Does C 4 Photosynthesis Occur in Wheat Seeds?

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Does C_4 Photosynthesis Occur in Wheat Seeds?

Dear Florian and Graham,

Our recent report of new evidence for C_4 photosynthesis in wheat seeds (Rangan et al., 2016) has been met with skepticism (Busch and Farquhar, 2016), maybe because this has been such a long-running controversy. Much of the confusion has been because early work did not distinguish photosynthesis in the pericarp of the seed (Fig. 1) from that in the covering glumes. Glumes, like leaves in wheat, capture CO_2 from the atmosphere by C_3 photosynthesis. However, RNA-Seq analysis demonstrates the expression of a complete C_4 pathway in the seed with a C_4-specific form of PEP carboxylase expressed specifically in the pericarp and aleurone capturing carbon released by respiration in the endosperm. Decarboxylation by a C_4-specific malic enzyme in the inner pericarp supplies CO_2 to a C_3 pathway. The decarboxylation of malate specifically in the pericarp is suggested by comparison of the levels of C_4-specific malic enzyme expression in the pericarp with the low levels in the endosperm. This provides a source of concentrated carbon dioxide for Rubisco in the pericarp, acting like the C_4 pathway in leaves that effectively concentrates CO_2 at the bundle sheath chloroplast for fixation in a C_3 process. Unlike the situation in wheat leaves, high concentrations of CO_2 in the endosperm cannot be fixed through photosynthesis due to the lack of light penetration, but capture as malate allows processing to generate higher CO_2 concentrations for the Rubisco expressed very specifically in the photosynthetic pericarp.

Analysis of the genome shows specific genes encoding the C_4 enzymes that are distinct from those encoding the C_3 pathway. These genes are expressed in a highly tissue-specific way in the pericarp and endosperm. Analysis of the specificity of wheat seed enzymes supports this interpretation. Labeling studies using ^{14}C-labeled CO_2 provide overwhelming support for the capture of carbon as malate in these tissues and the flow of this carbon into C_3 photosynthesis (Nutbeam and Duffus, 1976; Singal et al., 1986). Confusion on this issue has been deepened by a labeling study (Bort et al., 1995) that did not recognize the difference between glumes capturing external CO_2 and the inner seed capturing respired carbon. This study claimed evidence against C_4 photosynthesis based upon feeding CO_2 to the intact ear rather than respired carbon diffusing outwards from the endosperm despite very early work establishing the later as the source of carbon. Further misinterpretation may have resulted from an expectation that seed photosynthesis would use free CO_2 as a substrate. The endosperm of the developing seed maintains a high pH with a drop in pH only happening during germination. The native substrate in the developing seed tissues would be bicarbonate acting as a substrate for the large amounts of C_4-specific PEP carboxylase found in the aleurone and pericarp.

We therefore propose that there is no credible evidence opposing these reports of C_4 activity in wheat. Study of tissues outside the pericarp and a lack of recognition by some of respiration in the endosperm as the source of carbon have confused the issue.

Sincerely,
Robert, Parimalan, and Agnelo

Dear Robert, Parimalan, and Agnelo,

In your recent paper, you report interesting transcript profiles of wheat leaves and seeds, showing that the leaves express variants of genes encoding enzymes necessary for C_4 photosynthesis that resemble those found in C_3 plants, while the variants expressed in the seeds resemble those found in C_4 plants (Rangan et al., 2016). This is novel information that warrants a renewed and deeper look into the issue of whether wheat grains employ a C_4-type carbon concentrating mechanism, or more generally, why C_3 and C_4 plants have different isozymes of key photosynthetic proteins to begin with. In our commentary (Busch and Farquhar, 2016), we tried to raise the point that these expression profiles alone, however, are not sufficient to conclude on the existence of a functioning C_4 photosynthetic pathway.

Previous studies on this topic have been, as you pointed out, contradictory, and to discuss each of their virtues and flaws would go beyond the scope of this discourse. But whatever technique one chooses to establish the existence of a C_4 photosynthetic pathway, in our opinion the gold standard (and really the only standard) has to be to provide evidence of a flux through that pathway. As Hibberd and Furbank (2016) noted in their commentary, all the genes coding for proteins necessary for C_4 photosynthesis already exist and are expressed in C_3 plants, but are usually not used for that purpose. Just because a gene is expressed more highly does not imply it is translated more, or produces a higher activity of that protein, or, most importantly, that the protein catalyzes a reaction that directly contributes to a certain biochemical pathway.

Unfortunately, experimental evidence for or against a C_4 pathway in the wheat seed is scarce. Despite its shortcomings, in our view the most rigorous of these experiments support the idea that PEP carboxylase facilitates anaplerotic reactions and is not involved in a C_4 pathway (Bort et al., 1995). Furthermore, some of the data you presented in your recent work (Rangan et al., 2016) itself go against the existence of a C_4 pathway in the seed: The expression level of the gene for a critically important enzyme for C_4 photosynthesis, Rubisco, is almost negligible. If we assume for the moment (as you have) that expression of genes directly relates to protein

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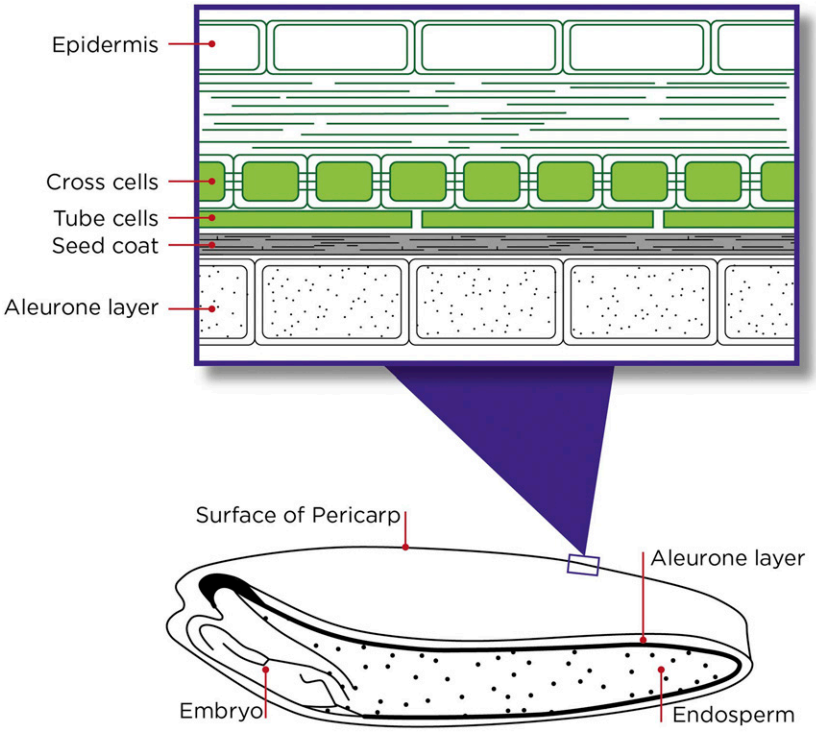

Figure 1. Schematic of a wheat seed with the outer layers detailed. The magnification of the pericarp shows the epidermis, cross cells (photosynthetic), and tube cells (photosynthetic) surrounding the endosperm with the outer (aleurone) layer depicted.

activity, most of the CO_2 fixed by PEP carboxylase would therefore have to be metabolized in a biochemical pathway other than photosynthesis.

Another aspect conflicting with a C_4 pathway in the seed is related to the compartmentation you mentioned: The CO_2 concentration is high in the endosperm but relatively low in the Rubisco-containing pericarp. One unifying feature of the C_4 pathway is the enrichment of CO_2 at the site of Rubisco relative to where the initial fixation occurs by PEP carboxylase. Why invest in an energy-intensive C_4 pathway if the CO_2 diffusing outward along its concentration gradient would arrive in the pericarp for free? In this sense, a C_4 carbon pump in the seed goes against intuition and therefore would need good experimental support based on carbon-flux measurements. To date, this evidence is lacking.

Sincerely,
Florian and Graham

Dear Florian and Graham,

The "gold standard" has been satisfied here by demonstration of flux through malate to C_3 intermediates in barley (Nutbeam and Duffus, 1976) and wheat (Singal et al., 1986). The C_4 versions of key genes have not only been shown to be expressed, but the proteins have been isolated and characterized as having the required specificity (Singal et al., 1986). Enzymes catalyzing the same reactions (e.g. PEP carboxylase) are found in both C_3 and C_4 plants, but the genes in C_4 plants encode versions with C_4 specificity. In wheat, we have demonstrated both C_3 versions expressed in the leaves and C_4 versions very specifically expressed in the seeds. The Rubisco level in the pericarp is far from negligible. The concentrations of Rubisco transcripts we reported were for the whole caryopsis including the endosperm. Our analyses of published data (Pearce et al., 2015) shows that Rubisco is very specifically expressed in the pericarp tissues of the caryopsis and not significantly in the endosperm, suggesting high concentrations of Rubisco in pericarp cells. The study of Bort et al. (1995) was designed largely to test glume photosynthesis as this seemed to be the most likely site of ear photosynthesis at the time. As a consequence, it could not have supplied significant labeled carbon via respiration to the pericarp and cannot be used to infer the pathway in the pericarp. However, it has erroneously been interpreted by some as evidence against C_4 in the pericarp.

The CO_2 concentrations in seed show very different patterns to those in a leaf. The leaf has light throughout, so it can use C_4 to capture low concentrations of CO_2, move the carbon inwards, and concentrate it for capture by Rubisco. The high CO_2 concentrations in the seed need to be moved out to a place that the light can reach. In the seed, the very active pericarp-specific decarboxylation of malate delivers concentrated CO_2 specifically at the site of the high concentrations of Rubisco in the pericarp with light penetrating the outer clear tissues to reach this point. If CO_2 was merely allowed to diffuse out, it would not reach the pericarp at a high concentration and could escape past this site during times of the day when sufficient light was not available. Indeed, the pathway of C_4 activity in seeds does act just like C_4 in leaves in that it delivers the required concentrations of CO_2 to Rubisco although the flux is outwards not inward.

We now have evidence at the gene (Rangan et al., 2016), transcript (Rangan et al., 2016), protein (Meyer et al., 1982), and metabolite (Nutbeam and Duffus, 1976) level for C_4 photosynthesis in wheat seeds. Many more aspects of these processes need to be explored. For example, it has been shown that wheat genotypes expressing higher levels of C_4 enzymes in the seed give a higher yield under stressed conditions, although the implications have not been recognized (Jia et al., 2015). The wide variation in the level of expression we have found suggests potential for selection of wheat genotypes for improved performance in hot or dry environments during grain filling.

Sincerely,
Robert, Parimalan, and Agnelo

Dear Robert, Parimalan, and Agnelo,

Previous studies estimating the carbon flux through a C_4 pathway in the seed have been contradictory, with a flux through malate to C_3 intermediates shown initially (Nutbeam and Duffus, 1976; Singal et al., 1986) but later refuted (Bort et al., 1995). The more recent study focused on photosynthetic pathways in the glume, although they also tested for ^{14}C-labeling of metabolites in the seed and found a similar labeling pattern to the C_3 flag leaf blade. You do have a valid point that the $^{14}CO_2$-labeling from the outside of the grain may result in different labeling patterns from what one would expect of labeled CO_2 released by respiration in the endosperm. However, Nutbeam and Duffus (1976) used isolated pericarps lacking the aleurone and endosperm with all its PEP carboxylase that could potentially be involved in C_4 photosynthesis. Consequently, their experiments also provide limited insight into a potential photosynthetic C_4 acid transport from the inside of the grain to the pericarp. The PEP carboxylase that does exist in the pericarp should fix CO_2 regardless of where it comes from as it lies on the periphery of the grain. It is therefore remarkable that Bort et al. (1995) did not find any involvement of PEP carboxylase in a C_4 pathway.

In addition—we have to emphasize this point again—the CO_2 respired in the endosperm that is not assimilated there via anaplerotic reactions would follow Fick's law and diffuse outwards, having to pass through the pericarp on its way. This means that the rate of CO_2 moving through the pericarp is equal to the rate of CO_2 release, independent of whether this happens via active transport as malate with subsequent release of CO_2 in the pericarp or via simple diffusion of CO_2. Therefore, the CO_2 concentration in the pericarp in both cases would equally be determined by the rate of CO_2 leakage from the pericarp to the outside air, which is the difference between the rate of seed respiration and that of CO_2 uptake by Rubisco, multiplied by the resistance to leakage of CO_2. This result is independent of whether malate transport occurs or not. The main difference between these two scenarios, corresponding to a C_4 pathway and a C_3 pathway, respectively, is the cost of two ATP per CO_2 fixed by PEP carboxylase in the former case (Hatch, 1987). It is not obvious how this investment would pay off, especially since the ATP and NADPH involved in this reaction have to be provided, as you pointed out, via respiratory processes due to lack of light in the endosperm. In this regard, it is also important to determine whether a potential C_4 pathway in the grain shuttles CO_2 from the cross cells in the periphery that have access to light to the tube cells, as you describe in your paper (Rangan et al., 2016), or from the inside of the endosperm outward, as you argue here.

This brings us back to the earlier statement that we still have much to learn about the photosynthetic properties of the wheat grain before reaching a definite conclusion about its photosynthetic type. With the gene expression analysis in your recent paper, you provided an exciting new piece of the puzzle as to how wheat grains may maximize their carbon gain. To turn this into solid evidence for or against C_4 photosynthesis, it needs to be supplemented by experiments that unequivocally determine the magnitude of the carbon flux through a potential C_4 pathway. Experiments that may help quantify this flux include thorough labeling studies as well as in vivo physiological measurements, such as the response of the CO_2 compensation point to the O_2 concentration. A detailed quantification of enzyme activities and in particular their location would significantly add to that understanding. We encourage everyone to look for this evidence with great scientific rigor.

Sincerely,
Florian and Graham

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