Final Progress Report for project no. DE-FG02-07ER64458
Genetic dissection of bioenergy traits in sorghum
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Specific Objectives:
1. To identify the gene(s) underlying a major QTL for stem sugar concentration located on chromosome 3.
2. To identify QTL for stem juice volume and stalk sugar concentration and to identify the underlying genes.
3. To classify 60 novel sorghum *bmr* mutants from the USDA TILLING population in allelic groups based on cell wall chemistry and allelism tests.
4. To select representative *bmr* mutants from each allelic group and selected NIR spectral mutants for their potential value as feedstock for ethanol production.
5. To clone and characterize those *Bmr* genes that represent loci other than Bmr12 and Bmr6 using a mapping and a candidate gene approach.

Objective 1
The experiments for this objective are largely complete and the data have been analyzed. Data interpretation and follow-up experiments are still in progress. A manuscript is in preparation and expected to be submitted this summer.
The main results are:
1) 16 cDNA libraries were prepared and sequenced at Cornell University. The libraries represent internode tissue and flag leaf tissue at booting, internode tissue and peduncle tissue at soft-dough stage, from two plants per sampling time with the Rio allele for the QTL on chromosome 3, and two plants with the BTx623 allele on chromosome 3 (4 tissues x 2 genotypes x 2 replicates)
2) 480 million 86-nucleotide reads were generated from four lanes of Illuminia HiSeqII
3) 74% of the reads could be mapped to the sorghum transcriptome, indicative of good sequence quality
4) Of the 216 genes within the QTL, 17 genes were differentially expressed among plants with and without the Rio QTL. None of these 17 genes had obvious roles in sucrose metabolism
5) Clustering algorithms identified a group of 721 co-expressed genes. One of these genes is a sucrose synthase gene. This cluster also contains 10 genes from the QTL.
6) Among these co-expressed genes are regulatory genes for which knock-out lines in Arabidopsis have been obtained. Analysis of these lines is in progress.

Objective 2
The experiments from this objective have been completed and the data were published in the journal Crop Science by **Felderhoff et al. (2012)** (see publication list for full details). The experiments were based on a mapping population derived from the sweet sorghum 'Rio' and the dry-stalk grain sorghum BTx3197. The main findings were:
1) The apparent juiciness of the sorghum stalk, based on the appearance of a cut stem surface (moist vs. pithy), is not representative of the moisture content of the stalk. This was surprising, as pithy stalks have been associated with low moisture content. This means that in order to assess 'juiciness', a different evaluation needs to be used, for example by removing juice with a roller press or by measuring the difference in mass between a fresh and dried stalk segment.
2) A total of five QTLs associated with juice volume (corrected for height) or moisture content were identified, but not all QTLs were detected in all environments, providing evidence for genotype x environment interactions. This finding complicates breeding for juice volume using marker-assisted selection.
3) The QTL for sugar concentration identified on chromosome 3, and the subject of Objective 1, was confirmed in this mapping population, but unlike in previous studies (Murray et al., 2008), the presence of this QTL was associated with negative impacts on agronomic performance (fresh and dry biomass yield, juice yield). Consequently, introgression of the Brix QTL from Rio as part of a commercial

breeding program will require monitoring of the precise impacts of this QTL on agronomic performance.

4) The absence of dominance effects for the Brix trait (= sugar concentration) indicated that Brix must be high in both parents to produce high Brix in hybrids. This means an extra constraint on the development of hybrid parents. With the results from Objective 1, the selection of progeny containing favorable alleles for sugar concentration is expected to be more efficienct.

Objectives 3 and 4

The experiments from these objectives have been completed. Some of the data have been published in the journal BioEnergy Research (**Sattler et al. 2012**) and in a book chapter on the utilization of sorghum biomass by **Vermerris and Saballos (2012)** (see publication list for full details). One manuscript is in progress and is expected to be submitted in the fall of 2012.

The experiments for these objectives were based on the characterization of a set of novel sorghum mutants identified in a TILLING population generated by Dr. Zhanguo Xin (USDA-RS, Lubbock, TX).

The main findings were:

1) Based on allelism tests of *bmr* mutants from the USDA TILLING population, there are three novel sorghum *bmr* loci, currently referred to as *bmr-20*, *bmr-100* and *bmr-1107*. This brings the total number of *bmr* loci to a maximum of seven (manuscript in preparation).
2) The biomass conversion properties of the novel *bmr* mutants are not significantly better than the wild-type control, limiting their utility for bioenergy production (manuscript in preparation). This also means that all three *bmr* loci that positively influence biomass conversion (*bmr2*, *bmr6* and *bmr12*) have been cloned. Two of these genes (*Bmr6* and *Bmr2*) were cloned with funding from this project.
3) Four novel mutant alleles of *bmr12* were identified and characterized. These mutants alleles are *bmr12-30*, *bmr12-34, bmr12-35*and *bmr12-820*, and they all contain missense mutations, leading to amino acid substitutions with varying effects on lignin content and lignin subunit composition (syringyl/guaiacyl ratio). One of the mutants, *bmr-35*, represents a phenotype that is intermediate between the wild type and the *bmr12-reference* mutant, which is a null mutant. This intermediate phenotype may offer a balance between enhanced biomass conversion properties and good agronomic performance (Sattler et al., 2012).
4) It is possible to identify sorghum mutants with altered biomass conversion properties using analysis of leaf segments by near infrared reflectance spectroscopy (NIRS). Approximately 10% of 200 M3 families contained spectral outliers suggestive of cell wall changes, and half of those showed variation in biomass conversion efficiency (Vermerris and Saballos, 2012).

Objective 5

The experiments from this objective were completed and the data were published by **Saballos et al. (2012)** in The Plant Journal. The main findings were:

1) The *Bmr2* gene encodes the main 4-coumarate CoA ligase in sorghum; the genetic proof consisted of showing how two independent mutations in this gene both resulted in the same phenotype, and by showing that these mutations were allelic. Reduced Bmr2 activity leads to reduced lignin content and brown vascular tissue.
2) Allele-specific molecular markers were developed so that the inheritance of these recessive alleles can be tracked in sorghum breeding programs aimed at improving biomass conversion.
3) Together with four other *bona fide 4CL* genes, the *Bmr2* gene is a member of a multigene family in sorghum. Based on phylogentic analysis, one of those genes is involved in flavonoid metabolism, the others in monolignol biosynthesis. Enzymatic activities for the enzymes encoded by *Bmr2* and its paralogs were determined.
4) Both *bmr2* mutations are missense mutations that result in the substitution of apolar amino acids with polar amino acids. In both cases, these substitutions are in hydrophobic domains, which destabilize the protein, leading to degradation. This is apparent from western blots and activity assays with heterologously expressed enzymes.
5) The plant tries to compensate for the reduced 4CL activity by increasing the expression of *Bmr2* and its paralogs. As a result of the higher expression levels of the paralogs, there is enough 4CL activity to minimize negative impacts on growth and development.

List of <u>all</u> publications to date in which the funding of this project is acknowledged

1) Vermerris W, Saballos A (2012) Genetic enhancement of sorghum for biomass utilization. In Paterson, A. (Ed.) *Genetics and Genomics of the Saccharinae*, Springer, New York, NY 391-428.

2) Felderhoff T, Murray SC, Klein PE, Sharma A, Hamblin MT, Kresovich S, Vermerris W, Rooney, WR (2012) QTLs for energy-related traits in a sweet × grain sorghum [*Sorghum bicolor* (L.) Moench] mapping population. Crop Science 52: 2040-2049.

3) Sattler SE, Palmer NA, Saballos A, Greene AM, Xin Z, Sarath G, Vermerris W, Pedersen JF (2012) Identification and characterization of four missense mutations in *Brown midrib12* (*Bmr12*), the caffeic acid *O*-methyltranferase (COMT) of sorghum. BioEnergy Research (in press) DOI 10.1007/s12155-012-9197-z

4) Saballos A, Sattler S, Sanchez E, Foster TP, Xin Z, Kang CH, Pedersen J, Vermerris W (2012). *Brown midrib2* encodes the major 4-coumarate:CoA ligase involved in lignin biosynthesis in sorghum (*Sorghum bicolor* (L.) Moench). The Plant Journal 70: 818–830. doi: 10.1111/j.1365-313X.2012.04933.

5) Vermerris, W (2011) Survey of genomics approaches to improve bioenergy traits in maize, sorghum and sugarcane. Journal of Integrative Plant Biology 53: 105–119

6) Saballos A, Ejeta G, Sanchez E, Kang CH, Vermerris W (2009) A genome-wide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [*Sorghum bicolor* (L.) Moench] identifies *SbCAD2* as the *Brown midrib6* gene. Genetics 181: 783-795.

7) Saballos A, Vermerris W, Rivera L, Ejeta G (2008) Allelic association, chemical characterization and saccharification properties of *brown midrib* mutants of sorghum (*Sorghum bicolor* (L.) Moench). BioEnergy Research 2: 193-204

8) Felderhoff TJ. (2012) QTLs for energy related traits in a sweet x grain RIL sorghum [*Sorghum bicolor* (L.) Moench] population. M.S. Thesis, Texas A&M University.

Publications in preparation (tentative titles)

9) Felderhoff T, Murray SC, Klein PE, Sharma A, Hamblin MT, Kresovich S, Vermerris W, Rooney, WL (2012) QTLs for biomass and juice composition in a sweet x grain sorghum [*Sorghum bicolor* (L.) Moench] mapping population.

10) Vermerris W, Fear J, Saballos A, Murray SC, Rooney WL, Kresovich S. Identification of candidate genes for sucrose accumulation in sweet sorghum using RNA-seq.

11) Sattler SE, Palmer NA, Saballos A, Xin Z, Vermerris W, Pedersen JF. Characterization of novel sorghum *brown midrib* mutants.

Presentations since last progress report

1) Saballos A, Fear J, Vermerris W, Felderhoff T, Murray S, Rooney W, Kresovich S (2012) Elucidating the genetic basis of sugar accumulation in sweet sorghum using high-throughput gene expression profiling of heterogeneous inbred families. 34th Symposium on Biotechnology for Fuels and Chemicals. 20 April – 3 May 2012, New Orleans, LA.

2) Sattler SE, Clemente TE, Pedersen JF, Funnell-Harris DL, Dowd PF, Prom LK, Huang Y (2012) Lignin modification to improve sorghum for cellulosic and thermal bioenergy. Midwestern Section Meeting of American Society of Plant Biologist. 24-25 March 2012, Lincoln, NE.

3) Sattler S (2011) Modifying lignin to improve sorghum for cellulosic and thermal bioenergy. Corn & Sorghum and Soybean Seed Research Conference 2011, American Seed Trade Association Seed Expo 2011. 7-9 December 2011, Chicago, IL.

4) Vermerris W (2011) Genetic dissection of complex bioenergy traits in maize and sorghum. Plant Breeding Department, Wageningen University, The Netherlands. 21 November 2011 (invited seminar).

5) Fear J, Saballos A, MurrayS, RooneyW, Kresovich S, Vermerris W (2011) Differential gene expression inside and out: QTL analysis using HiSeq. Florida Genetics 2011. 9-10 November 2011. Gainesville, FL.

6) Vermerris W (2011) Molecular dissection of complex bioenergy traits. Graduate Program in Molecular Plant Sciences. Washington State University, Pullman, WA. 19 October 2011

7) Felderhoff TJ, Rooney WL, Murray SC, Sharma A, Klein PE, Hamblin M, Vermerris W (2011) National Association of Plant Breeders 1st Annual Meeting. 23-25 May 2011. College Station, TX. P-12.

8) Vermerris W, Saballos A, Felderhoff T, Mitchell S, Rooney W, Murray S, Kresovich S, Pedersen J, Sattler S, Xin Z (2011) Genetic dissection of bioenergy traits in sorghum. Genomic Science Awardee Meeting IX and USDA-DOE Plant Feedstocks Genomics for Bioenergy Awardee Meeting. 10-13 April 2011. Washington, DC. Abstract 149, p. 109.

9) Murray SC (2011) Lessons from molecular quantitative genetics applied for maize and sorghum improvement. Department of Genetics, Texas A&M University. 14 April 2011 (invited seminar)

10) Murray SC (2011) Lessons from molecular quantitative genetics applied for maize and sorghum improvement. University of Florida Genetics Institute. 8 March 2011 (invited seminar)

11) Felderhoff, T, Rooney WL, Murray SC (2010) QTLs in Sweet by Grain Sorghum RILs. ASA/CSSA/SSA Annual Meeting. 31 October-4 November 2010, Long Beach, CA (poster)

12) Saballos A, Erickson J, Vermerris W (2010) Development of bioenergy sorghums for Florida. Florida Genetics 2010. 27-28 October 2010, Gainesville, FL. P-92.

13) Sattler S (2010) Sorghum *brown midrib* mutants: Genes to improve Sorghum forage utilization and bioenergy conversion. Dept. of Agronomy and Horticulture, University of Nebraska-Lincoln. 8 October 2010 (invited seminar)

14) Saballos, A., J. Erickson, and W. Vermerris. 11-12 August 2010. Beyond oranges: Development of bioenmergy sorghums for Florida. Sorghum Improvement Conference of North America (SICNA). Mead, NE.

15) Sattler S (2010) The effects of lignin biosynthetic mutants (*bmr6* and *bmr12*) on plant performance and cell wall chemistry. 2010 Sorghum Improvement Conference of North America (SICNA)/Great Plains Conference. 11-12 August 2010, Mead, NE.

16) Sattler S (2010) Sorghum *brown midrib* mutants: genes to improve sorghum forage utilization and bioenergy conversion. SweetFuel Project Annual Meeting. 19-23 April 2010, Sete Lagoas, Brazil.

Following is our previous report of other notes concerning the project: We are working with JGI (Dan Rokhsar and colleagues) on a re-sequencing project of the sweet sorghum Rio, one of the parents of the recombinant inbred line population used in the funded project (the other parent is the line already sequenced). It is our understanding the sequencing is complete, but discussions on the accompanying publications have not yet led to a manuscript.