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## The Agavoideae: an emergent model clade for CAM evolutionary biology

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Crassulacean acid metabolism – or CAM photosynthesis – was described in the early to mid-20th century, and our understanding of this metabolic pathway was later expanded upon through detailed biochemical analyses of carbon balance. Soon after, scientists began to study the ecophysiological implications of CAM, and a large part of this early work was conducted in the genus *Agave*, in the subfamily Agavoideae of the family Asparagaceae. Today, the Agavoideae continues to be important for the study of CAM photosynthesis, from the ecophysiology of CAM species, to the evolution of the CAM phenotype and to the genomics underlying CAM traits. Here we review past and current work on CAM in the Agavoideae, in particular highlighting the work of Park Nobel in *Agave*, and focusing on the powerful comparative system the Agavoideae has become for studying the origins of CAM. We also highlight new genomics research and the potential for studying intraspecific variation within species of the Agavoideae, particularly species in the genus *Yucca*. The Agavoideae has served as an important model clade for CAM research for decades, and undoubtedly will continue to help push our understanding of CAM biology and evolution in the future.

**Key words:** Agavoideae, Asparagaceae, Crassulacean acid metabolism, photosynthesis, evolution.

### INTRODUCTION

Photosynthesis is a complex trait, one that is highly interconnected to other aspects of a plant's biology and thus is often thought to be relatively invariant – it is, after all, required for most plants to survive. However, plants have adapted to their environments through remarkable alterations to their photosynthetic pathways. One of the largest limitations for plant photosynthesis is the amount of CO<sub>2</sub> in the atmosphere, which is at relatively low concentration compared to other gasses, such as O<sub>2</sub>. The main enzyme in the C<sub>3</sub> photosynthetic pathway, Rubisco, can interact with both CO<sub>2</sub> and O<sub>2</sub>, and under certain environmental conditions, will preferentially fix O<sub>2</sub>. O<sub>2</sub> fixation in plants leads to a costly process called photorespiration, and results in no net carbon gain. Two major pathways – C<sub>4</sub> and Crassulacean acid metabolism (CAM) – have evolved as carbon-concentrating mechanisms; both C<sub>4</sub> and CAM increase the amount of CO<sub>2</sub> around Rubisco to minimize photorespiratory stress. CAM has evolved numerous times independently across diverse land plant lineages, yet *how* CAM plants evolved from a C<sub>3</sub> ancestor remains unclear. Several studies have compared C<sub>3</sub> and CAM species to understand physiological and genetic differences, but many of these studies relied on distantly related model species, and thus conflated changes due to evolutionary distance with those related to the evolution of CAM photosynthesis (Heyduk *et al.*, 2019b).

The Agavoideae, a subfamily of the Asparagaceae, is a promising clade for fine-tuning our understanding of the evolution of CAM. Species within the subfamily range from C<sub>3</sub> to CAM and intermediates between, allowing for comparisons between

closely related species across a diversity of CAM phenotypes. Historically, the Agavoideae has been important for research on the ecophysiology of CAM plants. Today, research within the subfamily has generated new evolutionary and genomic insights into the origins of CAM, and the Agavoideae is uniquely poised to provide a powerful, integrative framework for evolutionary studies on CAM photosynthesis.

### THE AGAVOIDEAE

Some of the most iconic species of the deserts of North America are members of the Agavoideae (Asparagaceae), including agaves and yuccas. In contrast to these familiar desert species, there are a number of species in the subfamily that inhabit more mesic habitats, including *Hosta* and *Camassia* (Fig. 1). Members of the Agavoideae, once treated as a separate family, are in the family Asparagaceae, and are probably sister to the Scilloideae (Chase *et al.*, 2009; The Angiosperm Phylogeny Group, 2009; Chen *et al.*, 2013). The Agavoideae includes genera with centres of diversity in Africa (*Anthericum*, *Behnia*, *Chlorophytum*, *Herreriopsis*), Asia (*Anemarrhena*, *Hosta*), Europe (*Paradisea*), South America (*Herreria*) and North America (*Echeandia*, *Agave sensu lato*, *Beschorneria*, *Furcraea*, *Hesperoyucca*, *Hesperaloe*, *Chlorogalum*, *Camassia*, *Hastingsia*, *Leucocrinum*, *Schoenolirion* and *Yucca*). Apart from *Echeandia*, all North and Central American genera fall within the so-called Agavoideae Bimodal Karyotype (ABK) clade along with *Hosta* (McKain *et al.*, 2012). The origin of the ABK clade is associated with a polyploidy event ~28 million



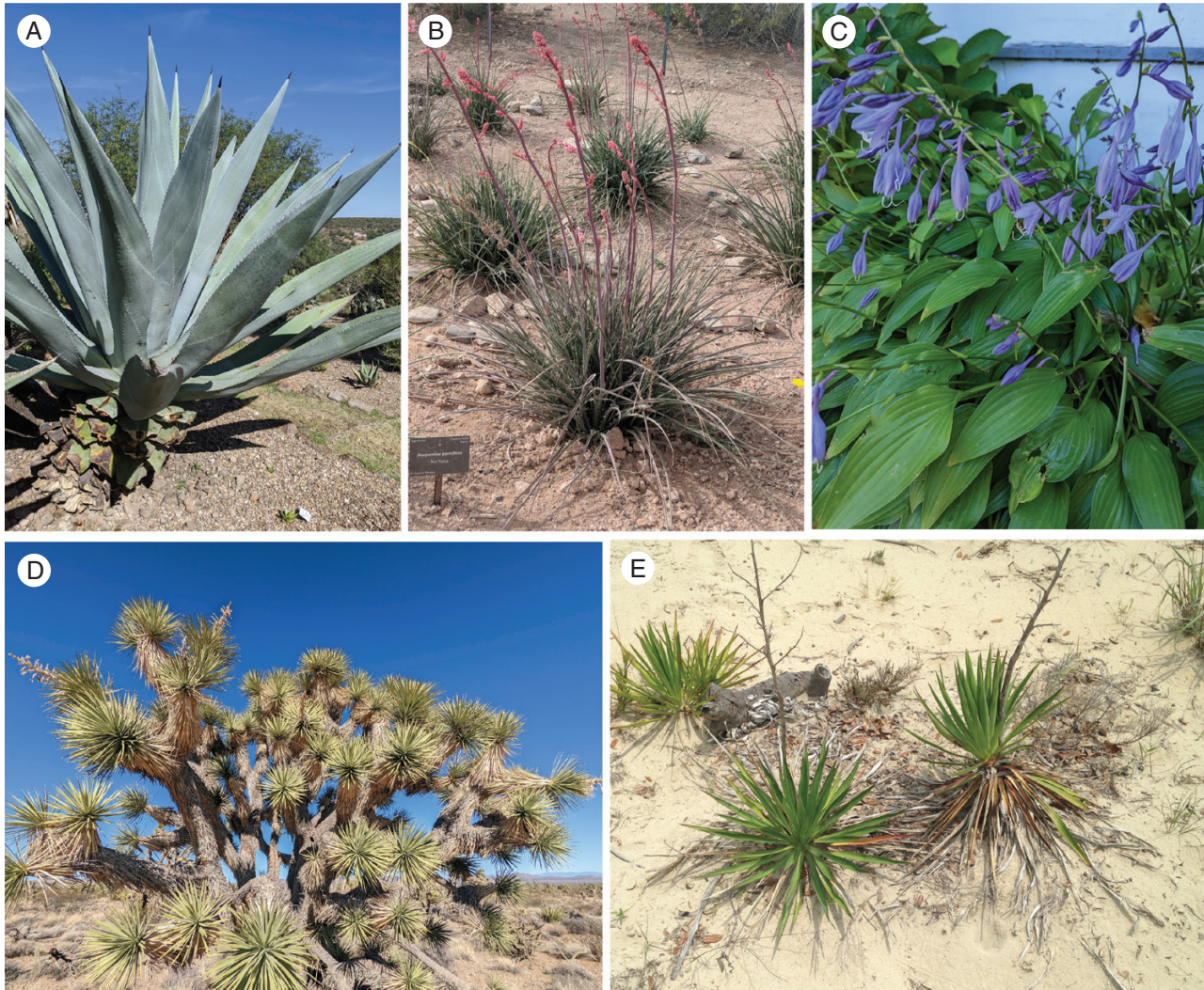


FIG. 1. Diversity of the Agavoideae. (A) *Agave* sp., (B) *Hesperaloe parviflora*, (C) *Hosta* sp., (D) *Yucca jaegeriana* (eastern Joshua tree) and (E) *Yucca gloriosa*. Photo credits: K. Heyduk.

years ago (Mya) (McKain *et al.*, 2012, 2016). Following an initial burst of diversification within the ABK clade crown group, increased diversification of the genus *Yucca* occurred ~20 Mya in association with the origin of the yucca–yucca moth pollination mutualism (Smith *et al.*, 2008; McKain *et al.*, 2016) and with the origin of the genus *Agave sensu lato* ~6 Mya in association with increased aridity in southwestern North America (Jiménez-Barron *et al.*, 2020). A second increase in *Agave* diversification occurred through the oscillating climatic conditions of the late Pliocene and Pleistocene (Jiménez-Barron *et al.*, 2020).

CAM is hypothesized to have evolved repeatedly within the ABK clade, presumably as an adaptive response to increased aridity (Fig. 2) (Heyduk *et al.*, 2016a). CAM photosynthesis is a modification to the primary  $C_3$  photosynthetic pathway that allows plants to conduct the majority of their gas exchange at night, when evapotranspiration rates are lower. Incoming  $CO_2$  is converted to malic acid and stored in vacuoles until the daytime, when stomata (mostly) close and the malic acid is decarboxylated, flooding Rubisco with high levels of  $CO_2$  and limiting

photorespiratory stress. By restricting most stomatal conductance to the night-time, CAM plants are especially successful in water-limited habitats, including deserts and seasonally dry tropical forests. CAM is often associated with succulent leaf tissue, thought to help store water in arid-adapted plants (von Willert *et al.*, 1990; Ogburn and Edwards, 2012; Griffiths and Males, 2017) and allow for large vacuoles in which to store malate, but succulence is also thought to limit diffusion and loss of  $CO_2$  out of cells, where it is highly concentrated in CAM plants (Zambrano *et al.*, 2014).

CAM involves multiple aspects of a plant's biology, from metabolism to leaf anatomy and to genetic regulation. To understand the evolution of CAM photosynthesis, model clades with comprehensive phylogenies are necessary to enable detailed comparative studies. Many of the early phylogenetic estimates in the Agavoideae focused on specific genera (Good-Avila *et al.*, 2006; Pellmyr *et al.*, 2007; Smith *et al.*, 2008; Halpin and Fishbein, 2013; Archibald *et al.*, 2015), or more broadly estimated relationships across the entire Asparagales (Chase *et al.*, 2009; Chen *et al.*, 2013). A more comprehensive phylogeny of

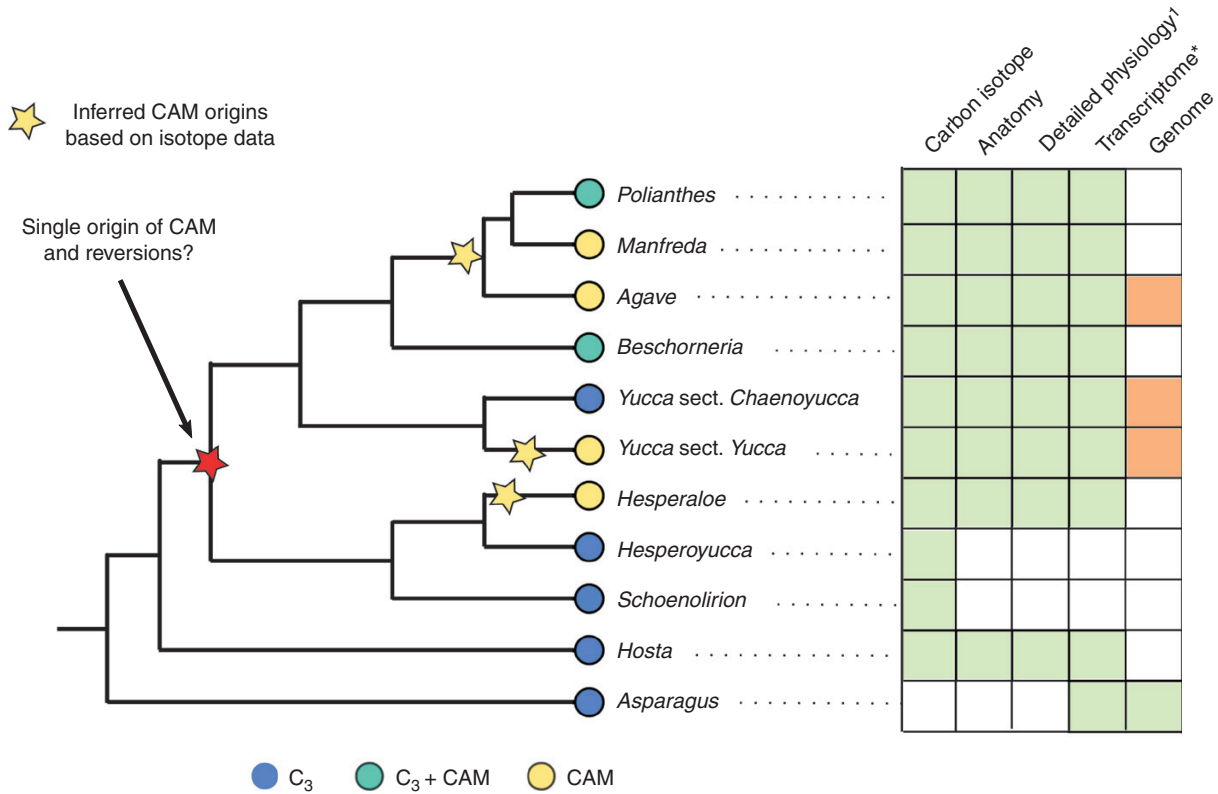


FIG. 2. Phylogenetic relationships of the major genera within the Agavoideae, with *Asparagus* as the outgroup; relationships are based on Heyduk et al. (2016a). Tip colours represent photosynthetic pathways, based on Heyduk et al. (2016a, 2018, 2022). The table on the right shows resources and information available for particular groups. Carbon isotopes (from Heyduk et al., 2016a) suggested three origins of CAM, shown in yellow stars on branches. New detailed physiology may instead suggest a single origin with subsequent losses (red star). <sup>1</sup>Detailed physiology indicates measurement of either daily gas exchange patterns or titratable acidities, typically on a single exemplar species per genus; \*transcriptomes were all sampled from a 24-h time course experiment with the exception of *Asparagus*. Orange boxes indicate work in progress.

60 species from the Agavoideae was estimated using sequence capture data, but still only represents ~10 % of the species diversity in the subfamily (Heyduk et al., 2016a). However, *Agave* is the largest genus in the Agavoideae with 287 recognized (and probably many more unrecognized) species (Plants of the World Online, 2023), but relationships within the genus have been difficult to resolve at the species level (Heyduk et al., 2016a; Jiménez-Barron et al., 2020). The polytomy within *Agave* is probably due to both a rapid radiation as well as ongoing hybridization; additional sampling within *Agave* is unlikely to lead to greater phylogenetic resolution. The sequence-capture-based phylogeny incorporated 25 *Agave* species and 19 *Yucca* species (of ~50 in total), representing the two largest genera in the subfamily. Relationships between genera were strongly supported across both nuclear and chloroplast phylogenies, though the placement of *Hosta*, thought to be sister to all other members of the ABK clade, instead had ingroup placement in the nuclear species tree estimation. Overall the phylogenetic placement of genera is strongly supported, and ancestral reconstruction of photosynthesis based on carbon isotope ratios indicated three origins of CAM in the subfamily: one in *Agave sensu lato*, one in *Yucca* and a third in *Hesperaloe* (Heyduk et al., 2016a) (Fig. 2).

The age of CAM lineages is largely consistent with late Miocene evolution, even across distantly related groups

(Arakaki et al., 2010; Ocampo and Columbus, 2010; Horn et al., 2014; Bone et al., 2015), probably associated with aridification of the planet. In the Agavoideae, the crown group ages for ABK clade CAM lineages *Agave*, *Yucca* section *Yucca* (the clade of *Yucca* species exhibiting CAM) (Thiede, 2019) and *Hesperaloe* have all been estimated at ~6 Mya (McKain et al., 2016; Jiménez-Barron et al., 2020) coincident with drying conditions in the American Southwest (Axelrod, 1948) and the Mexican highlands (Mastretta-Yanes et al., 2015). The presence and diversification of the Agavoideae in the deserts of North America has led to a long history of human use by Indigenous peoples. The Hohokam, for example, cultivated *Agave* species with rock mulching in the dry habitats of the Sonoran Desert; they used *Agave* for fibre, beverages and food, prepared by roasting in large ovens (Ortiz-Cano et al., 2020). The remnants of Hohokam cultivation can still be seen across the Arizona desert in the rock piles the Hohokam used as mulch (Ortiz-Cano et al., 2020), and the extensive cultivation of *Agave* by the Hohokam also led to domestication of *Agave* species (Hodgson et al., 2018). Today, *Agave* is also being explored as a potential biofuel feedstock source (Davis et al., 2011; Yang et al., 2015).

The Agavoideae includes iconic species across the desert landscapes of North America, with relevance to humans past and present. The diversity of this lineage has long intrigued biologists – including physiologists, ethnobotanists, systematists and



ecologists – and today research on the Agavoideae is expanding to include genomics and metabolomics. Here we review past and current research in the Agavoideae (particularly the ABK clade) that has made this clade an ideal system for investigating the genomic, molecular, physiological and ecological dimensions of CAM evolution.

#### EARLY WORK ON CAM IN THE AGAVOIDEAE

The pioneering research by Park S. Nobel documented the ecology and physiology of CAM in *Agave* and among cacti, shortly after the CAM pathway had been more fully described from the 1940s to the 1960s (Pucher *et al.*, 1947, 1949; Thomas, 1949; Black and Osmond, 2003). Using *Agave Hill* in the Boyd Deep Canyon Desert Research Area in southern California, Nobel studied not only the diurnal patterns of photosynthesis in *Agave*, but also how *Agave* species respond to their environment. For example, Nobel examined water relations in *Agave deserti* (Nobel, 1976), showed the degree to which *A. deserti* can down-regulate CAM when well-watered (Hartsock and Nobel, 1976), and explored the effect of leaf angle and light on CAM (Woodhouse *et al.*, 1980). Later, Nobel began estimating the remarkably high productivity values for some *Agave* species (Nobel, 1984; Nobel and Meyer, 1985; Nobel and Quero, 1986; Nobel and Valenzuela, 1987), compared water relations between C<sub>3</sub>, C<sub>4</sub> and CAM species (Nobel and Jordan, 1983), and finally showed that CAM plant productivity was nearly as high as in C<sub>3</sub> and C<sub>4</sub> plants, with total CAM productive dominance under certain conditions (Nobel, 1991). Nobel's focus on *Agave* species drew early attention to CAM in the Agavoideae, and his work contributed to the foundational studies assessing the general ecophysiology of CAM plants.

*Agave* is not the only genus in the Agavoideae studied from the photosynthetic perspective; numerous early studies on *Yucca* showed that, unlike *Agave*, a genus in which all species were presumed to be CAM, *Yucca* has both C<sub>3</sub> and CAM species. In the early 1980s a few studies focused on determining the photosynthetic pathway of iconic desert *Yucca* species, including Joshua trees (*Y. brevifolia*). While some, such as *Y. baccata* and *Y. toerreyi*, were found to use CAM, others, including *Y. brevifolia*, did not seem to use CAM at all (Kemp and Garetto, 1982; Smith *et al.*, 1983). These early studies, much like those in *Agave* by Nobel, also tested the effects of environmental conditions on the physiology and photosynthesis of *Yucca*, including the effects of seasonality, drought, elevated CO<sub>2</sub> and elevated temperatures (Szarek, 1976; Kemp and Garetto, 1982; Smith *et al.*, 1983; Szarek *et al.*, 1987; Huxman *et al.*, 1998). The ecological significance of CAM species in desert communities was also recognized in *Y. schidigera*; by transpiring at night, *Y. schidigera* has an inverse pattern of hydraulic lift, providing water to C<sub>3</sub> and C<sub>4</sub> plants during the day (Yoder and Nowak, 1999). Finally, species in *Hesperaloe* – another genus in the Agavoideae, sister to both *Yucca* and *Agave* – have also been shown to exhibit CAM photosynthesis (Ravetta and McLaughlin, 1993).

#### EVOLUTION OF CAM IN THE AGAVOIDEAE

Carbon isotope data from roughly 60 species in the Agavoideae pinpointed three potential independent origins of CAM in the

group (Heyduk *et al.*, 2016a) but obscured intermediate phenotypes of CAM. Isotopic signatures can differentiate between C<sub>3</sub> species and those that use CAM constitutively ('strong CAM', i.e. plants obtain most of their CO<sub>2</sub> via the CAM pathway, as in Edwards, 2019). However, several intermediate phenotypes of CAM have been described; by intermediate we do not mean intermediate in terms of an evolutionary trajectory, but rather in terms of the proportion of CO<sub>2</sub> obtained by either the C<sub>3</sub> or CAM pathway. For example, C<sub>3</sub> + CAM species are those that rely on both photosynthetic pathways (Edwards, 2019), but obtain most of their carbon from the C<sub>3</sub> pathway during the day. Facultative CAM species can upregulate CAM photosynthesis under drought or other abiotic stress, and then resume normal C<sub>3</sub> function when conditions become favourable again. These intermediate phenotypes should not be thought of only as an inevitable stepping stone in CAM evolution; instead, they should be considered as an evolutionarily stable photosynthetic phenotype. However, their intermediacy allows us to disentangle the evolutionary building blocks of CAM, from genomic perspectives to anatomical and biochemical. These intermediate phenotypes of CAM are important for understanding how CAM is assembled from a C<sub>3</sub> ancestor, but species that use intermediate forms cannot be detected by carbon isotope ratios, and thus are often overlooked and probably vastly undercounted in the flora.

Indeed, detailed physiological investigations have revealed a diversity of CAM phenotypes in the Agavoideae that were obscured by carbon isotope values. *Polianthes* is nested within *Agave* [and has recently been subsumed into the genus *Agave* (Thiede 2015; Thiede *et al.*, 2019)], but initial anatomical and carbon isotope data suggested it uses C<sub>3</sub> photosynthesis (Heyduk *et al.*, 2016a, 2018). Gas exchange experiments under well-watered and drought conditions revealed that *Polianthes tuberosa* is a C<sub>3</sub> + CAM species that can facultatively upregulate CAM photosynthesis under drought stress (Heyduk *et al.*, 2018). Similarly, *Beschorneria* is sister to *Agave sensu lato* and recycles respired nocturnal CO<sub>2</sub> into malate (known as CAM cycling) (Heyduk *et al.*, 2018). The diversity of photosynthetic types just within *Agave* and closely related genera – spanning strong CAM, to facultative CAM, to CAM cycling – allows for robust comparative analyses on the evolution of CAM photosynthesis. Furthermore, the new understanding regarding types of CAM employed across the Agavoideae raises questions about the evolution of CAM more generally in the group; isotope signatures suggested three origins, but the plethora of CAM in species previously designated C<sub>3</sub> has implications for the number of CAM origins in the clade (Fig. 2). For example, previous analysis of leaf anatomy highlighted a propensity across the entire clade for thicker, more succulent leaves, regardless of photosynthetic pathway (Heyduk *et al.*, 2016a). This conclusion was based on carbon isotope data, however, and our more nuanced understanding of CAM physiology in the clade is beginning to suggest an alternative hypothesis of a single origin of CAM and associated phenotypes, such as succulence, with multiple reversions to C<sub>3</sub>. Additional physiological investigations, particularly in lineages currently thought to be C<sub>3</sub>, would help better describe the evolutionary trajectory of CAM in the Agavoideae.

The genus *Yucca* on its own is a powerful comparative system within the Agavoideae, as species in section *Yucca* all appear to use CAM photosynthesis, while the remaining species are

presumably  $C_3$  (Heyduk et al., 2016a). By comparing closely related species in *Yucca* that differ in photosynthetic pathway, physiology and transcriptomic studies have been able to focus on changes probably associated with the evolution of CAM while minimizing stochastic evolutionary differences (Fig. 3). For example, RNA-sequencing (RNA-seq) in *Y. aloifolia* (CAM) and *Y. filamentosa* ( $C_3$ ) not only revealed differentially expressed genes in the CAM photosynthetic pathway, as expected, but also showed changes in the expression of carbohydrate metabolism and potentially photorespiratory-related redox pathways (Heyduk et al., 2019a). There are also instances of hybridization between  $C_3$  and CAM *Yucca* species: for example, intersectional hybrids have been reported in the International Four Corners region (Arizona and New Mexico in the USA, Sonora and Chihuahua in Mexico) (Lenz and Hanson, 2000a), and *Yucca gloriosa* is native to the south-eastern USA and is a hybrid species of *Y. aloifolia* (CAM) and *Y. filamentosa* ( $C_3$ ) (Rentsch and Leebens-Mack, 2012). *Yucca gloriosa* is photosynthetically intermediate between its parental species, exhibiting both  $C_3$  and CAM photosynthesis (Heyduk et al.,

2016b); its leaf anatomy likewise is intermediate, and anatomical traits such as cell size or leaf thickness were shown to be uncorrelated to CAM ability across a diverse set of *Y. gloriosa* genotypes (Heyduk et al., 2021a). The hybrid *Yucca* system is also proving important for understanding the genetics of CAM, and ongoing research is taking advantage of variability in strength and ability to upregulate CAM across genotypes of *Y. gloriosa* (see ‘Intraspecific Variation’ below).

#### AGAVOIDEAE IN THE GENOMICS ERA

Our understanding of the evolutionary relationships of species along the  $C_3$ -CAM continuum in the Agavoideae, together with data on the physiological and anatomical changes associated with the origin of CAM, sets the stage for powerful comparative genomic investigations. In particular, a number of molecular pathways are likely to be differentially regulated in CAM plants relative to  $C_3$ , including carbohydrate processing (Christopher and Holtum, 1996; Borland et al., 2016), stomatal regulation (Griffiths and Males, 2017), and connections

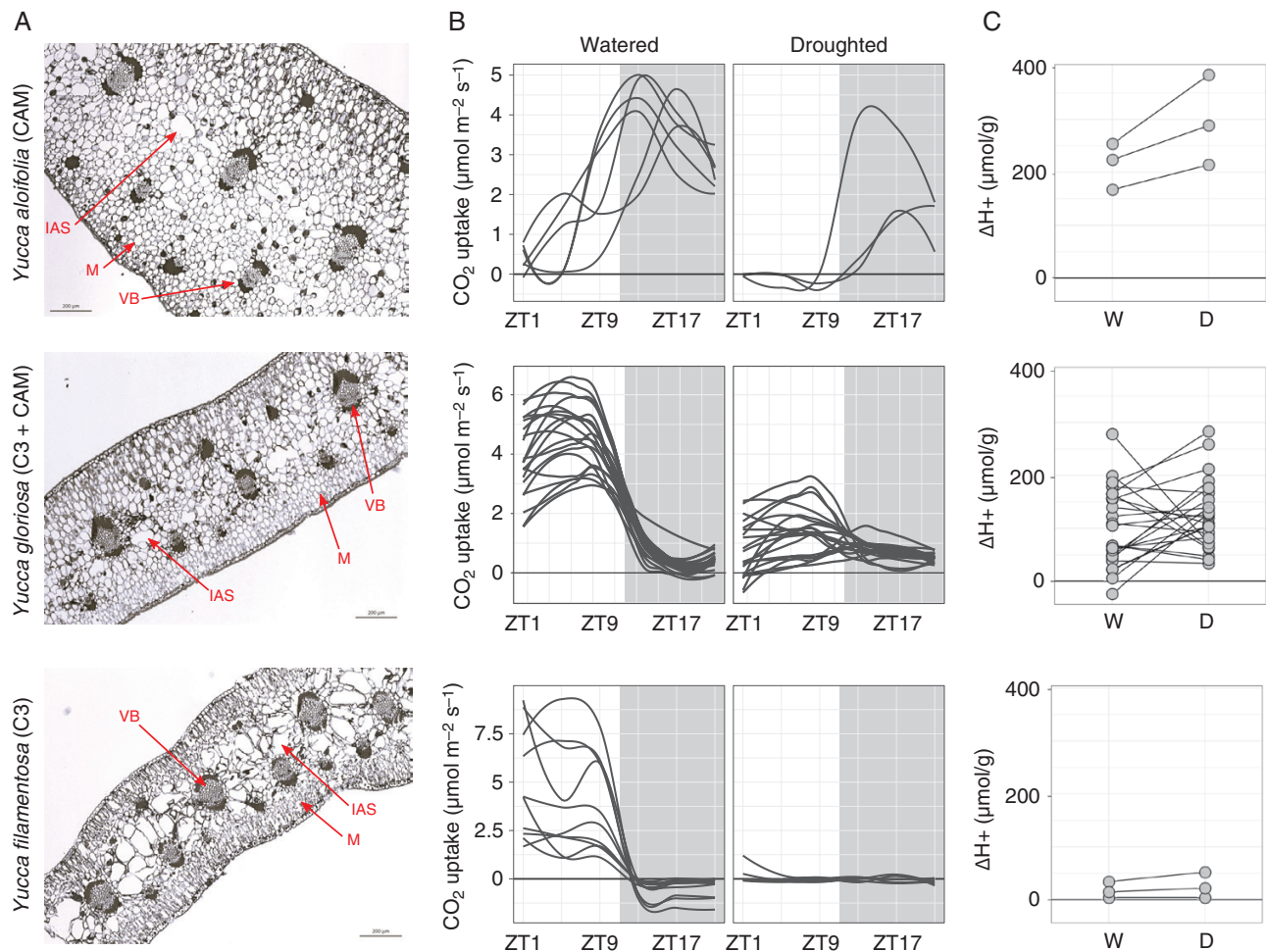


FIG. 3. (A) Anatomical cross-sections of leaves from *Yucca aloifolia* (top, CAM), *Y. gloriosa* (middle,  $C_3 + CAM$ ) and *Y. filamentosa* (bottom,  $C_3$ ). VB: vascular bundle, M: undifferentiated mesophyll, IAS: intercellular airspace, or the space between cells. (B) Gas exchange plots for the three species under well-watered and drought conditions, showing Loess smoothed curves across the day (white background) and night (grey background) for a number of individual genotypes of each species. ZT refers to zeitgeber time, or the number of hours after the lights turned on. (C) Amount of acid accumulated at night in genotypes of each of the three species, under both well-watered (W) and droughted (D) conditions. Lines connect genotypic means between treatments.

between circadian regulators and metabolic pathways (Taybi *et al.*, 2002; Hartwell, 2005; Davies and Griffiths, 2012; Boxall *et al.*, 2017). One of the earliest genomic studies from within the Agavoideae came from Gross *et al.* (2013), where *de novo* transcriptome assemblies from a range of tissue types were published for *Agave deserti* and *A. tequilana*. Although primarily a resource-generating endeavour, the transcriptomes provided evidence for transcriptionally active transposable elements (TEs). TE insertions are known to be a source of *cis*-regulatory motif variation, as has been documented for the evolution of C<sub>4</sub> photosynthesis (Cao *et al.*, 2016). Future work on TE activity and its functional implications for CAM evolution in the Agavoideae and other CAM lineages with published (Cai *et al.*, 2015; Ming *et al.*, 2015; Yang *et al.*, 2017) or forthcoming reference genomes should test whether TE insertions into *cis*-regulatory regions are contributing to the shifts in gene expression and the evolution of CAM traits.

While gene expression patterns obtained from RNA-seq experiments can provide valuable insight into biological processes, further insight can be gained by integrating protein and metabolite abundances to better understand post-transcriptional regulation and protein function. An analysis of transcripts, proteins and metabolites across a diel cycle in *Agave americana* compared to C<sub>3</sub> *Arabidopsis* revealed that *A. americana* has an inverted ‘redox poise’, where NADP<sup>+</sup> and NADPH are highly abundant at night (Abraham *et al.*, 2016). Additionally, 641 genes in *A. americana* were identified as having inverted expression profiles relative to their *Arabidopsis* orthologues. However, it is worth noting that this study measured *Arabidopsis* and *Agave* plants under different growth conditions, and that these two species are distantly related, confounding our ability to infer that any changes were due to photosynthetic differences. A separate time-course transcriptomic study comparing *A. americana* to *Arabidopsis* (C<sub>3</sub>) revealed altered expression patterns in both core CAM pathway genes as well as a single circadian regulator, REVEILLE 1 (RVE1) (Yin *et al.*, 2018); all other circadian regulatory transcripts had shared timing of expression between the two species. Both studies relied on distantly related C<sub>3</sub> and CAM species, and therefore any changes to the timing of expression could be related to either photosynthetic pathway or stochastic changes over evolutionary time.

In contrast, comparative analyses of the differentially expressed genes and metabolite accumulation between closely related *Yucca aloifolia* (CAM), *Y. filamentosa* (C<sub>3</sub>) and their hybrid *Y. gloriosa* (C<sub>3</sub> + CAM) revealed conservation of time-structured expression patterns of CAM genes between all three species (Heyduk *et al.*, 2019a). For example, a key gene in nocturnal CAM carbon fixation shows an increase in transcript abundance before the night period in both C<sub>3</sub> and CAM *Yucca*, though overall expression is 10-fold higher in the CAM species (Heyduk *et al.*, 2019a). The finding of conserved gene expression across photosynthetic types suggests these patterns were present in the ancestor of *Yucca* and may have facilitated the evolution of CAM within the genus (or, as discussed previously, provides additional evidence of a single origin of CAM and multiple losses). Despite the conservation of time-structured gene expression across the genus, metabolite accumulation differed between photosynthetic modes. Carbohydrate turnover is important for CAM species, because sugars or starches need to be broken down to provide building material for phosphoenolpyruvate (PEP), the substrate for carbon

fixation in the CAM pathway (Borland *et al.*, 2016). However, C<sub>3</sub> plants also store carbohydrates and undergo glycolysis to provide carbon for daytime photosynthesis and growth. In *Yucca*, overall starch content was relatively stable across the day in *Y. aloifolia* but depleted at night in *Y. filamentosa*, suggesting that starch is not the main source of PEP in CAM *Yucca* species, a result supported by similar findings in *Agave* (Abraham *et al.*, 2016). Instead, small fructans (as found in *Agave*) or triose-phosphates are probably providing the carbon required for PEP regeneration for CAM *Yucca* (Heyduk *et al.*, 2019a). Metabolite abundance differences between CAM and C<sub>3</sub> *Yucca* also highlighted areas where our understanding of CAM remains unclear. For example, the empirical evidence that CAM plants still experience oxidative stress during the daytime is mixed (Adams and Osmond, 1988; Griffiths *et al.*, 1989; Niewiadomska and Borland, 2008). It is thought that CAM plants have reduced photorespiratory activity, and thus might not be able to employ photorespiratory mechanisms as protection from oxidative stress (Kozaki and Takeba, 1996; Lüttge, 2010). However, *Y. aloifolia* was shown to have elevated daytime levels of ascorbic acid, which is an antioxidant hypothesized to be involved in protection from oxidative stress and may serve as an alternative sink to photorespiratory mechanisms for excess energy. Indeed, expression of genes and metabolites involved in photorespiration appear to be less abundant in *Y. aloifolia* (CAM) than in *Y. filamentosa* (C<sub>3</sub>) (Heyduk *et al.*, 2019a). Combined, the complementary transcriptomics and metabolomics work done in *Agave* and *Yucca* supports convergent evolution of photosynthetic and accessory pathways (e.g. carbohydrate metabolism) in these two independently evolved CAM lineages.

The emerging comparative genomics framework for the Agavoideae is also advancing understanding of environmental influences on photosynthetic strategies along the C<sub>3</sub>-CAM continuum, both within and among species exhibiting variation in photosynthetic mode. While CAM is often thought of as a drought-adapted physiology, it remains unclear how CAM plants will fare under climate change scenarios – including drought, higher CO<sub>2</sub> and higher temperatures (Heyduk, 2022). Studies in the Agavoideae are highlighting that CAM plants, like their C<sub>3</sub> counterparts, have environmentally induced responses, including water stress-induced changes in gene expression and carbon fixation. A comparison of leaves from well-watered and drought-stressed leaves of *Agave sisalana* identified 3095 genes that were differentially expressed under drought, with significant enrichment for gene ontology (GO)-categories associated with antioxidant response and osmotic adjustment (Sarwar *et al.*, 2019). A study of physiological and transcriptomic responses across the Agavoideae explicitly compared responses to drought stress in the three CAM lineages in the Agavoideae: *Agave sensu lato*, *Hesperaloe* and *Yucca* (Heyduk *et al.*, 2022). Gene expression in response to drought stress was not conserved across species, and instead varied widely across lineages in the number of genes differentially expressed (with nearly 20 % of genes in *A. attenuata* showing differences in drought-induced gene expression) (Heyduk *et al.*, 2022). Further, genes that were differentially expressed in response to drought were often unique to a particular lineage, which underlines the need to additionally study how CAM plants respond to environmental stressors.



The current body of genomic work in CAM plants has focused almost exclusively on transcriptomics, with a few proteomics or metabolomics studies. Moving beyond the gene–protein paradigm to understand other levels of gene regulation, such as transcriptional regulation and chromatin structure, will enhance our knowledge of the evolution of CAM, informing future efforts to engineer CAM into  $C_3$  plants. One current limitation toward this goal is how little is known about the *cis*-regulatory changes that are required for CAM gene expression. One hypothesis is that genes involved in CAM have transcription factor binding motifs (TFBMs) in their regulatory sequences that recruit circadian clock transcription factors. In pineapple, CAM genes were shown to be enriched in their promoters for known circadian clock gene TFBMs (Ming et al., 2015). In both *Y. aloifolia* (CAM) and *Y. filamentosa* ( $C_3$ ), phosphoenolpyruvate carboxylase (PPC) orthologues share the same time-structured expression pattern, but in *Y. aloifolia* the magnitude of expression is much higher at all time points (Heyduk et al., 2019a). The difference in magnitude of PPC expression may be due to recruitment of a unique set of transcription factors to the promoter of PPC in *Y. aloifolia* not found in the promoter region of *Y. filamentosa*. Methods that interrogate *cis*-regulatory elements such as ATAC-seq or DNase-seq should be applied to the Agavoideae and in other clades poised for comparative analyses to better understand the role of *cis*-regulation in CAM evolution.

#### INTRASPECIFIC VARIATION

Much of our knowledge about the photosynthetic mode for a species (both within and outside of the Agavoideae) is often based on a few specimens, with the goal of broadly understanding how many and which species use CAM photosynthesis. However, this approach may bias the field away from uncovering variation within species. Intraspecific variation in photosynthetic traits has been documented in crops such as maize (Heichel and Musgrave, 1969) and rice (Teng et al., 2004), as well as natural populations of Sikta alder and paper birch (Benowicz et al., 2000) and *Andropogon gerardii* (Johnson et al., 2015). Fewer studies have examined variation between photosynthetic pathways intraspecifically. Both *Flaveria* and *Alloteropsis* are examples of  $C_4$  systems where such variation has been studied (Brown et al., 1993; Teese, 1995; Lundgren et al., 2016; Olofsson et al., 2016, 2021). In CAM taxa, populations of *Puya chilensis* (Bromeliaceae) in South America grow along a latitudinal gradient that is correlated with precipitation. Populations in wetter areas (higher latitudes) exhibited less night-time acid accumulation and a more negative carbon isotope ratio ( $\delta^{13}C$ ) compared to low-latitude populations (Quezada et al., 2014). This work demonstrates that intraspecific variation for CAM photosynthesis exists and can be linked to relevant environmental parameters such as precipitation (Quezada et al., 2014). A follow-up field study suggested that CAM was selected for at drier sites (low latitudes) and selected against at wetter sites (high latitudes) (Quezada et al., 2017), although common gardens and/or reciprocal transplants would be necessary to bolster this finding. In *Portulaca oleracea*, a broadly distributed  $C_4$ –CAM species, subspecies vary in photosynthetic traits. Individuals from different subspecies of *P. oleracea* were subjected to drought, then re-watering,

within a growth chamber under controlled conditions (Ferrari et al., 2020). In well-watered conditions  $C_4$  photosynthesis was the primary mode across 11 geographically diverse subspecies of *P. oleracea* (Ferrari et al., 2020). Four subspecies showed night-time malate accumulation under well-watered conditions, whereas all subspecies showed acid accumulation under drought (Ferrari et al., 2020). However, while subspecies varied in the magnitude of malate accumulation during drought, the authors indicate that this variation was not correlated with the expression of key CAM photosynthesis genes (Ferrari et al., 2020).

Within the Agavoideae, *Yucca gloriosa* is an extensively studied example of intraspecific photosynthetic variation. *Yucca gloriosa* is a relatively recent hybrid species that formed between *Y. aloifolia* (CAM) and *Y. filamentosa* ( $C_3$ ) (Trelease, 1902; Rentsch and Leebens-Mack, 2012; Heyduk et al., 2021b). As a result of multiple hybridization events between parents with different photosynthetic modes (Heyduk et al., 2021b), *Y. gloriosa* is a  $C_3$  + CAM species with intermediate values (relative to parents) for gas exchange, night-time malate accumulation and cell size (Heyduk et al., 2016b). Subsequent work in *Y. gloriosa* showed a considerable amount of intraspecific variation in both gas exchange and malate accumulation (Fig. 3) (Heyduk et al., 2021a). Genotypes collected from across the range of *Y. gloriosa* exhibited variable upregulation of CAM photosynthesis under water stress. Variation in CAM upregulation may have long-term implications for the species as the effects of climate change alter precipitation and temperature in the south-eastern USA where this species grows. It is worth noting that if a single *Y. gloriosa* individual had been measured and used as diagnostic for the entire species, our understanding of photosynthetic diversity within the species would be extremely limited. Moreover, the intraspecific variation within *Y. gloriosa* for CAM photosynthetic traits and response to environmental cues can be used to disentangle the genetic components underlying facultative CAM physiology. By combining resequencing data and RNA-seq from the hybrids and detailed physiological measurements, we can interrogate the importance of parental-specific alleles and regulatory regions on CAM ability, as well as the role of latitude and environment in the variation in CAM traits seen in *Y. gloriosa*.

The genus *Yucca* has several instances of hybridization outside of the *Y. gloriosa* system described above. In the south-western USA, Joshua tree species *Y. brevifolia* and *Y. jaegeriana* hybridize in an area of sympatry despite the presence of an otherwise strong pollinator mutualism (Royer et al., 2016; Svensson et al., 2016). While Joshua trees are currently thought to be  $C_3$ , spatially variable environmental pressures (e.g. precipitation and temperature) throughout the distribution of the two species may warrant additional study to determine whether intraspecific variation for photosynthetic or other physiological traits exists. In an area centred around New Mexico, at least five species of *Yucca* appear to hybridize to form intermediate types based solely on morphological observations (Webber, 1953). However, these five species (*Y. angustissima*, *Y. baileyi*, *Y. glauca*, *Y. constricta* and *Y. elata*) are all  $C_3$  based on carbon isotope values (Heyduk et al., 2016a), and therefore any hybrids would be presumed to be  $C_3$  as well. Similarly, two CAM species, *Y. capensis* and *Y. valida*, hybridize in Baja California based on population genomic data

(Arteaga *et al.*, 2020). There are, however, examples of *Yucca* hybrids that have putative parents with different photosynthetic modes. *Yucca* × *schottii* currently refers to any hybrid between *Y. baccata*, *Y. elata* and *Y. madrensis* (Lenz and Hanson, 2000a, b); previous carbon isotope data from a single individual showed *Y. × schottii* uses CAM photosynthesis (Heyduk *et al.*, 2016a). All of the fleshy fruited section *Yucca* were inferred to be CAM (Heyduk *et al.*, 2016a), including *Y. baccata* and *Y. madrensis* (Lenz and Hanson, 2000a), whereas *Y. elata* uses C<sub>3</sub> photosynthesis (Heyduk *et al.*, 2016a). Hybrids have been identified on a morphological basis between all three pairs of parental species (Lenz and Hanson, 2000a). Additional research into *Y. × schottii* hybrids would allow for comparisons with the *Y. gloriosa*, *Y. aloifolia* and *Y. filamentosa* hybrid system to understand whether C<sub>3</sub> + CAM hybrid species converge on similar physiological traits and photosynthetic variation. Very recently a new species of *Yucca*, *Y. carrii*, was described as of putative hybrid origin in the Gulf Coast of Texas (Clary and Adams, 2021). The evidence of its hybrid origin was based on its inability to produce fruit and limited pollen viability, although it awaits molecular work to clarify its parentage and evolutionary origin (Clary and Adams, 2021). Since *Y. aloifolia* and *Y. gloriosa* are sympatric with *Y. carrii* (Clary and Adams, 2021), additional investigations into its mode of photosynthesis and genetic origin could provide additional data on photosynthetic pathway variation in a hybrid.

Hybridizing lineages are not the only way to investigate intraspecific photosynthetic intraspecific variation within Agavoideae. Many species in the Agavoideae have patterns of genetic differentiation that correspond to environmental clines. The genetic differentiation could indicate physiological differentiation as well, including in photosynthetic traits. For example, in the Tehuacán–Cuicatlán valley of Mexico, populations of *Agave kerchovei* exhibit extraordinarily high levels of genetic differentiation across heterogeneous environments (Aguirre-Planter *et al.*, 2020). While the authors attribute the patterns of genetic differentiation to historical climates and how they affected the range of *A. kerchovei* in the past (Aguirre-Planter *et al.*, 2020), the wide differentiation in environmentally variable populations may indicate variation in photosynthetic traits as well. Similarly, *Agave striata* exhibits significant genetic differentiation that correlates with the environment within the Chihuahuan desert (Trejo *et al.*, 2016). The substantial amount of genetic differentiation in *A. striata* is reflected in named subspecies (subsp. *striata* and subsp. *falcata*) and may even be an example of incipient speciation in progress (Trejo *et al.*, 2016). Since the habitats occupied by the two subspecies vary in environmental parameters such as annual precipitation, precipitation seasonality and temperature seasonality (Trejo *et al.*, 2016), *A. striata* is a promising group to test for intraspecific photosynthetic variation across subspecies. Outside of Mexico, recent work in Cuba has shown that *A. offoyana* can be found in areas that vary in water availability (Toledo *et al.*, 2020). While the focus of their work was not on photosynthesis, it and other similar studies highlight the importance of identifying habitat variability within a species' range as a means to generate hypotheses. The Agavoideae, with both hybridizing lineages and species with large ranges spanning environmental gradients, has untapped potential for understanding the origin and maintenance of intraspecific variation of CAM photosynthesis.

## FUTURE DIRECTIONS

From the original ecophysiological studies to recent advances in genomics, the Agavoideae is an important lineage for studying the evolution of CAM photosynthesis. The number of diverse CAM phenotypes across closely related species will be crucial for understanding how CAM evolves, and highlights the need to continue efforts to document CAM species through detailed physiology. With a robust set of emerging model systems, particularly in *Agave* and *Yucca* where both physiology and genomic resources are well developed, the Agavoideae can continue to serve as a model for understanding CAM plants in the future. Climate change is likely to alter precipitation patterns, average temperatures and CO<sub>2</sub> levels, but how CAM plants may respond to these novel environments is largely unknown. Continued expansion of genomic resources, including full genome sequences and systems where gene function can be tested, will make such studies more powerful, enabling us to connect phenotype to genotype. And as noted, the potential for exploring intraspecific variation in species of the Agavoideae is largely unexplored. Finally, the Agavoideae has closely related subfamilies that should likewise be further examined for potential studies on CAM evolution, including the Nolinoideae (Martin *et al.*, 2019). The emerging comparative genomics framework for the Agavoideae will undoubtedly advance the taxon's continued importance as a model for CAM research.

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