**DCT4—A New Member of the Dicarboxylate Transporter Family in C_4 Grasses**

Sarit Weissmann¹, Pu Huang¹†, Madeline A. Wiechert¹, Koki Furuyama², Thomas P. Brutnell³, Mitsutaka Taniguchi², James C. Schnable⁴*, and Todd C. Mockler¹,*

¹Donald Danforth Plant Science Center, St. Louis, Missouri, USA
²Graduate School of Bioagricultural Sciences, Nagoya University, Aichi, Japan
³Chinese Academy of Agricultural Sciences, Biotechnology Research Institute, Beijing, China
⁴Computational Sciences Initiative, Center for Plant Science Innovation, Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Nebraska, USA

†Present address: BASF Corporation, Durham, NC, USA
*Corresponding authors: E-mails: schnable@unl.edu; tmockler@danforthcenter.org.

Accepted: 24 November 2020

**Abstract**

Malate transport shuttles atmospheric carbon into the Calvin–Benson cycle during NADP-ME C_4 photosynthesis. Previous characterizations of several plant dicarboxylate transporters (DCT) showed that they efficiently exchange malate across membranes. Here, we identify and characterize a previously unknown member of the DCT family, DCT4, in Sorghum bicolor. We show that SbDCT4 exchanges malate across membranes and its expression pattern is consistent with a role in malate transport during C_4 photosynthesis. SbDCT4 is not syntenic to the characterized photosynthetic gene ZmDCT2, and an ortholog is not detectable in the maize reference genome. We found that the expression patterns of DCT family genes in the leaves of Zea mays, and S. bicolor varied by cell type. Our results suggest that subfunctionalization, of members of the DCT family, for the transport of malate into the bundle sheath plastids, occurred during the process of independent recurrent evolution of C_4 photosynthesis in grasses of the PACMAD clade. We also show that this subfunctionalization is lineage independent. Our results challenge the dogma that key C_4 genes must be orthologues of one another among C_4 species, and shed new light on the evolution of C_4 photosynthesis.

**Key words:** DCT4, new transporter gene, grass evolution, C_4 photosynthesis.

**Significance**

Dicarboxylate-transporter-2 (DCT2) plays a key role during C_4 photosynthesis in Zea mays. Its orthologs are assumed to function the same in related species, as Z. mays is the main C_4 reference species. We introduce a new gene, DCT4, that assumed the role of DCT2 in Sorghum bicolor and other C_4 grass species. By surveying related C_4 species, we propose that different members of the DCT family subfunctionalized for photosynthetic malate transport in the BS cells of C_4 grasses of the PACMAD clade. We suggest that rather than being static, biochemical adaptations continued after the divergence of the PACMAD lineages.

**Introduction**

Three subtypes of C_4 photosynthesis are generally recognized as defined by the primary decarboxylase in the bundle sheath (BS) cells: Chloroplastic NADP-dependent malic enzyme (NADP-ME); mitochondrial NAD-dependent malic enzyme (NAD-ME); and cytosolic phosphoenolpyruvate carboxykinase (PEPCK) (Hatch and Slack 1966; Hatch 1971; Rathnam and Edwards 1977). Different plant species may contain various...
combinations of these three subtypes (Hatch 1971; Chapman and Hatch 1979; Furbank 2011; Pick et al. 2011; Wang, Brautigam, et al. 2014). The movement and exchange of malate across membranes, by dicarboxylate transporters (DCTs/DITs), plays a significant role during photosynthesis in NADP-ME and NAD-ME C4 species (Ding et al. 2015). In C3 plants, DCTs are crucial to nitrate assimilation, such as the GS/GOGAT cycle and photorespiration (Linka and Weber 2010; Kinoshita et al. 2011). Taniguchi et al. characterized several plant DCTs that efficiently exchange malate across membranes (Taniguchi et al. 2002; 2004). The differential expression of C4 photosynthesis genes in mesophyll (M) and BS cells (John et al. 2014; Tausta et al. 2014; Wang, Czedik-Eysenberg, et al. 2014) suggests that different malate transporters may be needed to move malate out of the chloroplasts of M cells and into the chloroplasts of BS cells. In Zea mays, an NADP-ME C4 grass, dicarboxylate transporter-2 (ZmDCT2, GRMZM2G086258) moves malate into the chloroplast of BS cells during C4 photosynthesis (Weissmann et al. 2016). ZmDCT2 plays a critical role during C4 photosynthesis in Z. mays, and its absence severely impairs plant growth and development (Weissmann et al. 2016). The role of DCTs in C4 photosynthesis in other species, however, remains unknown.

Zea mays is the best characterized and functionally annotated C4 grass species. As such, it is a useful reference for identification of photosynthesis-related genes in poorly characterized C4 grasses and for resolving orthology (John et al. 2014; Ding et al. 2015; Huang et al. 2017). Microsynteny, the comparison of collinearity among related species, is a reliable approach to determine orthology and predict the function of a gene (Bennetzen and Freeling 1997; Chen et al. 1997; Tikhonov et al. 1999; Bennetzen 2000; Kumar et al. 2009; Jin et al. 2016). Davidson et al. (2012) showed that syntenic orthologs are likely to have conserved functions and expression patterns across lineages. Here, we identify a new member of the DCT family, DCT4, which is not syntenic to the photosynthetic gene ZmDCT2 and is not detected in the maize reference genome. We demonstrate that S. bicolor DCT4 (SbDCT4) efficiently exchanges malate across membranes, consistent with a malate transport role in C4 photosynthesis. We characterize the diverse expression patterns of DCT genes in leaves of multiple grass species. We also propose that subfunctionalization of DCTs in grasses of the PACMAD clade (Sanchez-Ken and Clark 2010) occurred during independent recurrent evolution of C4 photosynthesis.

**Results**

Identification of DCT4 in S. bicolor

To learn more about C4-related DCT in species evolutionarily related to maize, we identified the syntenic ortholog of ZmDCT2 in S. bicolor. Two genes, Sobic.007G226700 and Sobic.007G226800, are present at the predicted syntenic orthologous position on chromosome 7. We refer to them as SbDCT2.1 and SbDCT2.2, respectively (fig. 1). ZmDCT2 is abundantly expressed (table 1), and its expression is enriched in BS cells of maize leaves (fig. 2) (Li et al. 2010; Tausta et al. 2014; Ding et al. 2015). In contrast, the expression profiles of SbDCT2.1 and SbDCT2.2 in S. bicolor leaves are low (table 1). SbDCT2.1 expression is slightly enriched in the M cells.
whereas SbDCT2.2 is enriched in BS cells (fig. 2). We also analyzed the transcript levels of two other S. bicolor DCT, SbDCT1 (Sobic.002G233700) and SbOMT1 (Sobic.008G112300). These genes are the orthologs of the Z. mays genes ZmDCT1 (GRMZM2G040933) and Zm-oxoglutarate/malate transporter 1 (ZmOMT1; GRMZM2G383088), respectively. We found that SbDCT1 expression, similar to that of ZmDCT1, is relatively low (table 1), and only slightly differentially expressed in M cells relative to BS cells (fig. 2). The expression of both ZmOMT1 and SbOMT1 is relatively high (table 1), and both are slightly enriched in M cells (fig. 2).

In C₄ species, the expression of many photosynthetic genes is enriched in either BS and M cells (Li et al. 2010; John et al. 2014; Tausta et al. 2014; Weissmann et al. 2016; Rao et al. 2016). In NADP-ME species, two transporters, one within the chloroplast during C₄ photosynthesis (Brautigam et al. 2008; BS cells and another in M cells, move malate in and out of the cell. The expression of both ZmOMT1 and SbOMT1 is relatively high (table 1), and both are slightly enriched in M cells (fig. 2).

In C₃ species, the expression of many photosynthetic genes is enriched in either BS and M cells (Li et al. 2010; John et al. 2014; Tausta et al. 2014; Weissmann et al. 2016; Rao et al. 2016). In NADP-ME species, two transporters, one within the chloroplast during C₄ photosynthesis (Brautigam et al. 2008; BS cells and another in M cells, move malate in and out of the cell. The expression of both ZmOMT1 and SbOMT1 is relatively high (table 1), and both are slightly enriched in M cells (fig. 2).

In C₄ species, the expression of many photosynthetic genes is enriched in either BS and M cells (Li et al. 2010; John et al. 2014; Tausta et al. 2014; Weissmann et al. 2016; Rao et al. 2016). In NADP-ME species, two transporters, one within the chloroplast during C₄ photosynthesis (Brautigam et al. 2008; BS cells and another in M cells, move malate in and out of the cell. The expression of both ZmOMT1 and SbOMT1 is relatively high (table 1), and both are slightly enriched in M cells (fig. 2).

SbDCT4 is an Efficient Malate Transporter

To verify the ability of SbDCT4 to transport malate, we cloned coding sequences from the three sorghum DCT genes, SbDCT1, SbDCT2, and SbDCT4. We measured the malate transport activities of the recombinant proteins expressed in yeast. SbDCT4 was an efficient malate transporter (table 2). The Km of SbDCT4 was similar to that of SbDCT1, and the affinity for malate was highest in SbDCT2 among the three SbDCTs (table 2), consistent with the relative malate transport activities reported for maize DCT1 and DCT2 (Taniguchi et al. 2004).

Phylogenetic Distribution of DCT Genes in Grasses

To understand the relationship of SbDCT4 to other grass DCT genes, we searched the genomes of the grass species Setaria italica, Urochloa fusca, Brachypodium distachyon, and Dichanthelium oligosanthes. In S. italica, an NADP-ME C₃ species, we identified a DCT, Seita.9G375100, that showed no syntenic orthologous relationship with DCT genes in other available grass genomes. Phylogenetic analysis showed that this gene clustered with SbDCT4 but not with SbDCT1 and
from the de novo leaf transcriptome assemblies to generate a phylogenetic tree of the DCT family. The resulting phylogeny shows that DCT4 transcripts form a distinct subclade from the DCT1 clade (fig. 3). The absence of DCT4 transcript expression does not rule out the existence of the gene in the genome. We also used polymerase chain reaction (PCR) to survey for DCT genes in the genomes of grass species for which whole-genome assemblies were not available. We designed conserved primers (nondegenerate or minimally degenerate) to small regions unique to each of the three DCT genes using PrimaClade (Gadberry et al. 2005). We detected DCT1 and DCT2 in the genomes of all species tested (table 1, supplemental fig. 1, Supplementary Material online). DCT4, however, was detected only in the genomes of NADP-ME C4 species of the PACMAD clade, excluding Z. mays (table 1, supplemental fig. 2, Supplementary Material online).

Expression of Malate Transporter Genes in NADP-ME C4 Grasses

C3 species and U. fusca, a PEPCK C4 species, express both DCT1 and DCT2 at low levels in leaves (table 1). C4 NADP-ME species of the PACMAD clade generally express one DCT gene in leaves at a high level and also express one or two other DCT genes at low levels (table 1). We did not find an apparent lineage-specific pattern for the expression of the predominant DCT gene in the NADP-ME species we analyzed. This finding is consistent with random evolutionary processes underlying the subfunctionalization of members of the DCT family. Interestingly, Z. mays is the only species we examined in which DCT2 is the predominantly expressed DCT gene (table 1). We also examined the expression of the non-DCT malate transporter OMT1 gene in the leaves of grasses (table 1). Interestingly, we found that although OMT1 expression was generally abundant, there was no consistent pattern of relative expression between the DCT and OMT genes within the NADP-ME C4 species (table 1).

Discussion

Evolution of the DCT Gene Family in Grasses

We identified DCT4 as a new member of the DCT gene family in the grasses (fig. 1). Our analysis suggests that DCT4 is present in some C4 NADP-ME PACMAD grasses. DCT1 and DCT2 appear to have originated from a duplication of a single DCT gene after the monocot–eudicot split (Taniguchi et al. 2004) and DCT4 arose from a duplication of DCT1 at the root of the PACMAD clades (fig. 3). The expression of DCT genes in the grasses that we analyzed exhibited no clear lineage-specific patterns (table 1). Therefore, we propose that different members of the DCT family subfunctionalized for photosynthetic malate transport in the BS cells of C4 grasses of the PACMAD clade.

Table 2

| Gene   | Kₚm (mM) |
|--------|----------|
|        | DCT1     | DCT2     | DCT4     |
| Sorghum bicolor | 1.24 ± 0.14 | 0.71 ± 0.10 | 1.13 ± 0.10 |
| Zea mays | 1.1 ± 0.1 | 0.85 ± 0.44 | N/A |

The values are the means of three independent experiments ± SE.

*Kinetic values from a previous report (Taniguchi et al. 2004).

SbdC2 (fig. 3). We designated this gene SbdC4. We did not detect orthologs, syntenic or otherwise, in U. fusca, a PEPCK C4 species, or in the two C3 species. To expand the search for DCT4 in other grasses currently lacking genome assemblies, we examined leaf-derived transcript assemblies for Aristida congesta, Eriachne aristidea, Chasmanthium laxum, Danthoniopsis dinteri, Anthephora pubensis, Echinocloa esculenta, Paspalum vaginatum, and Arundinella hirta (Huang P, Mayfield-Jones D, Schnable J, Brutnell T, manuscript in preparation). We then used the predicted coding sequences of the DCT genes from available genomes and

Fig. 2.—Differential expression of malate transporters in Zea mays, and Sorghum bicolor leaves between the BS and M cells. The genome of Z. mays has one copy of DCT2 and does not contain DCT4, and 2mDCT2 is highly enriched in BS cells. Sorghum bicolor has two copies of DCT2 (DCT2.1, and DCT2.2) in the syntenic genomic location that are the result of gene duplication. Sorghum bicolor also expresses DCT4, which is highly enriched in BS cells. Both species express OMT1 and DCT1, which are only slightly enriched in the M cells. Red bars represent enrichment in the BS cells. Blue bars represent enrichment in the M cells. The white numbers inside the bars represent the significance (P-value) of the log2FoldChange.
This work also challenges the dogma that key C$_4$ genes must be orthologues of one another, among species, and show that they can be paralogs. This confirms the importance of including syntenic and expression data in assigning orthology across species, and of developing multiple models for C$_4$ photosynthesis in the grasses. For example, $\text{SiDCT4}$ was previously misannotated as the ortholog of $\text{ZmDCT2}$ (John et al. 2014), likely because of the lower expression level of $\text{SiDCT2}$ ($\text{Seita.9G375100}$) in leaf tissue. The use of different malate transporters, for example, DCT4 in $\text{S. bicolor}$ and $\text{S. italica}$, or DCT2 in $\text{Z. mays}$, suggests that multiple evolutionary paths resulted in the development of an active C$_4$ NADP-ME photosynthetic cycle. It is interesting to note that common origins of C$_4$ photosynthesis are often defined based on the predominant decarboxylase utilized, thus maize and sorghum are considered to have evolved from a common C$_4$ ancestor. This analysis suggests that rather than being static, biochemical adaptations continued after the divergence of maize and sorghum lineages. Thus, optimizations of C$_4$ activities may be continuous as breeding pressures or climate change alters ecological niches of individual species.

Various C$_4$ Subtype Combinations Have Different Transport Requirements

The variation of expression levels among the different malate transporters within each NADP-ME species (table 1) suggests different transport requirements during C$_4$ photosynthesis.

---

**Fig. 3**—A phylogenetic tree of the DCT family in the grasses showing that DCT4 is a subclade of DCT1. The DCT1, DCT2, and DCT4 gene lineages are black, blue, and red, respectively. The length of the branches represents the evolutionary distance between ancestor to descendent nodes. The numbers represent the confidence level of the specific branch.
This supposition is in agreement with the view that the three subtypes of C₄ photosynthesis are mixed rather than exclusive (Hatch 1971; Chapman and Hatch 1979; Furbank 2011; Pick et al. 2011; Wang, Brautigam, et al. 2014). For example, Z. mays utilizes both the NADP-ME (75%) and PEPCCK (25%) pathways to fix carbon (Chapman and Hatch 1979; Winger et al. 1999; Weissmann et al. 2016), and has similar expression levels of DCT2 and OMT1 and low expression of DCT1. Sorghum bicolor moves carbon through both malate and aspartate, although no PEPCCK activity was detected in its leaves (Chapman and Hatch 1979). Sorghum bicolor has similar expression levels for DCT4 and DCT1 and high expression of OMT1 (table 1). Other grass species may have DCT expression ratios that correspond to their unique combination of C₄ subsystems. For example, OMT1 is highly expressed in U. fusca, ~3- to 7-fold higher than DCT2 or DCT1, respectively. OMT1 transports dicarboxylates, excluding those containing an amino group (Taniguchi et al. 2002, 2004). Thus, in PEPCCK C₄ plants, OMT1 may move oxaloacetate into the mesophyll chloroplast, and 2-oxoglutarate out, to support the high production of aspartate needed to maintain the photosynthetic cycle (Rathnam and Edwards 1977). Interestingly, both zomT1 and sbOMT1 are only slightly differentially expressed in the M cells (fig. 2). As the loss of DCT2 in Z. mays prevents movement of malate into the BS chloroplast (Weissmann et al. 2016), OMT1 cannot be moving malate into the BS chloroplast alongside DCT2. But OMT1 may also have a role in organic acid metabolism in both cell types, such as shuttling reducing equivalents in organelles other than the chloroplast (Pleite et al. 2005).

Conclusions
Our results show that the newly identified member of the DCT gene family, SbDCT4, is an efficient malate transporter. Based on the expression patterns of malate transporters among the grasses, we suggest that different members of the DCT family may have evolved multiple roles in C₄ photosynthesis. Further studies will be needed to verify the subcellular localization of these proteins and to define their specific metabolic functions. Characterizing the various combinations of C₄ photosynthetic subsystems in grasses will facilitate the exploitation of DCT genes, through breeding or engineering, to improve the performance of crop plants and increase yield.

Materials and Methods
Identification of DCT4 Genes in Sorghum and Other Grasses
We used QUOTA-ALIGN (Tang et al. 2011) to identify syntenic orthologous regions in grass species with sequenced genomes, following the protocol described in Zhang et al. (2017). To find homologous genes at nonsyntenic locations, we used two complementary approaches. For species with sequenced genomes, we used LASTZ (Harris 2007) to align the coding sequence of the primary transcript annotated in Phytozome (https://phytozome-next.jgi.doe.gov, last accessed January 6, 2021) to the genome assembly. For species without assembled genomes, we used LASTZ to align the coding sequence of the primary transcript from Phytozome to transcript assemblies generated by Trinity (Grabherr et al. 2011).

Measurements of Malate Transport
We cloned each of the three SbDCT cDNAs between the promoter and terminator of yeast GAL2 in the pTV3e vector (Nishizawa et al. 1995). We transformed the plasmids into yeast LBY416 cells and selected transformants on tryptophan-deficient agar plates. We prepared a crude membrane fraction from the selected yeast transformants. We used a freeze-thaw technique to reconstitute liposomes for the measurement of the uptake of [14C]malate (Taniguchi et al. 2002).

Phylogenetic Analysis of DCT Homologs
DCT coding sequences for Z. mays, S. bicolor, S. italica, B. distachyon, O. sativa, D. oligosanthes, and U. fusca were from Phytozome (https://phytozome-next.jgi.doe.gov, last accessed January 6, 2021). We used BlastN (Altschul et al. 1990) to search de novo assembled leaf transcriptomes (Huang P, Mayfield-Jones D, Schnable J, Brutnell T, manuscript in preparation) from the C₄ grass species A. congesta, E. aristidea, C. laxum, D. dinteri, A. pubens, E. esculenta, P. vaginatum, and A. hirta with the DCT sequences from maize, Setaria, and Sorghum as queries. We used ProGraphMSA to generate a codon-based sequence alignment (Szalkowski 2012). We used MEGA6 (Tamura et al. 2013), with default parameters and the branch support values based on 1,000 bootstraps, to generate the phylogenetic reconstruction with the maximum likelihood method and based on the nucleotides in the third position of codons (Simmons et al. 2006).

Analysis of Gene Expression for Decarboxylase Transporters in Grasses
For species with published leaf transcriptome profiles (Ouyang et al. 2007; Li et al. 2010; Zhang et al. 2012; Schnable 2014; Wang, Czedik-Eysenberg, et al. 2014; Studer et al. 2016), gene expression levels were calculated and normalized, for each species, as Transcripts Per Million (TPM). For the other species, the normalized TPM values were based on de novo transcriptome assemblies (Huang P, Mayfield-Jones D, Schnable J, Brutnell T, manuscript in preparation). The values in table 1 only allow for intraspecies comparisons among the decarboxylase transporters.
**Table 3**

Primer and PCR Conditions for the Amplification of Grass DCT Genes

| Primer Pair | Forward 5'-3'* | Reverse 5'-3'* | Cycling Conditions |
|-------------|----------------|----------------|-------------------|
| DCT1        | CACCAACGAGGTGATCTTGG | AGTAGGGTGCAGTDCGGTC | 94°C C 3 min, [94°C 45 s, 58°C 30 s, 72°C 1 min] × 30, 72°C 10 min, 4°C ∞ |
| DCT2        | CTVTGGATGTCRAATTGTGTTG | TGGCTTGCAABADATAGTGAA | 94°C C 3 min, [94°C 45 s, 58-52°C (-0.5°C cycle) 30 s, 72°C 1 min] × 14, [94°C 45 s, 52°C 30 s, 72°C 1 min] × 16, 72°C 10 min, 4°C ∞ |
| DCT4        | CTTYGTTAAGGTGCTCGG | GACTTGATGATSAGCAGGA | 94°C C 3 min, [94°C 45 s, 60°C 30 s, 72°C 1 min] × 30, 32°C 10.0 min, 4°C ∞ |

*B = C + G + T, D = A + G + T, R = A + G, S = C + G, V = A + C + G, Y = C + T.

Identification of **DCT4** in Species without Sequenced Genomes

We aligned the coding sequences from each of the DCT genes from *Z. mays*, *S. bicolor*, *S. italica*, *U. fuscum*, *B. distachyon*, *O. sativa*, *D. oligosanthes*, *A. congesta*, *E. aristidea*, *C. laxum*, *A. pubensis*, *E. esculenta*, and *A. hirta* using PAL2NAL (Suyama et al. 2006). The resulting multiple sequence alignment enabled the design of non-degenerate or minimally degenerate PCR primers (table 3) using PrimaClade (Gadberry et al. 2005).

Jacob D. Washburn and J. Chris Pires (University of Missouri, Columbia) kindly provided genomic DNA from *A. congesta*, *E. aristidea*, *D. dinteri*, *A. pubensis*, *E. esculenta*, and *A. hirta* (Washburn et al. 2015). We used a CTAB-based method to extract genomic DNA from *C. laxum, P. vaginatum*, *Z. mays*, *S. italica*, and *B. distachyon* (Weissmann et al. 2016). *Zea mays* and *B. distachyon* were the negative controls for **DCT4** and the positive controls for **DCT1** and **DCT2**. *Sorghum bicolor* and *S. italica* were the positive controls for **DCT1**, **DCT2**, and **DCT4**.

We conducted amplification of **DCT** genes by PCR using a 25-μl reaction mix and an ABI 2720 Thermal cycler. The reaction mixture included 2.5 μl of 10× Buffer, 2.5 μl of 10 μM solutions of forward and reverse primers, 2 μl of 2.5 mM dNTP stock, 14 μl of nuclease-free water, 0.5 μl of Choice Taq enzyme, and 1 μl of 100 ng/μl DNA. We performed PCR reactions as described in table 3 with 5 μl of loading dye added to each reaction. Aliquots of 13 μl were loaded on 3% agarose gels (Invitrogen UltraPure Agarose 1000, 1× TAE buffer, Invitrogen SYBR Safe Gel Stain) and electrophoresed for 30 min at 100 volts. We based size estimates on 100 bp and 50 bp DNA markers (GoldBio).

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online.

**Acknowledgments**

We acknowledge funding from National Science Foundation (Award No. IOS-1546882) and Department of Energy BER (Award No. DE-SC0018277) to T.C.M. We would like to thank Dr Elizabeth Kellogg and Dr Doug Allen for their advice and consultations during the writing of this manuscript.

**Data Availability**

This work includes no new sequence data.

**Literature Cited**

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215(3):403–410.

Bennetzen JL. 2000. Comparative sequence analysis of plant nuclear genomes: microcotlinearity and its many exceptions. Plant Cell. 12(7):1021–1029.

Bennetzen JL, Freeling M. 1997. The unified grass genome: synergy in synteny. Genome Res. 7(4):301–306.

Brautigam A, Hoffmann-Benning S, Weber AP. 2008. Comparative proteomics of chloroplast envelopes from C3 and C4 plants reveals specific adaptations of the plastid envelope to C4 photosynthesis and candidate proteins required for maintaining C4 metabolite fluxes. Plant Physiol. 148(1):568–579.

Chapman KS, Hatch MD. 1979. Aspartate stimulation of malate decarboxylation in Zea mays bundle sheath cells: possible role in regulation of C4 photosynthesis. Biochem Biophys Res Commun. 86(4):1274–1280.

Chen M, et al. 1997. Microcotlinearity in sh2-homologous regions of the maize, rice, and sorghum genomes. Proc Natl Acad Sci USA. 94(7):3431–3435.

Davidson RM, et al. 2012. Comparative transcriptomics of three Poaceae species reveals patterns of gene expression evolution. Plant J. 71(3):492–502.

Ding Z, et al. 2015. Identification of photosynthesis-associated C4 candidate genes through comparative leaf gradient transcriptome in multiple lineages of C4 and C3 species. PLoS One 10(10):e0140629.

Furbank RT. 2011. Evolution of the C4 photosynthetic mechanism: are really three C4 acid decarboxylation types? J Exp Bot. 62(9):3103–3108.

Gadberry MD, Malcomber ST, Doust AN, Kellogg EA. 2005. Primaclide—a flexible tool to find conserved PCR primers across multiple species. Bioinformatics 21(7):1263–1264.

Grabherr MG, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 29(7):644–652.

Harris RS. 2007. Improved pairwise alignment of genomic DNA. Philadelphia (PA): Pennsylvania State University.

Hatch MD. 1971. The C4 pathway of photosynthesis. Evidence for an intermediate pool of carbon dioxide and the identity of the donor C4 dicarboxylic acid. Biochem J. 125(2):425–432.
