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Evolutionary implications of C_3-C_4 intermediates in the grass *Alloteropsis semialata*

Running title: C_3-C_4 *Alloteropsis semialata*

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Statement of authorship: MRL, PAC, and CPO designed the study. MRL generated and analysed data. EGE and RCL performed the PEPC quantifications. MRL, PAC, BSR, GB, and CPO contributed plant material. MRL, CL, PWH, and RPE contributed accession collection locations and isotope data. MRL, PAC, and CPO wrote the paper, with the help of all the authors.

ABSTRACT

C_4 photosynthesis is a complex trait resulting from a series of anatomical and biochemical modifications to the ancestral C_3 pathway. It is thought to evolve in a stepwise manner, creating intermediates with different combinations of C_4-like components. Determining the adaptive value of these components is key to understanding how C_4 photosynthesis can gradually assemble through natural selection. Here, we decompose the photosynthetic phenotypes of numerous individuals of the grass *Alloteropsis semialata*, the only species known to include both C_3 and C_4 genotypes. Analyses of $\delta^{13}C$, physiology, and leaf anatomy demonstrate for the first time the existence of physiological C_3-C_4 intermediate individuals in the species. Based on previous phylogenetic analyses, the C_3-C_4 individuals are not hybrids between the C_3 and C_4 genotypes analysed, but instead belong to a distinct genetic lineage, and might have given rise to C_4 descendants. C_3 *A. semialata*, present in colder climates, likely represents a reversal from a C_3-C_4 intermediate state, indicating that, unlike C_4 photosynthesis, evolution of the C_3-C_4 phenotype is not irreversible.

KEYWORD INDEX: C_3-C_4, C_2 metabolism, *Alloteropsis semialata*, grasses, complex trait, photorespiration, CO_2 compensation point, oxygen inhibition, stable isotopes

INTRODUCTION

C_4 photosynthesis is a complex trait that requires the accumulation and coordination of several anatomical and biochemical modifications to the ancestral C_3 pathway (Hatch 1987). This suite of novelties results in an efficient CO_2 concentrating mechanism (CCM) that separates CO_2 assimilation and reduction into two compartments, usually mesophyll and bundle sheath cells respectively, to increase the concentration of CO_2 around Rubisco (Hatch & Osmond 1976; von Caemmerer & Furbank 2003). The C_4 system consequently minimizes the oxygenation of RuBP and the associated costs of photorespiration, making it more efficient than plants that lack this complex trait under conditions where photorespiration would be limiting, such as hot, arid, saline, and/or high light environments. However, the extra energetic costs of the C_4 reactions make C_4 plants less competitive in cool and shady habitats where photorespiration rates are low (Chollet & Ogren 1975; Ehleringer 1978; Bauwe *et al.* 2010; Sage *et al.* 2012; Fernie *et al.* 2013). These environmental interactions under a low-CO_2 atmosphere are thought to have been the selection pressures underlying the independent origins of the C_4 trait in nearly 70 plant lineages across 19 angiosperm families (Sage *et al.* 1999, 2011; GPWG II 2012).

The evolutionary trajectory from the ancestral C_3 state to a derived C_4 trait is thought to gradually pass through a series of intermediate stages, all of which must confer an advantage, or at least little disadvantage, compared to the previous one (Heckmann *et al.* 2013; Williams *et al.* 2013). While the adaptive significance of many of these intermediate stages is still unclear, the glycine shuttle, or C_2 cycle, increases photosynthetic efficiency by recycling photorespired CO_2 through a weak mesophyll-bundle sheath CCM, while also beginning to establish the bundle sheath Calvin cycle and two-compartment coordination that are characteristic of C_4 photosynthesis (Hylton *et al.* 1988; Sage *et al.* 2013). Therefore, studies of plants using a C_2 cycle, alongside close C_3 and C_4 relatives, are important for uncovering the individual steps occurring along these evolutionary transitions and for understanding their adaptive significance (Christin *et al.* 2011; Muhaidat *et al.* 2011; Vogan & Sage 2011).

Naturally occurring plant assemblages that incorporate a high level of photosynthetic diversity are rare, but the grass *Alloteropsis semialata* (R. Br.) Hitchcock is known to include recently diverged C_4 and

phenotypically diverse non-C_4 populations, presenting remarkable intraspecific photosynthetic variation (Ellis 1974a, b; Ueno & Sentoku 2006; Lundgren *et al.* 2015). This diversity makes *A. semialata* an ideal system to study the evolutionary relationships between C_3 and C_4 phenotypes. The C_3 and C_4 photosynthetic types of *A. semialata* have been described in detail (Ellis 1974a, b; Brown 1975; Ellis 1981; Frean *et al.* 1983a, b; Gibbs Russell 1983). Plants grown under natural climatic conditions have previously presented typical C_3 and C_4 phenotypes (Ripley *et al.* 2007). However, the leaf anatomy and physiology of plants grown in controlled environments have not always aligned well with typical C_3 and C_4 types, to the extent of being described as C_3-like and C_4-like (Ueno & Sentoku 2006). Moreover, several studies have identified anomalous stable isotope signatures, leaf anatomy, and gross plant morphology in accessions collected from the wild, pointing to intermediate states between the two photosynthetic types (Table 1; Ellis 1981; Gibbs Russell 1983; Renvoize 1987; Hattersley & Watson 1992; Lundgren *et al.* 2015). Despite this compelling evidence for a gradient of photosynthetic phenotypes in the species, C_3-C_4 intermediates have not been clearly demonstrated in *A. semialata.* However, this is probably because previous physiological studies were focused on South African and Australian accessions and did not investigate central African plants, in which most of the anomalous phenotypes are now known to exist (Table 1).

Here we use accessions from across the species' range, including material collected from central and western Africa and Madagascar, as well as South Africa and Australia, to reveal the photosynthetic variation that exists within *A. semialata*, testing the hypothesis that there are C_3-C_4 intermediate forms within the species (Hattersley & Watson 1992). In doing so, we reveal the first known example of intraspecific photosynthetic variation that spans C_3, C_3-C_4, and C_4 photosynthetic types. We then decompose the photosynthetic phenotype into its anatomical and physiological components to gain insights into the gradual accumulation of C_4 characters among geographically isolated populations of the same species complex. Based on the phylogeographic history of the group (Lundgren *et al.* 2015), we discuss the possible transitions among distinct photosynthetic types, highlighting the diversity of evolutionary trajectories available to photosynthetically intermediate lineages.

MATERIALS AND METHODS

Plant material

This study included 22 accessions of *A. semialata* from 11 geographic origins spread across the range of the species (Table 2). The same accessions were analysed by Lundgren *et al*. (2015). Accessions from South Africa (MDG, SFD, CRL, EML, JMS, and KWT) and Tanzania (L01 and L04) were collected as cuttings from the field and established in controlled environment conditions at the University of Sheffield. Accessions from Australia (AUS), Burkina Faso (BF), and Madagascar (MAJ) were collected as seeds in the field and germinated under sterile conditions at 30°C, high light, and 65% RH in a controlled environment plant chamber (Sanyo, Bensenville, IL, USA). Once the field cuttings became established and the seedlings were mature, plants were maintained under well-watered conditions at 60% RH, 25/20°C day/night temperatures, and 500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) over a 14 hour photoperiod at ambient CO_2 concentrations (averaging 374 ppm over the span of the experiment) in a controlled environment growth chamber (Conviron Ltd, Winnipeg, Canada) and fertilized once a fortnight with Scotts Evergreen Lawn Food (The Scotts Company, Surrey, England). All plants were grown in large round plastic pots (sized between one and four litres to accommodate variation in root material) in potting compost (compost no. 2 and 3; John Innes Manufacturers Association, Reading, England). The Tanzanian plants were grown in higher nutrient potting compost (no. 3) to aid re-establishment of the cuttings. To test whether this difference in soil nutrients influenced the results, the Tanzanian plants were transplanted into the same moderate soil nutrient compost (no. 2) after the initial set of measurements and re-analysed (see Supplementary Methods and Results). Between one and four replicate plants were analysed per population (Table 2). For each plant, leaf physiology was measured first, then the same leaf was immediately sampled for subsequent carbon isotope discrimination and leaf anatomy analyses, as described below.

Carbon isotope discrimination

Tissue from the centre of the leaf blade was harvested, dried in an oven, and ground to a fine powder, of which 1-2 mg was analyzed using an ANCA GSL preparation module coupled to a 20–20 stable isotope analyzer (PDZ Europa, Cheshire, UK). $\delta^{13}C$ signatures are presented as isotopic ratios (per mil, ‰) relative to the isotopic standard Pee Dee Belemnite. $\delta^{13}C$ signatures of Panicoideae

grasses that use the C_3 pathway range between -31 and -24‰ while those that use C_4 photosynthesis range between -16 and -10‰ (Smith & Brown 1973).

As carbon isotope composition in plants raised in growth chambers may differ from natural populations (Farquhar *et al.* 1989), $\delta^{13}C$ signatures were measured on silica-dried leaves collected from the same population, and often the same plant, growing in the wild, when available (as reported in Lundgren *et al.*, 2015). To estimate a field $\delta^{13}C$ value in accessions lacking equivalent field material (i.e., AUS, BF, MAJ, and SFD populations), growth chamber $\delta^{13}C$ values were adjusted based on the difference (i.e. -2.8‰) between field (-11.1‰; Ibrahim 2007) and growth chamber (-13.9‰) plants measured on the MDG population.

Physiology

Leaf physiology measurements were made between two and seven hours into the photoperiod on the youngest fully expanded leaf of the tallest tiller of each plant using an open gas exchange system, incorporating an infra-red gas analyzer with leaf chamber fluorometer attachment (LI-6400XT and LI-6400-40, respectively; LICOR, Lincoln, Nebraska, USA). All light and CO_2 response curves were collected at 27°C leaf temperature (T_{leaf}), 250 μmol s^{-1} flow rate, and approximately 70% RH, after sufficient acclimation to chamber conditions to achieve steady state CO_2 and H_2O fluxes. Light response curves were collected at 400 μbar reference CO_2 concentration (CO_2R) at decreasing PPFD of 2000, 1750, 1500, 1250, 1000, 750, 500, 250, 100, 50, 25, and 0 μmol m^{-1} s^{-1} after reaching a steady state of CO_2 uptake at each light level. A/C_i curves were collected under ambient (~21%) and low (2%) O_2 concentrations at light saturation (1600 μmol m^{-2} s^{-1} PPFD) after reaching a steady state of CO_2 uptake at each CO_2R. Measurements were collected at 400, 250, 150, 120, 100, 85, 70, 50, and 35 μbar CO_2R, then a second measurement was collected at 400 μbar CO_2R to confirm no reduction in rate had been caused by exposure to low C_i (e.g. through Rubisco deactivation), and finally at 600, 800, 1000, and 1200 μbar CO_2R. The subambient portion of the A/Ci curve was repeated for C_3 accessions at 150 and 75 μmol m^{-2} s^{-1} PPFD, to calculate the CO_2 compensation point in the absence of mitochondrial CO_2 release not associated with photorespiration (Γ*) following Laisk (1977). The sub-ambient portion of the A/Ci curve was repeated under 2% O_2 after the IRGA was recalibrated for low O_2 measurements.

Light- and CO_2-response curves of photosynthesis were analysed following Bellasio *et al.* (2015) to generate values for CO_2 compensation point (CCP), maximal carboxylation efficiency (CE), CO_2 saturated net photosynthesis (A_{sat}), respiration in the light (R_{light}), gross assimilation (GA), light compensation point (LCP), the PPFD that half saturates GA ($PPFD_{50}$), and maximal quantum yield for CO_2 fixation (Φ_{CO2}). O_2 inhibition (OI) was calculated as the difference between CCP derived at ambient and low O_2 concentrations. Intrinsic water use efficiency (iWUE) is calculated as A/g_s, both measured at 400 µbar CO_2R and light saturation (1600 µmol m^{-2} s^{-1}).

To indicate deviation from typical C_3 metabolism, ^{13}C discrimination independent of the PDB standard ($\Delta^{13}C$) is plotted against the ratio of internal to ambient CO_2 concentrations (C_i/C_a). C_i/C_a values collected at growth light levels (500 µmol m^{-2} s^{-1} PPFD), 400 µbar CO_2R, and 27°C T_{leaf} were used. $\Delta^{13}C$ was calculated according to Farquhar *et al.* (1989), accounting for chamber air in this growth facility following Llorens *et al.* (2009), with the assumption that the ambient growth chamber CO_2 concentration is approximately 374 µbar (the average value measured in this chamber during the experiment). The theoretical relationship between $\Delta^{13}C$ and C_i/C_a is presented, according to Ubierna & Farquhar (2014), as:

$$\Delta^{13}\mathrm{C} = 2 + a + [b - a]\frac{c_i}{c_a} - f\frac{\Gamma^*}{c_a}$$

where the fractionation associated with the diffusion through air (a) = 4.4‰, by Rubisco carboxylation (b) = 27‰, and during photorespiration (f) =12‰. Averaged over the seven C_3 accessions, Γ* = 26.5 µmol mol^{-1} and C_a = 374 µbar. The theoretical relationship is adjusted by +2‰ to account for differences in discrimination measured online (as presented in Ubierna & Farquhar 2014) and in bulk leaf material (used here).

Leaf anatomy

Tissue samples 3-5 mm in length were collected from the centre of the youngest fully expanded leaf of each plant, fixed in 4:1 ethanol:acetic acid, and embedded in methacrylate embedding resin (Technovit 7100, Heraeus Kulzer GmbH, Wehrhein, Germany). Embedded leaves were sectioned between 6-8 µm thick on a manual rotary microtome (Leica Biosystems, Newcastle, UK) and stained with Toluidine

Blue O (Sigma-Aldrich, St. Louis, MO, USA). Stained leaf sections were photographed using microscopy imaging software (CellA; Olympus, Hamburg, Germany) and a camera mounted on a microscope (Olympus DP71 and BX51, respectively. Olympus, Hamburg, Germany). Images were stitched together (using DoubleTake, Echo One, Frederikssund, Denmark) to reproduce the whole width of the leaf blade in cross-section.

Anatomical traits were measured along segments of the leaf cross-sections using ImageJ (Schneider *et al.* 2012). A single segment was defined as the leaf portion between two 2° veins, which are large and contain metaxylem, while minor veins (e.g. tertiary, quaternary, and quinary veins) lack metaxylem. Only segments in the middle of the leaf blade were used and those immediately adjacent to the mid-rib and lateral edges of the cross-section were avoided. The total number of veins per segment, or vein frequency (VF), was counted and averaged along three segments per leaf. The minimum number of adjacent mesophyll cells separating vein pairs, or interveinal mesophyll cells (IVMC), was counted for ten pairs of veins per leaf and averaged. The width of one outer bundle sheath cell (OBS) and one inner bundle sheath cell (IBS) on both sides of a 2° vein were measured and averaged for three 2° veins per leaf. Only bundle sheath cells lying parallel to the leaf surface were chosen to maintain consistency across samples. To show chloroplast localization in mesophyll and bundle sheath cells, cross-sections of fresh leaves from a subset of accessions were cut by hand, mounted with distilled H_2O, then imaged as described above.

PEPC content

Nine of the *A. semialata* genotypes were sampled for PEPC content, alongside maize (C_4) and rice (C_3) controls. At the time of sampling, the *A. semialata* plants were growing under greenhouse conditions set to a 14-hour photoperiod at 25/20°C day/night temperatures at 60% RH and ambient light, augmented by 200 µmol m^{-2} s^{-1} when ambient light fell below 1000 µmol m^{-2} s^{-1} PPFD during the photoperiod. Maize and rice plants were grown in controlled growth chambers using a 12-hour (360 µmol m^{-2} s^{-1} PPFD, 25/17°C day/night) and 11-hour (270 µmol m^{-2} s^{-1} PPFD, 28/24°C day/night) photoperiod, respectively. To assess the assimilatory pathway used by the Tanzanian plants at the time that leaves were sampled for the PEPC assay, the sub-ambient CO_2 portion of the A/C_i curve was

measured again to calculate CCP and CE, as the slope (CE) and x-intercept (CCP) of the line connecting the data points.

Mature leaves were harvested at midday and flash-frozen in liquid nitrogen. Leaf tissue (250 mg) was ground to a fine powder in liquid nitrogen, homogenised in 1 mL chilled 200 mM Bicine-KOH (pH 9.8) and 50 mM dithiothreitol (DTT), and centrifuged at 16,800 *g* for 5 minutes at 4°C until supernatants appeared clear. Protein concentrations of the crude extracts were determined following Bradford (1976). A 1:1 ratio of crude protein to SDS-solubilisation buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue] was boiled at 100°C for 5 minutes, immediately cooled on ice, and then stored at -80°C. Two μg of protein was resolved by SDS-PAGE using a 4-20% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) blotting membrane using a wet transfer Trans-Blot tank (Bio-Rad, Hertfordshire, UK). Membranes were blocked for 2 hour in TBS-T [TBS (pH 7.4), 0.2% (v/v) Tween 20] containing 5% (w/v) skimmed milk and 3% (w/v) BSA, then probed with a monoclonal PEPC antibody raised against PEPC from maize sequences at 1:10,000 dilution. Immunoreactive polypeptides were visualised using horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) coupled with an enhanced chemiluminescence blotting kit and Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). To stain for whole proteins, 10 μg of protein was analysed by SDS-PAGE and stained overnight with dark incubation using SYPRO Ruby fluorescence stain (Invitrogen, UK), per manufacturer instructions. Gels were incubated in 10% (v/v) methanol and 7% (v/v) acetic acid for 30 minutes to remove excess stain, then visualised using a ChemiDock XRS+ UV transilluminator (Bio-Rad, Hertfordshire, UK).

RESULTS

Carbon isotope discrimination

Carbon isotope discrimination varied substantially among accessions of *A. semialata* (Table 2), reflecting the photosynthetic diversity of the species. A previous screening of 298 herbarium samples showed that all accessions from Asia and Australia had carbon isotopes values in the typical C_4 range (between -16.3 and -9.1‰), while African accessions encompassed C_4 and non-C_4 values (between -

34.1 and -9.3‰) and included 18 individuals with intermediate $\delta^{13}C$ signatures (between -23.9 and -18.6‰; Fig. 1; Lundgren *et al.* 2015). This geographic pattern is confirmed here. All accessions from Australia, Burkina Faso, and Madagascar had C_4 $\delta^{13}C$ signatures (Table 2). South African accessions were more diverse, with populations in the C_4 and non-C_4 ranges in plants grown in both the field and controlled environments (Table 2). The Tanzanian accessions had non-C_4 $\delta^{13}C$ signatures in both field and growth chamber conditions (Table 2).

Physiology

The gradient of carbon isotope discrimination values present in *A. semialata* is explained by its diverse physiology, as CCP, OI, and A_{sat} were negatively, and CE positively, associated with $\delta^{13}C$ signatures according to general linear model tests in R (Fig. 2a-d; version 3.1.1, R Development Core Team 2014). One-way analysis of variance (ANOVA) models performed in R show that accessions with C_4 $\delta^{13}C$ signatures had the lowest CCP (≤ 18.8 μmol mol^{-1}) and OI (≤ 4.6 μmol mol^{-1}), consistent with plants that use the C_4 photosynthetic pathway and are consequently referred to as such from here onward (Fig. 2; Tables 2 and 3). Accessions with non-C_4 $\delta^{13}C$ signatures were more variable. The four South African populations with the lowest $\delta^{13}C$ signatures (≤ -27.8/-27.1, in growth cabinet and field grown plants, respectively) had the highest CCP (≥ 43.1 μmol mol^{-1}) and OI (≥ 32.1 μmol mol^{-1}), which is typical of plants that use the C_3 pathway and, as such, are called C_3 from here onward (Table 2). The Tanzanian plants, however, had intermediate values of CCP (11.6 – 25.6 μmol mol^{-1}) and OI (10.3 – 22.7 μmol mol^{-1}), between those measured in C_3 and C_4 plants, and even overlapped in CCP with some C_4 accessions (Figs 2a-b; Tables 2 and 3). Despite these intermediate and C_4-like CCPs, the Tanzanian plants never had a greater CE than C_3 plants (Figs 2d and S1; Tables 2 and 3), and consistently showed a non-C_4 $\delta^{13}C$ signature (≤ -23.1‰; Table 2).

A/C_i curves of the C_3 and Tanzanian accessions were similar to each other (Fig. S1a), indicating commonalities in basic photosynthetic metabolism, but contrasting with the steep C_4 curve that quickly saturated. At 27°C, light response curves were similar among the C_3, C_4, and Tanzanian plants (Fig. S1b) and, as such, GA, PPFD50, and Φ_{CO2} did not differ across the species (Table 3). The C_3 and Tanzanian plants had greater iWUE than C_4 accessions (Fig. 2e, Tables 2 and 3). The Tanzanian plants

had higher LCP and R_{light} than both C_3 and C_4 plants, but this may result from the different soil conditions that the Tanzanian plants were growing in at this time, compared to the C_3 and C_4 plants.

Farquhar *et al.* (1982) show that $\Delta^{13}C$ in C_3 plants shows a linear dependence on C_i/C_a (Fig. 2f), depicting the iWUE-dependence of carbon isotope discrimination. As the stomatal limitation of gas exchange increases, C_i/C_a decreases and diffusion becomes increasingly important relative to Rubisco for discrimination against ^{13}C in photosynthesis, which decreases. In modelling $\Delta^{13}C$ against C_i/C_a, the data for C_3 and Tanzanian plants align along the theoretical line that reflects the relationship between these two parameters in C_3 plants. However, the data for Tanzanian plants show a lower discrimination at a given C_i/C_a value than the C_3 *A. semialata* plants (Fig. 2f), indicating that the difference in $\Delta^{13}C$ between these two types cannot simply be explained by differences in C_i/C_a.

Leaf anatomy

Leaf anatomy varied widely across *A. semialata* accessions, with between two and twelve veins per segment (VF), a range from one to nine mesophyll cells separating these veins (IVMC), and IBS cells that were between two-thirds smaller than, and one third larger than, OBS cells (Figs 3 and S2; Table 2). This anatomical variation can also partially explain the gradient of carbon isotope discrimination in this species (Figs 3a-c and S2). C_4 accessions had higher VF ($\geq$ 8), fewer IVMC ($\leq$ 2), and smaller OBS cell widths ($\leq$ 11.4 μm) than the other accessions (Fig. 3; Tables 2 and 3). In contrast, C_3 accessions had the lowest VF ($\leq$ 5), most IVMC ($\geq$ 6), and largest OBS cell widths ($\geq$ 16.0 μm; Figs 3a-c and S2; Tables 2 and 3). The Tanzanian accessions had intermediate VF (5-7), IVMC (4-5), and OBS cell sizes between those observed in C_3 and C_4 plants (11.6 – 14.4 μm; Figs 3 and S2; Tables 2 and 3). IBS cell size, however, showed a different trend across the $\delta^{13}C$ gradient, as the IBS cells of Tanzanian plants were not intermediate in size but were as large as those measured in C_4 plants (Figs 3d and S2; Tables 2 and 3).

Fresh hand-cut cross-sections showed that C_4 *A. semialata* had higher concentrations of chloroplasts in bundle sheath cells than in the mesophyll (Fig. S3e). As expected, C_3 *A. semialata* had abundant chloroplasts in the mesophyll; however, they also had a small number of chloroplasts present in the IBS (Fig. S3d). The Tanzanian plants had similar abundances of chloroplasts in the mesophyll and IBS,

which is consistent with them being plants using a C_2 cycle that run the Calvin cycle in both cell types. Small numbers of chloroplasts were also observed in the OBS of C_3, C_4, and Tanzanian accessions (Fig. S3).

PEPC content

The PEPC immunoblot showed that leaves of the two Tanzanian populations had PEPC in higher abundance than C_3 *A. semialata* and rice (Fig. 4), which suggests that the Tanzanian plants may be capable of using a C_4 cycle. In fact, the Tanzanian plants had similar PEPC content to C_4 *A. semialata* and maize (Fig. 4). Of the C_4 *A. semialata* accessions, the Madagascan plant had the most PEPC, while the Australian and South African accessions had lower PEPC content than maize.

At the time that leaves were sampled for the PEPC assay, the Tanzanian plants showed variable physiology. While one had an intermediate CCP of 18.1 μmol mol^{-1}, plants from the other population had C_4-like CCP values (4.3 and 1.1 μmol mol^{-1} in L04 replicates A and B, respectively), but CE remained low (>0.1 mol m^{-2} s^{-1}) in all three Tanzanian plants at this time. This suggests that the Tanzanian plants have variable physiologies despite high PEPC content.

DISCUSSION

C_3 and C_4 variants of *Alloteropsis semialata*

The carbon isotope discrimination, physiology, and leaf anatomy of most *A. semialata* accessions were largely consistent with those measured in typical C_3 and C_4 taxa, and consistent with earlier characterizations of individuals within this species (Ellis 1981; Renvoize 1987; Ueno & Sentoku 2006; Ibrahim 2007; Ripley *et al.* 2007; Osborne *et al.* 2008). We concluded that C_3 phenotypes were only identified in some South African populations, while accessions from Australia, Burkina Faso, Madagascar, and others from South Africa use C_4 photosynthesis (Table 2). However, typical C_4 plants have a CCP below 5 μmol mol^{-1} (Edwards & Ku 1987), whereas not a single C_4 accession in this study measured below this value and CCP was as high as 18.8 μmol mol^{-1} in one Madagascan plant. This suggests that some C_4 *A. semialata* populations may not be strongly optimized for C_4 function, being instead more C_4-like, as suggested by Ueno & Sentoku (2006), and there may be various degrees of C_4

optimization among C_4 accessions. Within the populations mentioned here, $\delta^{13}C$ signatures consistently fell within either C_3 or C_4 ranges in both field and controlled environment conditions (Table 2), indicating that the photosynthetic metabolism of these plants is not strongly influenced by the growth environment, and lending further weight to previous evidence that the C_3 and C_4 phenotypes of *A. semialata* are genetically fixed and not plastic (Ibrahim *et al.* 2008).

Discovery of C_3-C_4 intermediates in *Alloteropsis semialata*

The Tanzanian accessions presented in this study had values of CCP, OI, VF, IVMC, and OBS cell sizes that were intermediate between those of C_3 and C_4 plants, and abundant chloroplasts in both mesophyll and bundle sheath cells, consistent with plants using a C_2 cycle (Brown & Hattersley 1989; Ku *et al.* 1983). There are three possible explanations for the intermediate CCP values in Tanzanian accessions: operation of a C_2 cycle alone (i.e. *Type I C_3-C_4*), operation of a limited C_4 acid cycle alone, or operation of both C_2 and limited C_4 acid cycles (i.e. *Type II C_3-C_4*; Edwards & Ku 1987 or Type II C_2; Sage *et al*., 2012). Distinguishing between these possibilities is informed by our $\delta^{13}C$ results (Table 2) and the relationships between $\Delta^{13}C$ and C_i/C_a in *A. semialata* plants (Fig. 2f). First, Fig. 2f shows that Tanzanian plants discriminated against ^{13}C less at a given Ci/Ca value compared with C_3 plants. Second, the $\delta^{13}C$ values of Tanzanian plants, in either controlled environment or field grown plants, are almost consistently higher (less negative) than for C_3 *A. semialata* plants (Table 2). C_3-like $\delta^{13}C$ values are often observed in C_3-C_4 intermediate plants, partially as a result of the incomplete compartmentalization of photosynthetic enzymes that increases BS leakiness (Monson *et al.* 1988). However, by itself, this does not preclude the possibility that these plants have significant C_4 acid cycle activity. Hattersley & Watson (1992) note that modelling by Peisker (1986) and Monson *et al.* (1988) suggests that $\delta^{13}C$ signatures may not start to shift upward until more than 50% of CO_2 is fixed by C_4 acids. Together, the differences in stable isotope ratios indicate that there is less discrimination against ^{13}C during photosynthesis in the Tanzanian plants than in C_3 plants, which is likely due to a CO_2 concentrating mechanism between mesophyll and IBS cells. However, this CO_2 concentrating mechanism cannot be a C_2 cycle alone. If it was, the $\delta^{13}C$ values (Table 2) would be lower, and the values in Fig. 2f higher, for Tanzanian plants than for C_3 plants, because intermediate plants using a C_2 cycle, but no C_4 acid cycle, carry out double discrimination against ^{13}C by Rubisco in mesophyll and bundle sheath Calvin cycles (von Caemmerer 1989; von Caemmerer & Hubick 1989). We conclude

that (i) the photosynthetic carbon metabolism of the Tanzanian accessions is not typically C_3, (ii) the greater discrimination against ^{13}C during photosynthesis arises from some level of direct CO_2 fixation by PEPC in the mesophyll, and (iii) there is integration of at least a weak C_4 acid cycle. Despite the intermediate CCP and OI values, CE in the C_3-C_4 plants never increased beyond C_3 levels, a pattern that is typical of other C_3-C_4 intermediate taxa (Ku *et al.* 1983; Dai *et al.* 1996). Indeed, studies in C_3, C_3-C_4, C_4-like, and C_4 *Flaveria* show that more than 50% of CO_2 may be assimilated via a C_4 system before CE increases to values typically measured in C_4 species (Fig. S4; Ku *et al.* 1983; Dai *et al.* 1996; Vogan & Sage 2011). These *Flaveria* studies also show a strong relationship between CCP and the proportion of CO_2 fixed by the C_4 cycle (Fig. S4; Ku *et al.* 1991; Dai *et al.* 1996; Vogan & Sage 2011), indicating that the reduced CCP measured in the Tanzanian plants may result from an increase in C_4 cycle activity, which can be sustained by the high PEPC content observed in these plants.

If one accepts that a C_2 cycle is always present in plants intermediate between C_3 and C_4, then the Tanzanian plants have both C_2 and limited C_4 acid cycle activities, i.e. they are Type II C_3-C_4 intermediates, that probably fix a minority of CO_2 via the C_4 system, while the isotopically intermediate herbarium specimens identified in the literature (Table 1) likely engage more C_4 activity than the accessions measured in this study (Fig. S4; Monson *et al.* 1988). Thus, populations of *A. semialata* clearly exhibit a gradient of photosynthetic phenotypes, possibly encompassing C_3, C_3-like, C_3-C_4 (including C_2 cycles with various degrees of C_4 cycle activity), C_4-like, and C_4 states, making it the most photosynthetically diverse species known.

Evolutionary significance of C_3-C_4 intermediates within the species complex

Individuals with intermediate phenotypes might be interpreted as hybrids between C_3 and C_4 individuals. For example, hybrid crosses between C_3 and C_4 *Atriplex* congeners produce intermediate phenotypes (Osmond *et al.* 1980; Oakley *et al.* 2014). However, a hybrid origin of C_3-C_4 plants is not supported by the genetic data available for *A. semialata*, as all non-C_4 accessions so far sampled form a clade distinct from C_4 accessions in both plastid genome and nuclear rDNA phylogenies (Fig. S5; Lundgren *et al.* 2015). In the plastid phylogeny, all C_3 populations from South Africa form a monophyletic group, while the C_3-C_4 intermediates are placed in one of two other non-C_4 groups (Fig. S5). If the other accessions within these clades were also physiologically intermediate, the phylogenetic

pattern would suggest that the C_3 photosynthetic type present in South Africa results from an evolutionary reversal from a C_3-C_4 intermediate type, making the C_3 plants that colonized southern Africa the first documented case of an evolutionary loss of C_3-C_4 intermediate characters.

The evolutionary history of photosynthetic transitions within *Alloteropsis* has been discussed by previous authors, but the data available so far has not been conclusive (Ibrahim *et al.* 2009; Christin *et al.* 2010, 2012). However, the discovery of C_3-C_4 intermediates here changes this picture. If we accept that C_3 individuals arise from the loss of C_3-C_4 characters, one of the basal splits within *A. semialata* separates C_3-C_4 and C_4 plants, leaving two possible scenarios. Either the ancestor was C_4 and some of the descendants lost C_4 characters to reach a C_3-C_4 state, or this ancestor was C_3-C_4 and one or more of the descendants acquired more C_4-like characters. These two scenarios cannot be differentiated based on species relationships (Christin *et al.* 2010). However, discussion of the adaptive significance of lateral gene transfers within *Alloteropsis* raised the suggestion that the genus was initially C_3-C_4, and that the C_4 phenotype was independently realized on multiple occasions, via the co-option of C_4-like components present in C_3-C_4 plants (Christin *et al.* 2012). The presence of C_3-C_4 intermediates within the group supports this hypothesis, although comparative analyses of the genetic determinants of C_4 phenotypes within the group will be needed to definitively resolve this problem.

Conclusion

By comprehensively investigating carbon isotope discrimination, physiology, and leaf anatomy in accessions of *A. semialata* from across its geographic range, we demonstrate for the first time that C_3-C_4 intermediates, in addition to genetically fixed C_3 and C_4 types, exist in this taxon. These intermediates are not the result of hybrid crosses between the analyzed C_3 and C_4 genotypes, but form distinct lineages within *A. semialata*. The intermediates prosper in wooded savannas of Central Africa, where the origin of the species is inferred based on phylogeographic analyses. In these deciduous forests, conditions vary throughout the year in terms of light, temperature and nutrient availability. These varying conditions might have provided a selective edge to C_3-C_4 plants (Supplementary Methods and Results), which would then have constituted a reservoir of genes that could be co-opted to evolve more C_4 phenotypes. On the other hand, some lineages might have lost their C_3-C_4 characters when they migrated to southern latitudes of Africa, showing that C_3-C_4 evolution is reversible. In this

scenario, C_3-C_4 ancestors might have generated C_3, C_3-C_4 and C_4 descendants, leading to phenotypes spanning the C_3 to C_4 continuum in *A. semialata*, which constitutes the perfect system to study the origins of C_4 photosynthesis, in terms of genetics, physiology and ecology. The existence of this variation within a single species of grasses opens new avenues for the study of the intraspecific dynamics that allow the emergence of new physiological adaptations within plant species of ecological significance.

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FIGURE CAPTIONS

Figure 1. Photosynthetic variation across *A. semialata*. Frequency (A) and geographic (B) distributions of $\delta^{13}C$ signatures (‰) from 298 *A. semialata* accessions. Map points are color coded according to the color scheme used in the histogram.

Figure 2. Physiological variation in *A. semialata*. For individual accessions, variations in (A) CO_2 compensation point (CCP), (B) oxygen inhibition (OI), (C) CO_2 saturated photosynthesis (A_{sat}), and (D) carboxylation efficiency (CE) are shown across the $\delta^{13}C$ gradient. Panel E regresses stomatal conductance (g_s) against net CO_2 assimilation (A_{400}), both measured at a reference CO_2 concentration of 400 μbar, to show intrinsic water use efficiency (iWUE). Panel F shows carbon isotope discrimination ($\Delta^{13}C$) against the ratio of intercellular to ambient CO_2 (C_i/C_a), overlaid with the theoretical relationship for C_3 plants (see *Materials and Methods*). Data points are colored as C_3 (blue), C_4 (red), and the Tanzanian plants (green). Linear model adjusted R^2 and *p*-value results are shown, where relevant.

Figure 3. Anatomical variation in *A. semialata*. Variation in (A) vein frequency, (B) number of interveinal mesophyll cells (IVMC), (C) outer bundle sheath (OBS) cell size, and (D) inner bundle sheath (IBS) cell size. Data points are colored by C_3 (blue), C_4 (red), and the Tanzanian plants (green). Linear model adjusted R^2 and *p*-value results are shown.

Figure 4. PEPC content in C_3, C_3-C_4, and C_4 *A. semialata*. (A) Immunoblot indicating relative PEPC content after 10-s exposure and (B) whole protein staining across nine *A. semialata* accessions, with rice (C_3) and maize (C_4) controls. Along the whole protein stain, PEPC is indicated at 100 kDa.

TABLES

Table 1. Anomalous specimens of *A. semialata* in the literature.

Table 2. Details of individual accessions and corresponding stable isotope, anatomy and physiology results.

Table 3. ANOVA and post-hoc Tukey tests for physiology and anatomy traits.

Table 1. *Alloteropsis semialata* specimens with anomalous $\delta^{13}C$ isotope signatures (I), gross morphology (M), and/or leaf blade anatomy (A). The country, year of collection, and stable isotope values are show, where available.

Specimen	Country	Year	$\delta^{13}C$	Anomaly	Study[1]
Milne-Redhead 3021	Zambia	1937	-20.0[A] -20.7[D] -18.5[E]	I	A, D, E
Eyles 1920	Zimbabwe	1919	-10.3	M	B
Greenway 6290	Malawi			M	B
Milne-Redhead 3371	Zambia	1937	-18.6	I, M	B
Milne-Redhead & Taylor 8455	Tanzania	1956	-10.7	A	C
Astle 1137	Zambia			A	C
Norval 106	South Africa			A	C
Rattray 428	Zimbabwe		-11.4	A	C
Emson 340	Tanzania	1932	-21.4	I	D,E
Stowe 495	Zambia	1940	-20.8	I	D
Brzotowski 26	Tanzania	1944	-22.6	M, A	D
Robinson 4744	Zambia	1961	-22.7	M, A	D
Proctor 2165	Tanzania	1962	-23.0	M, A	D
Mbano DSM812	Tanzania	1969	-23.9	M, A	D
Proctor 2206	Zambia	1962	-23.9	M, A	D
Simon 932	Tanzania	1966	-25.3	M, A	D
Tanner 5076	Tanzania	1960	-26.3	M, A	D
Bogdon & Williams 238	Kenya	1947	-11.8	M, A	D
Symoens 14118	DRC	1971	-22.6	I	E
Lundgren & Christin 4	Tanzania	2014	-23.1	M, A	E
Bullock 1980	Tanzania	1949	-22.3	I	E
Bullock 1979	Tanzania	1949	-19.7	I	E
Ruffo & Kisena 2806	Tanzania	1987	-18.6	I	E
Shaunty 488	Zambia	1919	-20.7	I	E

[1]Studies refer to (A) Ellis (1981); (B) Gibbs Russell (1983); (C) Renvoize (1987); (D) Hattersley & Watson (1992); (E) Lundgren *et al.* (2015).

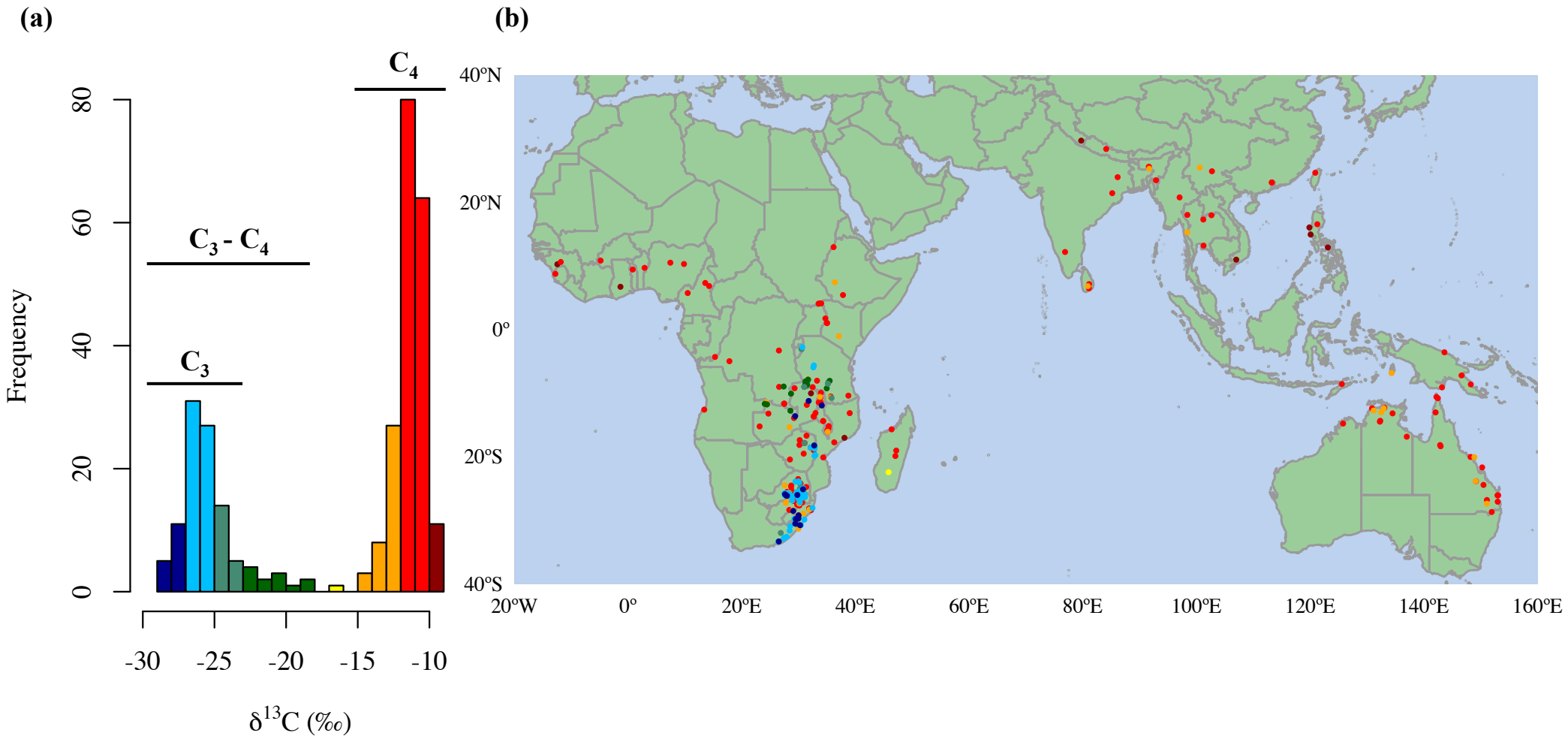
Table 2. Details of individual accessions and corresponding stable isotope, anatomy and physiology results.

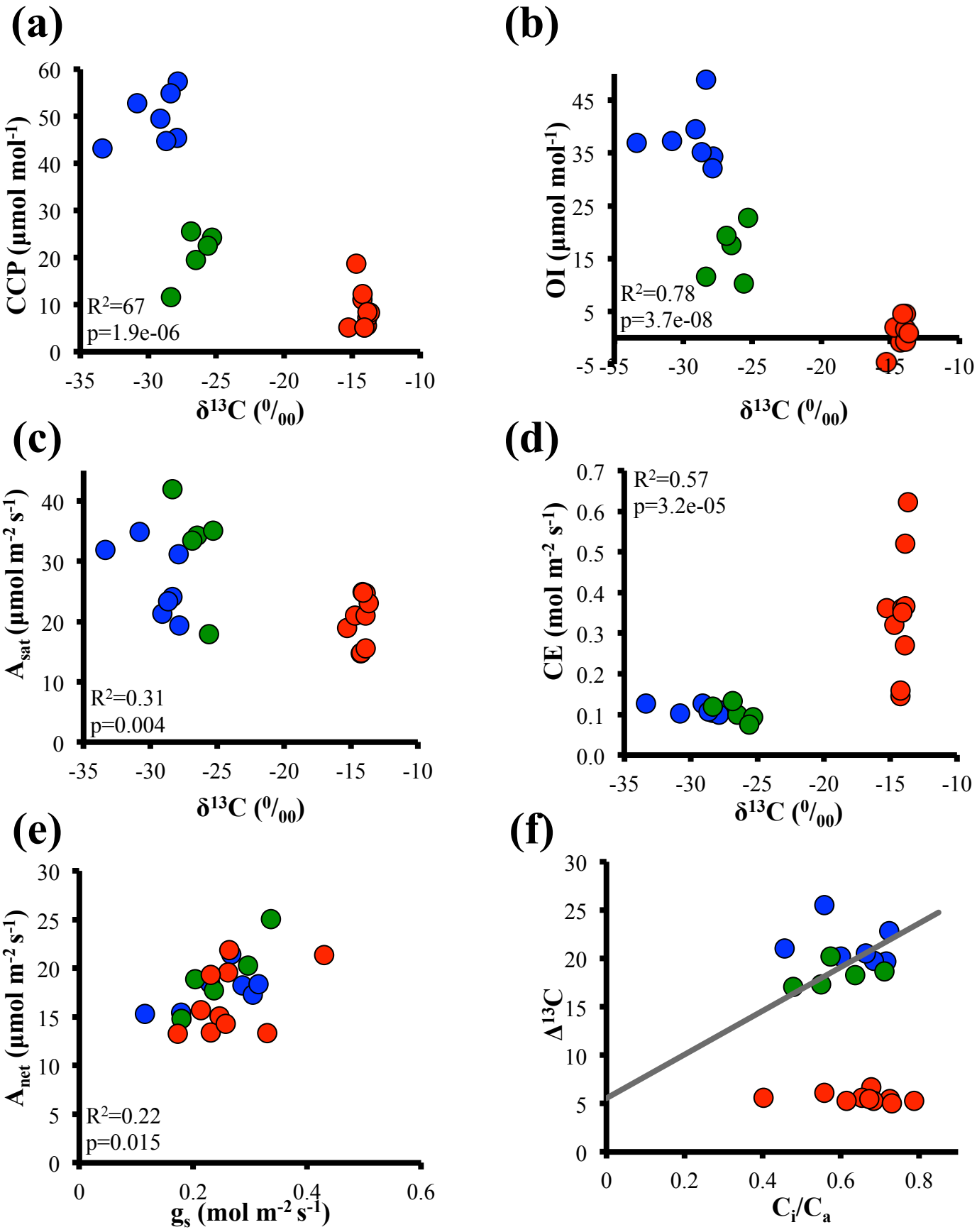
Accession	Pop	Country	Latitude	Longitude	$\delta^{13}C^{F}$ *	$\delta^{13}C^{C}$	VF	IVMC	IBS size (µ)	OBS size (µ)	CCP (µmol m^{-2} s^{-1})	CE (mol m^{-2} s^{-1})	OI	Type
CRL-2	CRL	South Africa	-25.74	30.24	-27.6	-27.8	2.7	5.9	7.1	15.8	57.4	0.11	34.3	C_3
CRL-4	CRL	South Africa	-25.74	30.24	-27.6	-28.3	4.0	7.4	10.7	19.6	54.8	0.10	48.9	C_3
EML-11	EML	South Africa	-26.29	30.00	-27.3	-29.1	3.3	7.2	7.5	16.0	49.5	0.13	39.6	C_3
JMS-201	JMS	South Africa	-33.32	26.44	-28.3	-30.8	4.7	8.6	7.3	18.2	52.8	0.10	37.2	C_3
JMS-202	JMS	South Africa	-33.32	26.44	-28.3	-27.9	4.7	7.7	7.9	17.3	45.4	0.10	32.1	C_3
KWT-3	KWT	South Africa	-32.70	27.53	-27.1	-28.7	4.0	7.8			44.7	0.11	35.2	C_3
KWT-5	KWT	South Africa	-32.70	27.53	-27.1	-33.4	3.3	6.8	7.5	17.3	43.1	0.13	37.0	C_3
L01-A	L01	Tanzania	-5.63	32.69	-26.3	-26.5	5.0	3.5	10.5	13.6	19.4	0.10	17.6	C_3-C_4
L04-A	L04	Tanzania	-8.51	35.17	-23.1	-25.3	5.3	5.1	11.6	14.4	24.3	0.09	22.7	C_3-C_4
L04-B	L04	Tanzania	-8.51	35.17	-23.9	-25.6	5.3	4.9	11.6	11.6	22.5	0.08	10.3	C_3-C_4
L04-D	L04	Tanzania	-8.51	35.17	-26.4	-26.9	5.3	5.4	12.5	13.2	25.6	0.13	19.3	C_3-C_4
L04-E	L04	Tanzania	-8.51	35.17	-25.0	-28.3	7.3	4.4	13.0	12.5	11.6	0.12	11.6	C_3-C_4
AUS-3	AUS	Australia	-19.62	146.96	-11.5 *	-14.3	8.3	1.5	9.5	7.7	11.1	0.15	1.0	C_4
AUS-4	AUS	Australia	-19.62	146.96	-12.5 *	-15.3	11.0	1.7	9.4	7.8	5.1	0.36	-4.6	C_4
BF-2	BF	Burkina Faso	10.85	-4.83	-11.1 *	-13.9	11.0	1.4	11.3	8.9	8.5	0.37	4.5	C_4
BF-3	BF	Burkina Faso	10.85	-4.83	-11.3 *	-14.1	11.0	1.6	9.5	8.3	5.2	0.35	1.2	C_4
MAJ-1	MAJ	Madagascar	-15.67	46.37	-11.4 *	-14.2	11.7	1.7	9.4	9.2	12.3	0.16	-0.8	C_4
MAJ-3	MAJ	Madagascar	-15.67	46.37	-11.9 *	-14.7	9.0	1.9	11.2	11.4	18.8	0.32	2.0	C_4
MDG-1	MDG	South Africa	-25.76	29.47	-11.1	-13.9	11.3	1.5	11.3	9.7	5.6	0.27	-0.6	C_4
MDG-2	MDG	South Africa	-25.76	29.47	-11.1	-13.9	11.7	1.3	12.2	9.9	7.2	0.52	1.6	C_4
SFD-1	SFD	South Africa	-28.39	29.04	-11.3 *	-14.1					5.2	0.36	4.6	C_4
SFD-3	SFD	South Africa	-28.39	29.04	-10.9 *	-13.7	10.0	1.5	10.9	8.7	8.3	0.62	0.9	C_4

* $\delta^{13}C$ measured on plants grown under controlled environment (C) or (F) conditions (in ‰). For accessions lacking field $\delta^{13}C$ data (*), controlled environment values were adjusted as described in *Materials and Methods*.

Table 3. Results of ANOVAs on physiological and anatomical variables. Accessions were grouped into C_3, C_4, and the two measurement sets of the Tanzanian plants. Trait abbreviations can be found in the Methods section. Variables with significant group effects were subjected to *post-hoc* Tukey tests and groups that differed significantly are denoted with different letters. *Post-hoc* Tukey tests for traits with significant population terms are presented in Table S1

		Group	**Population**	**C_3**	**Tanzanian**	**C_4**
	DF	*F (p-value)*	*F (p-value)*		*Mean^{Tukey}*	
Physiology						
CCP	2, 8	**187.0 (3.2e-09)**	1.8 (0.18)	49.69^{c}	20.68^{b}	8.73^{a}
CE	2, 8	**16.5 (4.9e-04)**	1.2 (0.40)	0.11^{a}	0.10^{a}	0.35^{b}
OI	2, 8	**116.2 (4.0e-08)**	0.5 (0.86)	37.73^{c}	16.30^{b}	0.98^{a}
A_{sat}	2, 8	**259.4 (0.014)**	29.9 (0.66)	26.56^{ab}	32.53^{b}	20.33^{a}
$_{i}$WUE	2, 8	**504.0 (6.6e-03)**	**11.7 (2.2e-04)**	79.72^{b}	78.60^{b}	65.69^{a}
R_{light}	2, 8	**6.9 (0.01)**	2.1 (0.12)	0.92^{a}	2.01^{b}	1.10^{a}
LCP	2, 8	**15.5 (6.4e-04)**	**4.6 (0.01)**	18.20^{a}	36.88^{b}	17.41^{a}
GA_{sat}	2, 8	0.1 (0.93)	1.8 (0.19)	18.63	17.82	18.64
$PPFD_{50}$	2, 8	1.0 (0.40)	1.5 (0.26)	281.42	217.27	217.87
ΦCO_2	2, 8	1.0 (0.42)	1.2 (0.40)	0.05	0.06	0.06
Leaf anatomy						
VF	2, 8	**85.7 (5.1e-07)**	0.8 (0.64)	3.81^{a}	5.66^{b}	10.56^{c}
IVMC	2, 8	**248.4 (3.0e-09)**	1.8 (0.19)	7.34^{c}	4.64^{b}	1.54^{a}
OBS width	2, 8	**76.3 (2.3e-06)**	0.8 (0.60)	17.34^{c}	13.07^{b}	9.07^{a}
IBS width	2, 8	**16.7 (9.4e-04)**	1.0 (0.49)	8.00^{a}	11.83^{b}	10.52^{b}


(a)
(b)
C4
C3 - C4
C3
Frequency
0
20
40
60
80
-30
-25
-20
-15
-10
δ13C (‰)
40°N
20°N
0°
20°S
40°S
20°W
0°
20°E
40°E
60°E
80°E
100°E
120°E
140°E
160°E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p=1.9e-06
δ13C (0/00)
(b)
OI (µmol mol-1)
R2=0.78
p=3.7e-08
δ13C (0/00)
(c)
Asat (µmol m-2 s-1)
R2=0.31
p=0.004
δ13C (0/00)
(d)
CE (mol m-2 s-1)
R2=0.57
p=3.2e-05
δ13C (0/00)
(e)
Anet (µmol m-2 s-1)
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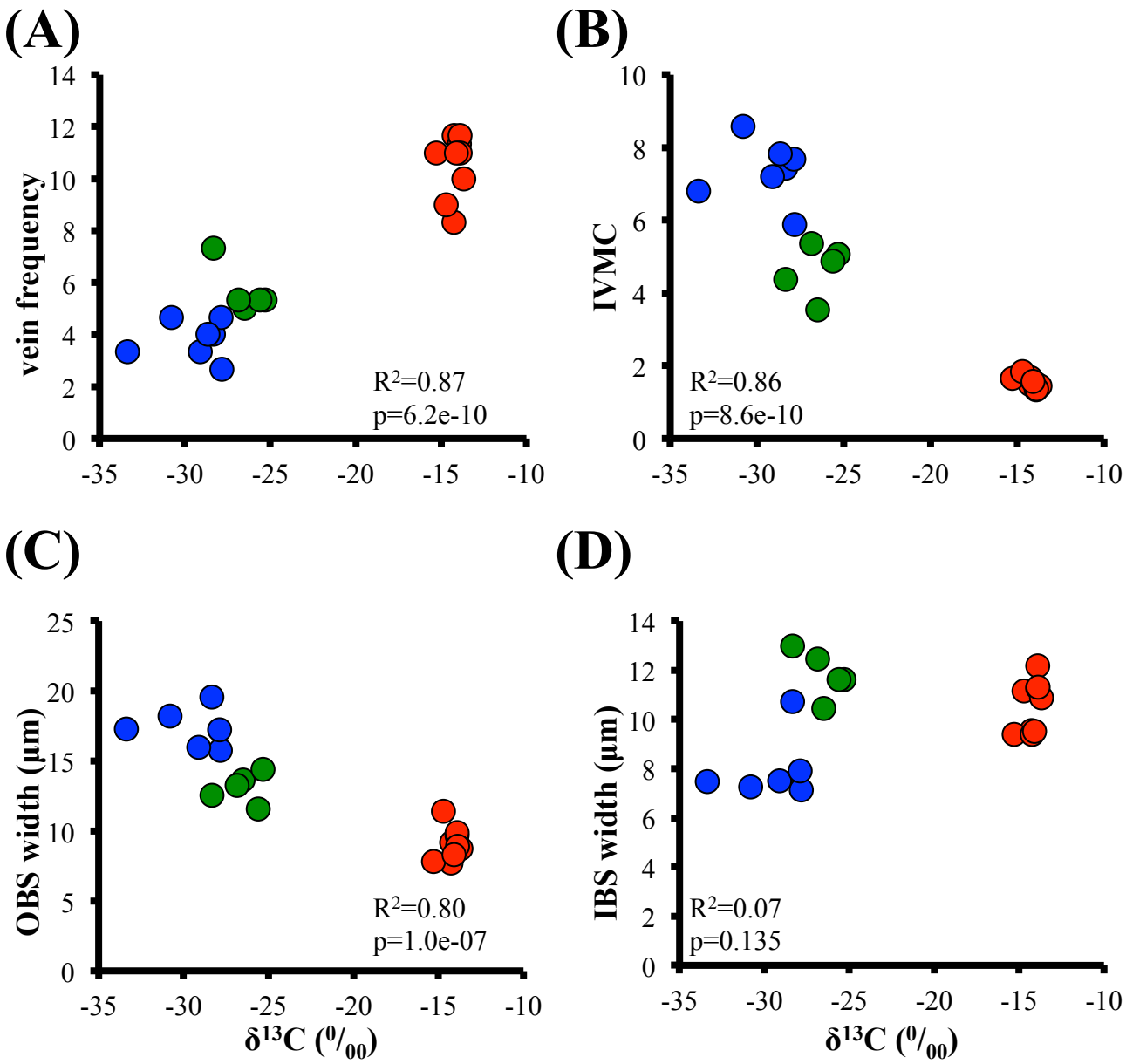
p=0.015
gs (mol m-2 s-1)
(f)
Δ13C
Ci/Ca


(A)
vein frequency
14
12
10
8
6
4
2
0
-35
-30
-25
-20
-15
-10
R²=0.87
p=6.2e-10
(B)
IVMC
10
8
6
4
2
0
-35
-30
-25
-20
-15
-10
R²=0.86
p=8.6e-10
(C)
OBS width (µm)
25
20
15
10
5
0
-35
-30
-25
-20
-15
-10
δ13C (0/00)
R²=0.80
p=1.0e-07
(D)
IBS width (µm)
14
12
10
8
6
4
2
0
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-30
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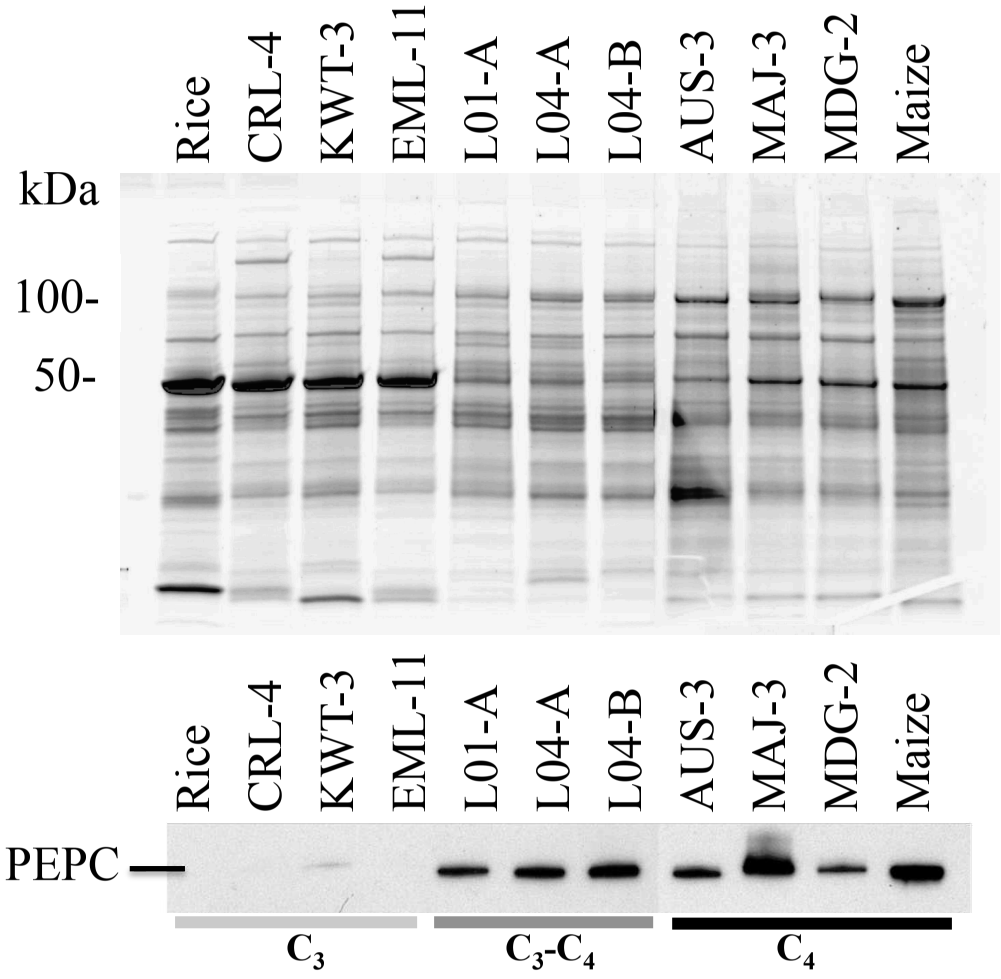
-15
-10
δ13C (0/00)
R²=0.07
p=0.135


Rice
CRL-4
KWT-3
EML-11
L01-A
L04-A
L04-B
AUS-3
MAJ-3
MDG-2
Maize
kDa
100-
50-
Rice
CRL-4
KWT-3
EML-11
L01-A
L04-A
L04-B
AUS-3
MAJ-3
MDG-2
Maize
PEPC
C3
C3-C4
C4

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS AND RESULTS

Response To Environmental Conditions

Rationale and Methods

While the C_3 and C_4 accessions in this study were grown in moderate nutrient compost (no. 2, John Innes Manufacturers Association, Reading, England), the Tanzanian plants were initially grown in higher nutrient potting compost (no. 3, John Innes Manufacturers Association, Reading, England) to aid re-establishment of the cuttings once they were imported into the UK. To test whether this difference in soil nutrients influenced the results, the Tanzanian plants were transplanted into the same moderate soil nutrient compost (no. 2) after the initial set of measurements and re-analysed after three months. At the end of the three months, stable isotope, physiology, and anatomy data were collected again on youngest fully expanded leaves that developed in the new soil environment. Isotope and anatomy methods used on these plants are identical to those described in the main text. However, only a subset of the physiology dataset was re-measured at this time, including only the sub-ambient CO_2R portion of the A/C_i curve, such that CE and CCP were calculated as the slope (CE) and x-intercept (CCP) of the line connecting these data points. WUE was calculated as in the main text.

Results

Growing the plants in higher nutrient soils did influence the leaf composition, as the percent of leaf nitrogen and carbon were greater, and the C:N ratio lower, in the higher soil nutrients compared to the moderate nutrient grown plants (Table S2). Despite this, the Tanzanian accessions consistently had non-C_4 $\delta^{13}C$ signatures (i.e. -28 to -23‰; Tables 2 and S2). It is interesting to note that CCP was no longer intermediate in these moderate soil nutrient conditions, but instead fell entirely within the range measure in C_4 *A. semialata* (i.e. ≤ 17.0 μmol mol^{-1}; Figs 2a and S6; Tables 3 and S3). However, CE did not improve under moderate soil nutrients (Fig. S6; Table S3).

DISCUSSION

There is some evidence that the degree of C_4-cycle activity is plastic in C_3-C_4 *A. semialata* plants, varying temporally or with soil environment. In particular, CCP in the Tanzanian plants was initially intermediate between C_3 and C_4 plants. However, when they were re-measured after being transplanted into lower nutrient soils, their CCP dropped to values similar to those of C_4 plants. In fact, CCP in L01A was initially intermediate in the high nutrient soil (i.e. 19.3 μmol mol^{-1}), then became C_4-like in the moderate nutrient soil (i.e. 6.3 μmol mol^{-1}), then became intermediate again when it was re-measured at the time of PEPC content leaf analysis (i.e. 18.1 μmol mol^{-1}), suggesting this accession can alternate back and forth between C_3-C_4 and C_4-like states in response to soil or atmospheric environments (Fig. S6). WUE also increased when these C_3-C_4 plants were in a C_4-like state (Table S3). Leaf anatomy, however, did not vary between these two states, suggesting that the anatomical phenotype of C_3-C_4 *A. semialata* plants may be suitable for both C_2 and C_4 metabolism. Indeed, IBS

cell sizes and IVMC of C_3-C_4 *A. semialata* fall within the range of the values measured across C_4 grass species as a whole (Lundgren *et al.* 2014). Thus, the observed physiological shift in CCP could result from plasticity in the underlying biochemistry, probably via enhanced C_4 cycle activity (e.g. from approximately 30 to 60%). However, follow-up pulse-chase work is needed to confirm this.

The C_2 cycle has been cited as an enabler of C_4 photosynthesis that may be a critical step in the transition from C_3 to C_4 (Hylton *et al* 1988; Heckmann *et al.* 2013; Williams *et al.* 2013; Mallmann *et al.* 2014; Sage *et al.* 2014). There are however several costs associated with running a C_2 cycle. First, this pathway inherently requires photorespiration to operate and, although photorespiratory CO_2 is recycled, the process still produces toxic by-products, consumes ATP and NADPH, and creates a cellular imbalance in N-metabolites (Mallmann *et al.* 2014). Second, establishment of the C_2 cycle requires construction costs to provision bundle sheath organelles and enzymes (Schuster & Monson 1990). Finally, when plants already using a C_2 cycle initiate a C_4 cycle, the C_4 biochemical functions are often inefficient and increase nitrogen requirements (Monson *et al.* 1986). This creates nitrogen sinks that offset the advantages gained by recycling photorespired CO_2 (Schuster & Monson 1990; Mallmann *et al.* 2014). Despite these costs, there are examples of C_3-C_4 plants that have persisted for millions of years (Christin *et al.* 2011), suggesting that the intermediate phenotype may be a better adaptation than the C_4 state under certain environmental conditions. Our data point to plasticity for C_4 cycle activity within C_3-C_4 genotypes, and we hypothesize that these intermediate states may maintain an inherent degree of biochemical flexibility, which allows them to shift between physiological states. If this is true, then phenotypic plasticity for traits important to photosynthetic efficiency may weaken selection pressures to fix the C_4 syndrome in these plants, maintaining them in an intermediate state. This could potentially explain the long-term persistence of C_3-C_4 species in some lineages. Supporting our hypothesis, high levels of physiological plasticity have been found in other C_3-C_4 species, including the species *Flaveria linearis*, which becomes C_4-like when grown in warmer conditions (Teese 1995). This apparent flexibility to transition between C_3-C_4 and C_4-like states (and perhaps also between C_3-C_4 and C_3-like states) would enable plants to acclimate their physiology to whichever environment they are currently experiencing, an ability that would be particularly advantageous in heterogeneous environments (West-Eberhard 1989).

The C_3-C_4 *A. semialata* identified here, as well as those isotopically intermediate accessions reported in the literature (Table 1), were all found in the densely wooded Miombo savannas of central eastern Africa (Fig. 1; Lundgren *et al.* 2015). These habitats are characterized by particularly heterogeneous light, precipitation, and soil nutrients. For instance, less than 30% of incident light penetrates a mature Miombo canopy (van der Meulen & Werger 1984), however these woodlands are dominated by deciduous tree species that shed their leaves during the dry season (Frost 1996), thereby exposing the perennial understory vegetation to both shady and full sunlight conditions at different times of the year. Moreover, these woodlands occupy areas averaging over 700 mm of annual precipitation, yet more than 95% of this occurs within the 5-7 month rainy season (Frost 1996), requiring Miombo vegetation to withstand precipitation extremes. Miombo woodland soils are generally nutrient-poor, however soil

quality overall is variable due to catenas (i.e. repeatable soil sequences along sloped landscapes), a variety of underlying bedrock, termite activity (e.g. from foraged litter, and thus nutrients, from the forest floor, but also from termite nests that create nutrient hot-spots), seasonal leaf fall (e.g. from the nitrogen-rich canopy trees which have N-fixing nodules), and anthropogenic history (e.g. slash and burn agriculture) (reviewed in Frost 1996). Perennial herbaceous species of the Miombo understory consequently must be able to tolerate heterogeneity in precipitation, light, and soil nutrients. Because of this, C_4 acid cycle activity may be optimal during portions of the year but more costly than only running a C_2 cycle at other times. The flexibility to shift the degree to which one type of carbon shuttle is used over another could be hugely beneficial in these heterogeneous environments, explaining the persistence of the C_3-C_4 intermediate phenotype within *A. semialata*.

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SUPPLEMENTARY FIGURES AND TABLES

Figure S1. A/C_i (A) and light response (B) curves showing means ± 1SE of C_3 (blue), C_4 (red), and Tanzanian plants (green).

Figure S2. Cross-sections of C_3, C_3-C_4 intermediate, and C_4 *A. semialata.* Examples of (A) C_3, (B) high nutrient Tanzanian, (C) moderate nutrient Tanzanian, and (D) C_4 accessions show differences in the number of veins per segment, the number of mesophyll cells separating the veins, and the relative sizes of inner and outer bundle sheath cells. See Supplementary Methods and Results for discussion of soil nutrients in the Tanzanian plants.

Figure S3. Hand-cut cross-sections showing chloroplasts in both mesophyll and inner bundle sheath cells of Tanzanian plants (A-C) compared to chloroplast localization primarily in the mesophyll of a C_3 plant (D) and the inner bundle sheaths of a C_4 plant (E).

Figure S4. Responses of (A) CO_2 compensation point (CCP) and (B) carboxylation efficiency (CE) to the percent of CO_2 fixed by C_4 acids in a range of C_3 (blue), C_3-C_4 (green), C_4-like (orange), and C_4 (red) *Flaveria* species. Data taken from Ku *et al.* (1991), Dai *et al.* (1996), and Vogan & Sage (2013).

Figure S5. Phylogenetic relationships among *A. semialata* accessions. This tree was obtained through Bayesian inference using chloroplast markers, and branch lengths are proportional to estimated divergence time, in arbitrary time units. Bayesian support values are indicated near branches. Tree modified from Lundgren *et al.* (2015), with permission.

Figure S6. Sub-ambient A/C_i curves measured on the Tanzanian plants in high nutrients (grey), moderate nutrients (black), and in the greenhouse at the time of PEPC assay leaf sampling (green).

Table S1. Means and post-hoc Tukey test results for variables with significant population term in ANOVA models presented in Table 3.

Table S2. Stable isotopes and leaf composition in the Tanzanian plants under high (H) and moderate (M) nutrient soils.

Table S3. Leaf anatomy and physiology of the Tanzanian plants under high (H) and moderate (M) nutrient soils.

Table S1. Means and post-hoc Tukey test results for variables with a significant population term in the ANOVA models presented in Table 2.

Group	Population	WUE	LCP
C_3	CRL	83.2^{b}	2.2^{a}
C_3	EML	132.7^{c}	10.7^{abc}
C_3	JMS	60.1^{ab}	23.6^{abcd}
C_3	KWT	69.37^{ab}	32.5^{bcd}
Tanzanian	L01	68.5^{ab}	51.1^{d}
Tanzanian	L04	81.1^{b}	33.3^{cd}
C_4	AUS	68.9^{ab}	25.4^{abcd}
C_4	BF	45.1^{a}	9.6^{ab}
C_4	MAJ	56.8^{ab}	12.4^{abc}
C_4	MDG	74.3^{ab}	15.4^{abc}
C_4	SFD	83.4^{b}	24.2^{abcd}

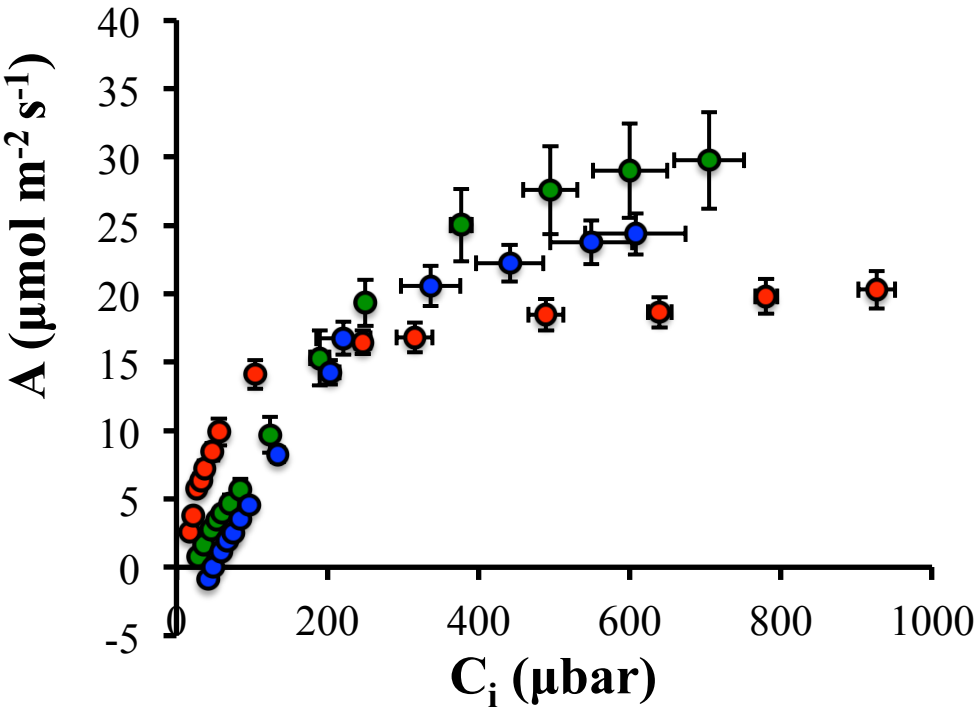
Table S2. Stable isotopes and leaf composition in the Tanzanian plants under high (H) and moderate (M) nutrient soils.

	^{13}C (‰)		^{15}N (‰)		Leaf nitrogen (%)		Leaf carbon (%)		Leaf C:N	
Plant	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*
LO1-A	-26.5	-28.3	2.3	9.4	3.8	2.0	48.8	44.0	12.9	22.1
LO4-A	-25.3	-25.6	1.8	7.6	4.0	1.9	48.0	39.8	12.0	21.1
LO4-B	-25.6	-22.8	2.2	8.6	4.2	2.0	49.6	45.3	11.9	23.0
LO4-D	-26.9	-26.7	0.4	5.5	3.4	2.2	48.7	54.4	14.5	24.5
LO4-E	-28.3	-23.7	2.6	7.6	3.7	1.4	48.6	49.4	13.1	36.6

Table S3. Leaf anatomy and physiology of the Tanzanian plants under high (H) and moderate (M) nutrient soils.

	VF		IVMC		IBS size (µm)		OBS size (µm)		A_{400} (µmol m^{-2} s^{-1})		gs_{400} (mol m^{-2} s^{-1})		$_{i}WUE_{400}$ (µmol mol^{-1})		CCP (µmol mol^{-1})		CE (mol m^{-2} s^{-1})	
Plant	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*	*H*	*M*
LO1-A	5.0	4.7	3.5	4.4	10.5	9.8	13.6	12.6	20.3	24.4	0.3	0.3	68.5	71.1	19.4	6.3	0.10	0.12
LO4-A	5.3	5.5	5.1	5.4	11.6	11.2	14.4	12.8	18.9	20.9	0.2	0.2	92.9	133.5	24.3	6.3	0.09	0.11
LO4-B	5.3	6.3	4.9	5.4	11.6	11.9	11.6	12.5	14.8	14.7	0.2	0.1	82.4	137.0	22.5	12.9	0.08	0.08
LO4-D	5.3	7.0	5.4	6.1	12.5	12.1	13.2	14.1	17.7	15.0	0.2	0.1	74.8	206.3	25.6	8.6	0.13	0.09
LO4-E	7.3	7.3	4.4	5.6	13.0	13.2	12.5	15.7	25.0	13.2	0.3	0.1	74.3	134.3	11.6	17.0	0.12	0.07

(A)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800
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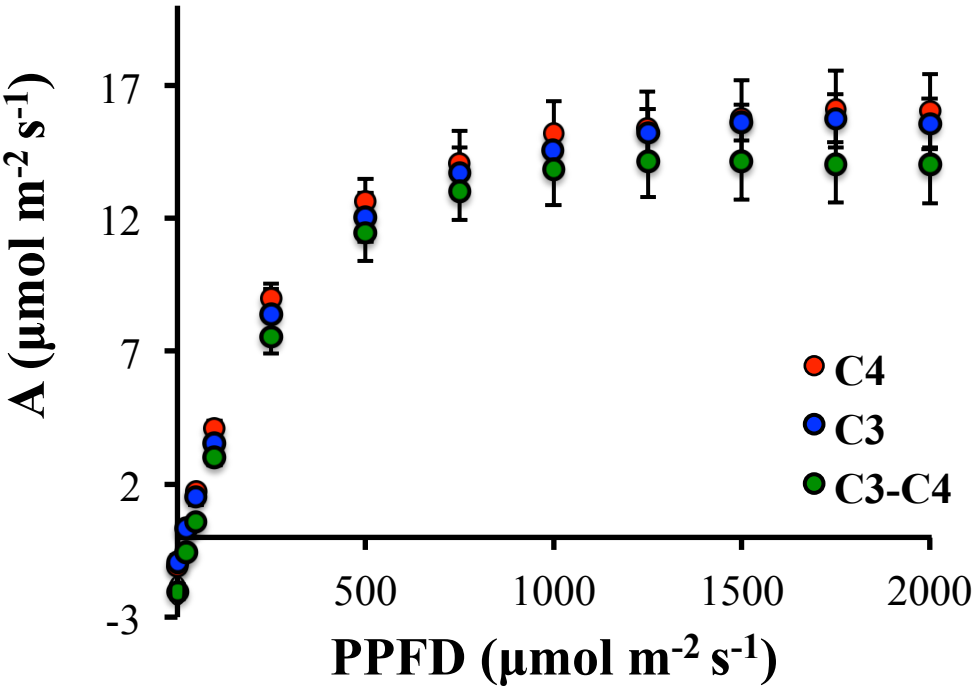
Ci (µbar)

(B)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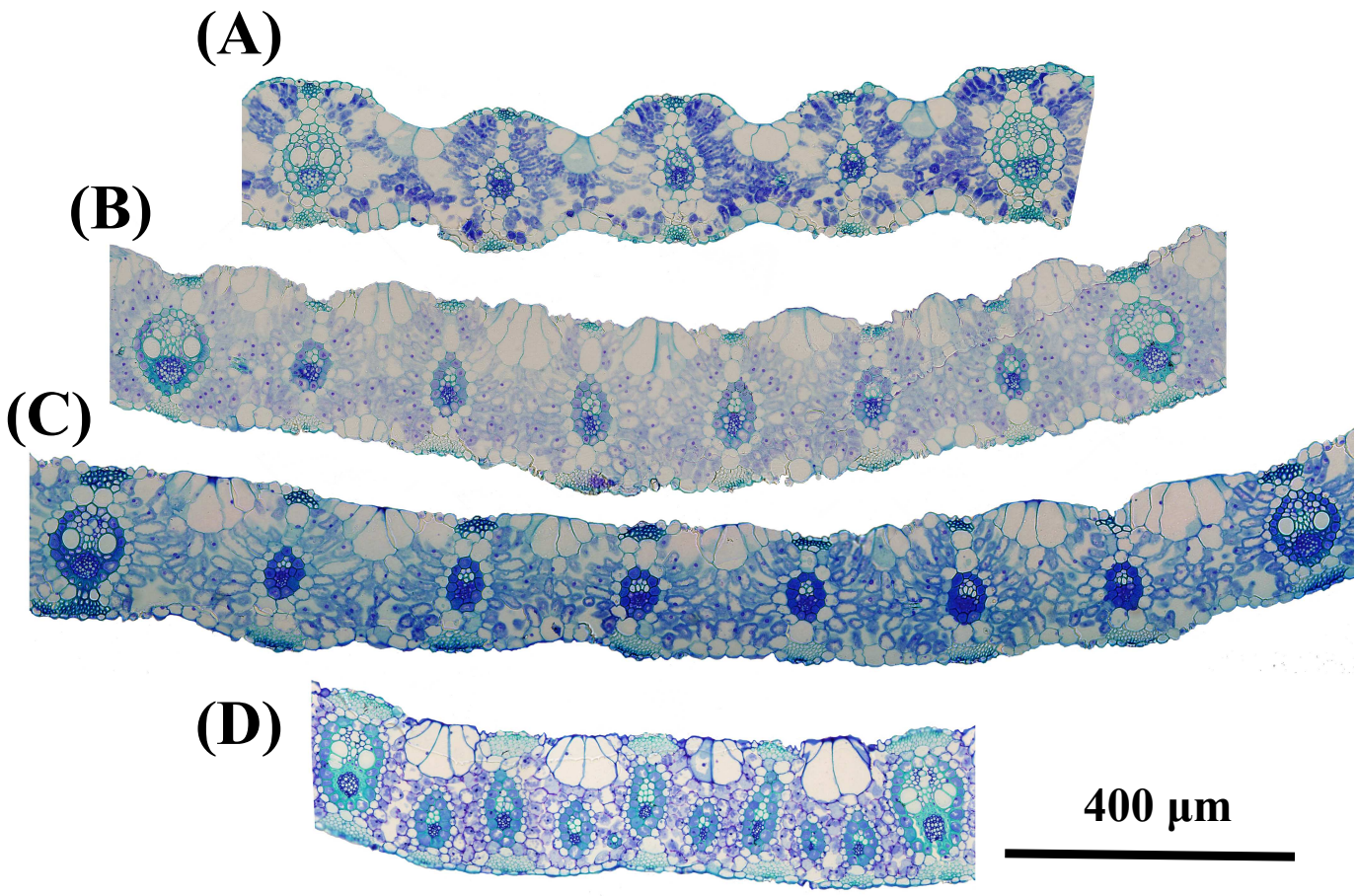
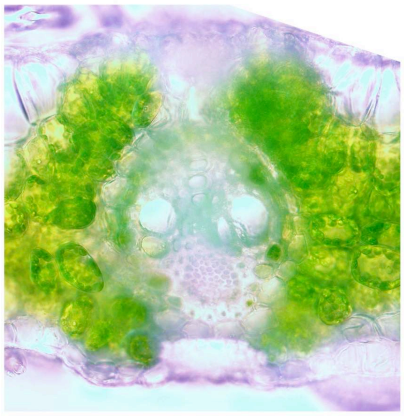
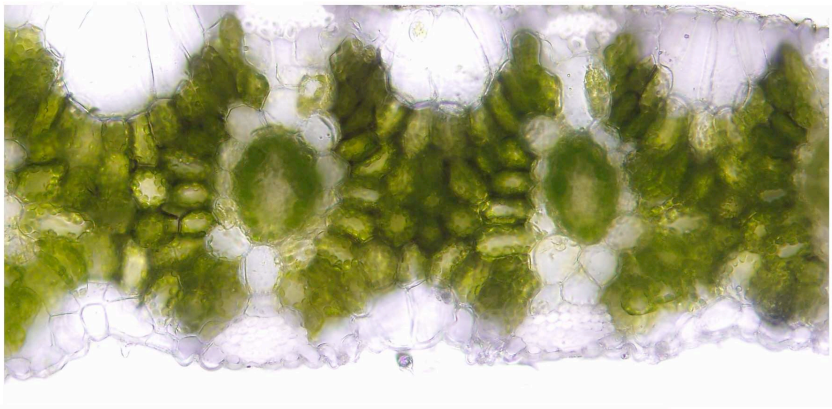
C3
C3-C4


(A)
(B)
(C)
(D)
400 µm

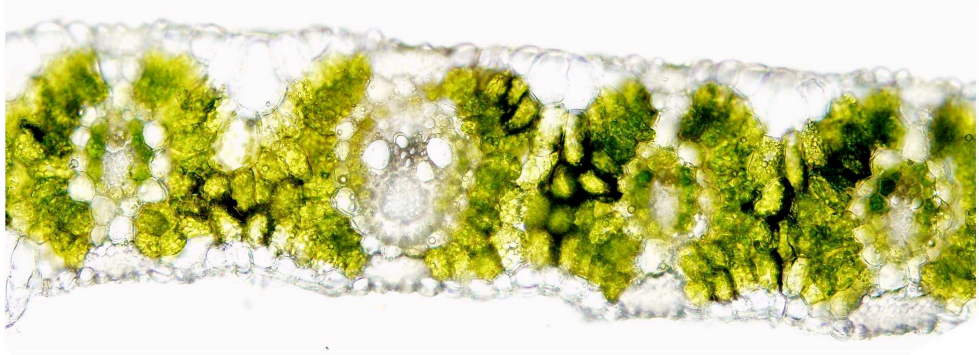
(A)



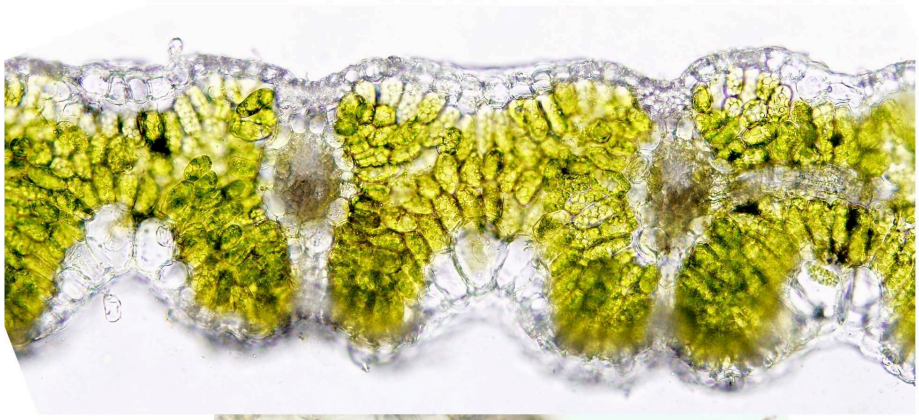
(B)



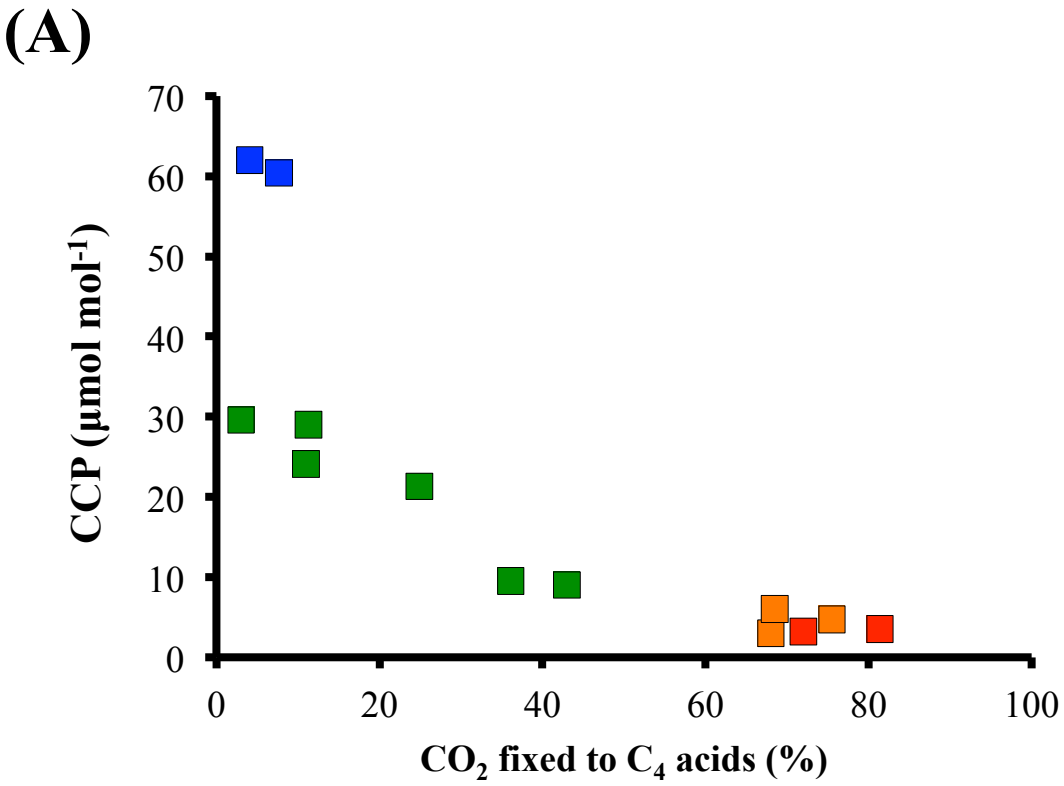
(C)

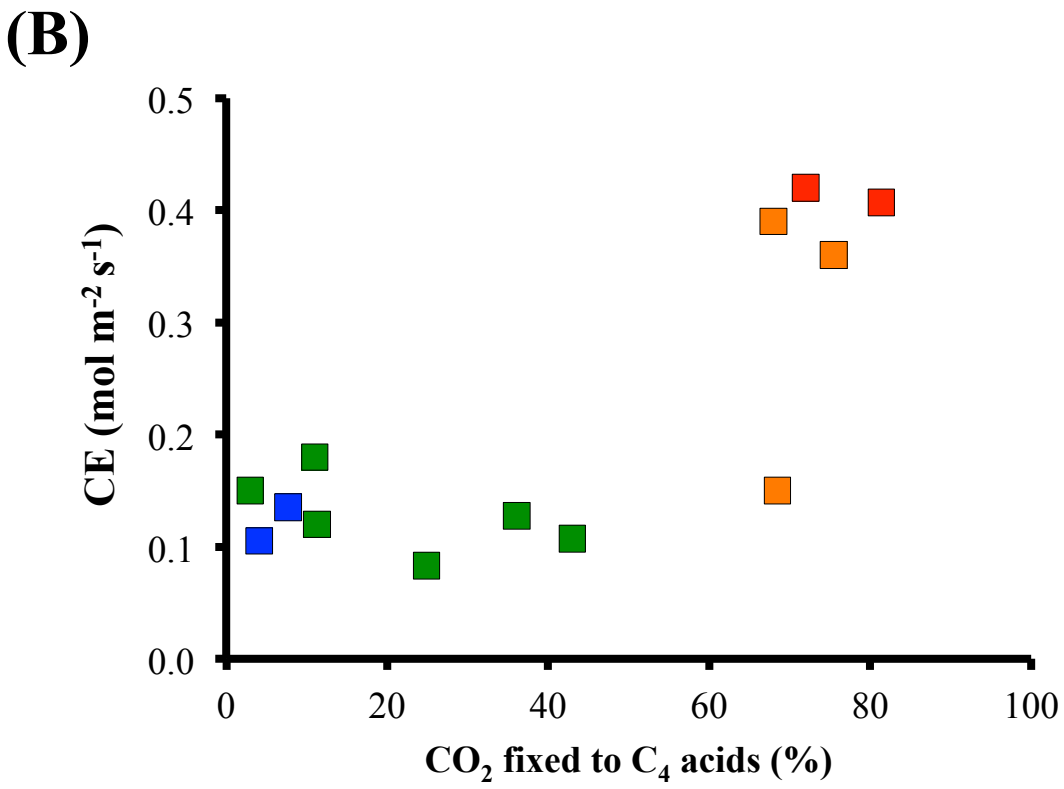


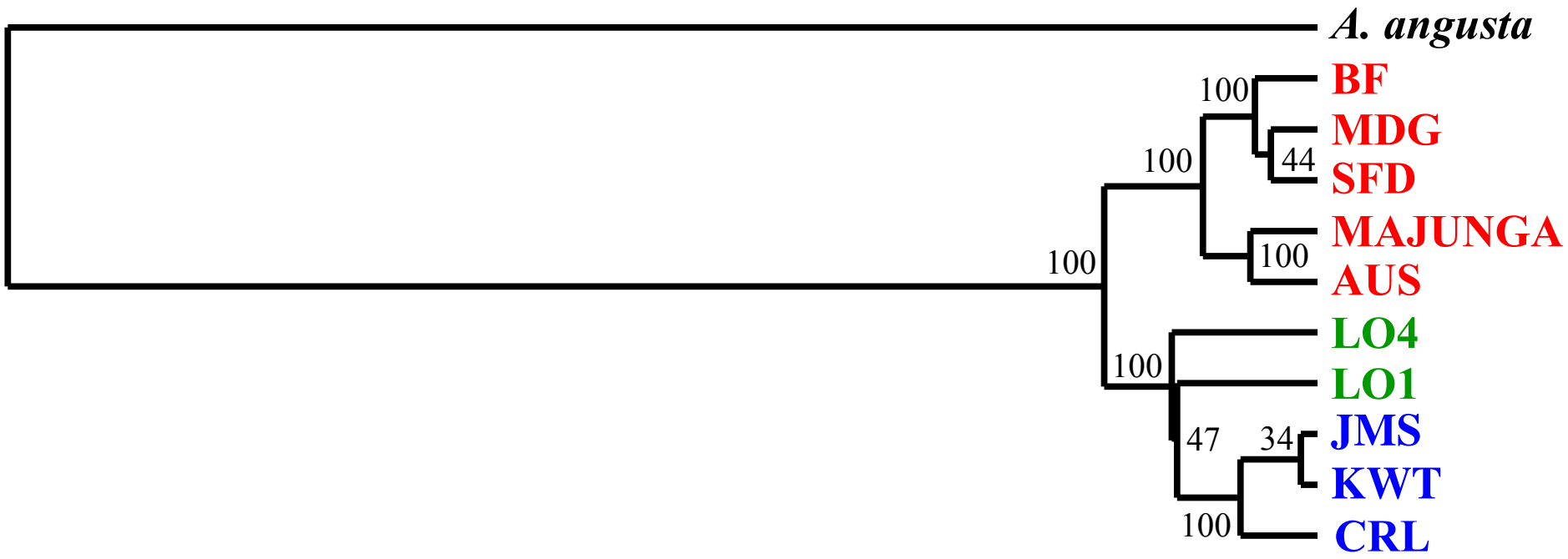
(D)

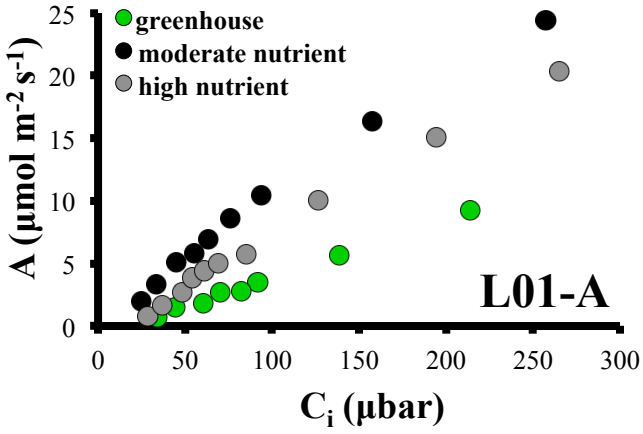


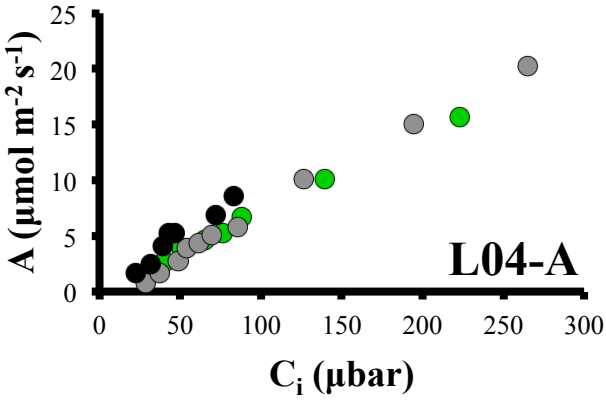
(E)

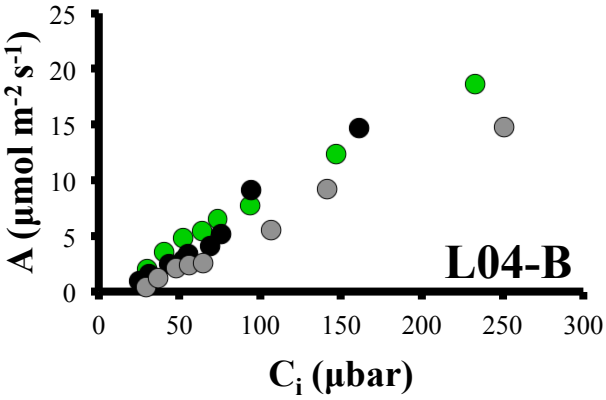

(A)
70
60
50
40
30
20
10
0
CCP (µmol mol-1)
0
20
40
60
80
100
CO2 fixed to C4 acids (%)

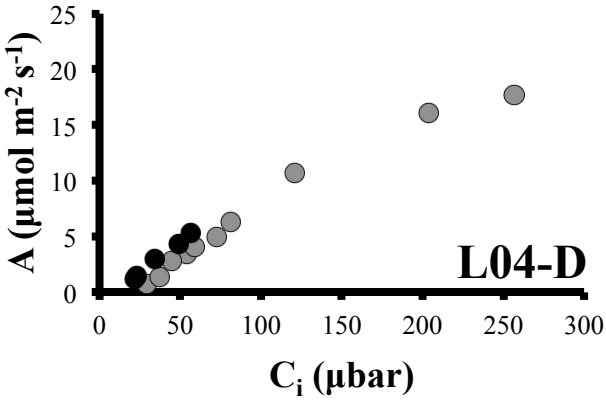

(B)
0.5
0.4
0.3
0.2
0.1
0.0
CE (mol m-2 s-1)
0
20
40
60
80
100
CO2 fixed to C4 acids (%)

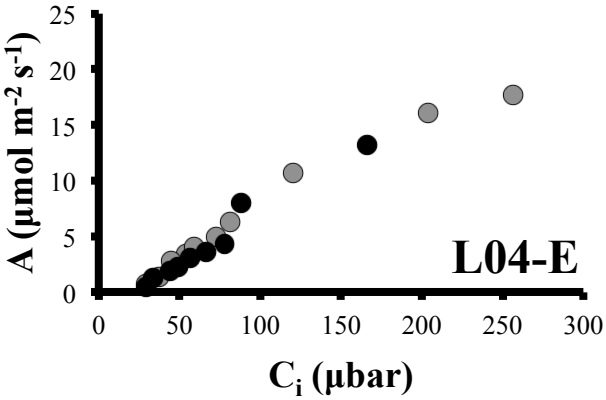

A. angusta
BF
MDG
SFD
MAJUNGA
AUS
LO4
LO1
JMS
KWT
CRL
100
100
44
100
100
100
47
34
100


greenhouse
moderate nutrient
high nutrient
A (µmol m-2 s-1)
Ci (µbar)
L01-A


A (µmol m-2 s-1)
Ci (µbar)
L04-A


A (µmol m-2 s-1)
Ci (µbar)
L04-B


A (µmol m-2 s-1)
Ci (µbar)
L04-D


A (µmol m-2 s-1)
Ci (µbar)
L04-E