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PNNL-29251

Programming Metabolite Exchange to Understand and Control Plant-Microbe Interactions

October 2019

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U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
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FY18 Laboratory Directed Research and Development

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Final Technical Report

Specific Aims

Many aspects of plant growth and survival are dependent upon beneficial interactions between plant tissues and bacterial species of the endophyte and rhizosphere. These associated microorganisms can contribute positively to plant growth, stress tolerance, metabolism and protection against plant pathogens. For example, an endosymbiotic bacterium *Burkholderia phytofirmans* PsJN was shown to colonize and promote growth of switchgrass under controlled conditions, suggesting it may be a promising candidate for improving bioenergy crop production (Kim et al., 2012). Despite the documented benefits of endophytic and rhizosphere microbes on plant fitness and photosynthetic productivity, there is still a gap in our understanding of specific molecular -and cellular-level processes that guide the development of beneficial plant-microbe interactions. As these interactions involve physical contact/metabolite exchange we also posit that directed co-localization of beneficial bacteria and plant cells will enhance these benefits. We propose to engineer bacterial species that co-localize with the plant tissue and then to measure cell growth as well as carry out –omics analysis to obtain information regarding the specifics of these beneficial interactions as well as their extent. In order to answer these questions the following Aims are proposed.

Aim 1. Engineer bacterial strains of the rhizosphere or endophyte that migrate toward plant cells expressing a specific molecular signal. **(A)** Using a beneficial bacteria strain of the endophyte or rhizosphere (we will start with *Bacillus subtilis* B26 but other strains may be used as well) we will engineer strains that activate a motility gene based on the presence and concentration of a molecular signal, in this case lysine. These cells will also express RFP so that they can be distinguished from wild-type cells. **(B)** Using the model grass plant *Brachypodium distachyon* Bd21 we will engineer plant cells that overexpress lysine constitutively. These cells will also express GFP so that they can be distinguished from wild-type cells. **(C)** Both wild-type and lysine expressing *B. distachyon* Bd21 cells will be cultured together and the preferential migration of *B. subtilis* or other bacterial species toward lysine-expressing *B. distachyon* Bd21 cells will be documented will fluorescent microscopy.

Aim 2. Determine the positive effects of beneficial bacteria on *B. distachyon* Bd21 plant cells during co-cultivation. **(A)** *B. subtilis* cells and *B. distachyon* Bd21 cells from Aim 1 will be cultured together and the cell growth and cell division of plant cells will be measure and compared to plant cells cultured without *B. subtilis* cells. **(B)** Transcriptomic profiling of both *B. subtilis* and *B. distachyon* Bd21 cells will take place with and without co-cultivation to identify genes responding to co-cultivation and likely involved in beneficial interactions between these organisms. **(C)** Based on previous knowledge and the results from RNA-seq analysis we will generate knockout strains of *B. subtilis* that lack genes hypothesized to be critical for beneficial interactions. The cell growth and cell division of *B. distachyon* Bd21 plant cells will then be examined with these knockout strains to identify the role of the missing gene in conveying benefits to plant cells.

Results and Accomplishments

Aim 1. Building lysine sensors for *B. subtilis*

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The process of building riboswitches involves the combination of a known aptamer (a region of RNA that binds to a small molecule), either naturally occurring or constructed, with a library of random switch sequences along with the gene to be controlled by the riboswitch. Certain combinations of aptamers and switch sequences will lead to the expression of the downstream gene as a function of presence or absence of the small molecule. A known aptamer for lysine already exists in *B. subtilis* (Sudarsan et al., 2003) that drives the expression of a lysine uptake system. We have used this aptamer and cloned it upstream of two separate motility genes in *B. subtilis*, *motAB* (Terahara et al., 2006) and *swrAA* (Ghelardi et al., 2012). Cloned between these two sequences (the aptamer and the motility gene) is a library of random switch sequences. We have carried out Sanger sequencing on these two constructs to confirm the random nature of the switch sequence (**Figure 1**) and have generated libraries of ~50,000 clones, each clone representing a single switch sequence. This library can now be screened to determine whether the addition of lysine induces expression of the motility gene, leading to a strain of *B. subtilis* that is motile in the presence of lysine and will move toward higher concentrations of this molecule. The current libraries we have built are a significant step in generating these motile strains.

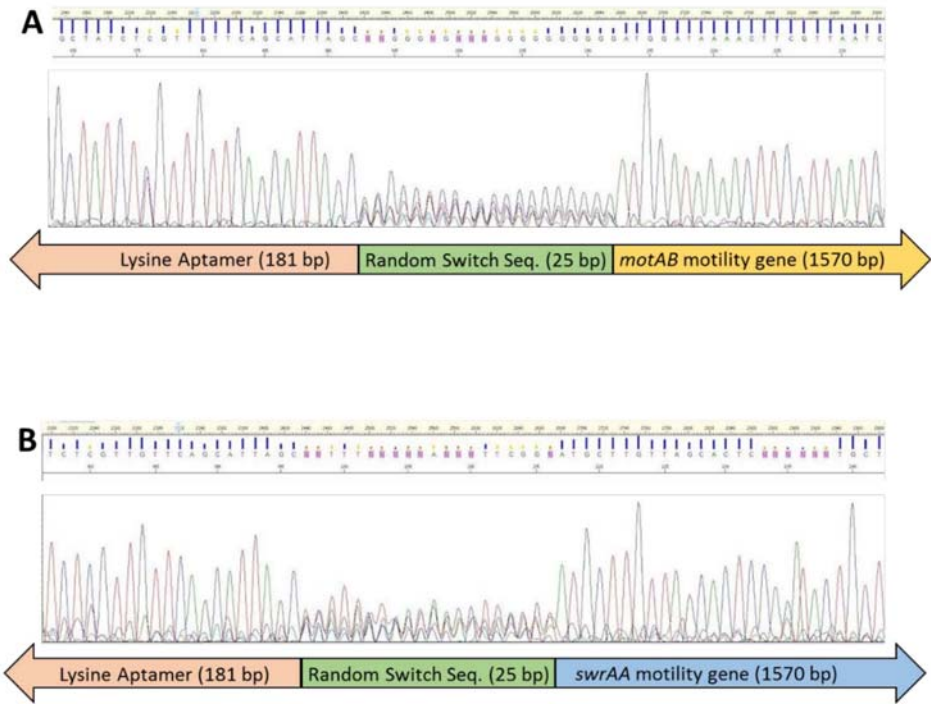

Figure 1. Lysine aptamer/motility gene libraries. (A) Sequencing profile of the lysine aptamer/random sequence/*motAB* gene cassette. Strong peaks in the lysine aptamer and *motAB* portions indicate high quality base calls for each position. Smaller peaks in the random sequence section indicate lower quality calls as this portion represents a random sequence and so no one base pair can be identified at each position. **(B)** Similar results for the *swrAA* motility gene.

Aim 2. Examining Interactions Between *B. subtilis* and *B. distachyon*

We have carried out a large experiment examining the role of *B. subtilis* during incubation of *B. distachyon* under high salinity conditions. High salinity represents a stress state for many plants that can be encountered during drought or other periods of water reduction. Previous studies have found a protective role of *B. subtilis* when *B. distachyon* is cultured under drought conditions but these studies have only looked at the phenotype of the plant (Gagne-Bourque et al., 2015). Here, we wanted to explore a new stress condition, salinity, and also gain a more detailed understanding of the interactions between these organisms by carrying out RNA-seq analysis to view the transcriptomic response of plant and bacteria both to co-incubation and to salt stress. *B. distachyon* plants were incubated with and without *B. subtilis* (6.0×10^6 cells per mL) for

18 days under sterile conditions before salt stress was started. Fluorescent microscopy during this time showed the association of *B. subtilis* with the roots of *B. distachyon* plants (**Figure 2A,B**). *B. distachyon* plants were then incubated with and without *B. subtilis* under both high (200 mM) salt conditions and control conditions with no salt for 10 days before phenotypic analysis and collection of RNA for sequencing of both the *B. subtilis* and *B. distachyon* transcriptome took place.

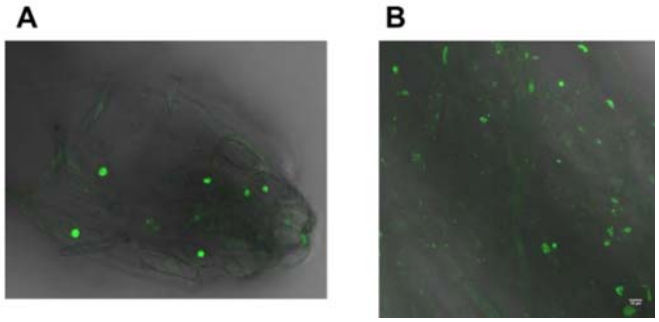

Figure 2: *B. subtilis* associates with *B. distachyon* roots. (A) A SYBR green stain of *B. subtilis* associated with the tips of roots of *B. distachyon* after inoculation. **(B)** Similar stain examining the upper root.

Phenotypic analysis showed a significant negative effect of salt stress on the growth of *B. distachyon* (**Figure 3A,B**). Under high salt conditions plants had significantly fewer tillers (side shoots of the main stem, 1.15 fold higher in control, *p*-value 0.015), shoots (leaves) were of smaller mass (1.37 fold higher in control, *p*-value < 0.001) and roots were of smaller mass (2.11 fold higher in control, *p*-value < 0.001). The phenotype of salt stress was ameliorated to a moderate degree upon co-incubation with *B. subtilis* (**Figure 3A,B**). Compared to treatment with salt only, treatment with salt along with co-incubation with *B. subtilis* led to more tillers (1.1 fold higher under *B. subtilis* treatment, p-value of 0.014) as well as a slightly higher root tissue weight, though this effect was not significant. The moderate protection provided by *B. subtilis* to salt treatment is well-suited to these studies as we wish to explore how this effect can be increased by guiding *B. subtilis* to the root tissue of *B. distachyon* using a lysine signal. It should also be noted that the main goal of this experiment was an analysis of the transcriptomic response of both organisms to co-incubation and salt stress. To that end high quality RNA has been collected from all treatments (4 biological replicates) and was sent to an outside party, GENEWIZ, for transcriptomic analysis of both *B. distachyon*. In

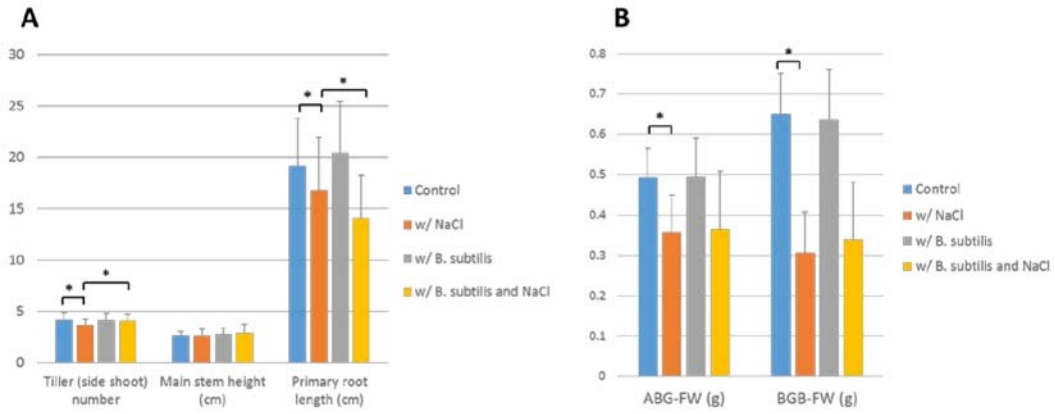

Figure 3. *B. distachyon* phenotypic response to salt stress and *B. subtilis*. (A) The number of tillers, the main stem height (cm) and the primary root length (cm) is shown for control *B. distachyon* (blue bars), *B. distachyon* with salt stress (orange bars), *B. distachyon* inoculated with *B. subtilis* (grey bars) and *B. distachyon* with salt stress and inoculated with *B. subtilis* (yellow bars). **(B)** The ABG-FW (leaf weight) and BGB-FW (root weight) is shown for control *B. distachyon* (blue bars), *B. distachyon* with salt stress (orange bars), *B. distachyon* inoculated with *B. subtilis* (grey bars) and *B. distachyon* with salt stress and inoculated with *B. subtilis* (yellow bars). An * indicates a *p*-value < 0.05

addition, high quality RNA was collected from co-cultivation conditions, with and without salinity stress and sent to GENEWIZ for analysis of *B. subtilis* RNA. For these RNA-seq experiments *B. subtilis* was added to *B. distachyon* whenever Hoagland's growth media was changed (every 3-4 days) to that higher amounts of *B. subtilis* could be maintained and sufficient depth of sequencing for this organism could be obtained. Resulting RNA-seq files were aligned to the genomes of each species to determine their transcriptomic response to co-cultivation and to salt stress. Analysis of the *B. distachyon* transcriptomic response to showed a significant response of the plant to both co-cultivation with *B. subtilis* and to salt stress (**Figure 4**).

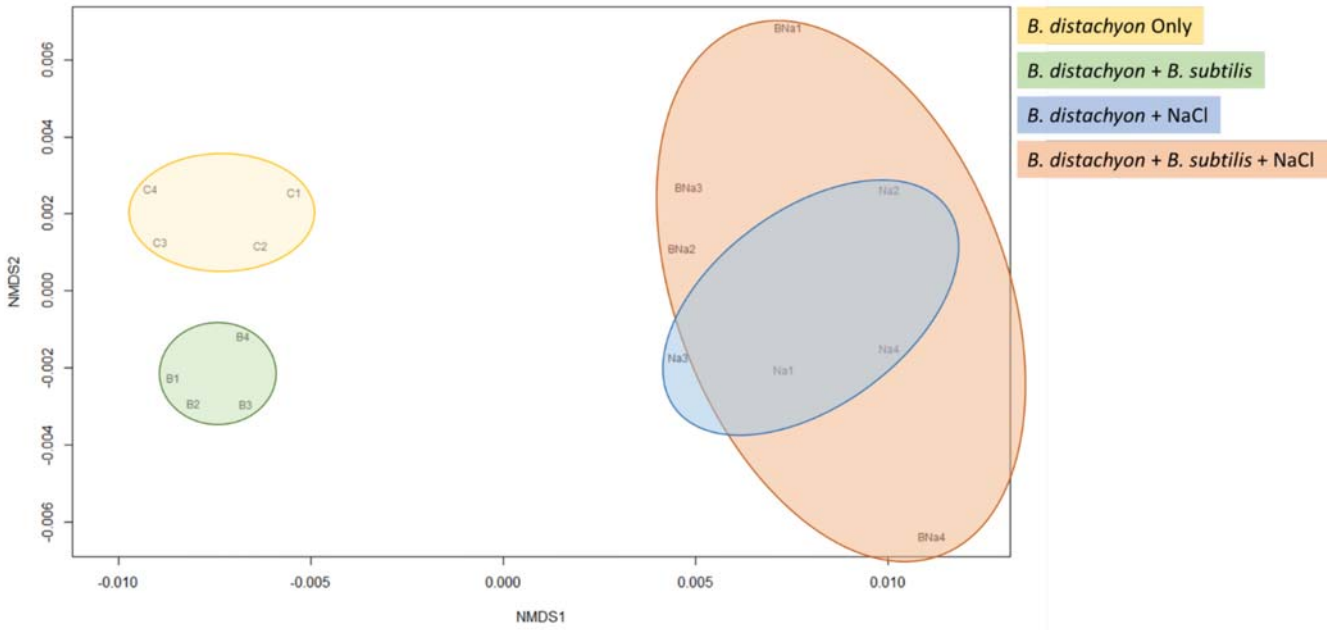

Figure 4. *B. distachyon* transcriptomic response to salt stress and *B. subtilis*. Transcriptomic samples were clustered using multi-dimensional scaling. The Yellow cluster indicates samples from *B. distachyon* grown only in Hoagland's solution with no salt stress or *B. subtilis*. The Green cluster indicates samples from *B. distachyon* grown in Hoagland's solution with *B. subtilis* added. The Blue cluster indicates samples from *B. distachyon* grown in Hoagland's solution with salt stress (200 mM NaCl). The final Orange cluster indicates samples from *B. distachyon* grown in Hoagland's solution with salt stress (200 mM NaCl) and *B. subtilis* added.

After completing this initial analysis, we next looked at what *B. distachyon* genes showed changed in expression as a result of salt stress, *B. subtilis* co-cultivation or both. During salt stress, without *B. subtilis*, *B. distachyon* responds through increased expression of flavonones biosynthesis, hormone degradation and flavonoid biosynthesis. During co-cultivation with *B. subtilis*, but without salt stress, *B. distachyon* responds with molybdenum metabolism, hormone synthesis (as opposed to degradation) and cell wall biosynthesis. When *B. distachyon* is co-cultured with *B. subtilis* under conditions of salt stress the plant responds with changes in expression of several amino acid biosynthesis pathways including tryptophan and alanine (suggesting that these may be provided by *B. subtilis* under stress conditions) (**Figure 5**).

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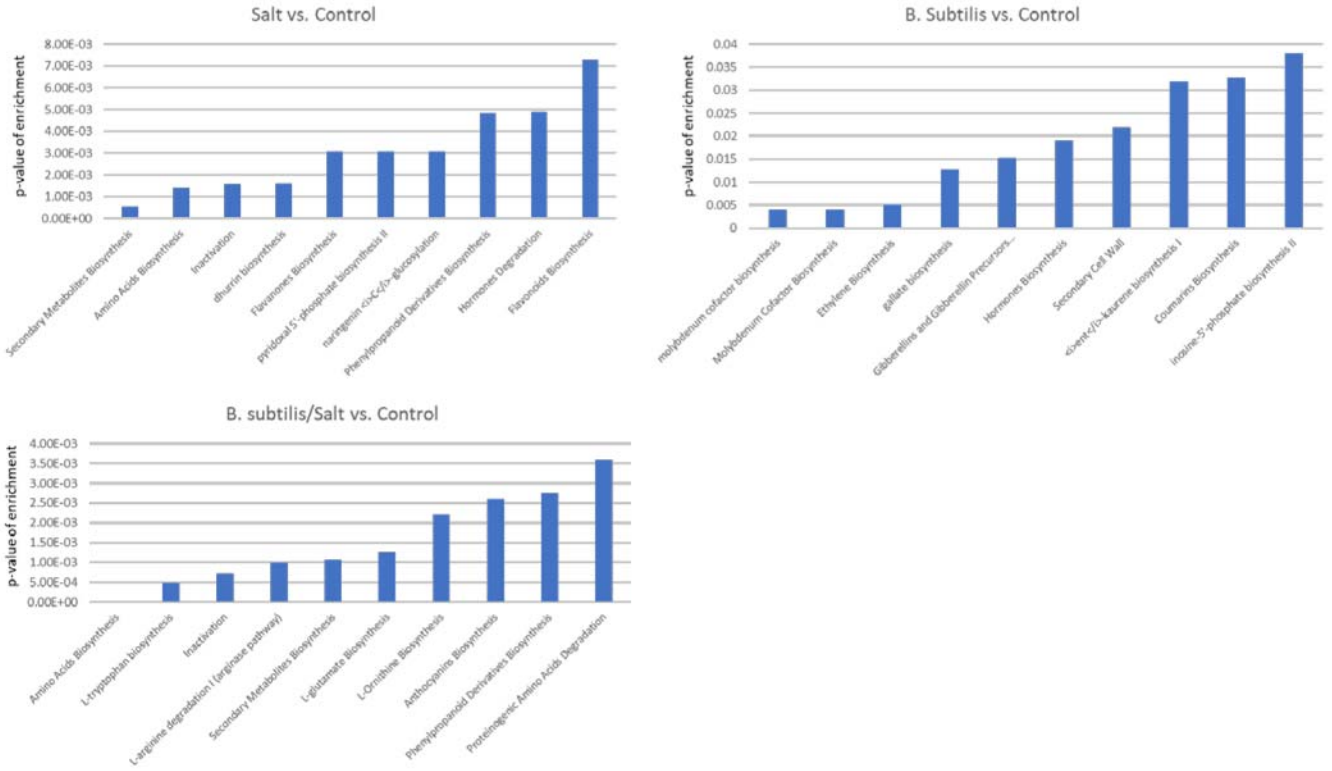

Figure 5. Changes in expression of *B. distachyon* genes in response to salt stress and *B. subtilis* co-cultivation. Gene categories are shown on the x-axis and p-values are shown on the y-axis, a lower p-value indicates a more statistically significant enrichment.

When looking at *B. subtilis* there were fewer genes showing significant regulation in response to salt stress and *B. distachyon* co-cultivation. The *pcp* gene of *B. subtilis* (BSU_02650) and the *alsD* gene (BSU_36000) showed downregulation during salt stress while nine genes showed upregulation under these conditions (**Table 1**).

Locus Tag	Gene Name	Product	Log2 FC (*B. distachyon* vs. *B. distachyon*/salt stress)	Adjusted p-value
BSU_00530	*pth*	peptidyl-tRNA hydrolase	-3.99	0.0997
BSU_02650	*pcp*	pyrrolidone-carboxylate peptidase	3.81	0.0306
BSU_04410	*ydbB*	Hypothetical protein	-5.21	0.0051
BSU_14880	*ctaB*	protoheme IX farnesyltransferase 2	-6.00	0.0306
BSU_19680	*yozE*	Hypothetical protein	-5.60	0.0012
BSU_31270	*tglL*	protein-glutamine gamma-glutamyltransferase	3.41	0.0707
BSU_33620	*yvaK*	carboxylesterase	-3.44	0.0589

BSU_35190	*yvkC*	phosphotransferase	-3.73	0.0500
BSU_36000	*alsD*	alpha-acetolactate decarboxylase	4.61	0.0608
BSU_36860	*atpE*	ATP synthase subunit c	-2.86	0.0997
BSU_37290	*arfM*	transcription regulator	-3.81	0.0306

Table 1. *B. subtilis* genes responding to co-cultivation with *B. distachyon* and salt stress.

Because we collected transcriptomic data from both *B. subtilis* and *B. distachyon* we were able to determine which gene pairs between these two species showed statistically significant co-expression across the conditions used. This may identify specific instances of interaction between the species. To that end we next inferred a network of *B. distachyon* and *B. subtilis* genes that showed had strong Pearson correlation co-efficient. This network showed that many *B. subtilis* genes occupy positions of high centrality and are linked to a large number of *B. distachyon* genes, this suggests that a few *B. subtilis* genes may be involved in several different *B. distachyon* metabolic pathways (**Figure 6**). We are currently looking at what pathways these high centrality genes are involved in and which they connect to in B. distachyon, revealing putative interactions between these species.

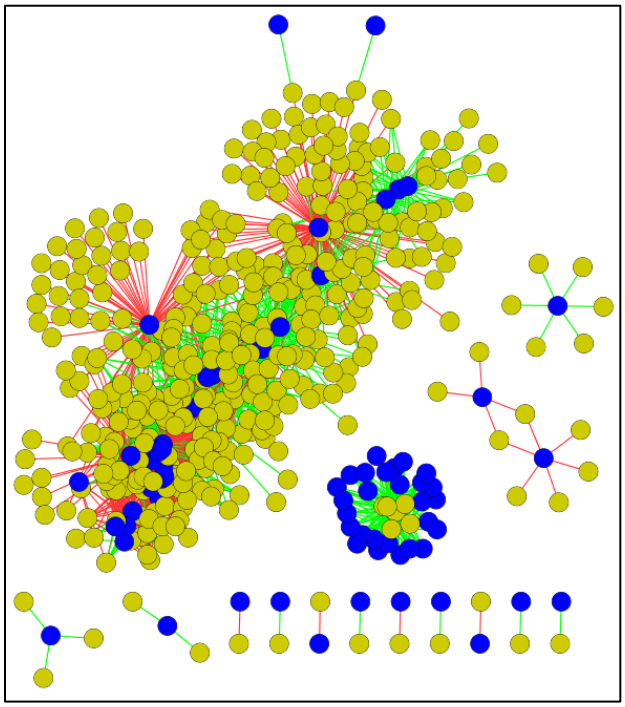

Figure 6. Gene co-expression network of *B. subtilis* and *B. distachyon* genes. Blue dots represent B. subtilis genes and yellow dots represent B. distachyon genes with lines connecting them indicating high co-expression. Red lines indicate negative co-expression and green lines positive co-expression.

Manuscript Milestones

The results of the transcriptomic analysis are being collected into a manuscript which is now under preparation. The tentative title is *Dual Transcriptomic Analysis of Bacillus subtilis and Brachypodium distachyon During High Salinity Stress.*

Mission Relevance

Biological and Environmental Research (BER)
Department Of Energy - Office of Science (DOESC)
Defense Advanced Research Projects Agency (DARPA)

The proposed project is at the nexus of the strategic plans of the Department of Energy (DOE) by addressing the current **Biological and Environmental Research (BER)'s areas of emphasis** including **Biosystems Design**: "*Develop the fundamental understanding of genome biology needed to design, modify, and optimize plants, microbes, and biomes for beneficial purposes*"; and **Bioenergy**: **"***The scope of research extends from gaining a basic understanding of plant and microbial biology necessary for developing and converting dedicated bioenergy crops to fuels and products to obtaining fundamental insights*

into the sustainability of bioenergy crop production". The proposed research also fits well with the recently DOE published Research for Sustainable Bioenergy Workshop Report: "…*to understand how genomic information is translated to functional capabilities, enabling more confident redesign of microbes and plants for sustainable biofuel production*…". This riboswitch aspects of this research also fulfill goals laid out by the **DARPA Biological Technologies Office** in their mission to investigate *programmable microbes* and *synthetic biology* as well as the **DOE Office of Science** in their goal to carry out *delivery of scientific discoveries and major scientific tools to transform our understanding of nature*.

New staff supported by this project
Yuliya Farris – Cloning and testing of motile *B. subtilis* strains
Tanya Winkler – Growth of *B. distachyon* and examination of the role of *B. subtilis* in protecting from salt stress.
Vimal Kumar – Analysis of *B. distachyon* RNA-seq data.
Tobias Flores-Wentz – Cloning of *B. subtilis*.

New projects and collaborations resulting from this project.
As a result of this project we have started collaboration with Rene Boiteau at Oregon State University. This collaboration led to the submission of a DOE proposal in response the Genome Sciences Call. This proposal was declined but the reviews indicate several positive avenues and we plan on re-submission.

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