Identifying Differences in Abiotic Stress Gene Networks between Lowland and Upland Ecotypes of Switchgrass (DE-SC0008338)
Final Report
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This final report represents activity covering the period from 1 August 2012 to 31 July 2016. The original project timeframe was August 2012 to July 2015, but a no-cost extension allowed us to work for an additional year until July 2016.

Summary

Switchgrass (*Panicum virgatum*) is a warm-season C_4 grass that is a target lignocellulosic biofuel species for use in the United States due to its local adaption capabilities and high biomass accumulation. Two ecotypes of switchgrass have been described. Members of the lowland ecotype are taller, have narrower leaf blades and generate more biomass compared to individuals from the upland ecotype. Additionally, lowland plants are generally found in the southern United States while upland switchgrass is more typically present in the northern United States. These differences are important as it is envisioned that switchgrass for biofuel production will typically be grown on marginal lands in the northern United States to supplement and diversify farmers' traditional crop incomes. While lowland switchgrass is more productive, it has poor winter survivability in northern latitudes where upland switchgrass is expected to be grown for biofuel use.

Abiotic stresses likely to be encountered by switchgrass include drought and salinity. Despite initially being described as preferring wetter environments, members of the lowland ecotype have been characterized as being more drought tolerant than plants of the upland ecotype. Nonetheless, direct trials have indicated that variation for drought tolerance exists in both ecotypes, but prior to this project, only a relatively small number of switchgrass lines had been tested for drought responses. Similarly, switchgrass cultivars have not been widely tested for salt tolerance, but a few studies have shown that even mild salt stress can inhibit growth.

The effects of drought and salt stress on plant growth are complex. Both drought and salinity affect the osmotic potential of plant cells and negatively affect plant growth due to reduced water potential and reduced photosynthesis that results from lower stomatal conductance of CO_2. Plants respond to drought and salt stress by activating genes that directly attempt to reduce the stress (e.g., transmembrane pumps that partition Na^+) and mitigate the effects of the stress (e.g., synthesis of osmoprotectant metabolites and stress-related signaling compounds). Prior to the start of this project, no gene expression analysis had been performed on switchgrass under conditions of drought or salt stress, and therefore, relevant gene networks responding to drought and salt stress were unknown in switchgrass.

In this project, we performed drought, salt and alkali-salt screens on 49 switchgrass cultivars (Liu et al 2014; Liu et al 2015; Hu et al 2015; Kim et al 2016).

These experiments demonstrated that a wide range of variation exists within switchgrass for drought, salt and alkali-salt tolerance and that, while the lowland ecotype of switchgrass is often considered more tolerant of abiotic stresses, there are some upland switchgrass lines that are also very tolerant of drought, salt and alkali-salt stress. We also conducted drought and salt time course experiments with Alamo and Dacotah. We have identified modules of coexpressed genes that differentiate Alamo and Dacotah drought responses. We are continuing to analyze these results and plan to submit manuscripts describing this work in early 2017. In an effort to show how drought- and salt-related gene modules could be dissected, we generated transgenic switchgrass overexpressing either *PvGTγ-1* or *ZmDREB2*. Increased expression of *PvGTγ-1* does confer increased salt tolerance, and we were able to identify genes that are induced and suppressed by *PvGTγ-1*. Overexpression of *ZmDREB2* increases drought tolerance in switchgrass. Analysis of the *PvGTγ-1* and *ZmDREB2* overexpression work is ongoing, and we plan to prepare manuscripts about these experiments for submission in early 2017.

Staffing

For this project, we had two postdoctoral associates. Yiming Liu was hired as a postdoctoral researcher by CoPI Zhao and CoPI Zhang at Virginia Tech. Because of his work on this project, Dr. Liu was able to obtain a faculty position at the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences / Key Laboratory of Crop Gene Resources and Germplasm Enhancement in Southern China. Additionally, Jeongwoon Kim was a postdoctoral researcher who was hired by PI Childs at Michigan State University and who worked half time on this project. Dr. Kim left her postdoctoral position to accept a job as a bioinformatician with Monsanto. Two undergraduate research assistants, Kaitlyn Heslip and Emily Hilliker, had also been employed by the project at Michigan State University. Both student workers have graduated. Ms. Heslip is now a laboratory technician with a biotech company, and Ms. Hilliker is a medical clinician.

During this project, PI Childs' position changed. At the start, he was a Fixed-Term Assistant Professor, which at MSU is a title for an investigator who is associated with the lab of a tenured faculty member. In 2014, Childs accepted an independent position at MSU as an Assitant Professor-Fixed Term. As a result of this position change, Childs' effort on the project was reduced from 50% to 20%. This effort reduction resulted in a change in salary expenditures that was partly responsible for the no-cost extension request that extended the project for an additional year.

Objectives

In our proposal, we had four main objectives.

Obj. 1. Phenotype 18 lowland and 25 upland switchgrass cultivars for physiological, morphological and metabolic responses to drought and salinity to identify cultivars that are tolerant and sensitive to these abiotic stresses.

Obj. 2. Profile gene expression patterns in lowland and upland switchgrass ecotypes and construct and compare gene coexpression network modules between drought and salt tolerant and sensitive cultivars. Physiological, morphological and metabolic phenotypes will be correlated with gene network modules to develop a systems level understanding of drought and salinity stress responses.

Obj. 3. Profile gene expression patterns in transgenic switchgrass plants overexpressing homologs of known drought and salt stress transcription factors and identify gene network modules controlled by the transgenes. Physiological, morphological and metabolic phenotypes will be correlated with transgene-regulated gene modules to characterize the targets of the selected abiotic regulatory genes.

Obj. 4. Expression and gene module data are highly informative as gene annotations and will be useful for switchgrass researchers. Incorporate gene coexpression network results into the existing Biofuel Feedstock Genomics Resource database.

Switchgrass Cultivars

At the time that we wrote the proposal, we were only confident that we would have access to 18 lowland and 25 upland cultivars. Later, these numbers changed, and Co-PI Zhao had been able to increase the number of cultivars to 49.

We used 9 lowland cultivars and 40 upland cultivars. However, all cultivars were not available for all of our screening experiments.

Lowlands: Alamo, TEM-SEC, TEM-SLC, TEM-LoDorm, Kanlow, T-2086, T-2101, BN-11357-63, BN-12323-69

Uplands: AM-314/MS-155, BN-13645-64, T16971, BN-18757-67, Summer, Cave-in-Rock, Shawnee, Caddo, Forestburg, Sunburst, Nebraska-28 (PI 477003), Dacotah, T4613, T4614, BN-10860-61, BN-8624-67, Genville-2, T-2100, Blackwell-1, Blackwell-2, Blackwell-3, Pathfinder, Trailblazer, Nebraska-28 (Grif 16408), Turkey, 70SG001, 70SG002, 70SG003, 70SG004, 70SG005, 70SG0016, 70SG0017, 70SG0018, 70SG0019, 70SG0020, 70SG0021, 70SG0022, 70SG0023, Central Iowa Germplasm

Objective 1

As described above, we used up to 49 switchgrass lines for screening for drought and salt tolerance. Our salt screening work is described in Liu et al (2014), Hu et al (2015) and Kim et al (2016).

The Liu et al (2014) study used 33 switchgrass lines that included five lowland cultivars. Plants were grown from seed hydroponically. Importantly, as switchgrass is highly heterozygous, the plants derived from seed are not genotypically identical to the parental plant. All measurements taken here were based on multiple plants in order to derive pooled responses. Two months after seeding, plants were treated with 250 mM salt or allowed to continue to grow in half-strength Hoagland's solution for 24 days. The effects of salt stress were determined by measuring physiological traits: leaf electrolyte leakage (EL), leaf photochemical efficiency (F_v/F_m), chlorophyll content, photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration rate (T_r). No metabolite analyses were performed for this screening. Using the physiological measurements, we calculated for each switchgrass line a salt tolerance index (STI) as the percentage of the physiological measurement under stress relative to the measurement under control conditions. Except for EL, calculated STI values were always less than 100%, indicating the negative effects of the applied salt stress on the plants physiological states. For EL, STI values above 100% indicate a stressed state. STI values varied greatly across the lines. For example, STI for P_n ranged from ~65% for TEM-LoDorm to ~5% for BN-18757-67. For T_r, the STI values ranged from ~85% for Kanlow to ~8% for Summer. Generally, lowland lines were more tolerant of the salt stress than upland lines, but some upland lines did perform well.

The Hu et al (2015) publication examined the effects of alkali-salt stress on switchgrass. Alkali-salt stress is the condition when soils contain high concentrations of Na^+ and also have high pH. This study was not directly specified in the original proposal. However, Guofu Hu was a visiting scientist with Bingyu Zhao during the project period, and as he was interested in screening a panel of switchgrass lines, it was decided that it was a good opportunity to add an additional abiotic stress to our work. Thirty switchgrass cultivars were used in this experiment. Plants were clonally propagated from parental material by taking tillers of similar developmental stages and transplanting to new pots. After two months of recovery, plants were treated with half strength Hoagland's solution with 0 mM Na^+ or nutrient solution with 150 mM Na^+ and a pH of 9.5. After 20 days of treatment, relative water content (RWC), EL, P_n, g_s and T_r were all measured. As with the hydroponic salt stress experiment, all cultivars show strong signs of stress in all physiological measures except RWC where the stress treatment had little to no effect on many lines. As expected, the most tolerant switchgrass lines were lowlands, and the most sensitive were upland switchgrass plants. Interestingly, for this study, clustering analysis was performed on all thirty lines using the physiological measurements. Three clear clusters of genotypes were found (high, medium and low tolerance). The most tolerant cluster contained four lowland lines and one unexpected upland line, 70SG0023. Furthermore, the least tolerant cluster contained two lowland lines, BN-13645-64 and AM-314/MS-155.

Our largest salt stress screen experiment involving 46 switchgrass lines is reported in Kim et al (2016). Plants were clonally propagated in pots in a greenhouse and allowed to recover until the shoots had five nodes. At that time, plants were watered with water or NaCl solutions for 30 days. Initial, NaCl treatments slowly increased in concentration over two days until 250 mM NaCl was being applied to the pots. After 30 days of treatment, RWC, EL, P_n, g_s, T_r, water use efficiency (WUE) and intercellular CO_2 concentration (Ci) were all measured. Additionally, proline was assayed from leaf tissues. As in the previous experiments, STI values were calculated for each physiological measurement and each switchgrass line. There was a wide range of responses from the assayed plants, and as expected, lowland cultivars tended to demonstrate more tolerance to the salt stress than upland switchgrass. However, there were upland lines that were as tolerant as the lowland lines. The tested lines were clustered based on STI measurements, and five distinct groups of cultivars were recognized. Two of the clusters contained relatively salt tolerant upland and lowland lines, and the remaining three clusters consisted only of upland genotypes. The plants in the two clusters that contained relatively tolerant upland and lowland lines all had similar

P_n, g_s, T_r, Ci and WUE STI values. The upland cultivars in the salt-tolerant clusters were T4613, Caddo, Pathfinder, T-2101, BN-18758-67, 70SG0021, 70SG0023 and 70SG0024. The two salt-tolerant clusters were distinguished by their EL and proline responses to salt stress. In one cluster, the EL STI values were relatively low (tolerant) and the proline STI values were moderate, but in the other cluster the EL and proline STI measurements were switched. These results are interesting as while proline levels are correlated with salt tolerance in some species there is also evidence that proline catabolism is actually required for salt tolerance (Aziz et al 1998; Sharma et al 2011; Szekely et al 2008). Observing salt tolerant switchgrass lines with both high and low levels of proline suggests that there may be multiple pathways available for switchgrass to tolerate salt stress.

Our final effort in support of Objective 1 of this project was to screen 49 switchgrass cultivars for drought (Liu et al 2015). Plants were clonally propagated from tillers in a greenhouse. After recovering from being transplanted, plants were subjected to drought treatment by reducing watering from 50% to 30% of evaporative water loss for 30 days. This reduction in water content corresponded to a reduction in soil water content from ~26% (well watered) to ~4% (30% of evaporative/transpiration water loss). Control plants were watered to compensate for 100% of evaporative/transpiration water loss. After 30 days of treatment, RWC, EL, P_n, g_s, T_r, WUE and Ci were all measured. Leaf tissues were also collected for analysis of abscisic acid, spermine, trehalose and fructose. Similar to STI calculations used in our salt stress screens, we calculated drought tolerance index (DTI) for each physiological measurement across all switchgrass cultivars as the percentage of the physiological measurement under drought relative to the measurement under control conditions. Lowland cultivars were generally more drought tolerant than upland switchgrass, but as with salt stress, some upland lines were as tolerant in some physiological measures as lowland plants. PCA analysis using the physiological measurements identified three clusters of switchgrass lines with the most drought tolerant cluster including several upland switchgrass: T4613, T-2102, T16791, Shawnee, Caddo, 70SG001, 70SG002, 70SG004, 70SG005, 70SG0018, 70SG0019, 70SG0020, 70SG0022, 70SG0023, Turkey, BN-8624-67. Metabolite analyses yielded an interesting observation. ABA, spermidine, trehalose and fructose levels increased in response to drought in the most drought tolerant lines, but only ABA showed any change in response to drought in the most drought sensitive lines. Control levels of these metabolites were similar in the most drought tolerant lines compared to the most drought sensitive lines. These metabolite observations suggest that drought tolerant switchgrass is at least partly drought tolerant due to the ability to regulate metabolites that are know to help mitigate the effects of drought (Zhang et al 2006; Naser et al 2010; Alcázar et al 2011; Ali et al 2011). The inability of some switchgrass lines to regulate these compounds during drought may contribute to the sensitivity of those lines to drought stress.

There are several main findings from our drought and salt screening experiments. 1. Large variation in drought and salt responses can be found in swithchgrass. 2. Lowland switchgrass lines tend to be more drought and salt tolerant than upland lines, but there are some upland switchgrass that are nearly as tolerant as lowland plants. 3. With regards to alkali-salt stress, strong sensitivity was observed in a few lowland cultivars. 4. Proline's role in tolerance of salt stress in switchgrass is probably complex. 5. Several metabolites (ABA, spermidine, trehalose and fructose) that are known to help mitigate drought stress were shown to be upregulated in drought tolerant switchgrass.

Within Objective 1, our biggest challenge was metabolite analysis. We had worked to detect 14 different metabolites, but we only reported results for five (proline, ABA, spermidine, trehalose and fructose). While we were able to detect all of our target metabolites, the variability in our measurements made the data unreliable in our minds. We only reported metabolite concentrations in the cases where we had confidence in the observed variation. While we did not identify the source of the observed variability, several possible sources of variability include the fact that plant material was grown and harvested at Virginia Tech, shipped on dry ice to Michigan State University (MSU), stored, processed and extracted at MSU and analyzed by HPLC or LC-MS at MSU. The large number of samples also meant that samples unavoidably sat in the auto-injection queue on the HPLC and LC-MS machines for a long time. The availability of heavy-isotope internal standards could have helped account for sampled degradation during processing and detection. Additionally, it is possible that handling fewer samples at one institution would have reduced possible metabolite degradation and measurement variation.

Objective 2

The goal of Objective 2 was to identify genes that respond to drought and salt stress in tolerant and sensitive plants and to then construct and compare gene coexpression network modules between drought and salt tolerant and sensitive cultivars. The correlation of physiological, morphological and metabolic phenotypes to the gene network modules would then allow us to develop a systems level understanding of drought and salinity stress responses. All samples were fully processed and sequenced before the end of the no-cost extension, and we are currently preparing data for publication.

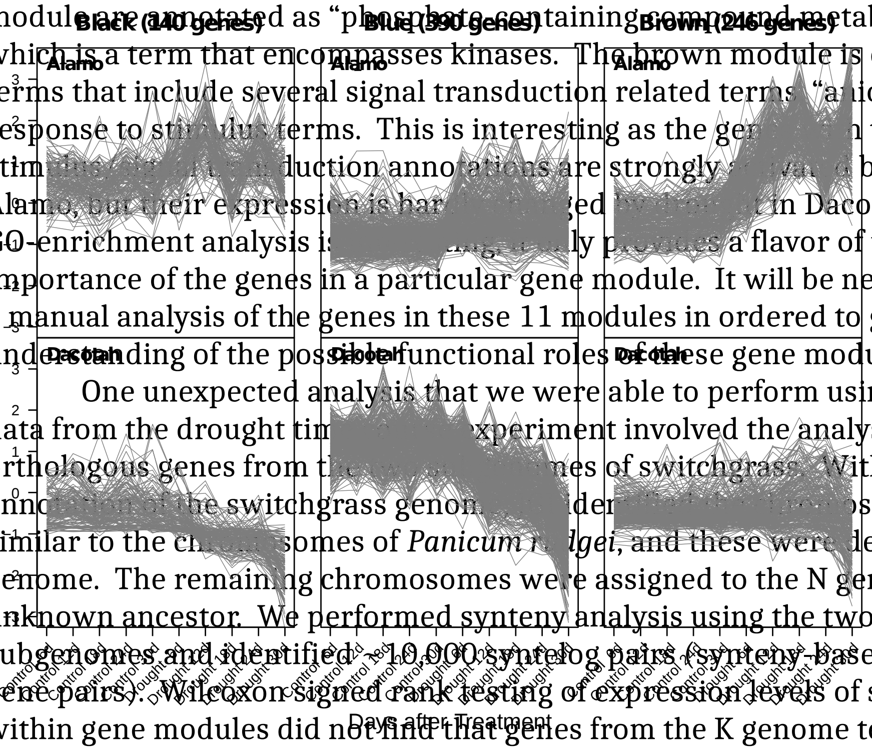
For the experiments that supported Objective 2, we elected to work with Alamo (lowland, drought/salt tolerant) and Dacotah (upland, drought/salt sensitive) lines of switchgrass. These two cultivars represent the extremes of the responses that we had observed from the large set of genotypes analyzed in Objective 1. Additionally, these cultivars are commonly used by switchgrass researchers, and so, they seemed like sensible choices for use in the experiments for Objective 2.

One experiment involved drought stressing Alamo and Dacotah plants for 30 days. Clonally propagated plants were grown in pots in a greenhouse. The drought stress treatment was performed as described in our drought screening manuscript (Liu et al 2015). We collected physiological data (RWC, P_n, g_s, T_r, Ci) and sampled leaf tissue at 0d, 6d, 12d, 18d, 24d and 30d from control and treated plants. Three replicates were used for all data points. Proline levels were also determined from leaf tissues. Other metabolites that were analyzed will not be considered for publication as variation in measured levels had been unacceptably high. RNA was extracted from leaf tissue, and RNA-seq was performed. RNA-seq reads were trimmed and cleaned and aligned to the latest JGI switchgrass genome assembly and gene annotation (version 3). We used the limma package in R to identify

Figure 1. Three gene modules identified from Alamo and Dacotah switchgrass drought time course experiment.

significantly differentially expressed genes (Law et al 2014). Genes with low counts across all samples were removed from analysis. Normalization factors were calculated, and samples were normalized using the voom procedure. A linear model was fit to the data (Y ~ genotype + treatment + time + genotype*treatment + genotype*time + treatment*time + genotype*treatment*time), and the eBayes function was used to calculate significance. Genes were determined to be differentially expressed if they had a $\log_2$-fold change >= 2 and a p-value <= 0.01. Most genes that passed our minimum read counts criteria were found to be differentially expressed based on genotype and treatment. However, it is the genotype-by-treatment interaction that is likely to be informative with regards to why Alamo and Dacotah switchgrass respond so differently to drought stress. Therefore, we focused on the 2,635 genes with expression patterns that are explained by the interaction of the genotype and treatment factors. In these cases, expression of the gene is different between control and drought treatments, and the gene expression is also different between Alamo and Dacotah. These 2,635 genes were subjected to coexpression analysis using Weighted Gene Coexpression Network Analysis (WGCNA; Langfelder and Horvath 2008). The coexpression analysis identified 11 modules of genes with highly correlated expression patterns. Figure 1 shows three of these gene modules. Analysis of the gene modules is ongoing, but some initial observations have been made. GO-enrichment indicates that the blue module is enriched for genes associated with various nucleic acid metabolism including ncRNA metabolic processing, tRNA aminoacylation. This is interesting as changes in tRNA charging have been shown to be important in stress responses in some species (Chan et al 2012). Nearly 10% of the genes in the blue module are annotated as "phosphate-containing compound metabolic process", which is a term that encompasses kinases. The brown module is enriched for GO terms that include several signal transduction related terms, "anion transport", and response to stimulus terms. This is interesting as the genes with these stimulus/signal transduction annotations are strongly activated by drought in Alamo, but their expression is hardly changed by drought in Dacotah. While the GO-enrichment analysis is interesting, it only provides a flavor of the functional importance of the genes in a particular gene module. It will be necessary to perform a manual analysis of the genes in these 11 modules in ordered to gain a better understanding of the possible functional roles of these gene modules.

One unexpected analysis that we were able to perform using the expression data from the drought time course experiment involved the analysis of expression by orthologous genes from the two subgenomes of switchgrass. With the most recent annotation of the switchgrass genome, we identified the chromosomes that are most similar to the chromosomes of *Panicum rudgei*, and these were designated as the K genome. The remaining chromosomes were assigned to the N genome from an unknown ancestor. We performed synteny analysis using the two switchgrass subgenomes and identified ~10,000 syntelog pairs (synteny-based orthologous gene pairs). Wilcoxon signed rank testing of expression levels of syntelog gene pairs within gene modules did not find that genes from the K genome tended to be more highly expressed than their N genome counterparts or vice versa. So, unlike in maize (Schnable et al 2011), no subgenome dominance is observed in switchgrass.



Using the syntelog pairs, we were able to see that, for many gene pairs from the K and N subgenomes, their expression patterns were similar even if the overall expression levels were different. However, this was not true for all syntelog pairs, and this suggests that gene subfunctionalization and/or neofunctionalization has occurred for some syntelog gene pairs. We will be submitting a manuscript on this work in early 2017.

A second experiment was performed involving salt stressing Alamo and Dacotah plants for 30 days. Similar to the drought time course experiment, plants were clonally propagated by transplanting tillers into pots and were grown in a greenhouse for two months before beginning treatments. The concentration of the NaCl treatment was gradually increased over two days to 250 mM. Control plants were treated with water. Physiological measurements (RWC, P_n, g_s, T_r, Ci) and leaf tissue samples were taken at 0h, 12h, 24h, 48h, 6d, 12d, 18d and 24d. RNA was extracted from the leaf tissues and sequenced. We have processed the RNA data, but have not yet finished analysis of these data. We expect to be able to prepare a manuscript on this experiment in early 2017.

Objective 3

When this project was first envisioned, we understood that the drought and salt time course experiments would not be completed until near the end of the grant period. However, had those experiments finished quickly, we would have wanted to focus on one or two relevant gene modules and to attempt to understand the genetic regulation of those modules. For example, in the Brown module in Figure 1, it is possible that some of the transcription factors in the module are in fact responsible for the drought induced expression of other genes in the module, or regulatory genes in the Brown module could be responsible for the drought-related down regulation of genes in other gene modules. To demonstrate this principle, the goal of Objective 3 was to profile gene expression patterns in transgenic switchgrass plants overexpressing homologs of known drought and salt stress transcription factors. We chose to work with maize *DRE-binding protein 2* (*ZmDREB2*), which is known to be involved in drought tolerance in maize (Wang et al 2011), and rice *GTγ-1*, which is a regulatory factor know to affect salt tolerance in rice (Fang et al 2010). For the *GTγ-1* gene, we were able to identify the switchgrass ortholog (*PvGTγ-1*) using synteny. Both *ZmDREB2* and *GTγ-1* were synthesized, placed into a transformation vector with a constitutive promoter and used to transform switchgrass callus culture tissue (Liu et al 2016). For each gene, multiple transformed plants were recovered and examined further.

Overexpression of *PvGTγ-1* (OE-*PvGTγ-1*) in switchgrass leads to improved salt stress tolerance. The OE-*PvGTγ-1* transgenic switchgrass plants have similar morphological characteristics as the non-transgenic controls. Under salt stress conditions (250 mM NaCl for 12 days), the OE-*PvGTγ-1* transgenic plants maintained better cell membrane integrity with less cell membrane electrolyte leakage. The OE-*PvGTγ-1* plants had a greater net photosynthetic rate than that of the non-transgenic controls. Overexpression of *PvGTγ-1* also induced a shift of hormonal balance resulting in differential stomatal regulation and lower senescence under the salt stress conditions. Higher levels of osmoprotectants were found in the transgenic plants than the non-transgenic controls under salt stress. RNA-seq gene expression profiling revealed 2,969 differentially expressed genes between OE-*PvGTγ-1* and control plants at six growth time points. Gene coexpression analysis identified 11 gene modules, and one module contains 604 genes that are specifically induced by OE-*PvGTγ-1*, while another module contains 506 genes that are suppressed by OE-*PvGTγ-1*. The expression of selected candidate genes was further validated by real time PCR analysis. Our results suggest that *PvGTγ-1* encodes a putative transcription factor and overexpression of *PvGTγ-1* improves switchgrass salt tolerance by regulating stress-related hormonal, osmotic metallization, and anti-senescence related gene expression. To our knowledge, this is the first report of GTγ-like a transcription factor altering the salt tolerance in switchgrass.

Expression of maize *ZmDREB2* in transgenic plants was tested by PCR, RT-PCR, and southern blot analysis. The *ZmDREB2* transgenic switchgrass plants have delayed flowering time, developed more tillers but smaller leaf blades compared to the non-transgenic controls. The *ZmDREB2* transgenic plants maintained greater net photosynthetic rate, but reduced stomatal conductance and transpiration rate than that of the non-transgenic control under drought stress conditions. Overexpression of *ZmDREB2* induced a shift of hormonal balance that we associated with increased stomatal closure and water conservation under the drought stress conditions. The transgenic plants also accumulated higher levels of osmoprotectants than the non-transgenic plants under drought stress. Real-time PCR analysis revealed several antioxidant-related genes are strongly induced by the ectopic expression of *ZmDREB2*. Therefore, our results suggest that overexpression of *ZmDREB2* improves switchgrass drought tolerance by regulating stress-related hormonal and antioxidant metabolism, as well as through improved osmotic adjustment. We were unable to perform gene expression analysis for the *ZmDREB2* experiment as funding for the project ran out.

We are currently working on completing the analyses of these data and plan to submit manuscripts for these two experiments in early 2017.

Objective 4

For Objective 4, we had intended to incorporate expression and gene module data into the existing Biofuel Feedstock Genomics Resource (BFGR) database (http://bfgr.plantbiology.msu.edu). Unfortunately, as we are still analyzing expression data from the Alamo and Dacotah time course experiments, we have been unable to add data to the BFGR database. When expression data is posted to the BFGR website, it will be provided as summary flat files of processed expression values across treatments. Additionally, tables of gene module membership will be provided. One concern is the long-term status of the BFGR database, which is no longer funded, and thus we will ensure that the large datasets generated in these remaining publications are archived via the Dryad Digital Repository and as supplemental datasets in published journals.

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