

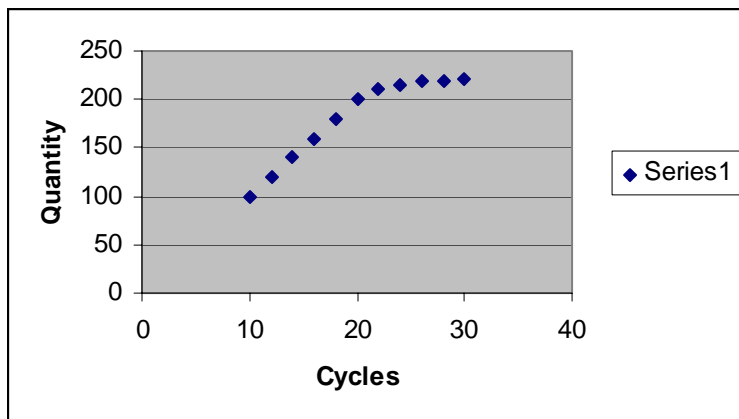
Module 4. General Background for Gene Expression.

“Gene expression” is often used loosely to describe the quantitative amount of mRNA present in a tissue or in response to stimulus. Strictly speaking, commonly used techniques measure mRNA abundance. The amount of steady state mRNA does not necessarily reflect transcriptional activity, translational activity, protein accumulation nor phenotypic expression. This is because transcription, mRNA stability and mRNA turnover affect message accumulation; translation, protein stability, and protein turnover affect protein accumulation; and enzymes are subject to regulation. There are specific genetic and biochemical methods for assaying various steps of “gene expression”, and studies aimed at understanding the regulation of expression therefore assay multiple steps in the process. For the purpose of this module, we will concentrate on measuring steady state mRNA levels.

RT-PCR. Reverse transcription (RT) of RNA coupled with PCR is a very sensitive method for detecting the accumulation of transcripts. We will extract RNA, and produce a cDNA copy using the enzyme reverse transcriptase. Reverse transcriptase requires a primer, and it is common to use a poly-T primer for eukaryotic messenger RNA. It is often desirable to use gene specific primers. The cDNA serves as a template for a specific amplification PCR reaction. For this technique to be accurate, RNA preparations must be free of DNA contamination, PCR amplification must be terminated at a cycle when amplification is in an exponential phase (linear), and an appropriate control gene must be identified. The control gene is usually chosen based on a consistent expression level across experimental conditions and is used to normalize data.

Quantitating RNA by RT-PCR. The quantity of a transcript is usually expressed relative to the control gene. For relative RT-PCR to be meaningful, the PCR must be terminated when the amplification of the control and the gene of interest are in the linear phase. Quantification can be obtained through a variety of techniques (radioactive labeling of the PCR reactions, quantification of pixels using the gel-doc, etc...).

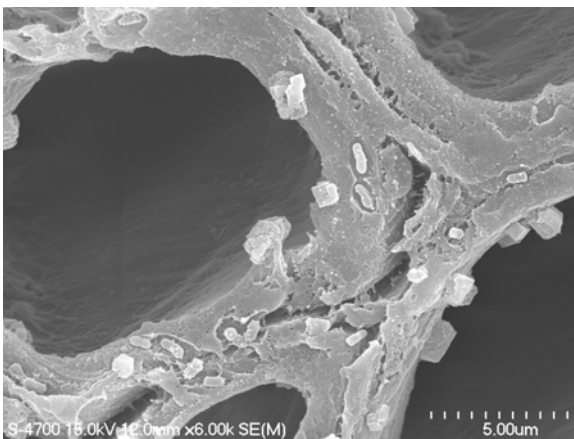
Determining Exponential Phase. A PCR master mix is prepared and split into aliquots that are then subjected to PCR. Aliquots are removed from the thermal cycler at intervals (10 cycles, 12 cycles, 14 cycles, etc...) and resolved by electrophoresis. The intensity of the products is quantified (e.g. using the gel doc) and cycle number is plotted against quantity.



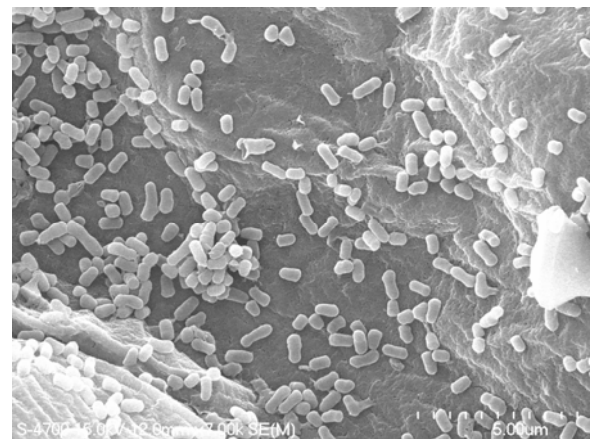
Specific Background:

We are interested in identifying genes that are expressed in the tomato plant during infection by the bacterial canker pathogen, *Clavibacter michiganensis* subsp. *michiganensis*. Both plant and pathogen genes play a role in modulating this host-pathogen interaction. Plant genes may be expressed in defense, as a result of pathogen induced stress, or because the pathogen is actively reprogramming plant gene expression. Bacterial genes expressed during infection may be required for pathogen growth and/or virulence. We can take a “global” or “specific” approach to identifying plant and pathogen genes that are regulated in response to infection. In a global approach we would design an experiment to track the expression of as many genes as possible. Large-scale gene expression studies are facilitated by “gene chip” technology, where the gene probes are affixed to a support (glass slides or silicone chip) and the slide is hybridized with labeled cDNA. Analysis of differentially expressed proteins using Mass Spectrometry has become another high throughput approach to studying gene expression. Given the extensive genomics resources for tomato and *Clavibacter*, such approaches are possible and increasingly accessible.

An example of a specific approach to studying mRNA accumulation is to target genes known to play a role in specific pathways. An analysis of tomato proteins that are differentially expressed during infection by Cmm suggests that genes involved in creating an oxidative burst are up-regulated in the plant. This information on host response and analogy to other host pathogen systems may provide a basis for identifying specific bacterial genes that are important to the infection process. Cmm is a gram-positive bacterium, and in other gram-positive pathogens (e.g. *Mycobacterium* species), catalase is a virulence factor that allows the pathogen to resist the oxidative defense of macrophages. A search of sequence databases for *Clavibacter* species indicates that Cmm contains a *furA*-catalase operon that is similar in structure to the virulence operon found in *Mycobacteria*. Because the plant appears to respond to infection with enzymes that will produce oxidative stress and because we have evidence that O_2^- is produced in infected tissue, we are interested in knowing if the Cmm catalase gene is expressed in the plant. Closely linked to the catalase gene is a pectate lyase gene that may be involved in breaking down host tissue. Again, we would like to know if this gene is expressed during infection.



Cmm in the cell walls of companion cells adjacent to infected vascular tissue. Plant cell walls are rich in pectin.



High populations of bacteria in Xylem tissue of infected tomato.

Table 1. Primers for RT and PCR.

		RT		PCR		
		Primer Temp.	Primer Temp.	Cycles	Product size	
Primers for amplifying pect. lyase from Cmm						
PectF2	AGCTGCACGACCTTGTCGTAGG			yes	60 C	? 278 bp
PectR2	GACTTCCACAACCAGGAGCTCAAC	yes	44 C	yes		
Primers for bacterial control						
Cms16sF2	GCCGTAAACGTTGGGAACCTA			yes	60 C	28 324 bp
Cms16sR2	TGCTGGCAACATAGAACGAG	yes	44 C	yes		
Primers for amplifying catalase from Cmm						
Fur2083R	GGTAGTCCTTCTGCGACCAG	yes	44 C			
Cmm1304F	GACCACTACCTCGTCGAGAAGCTC			yes	60 C	28 267 bp
Cmm1546R	GTAGTTGCCCTCGTCCGTGTAGAAC			yes		
Primers for tomato SOD						
SODP14831F	TCACCACAACCAGCACTACC			yes	60 C	? 202 bp
SODP14831R	CCCTTAAGGACAGCAACAGC	yes	44 C	yes		
Primers for tomato Pr3 (endochitinase)						
Endochitinase F2	AGGCAATCAAATGGGAAGTG			yes	60 C	? 450 bp
Endochitinase R	CAATCCGATCCTCCACTTGT	yes	44 C	yes		
Primers for tomato control						
CosOH41F	TGTCATTGTTGGTGCTGGAT			yes	60 C	28 256bp mRNA
CosOH41R	GCTCATGATGGTTGAGGTGA	yes	44 C	yes		750bp DNA

Target genes

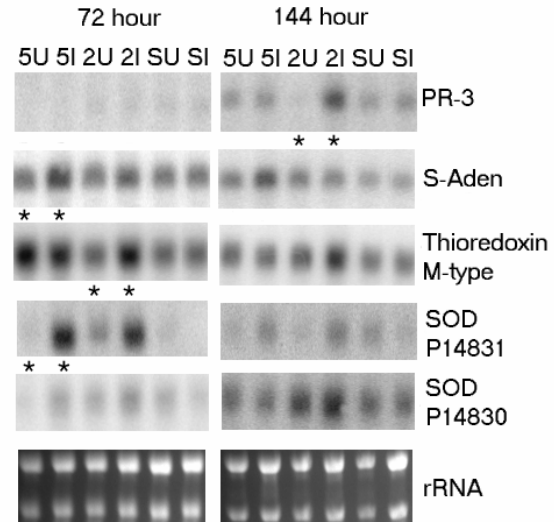
Two bacterial genes, Cmm Catalase and Cmm pectate lyase are of interest as potential “virulence factors”. For the Catalase gene, the primer, Fur2083R will be used for the RT reaction and primers Cmm1304F and Cmm1546R will be used for the PCR portion for the Cmm Catalase. For the second bacterial target, pectate lyase, we will use the same reverse primer for RT and PCR reactions. Plant target genes will be Super Oxide Dismutase (SOD) and the Pathogenesis related (Pr3) endochitinase.

Control Gene(s)

Control genes are used to normalize expression. In general they are chosen as “steady state” genes, that is genes that have a consistent level of expression under diverse environmental conditions. The bacterial control will be 16s rRNA. For the plant control, we will use one of Wencai’s “COSI” (Conserved Orthologous Sequence – Intron) genes. This control serves two purposes. To verify that no DNA is contaminating the RNA preparations we have designed the primers to span an intron. An amplification with RNA should give the expected mRNA fragment size (DNA minus intron), while amplification with DNA will give the larger fragment size (see hypothetical figures, page 5). The gene we will use, CosOH41, is a Thiazole biosynthetic enzyme, and for our purposes is a “steady state” gene.



Susceptible and Resistant plants after infection by Cmm.



Northern blot analysis of steady state mRNA accumulation for defense genes in resistant (5 and 2) and susceptible (S) varieties moc-inoculated (U) and inoculated (I) with Cmm. Expression levels that are significantly different are labeled “*”.

Experimental Design.

The interpretation of expression experiments is subject to experimental design, including controls and replication. An important part of this module will be for each group to plan an experiment and evaluate the expression of two to four genes. The plant control gene (CosOH41) and two plant test genes (SOD and Pr3), a bacterial control gene (16S rRNA) and bacterial test genes (Catalase and pectate lyase) are described above. Your group’s experiment must be designed to evaluate the expression of a set of these genes between treatments. Treatments may include genetic differences between accessions and varieties, differences between plant tissue types (leaves and stems), or differences between inoculated and moc-inoculated plants. You will need to make several decisions regarding experimental design prior to RNA extraction.

First, what is the hypothesis you are testing? Addressing this question will help you define your treatments. We will be somewhat limited by available reagents. No group should design an experiment that requires more than 18 RT reactions. For example I have outlined two experiments below.

Ho: Bacterial genes are differentially expressed in resistant and susceptible genotypes.

Treatments:

Inoculated Resistant plant

RNA extraction

4 RT reactions

(control plant, control bacterial gene, catalase, pectate lyase)

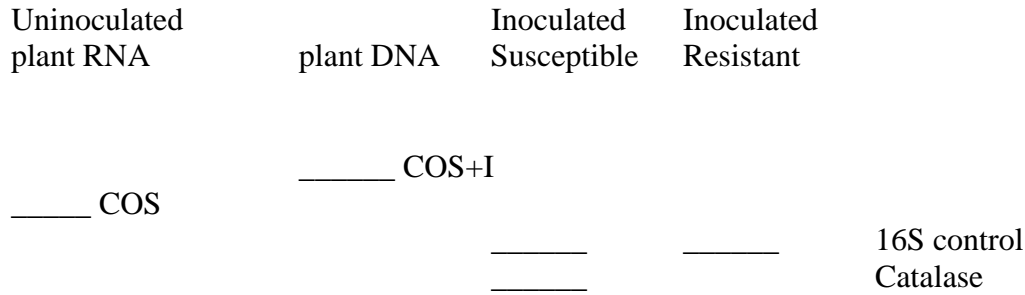
Inoculated Susceptible plant

RNA extraction

4 RT reactions

Adding a biological replicate: Four RNA extractions 16 RT reactions

Hypothetical figure:



Ho: Genes involved in plant defense are differentially expressed in infected and uninfected plants

Treatments:

Moc-inoculated Susceptible Plant

RNA extraction

3 RT reactions

(control plant, SOD and PR3)

Inoculated susceptible plant

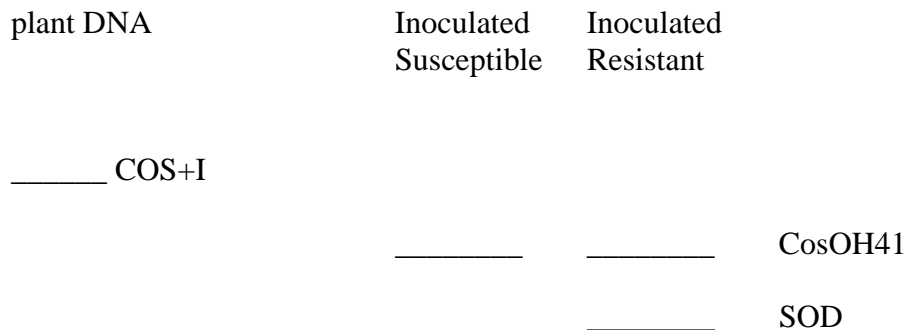
RNA extraction

3 RT reactions

(control plant, SOD and PR3)

Adding a biological replicate: Four RNA extractions 12 RT reactions

Hypothetical figure:



The next step in experimental design will be to make decisions about key details. If your hypothesis involves testing tomato germplasm as a treatment, you will need to discuss with the instructor which germplasm to use. We have a range of germplasm in the greenhouse that can be tested. There is a collection of ~25 accessions of *L. hirsutum* some of which are resistant and others of which are susceptible; there are cultivated types that are either resistant or susceptible; and there are cultivated types that have resistance derived from distinct wild progenitors. You will also need to decide which tissue type you will test. In our previous experiments, we have

detected bacterial gene expression in the stems of infected plants, but not in the leaves. We have detected differential expression of plant genes in leaf tissue, but we have never examined stem tissue. Please think about these issues prior to class, and be prepared to work in your group to design the experiment.

Constraints. In designing your experiments please follow the KISS approach (Keep it simple...). We will be limited with respect to reagents and no group should design an experiment that requires more than 18 RT reactions. Results should be evaluated statistically, and you will therefore need to incorporate replication.

References:

Chomczynski, P and Sacchi, N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem* 162:156

Coaker G., B. Willard, M. Kinter, E. J. Stockinger, and D. Francis. 2004. Proteomic analysis of resistance to bacterial canker of tomato. *Molecular Plant Microbe Interactions*. 17:1019-1028.

Freeman W.M., Walker S.J., Vrana K.E. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques*. 26:112-22, 124-5

Puissant and Houdebine (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *BioTechniques* 8:148-149.

Quantitative RT-PCR: pitfalls and potential (abstract)

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?holding=npg&cmd=Retrieve&db=PubMed&list_uids=9894600&dopt=Abstract

Clavibacter genome sequencing

http://www.sanger.ac.uk/Projects/C_michiganensis/

Animation describing RT-PCR

http://www.bio.davidson.edu/courses/Immunology/Flash/RT_PCR.html

RT-PCR: The basics (Ambion)

<http://www.ambion.com/techlib/basics/rtpcr/>