SPRINT- TTU Progress Report

Molecular mechanisms including micro RNA regulation of abiotic and biotic stress tolerance in sugarcane

Kameswara Rao Kottapalli, Center for Biotechnology and Genomics, Texas Tech University, Lubbock, TX-USA

Sonia Marli Zingaretti, Unidade de Biotecnologia, Universidade de Ribeirão Preto, Sao Paulo, Brazil

First year progress report : 07/01/2016 to 08/31/2017

PROJECT ABSTRACT

Abiotic factors such as water stress, increased average annual temperature, and aluminum toxicity are major limitations to agricultural production in most regions around the world. These effects are compounded by biotic stresses like diseases and limit agricultural productivity. Direct improvement in crop germplasm that results in increased yields under environmentally challenging conditions is critical if we are to meet future demands of food, fiber, and biofuel production.

Our long-term goals are to understand the molecular mechanisms of abiotic and biotic stress tolerance in crop plants and to develop stress-tolerant cultivars. For this proposal, we will 1) investigate the molecular basis of sugarcane's response to abiotic and biotic stresses, and 2) the role of miRNA's in this response. Hypotheses include: 1) sugarcane germplasm pools possess a wealth of novel genes and phenotypes for both abiotic and biotic stress response that can be exploited for improving stress tolerance and 2) a combination of molecular and next-gen sequencing methods will result in successful identification of molecular mechanisms that will assist in breeding improved stress tolerance.

Here, we propose to identify diverse sugarcane cultivars showing differences in aluminum toxicity and leaf scald disease resistance by greenhouse screening. Transcriptome analysis (RNA-Seq) will be used to identify molecular mechanisms underlying the stress response and fundamental differences between contrasting genotypes. This will be followed by global miRNA profiling by sequencing to identify post transcriptional gene regulation. Successful completion of this work will provide insight into the underlying molecular mechanisms controlling sugarcane abiotic and biotic stress responses. The generation of new transcriptome data will provide valuable sequence information to the sugarcane community and scoreable polymorphisms in parents for breeding abiotic and biotic stress tolerance.

ACHIEVEMENTS TILL DATE

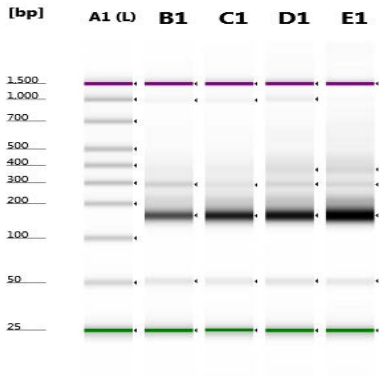
During this first year period two missions/visits did take place, one in Texas Tech University during August 2016 when Dr Sonia Zingaretti spent 10 days in Dr Kottapalli laboratory to process root RNA-Sequencing and had the opportunity to meet several others researchers from the Institute, working in different areas, and also meet his students. At this time Dr Sonia also participated in the "STEM Across Continents Workshop" held by Texas Tech University from August 31st to September 1st, and could present the research project approved by SPRINT program. In 2016, Dr Kottapalli also visited Brazil in November and spent a week in Sonia´s lab at Universidade de Ribeirão Preto. During his visit, he had the opportunity to meet the researchers from the University, discuss projects with students and present a formal talk to the students of the Biotechnology graduate program and faculty in the department.

During the first mission/project at Texas Tech University we processed sugarcane root samples (lyophilized tissue) generated from the experiments of ongoing grant (FAPESP 2014/79667-8) from Brazil, where two sugarcane cultivars, resistant and sensitive to Aluminum stress, had been selected for RNAseq. After growing at specific Al concentration root samples were collected from 2 time points and 3 reps for each time point from tolerant and susceptible sugarcane genotypes. Samples were processed and sequentially used for RNA extraction and sequencing as proposed.

A brief report on methodology and results obtained:

- Total RNA was extracted from lyophilized root samples using spectrum Total RNA kit, following manufactures protocols. The integrity of RNA was measured on a TapeStation 2200 (Agilent), also following the manufacturer's instructions.
- cDNA Libraries were prepared using True-Seq RNA library protocol (Illumina Inc, San Diego, CA, USA) and NEXTflex small RNA library prep protocol (BIOO-SCIENTIFIC). The libraries were quantified using a Qubit1 2.0 fluorometer (Life Technologies, Carlsbad, CA) and the quality was analyzed with the TapeStation 2200 for validating the purity and estimating the insert size (Fig 1).

Fig. 1 Library Validation Quality of sample libraries checked on Agilent TapeStation 2200. Final product at ~160 bp

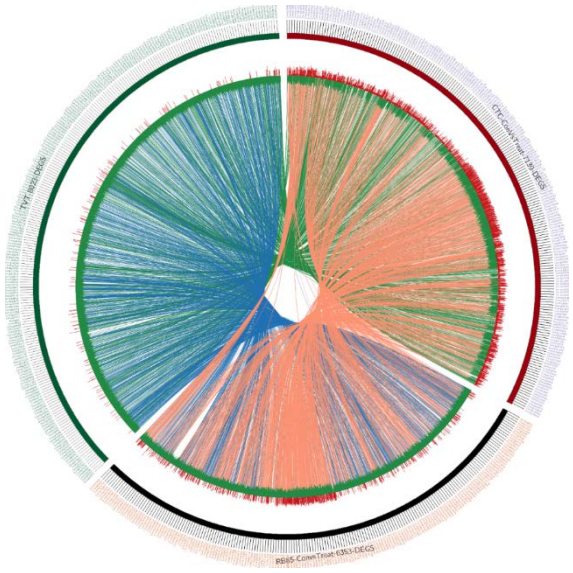


- To identify molecular mechanisms associated with Aluminum stress tolerance, sequencing was performed on HiSeq 2500 (Illumina Inc, San Diego, CA, USA) with 108 bp. paired end sequencing in rapid mode.
- Single end 35bp sequencing was followed for miRNA profiling using MiSeq (Illumina Inc, San Diego, CA, USA) instrument.
- Fast-QC was run to check sequencing read quality.
- Sequencing data analyses was performed on high quality reads.
- Reads were assembled using Trinity to generate a reference sugarcane transcriptome as described in Table 1.

Table 1 Sugarcane – Transcript assembly

Total Trinity 'genes'	**97335**
Total Trinity transcripts	162161
Percent CG	49.68
Median contig lenght	577
Average contig	836.46
Total assembled bases	135641186

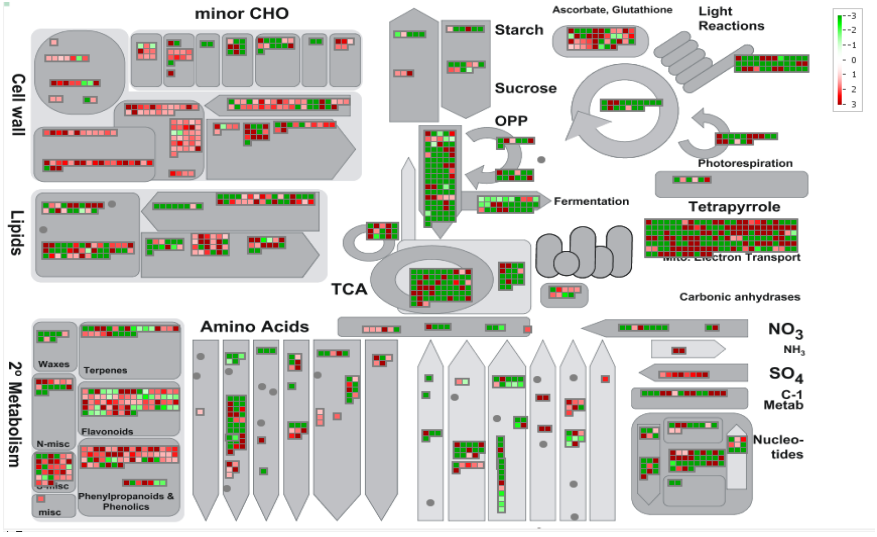
- Differential gene expression analysis was performed using Qseq software (DNAStar, MA, USA), and the output data was used as input to generate a CIRCOS plot (Fig 2).



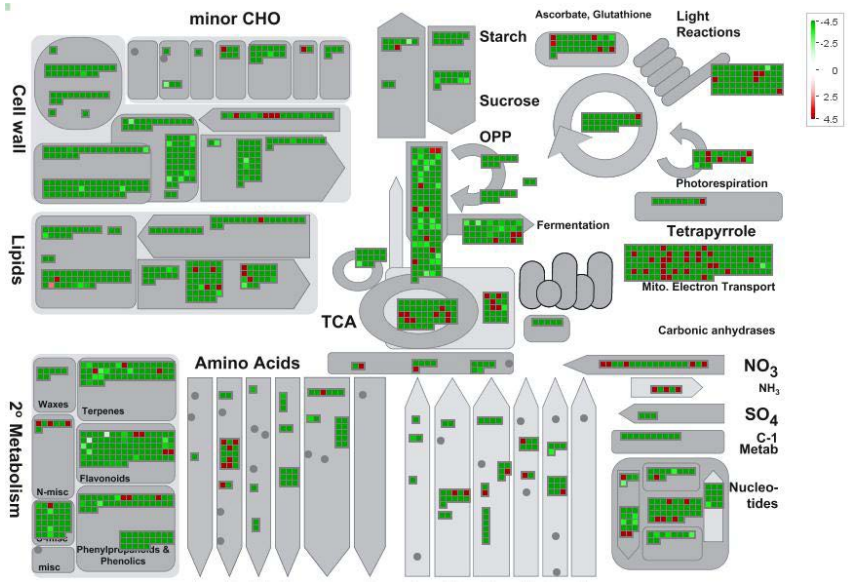
- The differently expressed genes were mapped in to biological pathways using MapMan software (http://mapman.gabipd.org) (Fig 3).

Fig 3 Differential gene expression. A- Induced and repressed genes identified in CTC a tolerant sugarcane cultivar under Stress and Control condition. B- Induced and repressed genes identified in RB a sensitive sugarcane cultivar under Stress and Control condition.

A- CTC- Stress/Control



B- RB85- Stress/Control

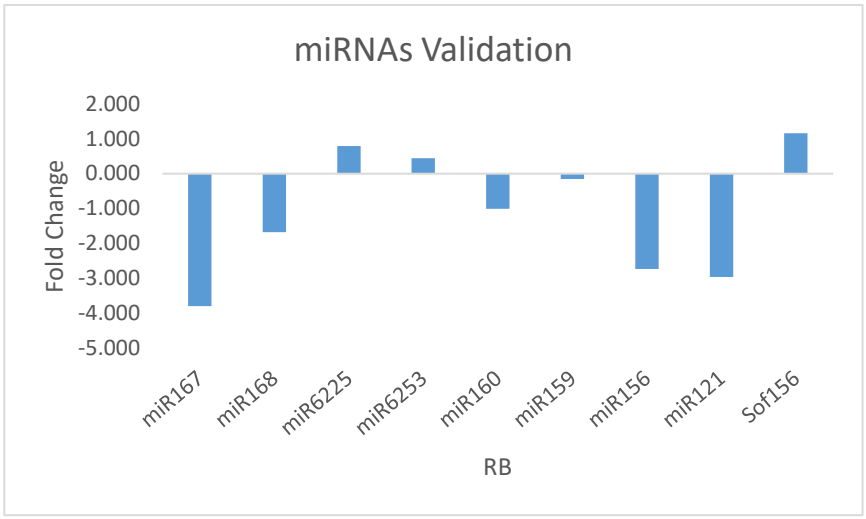


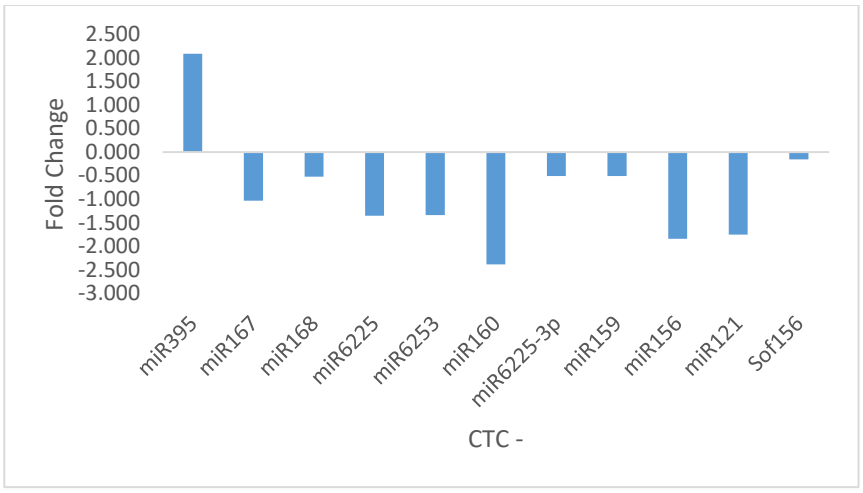
During the second mission/project/visit, when Dr Kottapalli visited Brazil and was hosted by Dr. Sonia the results from the mRNAs and miRNA sequencing were discussed and further experiments were planned. Also we chose a set of miRNAs and genes to be validated, as described bellow:

- Real time qRT PCR was ran on selected specific miRNAs to validate the miRNA-sequencing results as detailed below

We performed a qPCR analysis of eleven selected miRNA (miR395, miR167, miR168, miR6225, miR6253, miR160, miR159, miR6225-3p, miR156, miR121, Sof156) to validate our miRNA sequence data using the Stem-loop RT-qPCR technique (Varkonyi-Gasic; Hellens, (2011). RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used for cDNA synthesis following manufacture instructions. The reaction was performed in 20µL containing 200U of RevertAid M-MuLV Reverse Transcriptase, 20mM DNTPs, RiboLock RNase Inhibitor (20 U), 5X reaction buffer (Thermo Fisher Scientific), RT specific Primer 1 µM, dT primer (100 µM). cDNA was used as templates for PCR amplification using SYBR Green JumpStart Taq Ready mix (Sigma-Aldrich). 18SrRNA was used as housekeeping gene for normalization. The expression level were analyzed using MxPro QPCR Software versão 4.10 (Stratagene).

Fig 4 miRNAs validation by RT-qPCR.





On January 2017 we presented a poster at the Plant and Animal genome conference in San Diego, named ***Understanding Molecular Mechanisms Underlying Sugarcane Roots Response to Aluminum Stress, please see*** abstract bellow:

Sonia Marli Zingaretti[1], Pratibha Kottapalli[2], Kameswara Rao Kottapalli[2]

Sugarcane (*Saccharum* spp.) is an important source of sugar and ethanol and it is known that the global sugarcane production will increase by 21% by 2024. With increasing demand for energy, the sugarcane crop expansion is evident in Brazil. It is predicted that due to high demand for sugarcane and ethanol, the acreage under sugarcane will increase from 9.0 million ha to 64 million ha by the years 2018/2019. As a result more unconventional soils rich in minerals will be brought under cultivation. Aluminum ions (Al^{+3}) together with silicon and iron are the three most abundant mineral elements in soil. Although silicon and iron are required for plant growth, Al is toxic and its bioavailability is highest on acidic soils, resulting in inhibition of root growth and architecture leading to disruption of root elongation. Our ultimate goal is to understand the molecular mechanisms of abiotic stress

tolerance in sugarcane and the role of miRNA's in transcriptional regulation. Towards this goal, a relatively tolerant sugarcane cultivar CTC-2 and the susceptible RB855453 cultivar was subjected to Aluminum stress at 221 µMol. RNA-Seq was performed on 12 root tissue samples using 108 bp paired end sequencing on an Illumina HiSeq2500 sequencer. Pairwise comparisons between different treatments in tolerant cultivar identified 16,340 non redundant differentially expressed transcripts (DETs). Functional annotation of DETs revealed that AL^{+3} tolerance was controlled by several interacting pathways like calcium and G-protein coupled receptor mediated signaling, and regulation by WRKY and R2R3-MYB transcription factors. Some of these genes could be utilized by sugarcane breeders to improve Al^{+3} stress tolerance in field conditions. Additionally, we have sequenced the miRNAs isolated from root tissues and their interaction with transcripts will be discussed in our presentation.

At this point, a manuscript is under preparation where all the data will be presented and properly discussed. We are also plaining a new project that will be sent to FAPESP and also to a NSF-Dimensions of Biodiversity grant 2018. The due date will be February 2018 after the RFP is released sometime in October. In this proposal, up to two 5-year US-São Paulo Collaborative Research Project awards will be funded by NSF to the US components and by FAPESP (São Paulo Research Foundation) to the São Paulo components. NSF will fund its US researchers at a level up to $2,000,000. FAPESP will fund Thematic Project investigators at a level up to $2,000,000 (this total value includes both the overhead for researcher direct use and the overhead for institutional infrastructure).

It was a great opportunity for our lab's and my students to have Dr Sonia here and to have the privilege of discussing with her our ongoing projects and projects in Brazil. Next time Dr. Sonia is in USA, we will arrange for a presentation to our first year graduate MS Biotechnology students.

Future Projects/visits

In November 2017, Dr. Kottapalli will make a second visit to Brazil to discuss the sugarcane genome project and additional functional genomics projects. We are also planning to attend an International Conference to explore more collaborations and partners to expand the scope of our research and be more competitive for collaborative grants.