

Project Number: Machado-ma2-132

Proposal Title: **Analysis of transcriptome of citrus infected with *Ca. Liberibacter asiaticus* and *Ca. L. americanus*.**

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Methodology

The plants were put in a growth chamber (photoperiod of 16/8 hours, temperature 24/22 °C, at 60 % relative humidity) day/night, 22/6, 24/16), as described in the previous report, and monitored periodically for presence of bacteria and symptoms of HLB. After confirm the presence of both *Ca. Liberibacter asiaticus* (CLas) and *Ca. L. americanus* (CLam) the tissues of five plants were collected for RNA extraction. Since the infection of *Ca. L. asiaticus* progresses faster than the infection of *Ca. L. americanus*, sample were collected at different infection time. The tissues of symptomatic plants infected were processed as described in the previous report, and was later performed the extraction of total RNA (RNeasy Mini Kit, Qiagen). The RNA was then analyzed for integrity, quality and concentration. The next step was to send the samples to Roche NimbleGen for microarray hybridization as previously described.

Time course experiments - Since the proposal experiments were with *Ca. Liberibacter americanus* (there was publication with *Ca. L. asiaticus*), and considering the importance of time course experiments for a better understanding the gene network involved in the response to infection, we decide to set up a time course experiments with both bacteria.

Results

The first round of hybridizations were performed by Roche Nimblegen with a customized 385K chip containing 32,000 unigenes of *C. sinensis*. The raw data were pre-processed using the Robust Multiarray Average (RMA) by the NimbleScan software. Normalized data were imported to R package (Bioconductor) where statistical analyses were performed. A Bayesian moderated t-test statistic was calculated and p-values were adjusted for multiple comparisons by the false discovery rate correction. A total of 634 genes with *P-values* ≤ 0.05 , fold change $|\log FC| \geq 2.0$ and odds probability ≥ 0.95 were considered differentially expressed at a statistically significant level. From those, 419 were up- and 215 down-regulated in symptomatic plants. In order to identify the most representative metabolic pathways regulated in infected plants we applied a gene set enrichment analysis approach by using the information of Gene Ontology and KEGG annotation. We found 45 different biological processes, including those involved in response to jasmonic acid stimulus, signaling and metabolism. There is partial agreement between our results with *Ca. L. americanus* and Albrecht & Bowman (2008) results. They have found 279 to 515 differentially expressed genes according with the infection time. Although the statistical approaches are similar (R package, Bioconductor, RMA,

fold change, and False Discovery Rate), our results are based in wide DNA chips with only sequences of sweet orange. But the overall tendency is pointed out that the interaction of sweet orange with *Ca. Liberibacter americanus* is quite similar to the interactions with *Ca. L. asiaticus*.

The complexity and amount of information from microarrays experiments have been a challenge to be evaluated. But using the approach Gene Set Enrichment Analysis (GSEA) (Subramanian et al., PNAS 2005) several biological processes can be evaluated and integrated. When biological pathways (e.g. phytohormones, general metabolism, synthesis and transport of jasmonic acid, ethylene and silicic acid (SA), etc.) were evaluated there is a clear evidence of the activation of jasmonic acid (JA) pathway. JA seems to play an important role in interaction of sweet orange with both bacteria. Such results were confirmed when general defense processes against bacteria and fungus were evaluated. Genes of superoxide dismutase, PR4, chalcone synthase, and transcription factors (MYB and WRKY) were statistically up regulated. Plants infected with *Ca. L. americanus* had high gene expression of ion transport especially zinc transport. But several other transports were also up regulated.

Special attention has been given in the SA and flavonoid pathways. Transcripts of the begin and end of SA pathway (WRKY6 and PR) were also found but they need validation by RT-qPCR. Altogether there is a tendency for up regulation of genes involved with defense against biotic and abiotic stress. But only with the validation results such hypothesis can be confirmed.

Part of the results was presented in IOCV Conference (Brazil) and another part will be in the International Conference of HLB, in Orlando.

Next steps

- Analysis of time course experiments (underway);
- Validation of key genes putatively associated with the disease process.
- Submit the final manuscript for publication.