



EXPERT VIEW

Why is C₄ photosynthesis so rare in trees?

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Abstract

Since C₄ photosynthesis was first discovered >50 years ago, researchers have sought to understand how this complex trait evolved from the ancestral C₃ photosynthetic machinery on >60 occasions. Despite its repeated emergence across the plant kingdom, C₄ photosynthesis is notably rare in trees, with true C₄ trees only existing in *Euphorbia*. Here we consider aspects of the C₄ trait that could limit but not preclude the evolution of a C₄ tree, including reduced quantum yield, increased energetic demand, reduced adaptive plasticity, evolutionary constraints, and a new theory that the passive symplastic phloem loading mechanism observed in trees, combined with difficulties in maintaining sugar and water transport over a long pathlength, could make C₄ photosynthesis largely incompatible with the tree lifeform. We conclude that the transition to a tree habit within C₄ lineages as well as the emergence of C₄ photosynthesis within pre-existing trees would both face a series of challenges that together explain the global rarity of C₄ photosynthesis in trees. The C₄ trees in *Euphorbia* are therefore exceptional in how they have circumvented every potential barrier to the rare C₄ tree lifeform.

Keywords: C₄ photosynthesis, *Chamaesyce*, disjunct veins, *Euphorbia*, Euphorbiaceae, phloem loading, symplastic, trees, quantum yield.

Introduction

C₄ photosynthesis arises from anatomical and biochemical modifications to the ancestral C₃ photosynthetic machinery that serve to concentrate CO₂ around Rubisco (Box 1). This CO₂-concentrating mechanism (CCM) acts to elevate CO₂ assimilation, while functionally increasing the apparent specificity of Rubisco for CO₂, over O₂, thus minimizing oxygenation and resultant photorespiration (Tcherkez *et al.*, 2006). It follows that the evolution of C₄ photosynthesis is favoured by environmental conditions that would promote high rates of photorespiration in C₃ species, namely low ambient CO₂ concentrations, warmth, bright light, aridity, and salinity (Chollet and Ogren, 1975;

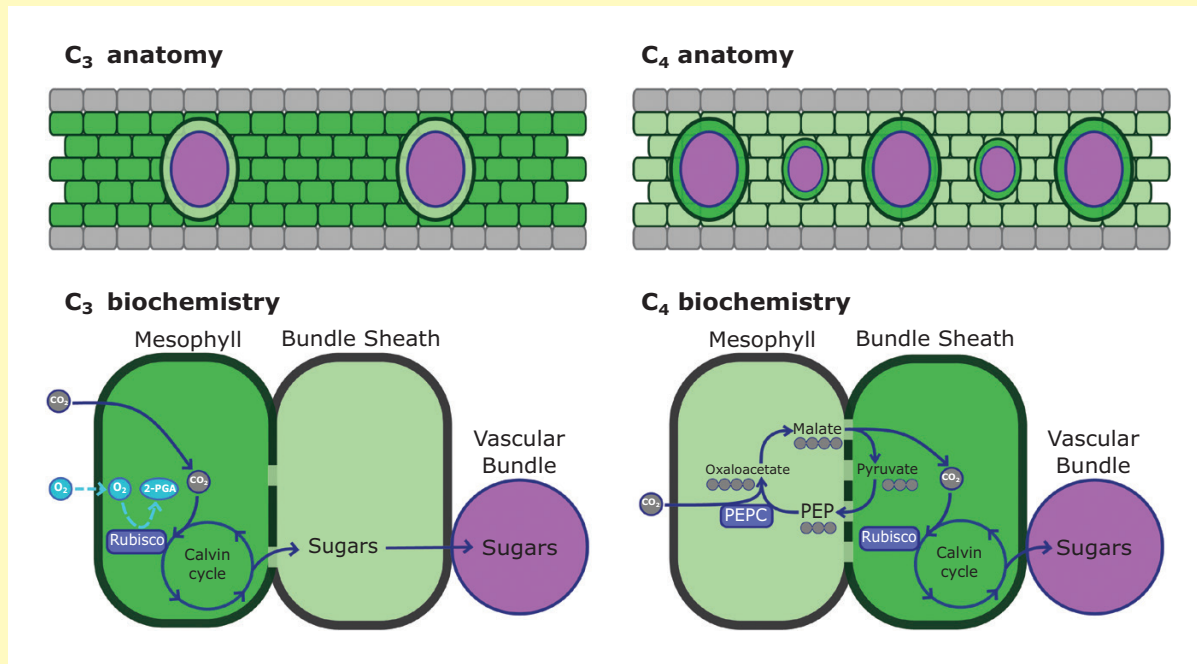
Ehleringer *et al.*, 1991; Sage *et al.*, 2018). Since C₄ photosynthesis was first observed >50 years ago (Kortschak *et al.*, 1965; Hatch and Slack, 1966), numerous studies have attempted to elucidate exactly which modifications are typically required to assemble the components of C₄ physiology (Box 2). To travel the path of C₄ evolution, an ancestral C₃ progenitor arrived in an environment selective for a C₄ benefit (Ehleringer *et al.*, 1997; Sage, 2001a; Sage *et al.*, 2018), and, starting from an initial set of genetic and anatomical pre-adaptations (Monson, 2003; Christin *et al.*, 2013, 2015; Griffiths *et al.*, 2013), evolved developmental and genetic modifications (Stata *et al.*, 2016; Moreno-Villena

Box 1. C₄ photosynthesis arises from both anatomical and biochemical modifications to the ancestral C₃ photosynthetic system

There are several anatomical and biochemical differences that arise during the transition from the C₃ photosynthetic system to the C₄ CO₂-concentrating mechanism (CCM). In C₃ plants, the majority of chloroplasts (and associated Rubisco) are localized to the mesophyll, which is largely exposed to ambient CO₂ and O₂ concentrations. The featureless nature of CO₂ and O₂ makes them enzymatically hard to distinguish, such that Rubisco has catalytic affinity for both molecules, and will catalyse the carboxylation and oxygenation of RuBP. Because the oxygen availability to Rubisco is high in C₃ plants, oxygenation and subsequently photorespiration occur at high rates, especially in high-temperature and low-CO₂ conditions. Some C₃ plants have evolved higher specificity of Rubisco for CO₂ over O₂; however, this comes at the cost of slower catalytic turnover (Tcherkez *et al.*, 2006).

The C₄ CCM allows plants to avoid this specificity–efficiency trade-off by increasing CO₂ concentrations at the Rubisco active site, leading to an apparent increase in the specificity of Rubisco for CO₂. In C₄ plants, CO₂ is biochemically shuttled from the mesophyll into the bundle sheath via the carbonic anhydrase–phosphoenolpyruvate carboxylase (PEPC) system, which builds up CO₂ concentrations around Rubisco within bundle sheath cells, drastically reducing the incidence of oxygenation and increasing net carbon assimilation.

To facilitate the C₄ cycle, C₄ plants have higher bundle sheath to mesophyll area ratios (Hattersley, 1984), often via increased density of vascular bundles (Lundgren *et al.*, 2019). In addition, the connectivity of the mesophyll and bundle sheath cells is enhanced in C₄ plants by an increased density of plasmodesmata at the cell interface, which allows for the increased flux of metabolites that is required for a functional C₄ cycle (Danila *et al.*, 2016).



Grey, leaf epidermis; dark green, cells with many chloroplasts; light green, cells with fewer chloroplasts; purple circles/ovals, vascular tissue; solid purple lines, photosynthesis; dashed light blue lines, oxygenation; dashed dark green line, mesophyll–bundle sheath interface.

et al., 2018; Dunning *et al.*, 2019a; Lundgren *et al.*, 2019), navigated energetic constraints (Bellasio and Lundgren, 2016), and underwent progressive optimization (Rondeau *et al.*, 2005; Christin *et al.*, 2009)—or, in some cases, potentially ‘cheated’ this lengthy final step via horizontal gene transfer (Christin *et al.*, 2012a, b; Dunning *et al.*, 2019b). Given that some version of this path has been repeatedly travelled nearly 70 times by diverse plant lineages spanning a wide range of lifeforms and ecological niches (Sage *et al.*,

2011a), it seems unusual that only a single group of true trees (i.e. defined here as tall, perennial, woody lifeforms with secondary growth) has evolved C₄ leaves (Percy and Troughton, 1975; Table 1). These exceptional trees are members of *Euphorbia*, a global genus of Euphorbiaceae spanning the semideserts of East Africa to the rainforests of the Pacific Islands, and encompassing growth forms from herbs to xerophytic stem succulents to trees of up to 30 m in height (Horn *et al.*, 2012).

Box 2. Key developments in understanding C₄ evolution

There are numerous evolutionary steps on the path from C₃ to C₄ (Stata *et al.*, 2019). To understand this complex evolutionary progression, it is most useful to examine it in study systems that contain individuals across the entire spectrum of photosynthetic types from C₃ to C₄. While there are many fully C₃ and C₄ *Euphorbia* species, there are only two known C₃–C₄ intermediates, and this limits the use of *Euphorbia* as a model for understanding C₄ evolution. However, study of the eudicot genus *Flaveria* has been crucial in understanding the evolutionary transitions from C₃–C₄ to C₄ in particular (Monson and Moore, 1989), while studies of the grass *Alloteropsis semialata* provide unparalleled insight into C₄ evolution owing to its extreme intraspecific photosynthetic diversity, which reduces the confounding effects of long divergence times in phylogenetic analyses and species comparisons (Lundgren *et al.*, 2016; Dunning *et al.*, 2019a). Key developments in these systems have started to pick apart the anatomical and biochemical components required to construct a functional C₄ photosynthetic system. The remarkable convergence of the C₄ trait across the plant kingdom means that the findings from *Flaveria* and *A. semialata* can be applied to other distantly related C₄ lineages, such as *Euphorbia*, despite differences in life history, to understand which stages on the path to C₄ might conflict with the tree habit.

- **High expression of key genes is important in the establishment of a weak C₄ cycle.** Moreno-Villena *et al.* (2018) demonstrated that highly expressed genes in grasses, and also possibly in *Flaveria*, were preferentially co-opted for C₄ photosynthesis regardless of tissue specificity. The importance of high expression levels of C₄ cycle genes was further shown by the observed increases in expression of the key C₄ genes phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) across the C₃ to C₃–C₄ transition (Dunning *et al.*, 2019a). These genes underwent duplication and a resultant dosage-dependent increase concurrently with their co-option for C₄ photosynthesis in *Alloteropsis semialata* (Bianconi *et al.*, 2018).
- **C₄ anatomy can evolve via a single developmental change: an increase in vein density.** Lundgren *et al.* (2019) demonstrated that an increase in vein density driven by proliferation of minor veins is sufficient, given necessary anatomical pre-conditions, to produce a functional C₄ leaf anatomy and create an evolutionary entry to more complex C₄ syndromes in *A. semialata*. Christin *et al.* (2013) present the necessary anatomical pre-conditions (or ‘enablers’) in grasses as a high (>15%) proportion of bundle sheath tissue (combination of a short distance between bundle sheaths and large bundle sheath cells), which, when combined with environmental changes, facilitated the emergence of the C₄ trait.
- **A reduction in chloroplast numbers and increased chloroplast size is associated with changes in C₄ metabolic activity.** Stata *et al.* (2016) showed that across C₃, C₃–C₄, and C₄ species in the genus *Flaveria*, chloroplast number and coverage of the mesophyll cell periphery increase with increased strength of C₄ metabolism, while increased C₄ cycle strength was associated with increased chloroplast size. The reduced chloroplast volume at the mesophyll cell periphery and associated increased cytosolic exposure to the atmosphere could enhance diffusion of CO₂ to PEPC, thus facilitating the incorporation of CO₂ into the C₄ metabolic cycle.
- **Lateral gene transfer is an important force in C₄ evolution, spreading functional genes among grasses.** A total of 59 genes in the genome of *A. semialata* were laterally acquired from other grasses, including those known to be involved in C₄ photosynthesis (Dunning *et al.*, 2019b; Olofsson *et al.*, 2019).

While these anatomical and biochemical modifications that assemble the C₄ trait were selected for by increased fitness in hot, dry environments, they have been retained by the entire Hawaiian *Euphorbia* across multiple transitions from open habitats into the moist forest understorey following the colonization of the Hawaiian Islands, and multiple transitions to the tree habit (Yang *et al.*, 2018). Considering these recent developments in our understanding of C₄ evolution, alongside the ecology and biogeography of the Hawaiian radiation, is key to understanding the overall evolutionary progression of the lineage.

Euphorbia is the only known genus to contain plants using crassulacean acid metabolism (CAM), C₃, C₃–C₄, and C₄ photosynthetic types (Yang and Berry, 2011). The C₄ lineage in *Euphorbia*, the largest single C₄ lineage among the eudicots, is found within subgenus *Chamaesyce*, a subclade of *Euphorbia* that includes C₃ and C₄ species, as well as C₃–C₄ evolutionary intermediates (Sage *et al.*, 1999a, 2011b; Yang and Berry, 2011; Box 3). Subgenus *Chamaesyce* underwent a

radiation on the Hawaiian Islands, resulting in 27 taxa that all use C₄ photosynthesis, as indicated by δ¹³C values spanning –14.5‰ to –12.0‰ (Percy and Troughton, 1975; Sporck, 2011). The Hawaiian species in *Chamaesyce* (hereafter referred to as Hawaiian *Euphorbia*, still recognizing that a separate colonization event led to the origin of a C₃ Hawaiian tree not in *Chamaesyce*, *Euphorbia haeleleana*) include a variety of growth forms, from sub-shrub to tree, which exist across a diverse

Table 1. Where are C₄ trees found?

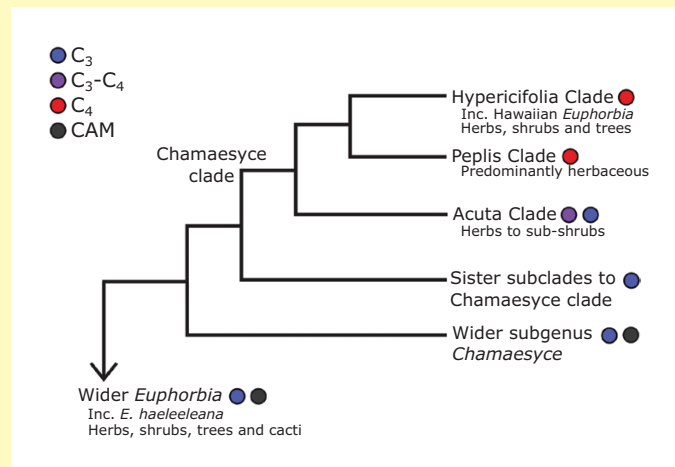
Species	Varieties	Form	Geography	Environment	Reference
Euphorbiaceae					
<i>Euphorbia olowaluana</i>		Tree (up to 9 m)	Open and subalpine forest	Dry	a, b, d, e, f
<i>E. herbstii</i> *		Tree (3–8 m)	Forest	Mesic to wet	a, c, d, e, f
<i>E. remyi</i>	<i>kauaiensis</i>	Small tree (2–3 m)	Forest ^{b,d,e}	Wet ^{b,d,e}	a, b, d, e, f
<i>E. rockii</i>	<i>rockii</i> ^{b,e} <i>grandifolia</i> ^{b,e}	Shrub to tree (1–8 m)	Open ridge to forest	Mesic to wet	a, b, d, e, f
<i>E. celastroides</i>	<i>lorifolia</i>	Shrub to tree (1–9 m)	Open forest	Dry	a, b, d, e, f
<i>E. atrococca</i>		Shrub to small tree (up to 3 m)	Forest	Dry to mesic	a, b, d, e, f
Chenopodiaceae (tribe Salsoleae <i>sensu stricto</i>)					
<i>Haloxylon persicum</i> **		Large shrub to tree (up to 8 m)	Desert	Dry	g, h, i
<i>H. ammodendron</i> **		Large shrub to tree (up to 8 m)	Desert	Dry	g, h, i

^aPearcy and Troughton, 1975; ^bKoutnik, 1987; ^cRobichaux and Pearcy, 1980; ^dSporck, 2011 (p70); ^eYang *et al.*, 2018; ^fYang, 2012; ^gSage, 2001a, b; ^hSage, 2016; ⁱPyankov *et al.*, 1999.

*formerly *E. forbesii*; **these species have C₄ photosynthetic stems, C₃ leaf-like cotyledons, and no true leaves, and become arborescent with age.

Box 3. Phylogenetic relationships in *Euphorbia* and closest relatives of Hawaiian taxa

C₄ photosynthesis is present in all species of the core *Chamaesyce*, which includes both the Hypericifolia and Peplis clades. A phylogeny of the *Chamaesyce* clade (*Euphorbia* subg. *Chamaesyce* sect. *Anisophyllum*) identifies four close relatives and possible progenitors of Hawaiian *Euphorbia*: *Euphorbia stictospora*, *E. velleriflora*, *E. mendezii*, and *E. leucantha* (Yang and Berry, 2011). These species are members of the Hypericifolia clade and are herbaceous annuals commonly found in the southern USA, northern Mexico, and/or the Caribbean. *Euphorbia haeleeleana*, a woody C₃ species that is part of *Euphorbia* subg. *Euphorbia*, represents a separate colonization of the Hawaiian Islands. The closest relatives of this taxa are the Australian succulents *E. plumerioides* and *E. sarcostemmoides*, also from subg. *Euphorbia* (Zimmermann *et al.*, 2010).



range of environments, from bright and arid habitats to mesic and wet forest understoreys (Table 1). Though there are some relatively shade-tolerant C₄ monocots (e.g. Lundgren *et al.*, 2015), shade-tolerant C₄ eudicots are rare (Sage *et al.*, 1999b) and thus the ubiquity of C₄ photosynthesis across the diverse habitats of Hawaiian *Euphorbia* is surprising (Pearcy and Troughton, 1975; Sage, 2016). Given that the entire Hawaiian radiation uses C₄ photosynthesis, it is likely that the progenitor for this radiation was also a C₄ species that arrived on the Hawaiian Islands. This idea is supported by recent phylogenetic work that suggests that the closest relatives of

Hawaiian *Euphorbia* are in fact C₄ herbs from the southern USA, Mexico, and/or the Caribbean, and that the woody state evolved after arrival on the Hawaiian Islands (Yang and Berry, 2011; Yang *et al.*, 2018; Box 3).

There has been periodic interest in Hawaiian *Euphorbia*, and the wider rarity of C₄ trees (e.g. Pearcy and Troughton, 1975; Ehleringer, 1978; Pearcy, 1983; Sporck, 2011; Sage, 2014; Sage and Sultmanis, 2016; Yang *et al.*, 2018); however, there has been little definitive progress towards understanding why C₄ trees are indeed so rare. Given that C₄ trees do exist, there cannot be any fundamental incompatibility between C₄ photosynthesis and the

tree habit, or any physiological explanation for the reduced competitive ability of a C₄ tree versus a C₃ tree that is true under all conditions. Therefore, in explaining the rarity of C₄ trees, all the factors that could limit, but not preclude, the evolution of the two syndromes in the same species must be considered, whether that evolution is via adaptation of a C₄ progenitor to an environment inhabited by trees, as is likely to be the case for Hawaiian *Euphorbia*, or via an existing tree traversing the adaptive landscape from C₃ to C₃-C₄ to C₄ (Yang and Berry, 2011). This review will consider the key steps on the path to C₄ photosynthesis, where these steps might conflict with the tree lifeform, and argue that Hawaiian *Euphorbia* present a unique opportunity to study the evolution of the C₄ trait in trees as a target for future research.

C₄ trees may perform poorly under a closed canopy

Rates of photorespiration increase in warm environments, making the C₄ pathway—which largely avoids photorespiration—superior to C₃ photosynthesis in plants with similar lifeforms (Ehleringer, 1978). As such, C₄ grasses frequently dominate in areas with warm climates where trees cannot grow, for example due to high levels of disturbance, while C₃ forests—and thus canopies—establish in warm areas where conditions are such that trees thrive. The theory that follows is that C₄ trees have failed to become widespread forest species due to their poor performance under canopies, where conditions are cool, shady, and often enriched in CO₂ (Sage *et al.*, 1999a). However, this theory may not hold true given insights into the physiological performance of *Euphorbia* species in the Hawaiian forest understorey (Robichaux and Pearcy, 1980; Pearcy *et al.*, 1985) and, more recently, C₄ photosynthesis under low light canopy conditions (Bellasio and Griffiths, 2014; Sage, 2014).

Reduced quantum yield as a limitation

Ehleringer (1978) proposed an early hypothesis that the quantum yield of photosynthesis, defined as the rate of photosynthesis relative to that of photon absorption, is important in determining the distribution of C₄ species, especially grasses. Maximum quantum yield is inherently lower in C₄ plants than in C₃ plants due to the greater energy requirements of the C₄ system, though the quantum yield of C₃ plants declines with increasing temperature while that of C₄ plants remains constant (Long, 1999; Monson, 1999). If below-canopy temperatures are sufficiently cool that the additional energy requirement of the C₄ system is greater than the light energy lost to photorespiration in an energetically inexpensive C₃ plant, then the quantum yield of the C₃ plant would be greater than that of a theoretical C₄ tree, and that tree would probably be outcompeted. Indeed, for *Atriplex* species native to grassland, desert, and coastal strand habitats, the temperature at which the quantum yields of C₃ and C₄ species are equal is 30 °C, at least at atmospheric CO₂ (at that time 325 ppm) and O₂ concentrations (Ehleringer and Björkman, 1977). Quantum yield also varies with CO₂ concentration, life history, and C₄ biochemical subtype. The lower quantum yield of eudicots compared

with monocots may contribute to the relative scarcity of shade-tolerant C₄ eudicot herbs compared with forest-shade grasses (Monson, 1999). Similarly, the higher quantum yields of plants using the NADP-ME biochemical subtype of C₄ photosynthesis (such as Hawaiian *Euphorbia*) compared with those using the NAD-ME subtype (Ehleringer and Pearcy, 1983; Pearcy and Ehleringer, 1984) may partially explain the shade tolerance of the understorey C₄ tree *Euphorbia herbstii* (formerly *E. forbesii*), whose quantum yield equals that of an equivalent C₃ tree at a leaf temperature of 22–23 °C (approximately the same value as the mean midday leaf temperature at the site where these plants were collected) (Robichaux and Pearcy, 1980).

Direct comparison between the quantum yields of C₃ and C₄ species, however, does not adequately address the question of whether or not quantum yield could limit the evolution of a C₄ tree: the quantum yields of intermediate species on the path from C₃ to C₄ must be considered. In the incipient C₃-C₄ phases of C₄ evolution in *Flaveria*, the poor integration of the C₃ and C₄ cycles causes futile cycling in the C₄ assimilation of CO₂ and thus reduced quantum yields (Monson *et al.*, 1986; Stata *et al.*, 2019). Inefficient transfer of CO₂ from the C₄ to the C₃ cycle may create an ‘adaptive trough’, expressed through reduced quantum yields in C₃-C₄ species, compared with fully coupled C₃ or C₄ taxa, which could act as a barrier to the evolution of C₃-C₄ traits in species native to shady habitats (Monson, 1989). Thus, the limitations of the C₃-C₄ intermediate state could make it difficult for the C₄ pathway to evolve in a tree under a forest canopy. However, this limitation does not apply where the transition to the forest understorey occurred subsequently to the evolution of the full C₄ trait, as was likely to have been the case for Hawaiian *Euphorbia* (Yang and Berry, 2011).

Poor ability to utilize sunflecks as a limitation

Sage *et al.* (1999a) proposed that C₄ plants may be maladapted to shady understorey environments due to their inefficient utilization of sunflecks, which represent the primary source of light available under the canopy. However, sunfleck use does not seem to be a limiting factor in *Euphorbia*, as the C₄ tree *E. herbstii* is as efficient in utilizing sunflecks as a comparative C₃ tree (Pearcy *et al.*, 1985). Similarly, study of the C₄ grass maize shows that, while it responds more slowly to short sunflecks, it otherwise has a similar sunfleck use efficiency to C₃ crop species (Krall and Pearcy, 1993), suggesting that C₄ plants may not be inherently limited by poor sunfleck exploitation.

Inability to meet the increased energy demands of C₄ in the shade

Under low light, C₄ plants suffer increased ‘bundle sheath leakiness’—the rate of diffusion of CO₂ out of the bundle sheath relative to that of phosphoenolpyruvate (PEP) carboxylation (Kromdijk *et al.*, 2008). This is driven by a slow down in the C₄ carboxylation process due to a reduction in ATP availability under low light. Thus, in the low-light forest interior, the higher leakiness would limit carbon gain and lead to poorer performance of a theoretical C₄ tree. However, maize plants grown under diffuse light can acclimate and thereby reduce

leakiness, possibly by allocating proportionally more energy to C₃ cycle activity to reduce CO₂ overcycling, optimizing scarce ATP resources, and then trapping a greater proportion of CO₂ in the bundle sheath (Bellasio and Griffiths, 2014).

Disjunct veins, which shade-tolerant Hawaiian *Euphorbia* species possess (Herbst, 1971), may be another mechanism adapted to tolerate shade. This modification to leaf anatomy allows these species to have a low density of functional veins (i.e. those that are connected to the vascular network) to reduce leaf costs, as is typical of shade species (Sporck, 2011), while establishing islands of bundle sheath tissue to increase the relative bundle sheath tissue area and maintain the close proximity of mesophyll and bundle sheath cells required for a functional C₄ system (Box 1). Therefore, it seems that optimization of C₄ physiology in combination with modifications to leaf anatomy should allow C₄ plants to thrive under the canopy, including C₄ trees such as *E. herbstii* and *E. rockii* (Table 1). However, not all C₄ lineages may be equally primed to adopt these modifications.

Evolutionary factors shaped the pathway to C₄ trees

There are several key historical factors and evolutionary ‘opportunities’ that have played a role in shaping the evolution of the C₄ trait, and subsequently the tree habit, in Hawaiian *Euphorbia*.

Evolution of the C₄ trait in *Euphorbia*

First, it is important to consider the age of the eudicot C₄ lineages and the timing of C₄ evolution relative to historical climatic changes. C₄ eudicots are not overall younger than C₄ monocots, and all lineages of C₄ plants evolved in the low CO₂ atmosphere that has shaped plant evolution over the last 30 million years (Christin *et al.*, 2011). This low CO₂ atmosphere was probably a key evolutionary opportunity for *Euphorbia* and is associated with the evolution of at least 17 independent CCMs, which are mostly CAM but also include the C₄ lineage (subsect. Hypericifoliae) of *Euphorbia* subg. *Chamaesyce* (Horn *et al.*, 2014; Sage, 2016).

Secondly, relatively short generation times in *Euphorbia*, owing to rapid flowering and high levels of reproductive output per plant, favour the comparatively fast evolution of traits, probably including those associated with C₄ photosynthesis, as well as the rapid accumulation of duplicated and neofunctionalized genes as a resource for C₄ evolution (Monson, 2003; Emms *et al.*, 2016; Bianconi *et al.*, 2018; Box 2). However, this rapid generation time probably offers less potential evolutionary benefit than lateral gene transfer, which is increasingly recognized as an important force in shaping C₄ evolution in the monocots, but has not been documented in C₄ eudicots (Christin *et al.*, 2012a, b; Dunning *et al.*, 2019b; Olofsson *et al.*, 2019; Box 2).

Thirdly, lifeform may have also played a role in the evolutionary potential of these plants. The ancestor of the C₄ lineage in *Euphorbia* was likely to have been herbaceous, while all

16–21 independent origins of CAM in *Euphorbia* occurred in woody ancestors (Horn *et al.*, 2014). While this is only a single example, it suggests that the woody ancestor of *Euphorbia* may have needed to undergo a transition to the herbaceous lifeform prior to the evolution to C₄ photosynthesis, and perhaps the evolution of C₄ is less favourable than that of CAM in a woody species. Furthermore, the herbaceous lifeform of the ancestor of Hawaiian *Euphorbia* facilitated its dispersal to the Hawaiian Islands (Yang and Berry, 2011). Indeed, much of the Hawaiian woody flora evolved from herbaceous ancestors, which had greater dispersal ability to reach the remote Hawaiian Islands (Carlquist, 1970; Panero *et al.*, 1999; Eggens *et al.*, 2007; Dunbar-Co *et al.*, 2008). Before European contact and the influx of invasive species, Hawaii had an environment with available niches along with topographic heterogeneity providing barriers to gene flow (Givnish *et al.*, 2009). Thus, C₄ trees or their progenitors (none of which are able to dominate the forest canopy) did not need to invade established C₃ communities or areas of high disturbance that typically occlude the tree lifeform, so providing an evolutionary opportunity for a C₄ ancestor to transition to the tree habit.

Finally, *Euphorbia* have a remarkably high degree of variation in morphological characteristics, level of adaptive plasticity, and species richness compared with other plant lineages of similar age (Horn *et al.*, 2012). In particular, there are several characteristics of the ancestral *Euphorbia* that may be synergistically associated with the origin of the C₄ lineage, and thus may have facilitated C₄ evolution. The combination of plagiotropic branches and a distichous leaf arrangement maximize the leaf area exposed to sunlight. The co-evolution of C₄ photosynthesis with these growth traits would maximize photosynthetic rates and minimize photorespiration rates in high-light, high-temperature environments. In addition, the high adaptive plasticity of *Euphorbia* may have facilitated the evolution of further adaptations in progenitors of the C₄ trees, for example the development of shade-tolerant leaves, circumventing constraints that the C₄ state places on phenotypic plasticity (Herbst, 1971; Robichaux and Percy, 1980; Sage and McKown, 2006; Horn *et al.*, 2012; Lundgren *et al.*, 2014). It is also worth noting that adaptive plasticity in Hawaiian *Euphorbia* specifically may have been furthered by their allopolyploid origin resulting in increased heterozygosity (Yang and Berry, 2011).

Evolution of the tree habit in C₄ *Euphorbia*

There are many factors that drive, and constrain, the evolution of the tree habit. These include, but are not limited to, protection from animal herbivory, improved dispersal, avoiding (self-) shading, and maintaining water balance. It is unclear what has driven the evolution of trees in *Chamaesyce*, as Hawaiian *Euphorbia* tree taxa do not appear to perform better than closely related shrub taxa by measure of abundance.

In terms of evolutionary constraints, in order to diversify to a forest understorey niche, such as that of *E. herbstii*, a C₄ tree must acquire some shade tolerance. However, shade tolerance does not universally constrain the evolution of the tree habit: C₄ shrubs can displace grasses in high-light scrubland, as is observed for the C₄ shrubs *Atriplex confertifolia* and *A. canescens* (Sage and

Sultmanis, 2016), and the C₄ tree *Euphorbia olowaluana* is a pioneer species on newly formed Hawaiian lava fields, occurring sparsely in high-light conditions (see fig. 1H in Yang and Berry, 2011). Therefore, the ability to develop shade-tolerant leaves alone does not dictate whether or not a C₄ plant can evolve the tree habit, and there may be other factors acting to constrain the evolution of trees in existing C₄ lineages, such as the age of these lineages (Sage and Sultmanis, 2016).

The evolution of the tree habit in a herbaceous C₄ ancestor requires sufficient evolutionary time following the appearance of the C₄ trait. C₄ trees, and C₄ shrubs that become arborescent with age, are found in two of the oldest C₄ eudicot lineages: *Euphorbia* (19.3 Mya) and tribe Salsoleae *sensu stricto* (Chenopodiaceae, 23.4 Mya), respectively (Table 1; Sage and Sultmanis, 2016). However, in the case of Hawaiian *Euphorbia*, 19.3 My is much longer than the time required for the transition from herbaceous to tree lifeform: the initial colonization event of the Hawaiian Islands was ~5 Mya and the true tree species themselves are ~1 My old (Yang *et al.*, 2018). Therefore, it may be more accurate to say that it is not evolutionary time, but the rate of evolution that can act to constrain the transition to a tree lifeform in a C₄ lineage. Many eudicot families that have C₄ lineages also have trees, but it may be that the C₄ state limits the rate at which the tree lifeform can be acquired within C₄ lineages by reducing adaptive plasticity (Sage and McKown, 2006; Bellasio and Lundgren, 2016). The aforementioned high adaptive plasticity of Hawaiian *Euphorbia*, or the fact that the lineage had woody ancestors that had previously undergone a transition to the herbaceous state, may have favoured a comparatively rapid evolution of the tree lifeform in this C₄ lineage (Horn *et al.*, 2014). Interestingly, the ancestor of the C₄ lineage in Salsoleae may have been a shrub or sub-shrub (Schüssler *et al.*, 2017), so while the transition to true tree has not been completed and thus is slower than that in *Euphorbia*, they may have been advantaged in this transition by acquiring C₄ photosynthesis in an already woody or semi-woody ancestor.

Passive symplastic phloem loading and/or hydraulic limitation negate the benefits of C₄ anatomy in trees

Tree height, and indeed the tree growth form, is limited by difficulties in sustaining water and sugar transport over the long pathlength (Ryan and Asao, 2014; Liesche *et al.*, 2017; Savage *et al.*, 2017). We propose that three elements of sugar and water transport design can contribute to limitations on the evolution of C₄ trees.

First, trees tend to exhibit high numbers of plasmodesmatal connections between mesophyll cells and minor vein cells that are devoted to phloem loading, a phenotype that is frequently associated with a passive symplastic loading mechanism (Davidson *et al.*, 2011). The persistently strong sugar sinks of trees, which have meristems and storage sites in their trunks and roots, and also high rates of photosynthesis and thus photosynthetic export from leaves, may actually select for passive symplastic phloem loading as it is less energetically demanding than active mechanisms (Turgeon, 2010). C₄ species, on the

other hand, have a high density of plasmodesmatal contacts between mesophyll and bundle sheath cells to allow for the flux of intermediate metabolites (Sowiński *et al.*, 2008; Danila *et al.*, 2016). In a C₄ passive phloem loader, the export of photosynthate would require a large number of plasmodesmatal contacts between bundle sheath and phloem cells. However, this would form a complete plasmodesmatal route from mesophyll to phloem, with C₄ intermediates moving from mesophyll to bundle sheath (and back), while sugar loading proceeds from the bundle sheath into the phloem. Due to the passive nature of this process and lack of compartmentalization, it would be difficult to regulate the flux of C₄ metabolites; that is, prevent leakage of C₄ intermediates into the phloem. Avoiding leakage of C₄ sugars may place a limit on phloem loading via plasmodesmata and thus on passive loading.

Secondly, the combination of C₄ intermediate diffusion with direct plasmodesmatal pathways for sugar transport from the mesophyll to the bundle sheath and into the phloem might be very difficult to sustain, given that sugar movement would be against the transpiration stream. Theoretical analysis has shown that transpiration-induced bulk flow from veins to stomata and passive sugar loading into the phloem by diffusion can co-exist (Rockwell *et al.*, 2018), but these analyses assumed very low plasmodesmatal fluxes between the bundle sheath and mesophyll, as is typical of C₃ species (i.e. without the extensive plasmodesmatal contact typical of C₄ species). More work is needed to determine if transpirational counterflow might present an obstacle to a C₄ passive loader.

Thirdly, taller trees tend to have upper canopy leaves with both lower leaf water potentials and a greater heterogeneity in cell water potentials, due to the tension associated with gravity, greater resistance pathlengths, and exposure of canopies to strong fluctuations in light and temperature (Zwieniecki *et al.*, 2004; Burgess and Dawson, 2007). Plasmodesmata may lose transport capacity when strong pressure differences are generated between adjacent cells or tissues (Oparka and Prior, 1992). Thus, the C₄ pathway, depending on plasmodesmatal transport, may not be feasible at very negative leaf water potentials and/or given the large heterogeneity of water status within the leaf, and so its evolution may be precluded in trees, particularly tall canopy-forming species unlike Hawaiian *Euphorbia* (Table 1).

Even if these three limitations could be overcome, the increased photosynthetic efficiency of the C₄ system combined with the reduced ratio of source tissue to phloem requires an increase in phloem loading efficiency to avoid accumulation of photosynthate in the leaves. In C₄ grasses, this is achieved by two mechanisms: first, by up-regulation of active transporters for bundle sheath sugar export, such as maize SWEET13, a transporter that was duplicated and retained during C₄ evolution (Emms *et al.*, 2016); and secondly, by increasing plasmodesmatal density at the interface of the bundle sheath and the vascular parenchyma to increase passive photosynthate transport in plants grown under high-light conditions (Sowiński *et al.*, 2007). Such adjustments would probably not be possible or effective in a C₄ tree, in the first instance owing to the absence of an active loading mechanism, and in the second instance due to the aforementioned limitations on plasmodesmatal transport. Inability to increase the maximum

rate of phloem loading and associated accumulation of leaf non-structural carbohydrates would result in a downward adjustment of photosynthetic capacity that could partially or fully negate the potential benefits of the C₄ system in a tree (Paul and Foyer, 2001; Paul and Pellny, 2003).

How the trees of Hawaiian *Euphorbia* have circumvented these limitations is unknown, and little is known of their phloem loading mechanism. Notably, the anatomical diversity of Hawaiian *Euphorbia* is exceptional, from their striking disjunct veins, which vary strongly across species, to their variation in growth form, leaf surfaces, and leaf cross-sectional anatomy (Herbst, 1971; Koutnik, 1987; Horn *et al.*, 2012). More work is needed to discover how phloem loading relates to this diversity, and if a specialized anatomy evolved that mitigates limitations on C₄ tree evolution dictated by phloem processes.

Conclusions

Previous commentary on the rarity of C₄ trees has pointed towards hypotheses of physiology and life history, and found that there is no single explanation that is satisfactory, with Hawaiian *Euphorbia* (and possibly *Salsola* *sensu stricto*, Table 1) acting as exceptions to every argument. Each hypothesis seems to have a caveat, whereby if a species is exceptionally shade tolerant, is markedly efficient at sunfleck use, utilizes a particular C₄ biochemical subtype, is notably morphologically diverse, evolved in an especially low competition environment, or has circumvented difficulties in phloem loading, then it may be the exception to the rule. Only by examining all of the interconnected aspects of a complex trait such as C₄ photosynthesis can we begin to understand why a few unique trees have travelled the pathway to C₄, whereas other would-be C₄ trees cannot complete the journey. New directions into understanding the rarity of C₄ trees also merit investigation, including comparative genomic approaches and investigations into any role that the characteristic latex and laticifers of *Euphorbia* plants may play in overcoming limitations imposed by passive symplastic phloem loading and hydraulic constraints. These Hawaiian *Euphorbia* represent a crucial resource in advancing our understanding in these areas. However, they are becoming increasingly threatened in their native habitat: 10 taxa are state and federally listed as endangered and several others are observed to be rare (MJS-K, unpublished data). The narrow endemism of these species and lack of appropriate, protected habitats for conservation mean they are vulnerable to fires, invasive species, human activity, and climate change.

With Hawaiian *Euphorbia* most probably arising from a herbaceous-to-woody transition in a C₄ ancestor (Yang and Berry, 2011), it is still unclear whether an existing tree could evolve the C₄ trait, and there are currently no known C₃–C₄ intermediate tree species to indicate that this is a possibility. Indeed, the low quantum yield of these C₃–C₄ intermediates could mean that, at least under an existing canopy, the transition to C₄ would require the traversal of an adaptive trough (Monson *et al.*, 1986; Stata *et al.*, 2019). Additionally, the occurrence of passive symplastic phloem loading could also act as a barrier to C₄ evolution in existing trees. While these considerations may not apply to the evolution of a tree habit in a C₄ ancestor, this alternative evolutionary path is still constrained

by adaptive plasticity (Sage and McKown, 2006; Bellasio and Lundgren, 2016). Thus, both routes to the evolution of a C₄ tree are potentially tortuous, which may together explain the global rarity of C₄ tree species.

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