Re-creation of a Key Step in the Evolutionary Switch from C₃ to C₄ Leaf Anatomy

Highlights
- Organelle development in rice vascular sheath cells is induced by maize GLK genes
- Cells with activated organelles form more plasmodesmatal connections than wild-type
- GLK-induced anatomy mimics a key step in the evolutionary transition from C₃ to C₄
- The intermediate proto-Kranz leaf anatomy appears to bear no fitness cost in rice

Authors
Peng Wang, Roxana Khoshravesh, Shanta Karki, ..., Robert Furbank, Tammy L. Sage, Jane A. Langdale

Correspondence
tammy.sage@utoronto.ca (T.L.S.), jane.langdale@plants.ox.ac.uk (J.A.L.)

In Brief
Wang et al. report that constitutive expression of maize GOLDEN2-like genes induces a suite of traits in C₃ rice leaves that is reminiscent of proto-Kranz anatomy, an intermediate state in the evolutionary trajectory from C₃ to C₄. A key step toward engineering C₄ rice has thus been achieved.
Re-creation of a Key Step in the Evolutionary Switch from C₃ to C₄ Leaf Anatomy

Peng Wang,1,9 Roxana Khoshravesh,2,9 Shanta Karki,3,7,9 Ronald Tapia,3,4 Robert Furbank,5,6 Tammy L. Sage,2,* and Jane A. Langdale1,10,*  
1Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK  
2International Rice Research Institute (IRRI), Los Banos 4030, Laguna, the Philippines  
3Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK  
4ARC Centre of Excellence for Translational Photosynthesis, Research School of Biology, Australian National University, Acton, ACT 2601, Australia  
5CSIRO, Canberra, ACT 2601, Australia  
6Present address: Ministry of Agricultural Development, Government of Nepal, Singhadurbar, Kathmandu, Nepal  
7Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON M5S3B2, Canada  
8These authors contributed equally  
9Lead Contact  
*Correspondence: tammy.sage@utoronto.ca (T.L.S.), jane.langdale@plants.ox.ac.uk (J.A.L.)  
https://doi.org/10.1016/j.cub.2017.09.040

SUMMARY

The C₄ photosynthetic pathway accounts for ~25% of primary productivity on the planet despite being used by only 3% of species. Because C₄ plants are higher yielding than C₃ plants, efforts are underway to introduce the C₄ pathway into the C₃ crop rice. This is an ambitious endeavor; however, the C₄ pathway evolved from C₃ on multiple independent occasions over the last 30 million years, and steps along the trajectory are evident in extant species. One approach toward engineering C₄ rice is to recapitulate this trajectory, one of the first steps of which was a change in leaf anatomy. The transition from C₃ to so-called “proto-Kranz” anatomy requires an increase in organelle volume in sheath cells surrounding leaf veins. Here we induced chloroplast and mitochondrial development in rice vascular sheath cells through constitutive expression of maize GOLDEN2-LIKE genes. Increased organelle volume was accompanied by the accumulation of photosynthetic enzymes and by increased intercellular connections. This suite of traits reflects that seen in “proto-Kranz” species, and, as such, a key step toward engineering C₄ rice has been achieved.

INTRODUCTION

The C₄ photosynthetic pathway evolved over 60 times independently in a diverse range of flowering plant species, with trajectories from C₃ to C₄ apparent in a number of lineages [1]. In most C₄ plants, photosynthetic reactions are compartmentalized between two cell types that are arranged in concentric wreaths around closely spaced veins. This cellular arrangement, which is referred to as Kranz anatomy [2, 3], facilitates initial fixation of carbon dioxide in the outer mesophyll cells followed by decarboxylation and refixation by ribulose bisphosphate carboxylase/oxygenase (RuBisCo) in the CO₂-enriched environment of the inner sheath cells (reviewed in [4]). In most C₄ species, the bundle sheath cells surrounding the vein are the site of refixation, but in some grasses an inner layer of mestome sheath cells with thick suberized cell walls play this role (either instead of or in addition to [5, 6, 7] the bundle sheath cells). A critical step in the evolution of C₄ was thus the functionalization of bundle sheath and/or mestome sheath cells for photosynthesis (reviewed in [8, 9]).

A long-term project is underway to introduce the C₄ pathway into the C₃ crop rice, with predictions of up to a 50% yield increase if successful [10, 11] (https://c4rice.com). One of the major challenges in this regard is the need to introduce Kranz anatomy into the rice leaf. Morphological analyses of extant species suggest that Kranz evolved in a stepwise fashion, with “proto-Kranz”- and “C₂”- type anatomies representing intermediate steps along the C₃-to-C₄ trajectory [9]. In both monocots [12] and eudicots [13–16], proto-Kranz anatomy is characterized by increased organelle volume in the bundle sheath and/or mestome sheath cells around the leaf vasculature (collectively referred to as vascular sheath cells), with chloroplasts accumulating the photosynthetic enzyme RuBisCo and mitochondria accumulating the photorespiratory enzyme glycine decarboxylase. In the grasses, proto-Kranz species exist in the “PACMAD” clade, in which 24 independent origins of C₄ have been identified [1, 12, 17, 18]. By contrast, there are no proto-Kranz or C₄ species in the sister clade, to which rice belongs. As such, a rational first step toward engineering C₄ rice is to induce proto-Kranz by activating chloroplast and mitochondrial biogenesis in vascular sheath cells.

GOLDEN2-like (GLK) transcription factors regulate chloroplast development in all land plant species examined [19–25], and...
ectopic expression of OsGLK1 activates chloroplast biogenesis in rice vascular sheath cells [26]. However, enhanced chloroplast development in overexpression lines is not sustained beyond the seedling stage [26], suggesting that ectopic OsGLK1 activity is suppressed by one of the post-transcriptional mechanisms that balance GLK-induced chloroplast development with leaf senescence [27–29]. On the basis of these findings, we postulated that sustained chloroplast development in the vascular sheath cells of rice might be achieved through the introduction of one of the two heterologous maize GLK genes (either ZmG2 or ZmGLK1 [20]). Here we validate that hypothesis and further demonstrate that enhanced chloroplast development leads to enhanced mitochondrial biogenesis, with both types of organelle accumulating metabolic enzymes. A proto-Kranz rice line has thus been generated.

RESULTS

Constitutive Expression of ZmG2 or ZmGLK1 in Rice Induces Greening of Callus and Roots

To determine whether maize GLK gene function is sufficient to promote enhanced chloroplast development in rice, ZmG2 or ZmGLK1 transgenes were introduced under the control of constitutive or cell type preferential promoters. The maize UBIQUITIN promoter (ZmUBIpro) was used to drive constitutive expression [30], the Zoysia japonica PHOSPHOENOLPYRUVATE CARBOXYKINASE promoter (ZjPCKpro) for preferential expression in bundle sheath cells [31], and the maize PHOSPHOENOLPYRUVATE CARBOXYLASE promoter (ZmPEPCpro) for preferential expression in mesophyll cells [32]. Constructs were transformed into both Oryza sativa spp. indica cultivar IR64 and spp. japonica cultivar Kitaake (Figure S1; Table S1). A phenotypic consequence of constitutive expression was immediately apparent in that transgenic calli were green, whereas wild-type calli and those transformed with cell type preferential promoter constructs remained yellow (Figures 1A–1G). The green calli were generated solely in the presence of the auxin analog 2,4-D, by-passing the requirement for cytokinin that is normally needed for greening of yellow callus. When cytokinin was added to the green calli, shoot regeneration occurred with a higher frequency than from wild-type callus (Figures 1H–1J). Embryos, shoots, and roots of germinating T1 seedlings that constitutively expressed ZmG2 or ZmGLK1 were also greener than wild-type (Figures 1K–1P). Constitutive expression of ZmG2 or ZmGLK1 in rice is, therefore, sufficient to induce greening in tissues where chloroplasts don’t usually develop (callus, embryos, and roots) and to enhance greening in shoots.
Maize GLK Gene Expression Significantly Increases the Volume of Functional Chloroplasts in Both Bundle Sheath and Mestome Sheath Cells of Rice

The enhanced greening phenotype in transgenic rice lines expressing maize GLK genes could reflect increases in chloroplast size and/or number. To distinguish these possibilities, quantitative measurements were taken from the fourth and seventh leaves of cv. Kitaake lines that either constitutively expressed ZmG2 or ZmGLK1 or mimicked the expression profile observed in maize (namely, ZmG2 expression preferentially in bundle sheath cells and ZmGLK1 expression preferentially in mesophyll cells). Individual leaf samples were first analyzed for total chlorophyll levels (Figure 2A) and for both transgene and endogenous OsGLK1/2 transcript levels (Figure S2). Both chloroplast size and number were then measured in bundle sheath cells that were isolated from the same leaves (Figure 2; Table S2). Chloroplast size was significantly increased in bundle sheath cells relative to wild-type at both stages of development and when either ZmG2 or ZmGLK1 was constitutively expressed (Figures 2A–2E; Table S2). Intriguingly the size increase induced by ZmGLK1 was manifest regardless of whether gene expression was driven by the constitutive ZmUBIpro or the mesophyll preferential ZmPEPCpro. This suggests either that the effect on bundle sheath chloroplast size was induced non-cell autonomously or that the ZmPEPCpro drove enough gene expression in the bundle sheath cells to have a phenotypic effect. Given that GLK proteins are known to act cell autonomously in Arabidopsis [33], it is most likely that the ZmPEPCpro drives gene expression in rice bundle sheath cells but that transcript levels are so low that promoter

Figure 2. Chloroplast Size, but Not Number, Is Increased in Bundle Sheath Cells of ZmG2 and ZmGLK1 Overexpression Lines

(A) Relationship between total leaf chlorophyll content and chloroplast planar area in bundle sheath cells of wild-type (WT) and T1 transgenic lines. Values are mean ± SEM (n = 3 individuals for chlorophyll content; n = 45 cells [15 cells/3 individuals] for chloroplast area). Chloroplast size was significantly increased in leaf 7 of all four transgenic lines relative to WT and in leaf 4 of lines constitutively expressing ZmG2 or ZmGLK1 (by a Games-Howell test of variance, p < 0.05). The phenotypic consequences of relatively weak transgene expression from the BS-preferential ZjPCKpro were less dramatic than with the constitutive promoters. (B–E) Representative bundle sheath (B and C) and mesophyll (D and E) cells from WT (B and D) and ZmUBIpro:ZmG2 (C and E) lines. Scale bars, 10 μm. (F and G) Boxplots showing chloroplast numbers in bundle sheath cells of leaf 4 (F) and leaf 7 (G) (n = 45 cells [15 cells from each of 3 individuals representing at least two independent lines] in each case). Boxplot whiskers indicate the range of observations. See Table S1 for details of transgenic lines used, Figure S1 for transgene copy number analysis, Figure S2 for transgene transcript levels, and Table S2 for a complete set of measurements.
activity has not been detected in previous reporter gene assays [32]. Quantification of chloroplast numbers per bundle sheath cell and of bundle sheath cell volume revealed no statistical difference between wild-type and any of the transgenic lines (Figures 2F and 2G; Table S2). Enhanced greening in transgenic lines is thus associated with an increase in chloroplast size that is unaccompanied by a decrease in chloroplast number, resulting in a larger chloroplast volume per bundle sheath cell relative to wild-type.

Transmission electron microscopy (TEM) of leaf sections from wild-type (Figure 3A) and ZmUBI_pro:ZmG2 transgenic (Figure 3B) lines confirmed that bundle sheath chloroplast volume is increased when ZmG2 is expressed and revealed a similar distinction in mestome sheath cells (Figures 3A and 3B; Table S2). In wild-type leaves, only small undifferentiated plastids (proplastids) are found in mestome sheath cells (Figure 3C), but highly organized thylakoid systems were apparent in mestome sheath chloroplasts of ZmUBI_pro:ZmG2 leaves (Figure 3D), with the area of chloroplast coverage per cell being equivalent to that seen in the bundle sheath cells (Table S3). Mesophyll cell chloroplast size was indistinguishable between wild-type and transgenic lines (Table S3). To determine whether enhanced chloroplast development in vascular sheath cells was accompanied by the accumulation of photosynthetic enzymes, antibodies against RuBisCo, RuBisCo activase, and fructose-1,6-bisphosphatase were reacted with tissue sections. Notably, all three enzymes could be detected in both bundle sheath and mestome sheath chloroplasts of ZmUBI_pro:ZmG2 transgenic plants (Figures 3C–3F; Figure S3), with levels in mestome sheath cells being significantly higher than in wild-type (Table 1). Constitutive expression of ZmG2 in rice is thus sufficient to induce the development of both bundle sheath and mestome sheath chloroplasts with photosynthetic capacity.

GLK-Induced Chloroplast Development in Rice Vascular Sheath Cells Is Accompanied by Enhanced Biogenesis of Mitochondria and Plasmodesmata

Qualitative analysis of TEM sections indicated that mitochondrial size and the frequency of plasmodesmata between cells were both increased in bundle sheath and mestome sheath cells of transgenic lines that constitutively expressed ZmG2 (Figures 4A–4D). Quantification of mitochondrial size confirmed this suggestion and also demonstrated that mitochondrial number did not differ between wild-type and transgenic lines (Figure 4E; Tables S3 and S4). As with chloroplasts, the significant increase in mitochondrial volume represented enhanced functional

Figure 3. Chloroplasts in Both Bundle Sheath and Mestome Sheath Cells of Lines that Constitutively Express ZmG2 Accumulate Photosynthetic Enzymes

(A and B) Transmission electron micrographs of minor veins in leaf 7 of wild-type (WT) (A) and ZmUBI_pro:ZmG2 transgenic (B) plants grown at 300 μmol photons m−2 s−1. The position of bundle sheath (BS), mestome sheath (MS), and mesophyll (M) cells is indicated, with arrows pointing to representative chloroplasts and arrowheads pointing to mitochondria in each cell type.

(C–F) Transmission electron micrographs of WT (C and E) and ZmUBI_pro:ZmG2 transgenic (D and F) lines showing plastid ultrastructure and immuno-gold labeling with RuBisCo antibody in MS (C and D) and BS (E and F) cells. Purple arrow, proplastid; red arrows, organized thylakoid stacks.

Scale bars, 2 μm (A and B) and 500 nm (C–F).

See Table 1 for quantification of immuno-gold labeling and Figure S3 for immuno-labeling with additional enzymes. See also Figures S1 and S2 and Tables S1 and S3.
The anatomical traits induced by constitutive ZmG2 or ZmGLK1 expression in rice mimic those seen in proto-Kranz species.

To assess the extent to which the vascular sheath cell phenotype of transgenic rice lines represents a transition along the C₃-to-C₄ trajectory, organellar composition was quantified in C₃, proto-Kranz, and C₄ species of both grass and eudicot clades. In proto-Kranz species, 4%–9% of the chloroplast planar area by a two-tailed t test ($p < 0.05$) translates into a 2- to 3-fold increase in the density of RuBisCo and RuBisCo activase per BS cell in ZmUBIpro:ZmG2 lines relative to WT. More substantial increases are observed in mestome sheath (MS) cells. See also Figures S2 and S3 and Table S3.

The evolutionary trajectory from C₃ to C₄ most likely occurred in a stepwise fashion, with the order of events differing between lineages [8, 16, 17, 37, 38]. Combined evidence from qualitative, quantitative, and modeling approaches suggests, however, that the unifying early step in all trajectories was the transition from C₃ to proto-Kranz [9, 12, 14, 15]. To recapitulate this step, organellar volume and photosynthetic/photorespiratory enzyme content in sheath cells surrounding the leaf veins must be increased. Constitutive expression of maize GLK genes in rice caused ectopic chloroplast development in cells around the vascular bundle, with photosynthetic enzymes accumulating to elevated levels in both bundle sheath and mestome sheath cells (Figures 1, 2, and 3; Table 1; Figure S3). Similarly, ZmUBIpro:ZmG2 and ZmUBIpro:ZmGLK1 transgenic lines have 4%–6% of leaf chloroplast area and 21% of leaf mitochondria area invested in vascular sheath cells, a 2.5- to 3-fold increase relative to wild-type (Figure 5; Table S5). As seen in other examples where C₃ and proto-Kranz species have similar vascular sheath cell sizes (e.g., [14, 15]), increased organellar area in the transgenic rice lines was not associated with altered cell size. Collectively, these results demonstrate that constitutive expression of either ZmG2 or ZmGLK1 is sufficient to induce proto-Kranz anatomy in rice, recapitulating one of the earliest steps in C₄ evolution.

**Proto-Kranz Anatomy in Rice Bears No Fitness Cost**

To determine whether the proto-Kranz anatomy that is induced by expression of ZmG2 or ZmGLK1 genes in rice has an impact at the whole-plant level, assays were conducted to measure photosynthetic efficiency and yield. These experiments were conducted in near-field conditions in the Philippines using cv. IR64 wild-type plus transgenic lines, in which expression from both constitutive and cell type preferential promoters had been confirmed (Figure S5). In all cases the transgenic lines resembled segregating wild-type plants in terms of gross morphology (e.g., plant height and tiller number) (Figures 6A–6C), and rates of leaf photosynthesis in response to both increased intercellular CO₂ concentration (A/Ci curve) and increased photosynthetically active radiation (light response curve) were not significantly different from wild-type (Figures 6D–6F). Similarly, the photosynthetic efficiency of photosystem II (Fv/Fm) (Figure S6C) and the non-photochemical quenching (NPQ) response (Figure S6D) were both similar to wild-type.

While the failure to detect altered rates of photosynthesis was initially surprising, the proportion of vascular sheath to mesophyll tissue is low in rice leaves (Table S5), and it is further likely that CO₂ fixed in vascular sheath cells is sourced from respiration rather than from diffusion through mesophyll cells and intercellular air spaces [35]. Regardless, prolonged photosynthetic activity was evidenced by a significant delay in both heading date and time to harvesting physiologically matured seeds (Figure 6D) and by enhanced greening of immature grains (Figures 6E–6J). Whereas similar delayed senescence phenotypes can lead to reduced yield [36], no consistent differences in the yield of viable seed were observed between wild-type and transgenic lines (Figure 6D). As such, there is no fitness cost of proto-Kranz anatomy in rice.

### DISCUSSION

The evolutionary trajectory from C₃ to C₄ most likely occurred in a stepwise fashion, with the order of events differing between lineages [8, 16, 17, 37, 38]. Combined evidence from qualitative, quantitative, and modeling approaches suggests, however, that the unifying early step in all trajectories was the transition from C₃ to proto-Kranz [9, 12, 14, 15]. To recapitulate this step, organellar volume and photosynthetic/photorespiratory enzyme content in sheath cells surrounding the leaf veins must be increased. Constitutive expression of maize GLK genes in rice caused ectopic chloroplast development in cells around the vascular bundle, with photosynthetic enzymes accumulating to elevated levels in both bundle sheath and mestome sheath cells (Figures 1, 2, and 3; Table 1; Figure S3; Tables S2 and S3). Activation of chloroplasts in these cell types was accompanied by an increase in mitochondrial size, a corresponding increase in levels of the photorespiratory enzyme glycine decarboxylase, and a higher frequency of plasmodesmatal connections with neighboring cells (Figure 4; Tables S3 and S4). These subcellular modifications did not alter...
photosynthetic parameters in optimal growth conditions, and they did not impact on yield (Figure 6; Figure S6). In combination, these results demonstrate that constitutive expression of maize GLK genes in rice is sufficient to induce the transition from C3 leaf anatomy to proto-Kranz (Figure 5) and that the transition bears no fitness cost.

GLK proteins are transcription factors that directly activate a large number of downstream target genes encoding chloroplast-localized or photosynthesis-related proteins [25]. The modified development of mitochondria and plasmodesmata in bundle sheath and mestome cells of ZmGLK overexpression lines, therefore, most likely occurred as an indirect consequence of activated chloroplast development. Several communication routes between chloroplasts and mitochondria have been proposed, with the best characterized being signaling via redox state (for example, [39–41]). A direct link between chloroplasts, mitochondria, and plasmodesmata has also been recognized with loss-of-function mutants in RNA helicases that are localized in mitochondria and chloroplasts having increased numbers of plasmodesmata [42–45]. These observations suggest the presence of intracellular signaling mechanisms that coordinate the biogenesis of chloroplasts, mitochondria, and plasmodesmata. The level of GLK gene activity is similarly regulated by intracellular signaling from the plastid, both at the transcriptional [25] and post-transcriptional levels [27–29].

In the lines generated here, developmental suppression of transgene expression by post-transcriptional regulation is evident (Figures S2 and S5); however, organelle enhancement in vascular sheath cells was sustained throughout. The lack of enhanced chloroplast development in mesophyll cells (Table S3), despite constitutive gene expression, suggests that endogenous feedback mechanisms moderated GLK transgene function in cells that were already photosynthetically active. Such feedback regulation would also explain why there is no direct correlation between transgene transcript levels (Figure S2) and the degree of phenotypic alteration in vascular sheath cells (Tables S2–S5). It is notable, however, that when constitutively expressed, ZmG2 (which is expressed preferentially in bundle sheath cells of maize) consistently induced more substantial changes in vascular sheath cells than ZmGLK1 (which is expressed preferentially in mesophyll cells of maize) (Figures 2, 3, 4, and 5; Tables S2–S5). This observation endorses the suggestion that the duplication of GLK genes in C4 lineages facilitated neo-functionalization for cell-type-specific roles [19].
The complexity of the anatomical and biochemical changes needed for the C3-to-C4 transition appears seemingly incongruent with the multiple independent origins of the pathway [1]. However, the results presented here suggest that one of the earliest steps in C4 evolution, the transition from C3 to proto-Kranz, could have resulted from modified activity of a single gene. During the course of land plant evolution, the probability of a C3-to-proto-Kranz transition presumably depended on the number of genes able to induce that change. Very few transcriptional regulators of chloroplast development have been discovered, but functional analyses with the cytokinin GATA transcription factor CYTOKININ-RESPONSIVE GATA TRANSCRIPTION FACTOR 1 (CGA1) [36, 46] and GROWTH-REGULATING FACTOR 5 (GRF5) [47] suggest that both genes can activate chloroplast development in non-photosynthetic tissues. Roles upstream (GRF5) [47] and downstream (CGA1) [48] of GLK position both genes as potential regulators of at least some aspects of the C3-to/proto-Kranz transition. Whether or not this potential is validated, with the discovery that modifications to the activity of a single gene can kick-start the C3-to-C4 transition, one of the most remarkable examples of convergent evolution becomes slightly less mysterious.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Plants
  - Microbes
- METHOD DETAILS
  - Gene cloning and construct design
  - Rice transformation
  - PCR screening
  - DNA gel blot analysis
  - Quantitative RT-PCR
  - Chlorophyll quantification
  - Isolation of single cells
  - Light and transmission electron microscopy
  - Immunohistochemistry
  - Gas exchange measurements
  - Chlorophyll fluorescence for Fv/Fm and NPQ measurements
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Experimental design, sampling and statistical methods

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and six tables and can be found with this article online at https://doi.org/10.1016/j.cub.2017.09.040.

AUTHOR CONTRIBUTIONS

Experimental Concept and Design, P.W., R.K., S.K., W.P.Q., R.F., T.L.S., and J.A.L.; Generation of Transformation Constructs and Genotypic, qPCR, and Phenotypic Analyses of Kitaake Lines, P.W.; Generation of IR64 Lines and Genotypic and Phenotypic Analyses of IR64 Lines, S.K.; Organelle Counts and TEM Analysis, R.K.; qPCR of IR64 Lines, R.T., C.P.B., and S.K.; DNA Gel Blot Analysis of IR64 Lines, C.P.B.; Data Analysis and Interpretation, P.W., R.K., S.K., A.B., W.P.Q., R.F., T.L.S., and J.A.L.; Initial Manuscript Preparation, P.W., R.K., S.K., T.L.S., and J.A.L. All authors contributed to the final version of the manuscript.

ACKNOWLEDGMENTS

We thank Sarah Covshoff for providing transformation vectors; Elizabete Carmo-Silva, Martha Ludwig, and Christine Raines for antibodies; and Andreas Weber and Steve Kelly for comments on the manuscript. We are grateful to Florencia Montecillo and Virginia Laluz for technical assistance with IR64 tissue culture; Menard de la Rosa for PCR analysis, seed threshing, and photography; Albert de Luna, Rey Vergara, Govinda Rizal, and Hisiang-Chun Lin for plant husbandry; Irma Canicosa, Kelvin Acebron, and Robert Coe for technical assistance with photosynthesis measurements; Gemma Lorenzana and Rona Lunar for PCR screening of IR64 lines; Jacque Dionora for microscopy of IR64 lines; Xueqin Wang for generation of Kitaake lines; Zara Lewis and Na Li for technical assistance with analysis of Kitaake lines; Julia Bull for general technical support; and John Baker for photography. Research was funded by a C4 Rice Project grant from The Bill & Melinda Gates Foundation to IRRI (2012–2015; OPPGD1394) and the University of Oxford (2015–2019; OPP1129902).

Figure 5. Percentage of Planar Organelle Area Invested in Vascular Sheath Cells Is Equivalent to that in Proto-Kranz Grass Species

Percentage of total planar chloroplast (top) and mitochondrial (bottom) area in vascular sheath cells of wild-type (WT) and transgenic rice lines, plus PACMAD grasses Dicanthelium oligosanthes (C3), Steinichisma laxum (proto-Kranz [PK]), S. hians (C3), and Homolepis aturensis (C2). Values are mean ± SEM (n = 3). The trend toward proto-Kranz correlates with levels of transgene expression. Both ZmUBI <sup>pro</sup> and ZmG2 and S. laxum (PK) have significantly greater investment of chloroplasts and mitochondria in vascular sheath tissue than WT by a one-tailed t test (p < 0.05). No significant differences were observed between ZmUBI<sub>pro</sub> and ZmG2 and S. laxum (PK) by one-tailed or paired t tests (p > 0.2). See Figures S1 and S2 and Table S1 for transgene details and transcript levels in lines examined, Table S5 for a complete set of measurements, and Figure S4 for equivalent graphs of C3, PK, and C2 Flaveria spp.
REFERENCES

1. Sage, R.F., Christin, P.-A., and Edwards, E.J. (2011). The C(4) plant lineages of planet Earth. J. Exp. Bot. 62, 3155–3169.

2. Haberlandt, G. (1896). Physiologische Pflanzenanatomie (Wilhelm Engelman).

3. Brown, W.V. (1975). Variations in anatomy, associations, and origins of Kranz tissue. Am. J. Bot. 62, 395–402.

4. Langdale, J.A. (2011). C4 cycles: past, present, and future research on C4 photosynthesis. Plant Cell 23, 3879–3892.

5. Hattersley, P.W., Watson, L., and Johnston, C.R. (1982). Remarkable leaf anatomical variations in Neurachne and its allies (Poaceae) in relation to C3 and C4 photosynthesis. Bot. J. Linn. Soc. 84, 265–272.

6. Ueno, O. (1992). Immunogold localization of photosynthetic enzymes in leaves of Aristida latifolia, a unique C4 grass with a double chlorenchymatous bundle sheath. Physiol. Plant. 85, 189–196.

7. Voznesenskaya, E.V., Chuong, S.D.X., Kotevya, N.K., Edwards, G.E., and Franceschi, V.R. (2005). Functional compartmentation of C4 photosynthesis in the triple-layered chlorenchyma of Aristida (Poaceae). Funct. Plant Biol. 32, 67–77.

8. Lundgren, M.R., Osborne, C.P., and Christin, P.-A. (2014). Deconstructing Kranz anatomy to understand C4 evolution. J. Exp. Bot. 65, 3357–3369.

9. Sage, R.F., Khoshravesh, R., and Sage, T.L. (2014). From proto-Kranz to C4 Kranz: building the bridge to C4 photosynthesis. J. Exp. Bot. 65, 3341–3356.

10. Hibberd, J.M., Sheehy, J.E., and Langdale, J.A. (2008). Using C4 photosynthesis to increase the yield of rice—rationale and feasibility. Curr. Opin. Plant Biol. 11, 228–231.

11. von Caemmerer, S., Quick, W.P., and Furbank, R.T. (2012). The development of C4 rice: current progress and future challenges. Science 336, 1671–1672.

12. Khoshravesh, R., Stinson, C.R., Staia, M., Busch, F.A., Sage, R.F., Ludwig, M., and Sage, T.L. (2016). C3-C4 intermediacy in grasses: organelle enrichment and distribution, glycine decarboxylase expression, and the rise of C2 photosynthesis. J. Exp. Bot. 67, 3065–3078.

13. Muhaidat, R., Sage, T.L., Frohlich, M.W., Dengler, N.G., and Sage, R.F. (2011). Characterization of C3-C4 intermediate species in the genus Heliotropium L. (Boraginaceae): anatomy, ultrastructure and enzyme activity. Plant Cell Environ. 34, 1723–1736.

14. Sage, T.L., Busch, F.A., Johnson, D.C., Friesen, P.C., Stinson, C.R., Stata, M., Sultmanis, S., Rahman, B.A., Rawsthorne, S., and Sage, R.F. (2013). Initial events during the evolution of C4 photosynthesis in C3 species of Flaveria. Plant Physiol. 163, 1266–1276.

15. Stata, M., Sage, T.L., Hoffmann, N., Covshoff, S., Ka-Shu Wong, G., and Sage, R.F. (2016). Mesophyll Chloroplast Investment in C3, C4 and C2 Species of the Genus Flaveria. Plant Cell Physiol. 57, 904–918.

16. Dunning, L.T., Lundgren, M.R., Moreno-Villena, J.J., Namaganda, M., Edwards, E.J., Nosil, P., Osborne, C.P., and Christin, P.-A. (2017).
Introgression and repeated co-option facilitated the recurrent emergence of C₄ photosynthesis among close relatives. Evolution 71, 1541–1555.

17. Christin, P.-A., Osborne, C.P., Chatelle, D.S., Columbus, J.T., Bensard, G., Hodkinson, T.R., Garrison, L.M., Vorontsova, M.S., and Edwards, E.J. (2013). Anatomical enablers and the evolution of C₄ photosynthesis in grasses. Proc. Natl. Acad. Sci. USA 110, 1381–1386.

18. Vicentini, A., Barber, J.C., Alsicioni, S.S., Giussani, L.M., and Kellogg, E.A. (2008). The age of the grasses and clusters of origins of C₄ photosynthesis. Glob. Change Biol. 14, 2963–2977.

19. Wang, P., Fouracre, J., Kelly, S., Karki, S., Gowik, U., Aubry, S., Shaw, M.K.K., Westphoff, P., Slami-Loedien, I.H.H., Quick, W.P., et al. (2013). Evolution of GOLDEN2-LIKE gene function in C₃ and C₄ plants. Plant 237, 481–495.

20. Rossini, L., Cribb, L., Martin, D.J., and Langdale, J.A. (2001). The maize golden2 gene defines a novel class of transcriptional regulators in plants. Plant Cell 13, 1231–1244.

21. Yasumura, Y., Moyal, E.C., and Langdale, J.A. (2005). A conserved transcription factor mediates nuclear control of organellar biogenesis in anciently diverged land plants. Plant Cell 17, 1894–1907.

22. Fitter, D.W., Martin, D.J., Copley, M.J., Scotland, R.W., and Langdale, J.A. (2002). GLK gene pairs regulate chloroplast development in diverse plant species. Plant J. 31, 713–727.

23. Powell, A.L.T., Nguyen, C.V., Hill, T., Cheng, K.L., Figueroa-Balderas, R., Aktas, H., Ashrafi, H., Pons, C., Fernandez-Munioz, R., Vicente, A., et al. (2012). Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. Science 336, 1711–1715.

24. Hall, L.N., Rossini, L., Cribb, L., and Langdale, J.A. (1998). GOLDEN 2: a novel transcriptional regulator of cellular differentiation in the maize leaf. Plant Cell 10, 925–936.

25. Waters, M.T., Wang, P., Korkaric, M., Capper, R.G., Saunders, N.J., and Langdale, J.A. (2008). Activity of a maize ubiquitin promoter in transgenic rice. Plant Cell Physiol. 49, 1381–1386.

26. Nakamura, H., Muramatsu, M., Hakata, M., Ueno, O., Nagamura, Y., Hirochika, H., Takano, M., and Ichikawa, H. (2009). Ectopic overexpression of the transcription factor OsoGLK1 induces chloroplast development in non-green rice cells. Plant Cell Physiol. 50, 1933–1949.

27. Rauf, M., Arif, M., Dortay, H., Matallana-Ramírez, L.P., Waters, M.T., Gil E1 (CDKE1) provides a cellular switch in plants between growth and stress responses. J. Biol. Chem. 286, 3439–3459.

28. Blanco, N.E., Guinea-Díaz, M., Whelan, J., and Strand, Å. (2014). Interaction between plastid and mitochondrial retrograde signalling pathways during changes to plastid redox status. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 20130231.

29. Kobayashi, K., Otegui, M.S., Krishnakumar, S., Minardos, M., and Zambrisky, P. (2007). INCREASED SIZE EXCLUSION LIMIT 2 encodes a putative DEVH box RNA helicase involved in photosmadesma function during Arabidopsis embryogenesis. Plant Cell 19, 1885–1897.

30. Stonebloom, S., Burch-Smith, T., Kim, I., Meinke, D., Minardos, M., and Zambrisky, P. (2009). Loss of the plant DEAD-box protein ISE1 leads to defective mitochondria and increased cell-to-cell transport via plasmodesmata. Proc. Natl. Acad. Sci. USA 106, 17229–17234.

31. Burch-Smith, T.M., and Zambrisky, P.C. (2010). Loss of INCREASED SIZE EXCLUSION LIMIT (ISE1) or ISE2 increases the formation of secondary plasmodesmata. Curr. Biol. 20, 989–993.

32. Burch-Smith, T.M., Brunskold, J.O., Choi, Y.G., and Zambrisky, P.C. (2011). Organelle-nucleus cross-talk regulates plant intercellular communication via plasmodesmata. Proc. Natl. Acad. Sci. USA 108, E1451–E1460.

33. Cornejo, M.J., Luth, D., Blankenship, K.M., Anderson, O.D., and Blechl, A.E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol. Biol. 23, 567–581.

34. Nomura, M., Higuchi, T., Ishida, Y., Ohta, S., Komori, T., Imaizumi, N., Miyao-Tokutomi, M., Matsuoka, S., and Tajima, S. (2005). Differential expression pattern of C₄ bundle sheath expression genes in rice, a C₄ plant. Plant Cell Physiol. 46, 754–761.

35. Matsuoka, M., Kiyokura, J., Shimamoto, K., and Kano-Murakami, Y. (1994). The promoters of two carboxylases in a C₃ plant (maize) direct cell-specific, light-regulated expression in a C₄ plant (rice). Plant J. 6, 311–319.

36. Waters, M.T., Moylan, E.C., and Langdale, J.A. (2008). GLK transcription factors regulate chloroplast development in a cell-autonomous manner. Plant J. 56, 432–444.
carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. Plant Physiol. 116, 539–546.

51. Hiei, Y., and Komari, T. (2006). Improved protocols for transformation of indica rice mediated by Agrobacterium tumefaciens. Plant Cell Tissue Organ Cult. 85, 271–283.

52. Yoshida, S., Forno, D.A., Cock, J.H., and Gomez, K.A. (1972). Routine Procedure for Growing Rice Plants in Culture Solution (International Rice Research Institute).

53. Toki, S., Hara, N., Ono, K., Onodera, H., Tagiri, A., Oka, S., and Tanaka, H. (2006). Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. Plant J. 47, 969–976.

54. Murray, M.G., and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321–4325.

55. Guillemaut, P., and Maréchal-Drouard, L. (1992). Isolation of plant DNA: a fast, inexpensive, and reliable method. Plant Mol. Biol. Report. 10, 60–65.

56. Langdale, J.A., Rothermel, B.A., and Nelson, T. (1988). Cellular pattern of photosynthetic gene expression in developing maize leaves. Genes Dev. 2, 106–115.

57. Amon, D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol. 24, 1–15.

58. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.

59. Khoshravesh, R., Lundsgaard-Nielsen, V., Sultmanis, S., and Sage, T.L. (2017). Light microscopy, transmission electron microscopy, and immunohistochemistry protocols for studying photorespiration. In Photorespiration: Methods and Protocols, A.R. Fernie, H. Bauwe, and A.P.M. Weber, eds. (Springer), pp. 243–270.

60. Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.

61. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

62. McKown, A.D., and Dengler, N.G. (2007). Key innovations in the evolution of Kranz anatomy and C₄ vein pattern in Flavenia (Asteraceae). Am. J. Bot. 94, 382–399.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-GLDP | Martha Ludwig | [12] |
| Rabbit polyclonal anti-RuBisCo 557 kDa hexadecamer | Agrisera, Martha Ludwig | AS07 218; RRID: AB_1031802 |
| Rabbit polyclonal anti-FBPase | Christine Raines | [49] |
| Rabbit monospecific anti-RuBisCo activase | Elizabete Carmo-Silva | [50] |
| 18nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (H+L) (min X Hu,Ms,Rat Sr Prot) (EM Grade) | Cedarlane Cat# 111-215-144 |
| **Bacterial and Virus Strains** |        |            |
| E. coli strain DH5α | Widely distributed | N/A |
| A. tumefaciens strain EHA105 | Widely distributed | N/A |
| A. tumefaciens strain LBA4404 | Widely distributed | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Glutaraldehyde 25% | Electron Microscopy Sciences (EMS) | Cat# 16210 |
| Paraformaldehyde (20%) | EMS | Cat# 15713 |
| Sodium cacodylate | EMS | Cat# 12300 |
| Osmium tetroxide 4% | EMS | Cat# 19150 |
| Low viscosity embedding kit (Dr. Spurr) | EMS | Cat# 14300 |
| LRW (medium grade) | EMS | Cat# 14381 |
| Pectinase from Aspergillus niger | Sigma-Aldrich | Cat# 17389 |
| **Critical Commercial Assays** |        |            |
| Gateway BP Clonase II enzyme mix | Invitrogen | Cat#11789020 |
| Gateway LR Clonase II enzyme mix | Invitrogen | Cat#11791100 |
| GoTaq Green Master Mix | Promega | M712 |
| GoTaq G2 Green Master Mix | Promega | M7822 |
| TURBO DNA-free Kit | Ambion | Cat#AM1907 |
| SuperScript III Reverse Transcriptase | Invitrogen | Cat#18080093 |
| TERRA PCR Direct polymerase mix | Clontech | Cat. No. 639270 |
| LightCycler 480 SYBR Green I Master (Real time PCR assays) | Roche | #0470516001 |
| SYBR Green PCR Master Mix | Applied Biosystems | Cat#4309155 |
| PCR DIG Probe Synthesis Kit | Roche | Cat. No. 1 636 090 |
| Random Primers DNA Labeling System | Invitrogen | Cat#18187013 |
| **Experimental Models: Organisms/Strains** |        |            |
| Oryza sativa spp. indica cultivar IR64 | IRRI | N/A |
| Oryza sativa spp. japonica cultivar Kitaake | IRRI | N/A |
| Oryza sativa IR64: ZjPCK::ZmG2 | This paper | IR64-IRS-784 |
| Oryza sativa IR64: ZmPEPC::ZmG2 | This paper | IR64-IRS-782 |
| Oryza sativa IR64: ZmUbi::ZmG2 | This paper | IR64-IRS-786 |
| Oryza sativa IR64: ZjPCK::ZmGLK1 | This paper | IR64-IRS-785 |
| Oryza sativa IR64: ZmPEPC::ZmGLK1 | This paper | IR64-IRS-783 |
| Oryza sativa IR64: ZmUbi::ZmGLK1 | This paper | IR64-IRS-787 |
| Oryza sativa Kitaake ZjPCKpro:ZmG2 | This paper | 57C1, 57C2, 57C3 |
| Oryza sativa Kitaake ZmUBIpro:ZmG2 | This paper | 57E1, 57E2, 57E3, 57E4 |
| Oryza sativa Kitaake ZmPEPC::ZmGLK1 | This paper | 58A1, 58A2, 58A3, 58A4 |
| Oryza sativa Kitaake ZmUBIpro:ZmGLK1 | This paper | 58E1, 58E2, 58E3, 58E4, 58E5 |

(Continued on next page)
**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Langdale (jane.langdale@plants.ox.ac.uk). Please note that the transfer of transgenic rice lines will be governed by an MTA, will be dependent on appropriate import permits being acquired by the receiver, and may be constrained by the size of available seed stocks.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Plants**

*Oryza sativa* spp. indica cultivar IR64 lines were grown in a transgenic greenhouse at the International Rice Research Institute (IRRI), Los Banos, Philippines (located at 14°14'53.58"S 121°15'32.19"E). The average day/night temperatures in the plant growth facility were 35 ± 3°C and 28 ± 3°C, respectively. Average light intensity and photoperiod were 824.75 ± 201.86 μmol photons m⁻² s⁻¹ and 13 hr day/11 hr night, respectively.

*Oryza sativa* spp. japonica cultivar Kitaake lines were grown in soil in a transgenic greenhouse in either Oxford, UK or Canberra, Australia. Day/night temperature was maintained at 30°C/22°C ± 3°C with a diurnal light regime of 16 hr light (supplemented to ~300 μmol photons m⁻² s⁻¹) and 8 hr dark.

All plants were self-pollinated by bagging inflorescences after flowering.

**Microbes**

*Agrobacterium tumefaciens* strains LBA4404 and EHA105 were cultured at 28°C in LB plus 100 mg L⁻¹ rifampicin and supplemented with 50 mg L⁻¹ kanamycin if harboring a plasmid for rice transformation.

**METHOD DETAILS**

**Gene cloning and construct design**

Full-length cDNAs of *ZmG2* (GenBank: AF318579) and *ZmGLK1* (GenBank: AF318580) were amplified by PCR from cDNA clones isolated previously [20] using Gateway compatible primers *ZmG2-cloningF, ZmG2-cloningR*, *ZmGLK1-cloningF* and *ZmGLK1-cloningR* (Table S6). The coding sequences were subcloned into the Gateway donor vector pDONR207 via a BP reaction, and the resulting entry clones were sequenced. Coding sequences were then cloned downstream of the *ZmPEPC*, *ZjPCK*, and *ZmUBI* promoters in the binary destination vectors pSC110, pSC210, and pSC310, respectively. The maize *UBIQUITIN* promoter (*ZmUBI_pro*) was used to drive constitutive expression [30], the *Zoisia japonica PHOSPHOENOLPYRUVATE CARBOXYKINASE*
promoter (ZjPCK<sub>pro</sub>) for preferential expression in bundle sheath cells [31] and the maize PHOSPHOENOLPYRUVATE CARBOXYLASE promoter (ZmPEPC<sub>pro</sub>) for preferential expression in mesophyll cells [32]. Six constructs were produced via a LR reaction (Figure S1A).

**Rice transformation**

*Oryza sativa* spp. *indica* cultivar IR64 and *spp. japonica* cultivar Kitaake were both used for transformation. For IR64, immature embryos were isolated from freshly harvested spikelets 8-12 days after anthesis. Embryos were inoculated with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid DNA of interest and then cultured and regenerated according to the protocol of Hiei and Komari [51]. Briefly, after one week of co-cultivation at 25 °C in the dark followed by growth on non-selective medium for 5 days, emerging resistant calli were selected on 30 mg L<sup>−1</sup> hygromycin B. Regeneration was subsequently carried out on 50 mg L<sup>−1</sup> hygromycin B for two weeks. Hygromycin-resistant regenerated T0 plantlets were transferred to Yoshida hydrosol solutions [52] for 2 weeks prior to transplanting into soil in 7 L pots. For Kitaake, calli induced from mature seeds were transformed with *A. tumefaciens* strain EHA105 carrying the construct of interest. Callus induction, transformant selection and seedling regeneration were performed at 32 °C under continuous light according to a protocol modified from [53] that can be downloaded at https://langdalelab.files.wordpress.com/2015/07/kitaake_transformation_2015.pdf. Hygromycin-resistant T0 seedlings that screened positive for the transgene by PCR were transplanted into soil (John Innes Compost No.2) in 0.73 L pots.

**PCR screening**

Regenerated T0 plants were subjected to genomic PCR using primers specific to the gene of interest or the cloning vector (Table S6; pVec8F (UBI<sub>pro</sub>), pVec8R (nosT), pSC110F (PEPC<sub>pro</sub>), pSC210F (PCK<sub>pro</sub>), HPTFpr3, HPTPr2, pSC1/2/310-R, ZmG2-F, ZmG2-R, ZmGLK1-F, ZmGLK1-R). For IR64 T0 lines, small leaf sections were harvested 10 days after germination. PCR amplification was performed in a total reaction volume of 10 μL using TERRA PCR Direct polymerase mix (Clontech). Plasmid DNA was used as a positive control and non-transgenic rice DNA and water were separately used as negative controls. PCR conditions were as follows: initial-denaturation for 2 min at 98 °C; 30 cycles of amplification consisting of denaturation for 10 s at 98 °C, annealing for 15 s at 60 °C and extension for 45 s at 68 °C; followed by a final extension step for 7 min at 72 °C. For Kitaake lines, genomic DNA was isolated using a modified cetyl trimethylammonium bromide (CTAB) method [54] (see below), and was tested in 10 μL PCR reactions containing 5 μL 2xGoTaq mix (Promega) and 2.5 μL 4M betaine. PCR conditions were: 95 °C for 5 min; 28 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 2.5 min; and 72 °C for 5 min.

**DNA gel blot analysis**

Genomic DNA was extracted from leaves of mature plants using potassium acetate (IR64 lines) [55] or CTAB (Kitaake lines) [54] methods. With the potassium acetate method, 1-2 g of rice leaves were ground to a fine powder in liquid nitrogen and suspended in 500 μL extraction buffer (100 mM Tris-HCl, 50 mM EDTA pH8.0, 500 mM NaCl, 0.07% b-mercaptoethanol). 1 mL 20% SDS was then added to the suspension, mixed well and incubated at 65 °C for 30 min. After that time, 5 mL 5 M potassium acetate was added and mixed gently by shaking, before incubating at 4 °C for 20 min. Samples were then centrifuged at 3500 rpm for 30 min, the supernatant was transferred to a fresh tube and nucleic acid was precipitated by adding 10 mL isopropanol followed by incubation at −20 °C for 20 min. Nucleic acid was pelleted by centrifugation at 5000 rpm for 15 min and then re-suspended in 500 μL H<sub>2</sub>O. Samples were digested with 1 μL RNase (10 mg/mL) at 37 °C for 10 min, extracted with 500 μL of chloroform: isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 10 min. Supernatants were transferred to fresh tubes and DNA precipitated by adding 75 μL 3 M Sodium acetate and 500 μL isopropanol. After centrifugation at 12000 rpm for 5 min, pellets were washed twice with 500 μL 70% ethanol and air-dried. DNA pellets were dissolved in 100 μL H<sub>2</sub>O and stored at −20 °C. With the CTAB method, leaves were first ground in liquid nitrogen, and suspended in 500 μL CTAB extraction solution containing 1.5% CTAB, 1.05 M NaCl, 75 mM Tris-HCl, 15 mM EDTA pH8.0. After incubation at 65 °C for 20 min, an equal volume of chloroform:isoamylalcohol (24:1) was added, samples were mixed by vortexing and then centrifuged at 12000 rpm for 5 min. Supernatants were transferred to fresh tubes, precipitated by adding 2.5 volumes of 100% ethanol, and centrifuged at 12000 rpm for 5 min. The pellets were washed with 800 μL 70% ethanol, and air-dried. DNA samples were dissolved in 50 μL H<sub>2</sub>O and stored at −20 °C.

For each plant, 6-8 μg of genomic DNA was digested with *Afl* II (ZmG2 samples) or *Hind* III (ZmGLK1 samples) restriction endonuclease. Digested DNA was electrophoresed and then transferred onto Hybond N+ membrane (GE Healthcare, UK). For IR64 lines, blots were hybridized with digoxigenin (DIG)-labeled gene specific probes synthesized using primers indicated in Figure S1A and Table S6, and the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany). Hybridization signals were detected using CDP-Star according to the manufacturer’s instructions (Roche Diagnostics). For the Kitaake lines, blots were hybridized as in [56], with <sup>32</sup>P labeled fragments of the hygromycin resistance gene synthesized using the Random Primers DNA Labeling System (Invitrogen), and exposed to autoradiography film.

Two independent IR64 lines with one or two transgene copies were obtained for each of the six constructs (Figures S1B and S1C; Table S1) and at least two independent Kitaake lines, with transgene copy numbers ranging from one to four, were obtained for ZjPCK<sub>pro</sub>:ZmG2, ZmUBI<sub>pro</sub>:ZmG2, ZmPEPC<sub>pro</sub>:ZmGLK1 and ZmUBI<sub>pro</sub>−ZmGLK1 constructs (Figures S1D–S1G; Table S1).
Quantitative RT-PCR

4th and 7th leaves were harvested from transgenic plants and wild-type controls when leaves were at the youngest fully expanded stage. RNA was extracted using TRIZOL reagent (Invitrogen) or a QIAGEN RNaseasy kit, and RNA integrity was confirmed by gel electrophoresis. For IR64 lines, total RNA was treated with RQ1 RNase free DNase (Promega) prior to use as template to synthesize cDNA with a first strand cDNA synthesis kit (Roche Diagnostics). Quantitative real time PCR was carried out with LightCycler 480 SYBR Green I Master mix in a final reaction volume of 20 μL (Roche Diagnostics). Primers specific to the gene of interest and actin (Table S6) were used. For Kitaake lines, TURBO DNA-free Kit (Ambion) was used for DNase treatment, SuperScript III Reverse Transcriptase (Invitrogen) was used for first strand cDNA synthesis, and SYBR Green PCR Master Mix (Applied Biosystems) was used for quantitative RT-PCR on a StepOnePlus System (Applied Biosystems).

Chlorophyll quantification

Leaf tissues of equal fresh weight were ground to a fine powder in liquid nitrogen, prior to submergence in equal volumes of 80% acetone. The chlorophyll content of the supernatant was measured at 663 and 645 nm using a spectrophotometer and quartz cuvettes. Total chlorophyll extracted (μg mL⁻¹) was calculated as follows: (8.02 × Absorbance@663 nm) + (20.29 × Absorbance@645 nm) [57], and adjusted per weight of fresh tissue sampled (g).

Isolation of single cells

2 mm sections of the recently fully expanded mid-region of leaves 4 and 7 were fixed (1 h) in 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and incubated (3 h) in 0.2 M disodium EDTA, pH 9.0 in a water bath at 55 °C. After incubation, samples were rinsed (20 min) in water and subsequently digestion buffer (20 min; 0.15 M sodium hydrogen phosphate, 0.04 M citric acid, pH 5.3). Leaf tissues were then incubated (1 h) at 45 °C in 2% pectinase in digestion buffer and rinsed (30 min) in digestion buffer. Bundle sheath cells could be distinguished from mesophyll cells by an elongated shape (Figures 2B and 2D). Isolated bundle sheath cells were viewed with Nomarski optics and bundle sheath cell volume and chloroplast size were quantified with ImageJ [58] from images captured on a Zeiss Axioplan equipped with Olympus cellSens imaging software.

Light and transmission electron microscopy

Leaves 4 and 7 were prepared for light and transmission electron microscopy (TEM), quantification of cellular features, and immunodetection of photosynthetic enzymes and glycine decarboxylase as described previously [59]. 2 mm leaf sections from the middle of recently fully expanded leaves were fixed in 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) overnight at room temperature, and post-fixed (2 h) in 1% osmium tetroxide. Fixed tissue was rinsed (2 × 30 min) in 0.1 M sodium cacodylate buffer. Samples were subsequently dehydrated in ethanol:H₂O with 10% increment increases from 10% to 100% ethanol (1 hr each increment) and two (1 hr each) changes of 100% ethanol. Dehydrated tissue samples were infiltrated in Spurr’s resin using 10% increment increases of Spurr’s in 100% ethanol (3 hr each increment) from 10 to 100% Spurr’s. After two changes in 100% Spurr’s (12 hr each), tissue was polymerized at 60 °C in a flat embedding mold. Fixation of leaf tissue for immunodetection of enzymes followed this same protocol except post-fixation with osmium tetroxide was omitted and specimens were infiltrated in London Resin White (LRW) using 1:3, 1:1, and 3:1 ratio of LRW to 100% ethanol (8 hr each increment), followed with 2 × 100% LRW (8 hr each). LRW infiltrated leaf samples were polymerized at 60 °C in an oxygen-free environment for 12 hr at 60 °C. Sections of 1.7 μm and 50-70 nm were collected for light and TEM, respectively. Images for light microscopy were captured on a Zeiss Axioplan equipped with Olympus cellSens imaging software and a Phillips 201 transmission electron microscope equipped with an Advantage HR camera system (Advanced Microscopy Techniques) was used to capture transmission electron micrographs.

Immunohistochemistry

50-70 nm sections of LRW embedded tissue were rehydrated in 0.01 M phosphate saline buffer (PBS) pH 7.4, blocked for 15 min with 0.4% or 0.5% bovine serum albumin (BSA) in PBS (for glycine decarboxylase and photosynthetic enzyme detection respectively), and then rinsed in PBS for 3 × 15 min before incubating for 3 hr in primary antibody at the following concentrations: 1:50 (anti-glycine decarboxylase), 1:100 (anti-RuBisCo and anti-RuBisCo activase), and 1:400 (anti-FBPase) in 0.1% BSA/PBS. Sections were then rinsed in PBS 3 × 15 min before incubation with secondary antibody (18nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG) for 1 hr at a concentration of 1:20 (glycine decarboxylase) or 1:40 (photosynthetic enzymes) in 0.1% BSA/PBS. Samples were then rinsed in PBS for 3 × 15 min, followed by ultrapure water for 3 × 15 min before being stained with 4% uranyl acetate for 10 min, and then lead citrate for 5 min [60].

Gas exchange measurements

Fully expanded 8th leaves were used to measure photosynthetic rates using a LI-6400XT portable photosynthesis system (LICOR Biosciences). Three individuals were sampled per line, and measurements were repeated three times. Measurements were made in the morning at a constant airflow rate of 400 μmol s⁻¹, leaf temperature of 30 °C and a leaf-to-air vapor pressure deficit of between 1.0 and 1.5 kPa. Leaves were acclimated in the chamber for approximately 30 min before measurements were made on the mid-section of the leaf blade. Net CO₂ assimilation rate (A) in response to intercellular CO₂ concentration (Ci) was measured at a light...
intensity of 2,000 μmol photons m^{-2} s^{-1} by increasing CO₂ concentration in the cuvette from 20 to 2,000 μmol CO₂ mol^{-1} air. Similarly, light response curves were produced by measuring net CO₂ assimilation rate (A) in response to increasing photosynthetic photon flux density (PPFD) from 20 to 2,000 μmol photons m^{-2} s^{-1} at a CO₂ concentration of 400 μmol CO₂ mol^{-1} air.

**Chlorophyll fluorescence for Fv/Fm and NPQ measurements**

For each individual, the three youngest fully expanded leaves of plants at maximum tillering stage were cut at the base and placed on a tray with the base of the leaves soaked in water to prevent drying and folding. Three individuals were sampled per line. The samples were dark adapted for 30 min prior to measurement. Fv/Fm and non photochemical quenching (NPQ) measurements were carried out using the PlantScreen Phenotyping System from Photon Systems Instruments (PSI), Czech Republic. Fluorescence images captured by the fluorometer were analyzed using the FluorCam7.0 software (PSI, Czech Republic).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Experimental design, sampling and statistical methods**

For all experiments, at least three individuals representing at least two independent transgenic lines were evaluated. Experiments to measure photosynthetic parameters and seed yield were carried out using wild-type and transgenic lines of *Oryza sativa spp. indica* cultivar IR64 (Figure 6; Figures S1, S5, and S6; Table S1). All other experiments were carried out with wild-type and transgenic lines of *Oryza sativa spp. japonica* cultivar Kitaake.

To assess the impact of developmental regulation: samples harvested from leaf 4 and leaf 7 of plants grown in the same environment were compared, measuring transgene transcript levels in the same leaves used for phenotypic analysis. For qRT-PCR (Figure S2) and TEM/immunolocalization (Figures 3, 4, and 5; Table 1; Figure S3; Tables S3–S5) experiments, samples were collected 3 h post-illumination. To minimize interference from starch granules, the same leaf was sampled 20 h later for single cell measurements (Figure 2; Table S2).

For quantification of heading date and seed weight, five individuals from each of two independent lines per construct were analyzed (Figure 6). Transgene transcript levels were quantified in leaf 4 and 7 of three individuals from each of those independent lines (Figure S5), and measurements of photosynthetic capacity were carried out in leaf 8 of three individuals from one of those lines for each construct (Figure S6).

qRT-PCR experiments were carried out using three experimental replicates per biological sample (Figures S2 and S5). The 2^{-ΔΔCT} method was used to quantify the relative abundance of transcripts using the CT value of OsActin as the internal control for normalization [61]. Primer pairs for ZmGLKs, OsGLKs and OsActin were tested in standard PCR reactions against maize and rice cDNA respectively to ensure specific targets were amplified. For each primer pair, a standard curve was generated to ensure amplification efficiency had a linear relationship with cDNA concentration, with all primer pairs giving a linear regression (R^2) value of 0.99, except for the primer pair for OsGLK2 that showed an R^2 value of 0.93. The overall primer efficiency values were between 96.8%–108.1%.

Average of individual chloroplast area and chloroplast numbers in isolated cells were quantified for 15 bundle sheath cells per individual (Figure 2; Table S2). 15 was established as the sample size based on preliminary experiments which showed that using more than 15 failed to alter the mean and variance for each individual. Three individuals were used per line as a standard minimum requirement. Bundle sheath cell volume was approximated using cell volume = [(W/2)^2] \times \pi \times \text{L}, where W = planar cell width and L = planar cell length. Cell volume was then used to quantify the number of chloroplasts/cell volume.

Planar cell area covered by total chloroplasts and mitochondria was quantified using ImageJ, sampling 15 mesophyll, bundle sheath or mestome sheath cells from TEM images of leaf 7 for each of three individuals from ZmPKCpro:ZmG2, ZmPEPCpro:ZmGLK1, ZmUBIpro:ZmG2, and ZmUBIpro:ZmGLK1 lines (Figures 2 and 4; Table S3). Similar leaf tissue and the same sample size was used for quantification of gold density on ZmUBIpro:ZmG2 and wild-type sections immuno-labeled for Rubisco and Rubisco activase (Table 1; Figure S3). The same leaf materials were tested to quantify the percentage of leaf cross section covered by mesophyll, bundle sheath, and mestome sheath cells. The percentage of organelles invested in each cell-type was then quantified as the proportion of organelar area in that cell-type, relative to the total photosynthetic organelar area (Figure 5; Figure S4; Table S5) (as in the three equations below):

1) Total planar area of photosynthetic tissue (TP) was quantified as:

\[
TP = T_M + T_{BS} + T_{MS}
\]

Where T = % cell-type area in cross section (T_M = % planar Mesophyll, T_{BS} = % planar Bundle Sheath, T_{MS} = % planar Mestome Sheath)

2) Total photosynthetic organellar area (PO) in planar cross section (%) was quantified as:

\[
PO = [(T_M/TP)O_M] + [(T_{BS}/TP)O_{BS}] + [(T_{MS}/TP)O_{MS}]
\]

Please cite this article in press as: Wang et al., Re-creation of a Key Step in the Evolutionary Switch from C3 to C4 Leaf Anatomy, Current Biology (2017), https://doi.org/10.1016/j.cub.2017.09.040
Where $O$ is the planar cell area covered by organelles (chloroplast or mitochondria)

3) The % of organelles invested in each cell-type was quantified as:

$$\% \text{ of organelle investment} = \frac{CO}{PO} \times 100$$

Where $CO$ is the % of TP occupied by the cell-type (M, BS, or MS) organelle area; e.g., $CO_M = \frac{TM}{TP} \times OM$

Similar quantifications were performed for six PACMAD grasses from the raw data in [12]. *Flaveria* organellar data were from [14, 15], and the values for cell type % in *Flaveria* species were extracted from M:BS ratios in Figure 4 of [62].

For all cellular measurements, statistical tests were performed using SPSS 20.0 and graphed using SigmaPlot 12.5. For continuous variables, a Shapiro-Wilk test of normality was used to determine whether the data were normally distributed. When the normality test failed, a General Linear Model (GLM) followed by a Games-Howell post hoc test was performed. An ANOVA and a Tukey’s test was used for normally distributed data. Presence or absence of plasmodesmata between bundle sheath-mesophyll and between bundle sheath-mesostome sheath cells was recorded as absence = 0, presence = 1. Relative frequency of plasmodesmata was calculated by dividing the number of cells which had plasmodesmatal connections with the adjacent cell with the total number of observed cells for that cell-type. A GLM for binary data was performed where presence and absence of plasmodesmata between cell-types was the dependent variable, line was the fixed factor, and bundle sheath planar chloroplast and mitochondria area per planar cell area were covariates, using line as the main effect and a confidence interval of 95%.

Standard errors for heading time, days to harvest and filled seed weight, and ANOVA were calculated using the Statistical Tool for Agricultural Research (STAR) 2.01 software.
Supplemental Information

Re-creation of a Key Step in the Evolutionary
Switch from C₃ to C₄ Leaf Anatomy

Peng Wang, Roxana Khoshravesh, Shanta Karki, Ronald Tapia, C. Paolo Balahadia, Anindya Bandyopadhyay, W. Paul Quick, Robert Furbank, Tammy L. Sage, and Jane A. Langdale
Figure S1. Transgene copy number in ZmG2 and ZmGLK1 lines. Related to Figures 1-6.
A) Constructs used for transformation. Arrows show primer positions used to amplify fragments for hybridization in (B) & (C). Blots in (D) - (F) were hybridized with the hygromycin resistance gene. Single Afl II and Hind III restriction sites within the constructs are indicated. LB = left border; RB = right border of T DNA. Numbers in red indicate construct reference for transformation into O. sativa spp. indica cultivar IR64, and in purple indicate construct reference for transformation into O. sativa spp. japonica cultivar Kitaake. B, C) Gel blot analysis of IR64 lines. Afl II digested DNA from wild-type (WT) plus 3 individuals of each of 2 independent ZmPEPC<sub>pro</sub>:ZmG2 (IRS782-043; -058), ZjPCK<sub>pro</sub>:ZmG2 (IRS784-018; -059) & ZmUBI<sub>pro</sub>:ZmG2 (IRS786-052; -088) T1 lines. All lines have individuals with a single transgene insertion site except for IRS786-052 which has two. In line IRS782-058, either two transgenes have segregated or there is a 3kb deletion in one of the three individuals (B). Hind III digested DNA from WT plus 3 individuals of 2 independent ZmPEPC<sub>pro</sub>:ZmGLK1 (IRS783-001; -099), ZjPCK<sub>pro</sub>:ZmGLK1 (IRS785-062; -063) & ZmUBI<sub>pro</sub>:ZmGLK1 (IRS787-050; -060) T1 lines. All lines have at least one individual with a single transgene insertion site except for IRS783-009 which has two. In line IRS783-001, segregation of two transgenes has resulted in one individual that is homozygous for one transgene (20), one that is homozygous for both transgenes (21) and one that is homozygous for one transgene and hemizygous for the second (22) (C). MW – molecular weight markers; P – plasmid. D-G) Cropped images of gel blot analysis of Kitaake lines. Afl II digested DNA from T1 plants (top) & T2 progeny (bottom) of lines transformed with ZjPCK<sub>pro</sub>:ZmG2 construct 57C. 6 individuals were derived from 3 independent transgenic events, one of which (C2) represents a single insertion site (D). Afl II digested DNA of T1 lines transformed with ZmUBI<sub>pro</sub>:ZmG2 construct 57E. 7 individuals display 4 independent transgene profiles, three of which (E1-E3)
represent a single insertion site (E). *Hind* III digested DNA of T1 plants (left) & T2 progeny (middle & right) of lines transformed with *ZmPEPC*<sub>pro</sub>:*ZmGLK1* construct 58A. 5 individuals display 4 independent transgene profiles, three of which (A2, A3 & A4) represent a single insertion site (F). *Hind* III digested DNA of T1 plants (left & middle) and T2 progeny (right) lines transformed with *ZmUBI*<sub>pro</sub>:*ZmGLK1* construct 58E. 6 individuals display at least 5 different transgene profiles, at least three of which (E2, E3 & E5) represent a single insertion site (G). Asterisks are positioned to the left of faint bands in (F) & (G).
Figure S2. Relative transcript levels in ZmG2 and ZmGLK1 T1 transgenic lines. Related to Figures 2-5, Table 1.

A-J) Relative levels of ZmG2 transgene, ZmGLK1 transgene, OsGLK2 and OsGLK1 in 4th (A-E) and 7th (F-J) leaves of T1 Kitaake lines grown at 300 µmol photons m⁻² s⁻¹. Transcript levels were measured by qRT-PCR and adjusted relative to actin transcript levels using the ΔCT value output from the StepOnePlus™ system. Error bars indicate standard error computed from the mean of three experimental replicates. Transgene transcript levels were higher in leaf 4 (regardless of promoter) than leaf 7, whereas endogenous OsGLK1 transcript levels were higher in leaf 7 than leaf 4. The bundle sheath preferential ZjPCK_pro consistently drove the lowest levels of transgene expression. Transgene copy number in each line is shown in Figure S1. Lines are those used for analyses in Figures 2-5 and Table 1 of the main paper.
Figure S3. Immunolocalization of photosynthetic enzymes and mitochondrial glycine decarboxylase in vascular sheath cells of wild-type and transgenic lines. Related to Figure 3, Table 1.

(A-P) Immuno-gold labeling of wild-type (WT) (A-D, I-L) and ZmUBI_pro:ZmG2 transgenic Kitaake (E-H, M-P) bundle sheath (A-H) and mestome sheath (I-P) cells. Sections were reacted with antibodies against chloroplast localized RuBisCo (A, E, I, M), RuBisCo activase (B, F, J, N) and fructose-1,6-bis-phosphatase (FBPase) (C, G, K, O) or mitochondrial localized P subunit of glycine decarboxylase (GLDP) (D, H, L, P). All sections were prepared from leaf 7 of plants grown at 300 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). Scale bars = 500nm; c, chloroplast; m, mitochondria; p, peroxisome; pl, proplastid. See Table S1 for details of transgenic lines used, Figure S2 for transgene transcript levels. Quantification of RuBisCo and RuBisCo activase labeling is shown in Table 1 of main paper.
Figure S4. Relative investment of organelles in bundle sheath cells of *Flaveria* species. Related to Figure 5.

Percentage of total chloroplasts (upper panel) and mitochondria (lower panel) invested in bundle sheath cells (on a planar area basis) in the photosynthetic tissue of C$_3$, proto-Kranz (PK) and C$_2$ *Flaveria*. Organellar data are from [S1,S2] and the values for cell type % are extracted from M:BS ratios in Fig. 4 of [S3]. Calculations are as in experimental design section of methods and Table S5.
Figure S5. Transcript accumulation levels in ZmG2 and ZmGLK1 overexpression lines. Related to Figure 6.

A-D) Relative levels of ZmG2 transgene (A), ZmGLK1 transgene (B), OsGLK2 (C) and OsGLK1 (D) transcripts in 4th and 7th leaves of the IR64 lines genotyped in Figure S1. Transcript levels were measured by qRT-PCR and adjusted relative to actin transcript levels using the 2^{-ΔCT} method. Error bars indicate standard error computed from the mean of three experimental replicates. As with equivalent Kitaake lines (Figure S2) ZmG2 Transgene transcript levels were higher in leaf 4 (regardless of promoter) than leaf 7, however, ZmGLK1 transcripts were similar at both developmental stages. A similar distinction was seen between the orthologous OsGLK2 and OsGLK1 transcripts, suggesting that sequence-specific post-transcriptional regulation may be operating. Lines are those used for analyses in Figure 6 of the main paper.
Figure S6. Overall photosynthetic parameters are not altered in rice lines expressing ZmG2 or ZmGLK1. Related to Figure 6.

A) A-Ci response curve  B) Light response curve  C) Quantum yield (Fv/Fm) measurements  D) Non-photochemical quenching (NPQ) response. Datapoints for each line are colour coded as in (A), with the line number indicated in parentheses. Error bars represent s.e.m. n= 9. For A & B three individuals were measured per line, with three experimental replicates in each case. For C & D, three different leaves were measured for each of three individuals per line. Transgene copy number and expression levels for each line are shown in Figure S1 and S5, respectively. Whole plant phenotypes and yield data for the same lines are shown in Figure 6 of the main paper.
| Cultivar | Original line number | # insertion sites | Leaf 4 single cell counts & qPCR | Leaf 7 single cell counts, TEM & qPCR |
|----------|----------------------|------------------|---------------------------------|----------------------------------|
| ZmPEPC<sub>pro</sub>:ZmG2 | IR64 | IRS782-043 | 1 |
| ZmPEPC<sub>pro</sub>:ZmG2 | IR64 | IRS782-058 | 1 |
| ZjPCK<sub>pro</sub>:ZmG2 | IR64 | IRS784-018 | 1 |
| ZjPCK<sub>pro</sub>:ZmG2 | IR64 | IRS784-059 | 1 |
| ZjPCK<sub>pro</sub>:ZmG2 | Kitaake | 57C1 | >3 | C1-47 | C1-70, 75 |
| ZjPCK<sub>pro</sub>:ZmG2 | Kitaake | 57C2 | 1 | C2-48 | C2-48 |
| ZjPCK<sub>pro</sub>:ZmG2 | Kitaake | 57C3 | >2 | C3-71 |
| ZmUBI<sub>pro</sub>:ZmG2 | IR64 | IRS786-052 | 2 |
| ZmUBI<sub>pro</sub>:ZmG2 | IR64 | IRS786-088 | 1 |
| ZmUBI<sub>pro</sub>:ZmG2 | Kitaake | 57E1 | 1 | E1-127 | E1-127 |
| ZmUBI<sub>pro</sub>:ZmG2 | Kitaake | 57E2 | 1 | E2-133 | E2-123 |
| ZmUBI<sub>pro</sub>:ZmG2 | Kitaake | 57E3 | 1 | E3-131 |
| ZmUBI<sub>pro</sub>:ZmG2 | Kitaake | 57E4 | 2 | E4-125 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | IR64 | IRS783-001 | 2 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | IR64 | IRS783-099 | 2 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | Kitaake | 58A1 | 2? | A1-104 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | Kitaake | 58A2 | 1 | A2-84,100 | A2-100 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | Kitaake | 58A3 | 1 | A3-85 |
| ZmPEPC<sub>pro</sub>:ZmGLK1 | Kitaake | 58A4 | 1 | A4-77 |
| ZjPCK<sub>pro</sub>:ZmGLK1 | IR64 | IRS785-062 | 1 |
| ZjPCK<sub>pro</sub>:ZmGLK1 | IR64 | IRS785-063 | 1 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | IR64 | IRS787-050 | 1 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | IR64 | IRS787-060 | 1 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E1 | 1 | E1-5 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E2 | 1 | E2-28 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E3 | 1 | E3-9 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E4 | 4 | E4-2 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E5 | 1 | E5-19 |
| ZmUBI<sub>pro</sub>:ZmGLK1 | Kitaake | 58E? | ? | E-16 |

Table S1. Summary of transgenic lines analyzed. Related to Figures 1-6.
| Line                      | Planar Chloroplast Area (µm²) | Chloroplast Number/BS Cell | Chloroplast Number/BS Cell Volume (µm³ × 10⁻³) | BS Cell Volume, (µm³) |
|---------------------------|-------------------------------|-----------------------------|-----------------------------------------------|------------------------|
| **Leaf 4**                |                               |                             |                                               |                        |
| WT                        | 10.58±0.37ᵃ                   | 14 (6-21)                   | 2.90±0.27ᵃ                                   | 5518±479ᵃ              |
| ZjPCK<sub>pro</sub>:ZmG2 (3) | 11.76±0.35ᵃ                   | 15 (7-26)                   | 2.84±0.14ᵃ                                   | 6386±539ᵃ              |
| ZmUBI<sub>pro</sub>:ZmG2 (3) | 15.85±0.69ᵇ                   | 13 (8-21)                   | 3.44±0.27ᵇ                                   | 5088±462ᵇ              |
| ZmPEPC<sub>pro</sub>:ZmGLK1 (2) | 11.68±0.36ᵃ                   | 15 (10-20)                  | 4.03±0.31ᵃ                                   | 5339±621ᵃ              |
| ZmUBI<sub>pro</sub>:ZmGLK1 (2) | 13.67±0.39ᵇ                   | 13 (9-19)                   | 3.08±0.22ᵃ                                   | 5076±335ᵃ              |
| **Leaf 7**                |                               |                             |                                               |                        |
| WT                        | 8.82±0.28ᵃ                    | 12 (9-19)                   | 3.26±0.29ᵃ                                   | 4803±320ᵃ              |
| ZjPCK<sub>pro</sub>:ZmG2 (2) | 10.94±0.32ᵇ                   | 15 (11-22)                  | 3.63±0.23ᵃ                                   | 4720±206ᵃ              |
| ZmUBI<sub>pro</sub>:ZmG2 (3) | 15.55±0.50ᶜ                   | 15 (11-21)                  | 4.05±0.30ᵃ                                   | 4606±324ᵃ              |
| ZmPEPC<sub>pro</sub>:ZmGLK1 (3) | 12.80±0.38ᵈ                   | 14 (11-20)                  | 3.77±0.23ᵃ                                   | 4541±322ᵃ              |
| ZmUBI<sub>pro</sub>:ZmGLK1 (3) | 13.30±0.57ᵈ                   | 15 (9-22)                   | 3.89±0.27ᵃ                                   | 4427±280ᵃ              |

**Table S2. Quantification of chloroplast size and number in bundle sheath cells. Related to Figure 2.**

Planar chloroplast area, number, and number per cell volume in isolated bundle sheath (BS) cells from leaves 4 and 7 of T1 transgenic Kitaake and wild-type (WT) lines grown at 300 µmol photons m⁻² s⁻¹. Transgene transcript levels in each leaf at the time of harvesting are shown in Figure S2. Mean ± s.e.m. (except chloroplast number which is represented by median and range), n = 45 i.e. 15 BS cells from each of three individuals representing at least 2 independent transgenic lines (number of independent lines in each sample is indicated in parentheses after construct name). Statistically distinct groups for each leaf are indicated by superscripted letters (p<0.05) as determined by a Games-Howell test of variance. Data correspond to Figure 2 in main text.
Table S3. Quantification of relationship between chloroplast and mitochondrial size in leaf 7 of wild-type and transgenic lines. Related to Figures 3 and 4, Table 1.

Planar chloroplast area/planar cell area (%)

|       | WT     | ZjPCKpro::ZmG2 | ZmUBIpro::ZmG2 | ZmPEPCpro::ZmGLK1 | ZmUBIpro::ZmGLK1 |
|-------|--------|----------------|----------------|--------------------|--------------------|
| BS    | 5.3±0.7<sup>a</sup> | 7.2±0.7<sup>ab</sup> | 12.9±0.7<sup>c</sup> | 9.3±0.7<sup>b</sup> | 7.0±0.7<sup>ab</sup> |
| MS    | 0.4±0.2<sup>a</sup> | 1.0±0.6<sup>ab</sup> | 16.3±1.9<sup>c</sup> | 1.3±0.3<sup>b</sup> | 7.2±1.3<sup>d</sup> |
| M<sup>*</sup> | 50.9±1.5<sup>a</sup> | 50.3±1.8<sup>a</sup> | 54.6±1.2<sup>a</sup> | 48.7±1.8<sup>a</sup> | 48.4±2.1<sup>a</sup> |

Planar mitochondria area/planar cell area (%)

|       | WT     | ZjPCKpro::ZmG2 | ZmUBIpro::ZmG2 | ZmPEPCpro::ZmGLK1 | ZmUBIpro::ZmGLK1 |
|-------|--------|----------------|----------------|--------------------|--------------------|
| BS    | 0.5±0.2<sup>a</sup> | 0.4±0.2<sup>a</sup> | 2.9±0.2<sup>b</sup> | 0.5±0.2<sup>a</sup> | 1.9±0.2<sup>b</sup> |
| MS    | 1.4±0.2<sup>a</sup> | 1.8±0.2<sup>ab</sup> | 2.3±0.2<sup>b</sup> | 1.5±0.2<sup>ab</sup> | 2.0±0.2<sup>ab</sup> |
| M     | 2.0±0.1<sup>a</sup> | 3.3±0.3<sup>b</sup> | 2.3±0.2<sup>ab</sup> | 1.3±0.1<sup>c</sup> | 1.9±0.1<sup>a</sup> |

Planar mitochondria #/planar cell area, µm<sup>2</sup> × 10<sup>3</sup>

|       | WT     | ZjPCKpro::ZmG2 | ZmUBIpro::ZmG2 | ZmPEPCpro::ZmGLK1 | ZmUBIpro::ZmGLK1 |
|-------|--------|----------------|----------------|--------------------|--------------------|
| BS    | 34.0±3.9<sup>ab</sup> | 26.1±3.9<sup>a</sup> | 44.5±3.9<sup>b</sup> | 48.4±3.9<sup>b</sup> | 29.3±3.9<sup>a</sup> |
| MS    | 107.5±18.4<sup>a</sup> | 103.7±15.3<sup>a</sup> | 136.6±12.1<sup>a</sup> | 118.5±14.3<sup>a</sup> | 154.4±14.1<sup>a</sup> |
| M     | 57.4±3.8<sup>a</sup> | 70.9±4.2<sup>a</sup> | 63.1±4.3<sup>a</sup> | 63.4±5.0<sup>a</sup> | 55.8±4.2<sup>a</sup> |

Table S3. Quantification of relationship between chloroplast and mitochondrial size in leaf 7 of wild-type and transgenic lines. Related to Figures 3 and 4, Table 1. Planar chloroplast and mitochondria area/planar cell area of bundle sheath (BS), mestome sheath (MS) and mesophyll (M) cells of leaf 7 from transgenic Kitaake and wild-type (WT) plants grown at 300 µmol photons m<sup>-2</sup> s<sup>-1</sup>. Transgene transcript levels in each leaf at the time of harvesting are shown in Figure S2. Cell and organelle areas were calculated from TEM images; values are mean ± s.e.m; n=45 i.e. 15 cells/individual, 3 individuals/construct. Individuals represent at least two independent transgenic events for each construct (see Table S1 for line numbers, and Figure S1 for transgene copy number in each line). Statistically distinct groups are indicated by superscripted letter within each row (p<0.05) by a Tukey’s test of variance for normally distributed data (asterisk) and a Games-Howell test of variance for the rest of the data. Data correspond to Figures 3 & 4 and Table 1 in main text.
Table S4. Relative frequency of plasmodesmata between cell-types in wild-type and transgenic lines. Related to Figure 4.
Percentage of bundle sheath cells that had plasmodesmata in walls shared with adjacent mesophyll (BS-M) or mestome sheath cells (BS-MS) in leaf 7 of transgenic Kitaake and wild-type (WT) plants grown at 300 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). Values were calculated from TEM images and are mean ± s.e.m; \( n = 45 \) i.e. 15 cells/individual, 3 individuals/construct. Individuals represent at least two independent transgenic events for each construct (see Table S1 for line numbers, Figure S1 for transgene copy number in each line and Figure S2 for transgene transcript levels). Asterisks indicate lines with frequency of plasmodesmata significantly higher than WT, as measured by a General Linear Model.

|        | WT     | \( ZjPCK_{\text{pro}} : ZmG2 \) | \( ZmUBI_{\text{pro}} : ZmG2 \) | \( ZmPEPC_{\text{pro}} : ZmGLK1 \) | \( ZmUBI_{\text{pro}} : ZmGLK1 \) |
|--------|--------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| BS-M   | 35.6±11.1 | 51.1±8.0                      | 64.4±5.9*                     | 60.0±6.7*                     | 56.7±3.3*                     |
| BS-MS  | 24.4±2.2  | 33.3±3.8                      | 44.4±11.8*                    | 42.2±9.7*                     | 28.9±2.2                      |
Table S5. Percentage of chloroplasts and mitochondria invested in each cell-type in wild-type rice, GLK transgenic lines, various PACMAD grasses and Flaveria. Related to Figure 5.

The % of organelles invested in each cell-type is quantified as the proportion of organellar area in that cell-type, as compared to the total organellar area across all photosynthetic tissues (see experimental design section of methods for full details). The total photosynthetic tissue area is defined as the sum of the planar leaf area covered by both mesophyll and vascular sheath cells. The total organellar area in all photosynthetic tissue is quantified as the sum of the fractional organelle area in each cell-type. Mean ± s.e.m., n = 3 for rice and each PACMAD grass. Dichanthelium, Steinchisma and Homolepis data are from [S4] Flaveria organellar data are from [S1,S2] and the values for cell type % in Flaveria species are extracted from M:BS ratios in Fig. 4 of [S3]. Data correspond to Figure 5 in main text and Figure S4.
| Primer name       | Sequence (5' to 3')                                                                 |
|------------------|--------------------------------------------------------------------------------------|
| ZmG2-cloningF    | GGGGACAAGTTTGTTACAAAAAAGCAGGCTATGCTTGAGGTGTCGAGTCGAGCTGACAGCTGCTTGAGGTGTCGACGCTG |
| ZmG2-cloningR    | GGGGACCTTTTGTTACAAAAAAGCAGGCTATGCTTGAGGTGTCGAGTCGAGCTGCTTGAGGTGTCGACGCTGACAGCTGCTG |
| ZmGLK1-cloningF  | GGGGACAAGTTTGTTACAAAAAAGCAGGCTATGCTTGAGGTGTCGAGTCGAGCTGCTTGAGGTGTCGACGCTGACAGCTG |
| ZmGLK1-cloningR  | GGGGACCTTTTGTTACAAAAAAGCAGGCTATGCTTGAGGTGTCGAGTCGAGCTGCTTGAGGTGTCGACGCTGACAGCTG |
| pVec8F (UBIpro)  | TTAGGGCTGCCTATACAG                                                             |
| pVec8R (nosT)    | ATTGCCAAATGTTTGGAACGA                                                             |
| pSC110F (PEPpro) | ACGACCTCCATCCCTATTT                                                             |
| pSC210F (PCKpro) | CTGCTGCTGTGCTGCTGCTC                                                             |
| pSC1/2/310-R     | AAGACCGGCAACAGATTTT                                                             |
| HPTFpr3          | AAAACTGTGATGGACGACACC                                                             |
| HPTRpr2          | CTATCACGACTTGTTGACG                                                             |
| ZmG2-F           | CATGGTGACGACACCTC                                                             |
| ZmG2-R           | CACATGGTTGCTCCACAGACC                                                             |
| ZmGLK1-F         | GGACCTGGATTTTCGACTTTCA                                                            |
| ZmGLK1-R         | CACTCCCTTTCTCCCTTC                                                              |
| Actin-F          | GCCACCACTCCCTCTACAAT                                                              |
| Actin-R          | CTCACACACTCAGAGTTT                                                             |
| OsGLK1-F         | AGCTGAGATTTTTCGCTC                                                              |
| OsGLK1-R         | ATAGCTGAGTTTTCGCTC                                                              |
| OsGLK2-F         | AGGGAGAGATTTTGGTTGAGG                                                         |
| OsGLK2-R         | TTTCCACGTTTTCCCTG                                                                |

Table S6. PCR primers used. Related to STAR Methods.
SUPPLEMENTAL REFERENCES

S1. Stata, M., Sage, T.L., Hoffmann, N., Covshoff, S., Ka-Shu Wong, G., and Sage, R.F. (2016). Mesophyll chloroplast investment in C₃, C₄ and C₂ species of the genus Flaveria. Plant Cell Physiol. 57, 904–918.

S2. Sage, T.L., Busch, F.A., Johnson, D.C., Friesen, P.C., Stinson, C.R., Stata, M., Sultmanis, S., Rahman, B.A., Rawsthorne, S., and Sage, R.F. (2013). Initial events during the evolution of C₄ photosynthesis in C₃ species of Flaveria. Plant Physiol. 163, 1266–1276.

S3. McKown, A.D., and Dengler, N.G. (2007). Key innovations in the evolution of Kranz anatomy and C₄ vein pattern in Flaveria (Asteraceae). Am. J. Bot. 94, 382–399.

S4. Khoshravesh, R., Stinson, C.R., Stata, M., Busch, F.A., Sage, R.F., Ludwig, M., and Sage, T.L. (2016). C₃–C₄ intermediacy in grasses: organelle enrichment and distribution, glycine decarboxylase expression, and the rise of C₂ photosynthesis. J. Exp. Bot. 67, 3065–3078.