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Recruitment of pre-existing networks during the evolution of C_4 photosynthesis

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During C_4 photosynthesis, CO_2 is concentrated around the enzyme RuBisCO. The net effect is to reduce photorespiration while increasing water and nitrogen use efficiencies. Species that use C_4 photosynthesis have evolved independently from their C_3 ancestors on more than 60 occasions. Along with mimicry and the camera-like eye, the C_4 pathway therefore represents a remarkable example of the repeated evolution of a highly complex trait. In this review, we provide evidence that the polyphyletic evolution of C_4 photosynthesis is built upon pre-existing metabolic and genetic networks. For example, cells around veins of C_3 species show similarities to those of the C_4 bundle sheath in terms of C_4 acid decarboxylase activity and also the photosynthetic electron transport chain. Enzymes of C_4 photosynthesis function together in gluconeogenesis during early seedling growth of C_3 *Arabidopsis thaliana*. Furthermore, multiple C_4 genes appear to be under control of both light and chloroplast signals in the ancestral C_3 state. We, therefore, hypothesize that relatively minor rewiring of pre-existing genetic and metabolic networks has facilitated the recurrent evolution of this trait. Understanding how these changes are likely to have occurred could inform attempts to install C_4 traits into C_3 crops.

This article is part of the themed issue 'Enhancing photosynthesis in crop plants: targets for improvement'.

1. Introduction

Photosynthesis has shaped life on the Earth by allowing the energy from sunlight to be harvested and used for the assimilation of carbon dioxide. The process of carbon assimilation via the Calvin–Benson–Bassham cycle [1] requires initial fixation of CO_2 by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to form the three-carbon molecule 3-phosphoglycerate (3-PGA). RuBisCO is thought to have evolved in bacteria under anoxic conditions approximately 3.5 billion years ago [2,3]. However, approximately 2.3 billion years ago, the proliferation of oxygenic photosynthetic organisms together with an increase in carbonate deposition due to weathering started to deplete atmospheric CO_2 concentrations [3–5]. Today, rather than RuBisCO being saturated by CO_2, it is now surrounded by 21% oxygen and only 0.04% CO_2. Under these conditions, O_2 competitively inhibits the carboxylation reaction of RuBisCO to produce 2-phosphoglycolate (2-PG) [6]. 2-PG is toxic and so is rapidly metabolized to prevent its accumulation [7]. The metabolism of PG is known as photorespiration and is energetically costly, especially at high temperatures when rates of oxygenation increase [8]. It has been proposed that high rates of oxygenation by RuBisCO led to the evolution of increased specificity for CO_2, but also that an inescapable trade-off between specificity and the rate of catalysis led to a lower turnover rate [9]. Owing to the relatively low rate of catalysis of RuBisCO, C_3 species are associated with significant losses of water via stomata, and large investments in nitrogen are required to produce the amounts of RuBisCO needed to maintain reasonable rates of photosynthesis [10].

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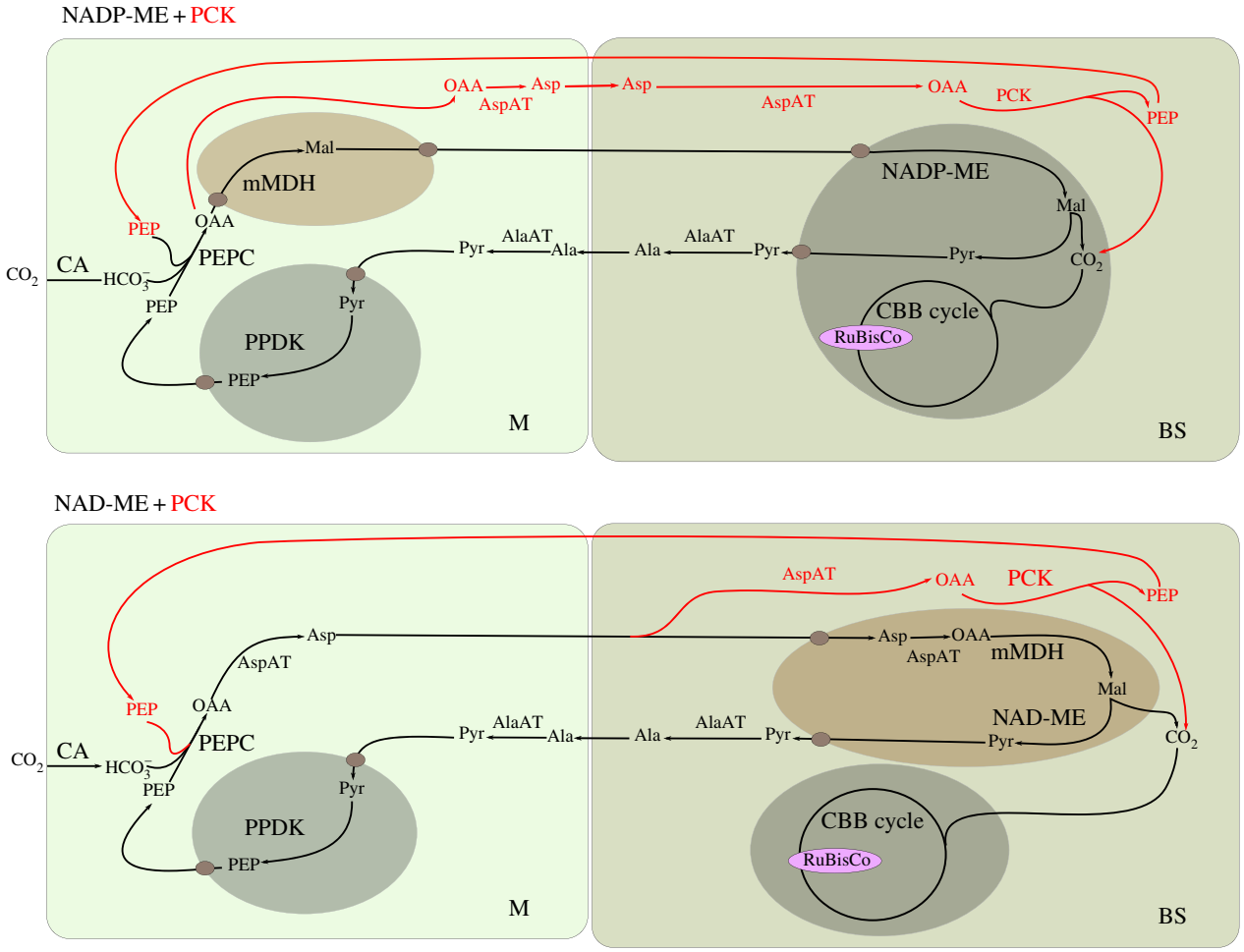

Figure 1. Biochemical subtypes of C_4 photosynthesis. Boxes represent the M and BS cells. Chloroplasts are in green and mitochondria brown. NADP-ME, NADP-dependent malic enzyme; PCK, phosphoenolpyruvate carboxykinase; NAD-ME, NAD-dependent malic enzyme; mMDH, mitochondrial malate dehydrogenase; CA, carbonic anhydrase; PEPC, phosphoenolpyuvate-carboxylase; PPDK, pyruvate,orthophosphate dikinase; AspAT, aspartate aminotransferase; RuBisCO, ribulose-1,5 bisphosphate carboxylase/oxygenase; AlaAT, alanine aminotransferase; CBB cycle, Calvin–Benson–Basham cycle; Asp, aspartate; Mal, malate; CO_2, carbon dioxide; HCO_3^-, bicarbonate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; Pyr, pyruvate.

It would therefore appear logical for photosynthetic organisms to have been subject to significant selection pressures to decrease rates of oxygenation at the active site of RuBisCO. Although there is considerable natural variation in the activity of RuBisCO [11] in photosynthetic lineages as diverse as the cyanobacteria, algae and land plants, it is thought that low CO_2 concentrations before the Anthropocene led to the evolution of carbon concentrating mechanisms. These include the carboxysome in cyanobacteria [12], the pyrenoid in algae and hornworts [13], as well as crassulacean acid metabolism [14] and C_4 photosynthesis in angiosperms.

The C_4 pathway results from a series of metabolic and structural adjustments to leaves that together concentrate CO_2 around RuBisCO. In doing so, photorespiration is reduced, less water is lost per unit of carbon fixed, and considerably lower amounts of RuBisCO and therefore nitrogen are accumulated per unit leaf area [15]. Despite its complexity, the C_4 pathway has evolved independently in more than 60 lineages that span 18 plant families [16], making it one of the most remarkable examples of convergent evolution found in biology. It is thought that the evolution of C_4 photosynthesis relied on a series of coordinated modifications to leaf anatomy, cell biology and biochemistry [17]. However, the basic components, including enzymes of the C_4 pathway, are present in species that use the ancestral C_3 pathway [18]. In this review, we summarize our current understanding of the role of C_4 proteins in C_3 species and the regulation of genes encoding these proteins. From these findings, we propose that rewiring of pre-existing metabolic and genetic networks has facilitated the evolution of this novel metabolic pathway.

2. The biochemistry and evolution of C_4 photosynthesis

In the majority of C_4 plants, CO_2 assimilation is divided between mesophyll (M) and bundle sheath (BS) cells [15]. CO_2 is first converted to HCO_3^- by carbonic anhydrase (CA) and then combined with phosphoenolpyuvate (PEP) by PEP-carboxylase (PEPC) in the M to generate the four-carbon acid oxaloacetate (OAA). Metabolism of OAA to either aspartate or malate is followed by diffusion to the BS where RuBisCO is localized. Decarboxylation of C_4 acids typically releases a three-carbon acid and high concentrations of CO_2 (figure 1). The three-carbon acid diffuses back to the M where conversion to PEP by pyruvate, orthophosphate dikinase (PPDK) allows the C_4 cycle to continue. O_2 does not react with PEPC and so CO_2 fixation occurs in the absence of oxygenation. Three different C_4 acid decarboxylase enzymes are known to operate in the C_4 pathway:

NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME) and PEP-carboxykinase (PCK) (figure 1). Although there is some dominance in the use of individual C_4 acid decarboxylases, apparently associated with different C_4 lineages, most species use a mixture of the three decarboxylases, the make-up of which varies depending on environmental conditions [19–21].

Most estimates suggest that in both monocots and eudicots, the earliest origins of C_4 photosynthesis occurred approximately 25–30 Ma during the mid-Oligocene [22,23]. An abrupt reduction in the concentration of atmospheric CO_2 during this period is thought to have favoured natural selection for the C_4 pathway [2,23]. However, over the next 20–30 Myr, the C_4 pathway continued to evolve in other lineages, suggesting that low CO_2 concentrations acted as a preconditioning event rather than the sole trigger for C_4 evolution [16]. Other factors such as high temperatures, salinity and fire frequency in tropical and subtropical regions have been proposed to contribute to the polyphyletic evolution of C_4 photosynthesis [24].

Gene duplication followed by selection or genetic drift are considered important sources for the appearance of new traits [25]. After duplication, most redundant genes tend to be lost as they do not reach sufficient frequencies to become fixed in a population [26,27]. However, those genes that are retained can acquire new functions (neofunctionalization) or mutate to control more than one function (subfunctionalization). Mechanistically, either can occur via changes in *cis*-regulatory control or through alterations to coding regions resulting in the production of new function [25,28–31]. Gene duplications may therefore have occurred prior to the appearance of the C_4 pathway and facilitated its evolution [2,32]. However, until recently the lack of genome sequences for closely related C_3 and C_4 species precluded accurate assessments of these phenomena, and so evidence for gene duplication followed by neofunctionalization playing a major role in the evolution of core C_4 genes was lacking [33–35]. Subsequently, approaches that accurately localized gene duplication events across gene families [36,37] have revealed that in monocotyledons, many C_4 cycle genes appear to have duplicated in the last common ancestor of lineages containing C_4 plants [38]. There is also evidence that C_4 photosynthesis is built on pre-existing components. For example, it makes use of M and BS cells, both of which exist in ancestral C_3 leaves. Furthermore, all the enzymes of the C_4 pathway identified to date operate in C_3 species [18]. Indeed, a number of models depicting evolutionary trajectories from C_3 to C_4 photosynthesis have been developed in recent years [2,16,39–41]. Although these models take contrasting approaches and focus on slightly different aspects of the C_4 system, overall they support the notion that anatomical modifications tended to precede a series of modular changes to metabolic networks that led to evolution of the full C_4 pathway. The ancestral role of C_4 enzymes in C_3 metabolism, from which these evolutionary changes take place, will next be discussed.

3. Characteristics of the C_4 pathway in C_3 plants

BS cells of C_3 species such as rice and barley are capable of carrying out photosynthesis and starch synthesis [42–46]. It is estimated that chloroplasts in BS and M cells of rice contain similar amounts of RuBisCO [47]. Downregulation of chlorophyll synthase in cells associated with the vasculature of C_3 *Arabidopsis thaliana* showed that photosynthetic capacity of these cells makes an important contribution to plant growth and seed production [48]. Thus, although the BS in C_3 species is most commonly associated with controlling fluxes of nitrogen, sulfur and water into and out of the leaf [49,50], these results suggest photosynthetic activity contributes significantly to plant fitness. In fact, in a number of species widely distributed from across the land plant phylogeny, cells associated with the vasculature show some characteristics of the C_4 pathway. In stems and petioles of celery and tobacco, cells of the mid-vein allow the decarboxylation of organic acids coming from the vasculature and thus release CO_2 around RuBisCO for use in photosynthesis [51]. These attributes have also been observed in *Arabidopsis* and rice leaves [52,53]. In each case, cells associated with veins are photosynthetically active and contain significant activities of C_4 acid decarboxylases [51–53]. In the case of rice, just as with the BS of certain C_4 species, linear electron transport from photosystem II to photosystem I is reduced in these veinal cells [53]. Thus, BS cells around veins of C_3 plants are photosynthetic, but they also contain multiple characteristics more commonly associated with the C_4 pathway.

4. The ancestral role of C_4 proteins in C_3 plants

The fact that core C_4 enzymes are present in C_3 species meant that they did not need to evolve de novo and so likely facilitated the recurrent evolution of the C_4 pathway across land plants. The role of these proteins in C_3 species prior to their recruitment into C_4 photosynthesis has been addressed recently [18,54]. We therefore next focus on discussing how groups of C_4 proteins could have been recruited from pre-existing metabolic networks occurring in C_3 species.

Gluconeogenesis is fundamental to all life, and in plants is particularly important in allowing conversion of storage lipids and proteins into sugars during germination and seedling establishment. Traditionally, it was considered that a single route meditated by PCK allowed the conversion of OAA to PEP, and thus for carbon to enter gluconeogenesis in plants [55–58]. However, disruption of PCK1 function in *A. thaliana* has only a small effect on early seedling growth [56]. Transcripts derived from the *PPDK* gene, which encodes the protein catalysing the last committed step of the C_4 pathway, are also abundant during seedling establishment [59], and the timing and location of expression within the germinating seed are broadly similar to those derived from *PCK* [60]. A double *ppdk-pck1* mutant showed compromised movement of labelled carbon from storage lipids and proteins into sugars compared with wild-type, and also compared with each single mutant. In addition, seedling establishment was compromised [60]. Based on these findings, it is concluded that two routes into gluconeogenesis operate in C_3 plants, both involving proteins associated with C_4 photosynthesis (figure 2). It therefore appears that expression of the *PCK* and *PPDK* genes is coordinated to ensure proper functioning of gluconeogenesis in C_3 plants. We propose that an ancestral gene regulatory system present in C_3 species is used to ensure their high and coordinate activity in C_4 plants. Clearly, this regulatory system must alter somewhat as C_4 evolves. First, it must become operational in mature leaves rather than cotyledons. Second, enhancers of expression must move from the internal promoter that drives expression of cytosolic PPDK in

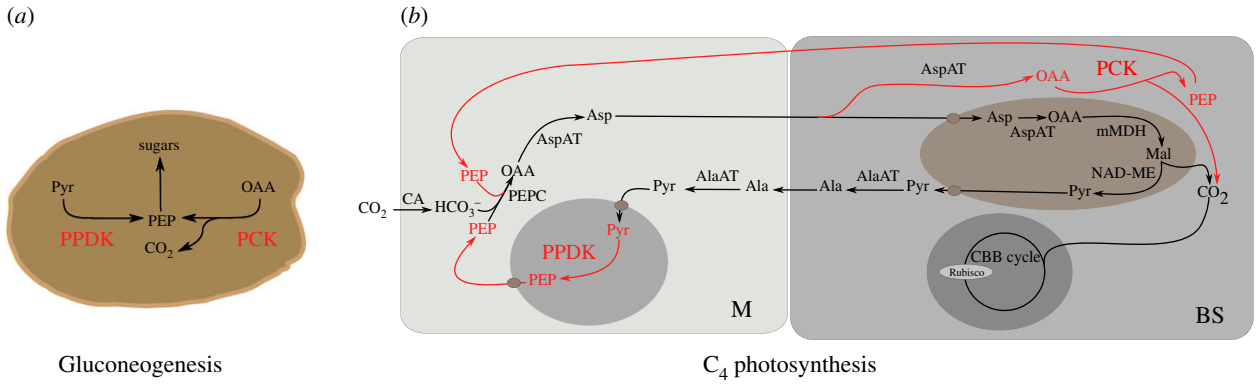

Figure 2. Genes associated with C_4 photosynthesis are coordinated in the ancestral C_3 state. (*a*) The enzymes PPDK and PCK (red) both act during gluconeogenesis in germinating *Arabidopsis* seedlings [60]. Both genes have been co-opted into the C_4 pathway (*b*) where PPDK regenerates PEP from pyruvate in the M and PCK acts as a C_4 acid decarboxylase releasing CO_2 around RuBisCO in the BS cells. Abbreviations as for figure 1.

C_3 seedlings to the distal promoter driving expression of chloroplastic PPDK in C_4 plants. Third, additional regulation must evolve to ensure that expression of the *PPDK* and *PCK* genes is restricted to M and BS cells, respectively. If additional genes encoding C_4 proteins are co-regulated in the ancestral C_3 state to allow the proteins they encode to function together in other metabolic pathways, this may well have further facilitated the evolution of this highly complex state. We next consider our understanding of mechanisms regulating C_4 genes in both C_3 and C_4 plants.

5. Recruitment of pre-existing gene regulatory networks

As with most traits, gene expression associated with the C_4 pathway is regulated at multiple levels, including epigenetic, transcriptional, post-transcriptional and post-translational [61,62]. However, it is unclear to what extent these mechanisms are already associated with C_4 genes in the ancestral C_3 state. It has long been clear that genes encoding proteins of the C_4 pathway respond to light [63–66]. Recently, it has become apparent that this key characteristic is found in the ancestral state. In C_3 *A. thaliana*, most genes encoding core C_4 proteins are regulated by light [67]. Furthermore, some C_4 genes are also subject to control by chloroplast-to-nucleus signalling [67]. Thus, two basic characteristics required for C_4 cycle genes to be coordinately expressed with other genes of C_3 photosynthesis are already in place in the ancestral C_3 state. Again, these networks need to be modified for an efficient C_4 system. First, compared with C_3 *A. thaliana*, in C_4 *Gynadropsis gynandra* (formerly designated *Cleome gynandra*), more C_4 cycle genes are controlled by the chloroplast. Second, although an existing system of light-regulation operates in C_3 species, this would need to be amplified in order that C_4 genes are expressed at sufficiently high levels in leaves undertaking C_4 photosynthesis.

In C_4 leaves, expression of C_4 genes is typically restricted to either M or BS cells [61]. For this to happen, *trans*-factors must recognize elements *in cis* in a cell-specific manner. For many years, it appeared that cell-specific expression in C_4 leaves was mediated by *cis*-elements that were not present in orthologous genes from C_3 leaves. For example, while the maize *PEPC* and *PPDK* genes are expressed in M cells, and *RbcS1A* expression is limited to BS cells, this was not the case for homologous genes in rice [64,68–71]. In addition, preferential expression of *PEPC* in the M cells of C_4 *Flaveria bidentis* is associated with two modifications *in cis* that generate an M-enhancing module (MEM1) [72]. However, it is now clear that multiple genes are expressed preferentially in M or BS cells of C_4 *G. gynandra* because of pre-existing *cis*-elements located in orthologous genes from *A. thaliana*. For example, both genes encoding the heterodimeric NAD-ME in *G. gynandra* contain elements in the coding sequence that determine BS expression, and these elements are found in the orthologues from *A. thaliana* [73,74]. The genes from *A. thaliana* are not preferentially expressed in the BS in the ancestral C_3 state, but they are when placed into leaves of C_4 *G. gynandra*. A similar situation has been found with *PPDK* and *CA* genes. Here, *cis*-regulatory elements located in untranslated regions generate preferential expression in M cells of C_4 *G. gynandra* [75,76]. Orthologous *CA* and *PPDK* genes from C_3 *A. thaliana* contain the same elements, and although they are silent in terms of cell specificity in C_3 leaves, when placed into C_4 *G. gynandra*, they lead to expression in the BS. In all these cases, the *cis*-elements are highly conserved in C_3 *A. thaliana*, suggesting that they carry out an important, but as yet undefined regulatory function. Taken together, these findings indicate that C_4 photosynthesis has on multiple occasions made use of *cis*-regulators found in C_3 species, and therefore that its evolution is based on alterations *in trans* as well as *in cis*.

Data accessibility. This article has no additional data.

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Competing interests. We declare we have no competing interests.

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References

1. Bassham JA, Benson AA, Kay LD, Harris AZ, Wilson AT, Calvin M. 1954 The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. *J. Am. Chem. Soc.* **76**, 1760–1770. (doi:10.1021/ja01636a012)

2. Sage RF. 2004 Tansley review: the evolution of C_4 photosynthesis. *New Phytol.* **161**, 30. (doi:10.1111/j.1469-8137.2004.00974.x)
3. Anbar AD *et al.* 2007 A whiff of oxygen before the great oxidation event? *Science* **317**, 1903–1906. (doi:10.1126/science.1140325)
4. Bekker A, Holland HD, Wang P-L, Rumble D, Stein HJ, Hannah JL, Coetzee LL, Beukes NJ. 2004 Dating the rise of atmospheric oxygen. *Nature* **427**, 117–120. (doi:10.1038/nature02260)
5. Canfield DE. 2005 The early history of atmospheric oxygen: homage to Robert M. Garrels. *Annu. Rev. Earth Planet. Sci.* **33**, 1–36. (doi:10.1146/annurev.earth.33.092203.122711)
6. Ubierna N, Sun W, Cousins AB. 2011 The efficiency of C_4 photosynthesis under low light conditions: assumptions and calculations with CO_2 isotope discrimination. *J. Exp. Bot.* **62**, 3119–3134. (doi:10.1093/jxb/err073)
7. Bauwe H, Hagemann M, Fernie AR. 2010 Photorespiration: players, partners and origin. *Trends Plant Sci.* **15**, 330–336. (doi:10.1016/j.tplants.2010.03.006)
8. Brooks A, Farquhar GD. 1985 Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406. (doi:10.1007/BF00392238)
9. Tcherkez GGB, Farquhar GD, Andrews TJ. 2006 Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc. Natl Acad. Sci. USA* **103**, 7246–7251. (doi:10.1073/pnas.0600605103)
10. Sage RF, Sharkey TD. 1987 The effect of temperature on the occurrence of O_2 and CO_2 insensitive photosynthesis in field grown plants. *Plant Physiol.* **84**, 658–664. (doi:10.1104/pp.84.3.658)
11. Sharwood RE, Ghannoum O, Kapralov MV, Gunn LH, Whitney SM. 2016 Temperature responses of Rubisco from Paniceae grasses provide opportunities for improving C_3 photosynthesis. *Nat. Plants* **2**, 16186. (doi:10.1038/nplants.2016.186)
12. Shively JM, Ball F, Brown DH, Saunders RE. 1973 Functional organelles in prokaryotes: polyhedral inclusions (carboxysomes) of *Thiobacillus neapolitanus*. *Science* **182**, 584–586. (doi:10.1126/science.182.4112.584)
13. Smith EC, Griffiths H. 1996 The occurrence of the chloroplast pyrenoid is correlated with the activity of a CO_2-concentrating mechanism and carbon isotope discrimination in lichens and bryophytes. *Planta* **198**, 6–16. (doi:10.1007/BF00197580)
14. Ranson SL, Thomas M. 1960 Crassulacean acid metabolism. *Annu. Rev. Plant Physiol.* **11**, 81–110. (doi:10.1146/annurev.pp.11.060160.000501)
15. Hatch MD, Slack CR. 1966 Photosynthesis by sugar-cane leaves. A new carboxylation reaction and the pathway of sugar formation. *Biochem. J.* **101**, 103–111. (doi:10.1042/bj1010103)
16. Sage RF, Sage TL, Kocacinar F. 2012 Photorespiration and the evolution of C_4 photosynthesis. *Annu. Rev. Plant Biol.* **63**, 19–47. (doi:10.1146/annurev-arplant-042811-105511)
17. Langdale JA. 2011 C_4 cycles: past, present, and future research on C_4 photosynthesis. *Plant Cell* **23**, 3879–3892. (doi:10.1105/tpc.111.092098)
18. Aubry S, Brown NJ, Hibberd JM. 2011 The role of proteins in C_3 plants prior to their recruitment into the C_4 pathway. *J. Exp. Bot.* **62**, 3049–3059. (doi:10.1093/jxb/err012)
19. Furbank RT. 2011 Evolution of the C_4 photosynthetic mechanism: are there really three C_4 acid decarboxylation types? *J. Exp. Bot.* **62**, 3103–3108. (doi:10.1093/jxb/err080)
20. Sommer M, Bräutigam A, Weber APM. 2012 The dicotyledonous NAD malic enzyme C_4 plant *Cleome gynandra* displays age-dependent plasticity of C_4 decarboxylation biochemistry. *Plant Biol.* **14**, 621–629. (doi:10.1111/j.1438-8677.2011.00539.x)
21. Wang Y, Bräutigam A, Weber APM, Zhu X-G. 2014 Three distinct biochemical subtypes of C_4 photosynthesis? A modelling analysis. *J. Exp. Bot.* **65**, 3567–3578. (doi:10.1093/jxb/eru058)
22. Christin P-A, Osborne CP, Sage RF, Arakaki M, Edwards EJ. 2011 C_4 eudicots are not younger than C_4 monocots. *J. Exp. Bot.* **62**, 3171–3181. (doi:10.1093/jxb/err041)
23. Christin PA, Besnard G, Samaritani E, Duvall MR, Hodkinson TR, Savolainen V, Salamin N. 2008 Oligocene CO_2 decline promoted C4 photosynthesis in grasses. *Curr. Biol.* **18**, 37–43. (doi:10.1016/j.cub.2007.11.058)
24. Edwards GE, Voznesenskaya EV. 2010 Chapter 4 C_4 photosynthesis: Kranz forms and single-cell C_4 in terrestrial plants. In C_4 photosynthesis and related CO_2 concentrating mechanisms (eds A Raghavendra, R Sage), *Adv. Photosynthesis Respir.* **32**, pp. 29–61. Dordrecht, The Netherlands: Springer.
25. Ohno S. 1970 *Evolution by gene duplication*, 2013th edn. Berlin, Germany: Springer Science & Business Media.
26. Thomas BC, Pedersen B, Freeling M. 2006 Following tetraploidy in an *Arabidopsis* ancestor, genes were removed preferentially from one homeolog leaving clusters enriched in dose-sensitive genes. *Genome Res.* **16**, 934–946. (doi:10.1101/gr.4708406)
27. Woodhouse MR, Schnable JC, Pedersen BS, Lyons E, Lisch D, Subramaniam S, Freeling M. 2010 Following tetraploidy in maize, a short deletion mechanism removed genes preferentially from one of the two homeologs. *PLoS Biol.* **8**, e1000409. (doi:10.1371/journal.pbio.1000409)
28. Arntz M, Delph L. 2001 Pattern and process: evidence for the evolution of photosynthetic traits in natural populations. *Oecologia* **127**, 455–467. (doi:10.1007/s004420100650)
29. Lynch M, Conery JS. 2000 The evolutionary fate and consequences of duplicate genes. *Science* **290**, 1151–1155. (doi:10.1126/science.290.5494.1151)
30. Moore RC, Purugganan MD. 2005 The evolutionary dynamics of plant duplicate genes. *Curr. Opin. Plant Biol.* **8**, 122–128. (doi:10.1016/j.pbi.2004.12.001)
31. Freeling M, Lyons E, Pedersen B, Alam M, Ming R, Lisch D. 2008 Many or most genes in *Arabidopsis* transposed after the origin of the order Brassicales. *Genome Res.* **18**, 1924–1937. (doi:10.1101/gr.081026.108)
32. Monson RK. 2003 Gene duplication, neofunctionalization, and the evolution of C_4 photosynthesis. *Int. J. Plant Sci.* **164**, S43–S54. (doi:10.1086/368400)
33. Gowik U, Westhoff P. 2011 The path from C_3 to C_4 photosynthesis. *Plant Physiol.* **155**, 56–63. (doi:10.1104/pp.110.165308)
34. van den Bergh E, Külahoglu C, Bräutigam A, Hibberd JM, Weber APM, Zhu X-G, Eric Schranz M. 2014 Gene and genome duplications and the origin of C_4 photosynthesis: birth of a trait in the Cleomaceae. *Curr. Plant Biol.* **1**, 2–9. (doi:10.1016/j.cpb.2014.08.001)
35. Williams BP, Aubry S, Hibberd JM. 2012 Molecular evolution of genes recruited into C_4 photosynthesis. *Trends Plant Sci.* **17**, 213–220. (doi:10.1016/j.tplants.2012.01.008)
36. Boussau B, Szollosi GJ, Duret L, Gouy M, Tannier E, Daubin V. 2013 Genome-scale coestimation of species and gene trees. *Genome Res.* **23**, 323–330. (doi:10.1101/gr.141978.112)
37. Emms DM, Kelly S. 2015 OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biol.* **16**, 157. (doi:10.1186/s13059-015-0721-2)
38. Emms DM, Covshoff S, Hibberd JM, Kelly S. 2016 Independent and parallel evolution of new genes by gene duplication in two origins of C_4 photosynthesis provides new insight into the mechanism of phloem loading in C_4 species. *Mol. Biol. Evol.* **33**, 1796–1806. (doi:10.1093/molbev/msw057)
39. Williams BP, Johnston IG, Covshoff S, Hibberd JM. 2013 Phenotypic landscape inference reveals multiple evolutionary paths to C_4 photosynthesis. *eLife* **2**, e00961. (doi:10.7554/eLife.00961)
40. Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber APM, Lercher MJ. 2013 Predicting C_4 photosynthesis evolution: modular, individually adaptive steps on a Mount Fuji fitness landscape. *Cell* **153**, 1579–1588. (doi:10.1016/j.cell.2013.04.058)
41. Mallmann J, Heckmann D, Bräutigam A, Lercher MJ, Weber APM, Westhoff P, Gowik U. 2014 The role of photorespiration during the evolution of C_4 photosynthesis in the genus *Flaveria*. *eLife* **3**, e02478. (doi:10.7554/eLife.02478)
42. Miyake H, Maeda E. 1976 Development of bundle sheath chloroplasts in rice seedlings. *Can. J. Bot.* **54**, 556–565. (doi:10.1139/b76-056)
43. Williams ML, Farrar JF, Pollock CJ. 1989 Cell specialization within the parenchymatous bundle sheath of barley. *Plant Cell Environ.* **12**, 909–918. (doi:10.1111/j.1365-3040.1989.tb01970.x)
44. Koroleva OA, Farrar JF, Tomos AD, Pollock CJ. 1998 Carbohydrates in individual cells of epidermis, mesophyll, and bundle sheath in barley leaves with changed export or photosynthetic rate. *Plant Physiol.* **118**, 1525–1532. (doi:10.1104/pp.118.4.1525)

45. Tsutsumi K, Taniguchi Y, Kawasaki M, Taniguchi M, Miyake H. 2006 Expression of photosynthesis-related genes during the leaf development of a C_3 plant rice as visualized by in situ hybridization. *Plant Prod. Sci.* **9**, 232–241. (doi:10.1626/pps.9.232)
46. Miyake H. 2016 Starch accumulation in the bundle sheaths of C_3 plants: a possible pre-condition for C_4 photosynthesis. *Plant Cell Physiol.* **57**, 890–896. (doi:10.1093/pcp/pcw046)
47. Yamane K *et al.* 2003 Bundle sheath chloroplasts of rice are more sensitive to drought stress than mesophyll chloroplasts. *J. Plant Physiol.* **160**, 1319–1327. (doi:10.1078/0176-1617-01180)
48. Janacek SH *et al.* 2009 Photosynthesis in cells around veins of the C_3 plant *Arabidopsis thaliana* is important for both the shikimate pathway and leaf senescence as well as contributing to plant fitness. *Plant J.* **59**, 329–343. (doi:10.1111/j.1365-313X.2009.03873.x)
49. Leegood RC. 2008 Roles of the bundle sheath cells in leaves of C_3 plants. *J. Exp. Bot.* **59**, 1663–1673. (doi:10.1093/jxb/erm335)
50. Griffiths H, Weller G, Toy LFM, Dennis RJ. 2013 You're so vein: bundle sheath physiology, phylogeny and evolution in C_3 and C_4 plants. *Plant Cell Environ.* **36**, 249–261. (doi:10.1111/j.1365-3040.2012.02585.x)
51. Hibberd JM, Quick WP. 2002 Characteristics of C_4 photosynthesis in stems and petioles of C_3 flowering plants. *Nature* **415**, 451–454. (doi:10.1038/415451a)
52. Brown NJ *et al.* 2010 C_4 acid decarboxylases required for C_4 photosynthesis are active in the mid-vein of the C_3 species *Arabidopsis thaliana*, and are important in sugar and amino acid metabolism. *Plant J.* **61**, 122–133. (doi:10.1111/j.1365-313X.2009.04040.x)
53. Shen W, Ye L, Ma J, Yuan Z, Zheng B, Chuangen LV, Zhu Z, Chen X, Gao Z, Chen G. 2016 The existence of C4-bundle-sheath-like photosynthesis in the mid-vein of C_3 rice. *Rice* **9**, 20.
54. Maier A, Zell MB, Maurino VG. 2011 Malate decarboxylases: evolution and roles of NAD(P)-ME isoforms in species performing C_4 and C_3 photosynthesis. *J. Exp. Bot.* **62**, 3061–3069. (doi:10.1093/jxb/err024)
55. Leegood RC, Ap Rees T. 1978 Phosphoenolpyruvate carboxykinase and gluconeogenesis in cotyledons of *Cucurbita pepo*. *Biochim. Biophys. Acta Enzymol.* **524**, 207–218. (doi:10.1016/0005-2744(78)90119-5)
56. Rylott EL, Gilday AD, Graham IA. 2003 The gluconeogenic enzyme phosphoenolpyruvate carboxykinase in *Arabidopsis* is essential for seedling establishment. *Plant Physiol.* **131**, 1834–1842. (doi:10.1104/pp.102.019174)
57. Penfield S, Rylott EL, Gilday AD, Graham S, Larson TR, Graham IA. 2004. Reserve mobilization in the *Arabidopsis* endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires *PHOSPHOENOLPYRUVATE CARBOXYKINASE*$_1$. *Plant Cell* **16**, 2705–2718. (doi:10.1105/tpc.104.024711)
58. Malone S, Chen Z-H, Bahrami AR, Walker RP, Gray JE, Leegood RC. 2007 Phosphoenolpyruvate carboxykinase in *Arabidopsis*: changes in gene expression, protein and activity during vegetative and reproductive development. *Plant Cell Physiol.* **48**, 441–450. (doi:10.1093/pcp/pcm014)
59. Parsley K, Hibberd JM. 2006 The *Arabidopsis* PPDK gene is transcribed from two promoters to produce differentially expressed transcripts responsible for cytosolic and plastidic proteins. *Plant Mol. Biol.* **62**, 339–349. (doi:10.1007/s11103-006-9023-0)
60. Eastmond PJ *et al.* 2015 *Arabidopsis* uses two gluconeogenic gateways for organic acids to fuel seedling establishment. *Nat. Commun.* **6**, 6659. (doi:10.1038/ncomms7659)
61. Hibberd JM, Covshoff S. 2010 The regulation of gene expression required for C_4 photosynthesis. *Annu. Rev. Plant Biol* **61**, 181–207. (doi:10.1146/annurev-arplant-042809-112238)
62. Reeves G, Grangé-Guermente MJ, Hibberd JM. 2016 Regulatory gateways for cell-specific gene expression in C_4 leaves with Kranz anatomy. *J. Exp. Bot.* **68**, 107–116. (doi:10.1093/jxb/erw438)
63. Collins PD, Hague DR. 1983 Light-stimulated synthesis of NADP malic enzyme in leaves of maize. *J. Biol. Chem.* **258**, 4012–4018.
64. Sheen J-Y, Bogorad L. 1987 Regulation of levels of nuclear transcripts for C_4 photosynthesis in bundle sheath and mesophyll cells of maize leaves. *Plant Mol. Biol.* **8**, 227–238. (doi:10.1007/BF00015031)
65. Langdale JA, Zelitch I, Miller E, Nelson T. 1988 Cell position and light influence C4 versus C3 patterns of photosynthetic gene expression in maize. *EMBO J.* **7**, 3643–3651.
66. Long JJ, Berry JO. 1996 Tissue-specific and light-mediated expression of the C_4 photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. *Plant Physiol.* **112**, 473–482. (doi:10.1104/pp.112.2.473)
67. Burgess SJ, Granero-Moya I, Grangé-Guermente MJ, Boursnell C, Terry MJ, Hibberd JM. 2016 Ancestral light and chloroplast regulation form the foundations for C_4 gene expression. *Nat. Plants* **2**, 16161. (doi:10.1038/nplants.2016.161)
68. Matsuoka M, Tada Y, Fujimura T, Kano-Murakami Y. 1993 Tissue-specific light-regulated expression directed by the promoter of a C_4 gene, maize pyruvate,orthophosphate dikinase, in a C_3 plant, rice (C_4 photosynthesis/gene evolution/transgenic rice). *Proc. Natl Acad. Sci. USA* **90**, 9586–9590.
69. Nomura M *et al.* 2000 The evolution of C_4 plants: acquisition of *cis*-regulatory sequences in the promoter of C_4-type pyruvate, orthophosphate dikinase gene. *Plant J.* **22**, 211–221. (doi:10.1046/j.1365-313x.2000.00726.x)
70. Nomura M, Katayama K, Nishimura A, Ishida Y, Ohta S, Komari T, Miyao-Tokutomi M, Tajima S, Matsuoka M. 2000 The promoter of *rbcS* in a C_3 plant (rice) directs organ-specific, light-dependent expression in a C_4 plant (maize), but does not confer bundle sheath cell-specific expression. *Plant Mol. Biol.* **44**, 99–106. (doi:10.1023/A:1006461812053)
71. Taniguchi M *et al.* 2000 The promoter for the maize C_4 pyruvate,orthophosphate dikinase gene directs cell- and tissue-specific transcription in transgenic maize plants. *Plant Cell Physiol.* **41**, 42–48. (doi:10.1093/pcp/41.1.42)
72. Gowik U, Burscheidt J, Akyildiz M, Schlue U, Koczor M, Streubel M, Westhoff P. 2004 *cis*-Regulatory elements for mesophyll-specific gene expression in the C_4 plant *Flaveria trinervia*, the promoter of the C_4 phosphoenolpyruvate carboxylase gene. *Plant Cell* **16**, 1077–1090. (doi:10.1105/tpc.019729)
73. Brown NJ, Newell CA, Stanley S, Chen JE, Perrin AJ, Kajala K, Hibberd JM. 2011 Independent and parallel recruitment of preexisting mechanisms underlying C_4 photosynthesis. *Science* **331**, 1436–1439. (doi:10.1126/science.1201248)
74. Reyna-Llorens I, Burgess SJ, Williams BP, Stanley S, Boursnell C, Hibberd JM. 2016 Ancient coding sequences underpin the spatial patterning of gene expression in C4 leaves. *bioR* **xiv**, 085795. (doi:10.1101/085795)
75. Kajala K, Brown NJ, Williams BP, Borrill P, Taylor LE, Hibberd JM. 2012 Multiple *Arabidopsis* genes primed for recruitment into C_4 photosynthesis. *Plant J.* **69**, 47–56. (doi:10.1111/j.1365-313X.2011.04769.x)
76. Williams B, Burgess S, Reyna-Llorens I. 2016 An untranslated *cis*-element regulates the accumulation of multiple C_4 enzymes in *Gynandropsis gynandra* mesophyll cells. *Plant Cell* **28**, 454–465. (doi:10.1105/tpc.15.00570)