SHARED GENE EXPRESSION IN C_3 AND CAM *YUCCA* SPECIES

Nocturnal expression of Crassulacean acid metabolism (CAM) genes predates the origin of CAM in the genus *Yucca*.

Karolina Heyduk[1], Jeremy N. Ray[1], Saaravanaraj Ayyampalayam[2], Nida Moledina[1], Anne Borland[3], Scott A. Harding[4,5], Chung-Jui Tsai[4,5], Jim Leebens-Mack[1].

1 –Department of Plant Biology, University of Georgia, Athens, Georgia, USA

2 – Georgia Advanced Computing Resource Center, University of Georgia, Athens, Georgia, USA

3 – School of Natural and Environmental Sciences, Newcastle University, Newcastle, United Kingdom

4 – Department of Genetics, University of Georgia, Athens, Georgia, USA

5 – Warnell School of Forestry, University of Georgia, Athens, Georgia, USA

List of author contributions: K.H. conducted the RNA-Seq experiments and most analyses; J.R. assisted with experiments and library preparation; S.A. assembled transcriptomes and assisted with aspects of bioinformatics; A.B. helped with interpretation of gene expression and metabolite data, as well as manuscript preparation; S.H. was responsible for the GC-MS for metabolomics; CJ.T. assisted in experimental design, particularly for metabolomics; J.L-M. helped guide experimental design, data analysis, and overall manuscript preparation.

Funding: This work was supported by NSF DEB 1442199 (to J.L-M.), a Society for the Study of Evolution Rosemary Grant to K.H., a UGA Graduate School Innovative and Interdisciplinary Research Grant to K.H., and a UGA Plant Biology Palfrey Grant to K.H.

Present address for Karolina Heyduk: Department of Ecology and Evolution, Yale University, New Haven, CT, USA

E-mail for contact author: karolina.heyduk@yale.edu

Abstract

Crassulacean acid metabolism (CAM) is a carbon-concentrating mechanism that has evolved numerous times across flowering plants and is thought to be an adaptation to water limited environments. CAM has been investigated from physiological and biochemical perspectives but little is known about how plants evolve from C_3 to CAM at the genetic or metabolic level. Here we take a comparative approach in analyzing time-course data of C_3, CAM, and C_3-CAM intermediate *Yucca* (Asparagaceae) species. RNA samples were collected over a 24-hour period from both well-watered and drought-stressed plants and were clustered based on time-dependent expression patterns. Metabolomic comparisons of the C_3 and CAM species link gene expression to carbohydrate metabolism and gene network co-expression analyses revealed compositional and functional changes to networks containing canonical CAM genes. Observed differences in carbohydrate metabolism and antioxidant response between the CAM and C_3 species reveal alternative sugar and starch degradation pathways, underscoring the need for more comparative metabolomic analyses to understand the evolution of CAM from C_3. Despite many differences in transcript and metabolite profiles between the C_3 and CAM species, shared time-structured expression of CAM genes in the C_3 species suggests ancestral expression patterns required for CAM may have predated its origin in *Yucca*.

Introduction

Crassulacean acid metabolism (CAM) is a carbon concentrating mechanism that can reduce photorespiration and enhance water use efficiency relative to plants that rely solely on the C_3 photosynthetic pathway. In CAM plants, stomata are open for gas exchange at night, when transpiration rates are lower, and incoming CO_2 is initially fixed by phosphoenolpyruvate carboxylase (PEPC) rather than RuBisCO. Carbon is temporarily stored as malic acid within the

vacuole, and during the day stomata close and the malic acid is decarboxylated in the cytosol, ultimately resulting in high concentrations of CO_2 around RuBisCO. The extra steps of CAM – carboxylation of PEP, decarboxylation of malic acid, transport into and out of the vacuole – come with extra energetic costs relative to than C_3 photosynthesis, but CAM plants have the advantage of acquiring carbon with increased water use efficiency (WUE). In addition, RuBisCO is able to act more efficiently with a high concentration of CO_2 and the risk of photorespiration is thought to be significantly minimized (Cushman and Bohnert, 1997; Schulze et al., 2013). CAM plants are therefore adapted to habitats where water stress is unavoidable and where the energetic cost of CAM is offset by reduced photorespiration under water limited conditions. CAM has evolved at least 35 independent times in flowering plants (Silvera et al., 2010), thus making it a remarkable example of parallel and convergent evolution of a complex trait. Moreover, integrated understanding of the ecological, genetic and molecular dynamics that have contributed to these many independent origins of CAM may inform efforts to engineer CAM into C_3 fuel-crop species (Borland et al., 2014; Borland et al., 2015; Cushman et al., 2015; Yang et al., 2015a; Yang et al., 2015b).

CAM has been studied from a biochemical standpoint for decades, and much is known about the carbohydrate turnover, starch cycling, and enzymatic machinery of CAM plants (Cushman and Bohnert, 1997; Chen et al., 2002; Dodd et al., 2002). Additionally, physiological studies of CAM plants have revealed the importance of succulence and large cells (Kluge and Ting, 1978; Nelson et al., 2005; Nelson and Sage, 2008; Zambrano et al., 2014). In terms of the basic machinery required for CAM, carbonic anhydrase (CA) is required to covert CO_2 to HCO_3^- at night (Fig. 1A). PEPC fixes the carbon from CA into oxaloacetate (OAA), but its activity is regulated by a dedicated kinase, PEPC kinase (PPCK). Phosphorylated PEPC is able to fix

carbon in the presence of its downstream product, malate, whereas the un-phosphorylated form is sensitive to malate (Nimmo, 2000; Taybi et al., 2000). As day approaches, PPCK is down-regulated by a combination of two mechanisms: circadian regulation (Carter et al., 1991; Hartwell et al., 1996) or through metabolite control of transcription which results from elevation of cytosolic malate(Borland et al., 1999). During the day, the stored malic acid exits the vacuole and is decarboxylated by either phosphoenolpyruvate carboxykinase (PEPCK) and/or NADP/NAD-malic enzyme, depending on CAM species (Holtum and Osmond, 1981). NADP/NAD-ME CAM plants additionally have high levels of PPDK, which converts pyruvate to PEP. This final step is important for CAM plants, as PEP is then used in the gluconeogenesis pathway to synthesize carbohydrates.

A daily turnover of sugars or starch for PEP generation is a defining characteristic of CAM plants. Carbohydrates that are laid down during the day must be broken down to PEP at night to provide substrate for CO_2 fixation via PEPC. The nocturnal demand for PEP represents a significant sink for carbohydrates which CAM plants must balance with partitioning of carbohydrates for growth and maintenance (Borland et al., 2016). The interplay between carbohydrate metabolism and CAM is clearly an important regulatory mechanism; previous work has shown that plants with reduced carbohydrate degradation have decreased CAM function at night (Dodd et al., 2003; Cushman et al., 2008a). The evolution of temporally integrated carbon metabolism in CAM plants presumably involves rewiring of gene regulatory networks to link these processes with the circadian clock. Although timing of photosynthetic gene expression is to some degree circadian controlled in both C_3 and CAM species, the links between metabolism genes in CAM and circadian clock oscillators may be stronger (Hartwell, 2005; Dever et al., 2015).

Despite the apparent complexity of the CAM phenotype, the photosynthetic modification has evolved numerous times independently across the flowering plant phylogeny. What facilitates this frequent transition in diverse lineages is unknown. It is commonly thought that all CAM pathway genes need to have their expression timing altered from the C_3 state, which might require many changes in promoters or in gene body amino acid sequences. Another possibility is that all C_3 plants have low level organic acid cycling at night (Bräutigam et al., 2017), allowing CAM to evolve using this pre-existing pathway when environmental stresses (prolonged drought, for example) are present. If all C_3 species do indeed have low flux through a nocturnal organic acid cycling pathway, it further strengthens the possibility of a CAM continuum, where plants can be C_3, CAM, or a combination of both pathways (Silvera et al., 2010; Winter et al., 2015). Further, intermediate CAM species (those between full C_3 and full CAM) should exhibit mixed phenotypes at both physiological and genomic scales, and are potentially powerful systems to exploring the transition from C_3 to CAM

Our understanding of the genetics and genome structure of CAM has come predominantly from studies that involve comparisons between C_3 and CAM tissues sampled from evolutionarily distant species, or from samples taken from one species under different age or environmental conditions (Taybi et al., 2004; Cushman et al., 2008b; Gross et al., 2013; Brilhaus et al., 2016a) (but see (Heyduk et al., 2017)). Recent studies have profiled gene expression before and after CAM induction in *Mesembryanthemum crystallinum* (Cushman et al., 2008b) and in *Talinum* (Brilhaus et al., 2016b). These studies, together with comparison of transcript abundance profiles in photosynthetic (green) and non-photosynthetic (white) parts of pineapple (*Annanas comusus*) leaf blades have also provided insights into the regulation of canonical CAM genes (Zhang et al., 2014; Ming et al., 2015). However, RNA-seq of closely

related C_3 and CAM species, as well as intermediate C_3-CAM lineages, are lacking.

In this study, we compared transcript profiles among three closely related *Yucca* (Agavoideae, Aspargaceae) species with contrasting photosynthetic pathways: *Y. aloifolia* consistently has nighttime uptake of CO_2 with concomitant malic acid accumulation in leaf tissue, as well as anatomical characteristics indicative of CAM function; *Y. filamentosa* has typical C_3 leaf anatomy and showed no positive net CO_2 uptake or malic acid accumulation at night; *Y. gloriosa*, a hybrid species derived from *Y. aloifolia* and *Y.filamentosa*, acquires most of its CO_2 from the atmosphere through C_3 photosynthesis during the day with low-level CO_2 uptake a night, but when drought stressed transitions to 100% nighttime carbon uptake (Heyduk et al., 2016). *Yucca gloriosa's* leaf anatomy is intermediate between the two parental species, and to some extent may limit the degree of CAM it can employ (Heyduk et al., 2016). Clones of all three species (Supplemental Table S1) were grown in a common garden setting under both well-watered and drought stressed conditions and assayed gene expression and carbohydrate metabolism differences between species over a 24-hour diel cycle.

Results

Photosynthetic phenotypes

As described in previous work, *Y. aloifolia* conducts atmospheric CO_2 fixation at night via CAM photosynthesis, while *Y. filamentosa* relies only on daytime CO_2 fixation and the C_3 cycle. *Yucca gloriosa*, a C_3-CAM intermediate species, uses mostly daytime CO_2 fixation with low levels of nocturnal gas exchange under well-watered conditions, then relies solely on CAM photosynthesis under drought stress (Fig. 1B). Gas exchange and titratable acidity measurements shown in Fig. 1 are from prior work when RNA was sampled, though gas exchange patterns were largely consistent in *Y. aloifolia* and *Y. filamentosa* during a second round of sampling for

metabolites (Supplemental Figure S1).

Assembly and differential expression

After filtering to remove low abundance transcripts (FPKM<1) and minor isoforms (<25% total component expression), an average of 55k assembled transcripts remained per species. Transcripts were then sorted into gene families (orthogroups) circumscribed by 14 sequenced plant genomes and removed if their length was shorter than the minimum length for a gene family. Considering only transcripts that sorted into a gene family and had the proper length, transcriptome sizes were reduced further: 19,399, 23,645, and 22,086 assembled transcripts remained in *Y. aloifolia, Y. filamentosa,* and *Y. gloriosa,* respectively. SNPs showed greater variation between species rather than among genotypes (Fig. 2A), although *Y. filamentosa* exhibited more SNP variation among genotypes than the other two species. *Yucca gloriosa* genotypes used in this study were found to be slightly more similar to *Y. aloifolia* than *Y. filamentosa* based on PCA analysis of SNP distances; this is likely a consequence of choosing *Y. gloriosa* accessions that showed a propensity for CAM (and therefore were potentially more similar to *Y. aloifolia*) under drought stress for RNA-seq.

Differential expression analysis at each time point between well-watered and drought-stressed samples showed distinct patterns in the three species (Fig. 2B). The effect of drought on expression was greatest one hour after the start of the light period in the CAM species *Yucca aloifolia,* but just before light in the C_3 species *Y. filamentosa*. *Yucca gloriosa* (C_3-CAM intermediate) had near constant levels of differentially expressed transcripts across the entire day/night cycle. Gene Ontology (GO) enrichment tests showed general processes, such as metabolism and photosynthesis, as being commonly enriched in the differentially expressed transcripts (Supplemental Table S2).

Transcripts of each species were classified as time structured if their expression across time under either well-watered and drought conditions could be better described with a polynomial regression, rather than a flat line, with significance-of-fit corrected for multiple tests. There were 612, 749, and 635 transcription factor annotated transcripts with time-structured expression profiles in *Y. aloifolia*, *Y. filamentosa,* and *Y. gloriosa*, respectively. Of those, 92, 62, and 83 were differentially expressed in *Y. aloifolia, Y. filamentosa,* and *Y. gloriosa,* respectively, under drought (Supplemental Table S3). Putative CAM pathway genes largely showed the expected expression patterns in *Y. aloifolia* (Fig. 1), and additionally all three species shared time-structured gene expression patterns for some canonical CAM genes regardless of photosynthetic pathway. In all three *Yuccas*, PEPC, its kinase PPCK (Fig. 3A and B), as well as decarboxylating enzymes NAD/P-me, PPDK and, PEPCK showed time-structured expression (Supplemental Figure S2). PEPC and PPCK (Fig. 3A and B) exhibited time-structured expression in all three species, though they were only differentially expressed between well-watered and droughted treatments in *Y. gloriosa*. PEPC expression in *Yucca* peaks in the afternoon, much before the peak of PPCK. Expression of PEPC in *Y. filamentosa* was much lower in terms of TPM (transcripts per kilobase million), but had the same temporal pattern as both *Y. aloifolia* and *Y. gloriosa* (well-watered: $F_{(12,42)} = 1.38$, $p < 0.212$; drought: $F_{(12,42)}=0.53$, $p < 0.866$). In all 3 species, PPCK was most highly expressed at night, consistent with its role in activating PEPC for dark carboxylation, and showed no difference in temporal expression across species (well-watered: $F_{(12,42)} = 0.85$, $p < 0.605$; drought: $F_{(12,42)}=1.21$, $p < 0.309$). Carbonic anhydrase (CA), involved in conversion of CO_2 to HCO3-, had only 3 transcripts that were temporally structured in their expression in *Y. aloifolia*; two α-CA and one γ. In none of these cases did expression increase at night as might be expected (Supplemental Figure S3).

Species-specific gene co-expression network analysis was conducted on each PEPC cluster, combined with the corresponding cluster that had the strongest median negative correlation to the PEPC cluster (anti-PEPC). PEPC and anti-PEPC clusters were combined due to the use of a signed clustering algorithm: only transcripts with positively correlated expression patterns are co-clustered. Therefore, negative interactions would not be detected by examining a network of a single cluster. PEPC and anti-PEPC clusters in *Y. aloifolia* show dense networks within each cluster (Fig. 3C), and few connections between. *Yucca filamentosa* had well separated PEPC and anti-PEPC clusters (Fig. 3D), while *Y. gloriosa* had nearly joined PEPC and anti-PEPC clusters (Fig. 3E). To explore mechanisms of regulation of PEPC, significant connections to PEPC were extracted from each species' network (Table 1). *Yucca gloriosa* had the greatest number of gene connections to PEPC, despite similar numbers of nodes in both *Y. gloriosa* (2,550) and *Y. aloifolia* (2,311). Additionally, five PEPC-connected genes in *Y. gloriosa* displayed differential expression in well-watered vs. drought-stressed conditions, compared to only one connection in *Y. aloifolia* and none in *Y. filamentosa*.

Metabolomics

Of the 214 metabolites that were present in at least 25% of samples, 87 had a significant fit to a polynomial regression line (Fig. 4), with 16 having significant differences in either abundance or temporal regulation between the *Y. aloifolia* and *Y. filamentosa* (R^2>0.5) (Supplemental Table S4). Starch degradation is one possible route CAM plants can use for the nightly regeneration of PEP. Whilst starch content overall was comparable between the C_3 and CAM species, there was no net dark depletion of starch in the CAM species, suggesting little reliance on starch for nocturnal generation of PEP in the CAM Yucca (Fig. 5A). In contrast, starch is degraded at night in the C_3 species and hybrid, with increased levels of α-glucan

phosphorylase (PHS), a gene responsible for phosphorolytic degradation of starch (Smith et al., 2005; Borland et al., 2016). Maltose levels, a starch-derived breakdown product, were substantially elevated in the C_3 species compared to the CAM (Fig. 5B). The difference in maltose content was reflected by higher expression of the maltose exporter MEX1 gene in *Y. filamentosa* (Fig. 5B). Malic acid was much higher in *Y. aloifolia* as expected (though without day-night variation, perhaps because of two missing time points), as was transcript abundance of malate dehydrogenase (MDH), responsible for interconversion of malic acid and oxaloacetate (Fig. 5C).

An alternative source of carbohydrates for PEP can come from soluble sugars. Several soluble sugars had higher abundance in *Y. filamentosa*, including fructose and glucose (Fig. 6A). Fructose (but not glucose or sucrose) had a significant temporal difference between *Y. aloifolia* and *Y. filamentosa* (Supplemental Figure S4), with concentrations in *Y. filamentosa* decreasing during the dark period while concentrations in *Y. aloifolia* remained flat. Both species accumulate similar amounts of sucrose (Fig. 6A), indicating no difference in the amount of hexoses dedicated to sucrose production. However, there is a slight temporal change across the day-night period in *Y. aloifolia*, but it was not significant based on polynomial regression analysis. Gene expression also does not implicate conversion of hexose to triose phosphates as a mechanism for generating differences in hexose concentrations: Both *Y. aloifolia* and *Y. filamentosa* express fructose 1,6-bisphosphate aldolase (FBA) at equivocal levels, although different gene copies are used in *Y. aloifolia* vs. *Y. filamentosa* (Fig. 6A) and *Y. filamentosa* has a significantly different temporal pattern to expression under both well-watered ($t(42) = -4.293$, $p = 8.99e-05$) and drought-stressed conditions ($t(42) = -6.79$, $p = 3.55e-08$). The different FBA paralogs in *Y. aloifoia* and *Y. gloriosa* compared to *Y. filamentosa* represent alternative

localizations; the FBA homolog expressed in *Y. filamentosa* has an *Arabidopsis* ortholog which localizes to the chloroplast, while the copy expressed in *Y. aloifolia* and *Y. gloriosa* has cytosol localized *Arabidopsis* ortholog. FBA in the chloroplast is responsible for the production of metabolites for starch synthesis, implicating starch synthesis and breakdown in *Y. filamentosa*, consistent with this species' increase in maltose production. The cytosolic version found in the CAM and C_3-CAM intermediate species is thought to be involved in glycolysis and glucogenesis. Triose phosphates are too small to measure through GC-MS metabolomics methods, but genes associated with interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (triose phosphate isomerase, TPI) as well as genes involved in transport (triose phosphate transporter, APE2) show higher expression in *Y. aloifolia* compared to both *Y. filamentosa* and *Y. gloriosa* (Fig. 6B), though genes do not significantly differ in temporal expression pattern based on post-hoc linear model tests. G3PDH (glyceraldehyde-3-phosphate dehydrogenase), a gene involved in downstream branches of glycolysis, likewise has highest expression in *Y. aloifolia;* accounting for species in the linear model of gene expression significantly increases fit of the model under both well-watered ($F_{(12,46)} = 2.45$, $p < 0.015$) and drought-stressed conditions ($F_{(12, 42)} = 4.94$, $p < 4.97e\text{-}5$).

Another class of metabolites with large differences between *Y. aloifolia* and *Y. filamentosa* were those involved in reactive oxygen species (ROS) scavenging pathways. Vitamin C, or ascorbic acid, was present at much higher levels in *Y. aloifolia* (Fig. 7A), as was the oxidized form dehydroascorbic acid (Fig. 7B). Neither had a temporal expression pattern, however, indicating constant levels of both metabolites across the day-night period. Previous work has implicated increases in ascorbic acid as a method for CAM plants to remove ROS produced by high nocturnal respiration (Abraham et al., 2016), but genes involved in

mitochondrial respiration (cytochrome-C, CYTC) are only slightly elevated in *Y. aloifolia* at night (Fig. 7C). The biogenesis of ascorbate through galactono-gamma-lactone dehydrogenase (GLDH) does not seem to differ between the two parental *Yucca* species either, based on gene expression (Fig. 7C). Alternatively ROS might be produced from daytime activities, however whether or not CAM plants reduce oxidative stress (via reduced photoihibition, (Adams and Osmond, 1988; Griffiths et al., 1989; Pieters et al., 2003)) or instead produce high levels of O_2 (from increased electron transport behind closed stomata, (Niewiadomska and Borland, 2008)) remains unclear in the literature. Regardless, the photorespiratory pathway has been proposed as a means of protection from oxidative stress (Kozaki and Takeba, 1996). Kinetic modeling of RuBisCO oxygenase/carboxylase activity suggested CAM plants have either equivalent levels of photorespiration to C_3 species or reduced by as much as 60% (Lüttge, 2010). An increase in antioxidants like ascorbic acid would therefore be beneficial in CAM plants if photorespiration is reduced relative to C_3 while O_2 and ROS are still produced at the same rate. In support of this hypothesis, phosphoglycolate phosphatase (PGP) – a gene involved in the first step in breaking down the photorespiratory product 2-phosphoglycolate – is elevated in *Y. filementosa* and *Y. gloriosa* relative to the CAM species (Fig. 7F). Metabolites that take part in photorespiration, including glycine and serine, peak in the day period in C_3 *Y. filamentosa*, and are generally much higher in *Y. filamentosa* than seen in *Y. aloifolia* (Fig. 4) (Scheible et al., 2000; Novitskaya et al., 2002), suggesting CAM *Y. aloifolia* may instead rely on increased ascorbic acid concentrations to reduce ROS stress.

Discussion

CAM pathway genes

Time-structured expression of key CAM genes in a C_3 species of *Yucca* suggests

ancestral expression patterns required for CAM may have predated its origin in *Yucca*. This important observation is in line with recent suggestions that the frequent emergences of CAM from C_3 photosynthesis was facilitated by evolution acting directly on a low flux pathway already in place for amino acid metabolism (Bräutigam et al., 2017). Flux analysis using ^{13}C labelled substrates has shown that C_3 plants can store organic acids at night to fuel daytime amino acid synthesis, and that a portion of the stored organic acids are decarboxylated during the day (Gauthier et al., 2010; Szecowka et al., 2013). This implies that the evolution of CAM just required increasing the flux capacity of existing nocturnal CO_2 uptake and day/night turnover of organic acids in C_3 plants, without the need for extensive re-wiring or diel rescheduling of flux capacity (Bräutigam et al., 2017). This concept is consistent with the notion that CAM may be described as a continuum or spectrum, with full CAM at one end, C_3 at the other, and various intermediate forms between (Winter et al., 2015). Further support for this 'CAM continuum' is provided by the transcriptome data presented here, with the C_3-CAM intermediate Y. gloriosa showing several genes with the levels of transcript abundance intermediate between those in the C_3 and CAM Yuccas.

Expression of carbonic anhydrases, which are thought to be required for conversion of CO_2 to HCO_3^- for carboxylation of PEP via PEPC, were heavily skewed toward the morning for both α-CA and β-CA types in all three species; although this gene expression pattern is not consistent with expected nocturnal requirements for CA, it is possible transcription and translation are separated by hours, or that CA proteins are able to subsist in the cell for long periods of time. The α-CA are typically not involved in CAM photosynthesis (but see (Heyduk et al., 2017)) and indeed are most highly expressed midday in the C_3 species. The β-CA gene family has highest expression just after lights turned on, though notably *not* in the CAM *Y.*

aloifolia. Despite the lack of a clear expression pattern implicating CA in CAM, members of both α-CA and β-CA are differentially expressed in the C_3-CAM *Y. gloriosa* and increase in expression under drought stress; this data alone suggests a functional role in CAM activity, since nocturnal fixation of CO_2 in the hybrid increases under drought stress.

Regulatory reconfigurations

Network analysis was conducted on clusters containing genes positively co-expressed with PEPC ("PEPC cluster") and negatively co-expressed with PEPC ("anti-PEPC"). *Yucca aloifolia* has few links between the PEPC cluster and the anti-PEPC cluster, suggesting little connectivity between the two clusters (Fig. 3C). Low connectivity between clusters suggests that PEPC is likely to be regulated by a few genes expressed at the same time (afternoon), rather than through a mix of positive and negative feedbacks from genes in both PEPC and anti-PEPC clusters; this result is consistent with the idea of clock regulation of CAM pathway genes acting on both nighttime and daytime expression. *Yucca gloriosa* had the shortest distance between the PEPC and anti-PEPC cluster, indicative of correlations between the two clusters that suggest some form of regulation and antagonistic gene interactions. As a hybrid, *Yucca gloriosa* may very well have novel genomic and phenotypic traits, including those influenced by the strength and consequences of gene interactions. Differences in regulation of CAM genes relative to the CAM parental species may be contributing to the limited amount of carbon fixed through CAM in *Y. gloriosa* compared to *Y. aloifolia* (Heyduk et al., 2016). Additionally, *Y. aloifolia*'s network consists of many larger hub genes (compare Fig. 3C and Fig. 3E), indicative of a more highly connected core network especially in the PEPC cluster. Hub genes with many connections are typically genes with a higher regulatory potential and importance in biological networks (Jeong et al., 2001; Hahn and Kern, 2005; Seo et al., 2009), and are also more likely to be functionally

constrained (Masalia et al., 2017). Genes with direct connections to PEPC varied in their function and response to drought stress between the three species, with the greatest number of connections occurring in the hybrid *Y. gloriosa* (Table 1). The hybrid also had the largest number of those connections responding to drought stress via differential expression, suggesting increased lability in the hybrid in general, and specifically with PEPC response to drought.

Carbohydrate metabolism

To provide the nightly supply of PEP needed as substrate for CO_2 + PEPC, CAM plants either break down soluble sugars (including polymers of fructose in fructans) or starches to regenerate PEP via glycolysis. Work in the closely related genus *Agave* indicates that soluble sugars are the main pool for nightly PEP regeneration (Abraham et al., 2016). Here we investigated differences in the metabolite profiles in C_3 *Y. filamentosa* and CAM *Y. aloifolia.* As seen in *Agave*, the CAM *Yucca* species uses soluble sugars as a carbohydrate reserve for PEP requirements, while C_3 *Y. filamentosa* likely relies on starch pools. Although starch concentrations were largely equal in *Y. aloifolia* and *Y. filamentosa*, degradation of starch to form maltose was significantly higher in *Y. filamentosa*. The lack of MEX1 and PHS1 expression in *Y. aloifolia* further suggests that starch degradation was recently lost (or gained in *Y. filamentosa*) as *Y. aloifolia* and *Y. filamentosa* diverged only 5-8MYA (Good-Avila et al., 2006; Smith et al., 2008). *Yucca gloriosa* has intermediate expression of MEX1 and PHS relative to its parental species, indicating some reliance on starch for carbohydrates like its C_3 parental species.

Soluble sugars, such as glucose, fructose, and sucrose, can serve as an alternative source of carbohydrates for glycolysis. In *Agave*, fructans (chains of fructose monomers) are the predominant source of nocturnal carbohydrates for PEP (Wang and Nobel, 1998; Arrizon et al., 2010). *Agave*, relative to *Arabidopsis*, has temporal regulation of soluble sugar production and a

10-fold increase in abundance (Abraham et al., 2016). In general there was a lack of diel turnover in soluble sugars in *Y. aloifolia*, although it is possible unmeasured fructans constitute the majority of the carbohydrate pool. With one exception neither species shows temporal fluctuation of abundance of soluble sugars (*Y. filamentosa* exhibits time-structured variation in fructose concentrations, Supplemental Figure S4). Sucrose concentrations are largely equal between the two species, while glucose and fructose are elevated in C_3 *Y. filamentosa*. Glucose and fructose are the building blocks of sucrose, but it is unclear from the metabolite and transcript data alone whether these are elevated in *Y. filamentosa* due to degradation of sucrose, or for some other purpose. Equivalent sucrose concentrations between the two species may suggests they are not the byproducts of sucrose degradation, and instead that these abundant sugar monomers are being used for processes in addition to sucrose production in the C_3 species.

Many of the genes involved in glycolytic processes had much higher expression in *Y. aloifolia*, suggesting that the breakdown of triose phosphates into PEP is occurring at a higher rate in CAM *Yuccas*. Fructose bisphosphate aldolase (FBA) acts as a major control point for glycolysis by converting fructose 1,6-bisphosphate into triose phosphates and is also involved in the reverse reaction in the Calvin Cycle (formation of hexose from triose phosphates). FBA expression is initially high in both parental species (Fig. 6A), then rapidly drops in the C_3 species and is sustained throughout the day period in both *Y. aloifolia* and *Y. gloriosa*, although alternative copies of this gene are used in CAM and C_3 parental species. FBA is thought to be driven toward triose phosphate production within the cytosol (the gene copy expressed in the CAM species), whereas the chloroplastic copy expressed in the C_3 species is involved in Calvin Cycle carbohydrate synthesis. Therefore it is likely that while *Y. aloifolia* expresses FBA for production of triose phosphates for glycolysis and PEP regeneration, *Y. filamentosa* uses the

reverse reaction to synthesize greater concentrations of soluble sugars.

In total, metabolite data and gene expression suggest soluble sugar pools in and of themselves are not the critical part of carbon metabolism for CAM in *Yucca*; instead, it is more likely that flux through the system, particularly through glycolysis, is important for the maintenance of PEP and thus effective CAM function. The seeming variation in *which* carbohydrate pool is used – starch for C_3, soluble sugars for CAM – is surprising, given the relatively short evolutionary distance between the two species. The functional importance of large glucose and fructose accumulation and retention in *Y. filamentosa* relative to *Y. aloifolia* is unclear. Roles for the hexoses glucose and fructose in C_3 plants include hormonal signaling (Zhou et al., 1998; Arenas-Huertero et al., 2000; Leon and Sheen, 2003), plant growth and development (Miller and Chourey, 1992; Weber et al., 1997), and gene expression regulation (Koch, 1996); because CAM plants undergo all of the same metabolic processes, the stark difference in concentrations of these hexoses in C_3 and CAM *Yucca* remains to be investigated. Similarly, studies to describe the parental C_3 and CAM species metabolomes behave under drought stress, as well as the metabolic profile of the C_3-CAM *Yucca* hybrid, will provide a greater understanding for the links between metabolites, carbon metabolism, and photosynthesis.

Antioxidant response in CAM

Previous work in *Agave* discovered high levels of ascorbate and NADH activity relative to C_3 *Arabidopsis*, and was thought to be due to increases in mitochondrial activity at night in CAM species relative to C_3 (Abraham et al., 2016). Similarly, *Yucca aloifolia* has much higher levels of ascorbic acid and dehydroascorbic acid relative to its C_3 sister species, but gene expression of GLDH, which is responsible for production of ascorbic acid, is not much different between the two parental species. The clear difference in abundance of ascorbic acid between C_3

and CAM *Yucca* does imply differential need for antioxidant response between the two species. Respiration rates might be expected to be higher in CAM species at night to sustain the active metabolism. Although citric acid abundance is nearly identical in C_3 and CAM *Yucca* species, expression of cytochrome-C, a part of the mitochondrial electron transport chain, is higher in the CAM *Y. aloifolia*. Alternatively, due to inhibited photorespiratory response in the CAM species, an alternative form of ROS scavenging may be needed to regulate oxidation in the cells resulting from either photoinhibition or O_2 accumulation from electron transport behind closed stomata during the day. It is possible CAM species are using antioxidant metabolites like ascorbic acid to prevent oxidative stress, rather than relying on photorespiration. Indeed genes (PGP) and metabolites (glycine and serine) involved in photorespiration were more lowly expressed and found in lower abundance, respectively, relative to C_3 *Y. filamentosa.* Whether or not increased antioxidant response is required for CAM to efficiently function in plants is unknown, and future work discerning ROS production and mitigation – particularly in the hybrid *Y. gloriosa* – will inform understanding of the role of ROS scavenging and its impact on photosynthetic functions.

Conclusions

Transcriptomics and metabolomics of the parental species *Y. aloifolia* and *Y. filamentosa* revealed many changes to regulation, expression, and abundance. The most notable differences included degree of expression of core CAM genes and fundamental differences between the C_3 and CAM species in starch and soluble sugar metabolism. Most notably, the increased reliance on soluble sugars in the CAM species which is not shared with the C_3 *Y. filamentosa* indicates a recent alteration to carbohydrate metabolism after the divergence of these two species and coincident with reliance on the CAM pathway. The diploid hybrid species, *Y. gloriosa*, exhibited gene expression profiles more similar to its CAM parent, *Y. aloifolia*, than the C_3 parent, *Y.*

filamentosa. At the same time differences in CAM-associated gene expression correlation networks inferred for *Y. gloriosa* and *Y. aloifolia* suggest that CAM genes may not be regulated in the same way in these species. Additionally, the CAM species *Y. aloifolia* had heightened antioxidant response (both in metabolites and gene expression) relative to *Y. filamentosa*, indicating that the operation of CAM imposes a significant oxidative burden, relative to that in C_3. Despite these differences, similarities exist in levels of gene expression of a few CAM genes (PEPC, for example) between the C_3 and CAM *Yuccas* studied here, perhaps indicated shared traits in an ancestral genome that may have facilitated the parallel evolution of CAM photosynthesis multiple times within the Agavoideae. These results are in line with recent work suggesting that CAM lineages exploited existing low-level pathways for organic acid accumulation and daytime decarboxylation present in C_3 species (Bräutigam et al., 2017). Future work on metabolomics, as well as detailed sampling of a variety of genotypes of *Y. gloriosa*, will help us better understand the evolution of CAM-related regulatory networks and the evolutionary link between carbohydrate metabolism and photosynthesis.

Materials and Methods

Plant material and RNA sequencing

RNA was collected during experiments described in (Heyduk et al., 2016), with geographic locality information in Supplemental Table S1. Briefly, clones of the three species of *Yucca* were acclimated to growth chambers with a day/night temperature of 30/17ºC and 30% humidity in ~3L pots filled with a 60:40 mix of soil:sand. One clone was kept well-watered for 10 days while the second clone was subjected to drought stress via dry down beginning after the end of day 1. Clones of a genotype were randomly assigned to watered and drought treatment. On the 7th day of the experiment, after plants had water withheld for the five previous days, RNA

was sampled every four hours, beginning one hour after lights turned on, for a total of 6 time points; very old and very young leaves were avoided, and samples were taken from the mid-section of the leaf blade from leaves that were not shaded. RNA biological replicates consisted of three genotypes of *Y. gloriosa* and four genotypes of both *Y. filamentosa* and *Y. gloriosa* (Supplemental Table S1). Due to size limitations of growth chambers, genotypes from the three species were randomly assigned to three different growth chamber experiments conducted in July 2014, October 2014, and February 2015. Well-watered and drought stressed clonal pairs were measured in the same experimental month. RNA was isolated from a total of 130 samples (n=36, 47, and 47 for *Y. aloifolia*, *Y. filamentosa*, and *Y. gloriosa*, respectively) using Qiagen's RNeasy mini kit (www.qiagen.com). DNA was removed from RNA samples with Ambion's Turbo DNAse, then assessed for quality on an Agilent Bioanalyzer 2100. RNA libraries were constructed with 1ug of input RNA using Kapa Biosystem's stranded mRNA kit and a dual-index barcoding scheme. Libraries were quantified via qPCR then randomly combined into 4 pools of 30-36 libraries for PE75 sequencing on the NextSeq 500 at the Georgia Genomics Facility.

Assembly and read mapping

Reads were cleaned using Trimmomatic (Bolger et al., 2014) to remove adapter sequences, as well as low-quality bases and reads less than 40bp. After cleaning and retaining only paired reads, *Y. aloifolia* had 439,504,093 pairs of reads, *Y. filamentosa* had 675,702,853 pairs of reads, and *Y. gloriosa* had 668,870,164 pairs. Due to the sheer number of reads for each species, a subset of reads was used to construct reference assemblies for each species. Randomly down-sampling 14% of the total reads for each species was used, resulting in an average of 83 million pairs of reads per species for assembly. This prevented erroneous reads from piling up to

create false support for a mis-sequenced basepair, and allowed for more efficient assembly (Haas et al., 2013). Trinity v. 2.0.6 (Grabherr et al., 2011) was used for digital normalization as well as assembly. The full set of reads from each species library were mapped back to that species' transcriptome assembly with Bowtie (Langmead et al., 2009); read mapping information was then used to calculate transcript abundance metrics in RSEM v.1.2.7 (Li and Dewey, 2011; Haas et al., 2013). Trinity transcripts that had a calculated FPKM < 1 were removed, and an isoform from a component was discarded if less than 25% of the total component reads mapped to it.

To further simplify the assemblies and remove assembly artifacts and incompletely processed RNA reads, the filtered set of transcripts for each species was independently sorted into orthogroups, or inferred gene families, that were circumscribed using OrthoFinder (Emms and Kelly, 2015) clustering of 14 sequenced genomes (*Brachypodium distachyon, Phalaenopsis equestris, Oryza sativa, Musa acuminata, Asparagus officinalis, Ananas comusus, Elaeis guiensis, Acorus americanus, Sorghum bicolor, Vitis vinifera, Arabidopsis thaliana, Carica papaya, Solanum lycopersicum*, and *Amborella tricopoda).* First, transcripts were passed through Transdecoder (http://transdecoder.github.io/), which searches for open reading frames in assembled RNA-sequencing data. Transdecoder reading frame coding sequences for each species were individually matched to a protein database derived from gene models from the monocot genome dataset using BLASTx (Altschul et al., 1990). Best hit for each query sequence was retained and used to sort the *Yucca* transcript into the same orthogroup as the query sequence. Assembled *Yucca* sequences were further filtered to retain only putatively full length sequences; *Yucca* transcripts that were shorter than the minimum length of an orthogroup were removed. Transdecoder produces multiple reading frames per transcript, so only the longest was retained. Scripts for orthogroup sorting and length filtering are available at

www.github.com/kheyduk/RNAseq/orthoSort. Read counts for the final set of orthogrouped transcripts were re-calculated and analyzed in EdgeR (Robinson et al., 2010) in R 3.2.3, using TMM normalization to produce normalized read counts.

To assess variation between genotypes sequenced, SNPs were calculated from the RNAseq data by mapping reads from each genotype of all species to the *Y. aloifolia* transcriptome, which is the least heterozygous of the three species. SNPs were compiled using the *mpileup* command of samtools, followed by filtering in using bcfutils. SNP positions had to have coverage between 8 and 1000, and have at least 2 alleles to be used. Indels were ignored. Similarity between genotypes and species was assessed via PCA method using the SNPRelate (Zheng et al., 2012) package in R 3.2.3.

Expression analysis of differentially expressed genes

For each species, visual representations of read data were used to assess and remove outlier libraries. Two outliers for *Y. aloifolia* were removed, as they were divergent from biological replicates as determined by a multidimensional scaling plot of all data (not shown). Similarly, *Y. gloriosa* had two samples removed, while *Y. filamentosa* had a single outlier. In a given *Yucca* species, all libraries were separated by time point, then Student's T-test between treatments (watered and drought) was conducted in EdgeR to find the number of up and down regulated genes at each time point, using a p-value cutoff of 0.05 and adjusting for multiple testing with a Holm-Bonferroni correction. Gene Ontology annotations for individual genes were obtained from the TAIR10 ontology of each gene family's *Arabidopsis* members. GO enrichment tests were conducted for each time point per species, comparing GO categories of DE genes in well-watered vs. drought-stressed treatments, using a hypergeometric test within the phyper base function in R 3.2.3.

Temporal profile clustering of gene expression

To assess larger patterns in the expression data while taking into account temporal patterns across time points, we employed maSigPro (Conesa et al., 2006), using options for read count data (Nueda et al., 2014). maSigPro is a two-step algorithm for profile clustering; the first step involves finding transcripts with non-flat time series profiles by testing generalized linear models with time and treatment factors (using a negative binomial error distribution) against a model with only an intercept (y~1); the second step involves assessing the goodness of fit for every transcript's regression model and assessing treatment effects. For GLM models, maSigPro estimates goodness of fit by evaluating the percent of the deviance the model explains (but it is still denoted as R^2). This two-step method is advantageous in that in rapidly reduces a large number of transcripts to only those that show significant variation across time, and it also readily allows users to select transcripts that have a clear expression profile (as assessed by goodness of fit of the model). For the *Yucca* data, gene regression models were initially computed on transcripts mapped per million (TMM) normalized read counts with a Bonferroni-Holm corrected significance level of 0.05. Gene models were then assessed for goodness of fit via the T.fit() function, which produces a list of influential genes whose gene models are being heavily influenced by a few data points (in this case, samples). Those genes were removed, and regression models and fit were re-calculated. Genes with significant treatment effects can either have a) different regression coefficients for the two treatments or b) different intercepts (i.e., magnitude of expression) between the two treatments. To cluster transcripts with similar profiles, we employed fuzzy clustering via the "mfuzz" option in maSigPro. The clustering steps require a user-defined value for *k* number of clusters. We assessed the optimal number of clusters for each species' data by examining the within group sum of squares for *k*=1:20 clusters. A *k* was chosen

where the plot has a bend or elbow, typically just before the group sum of squares levels off for higher values of *k.* A *k* of 9, 12, and 15 was used for *Y. aloifolia*, *Y. gloriosa*, and *Y. filamentosa*, respectively. To estimate *m*, the "fuzzification parameter" for fuzzy clustering, we employed the mestimate() function in the Mfuzz package. *m* of 1.06, 1.05, and 1.05 was estimated for *Y. aloifolia*, *Y. gloriosa*, and *Y. filamentosa.*

By default, the clustering steps in maSigPro are run on genes that are not only significant with regards to temporal expression (non-flat profiles across time), but also only on the subset of genes that are significantly different between treatments. We modified the code for the see.genes() function to fuzzy cluster all transcripts that had non-flat profiles, regardless of whether they showed a significant change in expression as a result of drought stress. Afterwards, we found transcripts that were significantly different between treatments with an R-squared cut off of 0.7. The modified code for the see.genes() function, as well as detailed guide to the steps taken for this analysis, are available at www.github.com/kheyduk/RNAseq.

For genes of interest, additional tests were done to assess whether species differed significantly in their temporal pattern of expression. Because count data cannot be accurately compared between species, we instead used TPM values. For each gene family of interest, we selected a single transcript per species, typically one that was highest expressed and had time-structured expression as determined by maSigPro. TPM values were scaled within each species' transcript, separately for well-watered and drought-stressed libraries, by the maximum TPM value. All TPM values for each gene for each treatment had a polynomial model fit with degree=5 without distinguishing species, and a second polynomial model that included species as a factor. Using ANOVA, we compared the fits of the two models. For genes that had a significantly better fit when species was treated as a factor, we report the t-statistic and p-value

for the species that had a significant coefficient.

Networks were constructed for PEPC and “anti-PEPC” clusters – those that had the highest negative correlation (mean of the median expression of all transcripts in cluster A, correlated to mean of the median expression of all transcripts in cluster B) to the cluster containing PEPC in each species. In *Y. gloriosa*, two clusters had nearly identical correlation values to PEPC, so both were included in the anti-PEPC cluster. Networks were constructed in ARACNE (Margolin et al., 2006) without assigning transcripts as transcription factors; instead, all transcripts were inputted equally into the program. Resulting networks were visualized in Cytoscape (Shannon et al., 2003), with highly connected nodes (based on number of edges) having a large hub circle representation.

Gene annotation and gene tree estimation

All transcripts were first annotated by their membership in gene families; gene family annotations were based on the *Arabidopsis* sequences that belong to the gene family, using TAIR10 annotations. Putative transcription factors were annotated based on their *Arabidopsis* ortholog in the PlantTFDB v. 3.0 (Jin et al., 2014). To address homology of transcripts across species, gene trees were constructed from protein-coding sequences of gene families of interest, and included *Yucca* transcript sequences as well as the 14 angiosperm sequenced genomes. Nucleotide sequences were first aligned via PASTA (Mirarab et al., 2014). Gene trees were estimated via RAxML (Stamatakis, 2006) using 200 bootstrap replicates and GTRGAMMA nucleotide model of substitution.

Metabolomics

Tissue for metabolic analysis was harvested in a separate growth chamber experiment conducted in February 2017 at the University of Georgia greenhouses. Growth conditions in the

chamber were identical to conditions used when harvesting tissue for RNA-Seq (above), and plants used were the same genotype, but not the same clone, as for RNA-Seq. Because we expect hybrid genotypes to be more variable than either parental species, we sampled metabolites from only *Y. filamentosa* and *Y. aloifolia.* Samples were collected every 4 hours starting 1 hour after the lights turned on from 6 replicate plants per species; replicates were from different genotypic backgrounds (see Supplemental Table S1). Samples for metabolomics were only collected under well-watered conditions, and gas exchange data was collected concurrently to ensure plants were behaving as when RNA was collected previously (Supplemental Figure S1). Collected leaf tissue was flash frozen in liquid N_2 then stored at -80°C until samples were freeze-dried. A 1:1 mixture of MeOH and chloroform (400µL) was added to 10mg of freeze-dried, ball-milled (Mini-beadbeater, Biospec products Bartlesville OK, USA) tissue along with adonitol as an internal standard. Mixtures were sonicated for 30 minutes at 8-10°C, equilibrated to room temperature, and polar metabolites recovered by liquid phase partitioning after 200µL H_2O was added to the extract. Ten µL of the aqueous-methanol phase was dried and derivatized for GCMS by adding 15µL methoxyamine hydrochloride and incubating at 30°C for 30 minutes, then by adding 30µL MSTFA and incubating at 60°C for 90 minutes. Derivatized samples were analyzed via gas chromatography as in Frost et al (2012). Chromatograms were deconvoluted using AnalyzerPro (SpectralWorks, Runcom, UK). Peak identities were based on NIST08, Fiehnlib (Agilent Technologies, (Kind et al., 2009)), and in-house mass spectral libraries. Peak matching between samples was based on the best library match according to AnalyzerPro and retention index (Jeong et al., 2004). Initial metabolite peak calls were filtered first by the confidence level of their best library match (>0.5) and then by raw peak area (>1000). Filtered metabolite peak areas were then normalized based on adonitol peak areas. Standard curves were run for ascorbate,

sucrose, malic acid, and citric acid to determine absolute concentrations in umol/g of dry weight (Supplemental Figure S5).

Normalized values were imported into R v. 3.3.3 and, where appropriate, multiple metabolite peaks were summed to obtain a single value per metabolite. Time points 3 and 6 (last day time point and last night time point) were removed from analysis due to errors in derivitization steps. Remaining values were filtered for sample presence, retaining only metabolites that were found in at least 25% of all samples. The resulting 217 metabolites were imported into maSigPro, where we tested for time-structured expression using species as a treatment in the design matrix, allowing for polynomials with degree=3, and using a quasipoisson distribution in the glm model.

Accession numbers: The datasets generated and/or analyzed during the current study are available in NCBI's Short Read Archive (RNA-seq data, BioProject #PRJNA413947), or at github.com/karohey/RNAseq_Yucca/C_3-CAM (for count matrices and raw metabolite data).

Supplemental Material

Supplemental Table S1 - Genotypes sequenced through RNAseq

Supplemental Figure S1 - Gas exchange data for metabolomics samples.

Supplemental Table S2 - GO term enrichment

Supplemental Table S3 - Differentially expressed transcription factors

Supplemental Figure S2 - Decarboxylation gene expression

Supplemental Figure S3 - Carbonic anhydrases gene expression

Supplemental Table S4 - Significantly different metabolites between C_3 and CAM *Yucca*.

Supplemental Figure S4 - Temporally variable metabolite regressions

Supplemental Figure S5 - Calibrated concentrations of key metabolites

Acknowledgements: Authors would like to acknowledge support from the University of Georgia and the National Science Foundation (DEB 1442199).

Table 1 – Direct gene-to-gene connections to PEPC in each of the three species. Bolded annotations are genes that were differentially expressed in well-watered vs. drought conditions.

Yucca aloifolia (CAM)		*Yucca gloriosa* (C_3-CAM)		*Yucca filamentosa* (C_3)	
double Clp-N motif-containing P-loop nucleoside triphosphate hydrolase	AT2G40130	two-pore channel 1	AT4G03560	staurosporin and temperature sensitive 3-like A	AT5G19690
RING/U-box	AT2G25410	protein of unknown function	AT4G14270	amino acid permease 2	AT5G09220
DEAD/DEAH box helicase, putative	AT1G70070	pumilio 4	AT3G10360	phosphofructokinase family protein	AT1G76550
CCL/CCR-like	AT3G26740	DEA(D/H)-box RNA helicase	AT2G07750	CTC-interacting domain 9	AT3G14450
octicosapeptide/Phox/Bem1p	AT5G63130	**gigantea protein (GI)**	AT1G22770	tudor/PWWP/MBT superfamily protein	AT5G40340
RNA helicase	AT3G62310	**mechanosensitive channel of small conductance-like 10**	AT5G12080	polynucleotidyl transferase, ribonuclease H-like superfamily protein	AT3G25430
protein of unknown function	AT3G32930	Mitochondrial transcription termination factor	AT4G19650	NOP2A/oligocellula2	AT5G55920
plastid developmental protein DAG, putative	AT3G06790	phosphofructokinase 2	AT5G47810	translocon at the inner envelope membrane of chloroplasts 20	AT1G04940
phototropic-responsive NPH3	AT4G37590	**major facilitator superfamily protein**	AT5G14120		
ABI-1-like 1	AT2G46225	**BTB and TAZ domain protein 1**	AT5G63160		
		DNA/RNA polymerases	AT2G24120		
		NAC domain containing protein 1	AT1G56010		
		protein of unknown function DUF829	AT2G15695		
		dual specificity protein phosphatase-related	AT4G18593		

Figure 1 – A) CAM pathway diagram. CA, carbonic anhydrase; PEP, phosphoenolpyuvate; PEPC, PEP carboxylase; PPCK, PEPC kinase; OAA, oxaloacetate; NADP-me, NADP-malic enzyme; PEPCK, PEPC carboxykinase; PPDK, orthophosphate dikinase. B) Net CO_2 accumulation on the same samples used for RNA-seq, with error bars representing 1 standard deviation from the mean. C) Delta H+ (the total titratable acid accumulated during the night) measured on samples used for RNA-seq from well-watered ("W") and drought-stressed conditions ("D"). Both gas exchange and titratable acidity plots are modified from data published in (Heyduk et al., 2016).

Figure 2 – A) PCA of SNP diversity from transcriptome data, and B) up/down differential expression between well-watered and drought stressed plants at each time point based on EdgeR, with counts as a proportion of total transcripts expressed.

Figure 3 – Heatmaps and gene trees of PEPC (A) and PPCK (B) in the day (white bar) and night (black bar), under both well-watered (blue bar) and drought-stressed (red bar) conditions. Maximum TPM for each gene shown below the gene tree; asterisks indicate transcripts with expression profiles that fit a polynomial (time-structured) regression model significantly better than a simple linear model and red coloring indicates a significant treatment effect. Some genes have low expression values and are displayed with faint shading, but can still have detectable time-structured expression. For PEPC, only the gene family that had high and temporal expression in *Yucca* is shown. Gene tree circles are color coded by species (black=*Y. aloifolia* (CAM), white=*Y. filamentosa* (C_3), light grey=*Y. gloriosa* (C_3-CAM). C-E) Network on PEPC (blue) and anti-PEPC (yellow) gene clusters in *Y. aloifolia* (C), *Y. filamentosa* (D) and *Y. gloriosa* (E), where larger nodes indicate more highly connected genes.

Figure 4 – Heatmap of abundance of the 89 metabolites that could be fit to a polynomial regression, shown for each species during the day (white bar) and night (black bar).

Figure 5 – Gene expression and related metabolites, shown over a day (white bar) and night (black bar) period, under both well-watered (blue bar) and drought-stress (red bar) in RNA-seq data only. Maximum TPM for each gene shown below the gene tree; asterisks indicate transcripts that were significantly time-structured, with red coloring indicating differential expression between watered and drought. Gene tree circles are color coded by species (black=*Y. aloifolia* (CAM), white=*Y. filamentosa* (C_3), light grey=*Y. gloriosa* (C_3-CAM). The colors are carried to the metabolite plots (black bars=*Y. aloifolia*, white bars=*Y. filamentosa*). A) Starch synthase 1 (SS1), involved in the production of starch; glucan phosphorylase (PHS), involved in degradation of starch B) Maltose exporter 1 (MEX1), transports maltose out of plastids. C) Malate dehydrogenase (MDH), responsible for interconversion of oxaloacetate and malic acid.

Figure 6 – A) Gene expression and related metabolites, shown over a day (white bar) and night (black bar) period, under both well-watered (blue bar) and drought-stress (red bar) in RNA-seq data only. Maximum TPM for each gene shown below the gene tree; asterisks indicate transcripts that were significantly time-structured, with red coloring indicating differential expression between watered and drought. Gene tree circles are color coded by species (black=*Y. aloifolia* (CAM), white=*Y. filamentosa* (C_3), light grey=*Y. gloriosa* (C_3-CAM). The colors are carried to the metabolite plots (black bars=*Y. aloifolia*, white bars=*Y. filamentosa*). A) Fructose bisphosphate aldolase, responsible for interconversion of fructose-6-P and triose phosphates, and sucrose phosphatase (SPP) produces sucrose from glucose and fructose molecules. B) Triose phosphate isomerase (TPI) interconverts the two forms of triose phosphates, APE2 is a triose phosphate transporter out of the plastid, and G3PDH is involved glycolysis.

Figure 7 – Abundance over the day (white bar) and night (black bar) period for A) ascorbic acid, B) dehydroascorbic acid, and C) citric acid. Gene expression for D) cytochrome-C (CYTC), E) galactono-gamma-lactone dehydrogenase (GLDH), and F) phosphoglycolate phosphatase (PGP) over the day (white bar) and night (black bar), under both well-watered (blue bar) and drought-stressed (red bar) conditions. Maximum TPM for each gene shown below the gene tree; asterisks indicate transcripts that were significantly time-structured, with red coloring indicating differential expression between watered and drought. Gene tree circles are color coded by species (black=*Y. aloifolia* (CAM), white=*Y. filamentosa* (C_3), light grey=*Y. gloriosa* (C_3-CAM). The colors are carried to the metabolite plots (black bars=*Y. aloifolia*, white bars=*Y. filamentosa*).

Literature Cited

Abraham PE, Yin H, Borland AM, Weighill D, Lim SD, De Paoli HC, Engle N, Jones PC, Agh R, Weston DJ, et al (2016) Transcript, protein and metabolite temporal dynamics in the CAM plant Agave. Nat Plants **2**: 16178

Adams WW, Osmond CB (1988) Internal CO(2) Supply during Photosynthesis of Sun and Shade Grown CAM Plants in Relation to Photoinhibition. Plant Physiol **86**: 117–23

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215**: 403–10

Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, León P (2000) Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev **14**: 2085–96

Arrizon J, Morel S, Gschaedler A, Monsan P (2010) Comparison of the water-soluble carbohydrate composition and fructan structures of Agave tequilana plants of different ages. Food Chem **122**: 123–130

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**: 2114–20

Borland AM, Guo H-B, Yang X, Cushman JC (2016) Orchestration of carbohydrate processing for crassulacean acid metabolism. Curr Opin Plant Biol **31**: 118–124

Borland AM, Hartwell J, Jenkins GI, Wilkins MB, Nimmo HG (1999) Metabolite Control Overrides Circadian Regulation of Phosphoenolpyruvate Carboxylase Kinase and CO2 Fixation in Crassulacean Acid Metabolism. Plant Physiol **121**: 889–896

Borland AM, Hartwell J, Weston DJ, Schlauch KA, Tschaplinski TJ, Tuskan GA, Yang X, Cushman JC (2014) Engineering crassulacean acid metabolism to improve water-use

efficiency. Trends Plant Sci **19**: 327–38

Borland AM, Wullschleger SD, Weston DJ, Hartwell J, Tuskan GA, Yang X, Cushman JC (2015) Climate-resilient agroforestry: physiological responses to climate change and engineering of crassulacean acid metabolism (CAM) as a mitigation strategy. Plant Cell Environ **38**: 1833–1849

Bräutigam A, Schlüter U, Eisenhut M, Gowik U (2017) On the Evolutionary Origin of CAM Photosynthesis. Plant Physiol **174**: 473–477

Brilhaus D, Bräutigam A, Mettler-Altmann T, Winter K, Weber APM (2016a) Reversible Burst of Transcriptional Changes during Induction of Crassulacean Acid Metabolism in Talinum triangulare. Plant Physiol **170**: 102–22

Brilhaus D, Bräutigam A, Mettler-Altmann T, Winter K, Weber APM (2016b) Reversible Burst of Transcriptional Changes during Induction of Crassulacean Acid Metabolism in Talinum triangulare. Plant Physiol **170**: 102–22

Carter PJ, Nimmo HG, Fewson CA, Wilkins MB (1991) Circadian rhythms in the activity of a plant protein kinase. EMBO J **10**: 2063–8

Chen L-S, Lin Q, Nose A (2002) A comparative study on diurnal changes in metabolite levels in the leaves of three crassulacean acid metabolism (CAM) species, Ananas comosus, Kalanchoe daigremontiana and K. pinnata. J Exp Bot **53**: 341–350

Conesa A, Nueda MJ, Ferrer A, Talón M (2006) maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. Bioinformatics **22**: 1096–1102

Cushman JC, Agarie S, Albion RL, Elliot SM, Taybi T, Borland AM (2008a) Isolation and Characterization of Mutants of Common Ice Plant Deficient in Crassulacean Acid

Metabolism. Plant Physiol. 147:

Cushman JC, Bohnert HJ (1997) Molecular Genetics of Crassulacean Acid Metabolism. Plant Physiol **113**: 667–676

Cushman JC, Davis SC, Yang X, Borland AM (2015) Development and use of bioenergy feedstocks for semi-arid and arid lands. J Exp Bot **66**: 4177–4193

Cushman JC, Tillett RL, Wood JA, Branco JM, Schlauch KA (2008b) Large-scale mRNA expression profiling in the common ice plant, Mesembryanthemum crystallinum, performing C3 photosynthesis and Crassulacean acid metabolism (CAM). J Exp Bot **59**: 1875–94

Dever L V, Boxall SF, Kneřová J, Hartwell J (2015) Transgenic perturbation of the decarboxylation phase of Crassulacean acid metabolism alters physiology and metabolism but has only a small effect on growth. Plant Physiol **167**: 44–59

Dodd AN, Borland AM, Haslam RP, Griffiths H, Maxwell K (2002) Crassulacean acid metabolism: plastic, fantastic. J Exp Bot **53**: 569–580

Dodd AN, Griffiths H, Taybi T, Cushman JC, Borland AM (2003) Integrating diel starch metabolism with the circadian and environmental regulation of Crassulacean acid metabolism in Mesembryanthemum crystallinum. Planta **216**: 789–797

Emms DM, Kelly S (2015) OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol **16**: 157

Frost CJ, Nyamdari B, Tsai C-J, Harding SA, Ende W Van den (2012) The Tonoplast-Localized Sucrose Transporter in Populus (PtaSUT4) Regulates Whole-Plant Water Relations, Responses to Water Stress, and Photosynthesis. PLoS One **7**: e44467

Gauthier PPG, Bligny R, Gout E, Mahé A, Nogués S, Hodges M, Tcherkez GGB (2010) In

folio isotopic tracing demonstrates that nitrogen assimilation into glutamate is mostly independent from current CO2 assimilation in illuminated leaves of Brassica napus. New Phytol **185**: 988–999

Good-Avila S V, Souza V, Gaut BS, Eguiarte LE (2006) Timing and rate of speciation in Agave (Agavaceae). Proc Natl Acad Sci U S A **103**: 9124–9

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson D a, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol **29**: 644–52

Griffiths H, Ong BL, Avadhani PN, Goh CJ (1989) Recycling of respiratory CO2 during Crassulacean acid metabolism: alleviation of photoinhibition in Pyrrosia piloselloides. Planta **179**: 115–122

Gross SM, Martin JA, Simpson J, Abraham-Juarez MJ, Wang Z, Visel A (2013) De novo transcriptome assembly of drought tolerant CAM plants, Agave deserti and Agave tequilana. BMC Genomics **14**: 563

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M, et al (2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc **8**: 1494–512

Hahn MW, Kern AD (2005) Comparative Genomics of Centrality and Essentiality in Three Eukaryotic Protein-Interaction Networks. Mol Biol Evol **22**: 803–806

Hartwell J (2005) The co-ordination of central plant metabolism by the circadian clock. Biochem Soc Trans **33**: 945–8

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG (1996) Higher plant

phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. Plant J **10**: 1071–1078

Heyduk K, Burrell N, Lalani F, Leebens-Mack J (2016) Gas exchange and leaf anatomy of a C3-CAM hybrid, Yucca gloriosa (Asparagaceae). J Exp Bot **67**: 1369–1379

Heyduk K, Ray JN, Ayyampalayam S, Leebens-Mack J (2017) Shifting gene expression profiles associated with the evoultion of Crassulacean acid metabolism. Am. J. Bot. In review:

Holtum J, Osmond C (1981) The Gluconeogenic Metabolism of Pyruvate During Deacidification in Plants With Crassulacean Acid Metabolism. Aust J Plant Physiol **8**: 31

Jeong H, Mason SP, Barabási a L, Oltvai ZN (2001) Lethality and centrality in protein networks. Nature **411**: 41–42

Jeong ML, Jiang H, Chen H-S, Tsai C-J, Harding SA (2004) Metabolic profiling of the sink-to-source transition in developing leaves of quaking aspen. Plant Physiol **136**: 3364–75

Jin J, Zhang H, Kong L, Gao G, Luo J (2014) PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. Nucleic Acids Res **42**: D1182–D1187

Kind T, Wohlgemuth G, Lee DY, Lu Y, Palazoglu M, Shahbaz S, Fiehn O (2009) FiehnLib: Mass Spectral and Retention Index Libraries for Metabolomics Based on Quadrupole and Time-of-Flight Gas Chromatography/Mass Spectrometry. Anal Chem **81**: 10038–10048

Kluge M, Ting IP (1978) Morphology, anatomy, and ultrastructure of CAM plants. *In* WD Billings, F Golley, OL Lange, JS Olson, eds, Crassulacean Acid Metab. Springer-Verlag, Berlin, pp 29–38

Koch KE (1996) Crabohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol **47**: 509–540

Kozaki A, Takeba G (1996) Photorespiration protects C3 plants from photooxidation. Nature **384**: 557–560

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol **10**: R25

Leon P, Sheen J (2003) Sugar and hormone connections. Trends Plant Sci **8**: 110–116

Li B, Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics **12**: 323

Lüttge U (2010) Photorespiration in Phase III of Crassulacean Acid Metabolism: Evolutionary and Ecophysiological Implications. Springer, Berlin, Heidelberg, pp 371–384

Margolin AA, Nemenman I, Basso K, Wiggins C, Stolovitzky G, Dalla Favera R, Califano A (2006) ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. BMC Bioinformatics **7 Suppl 1**: S7

Masalia RR, Bewick AJ, Burke JM, Irizarry R, Bertranpetit J, Laayouni H (2017) Connectivity in gene coexpression networks negatively correlates with rates of molecular evolution in flowering plants. PLoS One **12**: e0182289

Miller ME, Chourey PS (1992) The Maize Invertase-Deficient miniature-1 Seed Mutation Is Associated with Aberrant Pedicel and Endosperm Development. Plant Cell Online 4:

Ming R, VanBuren R, Wai CM, Tang H, Schatz MC, Bowers JE, Lyons E, Wang M-L, Chen J, Biggers E, et al (2015) The pineapple genome and the evolution of CAM photosynthesis. Nat Genet **47**: 1435–42

Mirarab S, Nguyen N, Warnow T (2014) PASTA: Ultra-Large Multiple Sequence Alignment. *In* R Sharan, ed, Res. Comput. Mol. Biol. 18th Annu. Int. Conf. RECOMB 2014, Pittsburgh, PA, USA, April 2-5, 2014, Proc. Springer International Publishing, Cham, pp

177–191

Nelson EA, Sage RF (2008) Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression. J Exp Bot **59**: 1841–50

Nelson EA, Sage TL, Sage RF (2005) Functional leaf anatomy of plants with crassulacean acid metabolism. Funct Plant Biol **32**: 409

Niewiadomska E, Borland AM (2008) Crassulacean Acid Metabolism: a Cause or Consequence of Oxidative Stress in Planta? Prog. Bot. Springer, Berlin, Heidelberg, pp 247–266

Nimmo HG (2000) The regulation of phosphoenolpyruvate carboxylase in CAM plants. Trends Plant Sci **5**: 75–80

Novitskaya L, Trevanion SJ, Driscoll S, Foyer CH, Noctor G (2002) How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. Plant, Cell Environ **25**: 821–835

Nueda MJ, Tarazona S, Conesa A (2014) Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. Bioinformatics **30**: 2598–2602

Pieters AJ, Tezara W, Herrera A (2003) Operation of the Xanthophyll Cycle and Degradation of D1 Protein in the Inducible CAM plant, Talinum triangulare, under Water Deficit. Ann Bot **92**: 393–399

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**: 139–40

Scheible W-R, Krapp A, Stitt M (2000) Reciprocal diurnal changes of phosphoenolpyruvate carboxylase expression and cytosolic pyruvate kinase, citrate synthase and NADP-isocitrate

dehydrogenase expression regulate organic acid metabolism during nitrate assimilation in tobacco leaves. Plant, Cell Environ **23**: 1155–1167

Schulze S, Mallmann J, Burscheidt J, Koczor M, Streubel M, Bauwe H, Gowik U, Westhoff P (2013) Evolution of C4 photosynthesis in the genus flaveria: establishment of a photorespiratory CO2 pump. Plant Cell **25**: 2522–35

Seo CH, Kim J-R, Kim M-S, Cho K-H (2009) Hub genes with positive feedbacks function as master switches in developmental gene regulatory networks. Bioinformatics **25**: 1898–1904

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res **13**: 2498–504

Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC (2010) Evolution along the crassulacean acid metabolism continuum. Funct Plant Biol **37**: 995

Smith AM, Zeeman SC, Smith SM (2005) Starch degradation. Annu Rev Plant Biol **56**: 73–98

Smith CI, Pellmyr O, Althoff DM, Balcázar-Lara M, Leebens-Mack J, Segraves KA (2008) Pattern and timing of diversification in Yucca (Agavaceae): specialized pollination does not escalate rates of diversification. Proc Biol Sci **275**: 249–58

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22**: 2688–90

Szecowka M, Heise R, Tohge T, Nunes-Nesi A, Vosloh D, Huege J, Feil R, Lunn J, Nikoloski Z, Stitt M, et al (2013) Metabolic fluxes in an illuminated Arabidopsis rosette. Plant Cell **25**: 694–714

Taybi T, Nimmo HG, Borland AM (2004) Expression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxylase kinase genes. Implications for genotypic capacity

and phenotypic plasticity in the expression of crassulacean acid metabolism. Plant Physiol **135**: 587–98

Taybi T, Patil S, Chollet R, Cushman JC (2000) A minimal serine/threonine protein kinase circadianly regulates phosphoenolpyruvate carboxylase activity in crassulacean acid metabolism-induced leaves of the common ice plant. Plant Physiol **123**: 1471–82

Wang N, Nobel PS (1998) Phloem Transport of Fructans in the Crassulacean Acid Metabolism Species Agave deserti. Plant Physiol **116**: 709–14

Weber H, Borisjuk L, Heim U, Sauer N, Wobus U (1997) A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. Plant Cell Online 9:

Winter K, Holtum JAM, Smith JAC (2015) Crassulacean acid metabolism: a continuous or discrete trait? New Phytol. doi: 10.1111/nph.13446

Yang L, Lu M, Carl S, Mayer JA, Cushman JC, Tian E, Lin H (2015a) Biomass characterization of Agave and Opuntia as potential biofuel feedstocks. Biomass and Bioenergy **76**: 43–53

Yang X, Cushman JC, Borland AM, Edwards EJ, Wullschleger SD, Tuskan GA, Owen NA, Griffiths H, Smith JAC, De Paoli HC, et al (2015b) A roadmap for research on crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. New Phytol **207**: 491–504

Zambrano VAB, Lawson T, Olmos E, Fernández-García N, Borland AM (2014) Leaf anatomical traits which accommodate the facultative engagement of crassulacean acid metabolism in tropical trees of the genus Clusia. J Exp Bot **65**: 3513–23

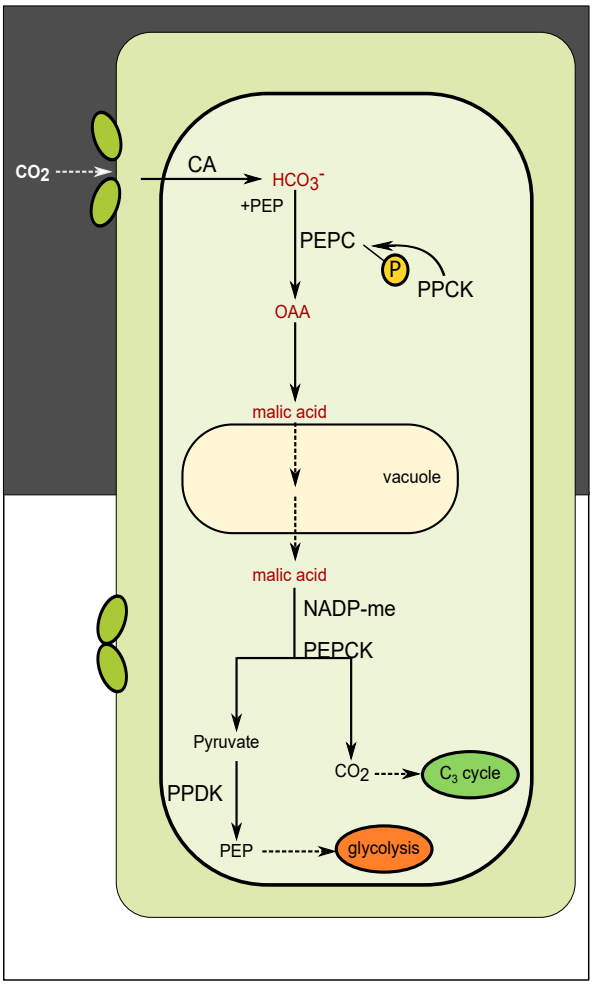
Zhang J, Liu J, Ming R (2014) Genomic analyses of the CAM plant pineapple. J Exp Bot **65**:

3395–404

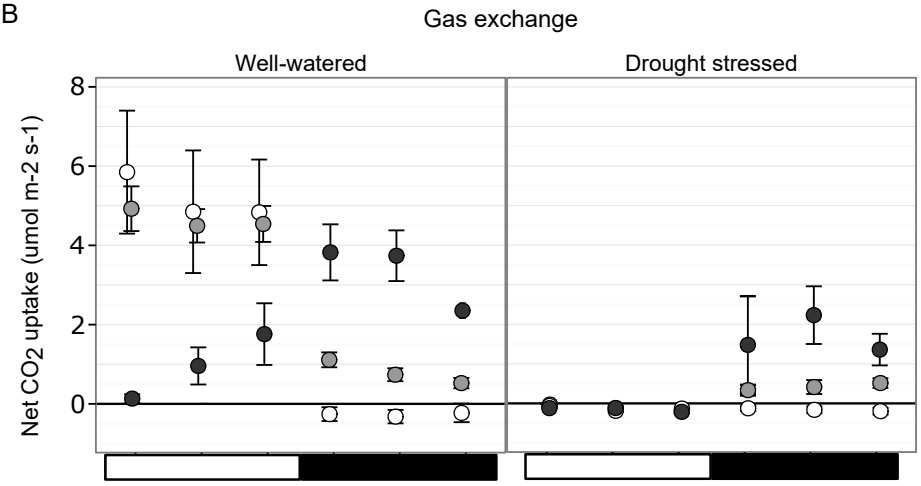
Zheng X, Levine D, Shen J, Gogarten SM, Laurie C, Weir BS (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. Bioinformatics **28**: 3326–3328

Zhou L, Jang JC, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci U S A **95**: 10294–9

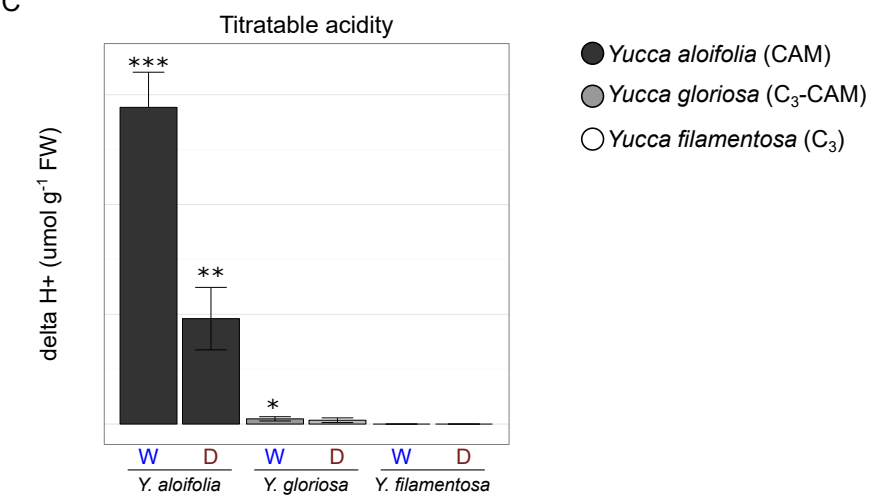
A

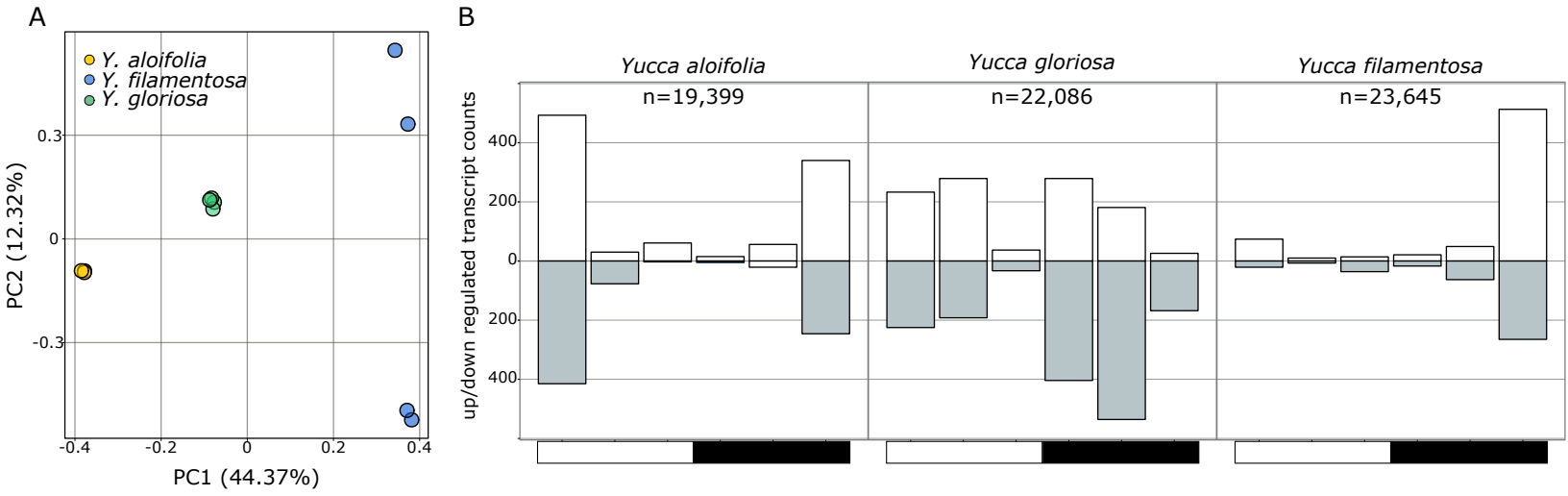

CO2
CA
HCO3-
+PEP
PEPC
P
PPCK
OAA
malic acid
vacuole
malic acid
NADP-me
PEPCK
Pyruvate
CO2
C3 cycle
PPDK
PEP
glycolysis

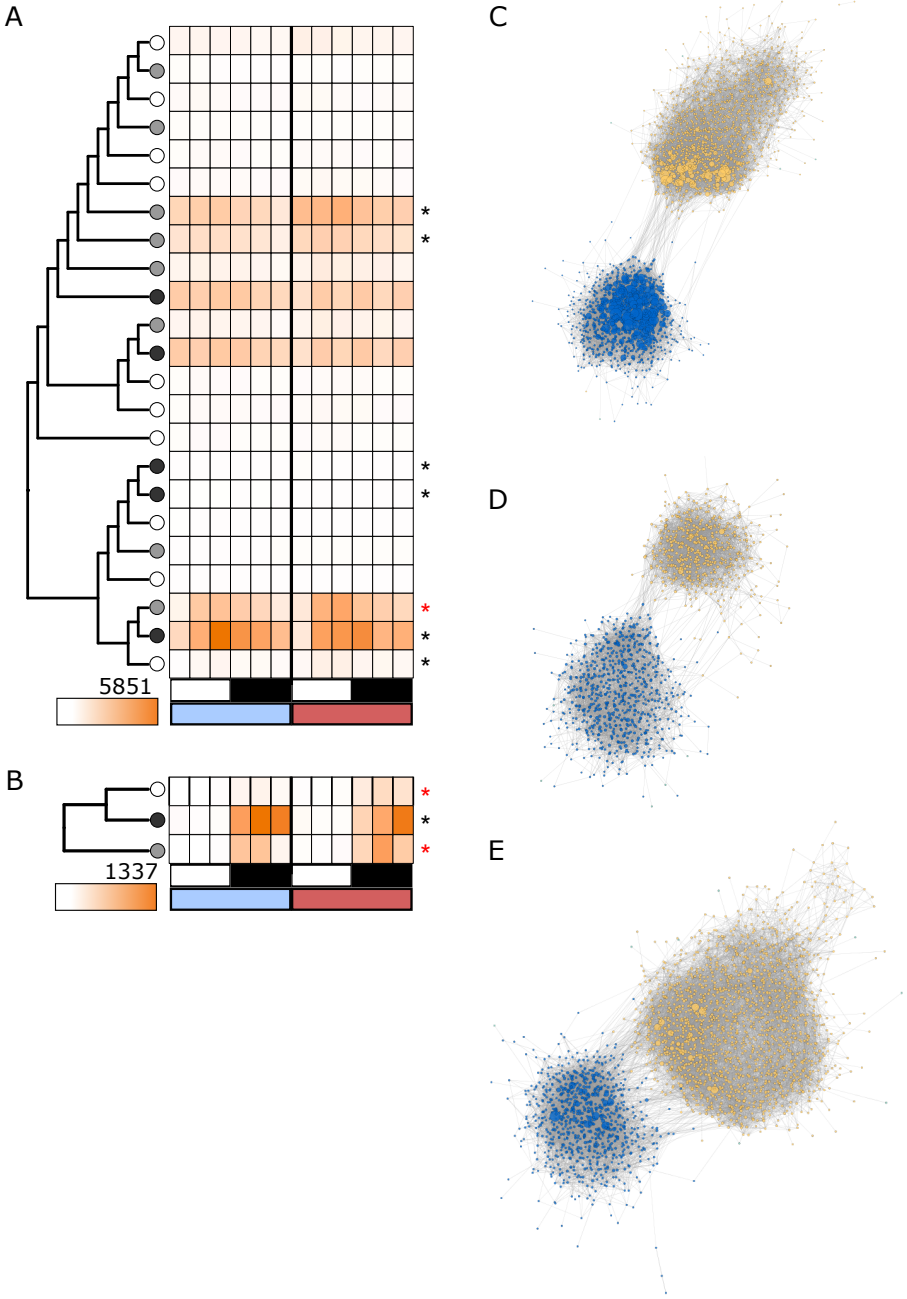
B

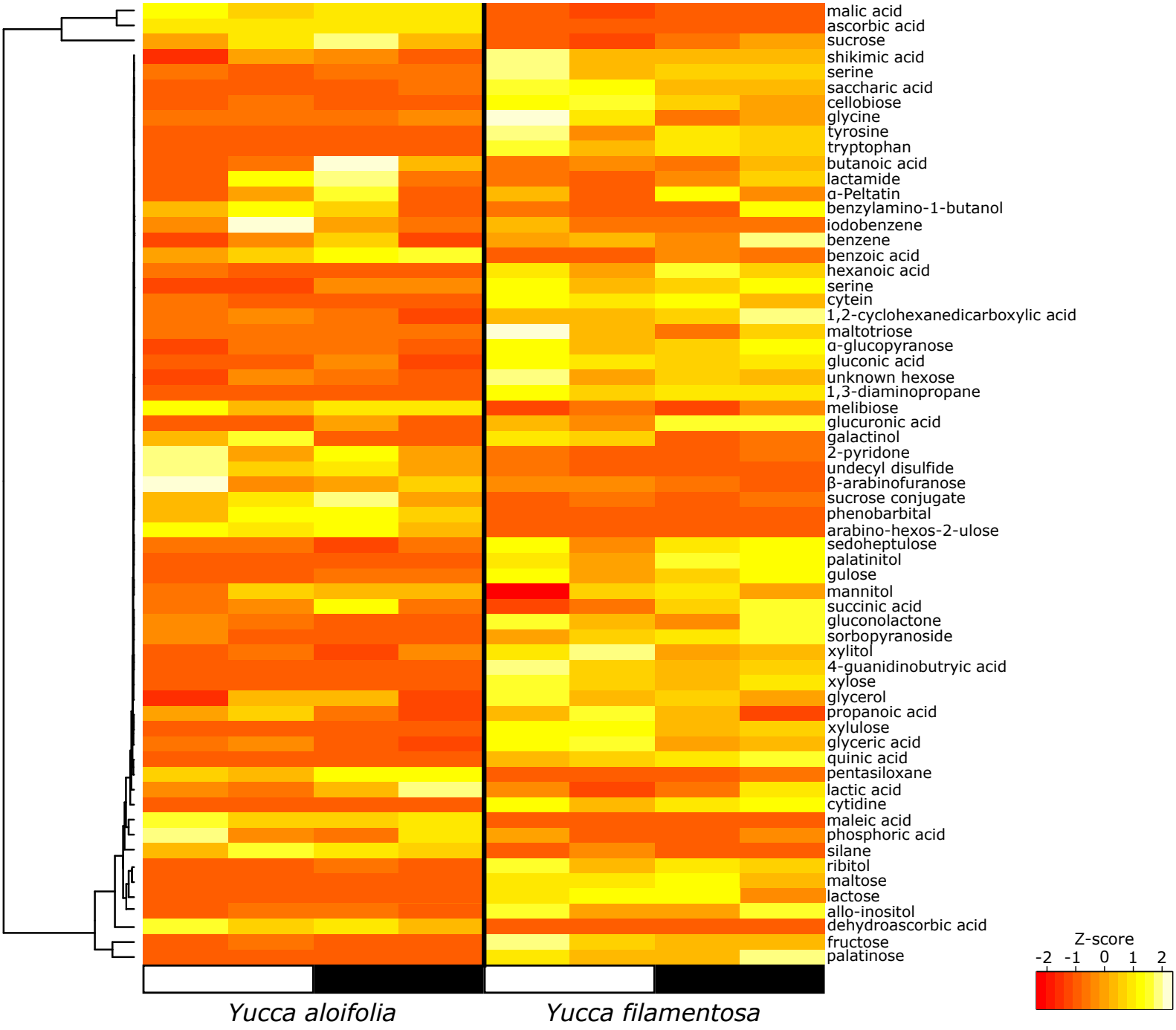

Gas exchange
Well-watered
Drought stressed
Net CO2 uptake (umol m-2 s-1)
8
6
4
2
0

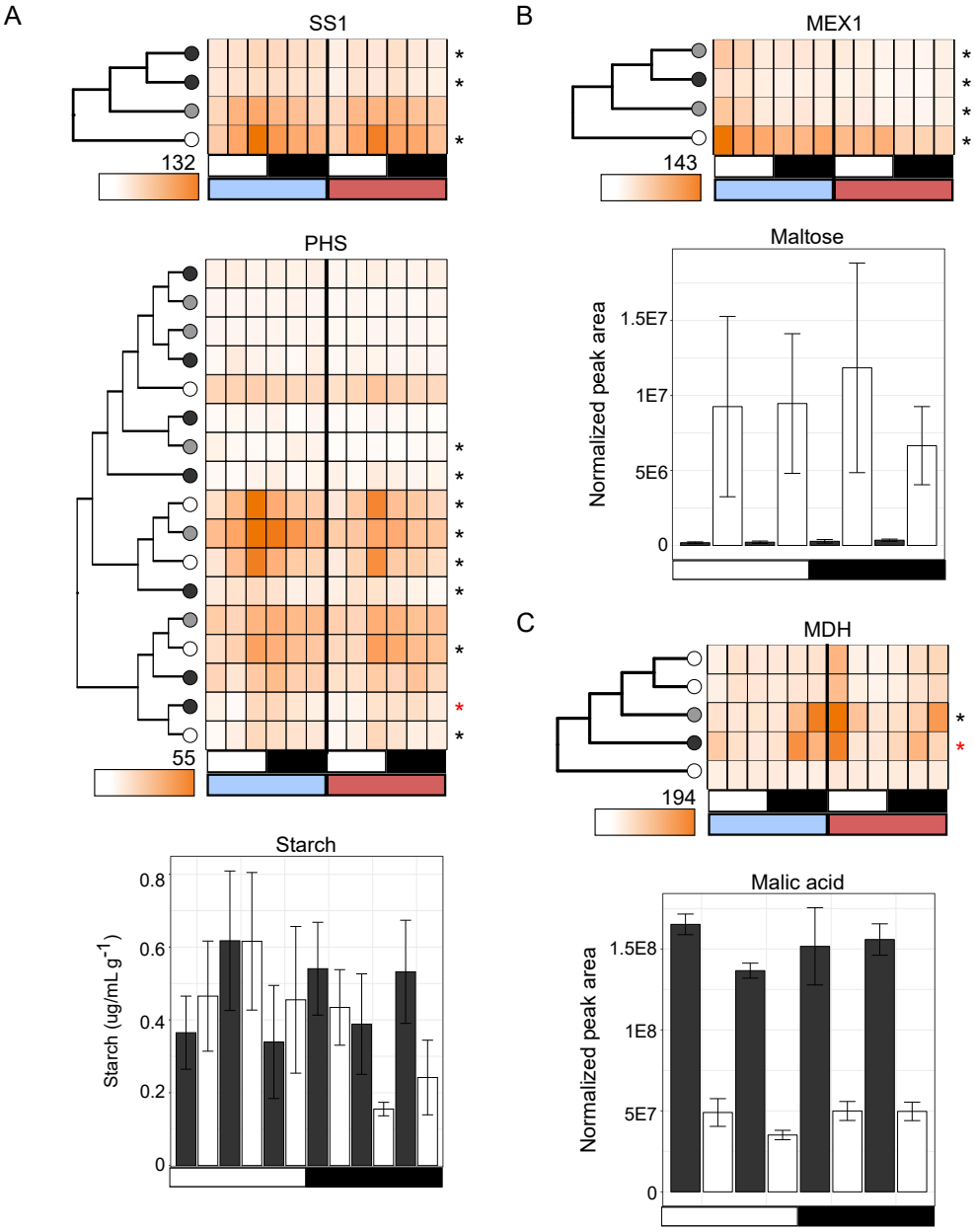
C

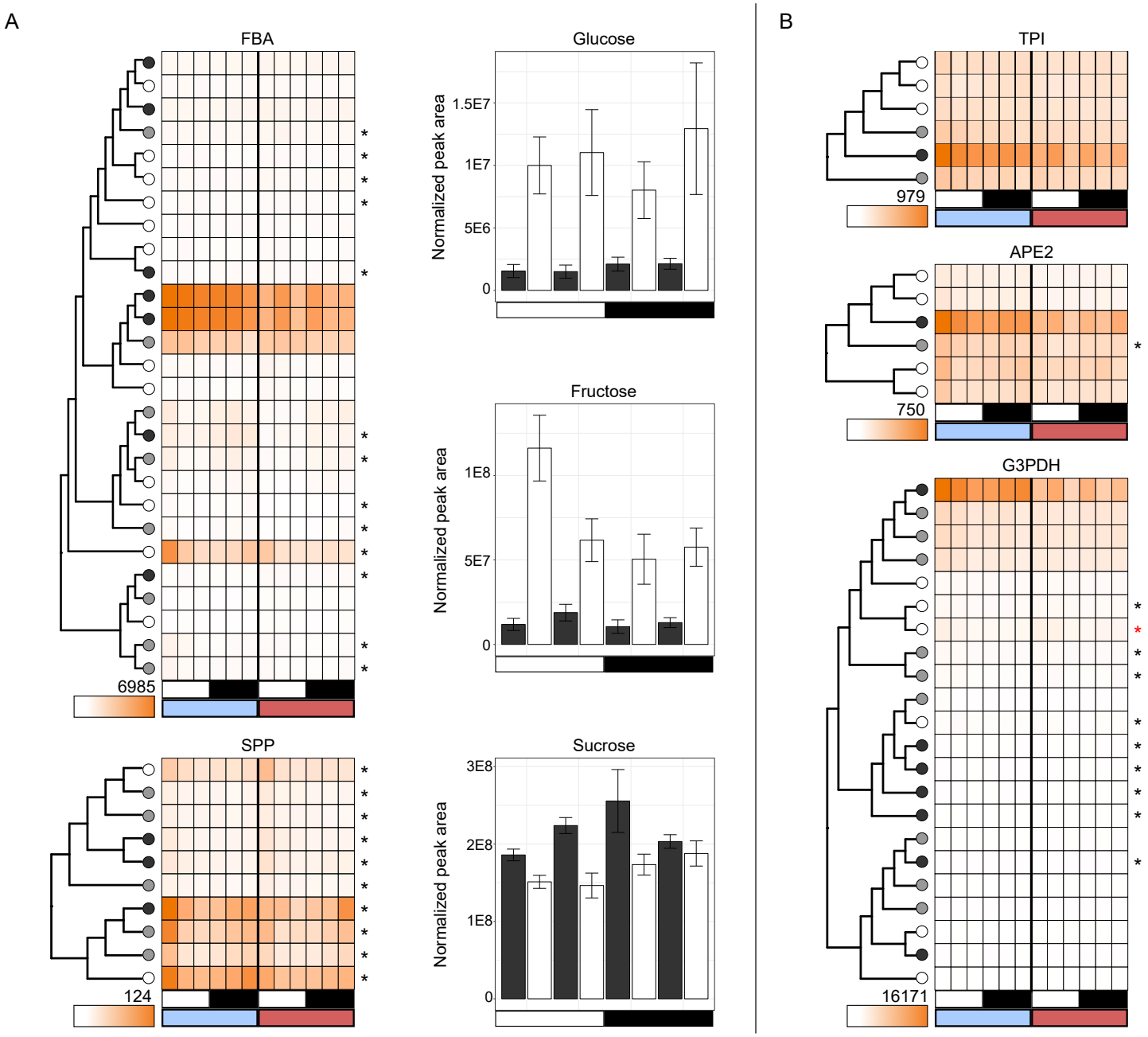

Titratable acidity
delta H+ (umol g-1 FW)
***
**
*
W
D
W
D
W
D
Y. aloifolia
Y. gloriosa
Y. filamentosa
Yucca aloifolia (CAM)
Yucca gloriosa (C3-CAM)
Yucca filamentosa (C3)

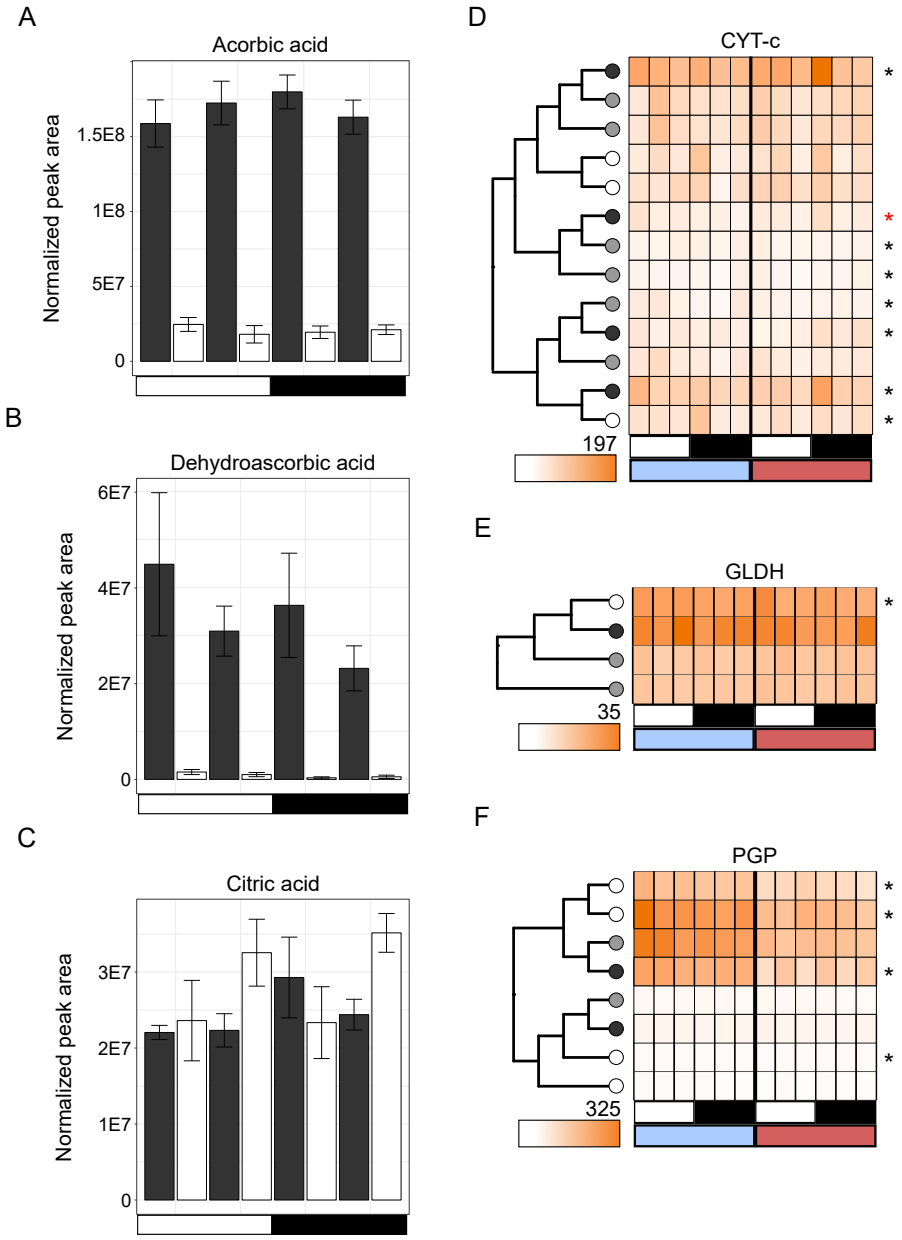

A
Y. aloifolia
Y. filamentosa
Y. gloriosa
PC2 (12.32%)
PC1 (44.37%)
0.3
0
-0.3
-0.4
-0.2
0
0.2
0.4
B
Yucca aloifolia
n=19,399
Yucca gloriosa
n=22,086
Yucca filamentosa
n=23,645
up/down regulated transcript counts
400
200
0
200
400


A
5851
B
1337
C
D
E


malic acid
ascorbic acid
sucrose
shikimic acid
serine
saccharic acid
cellobiose
glycine
tyrosine
tryptophan
butanoic acid
lactamide
α-Peltatin
benzylamino-1-butanol
iodobenzene
benzene
benzoic acid
hexanoic acid
serine
cytein
1,2-cyclohexanedicarboxylic acid
maltotriose
α-glucopyranose
gluconic acid
unknown hexose
1,3-diaminopropane
melibiose
glucuronic acid
galactinol
2-pyridone
undecyl disulfide
β-arabinofuranose
sucrose conjugate
phenobarbital
arabino-hexos-2-ulose
sedoheptulose
palatinitol
gulose
mannitol
succinic acid
gluconolactone
sorbopyranoside
xylitol
4-guanidinobutryic acid
xylose
glycerol
propanoic acid
xylulose
glyceric acid
quinic acid
pentasiloxane
lactic acid
cytidine
maleic acid
phosphoric acid
silane
ribitol
maltose
lactose
allo-inositol
dehydroascorbic acid
fructose
palatinose
Z-score
-2 -1 0 1 2
Yucca aloifolia
Yucca filamentosa


A
SS1
132
PHS
55
Starch
Starch (ug/mL g-1)
0.8
0.6
0.4
0.2
0
B
MEX1
143
Maltose
Normalized peak area
1.5E7
1E7
5E6
0
C
MDH
194
Malic acid
Normalized peak area
1.5E8
1E8
5E7
0


A
FBA
6985
SPP
124
Glucose
Fructose
Sucrose
Normalized peak area
1.5E7
1E7
5E6
0
1E8
5E7
3E8
2E8
B
TPI
979
APE2
750
G3PDH
16171


A
Acorbic acid
Normalized peak area
1.5E8
1E8
5E7
0
B
Dehydroascorbic acid
Normalized peak area
6E7
4E7
2E7
0
C
Citric acid
Normalized peak area
3E7
2E7
1E7
0
D
CYT-c
197
E
GLDH
35
F
PGP
325