 2-day branch ¹³CO₂ labeling experiment, a 1% O₂ atmosphere was used in order to suppress photorespiration³⁴. During the single 2-day ¹³CO₂ branch label experiment under a 1% O₂ atmosphere, a high precision O₂ cavity ringdown laser spectrometer (G2207-i, Picarro Inc.) was used to both verify the 1% O₂ concentrations in the dynamic branch headspace atmosphere and to demonstrate net O₂ production during photosynthesis in the light.

Verification of leaf methanol ¹³C-labeling during ¹³CO₂ photosynthesis by TD-GC-MS

During branch ¹³CO₂ labeling of potted *P. trichocarpa* plants, ¹³C-labeling of methanol was verified using thermal desorption gas chromatography-mass spectrometry (TD-GC-MS). Briefly, roughly 1.0 l of air sample from inside the dynamic branch enclosure was transferred to an empty Tedlar gas sample bag using a diaphragm pump set to 100 ml min⁻¹. The gas sample bags were connected to the air server-xr system (Markes International, UK) via tubing to allow the TD-GC-MS system to collect and analyze each sample for methanol ¹³C/¹²C analysis. Air samples (25 ml min⁻¹ × 10 min:

0.25 l) were first dried by passing the air sample through the Kori-xr held at -20°C before the methanol was quantitatively pre-concentrated onto the cold trap (Air toxics, Markes International, UK) held at -30°C with the sample flow path maintained at 150°C . Following the sample pre-concentration step, the collected volatiles were injected onto the analytical column, by rapidly heating the cold trap to 280°C for 3 min while back-flushing with carrier gas at a flow of 7.5 ml min^{-1} . In order to improve peak shape and further reduce the amount of water introduced into the GC-MS, 6.0 ml min^{-1} of this flow was vented through the split while the remaining 1.5 ml min^{-1} was directed to the column, temperature programmed with an initial hold at 40°C for 4 min followed by an increase to 70°C at $4^{\circ}\text{C min}^{-1}$. A post run temperature of 230°C was applied for 5 min. The mass spectrometer was configured for trace analysis (SIM Mode and 10X detector gain factor) with 25 ms dwell times for the target ions of methanol (m/z 15, 16, 28, 29, 30, 31, 33). Methanol showed a retention time of 7.8 min, verified using a standard.

^{13}C -labeling analysis of metabolites by LC-MS/MS

For three of the 2-day *P. trichocarpa* branch $^{13}\text{CO}_2$ experiments as well as control branches not exposed to $^{13}\text{CO}_2$, ^{13}C -labeling patterns of leaf metabolites were determined using hydrophilic liquid interaction chromatography and tandem mass spectrometry (LC-MS/MS). Following the $^{13}\text{CO}_2$ labeling period, the leaves were removed from the plant, flash frozen and ground with mortar and pestle under liquid nitrogen, and stored at -80°C . Frozen partially homogenized leaf tissues were freeze-dried (Labconco) overnight and then bead-milled using methanol washed 3.2 mm stainless steel beads in a Mini-Beadbeater-96 (BioSpec Products). Milling was done for a total of 45 s total in 5 s intervals separated by 10 s breaks to prevent overheating of sample; samples were re-cooled every 3 milling intervals. Powderized samples were refrozen and then suspended in 70% methanol with internal standards (see details in Supplementary Data 1) at $5\text{ }\mu\text{l/mg}$ dry tissue. Samples were then vortexed $2\times 10\text{ sec}$, bath sonicated in ice water for 15 min, and centrifuged at $10,000\text{ G}$ (5 min, 10°C); supernatants were $0.22\text{-}\mu\text{m}$ filtered in microcentrifuge filter tubes, and filtrates were transferred to amber glass vials for LC-MS/MS analysis. Metabolites were separated on an Agilent 1290 HPLC equipped with a hydrophilic liquid interaction column and detected with a Thermo QExactive Hybrid Orbitrap Mass Spectrometer; method parameters are described in Supplementary Data 1. Raw files were converted to MZML using ProteoWizard MSConvert 3.0.21265⁸² or ThermoRawFileParser version 1.4.2. Data were evaluated and peak heights for methionine and singly ^{13}C -labeled methionine were extracted using MZmine 2.0⁸³. MS2 spectra were extracted using the RaMS package version 1.3.1⁸⁴ and plotted in base R 4.3.0⁸⁵; vector-based PDFs were generated using Cairo 1.6. From a centroided mzml file, the most intense MS2 spectrum within a 5-ppm window of the precursor m/z for each file was selected and then filtered to remove background intensity fragment ions; a 15-ppm window was used to determine if sample fragment ions matched when comparing MS2 spectra (for example between leaf tissue and standard reference compound). Samples metabolite retention times, mass-to-charge (m/z) ratios and fragmentation spectra were compared with authentic reference standards when available.

^{13}C -labeling analysis of methyl esters from purified leaf cell wall extracts

Following a period of cumulative photosynthesis under a $^{13}\text{CO}_2$ atmosphere with individual leaves under optimal environmental conditions for photosynthesis and branches under diurnal light/dark periods, leaves were detached and immediately flash frozen in liquid nitrogen. For cell wall methyl ester ^{13}C -labeling analysis, two replicate leaves were labeled with $^{13}\text{CO}_2$ for 5 min, 15 min, 30 min, 1 h, and 3 h before flash freezing. In addition, leaves of two intact branches were labeled with $^{13}\text{CO}_2$ for 2 days and 5 days, respectively, before flash freezing all mature leaves (5–8 leaves) on the branch together in liquid nitrogen (leaf samples pooled into a single average branch sample). Two replicate leaves never exposed to the $^{13}\text{CO}_2$ atmosphere were also flash frozen as controls. Whole leaf cell walls were

isolated from the frozen leaf tissues through the generation of alcohol insoluble residues (AIR) as previously described⁸⁶. Briefly, control and $^{13}\text{CO}_2$ -labeled frozen poplar leaves were kept frozen on dry ice and ground with a pestle and mortar before placing one ground frozen leaf (0.2–0.3 g) in a 50 ml Falcon tube with 96% ethanol at 70°C . The Falcon tube was then immediately placed in a 70°C water bath for 60 min before cooling to room temperature. Homogenized tissue was allowed to settle, and the excess solvent was removed by pipetting and $\sim 250\text{ }\mu\text{l}$ of the resulting semi-solid ‘sludge’ was transferred to a 2-ml microcentrifuge tube and washed with 96% ethanol by vortexing for 1 min and centrifuging for 15 min at max speed (10,000–15,000 G). The supernatant was removed and the same solvent washing procedure was performed on all samples using 100% ethanol before washing with methanol:chloroform, 2 v:3 v two times in a shaker held at 4°C for 1 h. Leaf cell wall extracts were then washed with 100%, 65%, 80%, and again 100% ethanol before being dried at room temperature for 4 h using a speedvac to generate the AIR samples. Dried whole cell wall samples (AIR) were then stored at room temperature and shipped to Heidelberg University, Germany for analysis of methyl ester ^{13}C -labeling patterns as described in the methods section below, ‘Methyl ester ^{13}C -labeling analysis of isolated whole leaf cell walls by GC-C-IRMS’.

Methyl ester ^{13}C -labeling analysis of isolated whole leaf cell walls by GC-C-IRMS

Methyl ester ^{13}C -labeling analysis of leaf AIR samples was achieved by conversion to CH_3I using a 57% aqueous solution of hydriodic acid (Acros, Thermo Fisher Scientific, Geel, Belgium) followed by stable carbon isotope ratio analysis of CH_3I with gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) as previously described^{87,88}. Hydriodic acid (0.25 ml) was added to AIR samples (3–5 mg) in crimp-top glass vials (1.5 ml; IVA Analysentechnik, Meerbusch, Germany). The vials were sealed with crimp caps containing PTFE-lined butyl rubber septa (thickness 0.9 mm) and incubated for 30 min at 130°C . After heating, the samples were allowed to equilibrate at room temperature ($22 \pm 0.5^{\circ}\text{C}$) for at least 30 min before 30–50 μl of the headspace were directly injected into the GC using a 100 μl gas-tight syringe (SGE Analytical Science). $\delta^{13}\text{C}$ values of CH_3I were measured using an HP 6890 N gas chromatograph (Agilent, Santa Clara, USA) equipped with an auto sampler A200S (CTC Analytics, Zwingen, Switzerland), coupled to a MAT253 isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via an oxidation reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., with Cu/Ni/Pt wires inside (activated by oxygen), reactor temperature 960°C and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany). The GC was fitted with a Zebtron ZB-5MS capillary column (Phenomenex, Torrance, USA) (30 m \times 0.25 mm i.d., $df = 1\text{ }\mu\text{m}$) and the following GC conditions were employed: split injection (10:1), initial oven temperature at 40°C for 3.8 min, ramp at 50°C/min to 110°C . High-purity helium (5 N) was used as carrier gas at a constant flow of 1.8 ml min^{-1} . A tank of high-purity carbon dioxide (99.995% or N45, Air Liquide, Düsseldorf, Germany) was used as the monitoring gas. All stable carbon isotope ratios of methyl esters are expressed in the conventional ‘delta’ (δ) notation, meaning the relative difference of the isotope ratio of a substance compared to the standard substance Vienna Pee Dee Belemnite (VPDB). The $\delta^{13}\text{C}$ values were calibrated with linear regression using reference materials HUBG1 and HUBG2⁸⁹. Both materials were measured by EA-IRMS and calibrated to the VPDB scale using the reference material IAEA-603 ($+2.474 \pm 0.05\text{‰}$) and an in-house standard (acetanilide; $-30.06 \pm 0.20\text{‰}$), which, in turn, was calibrated against the two reference materials NBS22 ($-30.03 \pm 0.08\text{‰}$) and USGS44 ($-42.21 \pm 0.10\text{‰}$). The calibrated values for HUBG1 and HUBG2 are $-50.21 \pm 0.08\text{‰}$ ($N = 14$) and $+1.61 \pm 0.05\text{‰}$ ($N = 16$), respectively.

Diurnal observations of leaf water potential and leaf and branch gas exchange in field grown *P. trichocarpa* in Berkeley, CA, USA during the 2023 growing season

During three individual days during the growing season (25-April-2023, 19-May-2023, and 30-June-2023) diurnal measurements of leaf water potential

occurred together with leaf gas exchange including CO₂/H₂O fluxes with methanol and isoprene emissions. Leaf water potential (LWP, KPa) was manually measured from one leaf from five different California poplar trees during midnight (23:30 and 00:30), pre-dawn (5:00–6:00), mid-day (11:30–12:30), and afternoon (14:00–15:00) periods. The leaf was removed from the tree by cutting the petiole with scissors as long as possible. After cutting the petiole, LWP was immediately determined using a Scholander pressure chamber with nitrogen (Model 1005 with digital display, PMS instrument company). As soon as water emerged from the petiole top, the nitrogen pressure increase was immediately stopped, and the chamber pressure value (KPa) noted. LWP was recorded as negative value of the determined pressure. During the same three days, diurnal measurements of leaf gas exchange fluxes in the field were collected manually using the small leaf chamber (6 cm²). The Li6800 console was supplied with hydrocarbon free air generated by passing ambient air through a catalytic converter (ZA30-120, Aadco Inc.). Environmental conditions maintained in the leaf chamber throughout the day include constant air flow through the chamber (470 ml min⁻¹), leaf chamber CO₂ concentrations (400 ppm), and photosynthetically active radiation (red: 990 μmol m⁻² s⁻¹, blue 10 μmol m⁻² s⁻¹). Reference humidity was not regulated, and varied slightly based on the lab conditions between 6 and 12 mmol mol⁻¹. A fraction of the air exiting the chamber (75 ml min⁻¹) in the field was diverted to the PTR-MS in the laboratory through 1/8" O.D. Teflon PTFE tubing for continuous analysis of leaf methanol and isoprene concentrations. Leaf temperature was increased by 1 °C for each leaf inside the leaf chamber and ranged from a minimum of 20 °C for measurement at sunrise and up to 38 °C for the afternoon measurement in 30-June-2023. Leaf gas exchange was measured between 6:00 and 17:00 from a single California poplar tree and consisted of setting the leaf temperature without a leaf inside the chamber and collecting background isoprene and methanol concentration data (10 min) before placing a sun-exposed leaf inside the chamber and waiting for leaf gas exchange to stabilize (10 min) and logging net photosynthesis (A_{net} , μmol m⁻² s⁻¹), transpiration (E , mmol m⁻² s⁻¹), and stomatal conductance (mol m⁻² s⁻¹) data together with isoprene and methanol concentration data (ppb) for 15 min. Following this, the leaf was removed and the procedure was repeated on another sunlit leaf on the same tree. Leaf methanol and isoprene concentration changes were calculated by subtracting background concentrations (empty leaf chamber) from concentrations with a leaf in the chamber. Isoprene and methanol fluxes (nmol m⁻² s⁻¹) were calculated from the concentration changes, air flow rate through the chamber, and leaf area (6 cm²).

Branch gas exchange measurements of CO₂, H₂O, methanol, and isoprene fluxes from a single California poplar tree in the field were continuously quantified under ambient environmental conditions of light and temperature during the early (13–24 April), mid (25–31 May), and late (13–28 June) 2023 growing season. A dynamic branch enclosure (5 Liter Tedlar Bag, CEL Scientific Corporation, Los Angeles, CA, USA) was installed around a poplar tree branch together with a second empty Tedlar enclosure installed near the tree as a control. 5.0 l min⁻¹ hydrocarbon free air continuously passed through both branch and control enclosures with ambient levels of CO₂ and H₂O, with trace volatile organic compounds (VOCs) removed by catalytic oxidation of laboratory air (ZA30-120, Aadco Inc.). The enclosure was secured around the stem at the base with air entering the top through the 1/4" valve port and venting at the bottom where the enclosure was secured to the stem (4875 ml min⁻¹), with 125 ml min⁻¹ diverted to two real-time gas sensors for quantification of (1) CO₂ and H₂O branch headspace concentrations (Li7000, Li-Cor Biosciences, Lincoln, NE, USA), (2) methanol and isoprene branch headspace concentrations via high sensitivity quadrupole Proton Transfer Reaction-Mass Spectrometer (PTR-MS, Ionicon, Austria). The Li7000 was operated in absolute mode with the reference cell continuously provided 50 ml min⁻¹ of ultra-high purity nitrogen (5.0 purity, Praxair, Danbury, CT, USA) and designated as 0 ppm CO₂ and 0 mmol mol⁻¹ H₂O while 50 ml min⁻¹ of air from inside the branch enclosure was directed to the Li7000 sample cell with average CO₂ and H₂O concentrations recorded every min. Using an automated solenoid

valve, concentration measurements were made every hour of the empty branch enclosure (20 min) and the enclosure with the poplar branch inside (20 min). CO₂, H₂O, isoprene, and methanol fluxes each hour were determined by the difference in concentrations between the dynamic branch enclosure and empty chamber, the air flow through the chambers, and the enclosed leaf area, estimated by tracing the leaves paper and using the determined ratio of the paper area/weight ratio.

Co-occurrence analysis

We collected the protein sequences from Phytozome V13⁹⁰ for the following *A. thaliana* enzymes ATRBCS1A (RuBisCo small subunit), AtPGDH1, AtPGDH2, AtPGDH3, AtPSAT1, AtPSP, AtSHMT3, AtMTHFR1/2, and AtMS3 (Supplementary Data 2). All the genes except the *AtMTHFR* have a known/predicted chloroplast transit peptide and have proteomic experimental evidence confirming chloroplast localization^{60,61}. However, it should be noted that depending on specific gene expression patterns, proteins could be plastid-localized in heterotrophic cells and/or chloroplast-localized in autotrophic cells. Consistent with the 60-amino acid leader sequence acting as a transit peptide for transportation of *A. thaliana* PGDH to plastids⁹¹, all three proteins (AtPGDH1, AtPGDH2, AtPGDH3) were found to be present in the *A. thaliana* chloroplast proteome⁶¹ and are therefore included in the co-occurrence analysis. Using STRING-DB V12.0 (Search Tool for Retrieval of Interacting Genes/Proteins DataBase)⁵⁹, orthogroups of proteins of the above-listed genes in the C₁ pathway were identified across all organisms available in STRING-DB. The STRING-DB classified orthologous sequences into specific orthogroups and proteins from other species were extracted based on their specific groups. STRING-DB uses genomes available in the NCBI database and has significant representation of angiosperm genomes; however, only a single lycophyte, moss, and charophyte species was currently present in the STRING-DB. Based on their similarity score, a co-occurrence figure was made for selected species. For each protein, all sequences were manually confirmed to belong to a specific orthogroup. Protein absences of PSAT1, MTHFR, and MS in green algae, diatoms and red algae were confirmed manually using BLAST of *A. thaliana* proteins in Phycocosm⁹². Absences of cyanobacterial proteins were validated in Cyanobase⁹³ and sequences for present enzymes were extracted.

Phylogenetic analysis

To construct a phylogenetic tree, all protein sequences from the methionine synthase (MS) orthogroup were extracted from the STRING-DB⁵⁹ and manually curated to filter sequences specific to two diatoms, two green algae, one moss and 15 land plants (dicots and monocots). Two cyanobacterial MS sequences were extracted from Cyanobase⁹³. In total, 76 sequences from 22 species were used to construct the tree (see Supplementary Data 2). A multiple sequence alignment (MSA) of the selected protein sequences was performed using the `msa()` function⁹⁴ with the ClustalW method in R version 4.2.2. A maximum likelihood (ML) tree was generated using the `phangorn` package in R⁹⁵. Based on the MSA, various models were tested for the estimation of likelihood to the provided sequences with the `modelTest()` function and the best available model was selected to construct a ML tree using the `pml_bb()` function. To improve confidence, the tree was bootstrapped a 1000 times using the default ultrafast bootstrapping in the `phangorn()` package. The tree was plotted using the `ggtree` package in R⁹⁶. The sequences were checked for localization within the chloroplast by investigating the presence of transit peptide in TargetP⁹⁷ and prediction of plastid localization using DeepLoc2.0 (0.9 cutoff)⁹⁸. Sequences with either transit peptides or predicted plastid localization were manually annotated on the tree.

Field experiment

A field experiment was carried out throughout the 2023 growing season to explore the seasonal pattern in diurnal growth, methanol emissions, transpiration, and temperature in an established field plot adjacent to the

analytical trace gas laboratory. Detailed protocols used for 'Diurnal observations of leaf water potential and leaf and branch gas exchange' can be found in the supplementary information.

Field plants

Twenty California poplar saplings (*Populus trichocarpa*) were planted on May 24, 2021 in the Oxford Tract Experimental Facility field site. California poplar saplings were obtained from Plants of the Wild, WA, USA. Automated watering was configured during the growing season (April–December), such that 0.5 gal h⁻¹ was delivered to each tree for 1 h each day at 8:00. However, during the warm summer months (June through October) watering duration increased to 1.5–3 h in order to maintain relatively constant soil moisture. Prior to the commencement of experimentation in April 2023, the trees were grown in the field for 689 days (1 year, 10 months, and 20 days) yielding a height of 2.0–2.6 m.

Environmental monitoring of air temperature, humidity, and sap velocity

Starting on 31-Jan-2023 in the poplar tree field site, air temperature was continuously recorded at 1.5–2.0 m above the ground every 5 min using type-T thermocouples and a temperature data logger (OM-CP-OCTTC-TEMP-A2, Omega Engineering). Moisture of the soil (0–50%) was determined weekly throughout the field experiment manually for each tree using a Extech MO750 Soil Moisture Meter with Integrated 8" Heavy Duty Probe inserted 10 cm into the soil. In addition, ambient air relative humidity and temperature were continuously recorded every 10 min at 1.0 m height above the ground with a HOBO MX2300 Series Temp, RH data logger in the center of the field plot adjacent to the ambient air temperature thermocouple. Sap velocity was continuously monitored at 50 cm height on the main stem of one California poplar (CP02) using the heat ratio method (SFM1 sap flow meter, ICT International). The SFM1 sensor was configured as previously described³⁹. Briefly, heat pulses (30 J) were programmed every 15 min and stem sap velocity (sapwood) was recorded.

Software and Code

Commercial software used to control and collect data from the following instruments were used as follows;

- Online volatile organic compound concentrations were collected the commercial PTR-MS software for quadrupole mass spectrometers (PTR-MS Control software, Ionicon Analytic)
- Online ¹²CO₂, ¹³CO₂ (isotopic CRDS) and high precision O₂ CRDS concentrations data were collected using the commercial Picarro Surveyor software (Picarro Inc.)
- Online leaf photosynthesis, transpiration, and stomatal conductance data as well as controlled environmental variables were collected using Bluestem OS. Version 2.1.13 on a Li6800 portable photosynthesis system (Licor, Inc.)
- Branch level fluxes of leaf photosynthesis and transpiration were acquired using a Li7000 under computer control using the Li-7000 Control software (Licor, Inc.)
- Extracted leaf metabolite data using LC-MS/MS were collected using Thermo Xcalibur software.
- Verification of leaf methanol ¹³C-labeling by GC-MS was performed using Masshunter version 11.1 (Agilent)
- Data for analysis of methyl ester ¹³C-labeling patterns of isolated whole leaf cell walls by GC-C-IRMS was collected using Isodat (Thermo)
- STRING-DB V12.0 (Search Tool for Retrieval of Interacting Genes/Proteins DataBase) was used to identify orthogroups of proteins of the seven genes in the C₁ pathway were identified across all organisms available in STRING-DB.
- Phylogenetic tree construction: Protein sequences from the methionine synthase (MS) orthogroups were extracted from the STRING-DB and manually curated to filter sequences specific to two diatoms, two green algae, one moss and 15 land plants (dicots and monocots). Two cyanobacterial MS

sequences were extracted from Cyanobase. In total, 76 sequences from 22 species were used to construct the tree (see Supplementary Data 2).

- Environmental data (air temperature) during field studies were collected using Omega Data Logger Software (ODLS): A software platform for configuring, downloading, and analyzing data from the OM-CP-OCTTC-TEMP-A2 thermocouple data logger.

- Sap flow data was configured and downloaded from SFM1 sap flow sensors using Sap Flow Tool version 3.1.4 (ICT International).

Data analysis

- Leaf and branch gas exchange time series data were analyzed using Igor Pro software (Version 8.0).
- Leaf metabolite analysis: Raw LC-MS files were converted to MZML format using either ProteoWizard MSConvert (Version 3.0.21265) or ThermoRawFileParser (Version 1.4.2). Data were evaluated, and peak heights for methionine and singly ¹³C-labeled methionine were extracted using MZmine (Version 2.083). MS2 spectra were extracted using the RaMS package (Version 1.3.184) and plotted in base R (Version 4.3.0). Vector-based PDFs were generated using the Cairo package (Version 1.6).
- Statistical analysis of leaf metabolites (LC-MS/MS) was performed using R (Version 4.3.0).
- Phylogenetic tree construction for methionine synthase (MS): A multiple sequence alignment (MSA) of the selected protein sequences was performed using the `msa()` function with the ClustalW method in R (Version 4.2.2). A maximum likelihood (ML) tree was generated using the `phangorn` package. Various models were tested for likelihood estimation using the `modelTest()` function, and the best-fitting model was selected to construct the ML tree using the `pml_bb()` function. The tree was bootstrapped 1000 times to improve confidence using the ultrafast bootstrapping default in `phangorn`. The tree was plotted using the `ggtree` package in R. Protein sequences were further examined for chloroplast localization by identifying transit peptides with TargetP and plastid localization predictions using DeepLoc2.0 (0.9 cutoff). Sequences with either transit peptides or predicted plastid localization were manually annotated on the tree.

Statistics and Reproducibility

- For all leaf and branch experiments involving replicates, biological replicates were defined as independent samples derived from different individuals or experimental units (e.g., separate leaves or branches on a tree). All replicate studies were successful, and no data were excluded from the field study. Reproducibility was confirmed by performing independent repeats of experiments on different days under identical conditions or in the field by performing repeat experiments at different times throughout the growing season.
- 15 potted poplar trees were used for leaf (1–5 h) and branch (2–5 day) ¹³CO₂ labeling under conditions which promoted high rates of net photosynthesis while suppressing photorespiration. The group of 15 potted poplar trees grown in the greenhouse and used for leaf and branch ¹³CO₂ labeling studies were randomly selected for either leaf gas exchange and cell wall studies (detached branches) or branch gas exchange (intact branches) coupled with leaf metabolite analysis. During the branch gas exchange studies, a random potted tree was selected and moved into the analytical laboratory and placed under an LED grow light and automated watering system (and allowed to acclimate for several days). The branch selected for continuous 2–5 day gas exchange studies during ¹³CO₂ labeling was also randomly selected, but was required to be in the upper canopy so that it was not shaded by branches above it. All branches studied had a mix of young light green immature leaves (near the end of the branch) and darker green maturing leaves.

- Leaf ^{13}C labeling studies under constant environmental conditions for short time periods (up to 5 h) promoting high rates of photosynthesis lasting 1 h ($n = 5$), 2 h ($n = 2$), 3 h ($n = 1$), 4 h ($n = 2$), and 5 h ($n = 2$) were conducted together with online ^{13}C assimilation quantification and $^{13}\text{C}/^{12}\text{C}$ -methanol emission ratios analysis. In addition, short term (0, 5, 15, 30, 60, and 180 min) ^{13}C leaf labeling (replicated 2x for each time point) was conducted followed by flash freezing leaves at the end of the experiment for bulk cell wall pectin methyl ester ^{13}C -analysis. For cell wall methyl ester ^{13}C -labeling analysis, two replicate leaves ($n = 2$) were labeled with ^{13}C for 5 min, 15 min, 30 min, 1 h, and 3 h before flash freezing. In addition, following ^{13}C branch labeling for 2 days and 5 days, mature leaves (5–8 leaves) on each of the branches were flash frozen in liquid nitrogen for PME ^{13}C -labeling.
- Branch ^{13}C labeling studies were performed under diurnal light/dark cycles and 21% O_2 over 2 days with online ^{13}C assimilation quantification and $^{13}\text{C}/^{12}\text{C}$ -methanol emission ratios analysis and flash frozen at the end of the experiment for bulk cell wall pectin methyl ester and leaf metabolomics ^{13}C -analysis. A branch ^{13}C labeling study with gas exchange and leaf PME ^{13}C -labeling analysis was conducted over 5 days ($n = 1$) as well as under a 1% O_2 atmosphere over 2 days ($n = 1$).
- For leaf metabolomics ^{13}C -labeling analysis of PGA, serine, and methionine were compared between control leaves exposed to a natural abundance ^{12}C atmosphere ($n = 10$), and leaves exposed to ^{13}C over two diurnal cycles ($n = 12$). One tailed t test ($P < 0.001$) was used for comparisons between $^{13}\text{C}/^{12}\text{C}$ leaf metabolite labeling patterns among the ^{12}C and ^{13}C treated leaf samples and all statistical analyses of leaf metabolites.
- A population of 20 field grown poplar trees was used to study the temperature and hydraulic effects of methanol emissions. One tree was randomly selected for 1–2 week continuous branch gas exchange studies during April, June, and July 2023 as well as a single-day experiment at the leaf level on a separate individual to quantify sap velocity and leaf gas exchange together with leaf water potential (from 10 trees) at midnight, predawn, mid-day, and early afternoon.
- For the co-occurrence analysis of proteins of the photosynthetic C_1 pathway and phylogenetic analysis of methionine synthase across the photosynthetic tree of life, all orthogroups of proteins of the listed genes in the C_1 pathway were identified across all organisms available in STRING-DB.
- To construct a phylogenetic tree, all protein sequences from the methionine synthase (MS) orthogroup were extracted from the STRING-DB and manually curated to filter sequences specific to two diatoms, two green algae, one moss and 15 land plants (dicots and monocots). Two cyanobacterial MS sequences were extracted from Cyanobase. In total, 76 sequences from 22 species were used to construct the tree.

Sampling strategy

For all leaf and branch experiments involving replicates, biological replicates were defined as independent samples derived from different individuals or experimental units (e.g., separate leaves or branches on a tree). All replicate studies were successful, and no data were excluded from the field study. Reproducibility was confirmed by conducting independent repeats of experiments on different days under identical conditions or in the field at different times throughout the growing season.

- For each of the leaf and branch ^{13}C labeling experiments, one out of 15 potted trees maintained in the greenhouse was randomly selected for study. These 15 trees were grown in the greenhouse and randomly chosen for either leaf gas exchange and cell wall studies (detached sun-exposed branches in the upper canopy) or branch gas exchange (intact branches) coupled with leaf metabolite analysis. During the branch gas exchange studies, a random potted tree was selected, moved into the analytical laboratory, and placed under an LED grow light and

automated watering system (allowed to acclimate for several days). The branch selected for continuous 2–5 day gas exchange studies during ^{13}C labeling was also randomly selected but required to be in the upper canopy to avoid shading. All branches studied contained a mix of immature light-green leaves near the tip and more mature darker green leaves. All replicate leaf and branch ^{13}C labeling experiments showed a strict light-dependence of ^{13}C -labeling of pectin methyl esters in primary cell walls, leaf methanol emissions, and targeted leaf metabolites.

- Leaf ^{13}C labeling studies under constant environmental conditions for short time periods promoting high rates of photosynthesis lasting 1 h ($n = 5$), 2 h ($n = 2$), 3 h ($n = 1$), 4 h ($n = 2$), and 5 h ($n = 2$) were conducted together with online ^{13}C assimilation quantification and $^{13}\text{C}/^{12}\text{C}$ -methanol emission ratios analysis. All leaf samples showed a very tight linear correlations ($r^2 > 0.97$) between cumulative ^{13}C photoassimilation and the instantaneous $^{13}\text{C}/^{12}\text{C}$ -methanol emission ratio.
- For cell wall methyl ester ^{13}C -labeling analysis, leaves were labeled with ^{13}C for 5 min, 15 min, 30 min, 1 h, and 3 h under optimal conditions for net photosynthesis before flash freezing (replicated 2X per time point). In addition, leaves of two intact branches were labeled with ^{13}C for 2 days and 5 days, respectively, before flash freezing all mature leaves (5–8 leaves) on the branch together in liquid nitrogen (leaf samples pooled into a single average branch sample). Leaves on branches never exposed to the ^{13}C atmosphere were also flash frozen as controls for bulk cell wall pectin methyl ester ^{13}C -analysis ($n = 8$). All leaf samples showed a tight linear correlation ($r^2 > 0.97$) between $\delta^{13}\text{C}$ of leaf PMEs and light period duration.
- Long-term branch ^{13}C labeling studies (2 days) under diurnal light/dark cycles were repeated five times using randomly selected potted poplar trees. These experiments occurred under 21% O_2 , with conditions favoring photosynthesis over photorespiration (i.e. moderate light and temperature, high soil moisture, and elevated ^{13}C). RuBisCO oxygenation (V_o) was estimated to be less than 5% of carboxylation (V_c) under these conditions. Each replicate showed similar light-dependent ^{13}C -labeling patterns of methanol emissions, bulk leaf cell wall PMEs, as well as targeted leaf metabolites. To assess extended labeling, a single 5-day branch ^{13}C labeling study was performed on a randomly selected potted tree.
- A 2-day branch ^{13}C -labeling study under 1% O_2 was conducted to effectively eliminate photorespiration ($\text{V}_o < 0.5\% \text{V}_c$). Branch headspace was verified to contain 1% O_2 , and net O_2 production was confirmed using CRDS during the light period. Light-dependent ^{13}C -labeling patterns and methanol emissions, as well as ^{13}C -labeling of bulk leaf cell wall pectin methyl esters, were only slightly reduced relative to branches exposed to 21% O_2 . This slight decrease was attributed to reduced photosynthesis under 1% O_2 and the activation of fermentation metabolism.
- In the field experiment, 1–2 weeks of continuous branch gas exchange studies were conducted on a single randomly selected tree. These branch gas exchange studies were repeated in April, June, and July 2023 ($n = 3$). When branch data from each period were combined, a clear exponential dependence of methanol emissions on temperature was observed. During April and June 2023, a second tree was randomly selected for high temporal resolution observations of leaf gas exchange and sap velocity, with leaf water potential measurements taken across 10 individuals at midnight, predawn, mid-day, and early afternoon.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data needed to evaluate the conclusions in this paper including the source data of every figure and Supplementary Fig. is available for

download as a supplementary file. All raw LC-MS/MS data For LC-MS data used in Fig. 4 and supplementary Fig. S10 are available in the MassIVE repository (<https://massive.ucsd.edu/ProteomeSAFe/dataset.jsp?task=aabec0a91ce449e58e843e60ff3e5021>). Supplementary Data 1 (.xlsx file): Method details of the leaf methionine analysis from frozen leaf extracts separated using hydrophilic liquid interaction chromatography. Supplementary Data 2 (.xlsx file): 76 protein sequences from 22 species used to construct the phylogenetic tree shown in Fig. 7 with protein ID, Genus and species, and plant category. Supplementary Data 3 (.zip file): Data set for all data-based figures including Fig. 1a, Fig. 1b, Fig. 2, Fig. 3, and supplementary Figs. S1, S2, S3, S4, S5, S6, S7, S8, S9, S11, S12, S13, S14, S15, and S16.

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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