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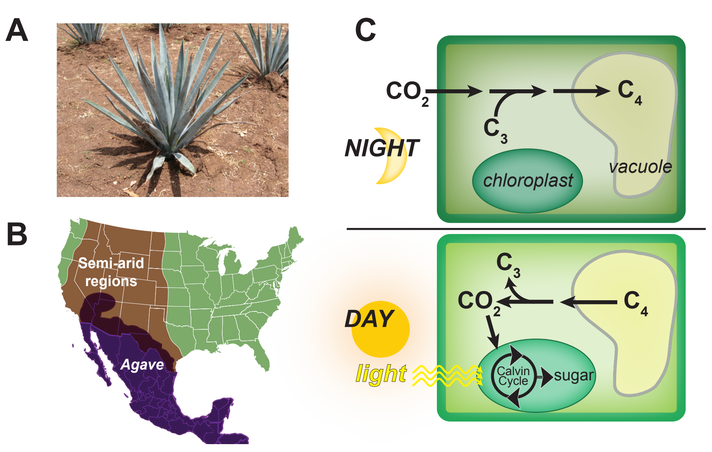
Abstract

Understanding drought and heat tolerance in plants will be critical for sustained agriculture and bioenergy production in a changing global climate. Because of their exceptional ability to thrive in arid, hot environments with minimal soil nitrogen, *Agave* species have been identified as a candidate bioenergy feedstock and provide a model for studies of adaptations to drought and heat. While some physiological mechanisms underlying these traits have been studied in detail, the molecular basis of the extreme heat and drought tolerance in these plants remains unclear. We have constructed *de novo* reference transcriptomes for *Agave tequilana*, an economically important species cultivated in Mexico for spirit distillation, and *A. deserti*, an extremely thermo- and drought-tolerant species native to the Colorado Desert, using 368 and 185 gigabasepairs, respectively, of Illumina short-read sequence data. These data sets enable cross-species comparative analysis of protein-coding sequences and tissue-specific gene expression levels. Based on these reference transcriptomes, we also perform quantitative expression profiling of agaves subjected to heat and drought stress in controlled greenhouse experiments, enabling a transcriptome-wide understanding of *Agave* stress responses. Complementary to plant-endogenous mechanisms, *Agave* plants are associated with microbes that may play important roles in mitigating abiotic stress in arid resource-limited environments. Using targeted sequence-based microbial community profiling, we study the microbiomes of cultivated *A. tequilana*, and wild *A. deserti* and *A. salmiana*. We also use deep metagenomic sequencing to gain further insight into *Agave* rhizosphere and phyllosphere microbe communities, and perform isolation and single-cell genome sequencing of individual microbial cells residing within *Agave* tissues. These studies aim to identify microbial species, genes, and pathways conferring additional stress resistance to agaves. Taken together, our work builds a robust platform to accelerate discovery of plant and plant-associated microbial adaptations to major abiotic stresses.

Overview

I. *Agave* can supplement other bioenergy feedstocks

Agave species, adapted to their native habitat in arid regions of Mexico and the United States, hold promise as a biofuel feedstock [1], capable of growing on marginal lands where other bioenergy plants cannot. The ability of agaves to withstand hot and arid conditions relies upon Crassulacean Acid Metabolism (CAM)—a specialized form of photosynthesis allowing agaves to keep leaf stomata (pores) closed during the day, minimizing water loss through evapotranspiration.



A. *Agave tequilana* cultivated in Mexico.

B. Semi-arid regions of the United States (brown) are unsuitable for cultivation of other bioenergy plants, which require temperate environments (green). Most *Agave* species are adapted to semi-arid regions in Mexico and the extreme southwestern USA (purple).

C. Crassulacean Acid Metabolism (CAM). CO_2 enters plant cells at night, joins with a 3-carbon molecule (C_3) and is stored in the vacuole as a 4-carbon molecule (C_4). During the day, C_4 molecules diffuse out of the vacuole, and CO_2 is relased and assimilated into sugar in the chloroplast.

II. Agaves are productive with minimal resources

Agaves are capable of producing lignocellulosic biomass with little water and nitrogen inputs. Species *A. salmiana* and *A. mapisaga* have been reported to produce up to 40 metric tonnes (Mg) of dry biomass per hectare per year [2].

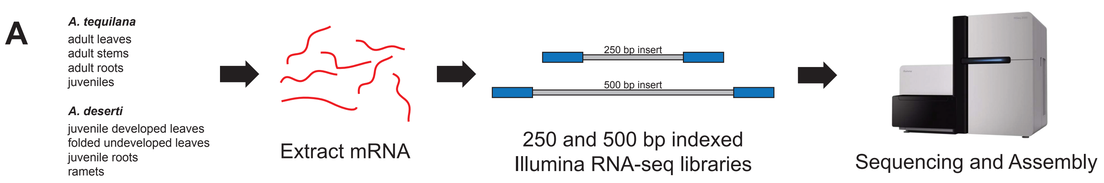
	Inputs			Outputs	
Feedstock	Water (cm yr^{-1})	Drought tolerance	Nitrogen (kg ha^{-1} yr^{-1})	Dry biomass (Mg ha^{-1} yr^{-1})	Ethanol (liters yr^{-1})
Corn grain	50–80	low	90–120	7–10	2900
Corn stover				3–6	900
Miscanthus	75–120	low	0–15	15–40	4600–12,400
Poplar coppice	70–105	moderate	0–50	5–11	1500–3400
***Agave* spp.**	**30–80**	**high**	**0–12**	**10–34**	**3000–10,500**

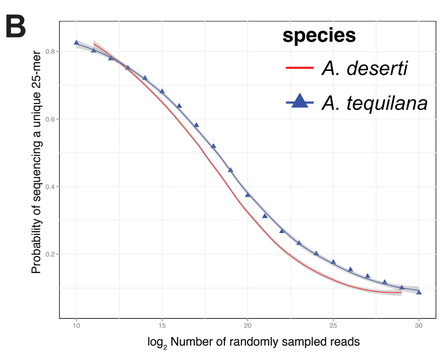
Comparison of inputs (water and nitrogen) and outputs (biomass and ethanol) of agaves and other biofuel feedstock species. Though agaves are harvested at several years of age, their annualized growth rate is comparable to *Miscanthus*. Table is modified from reference [4].

Building *Agave* reference transcriptomes

De novo assembly of *Agave* transcriptomes

Without sequence information, molecular studies of agaves are difficult. To address this need, we chose *A. tequilana*, which is currently cultivated for tequila production, and *A. deserti*, an extremely drough and heat tolerant agave, as our reference species. Agaves have large genomes (~4–7 Gb) [5, 6], so we focused on sequencing the protein-coding transcriptome using Illumina RNA-seq and *de novo* transcriptome assembly with Rnnotator [7], a *de novo* transcriptome assembly pipeline developed at JGI.





A. Illumina RNA-seq library production. Distinct tissues of agave were used as starting material. mRNA was purified from each tissue, fragmented to either 250 or 500 nt lengths, reverse transcribed into DNA, and ligated to Illumina indexed adaptors (blue). Our procedure preserves information about the mRNA source and strand specificity to aid transcriptome assembly.

B. Plot demonstrating sequencing depth, where the x-axis represents a number of randomly sampled Illumina reads, and the y-axis represents the probability of sequencing a unique 25-mer sequence.

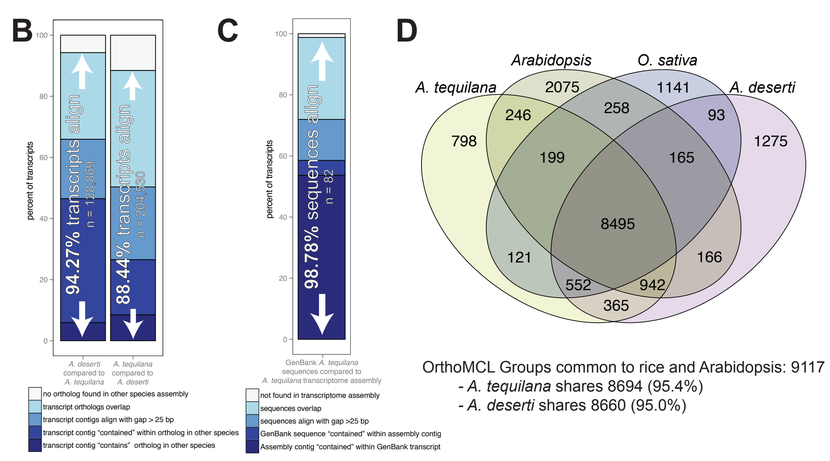
Robust transcriptome assemblies

A resource for *Agave* molecular biology

Transcriptome assemblies were filtered for artifacts and sequences from associated microorganisms. The resulting *Agave* transcriptomes, while not a 100% complete representation of *Agave* genes, contain a significant number of loci and encode the majority of proteins within other plant species.

A

Species dataset	Raw sequence	No. of loci	No. of transcripts	N50 transcript length	Assembled transcriptome size
A. tequilana	368 Gbp	139,525	204,530	1387 nt	204.8 Mbp
A. deserti	185 Gbp	88,718	128,869	1323 nt	125.0 Mbp



A. Summary of transcriptome assemblies for *A. tequilana* and *A. deserti*.

B. Comparing the *A. deserti* and *A. tequilana* transcriptomes by BLAT alignment. The majority of transcript in one species aligns to a transcript in the other.

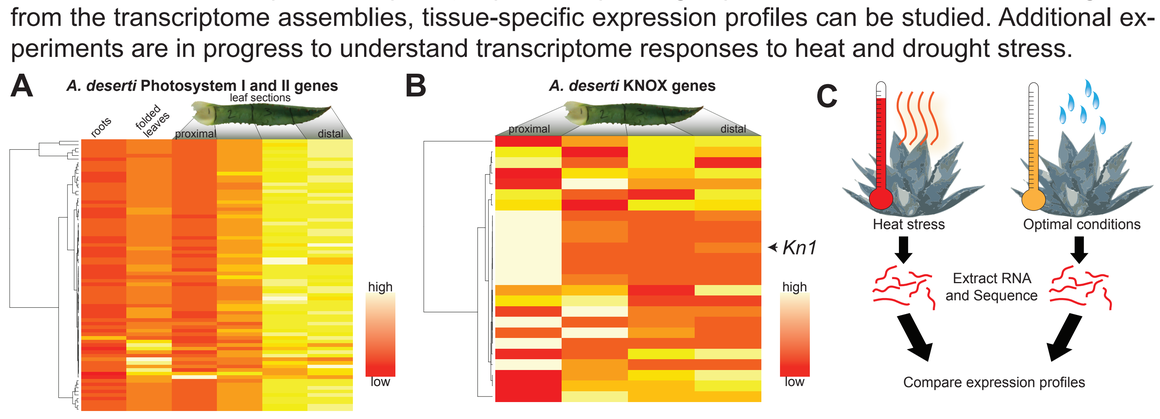
C. Alignment of 82 *A. tequilana* sequences in GenBank to the *A. tequilana* transcriptome assembly by BLAT. 81 of 82 sequences were found.

D. Shared OrthoMCL v5 [8] protein clusters between *Arabidopsis*, rice, *A. tequilana* and *A. deserti*.

Expression profiling of agaves

Adding functional data to annotation

With reference transcriptomes in place, expression profiling experiments can be initiated. Using data from the transcriptome assemblies, tissue-specific expression profiles can be studied. Additional experiments are in progress to understand transcriptome responses to heat and drought stress.



A. Heatmap of Photosystem I and II genes expressed in *A. deserti* roots, folded leaves, and sections along the proximal-distal axis of an unfolded leaf. Data indicates photosystem genes are most highly expressed in the distal half of the leaf, while the proximal end, lacking chlorophyll pigmentation, has relatively low expression of photosystem genes.

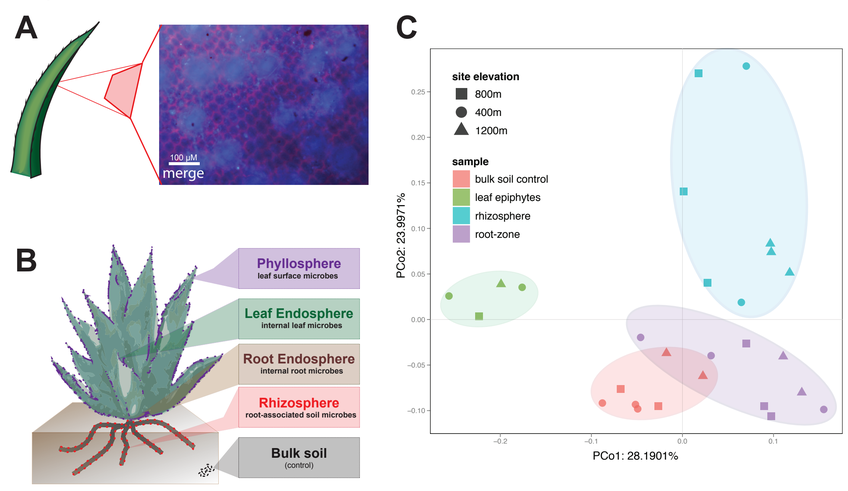
B. Heatmap of KNOX gene expression along the proximal-distal axis of an unfolded leaf. Arrow indicates the ortholog of *Zea mays Knotted1* (*Kn1*), expressed higher in proximal leaf sections than distal leaf sections.

C. Overview of ongoing expression profiling experiments, testing *A. tequilana* responses to prolonged drought and heat.

Agave microbiomes and adaptations to stress

Discovering the *Agave* microbial community

We have initiated sequence-based studies of the *A. tequilana, A. salmiana* and *A. deserti* microbiomes. Ultimately, we aim to identify microbes conferring stress and disease resistance to agaves. With both transcriptome and microbe sequences in hand, we will have a strong foundation for powerful plant-microbe interaction studies.



A. Microscopic examination of epiphytic microbes associated with the *Agave* phyllosphere (leaf surface). Red–stained plant cell walls, blue–epiphytic microbes.

B. Microbial communities of *Agave* to be examined by sequence-based techniques.

C. Fast UniFrac [9] PCoA analysis of *A. deserti* microbe communities collected during Summer 2011 in Southern California. Data demonstrate distinct microbial communities associated with different parts of the plant.

Acknowledgements and References

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