Introgression and repeated co-option facilitated the recurrent emergence of C₄ photosynthesis among close relatives

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The origins of novel traits are often studied using species trees and modeling phenotypes as different states of the same character, an approach that cannot always distinguish multiple origins from fewer origins followed by reversals. We address this issue by studying the origins of C₄ photosynthesis, an adaptation to warm and dry conditions, in the grass Alloteropsis. We dissect the C₄ trait into its components, and show two independent origins of the C₄ phenotype via different anatomical modifications, and the use of distinct sets of genes. Further, inference of enzyme adaptation suggests that one of the two groups encompasses two transitions to a full C₄ state from a common ancestor with an intermediate phenotype that had some C₄ anatomical and biochemical components. Molecular dating of C₄ genes confirms the introgression of two key C₄ components between species, while the inheritance of all others matches the species tree. The number of origins consequently varies among C₄ components, a scenario that could not have been inferred from analyses of the species tree alone. Our results highlight the power of studying individual components of complex traits to reconstruct trajectories toward novel adaptations.

KEY WORDS: Ancestral state, complex trait, co-option, reticulate evolution, species tree.

Inferences of transitions among character states along species phylogenies provide powerful tools to test specific hypotheses about the timing and rate of functional diversification, correlations among functional and ecological traits (e.g., Pagel 1999; Edwards et al. 2010; Danforth et al. 2013; Moreau and Bell 2013; McGuire et al. 2014; Halliday et al. 2016), and speciation rates (Rabosky et al. 2013; Cantalapiedra et al. 2017; Cooney et al. 2017). However, distinguishing between a single origin of a trait with subsequent losses versus multiple independent origins can be problematic (Whiting et al. 2002; Pagel 2004; Wiens et al. 2006; Gamble et al. 2012), particularly when some character states affect the rates of speciation and/or extinction, when rates of transitions are high and asymmetrical, or variable among clades and through time (Maddison 2006; Goldberg and Igic 2008; Beaulieu et al. 2013; Igic and Busch 2013; King and Lee 2015). Indeed, transition rates might be higher in taxonomic groups possessing evolutionary precursors that increase the likelihood of evolving a specific trait (Blount et al. 2008, 2012; Marazzi et al. 2012; Christin et al. 2013a, 2015; Werner et al. 2014). This can lead to an unbalanced distribution of character states across the tree, with clusters forming in certain clades. However, a low rate of origins would lead to similar patterns if the rate of reversals is high (Wiens 1999; Danforth et al. 2003; Trueman et al. 2004; Pyron and Burbink 2014). Difficulties worsen if hybridization and introgression disconnect the history of underlying traits from the species tree (Pardo-Diaz et al. 2012; Meier et al. 2017).

An alternative approach to analyzing the phenotypes as different character states is to decompose them into their constituent components.
photosynthesis is a complex phenotype that improves the efficiency of carbon fixation in warm and dry conditions when compared to the ancestral C$_3$ photosynthetic pathway (Sage et al. 2012; Atkinson et al. 2016). The C$_4$ advantages are achieved by increasing the concentration of CO$_2$ around Rubisco, the enzyme responsible for inorganic carbon fixation in the Calvin cycle of all photosynthetic organisms (von Caemmerer and Furbank 2003; Sage et al. 2012). To function, C$_4$ photosynthesis requires the coordinated action of numerous anatomical and biochemical components that lead to the emergence of a novel biochemical pathway, usually across two types of cells; the mesophyll and bundle sheath cells (Hatch 1987; Prendergast et al. 1987; Gowik et al. 2011; GPWGII 2012; Bräutigam et al. 2014). Besides the increased expression of genes coopted for a C$_4$ function, several other changes are known to occur during the evolution of C$_4$ photosynthesis, including an expansion of bundle sheath tissue, a concentration of chloroplasts within it, and the adaptation of the enzymes to the new catalytic context (Fig. 1; Bläsiing et al. 2000; von Caemmerer and Furbank 2003; McKown and Dengler 2007; Sage et al. 2012).

Despite its apparent complexity, C$_4$ photosynthesis evolved multiple times independently, and is present in distantly related groups of plants (Sinha and Kellogg 1996; Kellogg 1999; Sage et al. 2011). As with any complex trait, C$_4$ photosynthesis likely evolved in incremental steps, via stages that are functionally intermediate and gradually increase carbon assimilation in warm and dry conditions (Fig. 1; Sage et al. 2012; Heckmann et al. 2013; Williams et al. 2013; Mallmann et al. 2014; Christin and Osborne 2014). An increase in bundle sheath size and the relocation of the chloroplasts/Rubisco to these cells can sustain a photosynthetic bypass (Hylton et al. 1988; Bräutigam and Gowik 2016). Subsequent increases in C$_4$ enzyme abundances can generate a weak C$_3$ cycle, which assimilates some of the atmospheric CO$_2$, complementing the C$_3$ cycle in C$_3$+C$_4$ plants (referred to as "type II C$_3$-C$_4$ intermediates" in the specialized literature; Fig. 1; Heckmann et al. 2013; Mallmann et al. 2014). The transition to a full C$_4$ state involves further increases of the bundle sheath tissue and gene expression, while selective pressures adapt the C$_4$ enzymes for the new biochemical context (Fig. 1; Bläsiing et al. 2000; McKown and Dengler 2007).

In the angiosperm phylogeny, C$_4$ taxa form clusters, many of which have multiple C$_4$ clades that are separated by non-C$_4$ branches (Sage et al. 2011; GPWGII 2012). Thus, establishing past photosynthetic transitions is difficult when photosynthetic type is modeled as a simple binary character (Ibrahim et al. 2009; Christin et al. 2010; Hancock and Edwards 2014; Bohley et al. 2015; Fisher et al. 2015; Washburn et al. 2015). Overall, nonhomo-logy of key C$_4$ components among some closely related C$_4$ groups, including the cells, enzymes, and genes modified to generate the C$_4$ pathway (Prendergast et al. 1987; Soros and Dengler 2001; Bräutigam et al. 2014; Lundgren et al. 2014; Wang et al. 2014), points to a predominance of C$_4$ origins (Sinha and Kellogg 1996; Christin and Besnard 2009; Christin et al. 2010). However, the possibility of evolutionary reversals to a non-C$_4$ state is still debated (e.g., Kadereit et al. 2014; Bohley et al. 2015; Washburn et al. 2015). Furthermore, some components of the C$_4$ phenotype (e.g., expansion of bundle sheaths and migration of chloroplasts; Fig. 1) may have evolved relatively few times, and have then been recurrently used for independent transitions to C$_3$+C$_4$ or C$_4$ photosynthesis (Christin et al. 2011, 2013a).

One of the proposed candidates for an evolutionary reversal from C$_4$ to C$_3$ is in the grass genus Alloteropsis (Ibrahim et al. 2014). The use of distinct components can be interpreted as evidence for multiple origins, while reversals could leave a signature of the lost trait that can be detected when components are compared with those from species that never evolved it (Protaas et al. 2006; Christin et al. 2010; Oliver et al. 2012; Niemiller et al. 2013). Identifying the mutations that underlie a trait further helps to distinguish shared origins and reversals (Igic et al. 2006; Shimizu et al. 2008; Niemiller et al. 2013; Meier et al. 2017). Evaluating the number of origins of each component of a complex trait would reconstruct the order of modifications that led to the trait of interest. This approach is applied here to the photosynthetic diversity exhibited within a five-species taxonomic group.

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Within this genus, the species _Alloteropsis semialata_ contains _C_3, _C_3+ _C_4, and _C_4 genotypes (Ellis 1974; Brown 1975; Lundgren et al. 2016). In molecular phylogenies based on either plastid or nuclear markers, this species is sister to the _C_4 _Alloteropsis angusta_, and the two species form a monophyletic clade sister to the three remaining closely-related _C_4 species: _Alloteropsis cimicina_, _A. paniculata_, and _A. papillosa_ (Ibrahim et al. 2009; Christin et al. 2012; Olofsson et al. 2016). The _C_4 _A. semialata_ and _A. cimicina_ use different cell types for the segregation of _C_4 reactions (Renvoize 1987), which suggests independent realizations of _C_4 photosynthesis (Christin et al. 2010). However, the evolutionary origins of _C_4 biochemistry and the situation within the _A. angustal/A. semialata_ group remain largely unexplored.

In this study, we focus on the genus _Alloteropsis_ and its _C_3 outgroup, to test the competing hypotheses of multiple origins versus fewer origins followed by reversals, independently for each _C_4 component. A _C_4 phenotype generated via distinct cells, genes, or amino acid mutations would indicate independent origins. In contrast, a reversal may lead to a derived state that retains traces of its past _C_3 state when compared to the ancestral one (i.e., approximated by the _C_3 outgroup here). We combined different approaches to investigate different components of the complex _C_4 trait. (i) Focusing on anatomical characters, we evaluate the most likely number of episodes of movement of chloroplasts to the bundle sheath, and expansion of this tissue. (ii) Using transcriptome analyses to estimate gene expression, we then determine the most likely number of origins of a _C_4 cycle via the upregulation of known _C_4 photosynthetic genes. (iii) The number of episodes of enzyme adaptation for the _C_4 cycle is estimated using positive selection analyses, with scenarios corresponding to episodes of adaptation along different sets of branches. (iv) Finally, we compare divergence times across genes, to detect potential introgression of _C_4 components, as suggested within this genus for two _C_4 genes (Olofsson et al. 2016). Our multifaceted effort highlights the power of comparative analyses that directly consider genes and other components involved in the trait of interest, rather than modeling complex phenotypes as states of a single character. Using this approach, we show that recurrent origins of _C_4 photosynthesis in _Alloteropsis_ arose via a complex mixture of co-option of traits increasing _C_4 accessibility, hybridization, and independent adaptation of the phenotype.

### Methods

**TAXON SAMPLING**

The different datasets were obtained from plants grown under controlled conditions (See Supporting Information Methods 1.1 for detailed description of growth conditions), including one _Alloteropsis cimicina_ (_C_4) accession, one _A. paniculata_ (_C_4) accession, two _A. angusta_ (_C_4) accessions, and up to 10 different _A. semialata_ accessions collected from separate populations that encompass the global genetic and photosynthetic diversity of this species (one _C_3, two _C_3+ _C_4 intermediates with a weak _C_4 cycle, and seven _C_4 accessions; Table S1; Lundgren et al. 2016). The over representation of _C_4 _A. semialata_ accessions mirrors their natural abundance, with _C_4 accessions spread throughout Africa, Asia, and Australia, _C_3 accessions only reported in Southern Africa, and _C_3+ _C_4 individuals restricted to central East Africa (Lundgren et al. 2015). We also make use of species representing the _C_3 sister group to _Alloteropsis_ (Panicum pygmaeum and Entolasia marginata), previously identified using plastid markers (GPWGII 2012). Using the above taxa, we conduct four complementary sets of analyses, each providing insight into the origins or spread of distinct components of _C_4 in _Alloteropsis_.

**(i) COMPARING LEAF ANATOMIES AMONG PHOTOSYNTHETIC TYPES**

Leaf cross-sections were analyzed to identify the leaf compartment being used for the segregation of Rubisco and the modifications that increased the proportion of bundle sheath tissue in _C_3+ _C_4 and _C_4 accessions. Co-option of different tissues and distinct modifications among accessions would support independent origins, while a reversal should result in the leaves of _C_3 individuals having reverted to a state that retain traces of their past _C_4 state when compared to the ancestral condition (e.g., enlarged bundle sheath cells and/or chloroplasts in the bundle sheath).

We generated new anatomical data for nine _A. semialata_ accessions and _A. angusta_ (Table S2), which supplemented previously published anatomical data for _E. marginata_, _P. pygmaeum_, _A. cimicina_, and _A. paniculata_ (Christin et al. 2013a). Images of _A. semialata_ and _A. angusta_ leaves in cross-section were obtained by fixing the center portion of a mature leaf blade in 4:1 ethanol:acetic acid, embedding them in methacrylate embedding resin (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany), sectioning on a manual rotary microtome (Leica Biosystems, Newcastle, U.K.), staining with Toluidine Blue O (Sigma-Aldrich, St. Louis, MO), then photographing them with a camera mounted atop a microscope (Olympus DP71 and BX51, respectively, Olympus, Hamburg, Germany), as described in Lundgren et al. (2016).

All species used in this study have two bundle sheath layers, differentiated as inner and outer bundle sheaths, which create concentric circles around each vein (Fig. S1). The sheath co-opted for the segregation of Rubisco was identified by a concentration of chloroplasts producing starch. We also recorded the presence of minor veins, and measured the following traits on one cross-sectional image per accession, as described in Christin et al. (2013a), using ImageJ software (Schneider et al. 2012): the interveinal distance (IVD; the average distance between centers of consecutive veins), the number of mediolateral mesophyll cells...
between veins, the average width of all outer and inner bundle sheath cells within a leaf segment, and the ratio of outer to inner bundle sheath cell widths (OS:IS). One leaf cross-section was used per accession, with previous work showing the traits we are measuring exhibit little variation within populations (Lundgren et al. 2016).

(ii) Comparing Gene Expression Profiles Among Photosynthetic Types

We use RNA-Seq to identify the genes co-opted by the different accessions performing a C₄ cycle, as those encoding C₄-related enzymes that reach high abundance in C₄ leaves. Variation in the co-opted loci would support multiple origins of a weak C₄ cycle, while a reversal might lead to high expression of C₄-related genes in individuals without a C₄ cycle or loss of functions of genes previously used for the C₄ cycle.

For RNA-Seq, we sampled the highly photosynthetically active distal halves of fully expanded new leaves and fresh roots midway into the photoperiod, which were subsequently flash frozen. Two different photoperiods (i.e., 10 and 14 h) were used to ensure that the identification of the most highly expressed genes did not differ among light regimes. Data from root libraries were only used in this study for transcriptome assembly, while all leaf samples were used for both assembly and quantification of transcript abundances. For a full list of individuals, conditions, and tissues sampled see Table S3.

Total RNA was extracted, Illumina TruSeq libraries generated, and sequencing performed using standard laboratory procedures, and transcriptomes were assembled using available pipelines (see Supporting Information Methods 1.2 for a detailed description of RNA-Seq protocol and assembly statistics). For each assembled contig, the transcript abundance was calculated as reads per million of mapped reads (rpm). Using a previously developed phylogenetic annotation pipeline (Christin et al. 2013b, 2015), the transcript abundance was then calculated for each gene lineage encoding C₄-related enzymes. For each gene family, all sequences descending from a single gene in the common ancestor of grasses via speciation and/or duplication were considered as the same gene lineage (i.e., these are grass co-orthologs). These groups include potential lineage specific paralogs (i.e., also known as inparalogs). When different Alloteropsis genes were identified within the same group of co-orthologs through detailed phylogenetic analyses, the abundance of each group was estimated independently. In Alloteropsis, this is the case only for genes previously shown to have been acquired laterally from distantly related C₃ lineages (Christin et al. 2012; see Results). In short, the reference datasets, composed of Arabidopsis thaliana coding sequences annotated as encoding C₄-related enzymes, and homolog sequences from other completely sequenced plants including five grasses, were retrieved from Christin et al. (2013b; 2015), or generated following the same approach for additional C₄-related enzymes identified in more recent studies (Mallmann et al. 2014; Li et al. 2015; Fig. S2). Contigs with similar sequences from the transcriptomes generated here were identified using BLASTn, with a minimal e-value of 0.01, and a minimal matching length of 50 bp. Only the portion of the contig matching the references was considered to remove UTRs, potential introns, and other very variable segments. Each sequence retrieved this way was then aligned independently to the reference dataset using Muscle (Edgar 2004), and a phylogenetic tree was inferred using Phyml (Guindon and Gascuel 2003) with a GTR+G+I model, a model that fits the vast majority of genes (e.g., Fisher et al. 2016) and is appropriate to infer a large number of trees. Phylogenetic trees were automatically screened, and each contig was assigned to the previously identified gene lineages in which it was nested. The sum of rpm values of all transcriptome contigs assigned to the same gene lineage produced transcript abundance per group of grass co-orthologs or distinct genes within these groups, which were subsequently transformed into rpm per kilobase (rpkm) values. Rpkm values were then compared among accessions to identify similarities and differences in the expression of C₄ photosynthetic genes.

(iii) Gene Trees and Detection of Enzyme Adaptation for C₄ Photosynthesis

Phylogenetic trees were inferred for C₄-related genes that were highly abundant in the leaf transcriptomes of at least two C₄ Alloteropsis samples (identified from transcriptome data; see Results) and their co-orthologs in other C₃ and C₄ grasses (see Supporting Information Methods 1.3 for a detailed description of phylogeny construction). The inferred gene trees were used to verify that C₄-related genes were placed as expected based on the species tree, as opposed to a position suggesting an acquisition from distant C₄ relatives. In addition, the gene tree topologies were used for positive selection analyses to detect traces of past episodes of enzyme adaptation for the new catalytic context after the initial emergence of a C₄ cycle (Fig. 1; Blasing et al. 2000; Christin et al. 2007; Besnard et al. 2009; Wang et al. 2009; Heckmann et al. 2013; Mallmann et al. 2014; Huang et al. 2017). Positive selection on branches leading to each C₄ group would support independent transitions to a full C₄ cycle via enzyme adaptation, while an early origin followed by a reversal should result in positive selection in the common ancestor of all C₄ accessions and possibly in the lineages that reversed back to the previous state.

For each set of genes encoding core C₄ enzymes in at least two Alloteropsis accessions, identified via transcriptome analyses, we optimized several codon models (site and branch-site models) to test for adaptive evolution using codeml as implemented in PAML (Yang 2007). The best-fit model was identified among those that assume (0) no positive selection (M1a null model), and
the branch-site models that assume shifts in selection pressure, either to relaxed selection (model BSA) or to positive section (model BSA1), at the base of: (1) Alloteropsis (one round of enzyme adaptation), (2) both A. cimicina and A. angusta + A. semialata (two rounds of enzyme adaptation), and (3) A. cimicina, A. angusta, and A. semialata (three independent episodes of enzyme adaptation). Foreground branches for all models were specified as the branch leading to the identified node plus all descending branches (i.e., using a "$" sign as opposed to a "#"). Models involving positive selection in only one of the C₄ lineages were also considered (see Supporting Information Methods 1.3 for additional details of positive selection analysis). For each gene lineage, the best-fit model was identified based on the corrected Akaike information criterion (AICc), selecting the model with the lowest AICc after checking that its ΔAICc score was at least 5.22 units below that of the M1a null model. An ΔAICc score = 5.22 corresponds to a $P$-value threshold of 0.01 for a likelihood ratio test comparing these two models using 2 degrees of freedom (df).

C₄ species other than Alloteropsis were removed prior to analysis to avoid an influence of positive selection in these taxa affecting our conclusion. Analyses were repeated using only codons with fixed nucleotides within each lineage (i.e., A. angusta, C₄ A. semialata, C₃+C₄ A. semialata, and C₃ A. semialata), to verify that short terminal branches with unfixed mutations did not significantly inflate the dN/dS ratio, and therefore alter our conclusion. Finally, to assess the effect of gene tree topology on our conclusions, we repeated the positive selection analyses using 100 bootstrap pseudoreplicate topologies.

(iv) DATING THE DIVERGENCE OF ADAPTIVE LOCI TO IDENTIFY INTROGRESSION

To determine whether introgression has spread C₄ adaptations among species, we performed molecular dating of markers from across the transcriptomes, including those used for C₄ by at least two Alloteropsis accession and their paralogs. The divergence times between species estimated from introgressed genes are expected to be younger than those estimated from other genes (e.g., Smith & Kronforst 2013; Li et al. 2014; Marcussen et al. 2014; Li et al. 2016), resulting either in outliers (if few genes are introgressed) or a multimodal distribution of ages (if many genes are introgressed).

Groups of genes descending from a single gene in the common ancestor of Panicoideae (Panicoideae co-orthologs), the grass subfamily that includes Alloteropsis, were identified through phylogenetic analyses of our transcriptomes and completely sequenced genomes that were publicly available. Our automated pipeline started with gene families previously inferred for eight plant genomes (homologs: i.e., all the paralogs and orthologs; Vilella et al. 2009), including two Panicoideae grasses (Setaria italica and Sorghum bicolor), two non-Panicoideae grasses (Brachypodium distachyon and Oryza sativa), and four nongrass species (Amborella trichopoda, A. thaliana, Populus trichocarpa, and Selaginella moellendorffii). To ensure accurate annotation, we restricted the analysis to gene families that included at least one A. thaliana sequence. The coding sequences (CDS) from the above genomes were then used to identify similar sequences in our transcriptomes using BLASTn with a minimum alignment length of 500 bp.

Stringent alignment and filtering methods were used to ensure reliable alignments of the above sequences for each gene family for phylogenetic inference (see Supporting Information Methods 1.4 for full details). In total, 2,797 1:1 Panicoideae co-ortholog datasets were used for subsequent molecular dating, as implemented in Beast version 1.5.4 (Drummond and Rambaut 2007). For each dataset, divergence times were estimated based on third codon positions, to decrease the risk of selective pressures biasing the outputs. A log-normal relaxed clock was used, with a GTR+G+I substitution model, and a constant coalescent prior. The Sorghum sequence was selected as the outgroup and the root of the tree was fixed to 31 Ma (using a normal distribution with a SD of 0.0001), based on estimates from Christin et al. (2014). There is uncertainty around this date, and the low species sampling used here probably leads to overestimation of both divergence times and confidence intervals, but the use of consistent sampling and calibration points among markers allows for the comparison of relative (rather than absolute) ages, which is the point of these analyses. Each Beast analysis was run for 2,000,000 generations, sampling a tree every 1,000 generations after a burn-in period of 1,000,000. For nodes of interest, divergence times were extracted from the posterior distribution as medians.

Divergence times were also estimated for key genes used for C₄ photosynthesis in Alloteropsis (identified based on transcriptomes; see Results), using the same parameters. To guarantee a consistent species sampling, the taxa included in the transcriptome-wide analyses were retrieved from manually curated alignments for C₄-specific genes as well as other groups of orthologs from the same gene families, obtained as described above for C₄-specific forms. In addition, plastid genomes for the same species were retrieved from Lundgren et al. (2015), and reanalyzed with the same parameters. For each of these datasets, the median, 95% CI, and 0.25 and 0.75 quantiles were extracted from the posterior distribution, using the R package APE (Paradis et al. 2004).

Results

(i) DIFFERENT REALIZATIONS OF C₄ LEAF ANATOMY IN A. CIMICINA AND A. SEMIALATA/A. angusta

Grasses ancestrally possess two concentric rings of bundle sheath cells and either can be co-opted for C₄ photosynthesis (Brown
The closely related C₄ A. cimicina and A. paniculata co-opted the outer bundle sheath for Rubisco segregation, as evidenced by the proliferation of chloroplasts in this tissue (Fig. S1; Table S2). In these species, the overall proportion of outer bundle sheath tissue within the leaf is increased via enlarged outer bundle sheath cells. Indeed, the outer sheath is 7.8-fold larger than the inner sheath in C₄ A. cimicina and A. paniculata, compared to a 1.2- to 0.6-fold differences in C₃ A. semialata and A. angusta (Table S2). This contrasts strongly with the anatomy of the C₄ A. semialata and A. angusta (Fig. S1). Both of these species use the inner bundle sheath for Rubisco segregation and increase the overall proportion of this tissue via the proliferation of minor veins, and enlargement of the inner sheath cell size (Fig. S1; Table S2).

Staining by Toluidine Blue O indicates some starch production occurs in the inner bundle sheaths of both the C₃ and C₃+C₄ A. semialata (Fig. S1), which implies some Rubisco activity in these cells, confirming previous reports (Ueno and Sentoku 2006; Lundgren et al. 2016). The absence of minor veins in the C₃ and C₃+C₄ A. semialata results in a larger proportion of mesophyll compared to C₄ A. semialata (Table S2; Fig. S1). In the C₃ and C₃+C₄ A. semialata, the outer bundle sheath is slightly larger than the inner one (1.2- to 1.8-fold; Table S2), while the C₃ outgroup species P. pygmaeum and E. marginata have outer bundle sheaths that are considerably larger than their small inner sheaths (4.5- and 5.3-fold; Fig. S1; Table S2).

In summary, our comparative studies of leaf anatomy indicate that the C₄ A. cimicina and A. semialata/A. angusta use different tissues for Rubisco segregation and achieve high bundle sheath proportions via distinct modifications, supporting independent origins of C₄ anatomical components in these two groups. Some Rubisco activity is suggested in the inner sheath of the C₃ A. semialata, which supports an early origin migration of chloroplasts to this tissue (Fig. 2). In addition, a slight enlargement of the inner sheath, absent in the C₃ outgroup, is common to all non-C₄ A. semialata.

(ii) A. CIMICINA USES DIFFERENT ENZYMES AND GENES FOR C₄ BIOCHEMISTRY THAN A. SEMIALATA/A. ANGUSTA

All Allotropis C₃+C₄ and C₄ accessions have high expression abundance in their leaves of co-orthologs encoding phosphoenolpyruvate carboxylase (PEPC), the enzyme used for the initial fixation of atmospheric carbon into organic compounds in
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Figure 3. Expression of C₄-related enzymes in Alloteropsis.

For each gene encoding a C₄-related enzyme, the shade indicates the category of transcript abundance, using averages per group. For raw values, see Table S4. Note that ppc abundance varies among C₄ accessions of A. semialata (Fig. 4). The enzymes involved in core C₄ reactions (left column) are grouped by functional property, and gene names are written in italics on the right of the expression values.

C₄ plants. However, the gene lineage most highly expressed varies among accessions (Figs. 3 and 4). The close relationships between some of the genes for PEPC and one for phosphoenolpyruvate carboxykinase (PKC) isolated from Alloteropsis and those of distantly related C₃ species was confirmed by our phylogenetic analyses (Figs. S3 and S4), supporting the previous conclusion that these genes were acquired by Alloteropsis via lateral gene transfer (LGT; Christin et al. 2012). Based on the read abundance, A. cimicina uses ppc-1P3_LGT-M, while A. angusta uses ppc-1P4 (Fig. 4). There is variation within A. semialata, with C₃+C₄ and C₄ accessions using either one or a combination of several gene lineages several gene lineages (Fig. 4).

From the expression profiles (Fig. 3), the carbon shuttle of A. cimicina relies on enzymes and transporters associated with the most common form of C₄ photosynthesis (NADP-malic enzyme type; Gowik et al. 2011; Bräutigam et al. 2014; Mallman et al. 2014). This expression profile differs markedly from that observed in the C₄ A. semialata and A. angusta accessions. These two species mainly use the PKC decarboxylating enzyme, through the high expression of the same gene (pck-1P1_LGT-C; Fig. 4). There is little evidence in these species for an involvement of the auxiliary transporters observed in A. cimicina (Fig. 3; Table S4), and some of the core enzymes are not shared by A. cimicina and A. semialata/A. angusta (Fig. 3). Furthermore, even when the same enzyme family is used, it is not necessarily encoded by the same locus (e.g., A. cimicina expresses aspat-2P3 and A. semialata/A. angusta express aspat-3P4; Fig. 3).

The transcriptomes of the C₃+C₄ A. semialata show elevated levels of some of the genes used by the C₄ A. semialata, with a slightly higher abundance of those encoding the NADP-malic enzyme (nadpm-1P4; Fig. 3; Table S4). In terms of the expression levels of genes encoding C₄-related enzymes, the transcriptome of the C₃ A. semialata is not markedly different from that of the C₃ outgroup P. pygmaeum (Fig. 3; Table S4).

Figure 4. Leaf abundance of pck and ppc genes in the different accessions.

The shade indicates the relative expression (in rpkm) in the different accessions. For each accession, the averages are used. For raw values, see Table S4.

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Our comparative transcriptomics therefore indicate that *A. cimicina* uses different genes and different enzymes for the C₄ pathway than *A. semialata/A. angusta*, suggesting multiple origins of the C₄ cycle (Fig. 2). The only C₄-related genes used by some C₄ Allotroposis that are abundant in the C₃ *A. semialata* (bea-2P3 and tpr-1P1) are also highly expressed in the C₃ outgroup and in other distantly related C₃ taxa (Fig. 3; Külahoğlu et al. 2014; Ding et al. 2015), indicating that high levels in leaves is not specific to our group of species. For the C₃-related genes used by the C₃ *Allotroposis*, but not abundant in the outgroup, there is no evidence for high expression or pseudogenization in the C₃ *A. semialata*. Evidence is thus lacking that the C₃ *A. semialata* represent a reversal from an ancestor with a C₄ cycle.

**Table 1.** Results of positive selection analyses inferring the episodes of enzymatic adaptation in *Allotroposis*.

| Gene   | Number of sequences | Site model M1a | One origin | Two origins | Three origins | Only A. cimicina |
|--------|---------------------|----------------|------------|-------------|---------------|-----------------|
| aspat-2P3 | 14      | 0.00*          | 4.02       | 4.02        | 4.02          | 3.94 4.02       | 4.00 4.00       |
| nadpme-1P4 | 15      | 35.07          | 30.44      | 27.26       | 26.34 24.28   | 19.52 13.31     | 3.34 0.00*      |
| ppdk-1P2  | 15      | 29.45          | 32.00      | 36.35       | 32.31 27.17   | 26.37 23.37     | 3.55 0.00*      |
| alaat-1P5 | 14      | 0.00*          | 1.74       | 1.74        | 2.03 2.03     | 0.69 0.69       | 4.02 4.02       |

1The ΔAICc values compared to the best-fit model for that gene are shown. The most appropriate model is indicated with an asterisk, with the null model (M1a) only rejected if the ΔAICc was at least 5.22 (equivalent to a P-value of 0.01 with a likelihood ratio test with df = 2). Two branch-site models were used to test for a relaxation of purifying selection (BSA), and potential positive selection (BSA1).

**Table 2.** Results of positive selection analyses inferring the episodes of enzymatic adaptation in the *A. angusta/A. semialata* clade.

| Gene    | Number of sequences | Site model M1a | One origin | Two origins | Only A. angusta |
|---------|---------------------|----------------|------------|-------------|----------------|
| aspat-3P4 | 13      | 12.33          | 10.20      | 6.70        | 6.37 0.00*     | 5.45 5.29       |
| nadpme-1P4 | 14      | 10.19          | 14.19      | 9.66        | 13.52 0.00*    | 14.18 14.18     |
| ppc-1P3  | 9       | 72.43          | 66.62      | 66.58       | 11.70 9.85     | 5.66 0.00*      |
| ppdk-1P2 | 14      | 0.00*          | 4.01       | 4.01        | 4.01 3.91      | 3.91 3.91       |

1The ΔAICc values compared to the best-fit model for that gene are shown. The most appropriate model is indicated with an asterisk, with the null model (M1a) only rejected if the ΔAICc was at least 5.22 (equivalent to a P-value of 0.01 with a likelihood ratio test with df = 2). Two branch-site models were used to test for a relaxation of purifying selection (BSA), and potential positive selection (BSA1).

(iii) **INDEPENDENT EPISODES OF C₄-RELATED POSITIVE SELECTION IN EACH C₄ SPECIES**

The codon models do not support positive selection on any genes involved in C₄ photosynthesis at the base of *Allotroposis* or along the branch leading to the *A. angust/A. semialata* group (Table 1). In two cases (*nadpme-1P4* and *ppdk-1P2*), analyses including all *Allotroposis* accessions clearly point to changes in selective pressures specifically in the branch leading to *A. cimicina* (Table 1; Fig. S5). No evidence of positive selection was found for the two other genes analyzed on the three *Allotroposis* species (aspat-2P3 and alaat-1P5; Table 1). When testing for selection only in the *A. angust/A. semialata* clade, no positive selection was found on *ppdk-1P2*, while positive selection on *ppc-1P3* was identified only on the branch leading to *A. angusta* (Table 2). For the two other genes (*nadpme-1P4* and *aspat-3P4*), the model that assumes positive selection after the split of the two species was favored (Table 2). A majority of the amino acid sites identified as under positive selection by the Bayes Empirical Bayes analysis overlapped with those previously identified in other C₄ taxa (e.g., site 241 in *nadpme-1P4*; Fig. 5; Christin et al. 2009), or were shared with other C₄ species in our phylogenies (e.g., Fig. 5), supporting their link to C₄ photosynthesis. For *aspat-3P4*, more amino acid substitutions were fixed in *A. angusta* than in *A. semialata*. This variation among *A. semialata* C₄ accessions indicates repeated bouts of positive selection during the diversification of this species (Fig. S6). Conclusions based on the selection tests were also supported using only codons with fixed nucleotides within a lineage (i.e., photosynthetic types in *A. semialata*, and *A. angusta*), with the exception of *nadpme-1P4* for which no positive selection was inferred after removing the unfixed codons (Tables S5 and S6). Furthermore, gene tree topology had no effect on our conclusions, since all bootstrap replicates supported
Figure 5. Evolution of nadpme-1P4 genes in Alloteropsis and other Panicoideae.

This phylogenetic tree was inferred on 3rd positions of codons of nadpme-1P4 genes of Panicoideae. Bootstrap values are indicated near branches. Names of C₄ accessions are in bold. Amino acid at positions under positive selection are indicated on the right, with those associated with C₄ accessions in gray. Positions are indicated on the top, based on Sorghum gene Sb03g003220.1. Amino acid positions with a posterior probability >0.90 of being under positive selection are indicated on the right, asterisks indicate positions with a posterior probability >0.95.

(iv) GENES FOR PEPC AND PCK WERE SPREAD ACROSS SPECIES BOUNDARIES

The 2,797 groups of orthologs extracted from genomes and transcriptomes led to a wide range of estimated divergence times, with 95% of the medians falling between 6.51 and 17.92 Ma for the crown of Alloteropsis, and between 4.17 and 11.27 Ma for the split of A. semialata and A. angusta (Fig. 6). The peak of values...
Figure 6. Estimates of divergence times.
On the top, divergence times are shown for selected nuclear genes and plastomes for A) the split of *A. angusta* and *A. semialata* and B) the crown of *Alloteropsis*. For each marker, the median of the estimates is indicated by a square, with thick bars connecting the 25 and 75 percentiles and thin bars connect the 2.5 and 97.5 percentiles. The distribution of medians for the crown of *A. semialata* (left), the split of *A. angusta* and *A. semialata* (middle), and the crown of *Alloteropsis* (right) over 2,797 markers extracted from the transcriptomes is shown at the bottom. The scale is given in million years ago (Ma).

(i.e., 50% of the points) ranged between 9.38 and 13.07 Ma for the crown of *Alloteropsis* and 5.93 and 8.18 Ma for the split of *A. semialata* and *A. angusta* (Fig. 6). Finally, 95% of the markers estimated the crown of *A. semialata* between 1.88 and 7.77 Ma, with a peak between 3.12 and 5.07 Ma (Fig. 6). Note that monophyly of the groups was not enforced, and various combinations of *A. semialata* accessions were included across markers, contributing to the observed variation.

Most of the C₄-related genes, as well as the plastomes, provided age estimates ranging from 5.54 to 10.32 Ma for the split of *A. semialata* and *A. angusta*, which matches the distribution of estimates from the transcriptome-wide data (Fig. 6A), and indicates their transmission followed the species tree. The only exception is the gene *pck-1P1_LGT-C*, for which the last common ancestor of *A. semialata* and *A. angusta* was estimated at 2.77 Ma (Fig. 6A), which is smaller than all but four of the 2,797 estimates from the transcriptome-wide markers. While the confidence intervals of the estimate for this gene do overlap with those of almost all other markers, this estimate matches more closely the diversification of *A. semialata* accessions (Fig. 6A).

The different markers selected for detailed analyses similarly yielded estimates for the crown of *Alloteropsis* matching those obtained from transcriptome-wide data, between 9.38 and 16.46 Ma (Fig. 6B). The only exception is the gene *ppc-1P3_LGT-M*, for which the last common ancestor of *A. cimicina* and *A. semialata* is estimated at 3.25 Ma (Fig. 6B), which is smaller than all estimates based on markers extracted from the plastomes. The 95% CI of the divergence estimate based on this gene does not overlap with many of those based on other markers, and again matches closely with the diversification of *A. semialata* accessions (Fig. 6B).

Overall, our dating analyses support an introgression of these two genes among *Alloteropsis* species after their divergence, while the other genes were transmitted following the species tree (Fig. 2).

**Discussion**

**TWO INDEPENDENT TRANSITIONS FROM C₃ TO C₄**

The earliest split in *Alloteropsis* separates the lineage containing *A. cimicina* from *A. angusta* and *A. semialata* (Fig. 2). These two lineages co-opted different tissues for the segregation of Rubisco activity and achieved a large proportion of bundle sheath tissue via different modifications (Fig. S1). The evidence therefore strongly supports two independent origins of C₄ anatomical properties, which is generally accepted as the first step during the C₃ to C₄ transition (Fig. 1; Sage et al. 2012; Heckmann et al. 2013). Gene expression analyses show that the two clades use
different enzymes for parts of the C₄ cycle, express different genes encoding the same enzyme family when there is an overlap (Fig. 3), and positive selection analyses show that the enzymes were independently adapted for their C₄ function (Table 1). We therefore conclude that the different transitions to C₄ biochemistry occurred independently after the split of these two lineages (Fig. 2). The only exception to the distinctiveness of A. cimicina and the two other C₄ species is the gene *ppc-1P3_LGT-M*, used by both A. cimicina and some C₄ *A. semialata* accessions (Fig. 4).

This gene is absent from other accessions (Olofsson et al. 2016) and, as such, we previously concluded that it was acquired early during the diversification of the group and then recurrently lost (Christin et al. 2012). This hypothesis is falsified by our dating analyses here, which show that this gene was only recently transferred among species boundaries, likely as a result of a rare hybridization event (Fig. 6).

**ONE INDEPENDENT C₃ TO C₄ TRANSITION INCLUDES TWO SEPARATE C₃+C₄ TO C₄ SHIFTS**

The C₄ phenotype is realized in *A. angusta* and *A. semialata* via identical anatomical modifications, using the same enzymes, and the same genes encode these enzymes. Chloroplasts are present in the inner sheaths of all *A. semialata* and *A. angusta* accessions, independent of their photosynthetic type, which suggests that this characteristic represents the ancestral condition for the clade (Fig. 2). The C₄ cycle is realized using the same set of genes in *A. angusta* and *A. semialata*, which can be explained by convergent evolution (e.g., as indicated for other C₄ grasses; Christin et al. 2013b) or a single origin of a weak C₃ cycle (C₁+C₄), followed by a reversal to expression levels that resemble the ancestral condition in the C₃ accessions (Fig. 2). Differentiating these two scenarios would require retracing the origin of the mutations responsible for the increased expression of C₄ enzymes to identify where they occurred on the phylogeny. Unfortunately, the molecular mechanisms controlling C₄ gene expression are poorly known, and can involve both cis- and transacting elements (Gowik et al. 2004; Brown et al. 2011; Williams et al. 2016).

The positive selection analyses indicate that enzyme adaptation happened independently in *A. angusta* and *A. semialata* (Table 2). Together with the variation observed within the C₄ *A. semialata* (Fig. 4, S6), this evidence strongly suggests that the biochemical adaptation allowing the transition to a full C₄ cycle happened recently, and independently in the two species (Fig. 2). The dramatic increase in the proportion of the inner bundle sheath tissue via the proliferation of minor veins is limited to the C₄ *A. semialata* and *A. angusta* (Fig. S1). The genetic control of these features is unknown, preventing a comparison of the causal mutations. However, the distribution of anatomical characters among grasses indicates that the vast majority of C₄ lineages that co-opted the inner bundle sheath increased its proportion via the addition of minor veins (Renvoize 1987; Christin et al. 2013a).

With the current state of knowledge, we hypothesize that the common ancestor of *A. semialata* and *A. angusta* had chloroplasts in the inner bundle sheath, and that this facilitated the emergence of a weak C₄ cycle via the upregulation of some enzymes. Following their split, *A. angusta* strengthened its C₄ anatomy via the proliferation of minor veins, and enzyme adaptations led to a strong C₄ cycle (Fig. 2). In the *A. semialata* lineage, some isolated populations acquired mutations that added minor veins and adapted the enzymes, leading to a C₄ cycle. Other populations, potentially under pressures linked to the colonization of colder environments (Lundgren et al. 2015), might have lost the weak C₄ cycle by downregulating the genes (Fig. 2). However, the details of the changes leading to C₃ photosynthesis in some *A. semialata* will need to be confirmed by comparative genomics, when mutations regulating expression of C₄ enzymes and anatomy are identified.

**INTROGRESSION OF C₄ COMPONENTS AMONG SPECIES**

Our dating analyses suggest that the gene *pck-1P1_LGT-C* that encodes the decarboxylating enzyme PCK was introgressed among some members of *A. semialata* and *A. angusta* (Figs. 2 and 6). The C₄ cycle carried out before this event was likely based on NADP-malic enzyme, an enzyme still abundant in the C₃+C₄ *A. semialata* and some C₄ accessions (Fig. 4; Frean et al. 1983). The acquisition of *pck-1P1_LGT-C*, a gene already adapted for the C₄ context, probably added a PCK shuttle, which alters the stoichiometry of the pathway and the spatial distribution of its energy requirements, increasing its efficiency under some conditions (Bellasio and Griffiths 2014; Wang et al. 2014). This important component of the C₄ cycles of extant *A. semialata* and *A. angusta* populations first evolved its C₄-specific properties in the distantly related *Cenchrus* (Fig. S3; Christin et al. 2012), and therefore never evolved within *Allotheroposis*. Instead, it represents the spread of a component of a complex physiology across multiple species boundaries. Therefore, in addition to the possibility that the sequential steps generating a complex physiology can happen on different branches of a species phylogeny (Fig. 2), introgression among close relatives can disconnect the origins of key components from the species tree.

**ON THE INFERENCE OF TRANSITIONS AMONG CHARACTER STATES**

Inferences of transitions among character states are a key component of numerous macroevolutionary studies (e.g., Cantalapiedra et al. 2017; Cooney et al. 2017). However, species trees per se are not always able to disentangle the complex scenarios underlying the appearance or losses of multicomponent adaptations,
especially when complex phenotypes are modeled as different states of a single character (e.g., Goldberg and Igic 2008; Pardo-Diaz et al. 2012; Niemiller et al. 2013; Igic and Busch 2013; King and Lee 2015). In the case of photosynthetic transitions within *Alloteropsis* depicted here, considering the photosynthetic type as a binary character would lead to a single C₄ origin as the most plausible scenario (Ibrahim et al. 2009), and modeling photosynthetic types based on their category of C₄ cycle does not improve the inference (Washburn et al. 2015). For traits assumed to evolve via sequential stages, the accepted sequence of changes can be incorporated in the model (e.g., Marazzi et al. 2012). However, the power of character modeling remains inherently limited by the small number of informative characters. Decomposing the phenotype into its components can solve this problem, especially when the underlying genetic determinism is considered (Oliver et al. 2012; Niemiller et al. 2013; Glover et al. 2015; Meier et al. 2017), and good mechanistic models exist for the evolution of DNA sequences (Liberles et al. 2013). Violation of model assumptions can still mislead the conclusions, but the multiplication of sources of information, coupled with the possibility to track the history of specific genes independently of the species tree, limits the risks of systematic errors. We therefore suggest that efforts to reconstruct the transitions leading to important traits should integrate as many underlying components as possible. As progresses in genome biology increase data availability and improve our understanding of causal mutations, modeling phenotypes as the results of cumulative changes in genomes will be able to solve the problems raised by the paucity of informative characters.

**Conclusions**

In this study, we dissect the genetic and anatomical components of C₄ photosynthesis in *Alloteropsis*, a genus of grasses with multiple photosynthetic types. Our comparative efforts strongly support at least two independent origins of C₄ photosynthesis within this genus. The C₄ phenotype within these separate origins is realized via divergent anatomical modifications, the upregulation of distinct sets of genes, and independent enzyme adaptations. One of these lineages includes a range of photosynthetic types, and based on our analyses, we suggest that some C₄ components in this group evolved in the shared common ancestor, while others were acquired independently after the lineages diverged. The history of photosynthetic transitions within *Alloteropsis* is furthermore complicated by the introgression of C₄ genes across species boundaries. This disconnects the spread of C₄ components from the species tree, and means that the number of origins varies among the different components of the complex C₄ trait. This scenario is unlikely to have been inferred from traditional macroevolutionary approaches based on species trees alone.

We suggest that integrating genomic data and phenotypic details in future studies of character transitions might resolve similarly complicated scenarios in other groups, enabling a better understanding of the trajectories followed during the evolution of novel adaptations.

**AUTHOR CONTRIBUTIONS**

PAC, CPO, PN, and EJE designed the study, MRL, MN, and PAC secured plant material, MRL generated the anatomical data, JJMV generated the transcriptome data, LTD, MRL, JJMV, and PAC analysed the data, LTD and PAC wrote the paper with the help of all authors.

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**DATA ARCHIVING**

All raw RNA-Seq data have been deposited in the NCBI Sequence Read Archive (project identifier SRP072730), and transcriptome assemblies are deposited in the NCBI Transcriptome Shotgun Assembly repository (Bioproject PRJNA310121).

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Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Figure S1. Comparisons of leaf anatomy in Alloteropsis and relatives.
Figure S2. Phylogenetic trees for genes encoding three C4-related enzymes.
Figure S3. Phylogeny of pck-1P1 genes in Panicoideae.
Figure S4. Evolution of ppc-1P3 genes in Alloteropsis and other Panicoideae.
Figure S5. Evolution of ppdk-1P2 genes in Alloteropsis and other Panicoideae.
Figure S6. Evolution of aspat-3P4 genes in Alloteropsis and other Panicoideae.
Table S1. Alloteropsis semialata accessions used in this study.
Table S2. Leaf anatomical data for the study species and accessions.
Table S3. RNA-Seq data, NCBI SRA accession numbers, and growth conditions.
Table S4. Transcript abundance (in rpkm) for each C4-related gene and sample.
Table S5. Results of positive selection analyses inferring the episodes of enzymatic adaptation in Alloteropsis using only fixed differences.
Table S6. Results of positive selection analyses inferring the episodes of enzymatic adaptation in the A. angusta/A. semialata clade using only fixed differences.

Methods 1.1. Plant growth conditions.
Methods 1.2. RNA-Seq protocol.
Methods 1.3. Positive selection analysis.
Methods 1.4. Alignment and filtering.