nature portfolio

Peer Review File

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

Corresponding Author: Dr Kolby Jardine

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript could provide valuable information on the networks that connect the phosphorylated pathway of serine biosynthesis (most recently agreed to be named as phosphorylated and not phosphate pathway, in fact, authors named it once as “phosphorylated” and then changed to “phosphate”???) with photosynthesis and growth via methyl transferase reactions. The association of the phosphorylation pathway with growth has already been suggested by some authors working in the field (C3 plants increased their growth under high CO2 conditions even when the serine supply by photorespiration is severely restricted), although the responsible mechanism has not been fully investigated. The authors propose a new mechanism to explain the connecting links between the phosphorylated pathway and growth based on C labelling studies only, although no molecular or genetic evidence is provided.
Overall, the manuscript is well written and the supplemental animation is excellent. However, it has significant shortcomings in the introduction, and some references are missing or incorrectly cited. For instance, the authors state that little is known about the in vivo activity of the phosphorylated pathway. The most important literature in the field is missing in the introductions, particularly papers with mutants showing the effect of lack of the phosphorylated pathway activity on growth and development (see Benstein et al. 2013, 25: 5011–5029, Cascales-Miñana et al. Plant Cell 2013, 25: 2084–210, Toujani et al. Plant Physiology 2013, 163: 1164–1178; Rosa-Tellez et al. 2024, Plant Cell 36: 404–426), Additionally, some information provided in the introduction (and also in Figure 5 and S17) is incorrect: the SHMTs are not part of the phosphorylated pathway. On the contrary, the SHMT activity referred to in reference number 9 (which is a review and not an original paper) is SHMT1, which is the mitochondrial photorespiratory isoform. There are also other erroneous concepts along the text and figures, such as the assertion that MTHR is chloroplast localized. To my knowledge, in Arabidopsis and other species investigated, it is only localized in the cytosol (indeed the authors state in the methods section that the MTHFR has no chloroplast transit peptide). All these missing information and mistakes should be corrected before publication.
I agree with the authors that the importance of the serine phosphorylated pathway for growth and development remains unclear. The results from the manuscript will provide new insights in the field. Their main hypothesis linking the phosphorylated pathway with growth is that the Ado-Met (not measured here) required to methylation reactions comes directly from the phosphorylated pathway and from the Calvin-Benson cycle. To demonstrate this, the authors used a series of C labelling studies. They showed that C1 metabolism producing methanol is slightly dependent on photorespiration and comes directly from photosynthesis. They also showed that pectin methyl esters are synthesized in a photorespiration-independent manner. Then they hypothesized that the missing link between photosynthesis and C1 metabolism should be the phosphorylated pathway.
Several recent reports using metabolic flux analysis by isotopic labelling, modelling, and gas exchange measurements in wild-type plants under changing photorespiratory conditions indicated that a considerable amount of Gly and Ser could go out of the photorespiratory cycle (see Abadie et al., 2016, Nature Plants 2:15220 ; Busch et al., 2018, Nature Plants 4:46-54, Fu et al. 2023 Nat Plants 9: 169–178 and others) and that the photorespiratory pathway could importantly contribute to C1 metabolism (Rosa-Téllez et al 2024, Plant Cell 36: 404–426). It is also commonly accepted, although not necessarily true, that the phosphorylated pathway is most active at night, in non-photosynthetic organs versus photosynthetic organs, and in heterotrophic cells vs autotrophic cells. Many of the enzymes included in the author’s model are said to be chloroplastically located. However, having a transit peptide (as stated in methods) does not assure that a protein is chloroplast-localized. Depending on the specific expression pattern, it could be plastid-localized in a heterotrophic cell or chloroplast-localized in an autotrophic cell, or both, depending on the cell type. With all this important information in mind, a general discussion with biological meaning to be integrated into the manuscript would be required. For instance, why would the serine supplied by the glycolate pathway miss the proposed growth connections for this amino acid? Is it because it is produced in the

mitochondria? Serine is supposed to move freely between organellar compartments…How would the plant cell discriminate between the two sources of serine? Regarding this, Fig. 4 is very difficult to understand. This is why I am asking if the experiments described in this figure where only conducted under 21% O2? Since no 2-phosphoglycerate could be differentiated from 3-PGA, could the authors explain in detail, for a broader audience, how they were able to discriminate between photorespiratory serine and serine produced by the phosphorylated pathway?
Other comments.
What about the phosphorylated pathway in poplar? Is there any useful information in the genome databases regarding the number of genes and their expression patterns, how active it is, etc? Why was PGDH2 selected in Figure 6 instead of PGDH1, which is the essential gene at least in Arabidopsis where the phosphorylated pathway has been fully studied? Is it PGDH2 the PGDH1 Arabidopsis orthologue ?
Also some spelling mistakes such as "phosphogyceric" in figure 4 or "inccroporation" in line 191 should be corrected.

Reviewer #2

(Remarks to the Author)
This intriguing manuscript by Jardine et al. argues for a tight coupling between C1 metabolism and Calvin Cycle carbon reduction in plants. They furthermore argue that this connection provides the mechanistic underpinning for the relationship between methanol production/emission and growth. To my mind they do an excellent job of demonstrating the connection between C1 metabolism and the Calvin Cycle, rather than the more typically assumed C1-phoptorespiration link. Their data arguing for this novel connection more than justify the eventual publication of this paper in a high profile journal such as Communications Biology.

That said, there are minor elements of the paper that could be clearer and/or do not seem central to the primary exciting and novel result, and I think the paper could have a higher impact on the scientific community if they were clarified or excised. First of all, the link to global GPP seems non-essential and distracting. GPP since 1850 has risen for a variety of reasons, not only rising CO2. Indeed global changes in biomass and nitrogen (thanks to the Allies trying to starve the Germans in WWI) are arguably just as important. And the significance of agricultural advances beyond Haber Bosch cannot be ignored. I do not think the authors need this bit.
Second, the phenomenological connection between methanol production/emission and growth has been around for a while, and the link to PME and cell wall expansion tested by others such as Harley et al. and Oikawa et al. The highlight of the current ms. lies with excellent evidentiary basis presented for linking an early Calvin Cycle product to growth in a way takes into account water potential's control on growth. This integration of biochemistry and physiology is wonderful to see.
Third, and building from point two, Jardine knows (and as they allude to on page 8) as well as anyone in the world the complex relationship between stomatal conductance and methanol emission that arises out of the combination of methanol's low concentration relative to saturation and its high solubility in water. While I am sure that this relationship is accounted for in the author's production/emission calculations, seeing actual data on this point would make the paper easier to understand for many readers. Indeed, a paragraph contrasting water, methanol, and isoprene with respect to stomatal control over emissions would be very helpful (perhaps with the space saved by cutting much or all of the GPP discussion).
Finally, while I enjoy a good speculative handwave as much as the next person, the ending of the paper should keep the focus on the novel biochemistry elucidated and the links to plant hydraulics. I was unpersuaded by the globalchangish closing of the paper and don't think it necessary.

In short, this is one of the most interesting plant metabolism papers to have come across my desk in quite a while, and I think it highly suitable for Communications Biology. My suggestions above are offered as criticisms to heighten the paper's impacts and to help emphasize its novelty and importance.

Reviewer #3

(Remarks to the Author)
This paper explores the origin of 1-C groups in photosynthetic tissues, a topical and important subject. The main conclusion, however, that most these1-C units in methionine come from serine not derived from photorespiration, is not supported from the presented data (As outlined below).

The use of different CO2 concentrations could be better explained and seem to undermine the main premise of many of the experiments and the conclusions of the paper. For example, why was 900 PPM used in the case of diurnal labeling, but 750 ppm used for instantaneous leaf 13C methanol experiments? These high concentrations would also effectively limit much photorespiration by ~50% based on a rough estimate. This is especially problematic when enrichment of PME's was compared between 1000 PPM 13CO2 and 21% O2 and (presumably) 1000 PPM 13CO2 and 2% O2. This comparison was used to indicate that there was little photorespiratory-related contribution to PME's but under both conditions, photorespiration would be very minimal. Interestingly, there was a decrease in labeling under 2% O2, it would be interesting to calculate by how much photorespiration was decreased under these particular conditions since I would surmise it was pretty minimal as well. It is unfortunate that ambient CO2 concentrations were not used, where there would be a greater difference in photorespiration between 21% and 2% O2. The results do support the statement in line 175 that PME's come at least partly from non-photorespiratory carbon, but do not support the clam that the photosynthetic C1 pathway, rather than photorespiration, leads to the generation of light-dependent methionine under typical photorespiratory conditions.

What was the CO_2 concentration for the LC-MS/MS analysis of PGA, Serine, and methionine? Was this approach able to also pickup phosphoglycolate? It was not clear how the sulfur-carbon specific labeling of methionine supported the hypothesis that methionine synthesis was independent of photorespiration.

The presentation of the results, relative to the stated hypothesis, were unfocused. For example, how does the temperature response of 13C-methanol emission elucidate the source of carbon for methionine biosynthesis? Similarly, it was not clear what the temperature response of the in situ methanol emission rate added. Perhaps these could be grouped in a "temperature response" section to make their relevance clearer. The inclusion of this data was not tied well to the core hypothesis presented in the abstract and in the current state, served to distract from the core argument presented.

I appreciate the care that went into the graphical abstract 1 and video. This type of work is a great way to better communicate research findings to other audiences. This is even though I think the main conclusion is not supported entirely by the evidence.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In my opinion, the revised version of the manuscript has been substantially improved. Major concerns have been addressed, and the appropriate changes made where necessary.
Minor points:
• Although the authors stated that the name of the "Phosphorylated pathway" was changed throughout the manuscript, they still refer to the "Phosphorylated pathway" as the "Phosphate pathway" on page 2, line 60.
• Line 679: To my knowledge, the Arabidopsis PGDH1 and PGDH3 do have a transit peptide. Please confirm this.
• Spelling mistakes. Line 104: There is a spelling mistake—"Phosphoglyceric" should be used instead of "phosphogyceric."

Reviewer #3

(Remarks to the Author)
I appreciate the care to address my concerns about the elevated CO_2 conditions and in the time to help me (and future readers) get the point that the study is addressing most directly C1 metabolism in a reduced photorespiratory future and not necessarily what is currently the predominant source of 1C units under our more photorespiratory conditions. I still would like to see some clearer statements in the manuscript outlining this caveat since it is important.

For example, while there is a large difference in the photorespiratory conditions of 21 vs 1% O_2 under elevated CO_2 (vo less than 5% under 21% O_2 and then below 0.5% under 1% O_2), the 21% O_2 condition is still ~20% of ambient conditions, which were not measured. I think a clear statement in the discussion is needed that indicates that the predicted future conditions measured were very different from ambient conditions and can't be used to argue that under present conditions, photorespiratory C1 carbon flux is not a large source of C1 units. Of course the core conclusion of the paper that C1 flux can come from non-photorespiratory sources is still valid and interesting.

Minor:
Line 284: The papers cited here suggest that 20-30% of photorespiratory carbon is exported from the photorespiratory carbon, which is not really a "small" fraction. These numbers may be high but consider dropping "small" from this sentence since the papers cited suggest it is large.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
Thanks for considering my thoughts, I have no further concerns. - Berkley Walker

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29-July-2024
Manuscript title: "The 'Photosynthetic C_1 pathway' links carbon assimilation and growth in plants"
Point-by-point response to referees 1, 2, and 3

Referee #1 Comments and Responses

Referee #1: an expert in plant physiology, focusing on serine biosynthesis, plastidial glycolysis, and stress responses in Arabidopsis, tomato, and sugar beet.

Referee #1 Comment 1
This manuscript could provide valuable information on the networks that connect the phosphorylated pathway of serine biosynthesis (most recently agreed to be named as phosphorylated and not phosphate pathway, in fact, authors named it once as "phosphorylated" and then changed to "phosphate"???) with photosynthesis and growth via methyl transferase reactions. The association of the phosphorylation pathway with growth has already been suggested by some authors working in the field (C3 plants increased their growth under high CO2 conditions even when the serine supply by photorespiration is severely restricted), although the responsible mechanism has not been fully investigated. The authors propose a new mechanism to explain the connecting links between the phosphorylated pathway and growth based on C labelling studies only, although no molecular or genetic evidence is provided. Overall, the manuscript is well written and the supplemental animation is excellent.
Response 1: Many thanks to referee 1 for contributing their time and expertise in plant physiology, serine metabolism, and stress responses to the review of our manuscript. Throughout the main manuscript and supporting information document, we now refer to the most recently agreed name of "phosphorylated serine pathway" throughout the manuscript. We thank referee 1 stating that the manuscript is well written and for recognizing that the mechanisms presented here could explain recent observations cited in the article that C3 plants increased their growth under high CO_2 even when serine supply from photorespiration is severely restricted.

Referee #1 Comment 2
However, it has significant shortcomings in the introduction, and some references are missing or incorrectly cited. For instance, the authors state that little is known about the in vivo activity of the phosphorylated pathway. The most important literature in the field is missing in the introductions, particularly papers with mutants showing the effect of lack of the phosphorylated pathway activity on growth and development (see Benstein et al. 2013, 25: 5011–5029, Cascales-Miñana et al. Plant Cell 2013, 25: 2084–210, Toujani et al. Plant Physiology 2013, 163: 1164–1178; Rosa-Tellez et al. 2024, Plant Cell 36: 404–426).

Response 2
We greatly thank referee #1 for these very important references of mutagenesis studies showing critical roles of the phosphorylated serine pathway in growth and development. These studies add an important part of the story by describing links between the phosphorylated serine phosphate pathway and primary carbon metabolism, nitrogen assimilation, amino acid and secondary metabolite synthesis, and protein synthesis. In this revision, we now include the GS/GOGAT cycle for ammonia assimilation in the chloroplast linked directly with the phosphorylated serine pathway in the revised detailed metabolism figure (now **Figure 6**), to highlight the fact that the photosynthetic C_1 pathway is not only assimilating carbon, but also nitrogen, as two critical elements needed for plant growth and development.

Introduction, Line 39
"High expression levels of key enzymes of the phosphorylated serine pathway have been documented in light grown shoots[1] with the phosphorylated serine pathway directly linked to nitrogen assimilation by

providing 2-oxogluterate for ammonia fixation[2]. Disruption of the phosphorylated serine pathway leads to a reduction in nitrogen and sulfur contents in shoots and a general transcriptional response to nutrient deficiency[3]. While the in vivo activity of the 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 growth[4]. 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 growth[5] and mutants of the key enzymes phosphoglycerate dehydrogenase (PGDH) and phosphoserine phosphatase (PSP) resulted in an embryo-lethal phenotype[2,6,7]. 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[2,6]. While most of what is known about leaf serine derives from studies on photorespiration[8], the importance of the phosphorylated serine pathway as a source of serine was found to be particularly important under elevated ambient CO_2 concentrations associated with enhanced growth rates. Wild-type *A. thaliana* plants grown under elevated CO_2 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 observed[2]. These observations suggest that the serine phosphate pathway is particularly important under high CO_2 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 biosynthesis[9]."

Referee #1 Comment 3

Additionally, some information provided in the introduction (and also in Figure 5 and S17) is incorrect: the SHMTs are not part of the phosphorylated pathway. On the contrary, the SHMT activity referred to in reference number 9 (which is a review and not an original paper) is SHMT1, which is the mitochondrial photorespiratory isoform. There are also other erroneous concepts along the text and figures, such as the assertion that MTHR is chloroplast localized. To my knowledge, in Arabidopsis and other species investigated, it is only localized in the cytosol (indeed the authors state in the methods section that the MTHFR has no chloroplast transit peptide). All these missing information and mistakes should be corrected before publication.

Response 3: Thank you for pointing out that SHMTs are not part of the phosphorylated pathway, which has been stated more clearly in the introduction. We also include MTHFR in the cytosol in **Figure 5** and detailed updated metabolism **Figure 6** (previously supplementary Figure S17). The occurrence of MTHFR in the cytosol rather than the chloroplast is in agreement with the ^{13}C-labeling results of methionine and further support our suggestion that the photosynthetic C_1 pathway may accelerate together with the Calvin-Benson cycle under elevated CO_2. We made the following edits/additions to the main text and supplementary information document.

Line 72: "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 C_1 metabolism[10], especially under conditions like elevated CO_2 which suppresses photorespiration and enhance photorespiration."

In **Figure 5** and as well as the more detailed pathway schematic in now as **Figure 6**, we highlight the 'photosynthetic C_1' pathway that includes the enzymatic steps to convert the carbon of CO_2 to the methyl

group of methionine. This integrates the activity of the Calvin-Benson cycle, the phosphorylated serine pathway, and C_1 metabolism fueled by serine as the source of the methyl groups used in methionine synthesis, and subsequently C_1 metabolism.

Discussion Line 322: "Although six of seven enzymes of the 'photosynthetic C_1 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 transporters[11]. Thus, export of strongly ^{13}C-labelled 5,10-methylene-THF may be diluted by pre-existing 5,10-methylene-THF in the cytosol, resulting in a decrease in ^{13}C-lableing of the methyl group of methionine (~60%) relative to serine (> 90%)."

Discussion Line 475: "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-oxygluterate, requires ATP and NADPH (catalyzed by PSAT, see **Figure 6**)."

Supplementary Info Line 130: "The genomes of most land plant species encode several MTHFR isoforms which lack an obvious target sequence[12], suggesting the MTHFR subcellular localization in algae and land plant species is the cytosol[13]. However, it is important to keep in mind that MTHFR is the least understood enzyme of THF-mediated one-carbon metabolism in plants[12] and its subcellular location(s) require additional research. Here, we consider that 5-methyl-THF synthesis catalyzed by MTHFR occurs in the cytosol, with the export of 5,10-methylene-THF out of chloroplasts together with 5-methyl-THF import into chloroplasts mediated by one or more chloroplastic folate transporters[11]."

Referee #1 Comment 3
I agree with the authors that the importance of the serine phosphorylated pathway for growth and development remains unclear. The results from the manuscript will provide new insights in the field. Their main hypothesis linking the phosphorylated pathway with growth is that the Ado-Met (not measured here) required to methylation reactions comes directly from the phosphorylated pathway and from the Calvin-Benson cycle. To demonstrate this, the authors used a series of C labelling studies. They showed that C1 metabolism producing methanol is slightly dependent on photorespiration and comes directly from photosynthesis. They also showed that pectin methyl esters are synthesized in a photorespiration-independent manner. Then they hypothesized that the missing link between photosynthesis and C1 metabolism should be the phosphorylated pathway.
Response 3: Thank you for recognizing the new insights from this manuscript on the importance of the serine phosphate pathway during photosynthesis as part of a 'photosynthetic C_1' pathway linking central metabolism with growth and development. Note, that we give designate the light-dependent pathway name of the 'photosynthetic C_1 pathway' as a series of 3 different linked pathways including RuBisCO carboxylation and the Calvin-Benson cycle, the phosphorylated serine pathway, and C_1 metabolism mediated by THF intermediates giving rise to methionine in chloroplasts with severely, but naturally depleted S-bonded methyl carbon atom.

Referee #1 Comment 4
Several recent reports using metabolic flux analysis by isotopic labelling, modelling, and gas exchange measurements in wild-type plants under changing photorespiratory conditions indicated that a considerable amount of Gly and Ser could go out of the photorespiratory cycle (see Abadie et al., 2016, Nature Plants 2:15220 ; Busch et al., 2018, Nature Plants 4:46-54, Fu et al. 2023 Nat Plants 9: 169–178

and others) and that the photorespiratory pathway could importantly contribute to C_1 metabolism (Rosa-Téllez et al 2024, Plant Cell 36: 404–426). It is also commonly accepted, although not necessarily true, that the phosphorylated pathway is most active at night, in non-photosynthetic organs versus photosynthetic organs, and in heterotrophic cells vs autotrophic cells.

Response 4

This is a very interesting point that a small fraction of photorespiratory Gly and Ser could go out of the photorespiratory cycle contributing to C_1 metabolism. While we now acknowledge this and cite the references provided. However, the amount does not appear to be considerable. Precise quantification in Prof. Guillaume Tcherkez's group has shown that the flux is not zero, but is small (in the 1% range of photorespiratory flux). In this study, although our experimental conditions greatly promoted photosynthesis over photorespiration (moderate light and temperature, high CO_2 and 1% O_2 concentrations that strongly inhibited photorespiration), we acknowledge the possibility of photorespiration contributing to C_1 metabolism via serine and glycine. We now include the following text below in the conclusion section as it is a fascinating idea to consider potential interactions impacting C_1 metabolism between the photosynthetic C_1 pathway which includes the phosphorylated serine pathway, ammonia assimilation, photorespiration, and the oxidative C_1 pathway (methanol←→formaldehyde→formate→CO_2), which directly integrates with photorespiration (via formaldehyde and formate integration into C_1-THF pools) and photosynthesis (via CO_2).

Discussion Line 255: "These results are consistent with studies using metabolic flux analysis, modelling, and gas exchange measurements that suggest a small fraction of photorespiratory carbon can be exported as serine under photorespiratory conditions[14-16]. 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 cycle[3]."

Referee #1 Comment 5

Many of the enzymes included in the author's model are said to be chloroplastically located. However, having a transit peptide (as stated in methods) does not assure that a protein is chloroplast-localized. Depending on the specific expression pattern, it could be plastid-localized in a heterotrophic cell or chloroplast-localized in an autotrophic cell, or both, depending on the cell type. With all this important information in mind, a general discussion with biological meaning to be integrated into the manuscript would be required.

Response 5

We now clarify this important point.

Methods Line 638: "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."

Referee #1 Comment 6

For instance, why would the serine supplied by the glycolate pathway miss the proposed growth connections for this amino acid? Is it because it is produced in the mitochondria? Serine is supposed to move freely between organellar compartments…How would the plant cell discriminate between the two sources of serine? Regarding this, Fig. 4 is very difficult to understand. This is why I am asking if the experiments described in this figure where only conducted under 21% O2? Since no 2-phosphoglycerate could be differentiated from 3-PGA, could the authors explain in detail, for a broader audience, how they were able to discriminate between photorespiratory serine and serine produced by the phosphorylated pathway?

Response 6: We agree that serine should be free to move freely between organelles, and the carbon isotopic composition of serine produced during photorespiration should be very similar to that of serine produced from the phosphorylated serine pathway. As such, the plant likely utilizes serine from both pathways, and we were not able to discriminate serine produced form photorespiration versus the serine phosphate pathway in this study. As highlighted in the introduction however, recent studies demonstrated

that the phosphorylated serine pathway is indispensable for plant growth and its loss cannot be compensated by photorespiratory serine biosynthesis[9]. This is likely due to the main findings of this paper that the phosphorylated serine pathway is a biosynthetic pathway linked directly with photosynthesis via RuBisCO carboxylations. However, in our branch and leaf level experiments, gas exchange measurements were collected under conditions that promoted high rates of CO_2 assimilation while suppressing photorespiration (e.g. moderate light, optimal leaf temperatures, elevated $^{13}CO_2$, and 1% O_2). In Figure 1, the branch gas-exchange experiments presented were separately performed under 21 % O_2 (as well as the replicate 2-day and 5-day branch $^{13}CO_2$ labelling experiments shown in the supplementary information document) and 1% O_2. The main results show that diurnal methanol emission and ^{13}C-labelling patterns are similar under 1% O_2 (without photorespiration) compared with the $^{13}CO_2$ labeling experiments under 21% O_2 (that likely had low photorespiration due to the experimental conditions).

Conclusions and Perspectives Line 447: "Although we used conditions that promote high rates of photosynthesis while suppressing photorespiration, we were not able to discriminate serine produced form 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 synthesis[9]. 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 (**Figure 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 response[9]. 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^+)[17]."

Referee #1 Comment 7
What about the phosphorylated pathway in poplar? Is there any useful information in the genome databases regarding the number of genes and their expression patterns, how active it is, etc? Why was PGDH2 selected in Figure 6 instead of PGDH1, which is the essential gene at least in Arabidopsis where the phosphorylated pathway has been fully studied? Is it PGDH2 the PGDH1 Arabidopsis orthologue ?
Response 7:
Although limited information is available on the expression patterns of the seven genes of the photosynthetic C_1 pathway in poplar, we now include a citation of a study of poplar gene expression in natural trees in the field during the growing season. Our analysis of this dataset suggests that expression of most of the genes of the photosynthetic C_1 pathway showed a large increase during the growth phase.

Conclusions Line 444: "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. trichocharpa* 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)[18]."

In **Figure 7A**, the co-occurrence was with PGDH2 from Arabidopsis (not poplar). Thank you for pointing this out. We have updated **Figure 7A** (previously Figure 6A) to include PGDH1 and PGDH3 in addition to PGDH2. All three PGDH proteins were found in the Athal chloroplast proteome (Rowland et al 2022) despite only Athal PGDH2 having predicted chloroplast localization. Interestingly, each Athal PGDH protein had a different poplar PGDH protein with highest similarity. Initially, we used PGDH2 because it

had the predicted transit peptide localized to the chloroplast and was in the chloroplast proteome, and our proposed photosynthetic C_1 pathway is in the chloroplast. However, when we checked the *Arabidopsis* chloroplast proteome, it had PGDH1, PGDH2 and PGDH3. This is despite PGDH1 and PGDH3 not having the chloroplast predicted transit peptide, they are in fact localized in the chloroplast. Thus, we updated **Figure 7A** to include PGDH1 and PGDH3 in addition to PGDH2.

Line 679: "Although AtPGDH1 and AtPGDH3 do not contain a predicted chloroplast transit peptide, all three proteins (AtPGDH1, AtPGDH2, AtPGDH3) were found to be present in the Arabidopsis chloroplast proteome and are therefore included in the co-occurance analysis."

Referee #1 Comment 8
Also some spelling mistakes such as "phosphogyceric" in figure 4 or "inccroporation" in line 191 should be corrected.
Response 8: These spelling mistakes have now been corrected.

Referee #2 Comments and Responses
Referee #2: an expert in organismal ecologist, centering around questions regarding genomics and stress tolerance and trace gas exchange between plants and the atmosphere.

Referee #2 Comment 1
This intriguing manuscript by Jardine et al. argues for a tight coupling between C1 metabolism and Calvin Cycle carbon reduction in plants. They furthermore argue that this connection provides the mechanistic underpinning for the relationship between methanol production/emission and growth. To my mind they do an excellent job of demonstrating the connection between C1 metabolism and the Calvin Cycle, rather than the more typically assumed C1-phoptorespiration link. Their data arguing for this novel connection more than justify the eventual publication of this paper in a high profile journal such as Communications Biology.
Response 1: We greatly thank referee #2 for their strong support of our research article and its potential impacts on understanding serine metabolism in plants and its role in supporting growth via a 'photosynthetic C1 pathway', highlighted here for the first time.
Referee #2 Comment 2
That said, there are minor elements of the paper that could be clearer and/or do not seem central to the primary exciting and novel result, and I think the paper could have a higher impact on the scientific community if they were clarified or excised.
First of all, the link to global GPP seems non-essential and distracting. GPP since 1850 has risen for a variety of reasons, not only rising CO2. Indeed global changes in biomass and nitrogen (thanks to the Allies trying to starve the Germans in WWI) are arguably just as important. And the significance of agricultural advances beyond Haber Bosch cannot be ignored. I do not think the authors need this bit.

Response 2: The fertilization of net photosynthesis and plant growth by rising atmospheric CO_2 concentrations at the leaf, ecosystem, and regional scales is directly relevant to the study at hand, especially since this is linked with a suppression of photorespiration. While we agree that the magnitude of the CO_2 fertilization effect on terrestrial photosynthesis is uncertain because it is not directly observed and is confounded by climate variability like high surface temperatures, a strong CO_2 fertilization effect is detectable in globally distributed eddy covariance networks[19].

Thus, we wish to maintain the very brief section in the introduction (1 sentence) on the mechanisms of CO_2 fertilization of photosynthesis and growth, especially given the potential high importance of the photosynthetic C_1 pathway to link CO_2 assimilation and growth under elevated CO_2.

Referee #2 Comment 2
Second, the phenomenological connection between methanol production/emission and growth has been around for a while, and the link to PME and cell wall expansion tested by others such as Harley et al. and Oikawa et al. The highlight of the current ms. lies with excellent evidentiary basis presented for linking an early Calvin Cycle product to growth in a way takes into account water potential's control on growth. This integration of biochemistry and physiology is wonderful to see.

Response 3: Thank you for your support of our experimental approach which integrates the biochemistry and physiology of C_1 metabolism in plants. We agree that the link of methanol emissions to PMEs during growth is established, including at the whole leaf level where whole isolated cell walls were shown to emit methanol at fluxes comparable to those observed in leaf gas exchange studies[20]. Nonetheless, significant advances in this research area have been contributed in the current study. First of all the majority of studies evaluating the links between methanol emission and growth have been unable to resolve diurnal patterns in plant growth rates linked to methanol emissions due to stomatal closing at night. Due to continued growth processes at night, methanol is still produced within leaves while methanol emissions from leaves is severely restricted. Upon stomatal opening in the morning, large bursts of methanol emissions occurs. In the current study, we took advantage of the fact that *P. trichocarpa* displays high stomatal conductance at night, allowing diurnal methanol emission and growth dynamics to be studied for the first time. We show for the first time that methanol emissions begin to recover around mid-night increasing all night, potentially related to the recovery of leaf water potential. Moreover, we show that methanol emissions are suppressed in the hot afternoon periods, potentially related to enhanced leaf water potential stress as evidenced by the leaf water potential data. Thus, another key aspect of this paper is the potential discovery of hydraulic controls over plant growth and methanol emissions where the diurnal water status of the tissue strongly influences the temperature sensitivity of plant growth. For more details, please see the supplementary section, **Pectin demethylation, methanol emissions, and growth,** supplementary **Figure S17**: Graphical representation of diurnal growth and methanol emission processes in plants, and **Note S3:** Methanol emission: a metabolic biomarker of plant physiological status?

We have now added citations to this section of the two excellent papers by Harley and Oikawa et al. upon first mentioning methanol release from pectin methyl esters in the main text.

Referee #2 Comment 4
Third, and building from point two, Jardine knows (and as they allude to on page 8) as well as anyone in the world the complex relationship between stomatal conductance and methanol emission that arises out of the combination of methanol's low concentration relative to saturation and its high solubility in water. While I am sure that this relationship is accounted for in the author's production/emission calculations, seeing actual data on this point would make the paper easier to understand for many readers. Indeed, a paragraph contrasting water, methanol, and isoprene with respect to stomatal control over emissions would be very helpful (perhaps with the space saved by cutting much or all of the GPP discussion).
Response 4: It is important to note that methanol has only a moderate water solubility when compared with other oxygenated C_1 like formic acid, it is indeed the case that methanol emissions is partially under stomatal control (see Henry's law contents and a more detailed explanation here[21]) but also under the primary control of its production rates during growth. Thus, the nighttime (after 12:00 PM) increase in both ^{13}C-methanol and ^{12}C-methanol emissions cannot be explained by stomatal changes at night. We therefore attribute these changes to hydraulically driven growth at night as this is associated with a recovery of leaf water potential (see response 3 above). We dedicate text to the interacting factors of growth rates of primary cell walls and stomatal conductance in the supplementary introduction section on Pectin demethylation, methanol emissions, and growth**,** supplementary **Figure S17**: Graphical representation of diurnal growth and methanol emission processes in plants, and **Note S3**: Methanol emission: a metabolic biomarker of plant physiological status?

Referee #2 Comment 5
Finally, while I enjoy a good speculative handwave as much as the next person, the ending of the paper should keep the focus on the novel biochemistry elucidated and the links to plant hydraulics. I was unpersuaded by the global changish closing of the paper and don't think it necessary.
Response 5: We acknowledge that many papers these days jam in a global changish component that does not naturally fit and should be eliminated. However, in this case, the global rise in atmospheric CO_2 and the "seeding" of photosynthesis linked to global change processes is central to understanding the motivation and the broader impact of the current study on C_1 photosynthesis. This is because we view the photosynthetic C_1 pathway as potentially critical for understanding the evolution of oxygen photosynthesis (due to its existence of the phosphorylated serine pathway in cyanobacteria) and the future (due to the lack of ATP/NADPH requirements) theoretically allowing it to accelerate together with the Calvin-Benson cycle under an elevated CO_2 atmosphere.

Referee #2 Comment 6
In short, this is one of the most interesting plant metabolism papers to have come across my desk in quite a while, and I think it highly suitable for Communications Biology. My suggestions above are offered as criticisms to heighten the paper's impacts and to help emphasize its novelty and importance.
Response 6
We greatly thank referee 2 for making this very supportive statement and for recognizing our contributions in the elucidation of the photosynthetic C_1 pathway in plants.

Referee #3 Comments and Responses
Referee #3: an expert in plant physiology, focusing on resolving the biochemical, cellular and canopy-level mechanisms that determine photosynthetic fluxes of carbon and oxygen.

Referee #3 Comment 1
This paper explores the origin of 1-C groups in photosynthetic tissues, a topical and important subject. The main conclusion, however, that most these1-C units in methionine come from serine not derived from photorespiration, is not supported from the presented data (As outlined below).
Response 1
We thank referee #3 for the time spent reviewing our manuscript. We address the criticism of the main conclusion in our responses to the comments below. In the abstract and graphical abstract (the RuBisCO joke), we now soften the strong statement that only the photosynthetic C1 pathway can lead to methanol synthesis.

"**Abstract**: Using $^{13}CO_2$-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 C_1 metabolism, apparently unrelated to photorespiration, within minutes of light exposure."

"Graphical abstract 1: Under the conditions studied in poplar leaves, the photosynthetic C_1 pathway, rather than photorespiration, leads to the generation of light-dependent methionine synthesis used during methylation of innumerous substrates including new pectic polysaccharides in the Golgi apparatus that result in methanol emissions from the primary cell wall during growth processes."

Referee #3 Comment 2
The use of different CO_2 concentrations could be better explained and seem to undermine the main premise of many of the experiments and the conclusions of the paper. For example, why was 900 PPM used in the case of diurnal labeling, but 750 ppm used for instantaneous leaf 13C methanol experiments?

Response 2

There are two types of elevated $^{13}CO_2$ labelling experiments presented in the paper, long-term branch and short-term branch $^{13}CO_2$ labelling, each with its distinct advantages and disadvantages. The branch experiments were designed to allow for continuous $^{13}CO_2$ labelling over long periods of time (2-5 days) under optimal conditions for photosynthesis and growth (elevated $^{13}CO_2$, moderate light and temperature). The disadvantage is that gross $^{13}CO_2$ photoassimilation rates were not calculated due to the high variability of leaf biomass, leaf area, and leaf environmental conditions (light, temperature, humidity). During the diurnal branch labelling, 900 ppm $^{13}CO_2$ was the reference concentrations entering the branch chamber, and with a branch in the chamber in the dark. However, in the light, branch photosynthesis drew the $^{13}CO_2$ concentrations down to around 500 ppm as quantified by an isotopic CRDS for CO_2. In contrast, leaf level $^{13}CO_2$ labelling had the advantage of setting optimal environmental conditions for photosynthesis including elevated $^{13}CO_2$, optimal light, temperature, and humidity held constant for short-term periods (1-5 hours). In these leaf experiments, quantitative estimates of gross $^{13}CO_2$ assimilation were made to enable quantitative comparisons with ^{13}C-labelling of methanol and pectin methyl ester C_1 carbon pools. Due to the lower air residence time in the leaf chamber compared to the branch enclosure, $^{13}CO_2$ in the light was 700-900 ppm. The higher $^{13}CO_2$ acts to stimulate photosynthesis while suppressing photorespiration, the focus of the current study. The main result of the leaf experiments (supplementary **Figure S8)**, is the finding of a tight linear correlations between instantaneous $^{13}C/^{12}C$-methanol emission ratio and cumulative photosynthesis of $^{13}CO_2$ during 1-5 hour periods. This demonstrates that under elevated CO_2, a strong quantitative connection exists between growth (cumulative photosynthesis) and C_{1}-pool biomass accumulation including the biosynthesis of pectin methyl esters and the emission of methanol during cell wall expansion processes. We agree that future studies could directly evaluate the impact of ambient CO_2 including current ambient levels and sub ambient levels in the leaf headspace by performing A-Ci curves using $^{13}CO_2$.

Referee #3 Comment 4

These high concentrations would also effectively limit much photorespiration by ~50% based on a rough estimate. This is especially problematic when enrichment of PME's was compared between 1000 PPM $^{13}CO_2$ and 21% O_2 and (presumably) 1000 PPM $^{13}CO_2$ and 2% O_2. This comparison was used to indicate that there was little photorespiratory-related contribution to PME's but under both conditions, photorespiration would be very minimal. Interestingly, there was a decrease in labeling under 2% O2, it would be interesting to calculate by how much photorespiration was decreased under these particular conditions since I would surmise it was pretty minimal as well. It is unfortunate that ambient CO2 concentrations were not used, where there would be a greater difference in photorespiration between 21% and 2% O_2. The results do support the statement in line 175 that PME's come at least partly from non-photorespiratory carbon, but do not support the clam that the photosynthetic C_1 pathway, rather than photorespiration, leads to the generation of light-dependent methionine under typical photorespiratory conditions.

Response 4

We agree that photorespiration was already minimal in the 21% O_2 $^{13}CO_2$ labelling experiments. The same experiment under 1% O_2 effectively blocked photorespiration while retaining PME and methanol ^{13}C-labeling patterns, but with slightly reduced magnitude attributed to respiration/photosynthesis inhibitions at low O_2. However, we highlight the fact that the $^{13}CO_2$ concentrations during the branch $^{13}CO_2$ labelling studies under 21% and 1% O_2 was not too far above ambient (~500 ppm) during the day when branch photosynthesis drew down the concentration from the reference air (900 ppm). However, $^{13}CO_2$ labelling of individual leaves for 1-5 hours under optimal conditions for photosynthesis occurred at higher $^{13}CO_2$ in the light (700-900 ppm). Under 21% O_2 and 1000 ppm $^{13}CO_2$, with our range of values of photosynthesis (up to about 25 μmol/m²/s) and g_s (about 0.2 mol/m²/s), oxygenation (v_o) is estimated to be less than 5% of v_c while under 1% O_2 and 1000 ppm $^{13}CO_2$, it is reduced to below 0.5%, therefore, there is quite a difference between the two oxygen conditions. Thus, figure 1 effectively demonstrates that ^{13}C appearance in methanol is largely independent of photorespiration. We now include a more balanced

discussion on photorespiration versus the photosynthetic C_1 pathway in the conclusions and perspective section (see Response 6 to referee 1).

Referee #3 Comment 3
What was the CO_2 concentration for the LC-MS/MS analysis of PGA, Serine, and methionine? Was this approach able to also pickup phosphoglycolate? It was not clear how the sulfur-carbon specific labeling of methionine supported the hypothesis that methionine synthesis was independent of photorespiration.
Response 3
LC-MS/MS analysis of PGA, Serine, and methionine occurred following 2-day branch $^{13}CO_2$ labelling experiments with 900 ppm $^{13}CO_2$ at night in the branch chambers and ~500 ppm $^{13}CO_2$ during the day. Note that the analysis of 2-phosphoglycolate by LCMS is not an easy task, and required specific ionic LC we did not have.

The experimental conditions of the $^{13}CO_2$ labelling were optimized for high rates of net photosynthesis and low rates of photorespiration (moderate light and temperature), including elevated $^{13}CO_2$ (750 to 900 μmol mol^{-1} CO_2; under such CO_2 mole fraction, photorespiratory serine production is less than 5% of carboxylation). Thus, near complete labelling of PGA, serine, and sulfur-bound carbon of methionine supported the hypothesis that the metabolic origin of the methyl group of methionine was not strictly dependent on photorespiration, and could come from photosynthesis via a non-photorespiratory pathway. Unfortunately, LC-MS/MS analysis of leaf metabolites was not available during the branch $^{13}CO_2$ labelling under a 1% O_2 atmosphere to confirm that the ^{13}C enrichment was still observed in methionine even when photorespiration was suppressed. However, note that ^{13}C-labeling of PMEs and methanol emission were of with similar magnitude under 1% and under 21% O_2. Since AdoMet is the carbon source of methylation of new pectin in the Golgi apparatus prior to export to the primary cell wall, this result strongly suggests that under the conditions studied, the methyl group of methionine (methyl donor) primarily derived from CO_2 fixed during photosynthesis and not photorespiration.

However, as described more clearly in the introduction due to the very helpful comments and references from referee #1, the phosphorylated serine pathway is known from mutation studies to be indispensable for growth and development, is particularly important under elevated CO_2, and the severe impacts of its loss cannot be compensated for by photorespiratory serine production. This is consistent with our model where under conditions of high rates of photosynthesis and correspondingly low rates of photorespiration, the methyl group of methionine derives primarily from a non-photorespiratory source and we present the hypothesis of a photosynthetic C_1 pathway linking the Calvin-Benson cycle, the phosphorylated serine pathway, and methionine synthesis in chloroplasts with diverse AdoMet-dependent methyl transfer reactions, and AdoMet recycling in the cytosol. However, we acknowledge that this does not exclude the possibility that under photorespiratory conditions, the methyl group of methionine can also derive from photorespiratory serine as shown previously in sunflower via double $^{33}S/^{13}C$ labelling[22]. Presumably, the metabolic origin of C_1 units involves both photosynthetic C_1 metabolism (via chloroplastic serine synthesis) and photorespiration, depending on conditions and species. In the case of poplar under conditions that promote photosynthesis and suppress photorespiration, our results suggest that the photosynthetic C_1 pathway prevails.

Referee #3 Comment 4
The presentation of the results, relative to the stated hypothesis, were unfocused. For example, how does the temperature response of ^{13}C-methanol emission elucidate the source of carbon for methionine biosynthesis? Similarly, it was not clear what the temperature response of the in situ methanol emission rate added. Perhaps these could be grouped in a “temperature response” section to make their relevance clearer. The inclusion of this data was not tied well to the core hypothesis presented in the abstract and in the current state, served to distract from the core argument presented.

Response 4
We acknowledge this major point of the manuscript, which we now clarify. Growth and PME demethylation yielding methanol are highly temperature-sensitive processes and it therefore seemed to us that the effect of temperature had to be checked. The synthesis of pectin methyl esters via the 'photosynthetic C_1 pathway' is independent of the release of methanol from PMEs during temperature-stimulated (day) as well as hydraulically-driven (night) growth processes. Thus, the paper should be viewed with two separate lenses 1) Primary light dependent processes of C_1 photosynthesis, biosynthesis of C_1 pools such as PMEs, and 2) Light-independent processes including cell wall expansion during growth and development resulting in methanol production and emission. Thus, 1) occurs only during the day due to the strict light-dependence whereas 2) occurs during the day and night, but is impacted by temperature and plant hydraulics.

Discussion Line 274: "The results demonstrate that the synthesis of PMEs via the 'photosynthetic C_1 pathway' is independent of the release of methanol from PMEs during temperature-stimulated (day) as well as hydraulically-driven (night) growth processes. Thus, methanol emissions requires light dependent processes of C_1 photosynthesis leading synthesis of new PMEs 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."

Referee #3 Comment 5
I appreciate the care that went into the graphical abstract 1 and video. This type of work is a great way to better communicate research findings to other audiences. This is even though I think the main conclusion is not supported entirely by the evidence.
Response 5
Thank you for this as we indeed spent a lot of time and resources to carefully prepare the graphical abstracts and video which we appreciate helps communicate some of the main novel ideas presented on the links between a 'C_1 photosynthesis pathway', growth, and methanol emissions and likely also resolves the long-debated idea of plant methane emissions. To highlight this important aspect of our paper, we now mention this in the abstract and the conclusions/future perspective sections.

Conclusions and Perspectives Line 513: "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 mechanism for light-dependent production and emission of methane observed from cyanobacteria[23] and trees[24] as may be expanded with additional studies to other C_1 VOCs like methanethiol and dimethyl sulfide[25]. Thus, this resolves the controversy for many years that plants produce methane[26-28] versus the widespread view that vascular plants do not possess a biochemical pathway to produce methane[29], but rather only act as a conduit for microbially produced methane in soils to the atmosphere[30]. 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 photosynthesis[31], 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."

We also now cite a recently published paper in the discussion section that strongly supports a role of metabolic channeling of CO_2 to methionine via the photosynthetic C_1 pathway in algae.

Conclusions and Perspectives Line 507: "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 photorespiration[32]. 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 pyrenoid[33].”

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Point-by-Point response to reviewers (12-Sept-2024)

The 'Photosynthetic C_1 pathway' links carbon assimilation and growth in plants

Kolby J. Jardine[*1], Luiza Gallo[1,2], Melissa Roth[3], Shivani Upadhyaya[3], Trent Northen[4], Suzanne Kosina[4], Guillaume Tcherkez[5], Aymerick Eudes[4], Tomas Domigues[2], Markus Greule[6], Suman Som[1], and Frank Keppler[6,7]

Reviewer Comments and Responses
Reviewer #1: plant physiology, focusing on serine biosynthesis, plastidial glycolysis, and stress responses in Arabidopsis, tomato, and sugar beet.

Reviewer #1 Comment 1: In my opinion, the revised version of the manuscript has been substantially improved. Major concerns have been addressed, and the appropriate changes made where necessary.
Response 1: Thank you very much for the extremely helpful guidance through the review process which improved the manuscript including expanding the impact of the results beyond carbon assimilation to nitrogen assimilation.

Reviewer #1 Comment 2: Although the authors stated that the name of the "Phosphorylated pathway" was changed throughout the manuscript, they still refer to the "Phosphorylated pathway" as the "Phosphate pathway" on page 2, line 60.
Response 2: We replaced "Phosphate pathway" to the "Phosphorylated pathway" for consistency with the rest of the manuscript.

Reviewer #1 Comment 3: Line 679: To my knowledge, the Arabidopsis PGDH1 and PGDH3 do have a transit peptide. Please confirm this.
Response 3: We verified that Arabidopsis PGDH does have a plastid transit peptide. This was corrected in the text as follows:
Line 683: Consistent with the 60-amino acid leader sequence acting as a transit peptide for transportation of Arabidopsis PGDH to plastids[88], all three proteins (AtPGDH1, AtPGDH2, AtPGDH3) were found to be present in the Arabidopsis chloroplast proteome[61] and are therefore included in the co-occurance analysis.

Reviewer #1 Comment 4: Spelling mistakes. Line 104: There is a spelling mistake—"Phosphoglyceric" should be used instead of "phosphogyceric."
Response 4: "phosphogyceric" Has been corrected to "phosphoglyceric"

Reviewer #3: plant physiology, focusing on resolving the biochemical, cellular and canopy-level mechanisms that determine photosynthetic fluxes of carbon and oxygen.

Reviewer #3 Comment 1: I appreciate the care to address my concerns about the elevated CO2 conditions and in the time to help me (and future readers) get the point that the study is addressing most directly C1 metabolism in a reduced photorespiratory future and not necessarily what is currently the predominant source of 1C units under our more photorespiratory conditions. I still would like to see some clearer statements in the manuscript outlining this caveat since it is important.

Response 1:
Thank you for your thoughtful feedback, and we agree that this caveat is important to clarify. Please see our responses and edits made to the manuscript to reflect this.

Reviewer #3 Comment 2: For example, while there is a large difference in the photorespiratory conditions of 21 vs 1% O2 under elevated CO2 (vo less than 5% under 21% O2 and then below 0.5% under 1% O2), the 21% O2 condition is still ~20% of ambient conditions, which were not measured. I think a clear statement in the discussion is needed that indicates that the predicted future conditions measured were very different from ambient conditions and can't be used to argue that under present conditions, photorespiratory C1 carbon flux is not a large source of C1 units. Of course the core conclusion of the paper that C1 flux can come from non-photorespiratory sources is still valid and interesting.
Response 2: Thank you for this helpful point and the recognition of the validity and potential high importance of the core conclusion that C_1 flux can come from photosynthetic sources. To address this concern, we included a clear statement in the discussion:

Line 259: "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."

Reviewer #3 Comment 3:
Line 284: The papers cited here suggest that 20-30% of photorespiratory carbon is exported from the photorespiratory carbon, which is not really a "small" fraction. These numbers may be high but consider dropping "small" from this sentence since the papers cited suggest it is large.
Response 3: We agree and the word, "small" has been removed from this sentence.