The Setaria Model: Past, Present and Future

Andrew Doust

Species in the C4 panicoid grass genus *Setaria* have been domesticated multiple times in various parts of the world, but the only present day cereal crop is foxtail millet (*S. italica*), domesticated in Northern China approximately 11,000 years ago. Foxtail millet is grown in multiple growing regions of China as well as other countries, and there is a rich literature in Chinese on the cultivation and breeding of the crop. Its wild ancestor, green foxtail (*S. viridis*) is a worldwide weed, and much research has been put into how to control it. An important milestone in introducing the Setaria system was the comparative mapping of a cross between foxtail millet and green foxtail that demonstrated how the nine chromosomes of Setaria mapped onto those of the other mapped grass genomes (Gale and Devos, PNAS, 1998). That mapping population has proved useful to many for the elucidation of morphological and physiological changes, especially those concerned with domestication. Diversity collections and new mapping populations are expanding the genetic resources available, and a concentrated focus on improving transformation efficiencies in green foxtail has produced a model system that can be a versatile testing ground for basic biological questions raised by those researching maize and other crops that are less easy to manipulate crops (Doust and Diao, Springer, 2017). An increased focus on nutrition and stress resistance with climate change has also elevated the visibility of foxtail millet, especially for variable growing conditions. Questions remain about how the burgeoning Setaria community might best plan its growth, especially since our largest base of users may well be researchers whose main focus is on other crops. Problems faced by the world-wide Setaria community include a relatively poor connection between breeders and researchers (unlike that which has served the maize community so well), and scattered and inaccessible germplasm collections. I suggest that an important focus of the relatively young Setaria research community outside China should be on establishing viable cooperative strategies, and I hope that this will explicitly addressed at this conference.

Foxtail millet germplasm core collection- a source of multi-trait variation for food and nutritional security

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Foxtail millet (*Setaria italica* (L.) P. Beauv.) is an ancient C4 annual crops of dryland agriculture, distributed in the warm and temperate regions of the world. It is used as grain, forage or bird feed. Foxtail millet is valued for its drought tolerance, short duration, and its grains are nutritionally superior to other cereals such as rice and wheat. It has large within species racial diversity with three races and ten subraces. Globally >46,000 germplasm accessions are conserved in genebanks. At ICRISAT, to enhance use of the germplasm, core (155 accessions) and mini core (35) collections were established and 37 sets (21 core, 16 mini core) shared with scientists in eight countries. New sources of resistance to blast disease (15 accessions), drought (16), and salinity (10), and 21 accessions each for high calcium, iron, zinc, and protein, 27 for earliness and 40 for grain yield and combinations of multiple traits identified in the core collection. Our national partners conducted multilocation evaluation and large scale demonstrations of selected germplasm lines for release directly as cultivars, contributing to income to farmers, and nutritional and food security besides on-farm conservation. Genetic

structure analyses using SNPs revealed that foxtail millet accessions are structured along both on the basis of races and geographic origin, and the maximum proportion of variation was attributable to among individuals within populations. Accessions of race Indica were less diverse and are highly differentiated from those of Maxima and Moharia. Association studies have revealed marker-trait association for economic traits.

Cereal roots enact austerity measures during drought to bank water

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Shoot-borne nodal roots often called crown roots form the bulk of the root systems in cereal crops such as maize and rice. While this post-embryonic root system represents the major conduit for water uptake, little is known regarding what effect water availability has on its development. Data demonstrate that in the newly developed cereal crop model plant *Setaria viridis*, the crown locally senses water availability and suppresses post-emergence crown root growth under water deficit. This response was observed in field and growth room environments and in all cereal species tested. Luminescence-based imaging of root systems grown in soil revealed a shift in root growth from crown to primary-root derived branches, suggesting that primary-root-dominated architecture can be induced in *S. viridis* under certain stress conditions. Crown roots of maize and *Setaria italica*, domesticated relatives of teosinte and *S. viridis*, respectively, show reduced sensitivity to water deficit, suggesting that this response may have been influenced by human selection. Enhanced water status of maize mutants lacking crown roots suggests that, under water deficit, stronger suppression of crown roots may actually benefit crop productivity. Several approaches including forward genetics screens are currently employed to explore the interaction between crown root development and water availability.

Engineering the Setaria Genome

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By linking genotype with phenotype, genome engineering holds great promise for advancing functional genomics. Genome engineering is particularly valuable in organisms like Setaria where there are limited or underdeveloped genetic resources. Efforts are underway to develop robust genome engineering protocols for Setaria to create targeted gene knockouts, gene replacements and gene insertions. Initial efforts have used Agrobacterium-mediated transformation to introduce gene-editing reagents to cells; edited events are then recovered in plants regenerated from tissue culture. Novel methods to deliver reagents to plants are also being developed, including the use of virus-derived vectors. Specifically, geminivirus-based replicons – from Wheat Dwarf Virus – are being used to amplify nuclease-encoding cassettes and DNA repair templates. In addition, an RNA virus – namely Foxtail Mosaic Virus – is being used to deliver sgRNAs to Cas9-expressing, transgenic Setaria plants. The hope is that meristems will become infected and mutagenized through infection, and mutant plants will then be recovered in the next generation. Finally, we have developed a comprehensive, integrated vector system and toolkit for gene editing in diverse plants, including Setaria.

Brassinosteroids modulate meristem fate and differentiation of unique inflorescence morphology in *Setaria viridis*

Jiani Yang, Shuiyi Thames, Hui Jiang, Norman B. Best, Pu Huang, Brian P. Dilkes, Andrea L. Eveland

Inflorescence architecture is a key determinant of yield potential in cereal crops and is patterned by placement and developmental fate of axillary meristems. In grasses, flowers and grain are borne from spikelets, which differentiate in the final iteration of axillary meristem branching. In *Setaria spp*., inflorescence branches terminate in either a spikelet or a sterile "bristle", and these structures appear to be paired. In this work, we leverage *Setaria viridis* to investigate a novel role for brassinosteroids (BRs), a class of phytohormones, in specifying bristle identity and maintaining spikelet meristem determinacy. We report the cloning and characterization of the *bristleless 1* (*bsl1*) locus in *Setaria*, which encodes a rate-limiting enzyme in BR biosynthesis. Loss-of-function *bsl1* mutants fail to initiate a bristle identity program, resulting in the formation of spikelets in place of bristles. In addition, spikelet meristem determinacy is altered in the mutants, which produce two florets per spikelet instead of one. Both of these phenotypes provide avenues for enhanced grain production in cereal crops. Our results indicate that precise spatiotemporal regulation of BR biosynthesis during inflorescence development is necessary for specific meristem fate decisions and provide novel insight into the molecular basis underlying morphological variation in inflorescence architecture.

Population genetics and genome-wide association studies of Chinese Setaria accessions

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China has been considered as the center of origin and improvement of foxtail millet (*Setaria italica*), and over 80% of the world's Setaria accessions are conserved in the National Gene Bank of China. Assessment of Setaria collections, including green foxtail (*Setaria viridis*) and foxtail millet, can help to reveal the domestication history and potential for improvement of cultivated foxtail millet. Recently, the molecular diversity, genetic structure, eco-geographical distribution and selection history of foxtail millet cultivars has been revealed through large scale germplasm characterization, genomic analysis, and genome-wide association mapping of QTLs controlling agronomic traits. These achievements will provide fundamental resources and a platform for both genetic research and genetic improvement of foxtail millet.

C4 gene discovery in grasses and functional validations using *S. viridis* as a model

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One critical challenge facing human society is a growing population and the potential for food and energy insecurity. Plant science will provide solutions to this challenge through gains in higher crop productivity, stress resilience and environmental sustainability. In the past decade, advances in DNA sequence technology have created new genomic resources. Here, I demonstrate how these resources can be utilized to advance our understanding of both basic and applied aspects of plant science. Specifically, I will focus on the development of a cross species selection scan and highlight an application of this method to identify candidate genes relating to C_4 metabolism. I will also show how *Setaria viridis* serves as an ideal platform for genetic dissection of C_4 photosynthesis, and opens the potential for future engineering efforts.

Development and application of novel phenotyping techniques to understand the genetic control of productivity and drought traits in the model C4 grass Setaria

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http://foxmillet.org/

The ability to cheaply and quickly phenotype large mapping populations of C4 grass crops for complex traits related to productivity and drought tolerance severely limits efforts to understand genotype-to-phenotype associations under field conditions. Here we report the development and application of methods to assess: (1) above-ground biomass production from hemispherical imaging; (2) stomatal patterning from optical tomography; (3) leaf nitrogen status and allometry from hyperspectral reflectance; (4) drought-induced leaf curling from hemispherical imaging; and (5) canopy temperature by infra-red imaging as a proxy for crop water use. We demonstrate that these methods successfully capture the same genotype by environment interactions and reproduce quantitative trait loci analyses as traditional methods that are slower and more expensive. The combination of these advances in phenotyping capability and new knowledge of the genetic architecture of productivity and drought traits creates a research platform that can now be applied to biosystems design of more productive and ecologically sustainable biofuel and bioproduct crops.

The Status of *Setaria viridis* Transformation Methodologies: *Agrobacterium*-mediated to Floral Dip

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An overview of the various methods that have been reported for *Setaria viridis* transformation will be provided. The Van Eck lab utilizes an *Agrobacterium tumefaciens* transformation method based on infection of seed-derived callus. In brief, mature seeds from which the seed coats have been removed are cultured on an MS-based callus induction medium (CIM) that contains 2,4-D and kinetin. Following infection of 6-week-old callus and a cocultivation period of 3 days, the callus is transferred to selective CIM. The vectors they use contain the hygromycin phosphotransferase II selectable marker gene driven by either *Panicum virgatum* or *Zea mays* ubiquitin promoters. They have used the *Agrobacterium*-mediated method to generate transgenic lines for functional analyses of genes by groups utilizing overexpression, RNAi, and CRISPR/Cas9 vectors. To explore the possibility of bypassing the callus phase for transformation, Van Eck acquired a construct from DuPont Pioneer for the recently reported maize *Baby boom* and *Wuschel* genes system that induces a morphogenic response. Transgenic lines were recovered from direct infection of mature embryos and shoot tips harvested from seedlings. The Van Eck lab also investigated the two methods reported for floral dip transformation of inflorescences of young *S. viridis* plants and results will be presented.

Fonio Millet, a grain for the future

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Fonio (*Digitaria exilis*) is a staple grain widely cultivated in West Africa. This rainfed crop is well adapted to the semi-arid climates of the Sahel and the Northern Guinean savanna. Close

relatives include crabgrass, *Setaria*, switchgrass, sorghum, and maize. A major goal is to evaluate and develop fonio as a valuable crop in both the Sahelian and northern Guinean zones of Mali through breeding for shattering resistance, lodging and flowering using both traditional and molecular tools. Fonio research also has potential to increase our understanding on crop domestication and provide a nutritious food source with a low glycemic index. Essential to such research is developing an improved understanding on basic growth and morphology and developing an effective method for plant crosses. Initial characterization on the morphological and genetic diversity amongst selected landlines and strategies for crossing will be provided.

Using *Setaria viridis* to accelerate the characterization of candidate genes in Kranz anatomy development

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C_4 photosynthesis relies on both biochemical and anatomical adaptations; and while the biochemistry is well established, the regulatory networks underlying Kranz anatomy are largely unknown. Recently, a number of candidate genes underlying Kranz anatomy have been identified including a regulatory network involving the transcriptional module of SHORT-ROOT/SCARECROW (SHR/SCR). The interactions of SHR/SCR with IDDs have been hypothesized to determine cell identity in the leaves of C_4 species. The complexity of these gene networks and time to generate mutations in several genes in maize has hampered functional analyses of these candidates. We have thus pioneered the use of *Setaria viridis* as a model C_4 grass to dissect the function of genes involved in the establishment of Kranz anatomy. We have successfully applied CRISPR/Cas9 technologies to generate null alleles in *S. viridis*. In parallel, translational fusion constructs tagged with YPet were developed for cellular localization and ChIPseq. Confocal and light microscopy in single and higher order mutants is being used to evaluate defects in cellular patterning or differentiation of the vascular bundle in the independently evolved *Setaria viridis* and also in maize. We hypothesize that BS- or M-cells enriched IDDs act together with SHR and SCR define a network that contributes to the differentiation of photosynthetic cells in C_4 grasses.

Dissecting the genetic basis for meristem size control and branch initiation during grass inflorescence development using Setaria viridis as a model

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Inflorescence architecture at maturity directly impacts crop yield. Characteristics of the inflorescence, such as order of branches, pairing and number of spikelets can be reflected at early developmental stages. However, much remains to be discovered about the genetic controls of early inflorescence development. In this project, we are using *Setaria viridis* as a model to understand the genetic control of meristem maintenance and branch initiation, which are crucial to understand the mechanism of early inflorescence development. For this, we studied the evolution of CLE genes and are testing their function using in situ and CRISPR-CAS techniques in *Setaria*. CLEs are CLV3-like genes whose function in meristem maintainance has been examined in Arabidopsis but their function in grass inflorescence development remains poorly understood. In addition, we are investigating the role of a family of auxin importer proteins in

early inflorescence development. While function of auxin efflux carriers such as PIN1 have been extensively characterized during inflorescence development, the role of auxin influx carriers remains largely unstudied. We have shown that mutation in one of the auxin importer genes in *Setaria* results in initiation of fewer branches and thus fewer spikelets, suggesting a critical role of auxin importer genes in grass inflorescence development. RNAseq analysis and various imaging techniques have been employed to understand the functional mechanism of auxin importer genes. Together, these studies provide insights in understanding the molecular mechanisms of meristem control and branch formation during grass inflorescence development.

Mapping and function characterization of genes contribute to development and drought response in *Setaria*

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Map-based cloning is a classical approach for gene isolation and function analysis. A great number of important genes controlling plant growth and development were identified through this approach in Arabidopsis and rice. For years, our team have been making efforts to make map-based cloning works in *Setaria italica* which is an emerging model for Panicoideae grasses. A large-scale EMS-induced *S .italica* mutant library was constructed, and a number of polymorphic molecular markers were developed. On basis of this sound groundwork, our team discovered four functional genes contribute to plant development and drought response in foxtail millet to date. Take *SiAGO1b* for example, through map-based cloning, we found that mutation at the C-terminus of the *SiAGO1b* gene in foxtail millet disrupt the interaction between SiAGO1b and SiHYL1, and led to dwarfing stem, narrow and rolled leaves, smaller panicles, and lower rates of seed setting. Besides, we found the mutant is more sensitive to ABA and drought. Our research demonstrated that *Setaria* has the potential to serve as promising model for gene discovery and pathway engineering.

Characterization of EARLYFLOWERING 3 orthologs from model grasses reveals conserved molecular functions.

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The circadian clock broadly regulates plant responses to the changing environment. Many circadian clock factors have been well characterized in the model plant *Arabidopsis thaliana*. However, little is known about the molecular function of clock genes in other plant species. Here, orthologs of a key Arabidopsis clock gene *EARLY FLOWERING3* (*ELF3*) were identified from two model grass systems, *Seteria viridis* and *Brachypodium dystachyon*, and characterized at the molecular level. Despite comparably low sequence identity versus AtELF3, SvELF3 and BdELF3 complemented an *elf3* mutant in *A. thaliana*, rescuing hypocotyl elongation, flowering time and arrhythmic clock defects. Using affinity purification and mass spectrometry, SvELF3 and BdELF3 were found physically integrated into similar complexes and protein-protein interaction networks as AtELF3. Thus, we find that ELF3 likely forms and regulates similar networks in the grasses, despite 180 million years of separation. Furthermore, to identify both cycling genes and possible ELF3 functional orthologs in *S. viridis*, a circadian RNA-seq dataset and online query tool (Diel Explorer) was generated as community resource to explore

expression profiles of *Setaria* genes under constant conditions after photo- or thermo-entrainment. The Diel Explorer tool will aid in further exploration of the functional comparison of circadian regulated genes in *S. viridis.*

Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases

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Precise genome editing of plants has the potential to reshape global agriculture through the targeted engineering of endogenous pathways or the introduction of new traits. To develop a CRISPR nuclease-based platform that would enable higher efficiencies of precise gene insertion or replacement, we screened the Cpf1 nucleases from *Francisella novicida* and Lachnospiraceae bacterium ND2006 for their capacity to induce targeted gene insertions via homology directed repair. Both nucleases, in the presence of guide RNA and repairing DNA template, were demonstrated to generate precise gene insertions as well as indel mutations at the target site in the rice genome. The frequency of targeted insertions for these Cpf1 nucleases, up to 8%, is higher than most other genome editing methods reported to date. Further refinements and broad adoption of the Cpf1 genome editing technology has the potential to make a dramatic impact on plant biotechnology.

Influence of leaf rolling on canopy light environment and yield response to drought revealed by hemispherical imaging in *Setaria*

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In cereals, leaf rolling as a reversible means to adjust the canopy microenvironment is an under-improved behavior that has the potential to influence yield responses to drought stress. The proportion of light passing through the canopy (Global Site Factor, GSF), and the amount of plant tissue area per unit ground area (Plant Area Index, PAI) were estimated by hemispherical canopy imaging in *Setaria viridis* mapping populations. A RIL mapping experiment showed that PAI is an effective yield proxy on the basis of strong phenotypic correlation and QTL co-localization with traits describing biomass productivity. A GWAS drought experiment showed that diurnal changes in GSF correspond with plot-level rolling score and directly measured leaf-level rolling angle. PAI evaluated from dawn hemispherical images was used to estimate mid-season yield. Dawn PAI percent treatment differences correlated with percent time differences of drought-stressed plot-level rolling score (r^2=0.58) and leaf-level rolling angle (r^2=0.59). Initial GWAS analysis detected seven SNPs related to plot-level rolling score. Results demonstrate that leaf rolling is a good predictor of yield responses to drought. An upcoming greenhouse GWAS experiment will score rolling with higher temporal resolution and characterize the number and size of bulliform cells (osmotic motors that drive leaf rolling) with microscopy.

***SiASR4*, the target gene of SiARDP from *Setaria italica*, improves abiotic stress adaption in plants**

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The abscisic acid-, stress-, ripening-induced (ASR) proteins play important roles in protection of plants against abiotic stress. However, the regulatory pathway of the ASR encoding gene remains to be elucidated. In this study, the foxtail millet (*Setaria italica*) ASR gene, *SiASR4*, was cloned and characterized. SiASR4 localized to the cell nucleus, cytoplasm and cytomembrane, and the protein contained 102 amino acids, including an ABA/WDS (abscisic acid/water-deficit stress) domain. The abundance of *SiASR4* transcripts increased after treatment with ABA, NaCl and PEG in foxtail millet seedlings. It has been reported that the *Setaria italica* ABA-responsive DRE-binding protein (SiARDP) binds to a DNA sequence with a CCGAC core and that there are five DRE motifs within the *SiASR4* promoter. Our analyses demonstrated that the SiARDP protein could bind to the *SiASR4* promoter *in vitro* and *in vivo*. The expression of *SiASR4* increased in *SiARDP*-overexpressing plants. *SiASR4*-transgenic *Arabidopsis* and *SiASR4*-overexpressing foxtail millet exhibited enhanced tolerance to drought and salt stress. Furthermore, the transcription of stress-responsive and reactive oxygen species (ROS) scavenger-associated genes was activated in *SiASR4* transgenic plants. Together, these findings show that *SiASR4* functions in the adaption to drought and salt stress and is regulated by *SiARDP* via an ABA-dependent pathway.

A system biology approach for the study of abiotic stress tolerance in *Setaria viridis*.

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We previously studied the plasticity of *Setaria viridis* under water deficit and heat stress, and identified a number of accessions (both stress-tolerant and stress-sensitive) that could be used to study the mechanisms associated with stress tolerance. Moreover, these accessions could provide a tool for the characterization of the regulatory networks associated with the plant responses to abiotic stress in this C4 grass. We used *S. viridis* accessions, (A10.1; stress-tolerant) and (Ast-1; stress-sensitive) to characterize the functional role(s) and the physiological significance of a number of genes, shown to be associated with the plant response to abiotic stresses. In addition, we performed transcriptome and metabolome analyses. Our results show that processes such as photosynthesis, protein synthesis, carbon- and nitrogen-metabolism, etc. are differentially regulated during the response to water deficit stress. These results will be presented and discussed.

Large-scale screening of C_4 photosynthesis -related mutants in *Setaria italica*

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The C_4 photosynthesis pathway engineering in C_3 crops like rice is a hot research topic. *Setaria italica* and *S. viridis* is regarded as an ideal model grass system to investigate C_4 photosynthesis. In the past years, we screened 42,600 EMS-induced *S. italica* M3/M4 lines for C_4 photosynthesis related mutants. Considering the C_4 photosynthesis characteristics, we used the following methods for mutant identification: 1. Growth screening of mutants under low-CO_2 concentration. 2. Micro lens observation of the vein arrangements and I_2-KI staining of leaves. 3. Paraffin section and microscopy analysis of the 'Kranz structure' variation. 4. $\delta^{13}C$ value, chlorophyll content, and photosynthesis data measurements. According to these criteria, we totally identified

549 C_4 photosynthesis-related mutants to date. Future work will be concentrated on physiological, biochemical, and genetic analyzing the candidate mutants. Our efforts in identification of C_4 mutants in *Setaria* will provide valuable resources for C_4 photosynthesis engineering.

Investigating the role of the monocot circadian clock in agricultural biotechnology trait development

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Proper timing of daily physiological processes is critical to evolutionary and agricultural success. However, little is known about how daily rhythms can influence trait development in monocot crops. Here, we investigate two aspects of timing-related regulation in the model C4 monocot *Setaria viridis*: diel regulation of transcription and control of flower opening time (FOT). First, we are developing a toolkit of circadian-responsive promoters to facilitate analysis of circadian regulation and improve transcriptional control of transgenes. Second, we compared FOT in undomesticated *Setaria viridis*, wild and domesticated rice, and domesticated sorghum. In rice, FOT varies greatly across cultivars and wild accessions, while FOT in sorghum is restricted to a 30 min window after dawn. We hypothesize that this adaptation is a heat escape mechanism that increases heat stress resilience in sorghum. In contrast, two setaria accessions showed a FOT near dawn with many of the flowers opening prior to dawn, before the onset of light, indicating a potential role for the circadian clock in the regulation of FOT in setaria. Results from these studies will help elucidate the role of the monocot clock in regulating agricultural traits in crop species and will serve to improve trait development for C4 monocots.

Photoperiodic effects on flowering time and architecture in Setaria

Andrew Doust, Margarita Mauro-Herrera, John Hodge, Yisel Carrillo

In many plants, photoperiod variation is a potent signal for developmental change. In grasses, photoperiodic variation is crucial for induction of flowering (with or without previous vernalization), and most wild grasses show some degree of photoperiod sensitivity, with shorter days promoting flowering and longer days delaying it. The panicoid model C4 grass system Setaria is of temperate origin, and we are investigating the effect of different photoperiod regimes on flowering time and plant architecture in a RIL population created from a cross between a relatively photoperiod insensitive accession of foxtail millet (*S. italica*) and a photoperiod sensitive accession of green foxtail (*S. viridis*) . We find that the population generally flowers later, is taller, and has more phytomers, suggesting that long days induce a developmental delay in transition to flowering. However, the RIL population has a different pattern of response at 16 hours as opposed to 12 and 8 hours, suggesting distinct short and long day responses. QTL analysis supports this interpretation, with very similar QTL found in 8 and 12 hours, but a different pattern in 16 hours. Several QTL unique to the different regimes are being investigated, including ones that contain copies of known flowering time genes.

Early growth dynamics affecting height in Setaria.

Qing Li, Margarita Mauro-Herrera, John Hodge, Hao Hu, Andrew Doust

Plant height is an agriculturally important trait with a huge impact on biomass and on the ability of the plant to compete with other plants. Previous work in our lab has examined phenotypic components and genetic control of height at three different stages of growth (vegetative, flowering, post-flowering), in a RIL population derived from a domesticated foxtail millet by wild green foxtail RIL population, where we find highly heritable and shared QTL for height, as well as differences in initial growth rates between the wild and domesticated parents. We are now investigating the relative contributions of phytomer production vs. internode elongation to differences in height, with particular interest in early growth dynamics. Digital image analysis is being used to understand the fine-scale dynamics of leaf and stem growth that contribute to plant height in Setaria, and several RIL lines, chosen on the basis of our previous studies have been phenotyped. We find that the RILs vary in the differential expansion of the first few leaves and we suggest, based on both phenotypic and genotypic analyses, candidate genes for these effects.

Droplet Digital PCR for Measurement of Transgene Copy Number in Plants

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Plant transformation is an important strategy for studying plant gene function. Since the insertion of multiple transgene copies can trigger silencing, it is useful to identify single copy transgenic lines. The gold standard for copy number measurement is the Southern blot; however the Southern is time-consuming, can be expensive, often requires a large amount of genomic DNA, and typically involves the use of radioactively labeled probes. Alternatively, we developed a droplet digital PCR (ddPCR)-based method for transgene copy number measurement in maize, rice, wheat, citrus, potato, and tomato. Genomic DNA fragmented by restriction endonuclease digestion was added to a reaction cocktail containing primers optimized for duplex endpoint amplification of the transgene and an endogenous reference of known copy number. Prior to amplification the cocktail was partitioned into thousands of droplets. Detection and quantification of droplets in which either the transgene or reference gene was successfully amplified was possible due to inclusion in the cocktail of sequence-specific fluorescently labeled probes. Comparison of Southern blots generated from the same transgenic events utilized for ddPCR for rice, potato and Citrus validated that ddPCR rapidly and unambiguously measures transgene copy number. Finally, transgene zygosity in segregating transgenic wheat and tomato lines was clearly demonstrated.

Landmark-based semi-automated phenotyping for developmental traits

John G. Hodge, Qing Li, Andrew N. Doust

Screening phenotypes across development can provide key insights into subtle differences between morphological regulators. This is especially true for labile traits that may shift either in response to physiological stress or perceived changes in the environment. With this issue in mind an image analysis pipeline based around our algorithm, Acute, was developed using time series image data for quantifying patterns of growth based on pseudolandmarks. This method for identifying, extracting, and linking pseudolandmarks has broad applications for measuring plant stature and architecture between different genotypes, including mutants that may have subtle developmental defects. It will also be useful for characterizing large crossing populations. Tests

have been performed on a subset of recombinant inbred lines selected from a *Setaria italica* (foxtail millet) X *S. viridis* (green foxtail) mapping population, particularly focusing on pseudolandmarks associated with axillary branch outgrowth and elongation. Deviations in plant growth were characterized with greater temporal resolution and less subjectivity than manual measurements. This variation could be attributed to differences in lateral branch outgrowth and elongation of the main culm. Our method of high throughput phenotyping opens up possibilities for identifying subtle variations within populations that could otherwise be missed.

Widespread non-target-site resistance in *Setaria viridis* to four classes of herbicide

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Setaria viridis is a cosmopolitan, selfing weed that has evolved resistance to multiple classes of herbicide. Our goal was to assess the diversity in herbicide target genes and identify novel and predicted target-site mutations that lead to resistance. 235 *S. viridis* accessions from North America and Eurasia were exposed to commonly used herbicides inhibiting specific genes: **HRAC A**) acetyl CoA carboxylase, **HRAC B**) Acetolactate synthase, **HRAC G**) EPSP synthase and **HRAC H**) Glutamine synthetase. Each gene in 53 of our accessions was PCR-amplified and Illumina sequenced, and gene sequences from an additional 42 accessions were obtained from JGI whole genome sequences. Sequences were aligned to the target gene sequences from the *Setaria italica* V2.2 genome reference and mined for SNPs. There were resistant accessions to all of our herbicides. Almost 30% of our accessions survived to reproduction following application of at least one herbicide, and 13 accessions were resistant to two classes of herbicide. Although there were numerous SNPs and known resistance target-site mutations in our target genes, SNPs found only or predominantly in herbicide resistant genotypes, relative to susceptible, were largely intronic or synonymous. The broader pattern of herbicide resistance in *Setaria viridis* is likely driven by non-target mutations that detoxify or compartmentalize applied herbicides.

Identification of bacterial genes essential for colonization of *Setaria viridis* roots.

Fernanda Plucani do Amaral, Vania Carla Pankievicz, Emanuel de Souza, Joel Griffits and Gary Stacey

Plants interact with a wide range of soil microorganisms and this interaction can result in significant benefits to microbe and plant host. The effects of such ‘plant growth promoting bacteria’ (PGPB) have been well documented with a variety of plant species. However, the molecular mechanisms behind this growth promotion are still largely unclear. We have adopted *Setaria viridis* as a suitable model bacterial-plant system, inoculated with a plant growth promoting bacteria *Azoarcus olearius*. Inoculated plants show significantly increased root and shoot biomass. In addition, *Setaria viridis* roots were strongly colonized, endophytic and epiphytically by the bacteria. We employed the Tn-seq method to identify *A. olearius* genes essential for root colonization. Over 90 candidate genes were identified as either promoting or reducing successful root association. An example is quinoprotein ethanol dehydrogenase (ADH) in which mutations significantly reduced endophytic root colonization. Ongoing studies on several other genes are being conducted in order to understand the mechanism of this

association. Our long-term goal is to understand the mechanism of PGPB-plant association and ultimately to manipulate the association to maximize its benefits for crop production. For this purpose, Setaria has obvious utility and should prove to be a widely-adopted model.

Genome-wide identification and classification of basic Helix-Loop-Helix (bHLH) in Setaria spp. and functional characterization of vascular related transcription factors through CRISPR/Cas9 mutagenesis

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The bHLH proteins form the largest family of transcription factors in plants, and regulate a diverse range of biological processes. Recently, many bHLHs have been implicated in the determination of the C_4 Kranz anatomy. The aim of this study is to identify and classify the bHLH transcription factors in the *Setaria* spp. and to functionally characterize two bHLHs reported to control vascular development, an important trait in Kranz development – LHW and TMO5. The genome-wide identification of bHLHs identified 197 sequences for each *Setaria* spp. Bayesian inference successfully recovered the evolutionary history of this gene family, and from the other species analysed, *A. thaliana*, *O. sativa*, *B. distachyon*, *S. bicolor* and *Z. mays* . The *Setaria* species displayed great similarity, not only at the sequence level but also in genome synteny, suggesting that domestication did not affect the bHLH structure in the *S. italica* and *S. viridis* genomes. Finally, several independent CRISPR/Cas9 lines were obtained through *S. viridis* transformation. From those, six lines had a mutation in LHW-like2 with mendelian segregation; and other two lines in TMO-like1 and TMO-like3, but no homozygous plants were retrieved, suggesting lethality. Morphological analysis is ongoing to elucidate the role of these transcriptional factors in Kranz development.

Leaf-level hyperspectral reflectance accurately detects genotype-by-environment response of leaf allometry and nitrogen content to drought in a Setaria mapping population

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The physiological and genetic controls of C4 photosynthesis and the water and nitrogen resources used in association with this key plant process are poorly understood. Specific leaf area (SLA) and nitrogen content (N_{area}) are leaf functional traits that are strongly associated with photosynthetic capacity. Leveraging genomic tools to understand crop response to stress requires the development of reliable high-throughput phenotyping methods for physiologically relevant traits. Leaf-level hyperspectral reflectance has been used to predict genetic variation in photosynthetic traits in C3 plants (deciduous trees and soybean), and recently in the C4 crop, maize. The extent to which this tool can detect important genotype-by-environment interactions has not yet been demonstrated. Here leaf-level hyperspectral reflectance was tested as a tool for predicting SLA and N_{area} for a *Setaria viridis x S.italica* RIL mapping population field grown in well-watered and drought treatments. Reflectance-predicted values were strongly correlated with measured values for SLA (r^2=0.69) and N_{area} (r^2=0.71). Moreover, hyperspectral reflectance successfully predicted genotypic variation in drought effect on SLA (r^2=0.81) and leaf nitrogen (r^2=0.77). QTL for reflectance predicted values of SLA and N_{area} overlapped with QTL for their traditionally measured equivalent. This work demonstrates that leaf-level hyperspectral reflectance is a tool capable of accurately predicting physiologically relevant traits and detecting genotype-by-environment interactions.

A nonspecific *Setaria italica* lipid transfer protein gene plays a critical role under abiotic stress

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Lipid transfer proteins (LTPs), a class of cysteine-rich soluble proteins having small molecular weights, play important roles in pathogen and abiotic stress responses. A nonspecific LTP gene (*SiLTP*) was isolated from a *Setaria italica* suppression subtractive hybridization (SSH) library enriched for differentially expressed genes after abiotic stress treatments. *SiLTP* was expressed in all tissues and induced by NaCl, PEG and ABA. SiLTP was localized in the cytoplasm of tobacco leaf epidermal cells and maize protoplasts. Ectopic expression of *SiLTP* in tobacco resulted in higher salt and drought tolerance than wild type (WT). To assess the function of SiLTP, *SiLTP* overexpression (OE) and RNA interference (RNAi) transgenic foxtail millet were obtained. *SiLTP*-OE performed better under salt and drought stresses compared with WT, whereas the RNAi lines were much more sensitive. The ABA-responsive DRE-binding protein (SiARDP) could bind to DRE element of *SiLTP* promoter *in vitro* and *in vivo*. Moreover, the *SiLTP* expression levels were higher in *SiARDP*-OE plants. These results indicated that *SiLTP* play important roles in salt and drought tolerance in foxtail millet, and may partly be up-regulated by SiARDP. *SiLTP* may provide an effective genetic resource for molecular breeding in crops to enhance salt and drought tolerance levels.

Utilizing *Setaria viridis* as a Model for Molecular Characterization of Jasmonate-Mediated Growth and Defense Responses

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The plant hormone jasmonate (JA) and its derivatives control many important agricultural traits from growth and development to defense against biotic and abiotic stresses. Here we utilize *Setaria viridis* as a model system to study mechanisms underlying this process in economically important grass systems. The core JA signaling pathway consists of JASMONATE ZIM-DOMAIN (JAZ) repressors that interact with COI in the presence of bioactive JA. Upon COI-JAZ interaction, JAZs are targeted for degradation, resulting in activation of downstream transcription factors (TFs) that regulate JA-responsive gene expression. In this study, SvCOI-JAZ interactions were examined and unique interaction partners were identified. Cas9-mediated genome editing of *SvCOI*s with different JAZ-interaction patterns were generated and preliminary phenotypes were observed. To identify downstream TF partners of JAZ proteins, a wound treatment was applied to the *S. viridis* leaf and transcriptional responses measured over a developmental gradient. Clustering analysis of the RNA-seq data coupled with co-expression analyses led to identification of novel basic helix-loop-helix TFs that are candidates for regulating JA signaling outputs. Outcomes from this research provide insight into the dynamics and complex regulation of JA responses in grass systems, and provide opportunities to engineer bioenergy crops for enhanced stress resistance without compromising growth.

Brassinosteroids control inflorescence development in panicoid grasses

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Brassinosteroids (BRs) are phytohormones implicated in developmental control of plant architecture, including inflorescence architecture, an important agronomic trait in cereal crops. In maize, BR biosynthesis mutants (e.g. *na1*, *na2*, *brd1*) are dwarf and develop feminized tassels. Despite prominent phenotypes, little is known about the mechanisms by which BRs modulate panicle development. Here, using *Setaria viridis* as a model system, we cloned the *bristleless1* (*bsl1*) gene, which encodes a cytochrome P450 involved in BR biosynthesis; the syntenic ortholog of *Dwarf11* (*D11*) in rice. The *bsl1* mutant was identified because it exhibits severe reduction of bristles in its inflorescences. Bristles are modified, sterile branches unique to the "bristle clade" of grass species, and are produced along with spikelets in the *Setaria* inflorescence, apparently in place of paired spikelets characteristic of maize and sorghum. Our analysis of the *bsl1* mutant suggests that BRs act in the spikelet meristem fate decision to differentiate into a bristle or spikelet. In contrast to normal *Setaria* flowers, lower florets of *bsl1* spikelets do not abort and appear to develop normally, reminiscent of maize sex-determination mutants, such as *tasselseed2*. *In situ* hybridization in inflorescence primordia showed that *bsl1* transcripts localized to the adaxial side of developing primary branches and accumulated at lateral organ initiation sites in spikelet meristems, suggesting a function for *bsl1* in both meristem fate and lateral organ initiation. We further showed that the maize ortholog, *ZmD11*, was expressed in analogous patterns in ear and tassel primordia, suggesting a conserved role in maize. RNA-seq data also support a role for BRs in regulating inflorescence development through modulation of known regulators. The *bsl1* mutant therefore provides an ideal system for studying BRs in regulation of panicoid inflorescence development and their role in maize sex-determination.

Molecular manipulation of the microRNA396/*GROWTH REGULATING FACTOR* expression module in the model C_4 plant, *Setaria viridis*

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Plant microRNAs (miRNAs) are now well-established central regulators of gene expression, influencing development, pathogen defence and abiotic stress adaptation. Alterations, directed by molecular modulation or mutation, to the miR396/*GROWTH REGULATING FACTOR* (*GRF*) expression module, has produced highly desirable morphological and physiological phenotypes in a range of monocotyledonous and dicotyledonous species. One such phenotype is an enhanced adaptive response to drought stress. This project aims to utilise traditional and contemporary molecular genetic approaches, to characterise the phenotypic and physiological consequences of altering the miR396/*GRF* expression module in *Setaria viridis*, grown under both well-watered and drought stress regimes. More specifically, *Agrobacterium*-mediated transformation will be used to generate miR396 overexpression and knockdown mutants in *S. viridis* accession A10. In addition, a collection of *Setaria italica* ecotypes (≈ 200) sourced from 15 countries scattered across 4 continents, will be screened to identify the extremes of drought susceptible and drought tolerant accessions. A subset of accessions will be further characterised at a molecular level in an

attempt to establish linkage(s) between the drought response of each accession and measured changes of the miR396/*GRF* expression module.

The putative role of PIP1 aquaporins in regulating transpiration and root hydraulics of *Setaria viridis*

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Aquaporins (AQPs) constitute a large family of transmembrane proteins that function as water channels (i.e. increase the membrane water permeability). Until now, the role of this large and diverse family of channels in controlling the basic characteristics of the plant water balance (e.g. transpiration and root hydraulics) is not clear. We investigated the whole plant transpiration rates and root water hydraulics in two *S. viridis* natural accessions that showed differential behavior under normal conditions and conditions of water-deficit stress (Zha1- tolerant and Sha1-sensitive). Zha1 showed enhanced whole plant transpiration compared to Sha1 under all tested conditions. The higher transpiration rates were accompanied by higher root hydraulic conductivities of Zha1. Root *PIP1* expression in Sha1 exceeded the levels of expression in Zha1 under both normal and stress conditions. Our results suggest that root PIP1 contributes to Zha1 observed tolerance to water deficit by regulating root hydraulics and shoot gas exchange. We are currently modifying the expression of *PIP1* in *Setaria viridis* Zha1 and Sha1, in order to assess the role(s) of aquaporins in water deficit tolerance.

Accelerating gene discovery and translation from *Setaria viridis* to maize: from fine mapping to community resources

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Setaria viridis has become an excellent model system for C4 grasses due to its small stature, short life cycle, small genome size (~515Mb), ease of crossing and transformation. To accelerate gene discovery in *Setaria viridis*, we have developed a NMU mutagenized population consisting of 20,000 M2 families and sequenced 61 mutants to calculate mutation frequencies. On average there were 66 homozygous nonsynonymous mutations per family. Approximately 3000 M2 families were screened and two sparse panicle mutants *(spp1* and *spp3)* identified. Bulked Segregant Anlaysis (BSA) followed by deep sequencing enabled us to fine map the *spp* phenotype to *SvAux1*. Synteny comparisons identified a maize ortholog of SvAux1 and the characterization of a maize mutator line confirmed a role of ZmAUX1 in inflorescence and root development. These findings demonstrate the value of *Setaria viridis* as a tractable genetic model for rapid gene candidate identification. We have also assembled a collection of ~500 diverse *Setaria* accessions collected throughout the US and Canada. These accessions have been sequenced at JGI-DOE and a subset characterized for phenotypic traits and propagated for seed distribution at the USDA GRIN. We have also initiated the construction of seven recombinant inbred line (RIL) populations. One RIL population consisting of ~270 lines, which was derived from a cross between A10.1 and a drought tolerant line (Roche10106) has been genotyped and self-pollinated to generate an F8 populations through single seed descend (SSD). In collaboration with JGI, we have also generated a gene-atlas using RNA isolated from leaves, roots, and panicles of *S. italica* B100 and *S. virids* A10.1 at different developmental stages. Collectively, these genetic resources will help drive future genetic studies in Setaria and will be accessible to the public through the GRIN database and seed banks.

Evidence for a role of chloroplast gene regulation in C_4 cell fate

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The hallmark of C_4 photosynthesis is two differentiated leaf cell types, bundle sheath (BS) and mesophyll (M), each with specialized chloroplasts and cell-type specific proteins. Yet, molecular mechanisms that regulate this differentiation are amongst the most intensively studied, but most poorly understood in plant science. To identify factors involved in BS specificity of Rubisco, a mutant screen was conducted in *Setaria viridis* expressing YFP specifically in BS chloroplasts. Phenotypes sought included Rubisco deficiency, and mislocalization of YFP to M as well as BS chloroplasts. Chlorotic and Rubisco-deficient mutants mostly affected chloroplast gene regulation and translation rather than Rubisco assembly, and two of these mutants accumulated YFP in both BS and M chloroplasts. The causative mutations were determined to be in genes encoding a chloroplast RNA-binding protein, and a chloroplast-targeted exoribonuclease, both of which have essential roles in chloroplast rRNA maturation. Additional experiments suggested that in these mutants, YFP migrates freely from BS to M cells, consistent with modification to normal cell-to-cell communication mediated by plasmodesmata. We hypothesize that proper chloroplast ribosomal RNA maturation signals through a retrograde pathway to ensure correct development of C_4 morphology, possibly mediated by ABA.

***Setaria viridis* response to drought under high temperature**

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Water scarcity and higher temperature are major consequences of climate changes with potential devastating effects on plant productivity. Despite the fact that plant response to drought has been extensively studied, it is a complex phenomenon with endless experimental set ups, which in turn partially determines the output. Three days seedlings of *Setaria viridis* were transferred to a hydroponic system in modified Hoagland® nutritive solution for one week. The drought treatment was gradually established in polyethileneglycol 2.5% g/mL (-0.09 MPa) solution for three days, followed by 5% g/mL (-0.38 MPa) for another three days. Plants were under high temperature (30-37^{o}C max night-day). Photosynthetic performance was evaluated using imaging fluorescence (FluorCam800MF, PSI) and stomata conductance by a leaf porometer (SC-1, Decagon). Leaf and root morphology were evaluated using scanning electron and light microscopy. Plants under low-water availability showed: smaller adventitious roots, but no difference in the number/length of crown roots; smaller stomata and bundle sheath diameter; and increased deposition of pectin substances and lipids in the external wall of the epidermis. This relatively mild and gradual drought determined somewhat different response from that reported in the literature. Disagreements are probably related to longer and more intense drought treatment used in previous studies.

Accelerating product development in C4 crops using *Setaria* viridis as a trait discovery engine

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Accelerated genetic improvements and adoption of enhanced crop management practices have led to remarkable improvements in crop productivity over the last few decades. These strategies are however thought to be nearing their maximum potential for yield improvement. Arguably, improved photosynthetic performance, which has not been addressed by breeding, is believed to be one of the most important opportunities left to further realize major yield gains and guarantee food security over the next several decades.Strategies to enhance photosynthetic performance in C4 crops have been impeded by the lack of accessibility to a relevant, rapid cycling, and easily transformable model species. Over the last decade and a half, the discovery platforms of the major crop biotechnology players have relied exclusively on the transformation and testing of transgene candidates in C3 model backgrounds like *Arabidopsis*, tobacco and rice. Attempts, using these model species, to improve yield in C4 crops like maize, through biotechnology, have failed to deliver successful outcomes.At Benson Hill Biosystems, we have developed *Setaria viridis* as the model system at the heart of our trait discovery and design program. Combined with a internally-developed, world-class computational and analytics platform, this capability enables us to screen photosynthesis trait candidates in a high throughput manner, allowing us to quickly identify and prioritize positive trait leads for further validation and downstream product development in C4 crops. Many trait leads identified through this approach have been demonstrated to not only drive significant improvement in crop productivity but also impart measurable increases in photosynthesis efficiency of the positive transgenic events. Some of these early leads have been successfully validated in maize hybrid trials and are advancing in our product development pipeline.

KAZU buffer for plant DNA extraction

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KAZU buffer is a low-cost, novel and non-toxic buffer for chemical-free plant DNA extraction. We have developed robust protocols for both low and high throughput DNA extractions that are of suitable quality for many downstream molecular biology applications. It is faster and safer than traditional CTAB DNA extractions for plant material. We distributed KAZU buffer to several groups within the Donald Danforth Plant Science Center (DDPSC) and the Boyce Thompson Institute for Plant Research (BTI). We successfully used KAZU buffer for high through-put genotyping in *Seteria*. We also present several product comparisons – demonstrating the strengths and weaknesses of KAZU buffer. KAZU buffer is currently sold by Kerafast (http://www.kerafast.com/p-2122-kazu-dna-extraction-buffer.aspx).