TRANSCRIPTIONAL PROFILING ON TREES AFFECTED BY CITRUS BLIGHT AND IDENTIFICATION OF AN ETIOLOGICAL CONTRAST POTENTIALLY ASSOCIATED WITH THE DISEASE

By

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This work is dedicated to my wife Darlene, to my daughters Luísa and Lígia, to my parents Norival and Maria, to my aunt Josefa, and to all my friends and family that shared their love and care. Along the way, many things were surpassed and our dear aunt Neula, uncles Otávio and Waldemar, and friend Beatriz Nielsen will always be in our hearts.

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I started in the Department of Plant Pathology and I thank Dr. Bill Zettler and Dr. George Agrios for the attention and dedication that I received during the application process and as a new graduate student.

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TRANSCRIPTIONAL PROFILING ON TREES AFFECTED BY CITRUS BLIGHT AND IDENTIFICATION OF AN ETIOLOGICAL CONTRAST POTENTIALLY ASSOCIATED WITH THE DISEASE

By

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Cochair: Dr. Kenneth S. Derrick
Major Department: Plant Molecular and Cellular Biology

Citrus blight is an important citrus disease in Florida (USA) and São Paulo (Brazil), primarily affecting yield in adult plants and compromising maintenance of entire commercial blocks. Blight is associated with rootstock choice, but, at present, is of unknown etiology. Therefore, the objectives of this study were to identify differentially transcribed genes and uncover etiological contrasts under non and citrus blight conditions. Roots of healthy and blighted Rough lemon (*Citrus jambhiri* Lush) rootstock supporting Valencia sweet orange (*Citrus sinensis* L. Osbeck cv. Valencia) canopy were collected from a central Florida area. Total RNA was obtained and RT-PCR was performed enriching for messenger transcripts. Subtracted cDNA libraries were created and around 140 clones were arrayed onto nylon membranes. Independent RNA sources from feeder roots of ten healthy and ten blighted trees were labeled with P33, hybridized overnight with the membranes and analyzed under an imager system. Selected clones

were validated by RT quantitative real time PCR. The results indicated that citrus blight was able to affect the transcriptional levels of certain genes in a similar pattern among different replicates. The level of response was dependent of the assessed group of plants. One of the clones had sequence similarities to a citrus EST and to a potential ubiquitin subunit. Another one had similarities to a citrus chitinase, helping to deduce a candidate sequence for the blight associated P5 gene. Three genes had higher transcriptional levels under blight condition and did not respond to cold and drought stresses. The blight associated P12 had higher levels in mildly than in fully blighted trees. Further characterization of these genes may contribute to the understanding and control of citrus blight. In another experiment, citrus tristeza virus (CTV) genes were observed in the libraries. The transcriptional level of the P27 gene (divergent coat protein gene) of CTV was far more abundant in roots of blighted than in healthy Carrizo citrange, which is considered to be resistant to variant forms of CTVs. It remains to be investigated if CTV causes or enhances blight, or only grows better in feeder roots of already affected trees.

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CHAPTER 1
INTRODUCTION

The importance of one industry in the agriculture segment and the context where science exists to help society were considered in this study. The result was a true excitement not only because the focus of this research is (still) an unresolved real problem of citrus, but also because the search for information involved molecular biology and related fields.

Importance of Citriculture

In addition to tasting good, citrus is a well known source of vitamin C and antioxidants. It is also believed to have anti-cancer properties (Rafter, 2002), and processed peel and pulp are frequently used for animal feed (Fegeros et al., 1995). Because of its nutritional relevance, citrus is an important industry world-wide, raising economies at macro and local levels by supporting social development directly with jobs and with secondary industries and services. Citrus is commercially present in more than a hundred countries in all five continents, primarily within tropical and subtropical regions. The total production has consistently increased in the last 40 years, and, more recently, reached one hundred million tons yearly (Figure 1-1). The early citrus pioneers would probably be astonished by this level of production, but the modern industry has through the years achieved efficiency, flexibility and high quality standards.

Citrus was first brought to the new world by the Portuguese and Spanish explorers at the beginning of the XVI century (Moreira, 1980; Allen, 2000). By the second half of the XIX century, the USA and Brazil had established fresh fruit companies, and the

frozen concentrated technology, developed in the 1940s in Florida (Lewandowski, 2000), increased the demand for citrus juices.

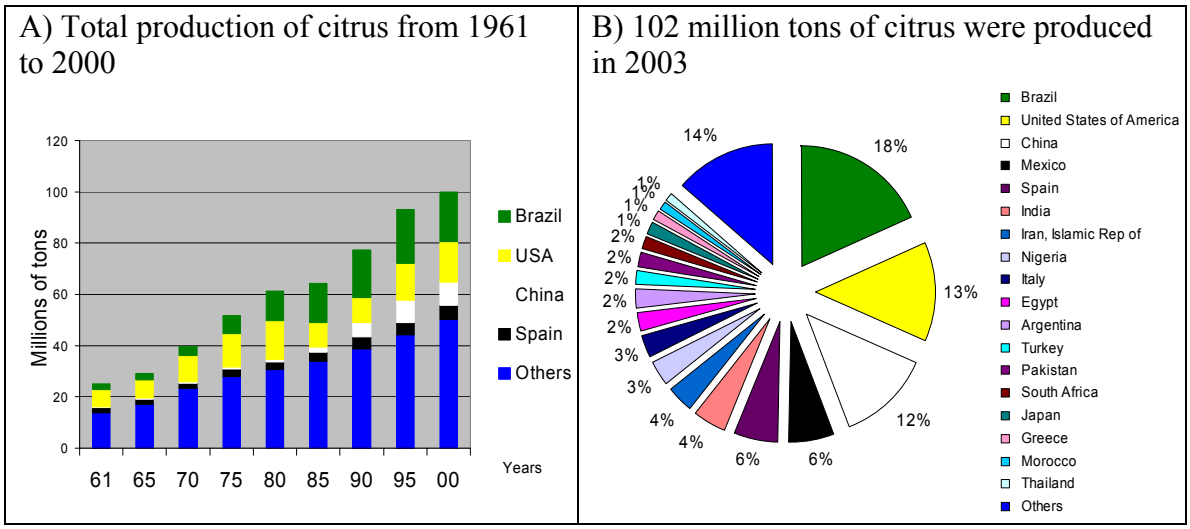


Figure 1-1. Citrus around the world. A) The total production of citrus has increased almost 5 fold in the last 40 years. Brazil took the lead in production over the USA only after the Florida freezes during the 1980s. B) Citrus is commercially present in 138 countries according to FAO, with China, Mexico and Spain ranking respectively 3rd, 4th and 5th in total production in 2003. (Source: FAOSTAT data, 2004, http://apps.fao.org , last accessed April 07, 2004).

Many changes in production systems have been necessary to meet the ongoing needs of growing markets and the demands of new challenges, such as changes in organoleptical concerns, unexpected drought and cold stresses, outbreaks of pests and diseases and raises of political barriers, to name just a few. Consequently, production constraints have been overcome by the use of grafted plants to replace seedlings, changes in rootstocks, selection of new cultivars, relocation of production fields and charges in diplomatic battles (Fawcett and Lee, 1926; Moreira, 1980; and others). Literature is available for all transitions suggesting that the need for research in citriculture is not a

recent event. In fact, despite frequent new challenges, in the last decades citrus has surpassed other important fruit crops such as bananas, apples and grapes in production according to FAO (Source: FAOSTAT data, 2004, http://apps.fao.org , last accessed April 07, 2004).

Citrus in São Paulo and Florida

The two major citrus producing areas in Brazil and the USA are the states of São Paulo and Florida (Figure 1-2).

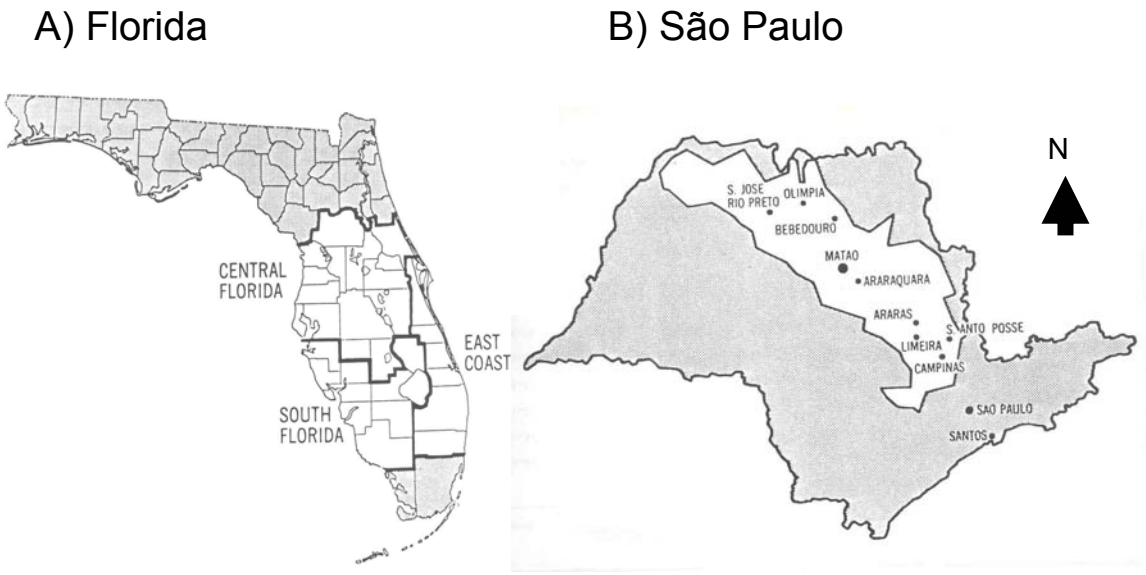


Figure 1-2. The citrus belts in Florida and São Paulo. A) The industry in Florida has moved to warmer southern areas after the freezes of the 1980s, and the major producers are nowadays Polk and Hendry counties, respectively, in the central and southwestern regions. B) In São Paulo, the northern region is drier and warmer than the southeastern areas and the major citrus companies have their headquarters around Araraquara city.

Supported by good environmental conditions, efficient systems and high quality products, both Florida and São Paulo have industries primarily devoted to processed citrus juice, especially oranges, supplying most of the world demand for this commodity.

While Florida has focused its efforts on the North America market, São Paulo dominates the European one. The social and economic consequences of abrupt changes in the citrus industry of both São Paulo and Florida would affect, at least to a certain extent, the around 400,000 jobs and 3 billion dollars in exports yearly in Brazil, and the 89,000 jobs and 4 billion dollars in sales in the United States. More details and other statistics can be seen at ABECITRUS (Source: http://www.abecitrus.com.br , last accessed April 07, 2004), FUNDECITRUS (Source: http://www.fundecitrus.com.br , last accessed April 07, 2004), CREC (Source: http://www.lal.ufl.edu , last accessed April 07, 2004), and USDA (Source: http://www.nass.usda.gov/fl , last accessed April 07, 2004).

Both socially and economically, there are similarities and also differences in citrus industries worldwide. However, all contribute to supply different markets with distinguished quality fruits: citrus fruits. Therefore, citrus is an important segment of agriculture and demanding of research, which, ultimately, may help in the stabilization of society.

CHAPTER 2
IMPORTANCE OF CITRUS BLIGHT AND REASONS FOR ITS CONTROL

There are many constraints to citrus production around the world and citrus blight, in Portuguese "declínio", is one of them. It has caused impressive annual losses of around 60 million dollars in São Paulo and 100 million dollars in Florida (Derrick and Timmer, 2000). Since its appearance in Florida during the second half of the 1800s, it has been a top concern for the Florida citrus industry, probably only approached by the damage caused by freezes in the past. Swingle and Webber mentioned that blight was first reported by Underwood in 1891, and also, that affected trees had been seen two decades earlier (Swingle and Webber, 1896). Symptomatic plants undergo a slow tree decline, rather than a sudden disruption in development, as suggested by the given name in English.

Citrus blight has distinct characteristics not observed in declines caused by other diseases, such as *Phytophthora* sp., Tristeza, and others. Early symptoms, which occur in mature productive trees normally older than five to six years, include loss of the intense color of leaves and other green tissues; the expression of specific proteins (Bausher, 1990; Derrick et al., 1990; Taylor et al., 1996; Paiva et al., 1997); and complexation and translocation of zinc from leaves to trunk bark tissues (Albrigo and Young, 1980);(Taylor et al., 2002). Affected plants have their xylem vessels gradually blocked by amorphous and filamentous particles (Cohen et al., 1983), which probably leads to the visual symptoms of overall decline: smaller tree size, twig die back, smaller yield and fruit size and general mineral unbalance (with the clear pattern for zinc deficiency on leaves).

Major physiological changes also occur (Albrigo et al., 1986), such as off season flowering, longer flowering periods and shooting of sprouts inside of the canopy, as if the affected plant was attempting to keep its vital functions (under normal levels). It is common to have less water uptake into the trunk of the affected plants due to xylem blockage (Cohen, 1974; Lee et al., 1984). Affected plants rarely die but are often pushed out and replaced by a young tree. Thus, not only direct losses because of tree failure and replacement, but other costs in grove management are always incurred. Reset groves pose serious difficulties in horticultural maintenance programs, since they now have trees of different ages, sizes and possibly on different rootstocks, all with different needs. They have also increased the risk of other diseases that can be spread on the new young trees. The Figure 2-1 displays the described progress of citrus blight often seen in Florida and in São Paulo groves.

In addition to the noted annual losses, a simple and dramatic way to see the impact of citrus blight is by just driving through the citrus areas in Florida and São Paulo and paying attention to symptomatic trees and reset groves.

Citrus blight also occurs in many other countries (Wutscher et al., 1980), except those with Mediterranean and desert climates where it has not yet been reported.

Citrus Blight Etiology

Citrus blight is an unresolved problem and its origin and etiology are still matters of debate. Several characteristics of a plant disease, meaning the outcome between a host and a pathogen under a proper environment, are present in affected trees, except the presence of a confirmed pathogen. However, it is worthy to note that (Agrios, 1997)

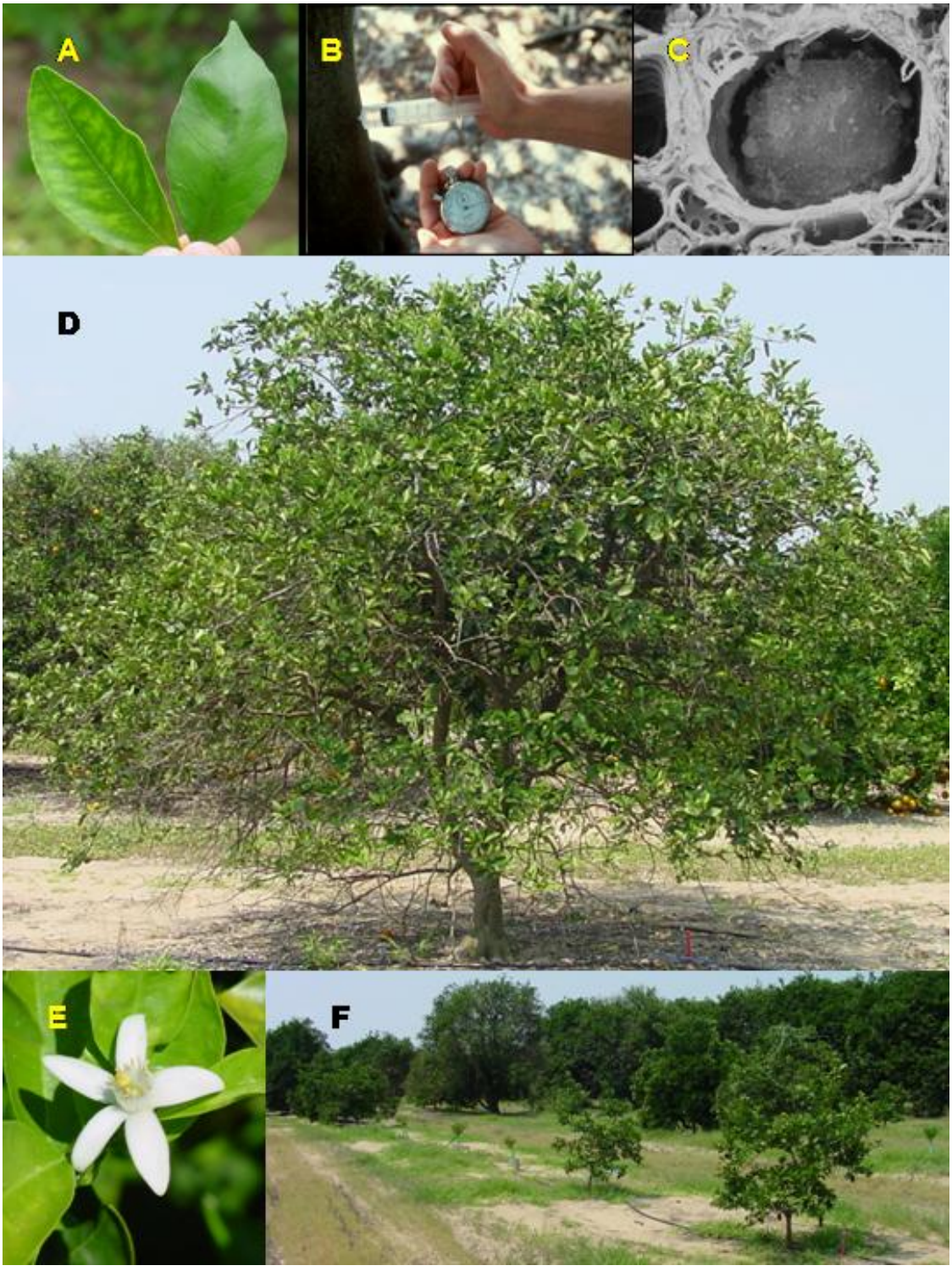


Figure 2-1. Characteristics and symptoms of citrus blight. A) Zinc deficiency on leaves. B) Water uptake test into the trunk of the affected plant. C) Xylem blockage. D) Overall plant decline. E) Off season flowering. F) A reset grove has trees with different ages and needs.

defines plant disease as: "….whenever the ability of the cells of a plant or plant part to carry out one or more of these essential functions is interfered with by either a pathogenic microorganism or an adverse environmental factor, the activities of the cells are disrupted, altered, or inhibited, the cells malfunction or die, and the plant become diseased…". Thus, this study considers blight as a plant disease of citrus. In addition, the process seems to be infectious in nature (Timmer et al., 1992), since transmission by root grafting was achieved in experiments done in the USA (Tucker et al., 1984), in South Africa (Marais and Lee, 1990) and in Brazil (Rossetti et al., 1991). However, the etiology remains unconfirmed and transmission has not occurred by either grafting canopy branches (Albrigo et al., 1993), by bud grafting, or by soil replacement (Timmer and Graham, 1992). Figure 2-2 summarizes the experimental transmission of citrus blight obtained by root but not by canopy grafting.

Citrus blight is also present in mature seedling trees, eliminating the possibility of scion/rootstock incompatibility as a cause. The pattern of spreading seems to be towards adjacent trees in the same planted row, rather than across rows, as often noted by farmers and also by (Swingle and Webber, 1896), who first raised the hypothesis of infectious spreading pattern. Over time, the progression of the disease seems not epidemic, following a pattern of incidence that can be described by a linear model, which is closer to abiotic abnormalities (Berger, 1998). (Swingle and Webber, 1896) recommended eradication of affected trees, fearing further spread of the problem. That was not implemented. Since then, candidates for causal agent and other theories have been examined, such as a non-parasitic origin (Rhoads, 1936), the initial transmission trials

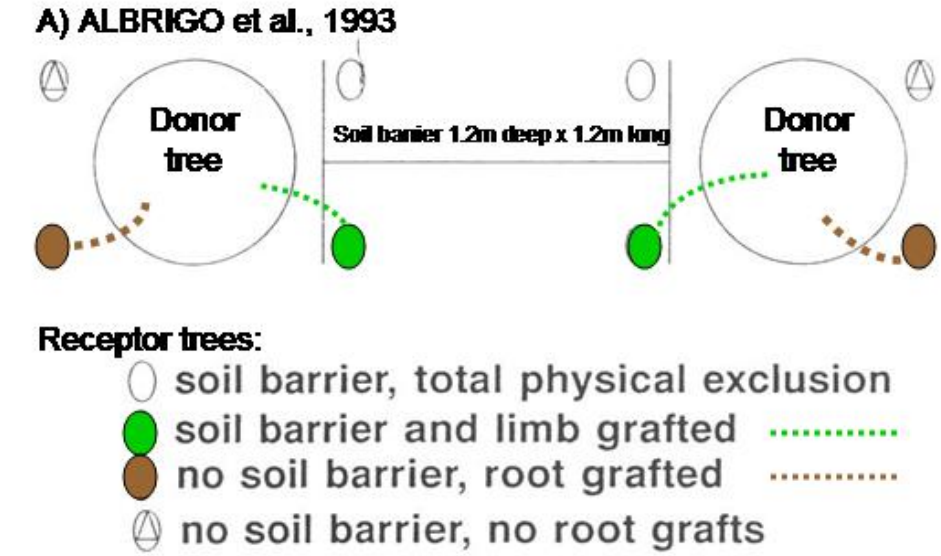


Figure 2-2. Transmission of citrus blight. A) Layout of the work done by (Albrigo et al., 1993), where the receptor trees that were root grafted to blighted trees displayed symptoms 6 years after that, while no limb grafted plant displayed symptoms during the same period; B) Receptor trees used by (Rossetti et al., 1991) showing symptoms of blight after the root grafting work done in Brazil, confirming that blight is transmitted by roots as first demonstrated by (Tucker et al., 1984).

(Cohen, 1968), soil born candidate pathogens (Nemec, 1994), the vascular limited bacterium *Xylella fastidiosa* (Hopkins, 1987), nutritional related factors (Wutscher and Hardesty, 1979), and others, but all remain unconfirmed (Derrick and Timmer, 2000).

Citrus Blight is Affected by the Employed Rootstock

Resistance to citrus blight is dependent upon rootstock choice, and even though rootstock replacement has been a necessity for decades, the overall solution for citrus blight has not yet been achieved. Resistance to citrus blight is apparently less common than initially thought (Young et al., 1982).

Rough lemon (*Citrus jambhiri* Lush), having excellent yield and drought tolerance but susceptible to citrus blight, was once the major rootstock in central Florida. Extensive plantings on this rootstock in the 1940s, replacing sour orange (*Citrus aurantium* L.), was probably the reason blight has ranked as one of the most common citrus diseases in the state since then. Volkamer Lemon (*Citrus volkameriana* Ten. and Pasq.) was another rootstock option in the past for similar reasons. However, replacement of both with other rootstocks more resistant to citrus blight contributed to major changes in citriculture, since the replacements were normally less vigorous and sustained lower yields (Young et al., 1982). Among other consequences were increases in grove maintenance needs such as more attention to grove fertilization, to tree density per area, to other disease susceptibility, and to increases in irrigation, which ultimately contributed to the urbanist concerns in water usage in Florida (Callies, 2000).

In Florida, replacement of Rough lemon (*Citrus jambhiri* Lush) by Carrizo citrange (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) was not as effective as anticipated because Carrizo proved to be almost as susceptible to blight as Rough lemon (Young et al., 1982). Sour orange (*Citrus aurantium* L.) never matched Rough lemon yield in

central Florida, and it also succumbs to citrus tristeza virus. Cleopatra mandarin (*Citrus reticulata* Blanco) yields fruit later than Rough lemon and when in full production the fruit is smaller and the yield lower, primarily when supporting sweet orange and grapefruit cultivars. Sweet orange (*Citrus sinensis* L. Osbeck) and Sunki tangerine (*Citrus sunki* L.) are considered more resistant to citrus blight, but are not widely used because of high susceptibility to gummosis of *Phytophthora* sp. and also to drought. Swingle citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.), largely used nowadays, will probably be of restricted use in the near future because some groves on this rootstock have become affected by citrus blight. In São Paulo, rootstock replacement is also an unresolved issue, with no obvious alternative to Rangpur lime (*Citrus limonia* Osbeck), another rootstock susceptible to citrus blight, and more recently to citrus sudden death disease. Able to grow on non-irrigated areas, on soils with high aluminum, and in low input production systems, Rangpur lime is still the rootstock of choice in more than 80% of groves in the São Paulo area, despite citrus blight losses (Pompeu JR., 2001). Thus, groves that normally would last naturally for 50 years or more are being replaced within 10 to 15 years, or less. The consequence is a fast and expensive turnover of trees for big companies and the end of business for medium and small farmers. Just the return on investment in citrus normally takes 8 to 9 years after planting.

Therefore in all aspects examined, citrus blight is an important concern for citrus industries, wherever it happens. The complexity and importance of citrus blight demands broad inter-institutional scientific cooperation on efforts to examine its etiology and gain insights into what causes the plant to block its own xylem and decline, what is transmitted from plant to plant (and why only by roots), how the plant responds and what

is changed in the metabolism of the affected plant, why young non-bearing plants are normally not affected, and more. But these are overwhelming questions. The present study, more conservatively, seeks to uncover molecular and etiological contrasts between healthy and blighted trees.

Objectives

The major objectives of this study were to identify differentially transcribed genes under non and citrus blight conditions and contribute to its understanding and control, which is the long term goal of the citrus blight research at this institution.

The specific objectives were

- to build subtracted cDNA libraries from root samples of non and blighted trees.
- to identify differentially transcribed genes using cDNA arrays.
- to obtain quantitative information about the transcriptional level of the selected genes using reverse transcriptase real time PCR.
- to evaluate the presence of potential causal agents found in the subtracted libraries.

CHAPTER 3
BUILDING THE CITRUS BLIGHT SUBTRACTED LIBRARIES

For research on citrus blight, several promising technologies were considered. Genome wide approaches and microarrays have offered great perspectives in several biological systems, but sequence information is limited for citrus. Thus, cDNA subtractive hybridization (Diatchenko et al., 1996) was undertaken, with the objective to obtain enriched cDNA libraries for each considered condition: healthy and blighted plants. Subtracted libraries can uncover genes up or down regulated under a specific condition, thus being a useful source of candidate clones for further studies. Similar approaches taken with rice (Xiong et al., 2001) and soybean (Colebatch et al., 2002) encourage such efforts, because genes differentially regulated under disease pressure and protein synthesis responses were identified.

Root Samples and cDNA Synthesis

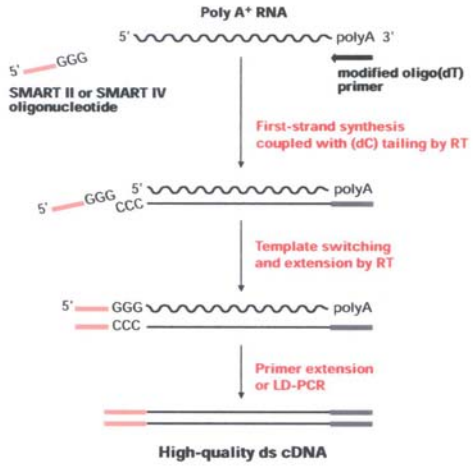
Since blight dissemination is associated with the root system (Timmer et al., 1992) and morphological changes are seen in the roots of affected plants (Lindbeck and Brlansky, 1998), molecular responses are also expected to happen; therefore, root tissues were chosen for subsequent procedures. Superficial roots of Rough lemon (*Citrus jambhiri* Lush) rootstock supporting Valencia sweet orange (*Citrus sinensis* L. Osbeck cv. Valencia) canopy were collected and total RNA was extracted the same day from feeder root tissues using Qiagen RNeasy protocol, with DNase digestion (Source: http://www1.qiagen.com , last accessed July 15, 2001). It was done during the summer of 2001. Rough lemon was chosen because of its known susceptibility to citrus blight.

Feeder roots, also called fibrous roots, of around 2 to 4 mm in diameter, were chosen because they display xylem plugging and internal anatomic differences in affected trees compared with healthy ones (Lindbeck and Brlansky, 2000), similar to what happens in canopy tissues, suggesting that molecular contrasts may also be present. Samples were taken from healthy and fully blighted trees previously diagnosed by typical visual symptoms, by the water uptake test (Lee et al., 1984), and by the P12 based immunoassays on leaf tissues (Derrick et al., 1990). The chosen trees were in a 10-year-old block in the Lake Alfred area, Florida (USA). Reverse transcriptase (RT) polymerase chain reaction (PCR) was performed and cDNA was synthesized using Clontech procedures, enriching for messenger RNA transcripts (Source: http://www.bdbiosciences.com/clontech/ , last accessed September 15, 2001). An experiment was performed to determine the number of PCR cycles necessary to normalize the cDNA synthesis of both samples (Figure 3-1).

Building the Suppressive Subtracted cDNA Libraries

The healthy and blighted cDNA samples were digested with *Rsa* I restriction enzyme to remove the Clontech Smart oligos at the terminal ends of the amplified cDNAs, generating blunt ends. After purification, different Clontech adaptors (1 and 2R) were ligated to split sets of cut healthy and blighted cDNAs. Healthy cDNAs ligated to each adaptor were hybridized with an excess adaptor-free blighted cDNA and with each other. The same procedure was done with blighted cDNA samples and PCRs were performed in both cases, enriching for differentially transcribed genes under each condition, following the Clontech PCR-Select cDNA subtraction procedures (Source:

A



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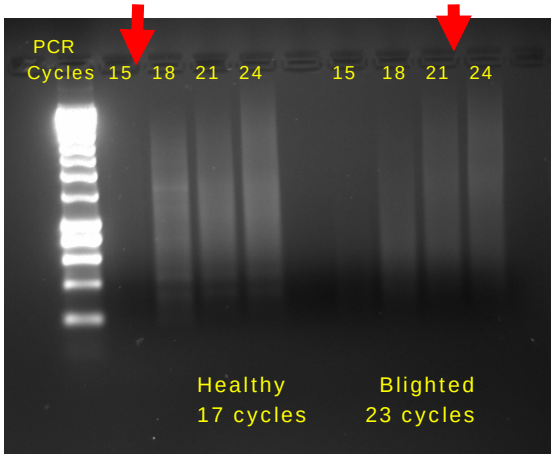


Figure 3-1. cDNA synthesis enriching for mRNA. A) Scheme of cDNA synthesis following the Clontech Smart cDNA procedures (Source: http://www.bdbiosciences.com/clontech/ , last accessed April, 15, 2004). The 3'-PolyT-oligo-5' binds to the poly-A tail of the mRNA and the final extension of the 1st cDNA strand leaves an overhang of Cs, which is bound by the 5'-oligo-GGG-3'. After template switching both ends are filled by polymerization of the complementary strands, and PCR starts, using the primer extension sites present at both oligos. B) Different cycles of PCR were performed on each sample to achieve tentative uniform cDNA accumulation before reaching the plateau of the reaction, following the manufacturer recommendations. Healthy samples were considered optimized at the 17th cycle, while for blighted ones, 23 cycles were run.

http://www.bdbiosciences.com/clontech/ , last accessed September 15, 2001). After PCR, only molecules with different adaptors were exponentially amplified, in theory, while others could only have linear amplifications or impaired products due to hairpin structures. This technique was developed by Diatchenko et al. (1996). Thus, subtracted healthy minus blighted (H – B) and blighted minus healthy (B – H) libraries were generated, in contrast to unsubtracted ones (H and B) and controls (+ and -), Figure 3-2.

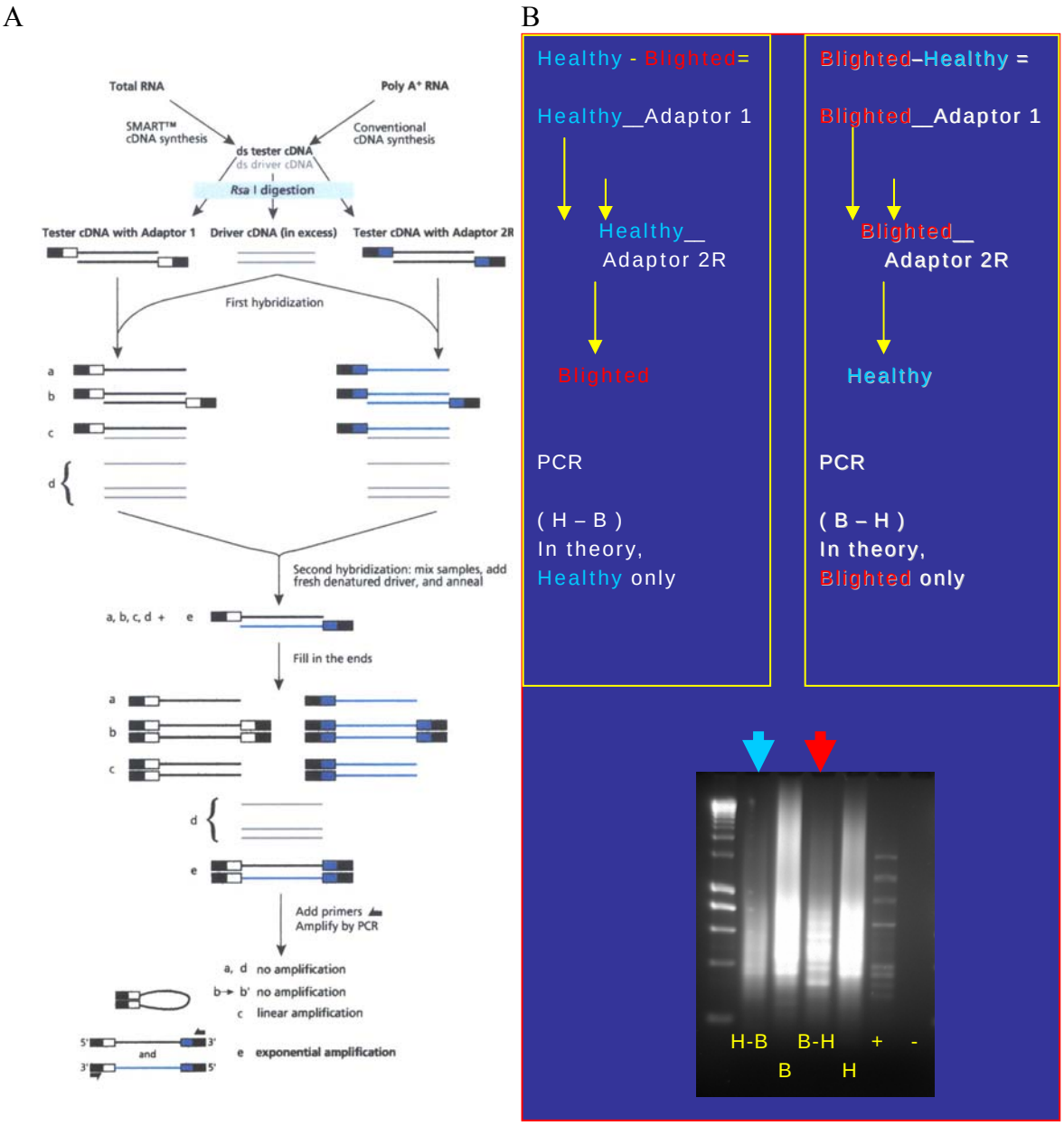


Figure 3-2. Making the subtracted "healthy" and "blighted" libraries. A) Scheme of cDNA subtractive hybridizations following Diatchenko et al. (1996). Two sets of the same cDNA (called 'tester') are ligated to different adaptors and hybridized with in excess adaptor free cDNA (called 'driver') and with each other. PCR was performed in both cases, enriching for differentially expressed genes in both directions (called 'forward' and 'reverse' libraries). B) The same procedure was done with blighted and healthy cDNA samples. Thus, subtracted Healthy minus Blighted (H – B) and Blighted minus Healthy (B – H) libraries were generated, in contrast to unsubtracted ones (H and B) and controls (+ and -).

The differentially enriched cDNAs, (H – B) and (B – H), were cloned following the Promega protocol for the pGMTeasy vector (Source: http://www.promega.com/vectors/ , last accessed on September 15, 2001) and around 500 clones were randomly selected for sequencing from both libraries (400 from the B-H library and 100 from the H-B library).

Sequence Analysis and Selection of Clones

The obtained sequences were trimmed of vector and adaptor sequences used for cloning until no more matches were found using the VecScreen search software (Source: http://www.ncbi.nlm.nih.gov/VecScreen/ , last accessed April 10, 2002). The sequences were run in the automated system of the University of Florida sequencing core (Source: http://www.biotech.ufl.edu/ , last accessed April, 15, 2003) but the Clontech adaptors were not hidden in many cases, requiring manual trimming to avoid contamination of vector sequences. To reduce redundancy of clones that may represent the same gene, the sequences were analyzed using all available Genebank sequences (all-sequences and non-human non-mouse ESTs) and the Brazilian Citrus-EST project as reference sequences for Blast searches (Altschul et al., 1997). With all sequences individually grouped by similar blasted match outcomes, further clusterization and alignment assemblies were made using the Vector NTI software assisted by Microsoft Office applications and other web-based tools (Source: http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html , last accessed April, 10, 2003). The Figure 3-3 displays examples of the four steps performed for trimming and clustering all the 500 individual sequences. After that, the longest clone within the homolog group was the one considered for further studies.

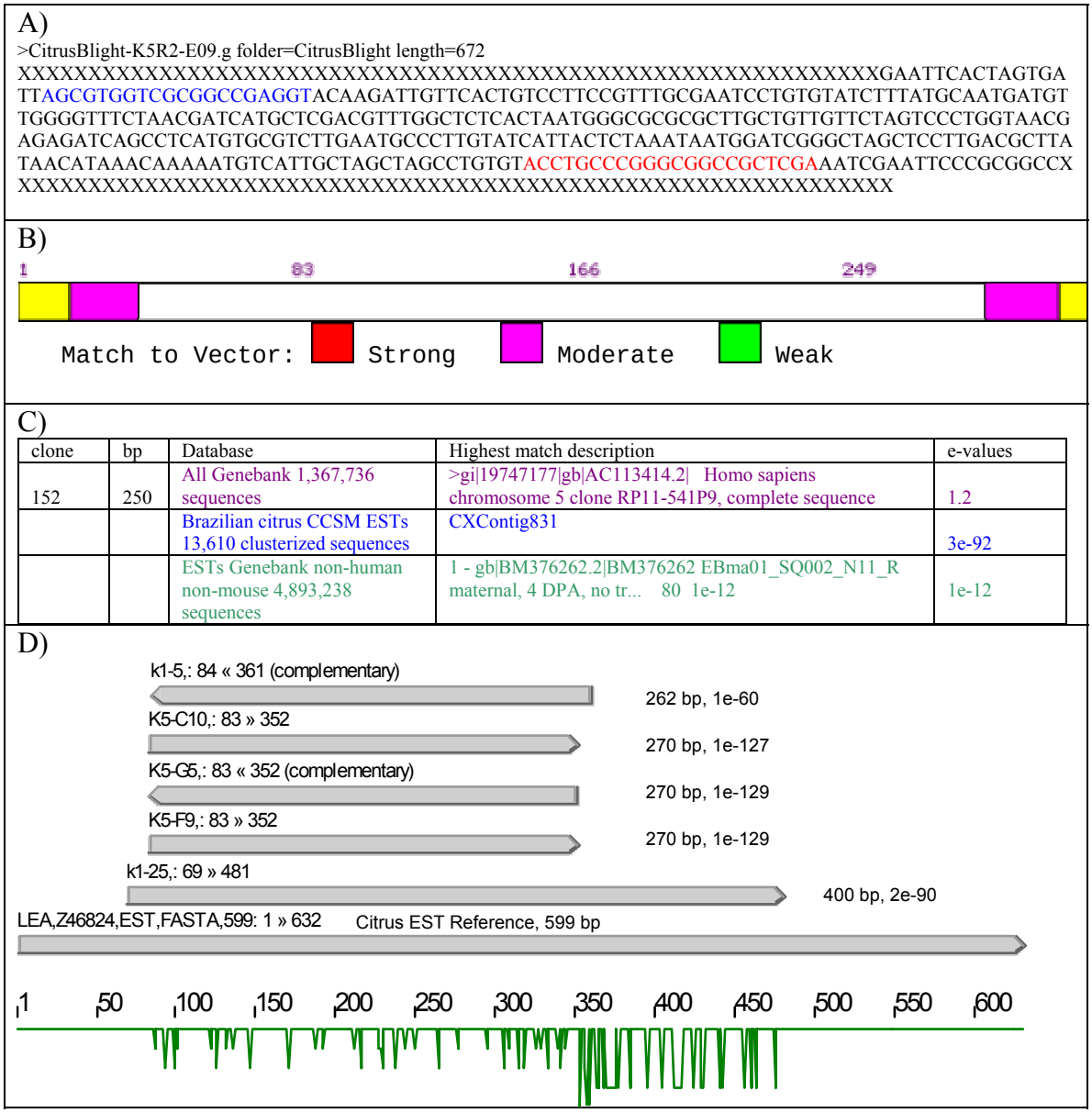


Figure 3-3. Analyses performed for each sequenced clone. A) Looking for vector sequences, such as the Clontech adaptors highlighted in blue and red letters, after the sequences had been masked by the sequencing core system as Xs. B) VecScreen analysis were run for all available clones, confirming in many cases the need of further trimming. C) Blast-N analysis in 3 databases. Many clones had only poor matches in the Genbank search, but had good sequence homology in the citrus and in the non-human-non-mouse EST databases. D) Alignment of the homolog clones against a reference sequence. The green plot, underneath the bars representing the clones, reflects the sequence variability per region.

This step was an intensive computational effort aimed at reducing potential future nonspecific variation from different clones that, in reality, might be representing a transcriptional pattern of only one gene. The average length of the selected clones reached 240 base pairs. Then, it was possible to narrow down all sequences to around 140 tentative unique clones. The Table A-1 in the appendix contains information about these clones and displays the tentative functions and other characteristics based on blast analysis of homolog sequences. It is worthy to note that several clones had high matches with different genes of citrus tristeza virus.

CHAPTER 4
IDENTIFICATION OF DIFFERENTIALLY TRANSCRIBED GENES UNDER NON AND CITRUS BLIGHT CONDITIONS

In order to evaluate the transcriptional pattern of the genes represented by the clones selected from the subtracted libraries, a cDNA array experiment was assembled and run. The major objective was to compare those clones under non and citrus blight conditions.

The DNA array technology has been used in plants with success. There are examples studying defense reactions (Scheideler et al., 2002), stress responses (Rizhsky et al., 2002), and others. Reviews can also be seen at Seki et al. (2003) and at Donson et al. (2002). However, in general, one arduous step in DNA array technologies is to compare information between different experiments (Stoeckert et al., 2002). With a similar concern, Brazma et al. (2001) proposed the MIAME protocol, which stands for 'Minimum Information About Microarray Experiment'. The intrinsic principles of the MIAME apply primarily to define the information that should always be included in microarray repository databases, allowing other researchers to understand the experiment and the data. The ultimate goal of the MIAME protocol is to establish a standard for recording and reporting microarray data based on detailed annotation of six experimental sections: 1) experimental design; 2) array design and spot features; 3) sample preparation and labeling; 4) hybridizations and parameters; 5) measurements and specifications; and 6) normalization and controls. The MIAME protocol does not specify the format in which the information should be provided, but only its content. In the present cDNA array

study, much more modest than the commercially available microarrays, those guidelines were observed attempting to induce uniform experimental conditions and effective ways to analyze the data. Annotations during all experimental steps were recorded and are summarized below.

Experimental Design

The tentative non-redundant clones from the subtracted libraries, (H – B) and (B – H), described at the appendix A, plus ribosomal clones and other tentative controls, were standardized at the same concentration (160ng/ul) after PCR and purification steps. That was possible because all the clones had the same flanking sequences at the Clontech 1 and 2R adaptor regions. Figure 4-1 displays the initial PCR efforts for some of the clones used in this study. Most of the PCR products were in the range from 200 to 400 bp, and some clones had to be amplified more than once to achieve the standards above.

Array Design and Spot Features

The clones were uniformly arrayed on nylon membranes, by printing duplicated sets of each using the robot printer of the ICBR-UF (Interdisciplinary Center for Biotechnology Research of the University of Florida). Each membrane contained 188 double spotted probes ready for further quantitative assays (Figure 4-2). The description of each clone is given in the appendix.

For positional landmarking, ribosomal genes were used. They were clones with sequence homology to a citrus ribosomal gene, Accession X05910.1. Controls for quality evaluations included detection of water spots for background assessments, plasmid with GFP for nonspecific hybridizations and aerial plant stress related genes, such as the cold responsive gene CORc115 (panel G12, spot 180), for variation determinations. Membranes were UV-cross linked and stored at around 6^{o}C.

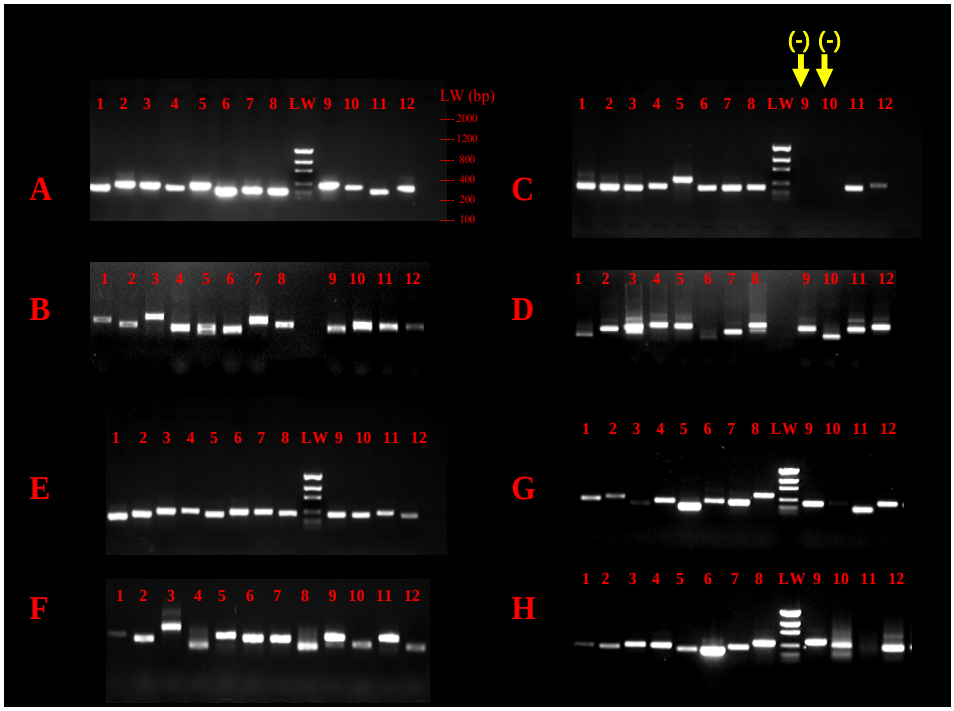


Figure 4-1. Preparing individual clones for the cDNA array. Selected clones were amplified by PCR from the subtractred libraries and prepared to be arrayed on nylon membranes (LW for low DNA mass ladder from Invitrogen; (-) for no-template control).

Sample Preparation and Labeling

The membranes were hybridized with independent "healthy" and "blighted" RNA sources. Total RNA samples were extracted under the same conditions from feeder roots from adult groves of Valencia sweet orange (*Citrus sinensis* L. Osbeck cv. Valencia) on Rough lemon (*Citrus jambhiri* Lush), Carrizo citrange (*Citrus sinensis* L. Osb. *x Poncirus trifoliata* L. Raf.), and Swingle citrumelo (*Citrus paradisi* Macf. *x Poncirus trifoliata* L. Raf.) rootstocks from the central Florida region, using Qiagen RNeasy procedures with DNase treatment. Plants had been previously diagnosed using the visual

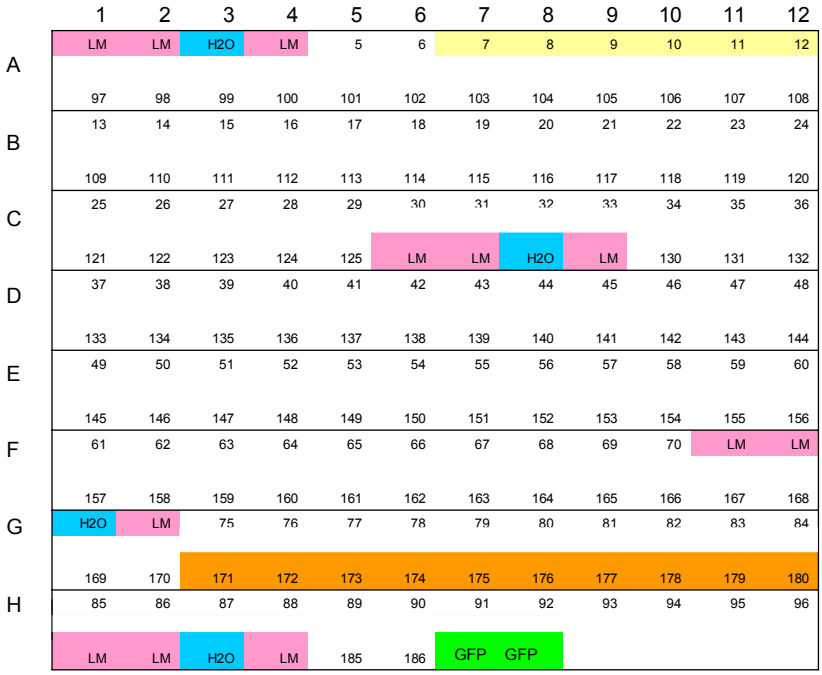


Figure 4-2. Array design. Colored spots indicate different types of clones or controls: pink (LM) for landmark positing; blue (H2O) for water controls; yellow (spots 7 to 12) for probes from citrus leaf tissue; orange (spots 171 to 180) for probes from stress related genes; and green (GFP) for hybridization control. Those controls accounted for thirty four spots. Other spots were printed with clones from the subtracted libraries of citrus root tissue. Seventeen clones were from the H - B library (spots 5 and 6; from 53 to 63; and from 67 to 70). All the remaining one hundred and fifty four clones were from the B - H library.

identification of typical symptoms in the canopy and the water injection test in the trunk (Lee et al., 1984). All samples were uniformly handled, using tissues from pairs of healthy and fully blighted trees of the same genotype, grown under the same condition and from the same grove. Some membranes were used to determine the best amount of RNA, type of primer for RT (reverse transcriptase) reaction, and RNA sources, comparing total versus messenger RNAs. After optimizations, samples with 2ug of total RNA had the 1st strand cDNA labeled with dATP33 using random primers following procedures similar to that of the Ambion Strip-EZ kit (Source: http://www.ambion.com/ ,

last accessed Octpber 10, 2003). The RT efficiency was evaluated by counting the scintillation of the labeled samples.

Hybridizations and Parameters

All membranes were exposed to the same amount of labeled probe, based on the scintillation reading of one million counts/minute/ml of buffer. The samples were hybridized overnight at 64^{o}C with individual membranes, washed, and analyzed under the Typhoon Phosphoscreen Imager System (Source: http://www.amershambiosciences.com, last accessed April 15, 2004), always uniformly manipulated in pairs of healthy and blighted samples to allow further pairwise comparisons. The employed imager system evaluates the fluorescence of each spot represented on the screen. Changes of the oxi-reductive status of a screen component, made by crystals of barium (Ba), fluorine (F), bromine (Br) and europium (Eu), are induced by the radiation of the samples after overnight exposure. Then, a laser beam from the reader induces excitation and subsequent fluorescence of each spot represented on the screen, proportionally to the initial radiation emitted from the labeled sample. The image of each membrane was captured by the scanner and the quantitative information was obtained using the software ImageQuant (Source: http://www.amershambiosciences.com, last accessed April 15, 2004). All membranes were treated as uniformly as possible to minimize other sources of variation in the subsequent analyses.

Measurements and Specifications

The membranes were framed and had the image contrast optimized for better visualization using the ImageQuant, Microsoft PhotoEditor and PowerPoint softwares. The quantitative information of each clone was then evaluated. The parameter for quality

of each membrane was verified looking at the ratio (Ratio) between the averaged absolute values, intensity of the fluorescence per pixel in the screen, of all the genes (AveGene) represented by the clones, over the average of all the "water" spots (AveH2O), used as negative controls for the hybridizations, and therefore considered as background or noise, as

$$\text{Ratio} = \text{AveGene} / \text{AveH2O}$$

Seventy-one membranes were printed and run. The expected true ratio was calculated to be within the confidence interval of 2.33 and 3.27, for $p < 0.01$. Therefore, individual membranes with a ratio smaller than the low limit of 2.33 were considered inappropriate for analysis and were discarded. One membrane that had nonspecific hybridization, measured by the spots with GFP genes, was also discarded. Forty unbiased membranes passed those criteria and were saved. Among those, twenty individual membranes represented ten pairs of uniformly manipulated healthy and blighted sampled trees. Each pair of samples was collected at the same day, from the same grove, and had their RNA extracted, labeled and hybridized under the same conditions, as uniformly as possible. Thus, membranes of any considered pair of healthy and blighted samples displayed an averaged absolute value (intensity/pixel) for all clones similar to the other membrane, in most of the cases, and both were respectively higher than their background and nonspecific hybridization controls by at least 2.33 fold. Root samples from trees on Carrizo were used on the pairs made by the membranes 1x2, 5x6, 67x54 and 65x66.

From Lemon, on the pairs 9x10 and 61x58. From Swingle, on the pairs 29x30, 31x34, 51x52 and 69x70 (Figure 4-3).

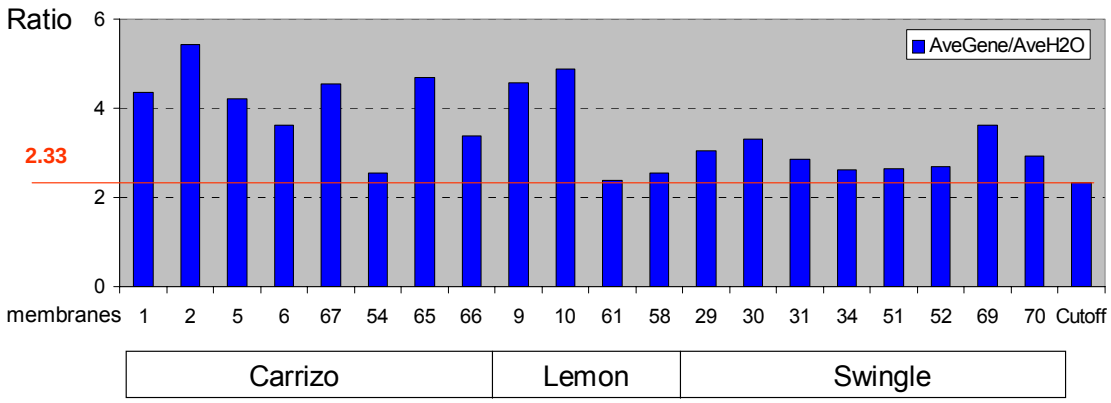


Figure 4-3. Quality parameter for each membrane. The unitless ratio represents the proportion between the averaged absolute value for all clones (AveGene) in the membrane over the averaged absolute reading for the background level (AveH2O) of the same membrane. Membranes with a ratio smaller than 2.33 were discarded.

Normalization and Controls

The readings of the duplicated spots for each clone were averaged. All "water" spots (AveH2O), previously calculated for each membrane, were used to subtract the background from each clone (C). Negative values implied that the reading of the clone was smaller than the background value. Those values were discarded. Then, each clone was normalized (n) against the new mean of the clones (MeClone) in the membrane, rather than against one or two genes, such as a ribosomal or other ones, as follows:

$$n = [(C - AveH2O) / MeClone] * 100$$

The normalization method should reflect similar results comparing to the visual evaluation of the studied membranes. The mean of the clones was used in this study because it met this criteria better than other measures of central tendency, such as the median of the numbers. Normalization against a measure of variance, using the standard deviation, was also tried but no improvements were observed either.

Besides the visual evaluation of the membranes, two other types of analyses were performed. To study the relative levels of transcription of the same clone comparing healthy and blighted samples from uniform conditions, cluster analysis was done. To study the variance of each clone, contrast of means analysis was done.

The Visual Evaluation of the Membranes

The tentative visual differences for each clone were highlighted in each framed membrane. This procedure helped to identify clones not previously seen under the initial visual evaluations. The three evaluated genotypes (a lemon, a citrange and a citrumelo) displayed differences across the species. But looking at one rootstock per time, some visual differences between healthy and blighted samples were also seen (Figure 4-4).

Under the citrus blight condition, Carrizo citrange displayed stronger signal for the genes represented by the clones on the panels B1 (clone 109), C1 (clone 25), D4 (clone 136), F1 (clone 157), G7 (clone 79), and maybe others. Lemon had differences on the panels C1 (clone 25), C10 (clone 34), G7 (clone 79) and G8 (clone 80). Swingle had differences mainly on the panel C1 (clone 25). Very few clones displayed more signal on the healthy than on blighted samples: the panel A6 (clone 6) shows a tentative candidate of that on Carrizo and the panels E8 (clone 152) and E9 (clone 153) on Lemon.

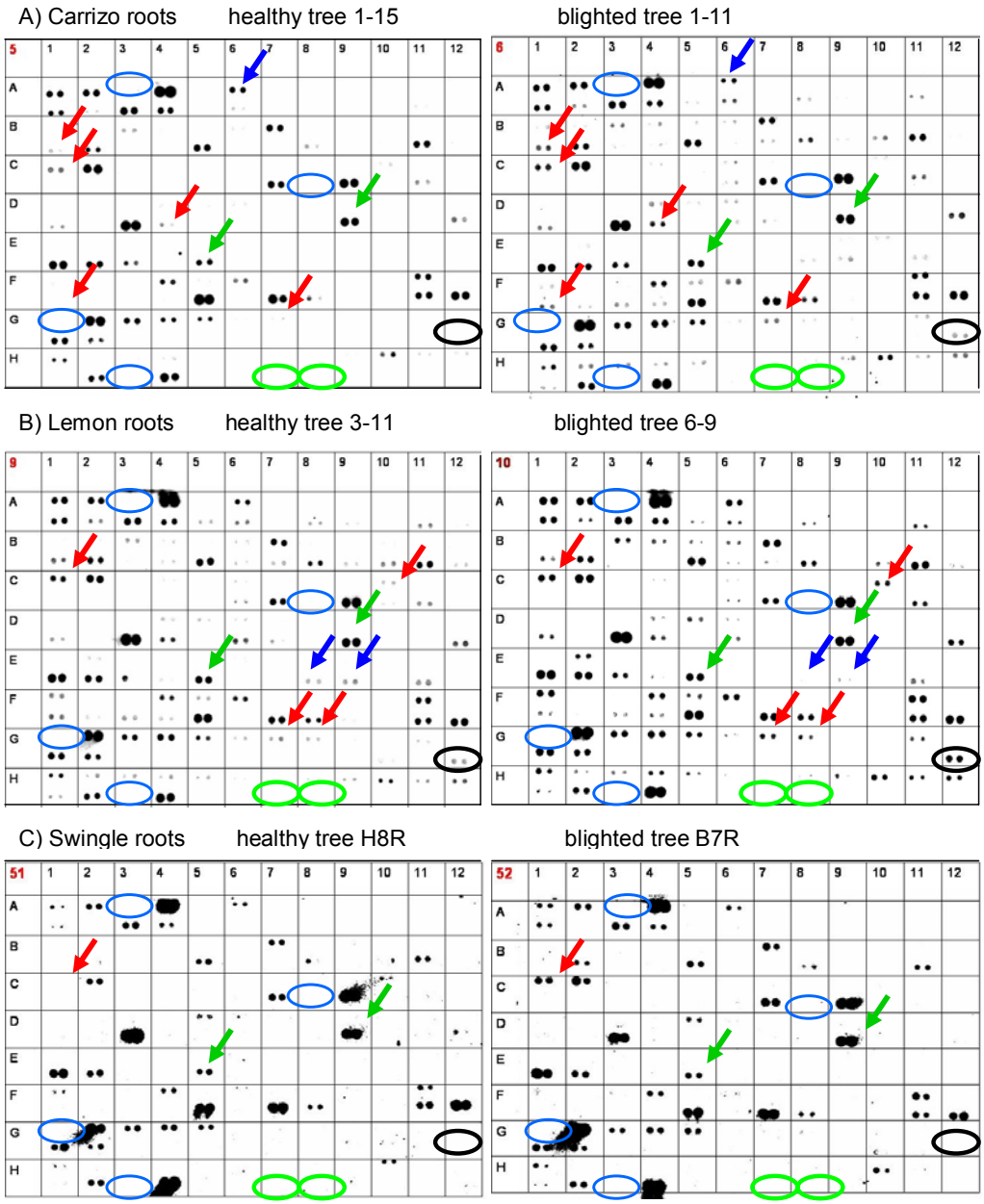


Figure 4-4. Visual evaluation of the membrane pairs. Membranes were hybridized with samples labeled with P33, using feeder roots of 'Valencia' tree on different rootstocks. A) on Carrizo citrange (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.). B) on Rough lemon (*Citrus jambhiri* Lush). C) on Swingle citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.). Each panel has two clones horizontally replicated. Red arrows represent potential more signal on blighted samples. Blue arrows, on healthy samples. Green arrows, near identical. Blue circles represent spots filled only with water, for background control. Green circles, with GFP, for non-specific hybridization control. Black circles, with CORc115, for observations of a strees related gene.

Nearly unchanged genes were seen in all samples and the panels D9 (clone 141) and E5 (clone 149) represent examples of those, in all three rootstocks. The Figure 4-4 shows one pair of membranes for each rootsctock.

The Cluster Analysis

The transcriptional levels of the normalized Healthy (nH) and Blighted (nB) samples were compared following the Log2 function, to allow equidistant visualization of the fold (F) induction or repression for each gene, as:

$$F = \text{Log2} [(nB+0.01) / (nH+0.01)]$$

Gaasterland and Bekiranov (2000) describe two major types of analysis, the supervised and the unsupervised approach. In the supervised analysis, a particular context of each measurement is known and the end result is a list of individual genes behaving differently in each of the different contexts. In this study, the supervised model was accepted and the null hypothesis considered no differences in the transcriptional levels of the candidate gene under healthy and blighted conditions.

Then, the cluster analysis and the graphic visualization for each clone was done employing respectively the Cluster and the TreeView softwares (Eisen et al., 1998). The Cluster software works on a random assignment of vectors for similarities found within the numbers. Subsequent new layers of vectors allow further clusterization of all samples. To test the significance of the differences, t-Tests were employed. Induced and repressed candidate clones were nominated, after analyzing the outcome of the ten independent pairs of healthy and blighted trees (Figure 4-5).

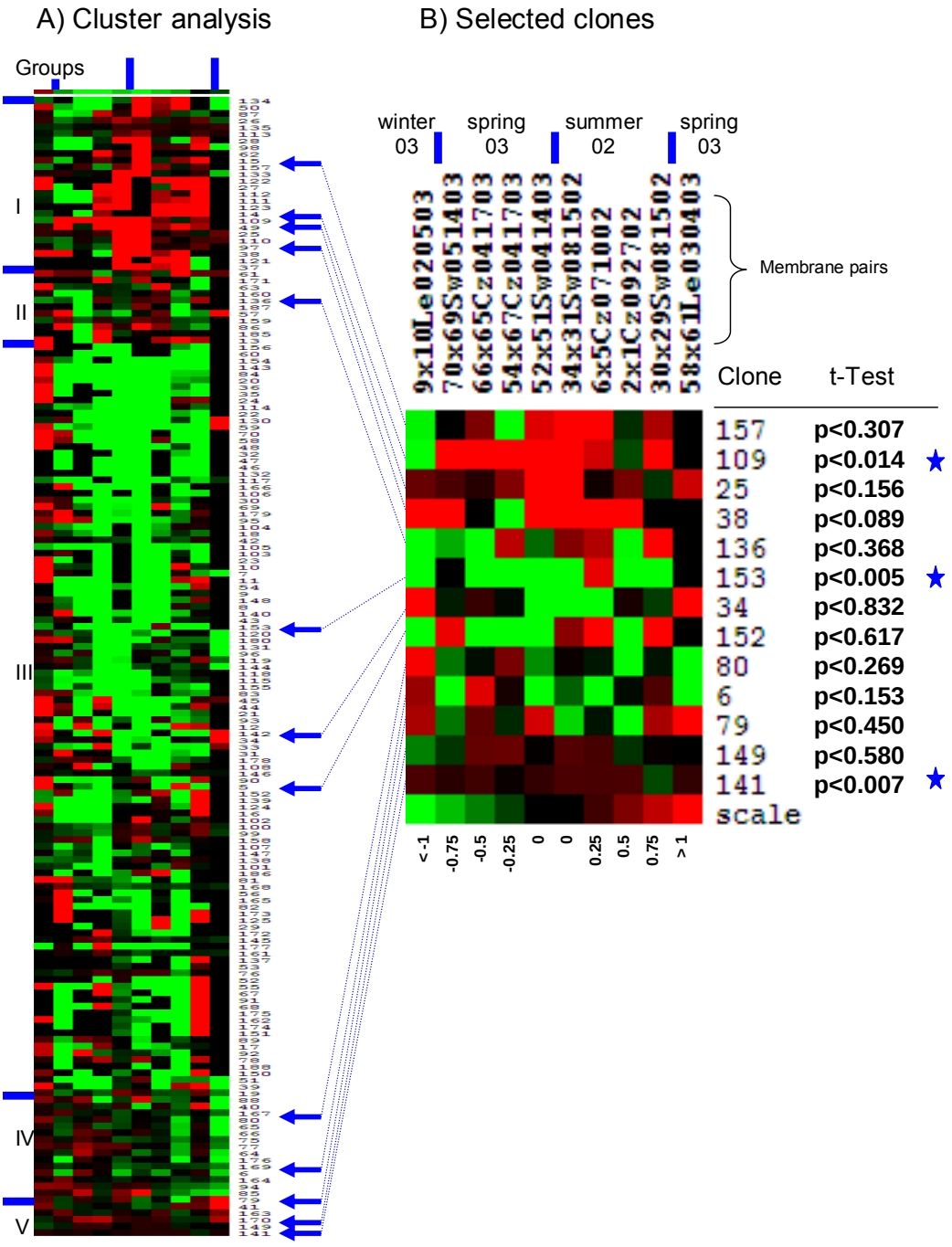


Figure 4-5. Transcriptional profiling of the candidate genes. A) Outcome of the ten pairs of trees under healthy and blighted conditions, being four pairs on Carrizo citrange, two on Rough Lemon and four on Swingle citrumelo rootstock. Each spot represented the ratio of the normalized blighted (nB) over the healthy (nH) sample given by Log2[(nB+0.01)/(nH+0.01). Green indicates negative ratio (more healthy than blighted) and red positive ratio (more blighted than healthy). B) Visualization of the individual clones and their 'p' values for the t-Test, with the significant ones ($p<0.05$) being highlighted with a nearby star.

After the cluster analysis, all membranes were visually re-evaluated. Clones that had $p<0.05$ from the group I, mostly upregulated candidates under the citrus blight condition, were located again. This process helped to spot differences not previously seen in the membranes. Similar approach was taken for the clones of the groups II, III and IV, mostly downregulated candidates under the citrus blight condition. However, it is possible that, for these clones, a more sensitive detection method would be more effective, because few visual differences were re-confirmed. The nearly unchanged genes of the group V, displayed the expected clones 141 and 149. Albeit small, the clone 141 did have a significant variation, with $p<0.007$, leaving the clone 149 alone as a candidate for an unchanged gene under citrus blight condition.

These ten paired samples were collected and run from July of 2002 to May of 2003. Closer patterns can be observed comparing the Carrizo and Swingle samples from the summer of 2002 and the spring of 2003. However, more replicates would be needed to better study the real contribution of the season of the year on the studied model.

The Contrast of Means Analysis

Although the previous analysis is powerful for initial screening, it brings a natural chance of error by selecting potential false positives, since a clone with initial reading value (C) that approaches the background level (W) will generate a normalized number (nH or nB) that approaches zero, complicating any further conclusions for the pairwise or cluster analyses. On the other hand, simply discarding those clones may penalize the final outcome by leaving behind clones that may be indeed positives. Thus, besides exercising caution on apparently extremely induced or repressed clones, those were the reasons to perform the contrast of means analysis. For that, each clone was assessed individually

within each uniform pair of plants (healthy and blighted), from the same rootstock type and from the same block of trees, as previously described.

The assumed model for variance in this case implies that the variance of a clone (x) is a function of its averaged value (^x), plus the contribution of the membrane (M), plus the contribution of the uncontrolled residual factors (e), as

$$x = \hat{x} + M + e$$

Other assumptions for the model above were also considered, as having additive factors, normal distribution of the data, independence between treatments and homocedasticity-also called uniformity of variance (Banzatto and Kronka, 1992). Additive contributions of each factor can be explained by the assumed model itself, since the outcome of each clone was the sum of those factors. Normality was only partially observed because the data aggregated around the mean, for the most part, but not perfectly. Independence of treatments in this biological system could not be fully accepted because that implies that the genes assessed by each clone operate independently from each other. Since metabolic pathways are complexes and regulated by counterpart genes, this is probably not true in this system either. Homocedasticity was not verified and the data was checked for transformation options, such as, RootSquare, Log and other functions. The smallest discrepancy in variances was obtained using the Log transformation of the previous normalized data (n), giving to each clone its final value (V), as

$$V = \text{Log2}\ (n + 1.5)$$

The normalization for the contrast of means was similar to the initial normalization for the cluster analysis (nH and nB) previously described, except for considering individual values of all spots and adding the value 1.5 to avoid zeros in the computation. Once the transformation was observed, t-Test was employed on the established contrast of means, where the estimate of the healthy mean (Hm) and the estimate of the blighted mean (Bm) generated the estimate of the contrast (^Y) for each clone, as

$$\hat{Y} = Hm - Bm$$

Then, the relation between the absolute value for the estimate of the contrast (^Y) and its standard error, which is a function of the number of replicates (rH or rB) and the standard deviations (sH or sB) for healthy and blighted samples, gives the t-Test value (t) for each clone (Banzatto and Kronka, 1992).

Subsequent comparisons against standard t-values determined at which level of probability (p) the null hypothesis, claiming no difference in the transcriptional level of the studied clone, can be accepted or rejected, in favor of the alternative hypothesis, claiming significant differences between healthy and blighted transcriptional levels for each clone.

Some of the clones were variable but not at a level considered significant (t-Test) for this cDNA array system ($p<0.05$). However, other clones displayed significant

response to citrus blight, suggesting that citrus blight is apparently able to affect the transcriptional level of certain genes in affected plants (Figure 4-6).

In plants with citrus blight, the clone 6 was significantly downregulated ($p<0.05$) in three out of the ten pairs of evaluated trees. However, it was also significantly upregulated twice, implying in a great non-specific variation.

The clone 25 was significantly upregulated ($p<0.05$) only in two studied pairs, but displayed higher averaged values on blighted samples in nine pairs of plants, and a strong significant difference on the pair 51x52.

The clone 38 was significantly upregulated in two pairs of plants. It also had a higher averaged transcriptional value in three other pairs of plants.

The clone 109 was significantly upregulated ($p<0.05$) in three out of the ten pairs, and had other three higher averaged values on blighted than on healthy samples.

The clone 149 was nearly unchanged, however it had one significant difference ($p<0.05$) comparing the affected and the healthy plants on the pair 9x10.

The clone 153 was significantly downregulated ($p<0.05$) in only two paired samples, but had higher averaged values on healthy than on blighted samples in other five pairs.

The clone 157 had a higher averaged transcriptional level on affected than on healthy plants, but a large variance too, being significantly downregulated ($p<0.05$) in one pair of plants.

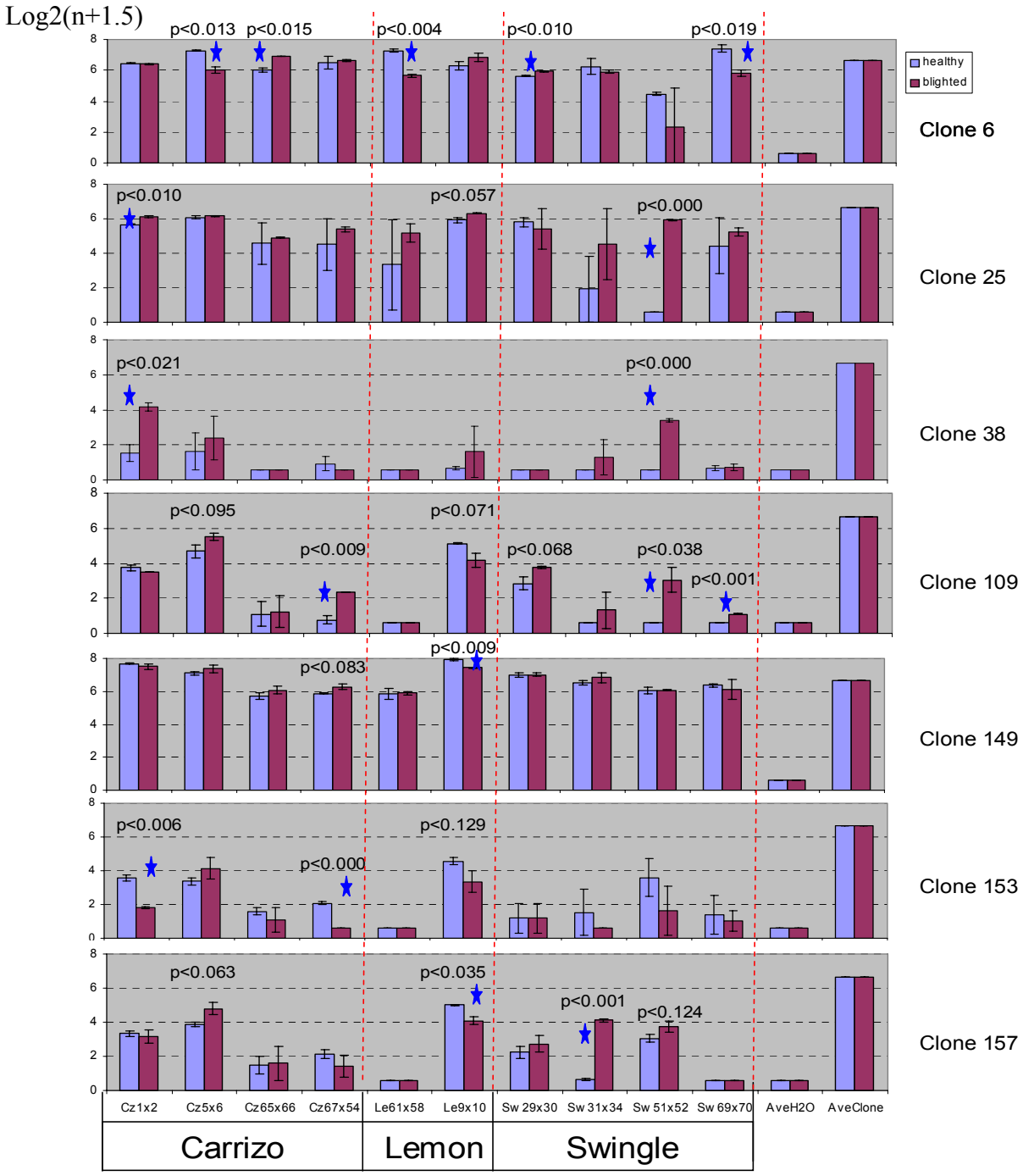


Figure 4-6. Contrast of means analysis of selected clones. Pairs of healthy (blue columns) and blighted (purple columns) trees on Carrizo (Cz), Lemon (Le) and Swingle (Sw). The 'p' value represents the significance of the t-Test, helping in the decision of rejecting or accepting the null hypothesis. The blue stars indicate significant differences when $p<0.05$. The bars are the standard deviation of each studied condition: healthy or blighted samples.

Comparing the Evaluation Methods

All three methods used to evaluate the transcriptional pattern of the genes represented by the clones displayed positive and negative aspects. The visual evaluation of the membranes required neither normalization nor computation and it was good for the major differences, besides confirming, or not, the results of the quantitative analysis. However it did not allow an easy identification of all the candidate clones in the first attempts. The cluster analysis was good for combining all clones from the available samples under the pairwise based approach. Inferences about the averaged fold induction, or repression, and about the aggregation group of each gene could be estimated. But not all quantitative information matched the visual evaluation of the membranes. The contrast of means was positive to reveal the significance of the differences within each pair of trees. It represented an unfolded view of the cluster analysis replicates. However, since the data was transformed, further inferences on the relative transcriptional levels within each pair of plants were no longer precise.

Combining the Results for the Selected Clones

Several clones displayed either visual or quantitative differences between the evaluated samples. The Table 4-1 displays the summarized results for those selected clones.

Among the clones that displayed some differences in the cDNA array experiment, the clone 6 is from the healthy minus blighted (H-B) enriched library and displayed mostly lower transcriptional levels under the citrus blight condition. It is 325 bases long and has sequence homology (Table 4-2) with an *Arabidopsis thaliana* EST (e-value of

Table 4-1. Transcriptional pattern of the selected clones. Observations from the three different types of analysis, comparing healthy (H) and blighted (B) samples.

Clone (library)	Visual differences	Cluster analysis (averaged Log2*; p-value)	Contrast of means analysis (number of significant differences, for p<0.05)
6 (H-B)	H > B	-0.46; p<0.153	3 (H > B) and 2 (H < B)
25 (B-H)	H < B	+1.82; p<0.156	2 (H < B)
38 (B-H)	rare	+2.44; p<0.089	2 (H < B)
109 (B-H)	H < B	+ 3.12; p<0.014	3 (H < B)
149 (B-H)	H ~ B	+0.05; p<0.580	1 (H > B)
153 (B-H)	H > B	-3.13; p<0.005	2 (H > B)
157 (B-H)	H < B	+1.13; p<0.307	1 (H > B) and 1 (H < B)

* Log2 stands for Log2[(nB+0.01)/(nH+0.01)]

1e-180) in the Genbank and with a citrus EST (7e-11) in the Brazilian databank. It has an unknown function based on the translated query (Figure 4-7) to the protein databank (4e-30) and a high nucleotide homology to a mitochondrial sequence (1e-180), raising the question whether the clone 6 represents an open reading frame (ORF) of a gene or not.

```
Query: 41  CRRQRGSRYTIRAGRYLCDKEFRYLRTVRVTAAVYRGFHSKLITLLLLTFQHRACVRLYT 220
           C RQRGSRY IRAGR L DKEFRYLRTV VTAAVYRGF+S ++  LLLTF+HRA VR YT
Sbjct: 4   CWRQRGSRYAIRAGRNLPDKEFRYLRTVIVTAAVYRGFNS-VLAHLLLTFRHRAGVRPYT 62

Query: 221 SCYHLAESCVFNKQSLPPGMCRFPNQKIGEHPFSR 325
           SCYH AESCVFNKQSLPPG+C          PFS+
Sbjct: 63  SCYHFAESCVFNKQSLPPGLCHLALVAQHRSPFSQ 97
```

Figure 4-7. Blast-X of the clone 6 using the non-redundant protein database. Sequence of an environmental protein Accession EAI39113.1 (subject), with unknown function, had similarities (4e-30) to the translated version of the clone 6 (query).

But because of its variance (Figure 4-6), the clone 6 is considered to represent a false positive outcome, not representing a true downregulated gene under the citrus blight condition.

The Table 4-2 displays the accession numbers of the first match on the blast search analysis, using different databases, for the selected clones.

Table 4-2. Blast search analysis for some clones. The search for homolog sequences was done using different databases.

Clones	e-values	Highest match on sequence search analysis
6	$1e^{-180}$ $7e^{-11}$ $4e^{-30}$ $1e^{-180}$	*Arabidopsis thaliana* EST, Accession CF653082 Citrus EST, CCSM, Brazil, Contig 204 unknown protein, environmental sequence, Accession EAI39113.1 *Arabidopsis thaliana* mitochondrial genome, part A, Accession Y08501.1
25	$1e^{-91}$	Citrus EST, CCSM, Brazil, Contig 416
38	$8e^{-88}$ $1e^{-88}$ $3e^{-95}$	Citrus chitinase class II, Accession Z70032.1 Citrus chitinase class I, Accession AB081944.1 Citrus EST, CCSM, Brazil, Contig 1434
109	$9e^{-56}$ $9e^{-44}$ $(2e^{-65})$*	Citrus EST, Accession CK935651 Citrus EST, Accession CB293790 *Arabidopsis thaliana*, ubiquitin ligase SCF complex subunit, Accession NP568603.1
149	$2e^{-54}$	Citrus EST, CCSM, Brazil, Contig CXJE02
153		Poor sequence information
157		Poor matches in the searched databases

* e-value of the citrus EST Accession CK935651 translated query on the protein databank

The clone 25 is from the blighted minus healthy (B-H) enriched library and displayed higher transcriptional levels under the citrus blight condition in some of the tested pairs of plants. It is 300 bases long and has sequence homology with a citrus EST (1e-91) in the Brazilian databank, but only poor matches in Genbank. It also had a strong visual difference on the membranes of Swingle (panel C1, figure 4-4C). That difference was significant ($p<0.05$) in one out of the four tested pairs of plants on Swingle (Figure 4-6). However, considering all rootstocks, the overall transcriptional fold induction under the blight condition was around three and a half times, Log2[(nB+0.01)/(nH+0.01)=1.82, but with a poor 'p' value of only 0.156 (Table 4-1 and Figure 4-5B). It is possible that the

clone 25 represents a true upregulated gene under the citrus blight condition, but with higher transcriptional responses in Swingle citrumelo.

The clone 38 is from the B-H library and is 235 base pairs long. It represents a citrus chitinase gene (Figure 4-8).

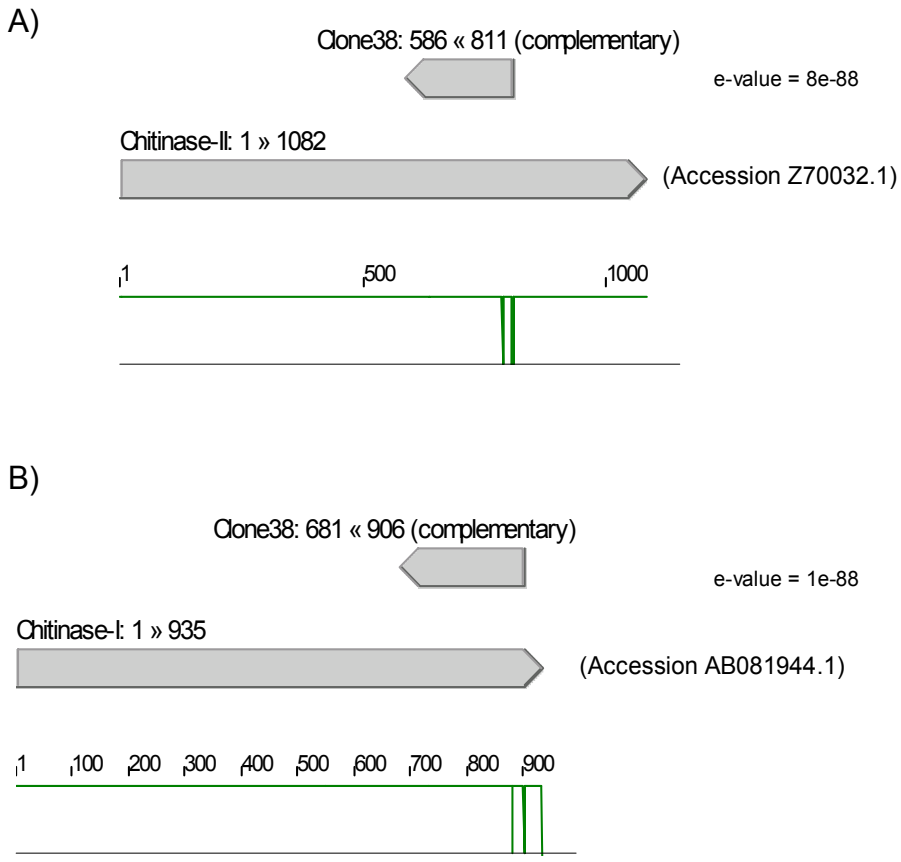


Figure 4-8. Clone 38 has sequence homology to citrus chitinases. Blast-N showed high similarities to (A) a class II chitinase; and to (B) a class I chitinase.

The clone 38 had a transcriptional fold induction under the blight condition of around five times, Log2[(nB+0.01)/(nH+0.01)=2.44 (Table 4-1); but a strong visual difference within the membranes was not seen (panel D2, Figure 4-4A). Looking at the

contrast of means (Figure 4-6), two pairs of trees displayed significantly ($p<0.05$) more transcripts of the gene represented by the clone 38 in blighted than in healthy trees. However, five pairs of trees responded only at a very low level, close to the background (AveH2O). It is possible that the clone 38 represents a responsive gene to citrus blight, but had poor printing on the membrane surfaces for those five abnormal readings. To test that hypothesis, a new series of membranes were printed and run. The figure 4-9 displays the results.

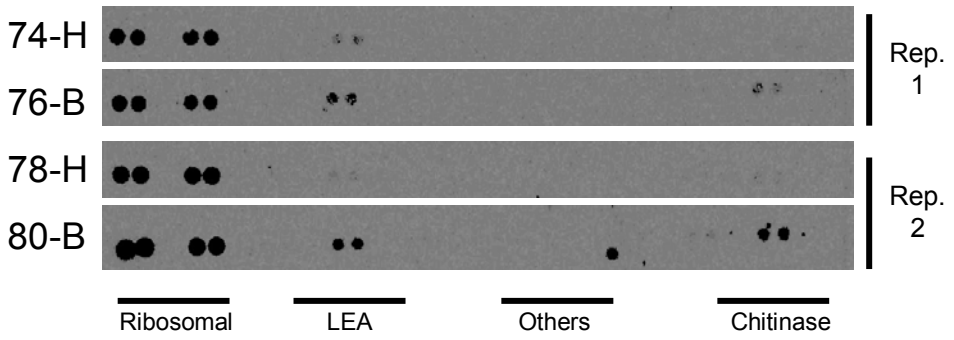


Figure 4-9. Transcriptional profiling of the clone 38 (a chitinase homolog) and other clones. The membranes were manipulated and hybridized under similar conditions, using 5ug of total RNA labeled with P33, from feeder roots samples of a healthy (H) and a blighted (B) tree on Carrizo citrange. The process was repeated twice (rep.1 and 2) generating four membranes. The quantitative difference for the chitinase homolog clone, comparing the healthy and blighted samples (t-Test), was significant ($p<0.01$).

Therefore, the clone 38 is considered to represent a true upregulated gene under the citrus blight condition.

The clone 109 is also from the B-H enriched library and displayed mostly higher transcriptional levels under the citrus blight condition. It is 176 bases long and has sequence homology with citrus ESTs in the Genbank using the non-mouse and non-

human entry (Figure 4-10), but only poor direct matches as a translated query on the protein databank.

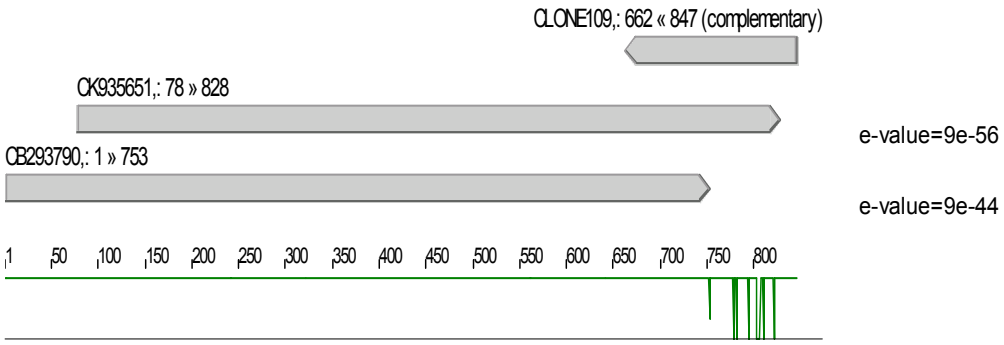


Figure 4-10. The clone 109 had sequence homology to citrus ESTs. The citrus EST Accession CK935651 was obtained from a library using citrus fruit developing tissues. The CB293790, from a library using citrus cold acclimated tissues.

However, blasting the longer citrus EST homolog (Accession CK935651) as a translated query gives a high match (2e-65) with an 'E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 (At2)/UFO-binding protein (UIP2)' of *Arabidopsis thaliana* (Accession NP568603.1). The clone 109 had visual differences in the membranes (panel B1, Figure 4-4A); had an averaged transcriptional fold induction of around eight times, Log2[(nB+0.01)/(nH+0.01)] =3.12, with $p<0.014$ (Table 4-1 and Figure 4-5B); and it was significantly upregulated in three out of the ten studied pairs of samples. Therefore, the clone 109 is considered to represent a true upregulated gene under the citrus blight condition.

The clone 149 is 144 bases long and has only poor matches in Genbank either as a nucleotide query or as a translated query, but it has a good match with a citrus EST (2e-54) in the Brazilian databank. It is from the B-H enriched library. The clone 149 probably

represents an unchanged gene under the citrus blight condition, considering the three methods used to evaluate its transcriptional pattern.

The clone 153 is from the B-H enriched library, but probably represents a true downregulated gene under the citrus blight condition. It displayed visual differences in the membranes (panel E9, Figure 4-4B); significant differences in the cluster analysis with $p<0.005$ (Figure 4-5B); and two significant differences in the contrast of mean analysis (Table 4-1 and Figure 4-6). However, the sequence information available was poor, with low phred-quality parameter. It was re-sequenced twice with no further improvements.

The clone 157 is 195 bases long but had no good match in all three searched databases. Because of its variance (Figure 4-6), it is considered to be another false positive outcome, not representing a true upregulated gene under the citrus blight condition.

CHAPTER 5
THE RELATIVE TRANSCRIPTIONAL RESPONSES OF THE SELECTED GENES UNDER DIFFERENT INCIDENCES OF CITRUS BLIGHT

The process of building cDNA arrays is marked by many steps that go from the collection of samples to make the libraries until the manipulation of the membranes. As a consequence, the final data can carry cumulative sources of nonspecific variation, which may compress or hide legitimate up or down regulated genes under study. With that concern, Chuaqui et al. (2002) stated that researchers must be aware whether the results in microarray based experiments are accurate and the data fundamentally describe the phenomenon being investigated. In addition to employing replicates as used in this study, Chuaqui et al. (2002) considers the need of a validation process. Therefore, an independent RNA assessment method was used to confirm, or not, the transcriptional level of the previously identified genes under non and citrus blight conditions. The effect of cold and drought stresses was also tested.

Quantitative Real Time PCR was the Method of Choice

Among the available methods, quantitative real time PCR was the method of choice, because of its sensitivity and reliability. It has been used under different platforms and for different purposes, such as confirmation of microarray data in human cancer research (van den Boom et al., 2003), and quantification of a citrus pathogen (Oliveira et al., 2002). It is based on the determination of a fluorescent signal produced during the PCR cycles, allowing the quantification of the amplified product at a real time and the subsequent estimation of its relative original template concentration (Mackay et al.,

2002). According to Dorak (source: http://dorakmt.tripod.com/genetics/realtime.html, last accessed on January 10, 2004), real time PCR is a preferable alternative to study transcription to other forms of reverse transcriptase (RT) PCR, which only detect the final amount of the amplified product. The chemistry employed in real time PCR detection system is the key to the process. There are two general methods for the quantification of the PCR product: the DNA binding reagents (i.e. SYBR Green) and the fluorescent probes (i.e. TaqMan). Characteristics of each detection type and more details about the technique can be seen in Mackay et al. (2002). The TaqMan probe relies on the fluorescence resonance energy transfer (FRET) for quantification. TaqMan probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. Normalization is done against an active internal control such as a ribosomal or other gene. Passive controls, compounds that do not participate in the PCR reaction, are used to adjust the reaction level and background. Other important parameter is the threshold cycle, or Ct, obtained by an arbitrary threshold line chosen within the linear phase of the PCR reaction, that gives the PCR cycle for that fluorescence value. The Ct values are

used to compare different PCR reactions, allowing the quantitative estimation of the initial target template.

Compared to DNA dyes, the TaqMan method also has the benefit of producing an amplified target with longer sequence specificity, since it is the product of the hybridization of two primers plus a probe, in a total length that normally stays around 150 bases. Real time PCR using dyes (i.e. SYBER Green) relies only on the specificity of the primers. Considering the described characteristics, the TaqMan probe was the method of choice to validate (or not) the initial results of the cDNA array experiment.

The Selected Clones and the Characteristics of the Probes

The selected clones observed in the cDNA array were used in this experiment. The clones considered to represent up-regulated genes under the citrus blight condition were the clones 25, 38 and 109. The clone 149 appeared to be nearly unchanged. The clone 153 was considered to be down-regulated; however the sequence information for this clone was poor, even after re-sequencing. Thus, the clone 6 was included in this experiment as a candidate to represent a down-regulated gene under the citrus blight condition. Those clones were chosen because genes with altered transcriptional levels under the citrus blight condition can be valuable tools on further comprehensive experiments. Genes with unaltered patterns can be useful references for normalizations or for other biological needs as well.

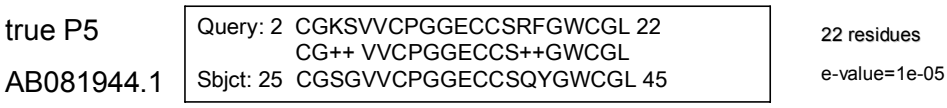
Based on sequence homology, the clone 38 represents a citrus chitinase gene (Table 4-2, chapter 4). Chitinases are known to respond to different forms of stresses, such as fungal pathogens in citrus plants (Fanta et al., 2003). Citrus blight causes mineral unbalance with a clear pattern for zinc deficiency in leaves. Taylor et al. (1996), identified a small protein, named P5 (Accession AAB46813), that has sequence

similarities to chitin binding proteins and functions complexing zinc in affected plants. It is probably associated with the known translocation of zinc from leaves to trunk tissues (Albrigo and Young, 1981). In addition to the clone 38, chitinase homologs were found in our libraries and in combination with the class I Chitinase (Accession AB081944.1) as reference, a putative nucleotide sequence for a P5 candidate gene was deduced and was used in this validation experiment (Figure 5-1).

A)

protein	e-values	Highest match on sequence search analysis
true P5	$1e^{-05}$	Citrus chitinase class I, Accession AB081944.1

B)



C)

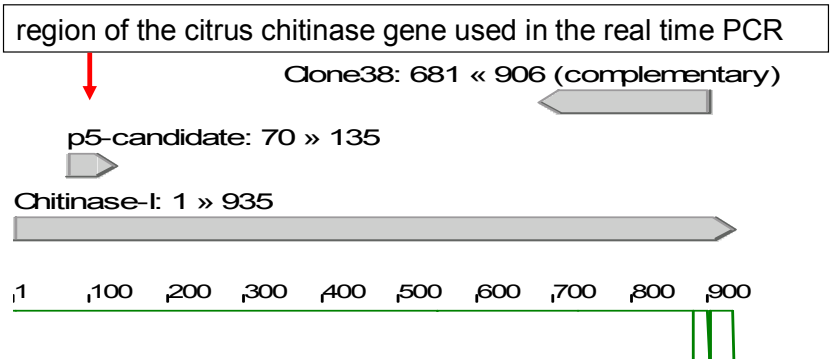


Figure 5-1. Designing a candidate sequence for the P5 gene. A) The true P5 protein has homology to the translated version of the citrus chitinase class I Accesion AB081944.1. B) The aminoacid identities was 80%, with an e-value of 1e-05. C) Based on the reference chitinase class I (Accession AB081944.1), which also had high homology to the clone 38, a candidate sequenced was designed for the P5 gene and was used in the real time PCR experiment.

Another gene associated to citrus blight is the P12 (Accession AF015782). It is up-regulated in affected plants (Derrick et al., 1990) and has sequence similarities to expansins (Ceccardi et al., 1998), but has so far an unknown role in the decline of the trees. P12 was also included in this experiment.

The Table 5-1 displays the characteristics of the chosen probes for each of the candidate clones and P12.

Table 5-1. Characteristics of the chosen probes. The primers and probes were designed using the PrimerExpress Software (Source: Applied Biosystems Resources, http://www.appliedbiosystems.com/index.cfm, last accessed December 10, 2003).

Clone name	Start (bp)	Length (bp)	Tm (°C)	GC (%)	Taqman probe architecture (flourochrome-5'sequence 3'-quencher)
6	136	29	69	45	fam6-TCGCTACTTATGCGACAAGGAATTTCGCT-tamra
25	192	32	69	41	Fam6-TTGAAGGCAAGTTAGGAAATTAGCAAAGCCAG-tamra
109	94	39	69	33	fam6-ATGATACAGAGAAGGTTGGGATGATATGACATTAAAACA-tamra
149	213	26	69	42	fam6-CTGTATCATCTTACTTTACGCTTCCC-tamra
P5 candidate	78	18	68	67	fam6-AAGCGGCGTTGTGTGCCC-tamra
P12	179	25	69	56	tet-TGGAGTCATGATAGCCGCAGCAAGC-tamra

Two major calculation methods are possible, the absolute and the relative quantification of the target gene (Source: bulletin # 2, Applied Biosystem, http://www.appliedbiosystems.com/index.cfm, last accessed December 10, 2003). This study employed the relative method. Then, the 18S gene was chosen as the active internal control, or normalizer, because it is considered to be non altered under different conditions (Dorak, 2004, source: http://dorakmt.tripod.com/genetics/realtime.html, last accessed on January 10, 2004). However, no previous information was seen about the 18S levels in feeder root samples of citrus hybrids under non and citrus blight conditions. It was assumed that the transcriptional level of the 18S gene was not altered by citrus blight.

To test the efficiency of the amplification between the target gene and the normalizer, a RNA 2 fold dilution series was evaluated, from 3.9 to 1000 ng of a mixed pool of RNA templates. Under a similar efficiency, the graphic of the log input amount of the total RNA and ΔCt (difference between the chosen thresholds, the Ct of the target minus the Ct of the normalizer) would give a straight line, nearly parallel to the abscissa. Small deviations from that are accepted and the bulletin # 2 of Applied Biosystem (Source: http://www.appliedbiosystems.com/index.cfm, last accessed December 10, 2003) recommends that the absolute value of the slope (of the estimated linear function) should be equal or smaller than 0.1. Figure 5-2 displays the outcome for the clone 109, which had a slope of 0.1055, for the tested range of 3.9 to 1000ng of RNA template.

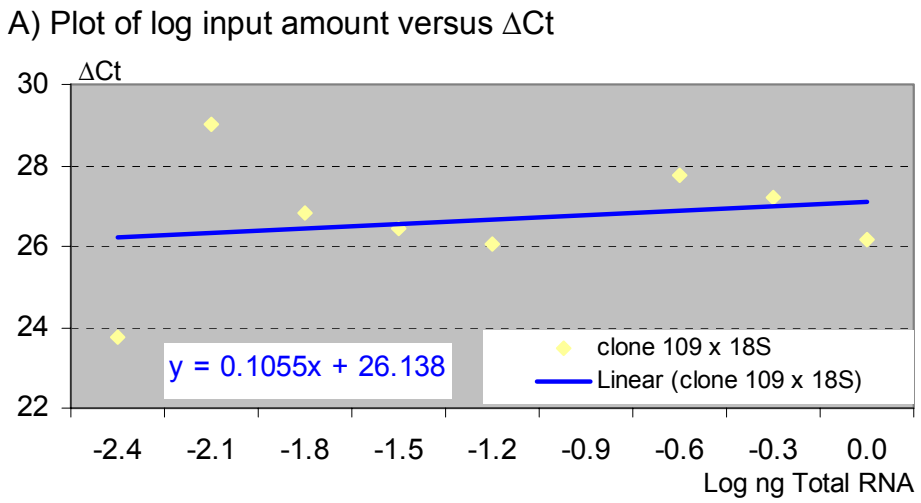


Figure 5-2. Relative PCR efficiency plot of the clone 109 against the normalizer 18S.

In the same type of analysis, the clone 6 had a slope of 0.0104; the clone 25, 0.1029; the clone149, 0.0602; the P5 candidate, 0.0811; and the P12, 0.0196.

Collecting and Preparing the Samples

The same citrus groves in central Florida area, used to collect root samples for the cDNA array experiment, were revisited, with the expectation to re-assess the same trees. However, most of the previously healthy trees had started to display some blight symptoms and some of the affected trees had been pushed out. Therefore, some other plants were used in the real time PCR experiment, after verification of the typical symptoms and the water test (Lee et al., 1984) for diagnostic purposes. Three classes of plants were sampled:

- Healthy: with no visual symptoms in the canopy, and with a minimum uptake of water, manually injected into the trunk by a syringe, of 3 ml/10 seconds.
- Mildly affected: initial symptoms in the canopy, such as opaqueness of leaves and few twig dye backs on the top. Uptake of water from 1 to 2 ml/10 seconds.
- Blighted: fully symptomatic canopy. Uptake of water smaller than 0.5ml/10 seconds.

Tentative different stages of the process (healthy, mildly and fully affected trees) were used because they may offer more information than only comparing healthy and fully declined trees, as done on the cDNA array experiment.

Feeder root tissues from each tree were once again collected and the RNA extraction procedures had this time double DNA digestion step, using an adaptation of the Qiagen RNeasy kit (Source: Qiagen, http://www1.qiagen.com/Default.aspx, last assessed January 4, 2004). No DNA contamination was observed when verifying RNA quality on formaldehyde gels. All sampling and extracting procedures were done as uniformly as possible. The sampled trees were Valencia sweet orange (*Citrus sinensis* L. Osbeck cv. Valencia) on the rootstock Carrizo citrange (*Citrus sinensis* L. Osb. *x Poncirus trifoliata* L. Raf.) of around 10 years old. This choice was considered because

trees of Valencia on Carrizo have been largely used in the Florida citrus industry. The combinations of Valencia on Rough lemon and on Swingle citrumelo were also initially considered. But the sampled trees were of different ages and had other non uniform conditions. Therefore, they were no longer considered.

Reverse Transcriptase (RT) and PCR Reactions

Duplicated RT reactions and two PCR reaction sets per each sample were used to minimize pipeting and other reaction errors, resulting initially, in four mechanical replicates per each biological sample. Both RT reactions for each sample were done at the same time and under the same conditions, using a 96 well plate and the protocol given by the Applied Biosystem RT kit (Source: http://www.appliedbiosystems.com/index.cfm, last accessed January 10, 2004). Other conditions were also as uniform as possible. The RT plate was then stored at -20^{o}C.

The 7700 Applied Biosystem sequence detection system was employed for all set of PCR reactions. The PCR conditions were 50^{o}C/2minutes, 95^{o}C/10minutes, and 45 cycles of 95^{o}C/15seconds plus 60^{o}C/1minute.

An arbitrary threshold cycle (Ct) was chosen for each amplification plot. The data was analyzed using the baseline computation method on the Apple based Sequence Detection System (SDS) v1.9 software (Source: http://www.appliedbiosystems.com/index.cfm, last accessed April 10, 2004).

Three controls were employed in all real time PCR plates: the NTC (no template control, to verify non specific amplifications), the NRT (no reverse transcriptase control, to verify DNA contamination) and the NAC (no amplification control, to verify non specific readings). After the reactions, none of the NACs gave integral fluorescent readings. The great majority of the NTCs did not reach the chosen threshold level (Ct) for

the target gene at the 40th PCR cycle. The reactions that did, were discarded. All samples that had a difference in the ΔCt (the Ct of the sample minus the Ct of the NRT) for the reading of the 18S gene, smaller than 10 cycles were also discarded. The Applied Biosystem (Source: Taqman ribosomal RNA control reagents protocol, http://www.appliedbiosystems.com/index.cfm, last accessed April 10, 2004) recommends this cut off level to avoid significant contribution of non-target templates to the measured gene. The remaining healthy, mildly affected and blighted samples were further analyzed. Figure 5-3 displays examples of amplification plots for the clone 109, P5 candidate and P12.

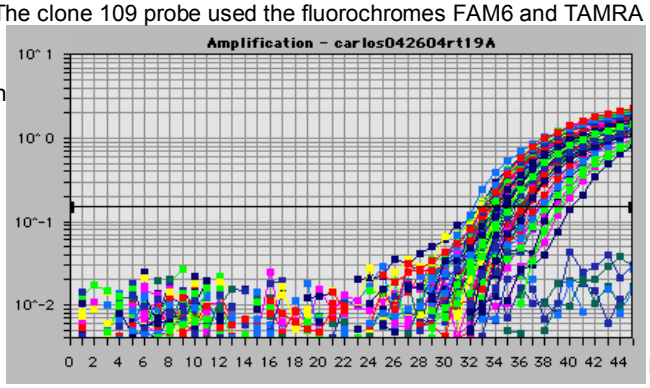
Relative Quantification of the Transcriptional Levels of the Selected Genes

All samples were analyzed in groups according to their similar levels of 18S, measured by the threshold (Ct) in each reaction. Four replicates, groups of independent healthy, mildly affected, and blighted trees, were analyzed, in a total number of twelve different sampled trees.

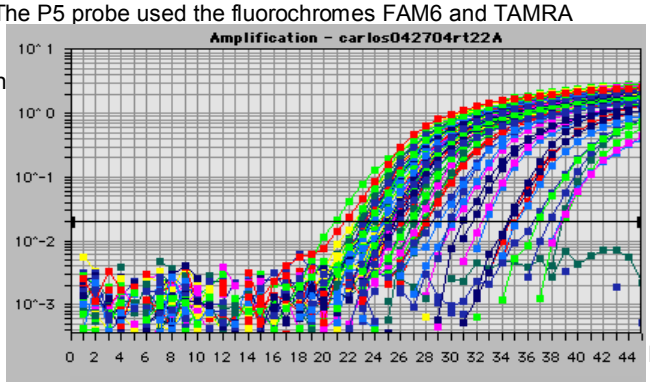
Each clone was evaluated individually. The contrast to compare 'healthy-mildly affected-fully blighted conditions' is not orthogonal. Therefore, the t-Test can not be used. The residual standard deviation (s) was chosen to indicate the non specific variation within each group of plants, or replicates. This measure of variation indicates the effect of non controlled factors, and therefore, seems adequate to estimate the contribution of errors in the experiments. It was calculated using the mean square of the residue (MSres), after the ANOVA (analysis of variance) for each group of plants, as:

$$s = \sqrt{(MSres)}$$

A) The clone 109 probe used the fluorochromes FAM6 and TAMRA



B) The P5 probe used the fluorochromes FAM6 and TAMRA



C) The P12 probe used the fluorochromes TET and TAMRA

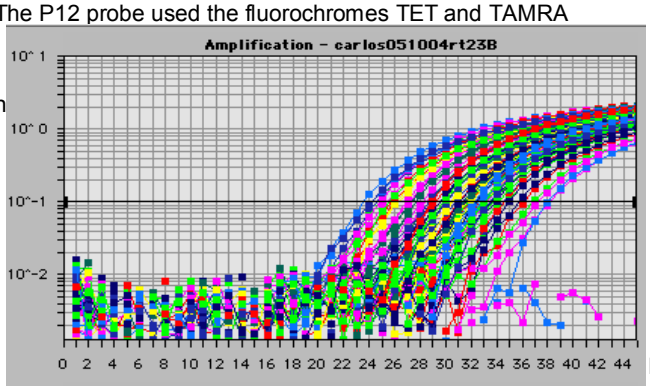


Figure 5-3. Examples of real time PCR reactions. A) For the clone 109. B) For the P5 candidate gene. C) For the P12. The reactions were done with samples from all the tested conditions and reflect the differences in the transcriptional level of the target gene according to each sample.

Figure 5-4 displays the relative transcriptional levels of the selected clones. Within each group of plants, regression analysis was performed and a polynomial equation was shown. The three evaluated stages of the disease (healthy, mildly and fully blighted trees) are certainly not enough for better inferences about the progress of the disease, neither blight can be precisely estimated, since no pathogen is known to cause the problem. But an initial tendency of the relative transcriptional levels compared to the healthy samples, was observed for some of the clones.

The clone 6 can probably be considered a mistaken choice taken from the cDNA array experiment. The tentative down-regulated pattern was not observed in the real time PCR experiment. Moreover, it seems to be up-regulated in affected comparing to healthy plants. More replicates, or maybe other techniques, would probably be needed for further elucidation

The clone 25 was considered to be up-regulated in affected plants, especially when Swingle rootstock was used (panel C1, Figure4-4C). This pattern was also observed in the real time PCR experiment using Carrizo rootstock, confirming the previous expectation. In the group D of plants, the fully blighted tree displayed similar transcriptional levels to the healthy tree, but both were lower than the mildly affected one. It is possible that fully blighted trees reduce some of their metabolism affecting the transcriptional level of certain genes. That was apparently the case for this gene in this studied blighted tree. In the other three groups of plants, the relative amount of the transcripts of the gene represented by the clone 25 were more abundant in fully blighted than in healthy or mildly affected plants. Therefore, the clone 25 was considered to represent an up-regulated gene under citrus blight conditions.

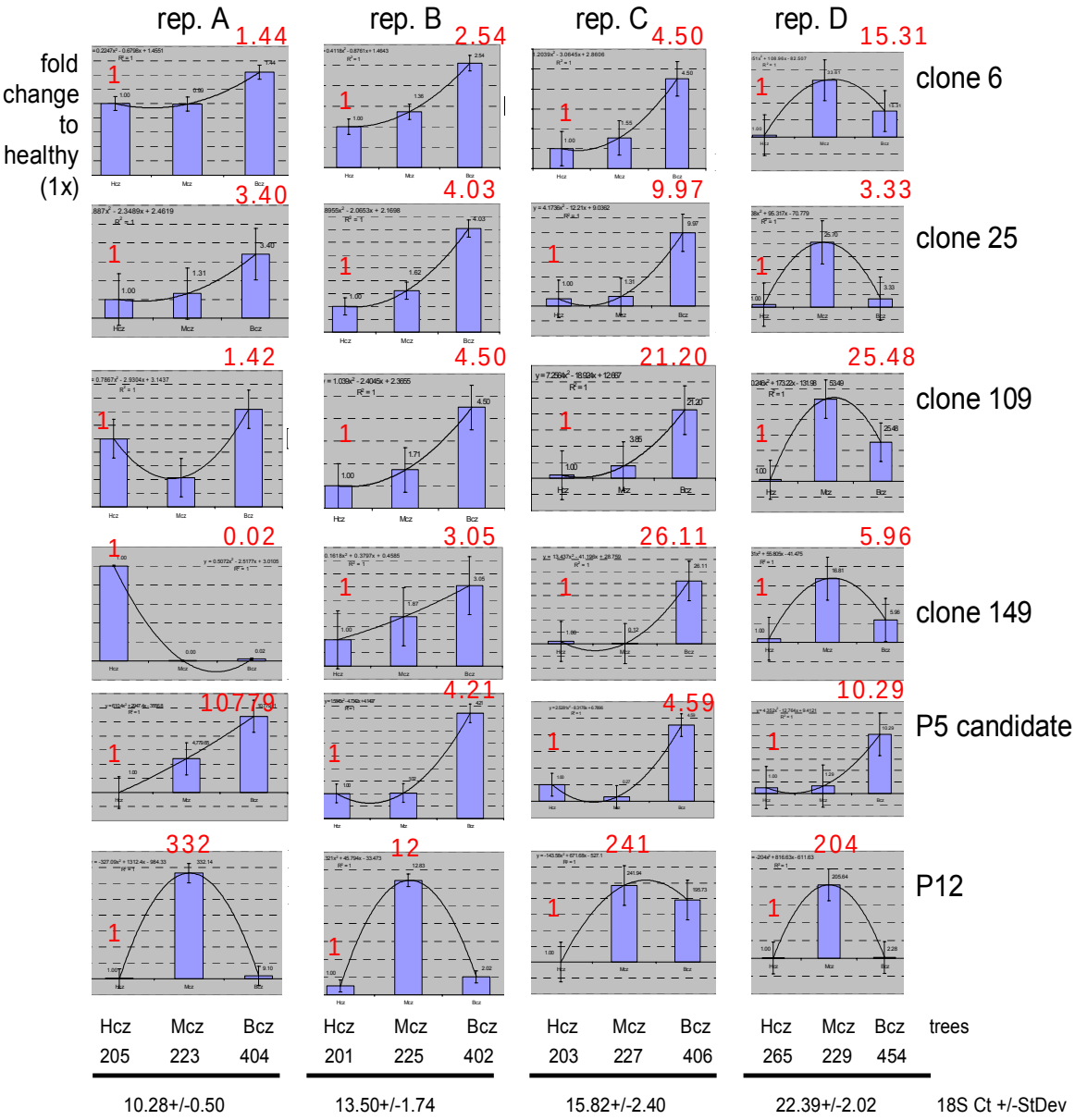


Figure 5-4. Relative transcriptional levels of the selected genes. Twelve individual plants were analyzed in four groups (rep. A, B, C and D), based on similar Ct values for the 18S readings. Feeder root samples of healthy (H), mildly affected (M) and fully blighted (B) trees on Carrizo (cz) rootstock were numbered (201 to 454) and used in this experiment. The columns represent the relative transcriptional level of each clone, comparing to healthy samples. The bars represent the residual standard deviation estimated for each group of plants.

The clone 109 was up-regulated in three out of the four replicates, comparing the healthy to any of the two blighted conditions. The pattern was similar in the groups B and C. The mildly affected plant of the fourth replicate (group D) displayed even higher transcriptional level than the fully blighted tree. In addition, this pattern of the second and third replicates (groups B and C of plants) may represent only an initial or partial outcome for a latter blight development that has yet to come, and could reduce the transcripts of the gene represented by the clone 109 to a similar level observed in the group D of plants. That seems possible because citrus blight can not be precisely quantified so far, and therefore, the progression of the disease can not be completely pursued either. The first replicate (group A of plants) displayed a non expected result. The relative transcriptional level of the mildly affected plants was reduced to a range of 40% comparing to the healthy trees. To test whether or not that was an artifact of the method, a new RT and PCR reactions were run with the same RNA samples. No further discrimination was observed, however the residual standard deviation was higher. The mildly affected tree still had around 40% of the transcripts compared to the healthy sample while the blighted reached 1.4 the healthy sample. It is possible that this result may represent noise caused by blight itself, or by another factor not studied in the system. New samples from the field were not collected and tested because that would add a contribution of the season of the year and temperature to the present model. In addition, the sampled plants are probably in different stages of blight at this point, because all studied samples were collected and processed six months ago, from January 9th to the 15th of this year.

Considering that the clone 109 was up-regulated in three out of four replicates; that a similar pattern was observed; and that the level of induction compared to healthy plants was above twenty fold in the groups C and D of plants; the clone 109 was considered to represent a gene that is upregulated under citrus blight conditions, confirming the expectation from the cDNA array experiment.

The clone 149 was variable but with no defined pattern considering the outcome of the four groups of plants. On the cDNA array, the transcriptional level of the gene represented by this clone was nearly unchanged by citrus blight. However, since the real time PCR is more sensitive, discrepancies towards either way, being up or down-regulated according to individual organisms, could be unfolded because in the cDNA arrays the data tends to be compressed. That was apparently the case, and the clone 149 does seem variable, but according to other factor not studied.

The P5 candidate gene was responsive to citrus blight with a similar pattern in all four replicates. It was more abundant in fully blighted trees than in any other studied condition. Apparently the gene represented by the P5 candidate displays an ascendant transcriptional response towards the fully blighted tree. This result may not parallel the initial complexation of zinc seen in leaves and trunks of mildly affected trees (Albrigo and Young, 1980). Therefore, the chitinase gene represented by the P5 candidate is considered to be upregulated under citrus blight condition, confirming the similar expectation observed with the clone 38 in the cDNA array experiment; however, it remains to be investigated whether or not this candidate gene is the true P5 identified by Taylor et al. (1996). It is worthy to note that the behavior of the true P5 is not known in feeder root tissues, as used in this experiment.

The P12 displayed a significantly higher transcriptional level on mildly affected plants compared to healthy or fully blighted trees. The pattern was similar in all four groups of tested plants. It is possible that if more stages of blight were available, a wider range of conditions would be better evaluated, and maybe a different polynomial equation would better describe the phenomenon. But the pattern for a higher transcriptional level in mildly affected trees was clear. The lower amount of P12 transcripts in fully blighted trees may account for the previous failure in detecting P12 in the subtracted libraries and also in the cDNA array experiment. Both procedures employed samples from fully symptomatic trees.

Although patterns were observed, the intensity of the responses was related to the group of studied plants. Therefore, a general, or averaged, transcriptional level for each gene was not estimated, because it may not meaningful.

The Potential Biological Meaning of the Clone 109

Regardless of what causes citrus blight, affected plants visually go to a declining condition during the whole process. A closer look at the clone 109 reveals sequence homology to a citrus EST annotated as a cold acclimated responsive gene from the Washington Navel sweet orange (*Citrus sinensis* L. Osbeck cv. Bahia), in experiments done in California (Close et al., 2003; in press; assession number CB293790.1), shown in Figure 4-10 (Chapter 4), with an e-value of 9e-44.

It is known that plants respond to cold and to drought periods activating and repressing responsive genes. Seki et al. (2001) found 5 drought specific inducible genes, 2 cold specific inducible genes and 16 drought and cold inducible genes using a microarray with around 1,300 full length cDNAs of *Arabidopsis thaliana*. Citrus blight is a xylem blockage problem, which ultimately may lead to water deficiency, and maybe,

that gene represented by the clone 109 is involved in this process. In addition, the clone 109 displayed mostly higher transcriptional levels under citrus blight condition, using samples from different seasons of the year.

An alternative hypothesis could claim specific response to the citrus blight process, rather than being induced only as an indirect effect of the internal water stress caused by citrus blight. The clone 109 also had potential similarities to an ubiquitin subunit (Table 4-2, chapter 4) of the SCF complex (Skp1-Cullin-F-box protein). This complex is involved in the ubiquitination of proteins and cell cycle regulation. The citrus blight process can be seen as an accelerator of senescence. Young affected trees, of 5 to 10 years old, start to display an overall decline and lack of vigor, normally only seen on healthy trees older than 50 years. The clone 109 could function in the ubiquitination process, targeting other proteins for degradation, and or, impairing the normal cell cycle in affected trees.

Experimental confirmation is certainly needed either way regarding its function and involved pathways. But in the first scenario, if cold acclimation is a feasible goal in plant genetic engineering programs, maybe ‘hardening’ rootstocks for citrus blight could be as well. In the second scenario, experimental effort on the cell cycle regulatory process in citrus can eventually offer perspectives to control citrus blight.

A Tentative Test to Verify the Effect of Cold and Drought Stresses

In order to evaluate whether or not the gene represented by the clone 109 responds to cold and drought stresses, another experiment was assembled and run. Other clones were also included. The major objective was to compare the transcriptional levels of the selected genes, and determine whether they seem to be a specific response to citrus blight, or only a secondary effect of the stress caused by the disease. However, moving

adult citrus plants to controlled conditions was not attempted. Greenhoused young trees, of about one year old were used instead. They were exposed to cold (4^{o}C for 52 hours) and drought stresses (no water for one week plus additional root airing for 24 hours). Seedlings of Carrizo citrange (*Citrus sinensis* L.. Osb. *x Poncirus trifoliata* L. Raf.) were chosen to equal the genotype evaluated in the previous real time PCR experiment. The references, or control plants, were from the same lot of seedlings, but kept under normal greenhouse conditions for the same period of time. Four independent replicates (i.e. different seedling plants) were evaluated for each treatment. Feeder root tissues were once again used. Figure 5-5 shows the results. The contrast of interest, to compare the control, cold and drought treatments, is not orthogonal, and therefore, the t-Test can not be used. However, the number of replicates was uniform, allowing the application of a test to compare the means. Among the options, the Tuckey test was employed because of the better discrimination of the means compared to other tests, like Sheffeé, Dunnett or Duncan (Banzatto and Kronka, 1992). The null hypothesis was no difference among the treatments.

The clone 109 did not respond to cold and drought stresses under the studied conditions. No significant effect ($p<0.05$) was seen for most of the other clones either, including P12. The drought treatment only affected the gene represented by the clone 25. Its transcriptional level was reduced to around one-third compared to the control. This level of significance ($p<0.05$) implies that the maximum estimated chance of having similar results caused only by chance is of only 5%. Lowering this probability to 1% ($p<0.01$) makes this difference became not significant. In spite of which level of probability should be considered, this reduction of the transcriptional level was not

expected. Blight induces xylem blockage; and consequently, a potential internal water stress. Since the clone 25 was considered to be up-regulated in the cDNA array and in the real time PCR experiment, it would also be expected to be unchanged or up-regulated

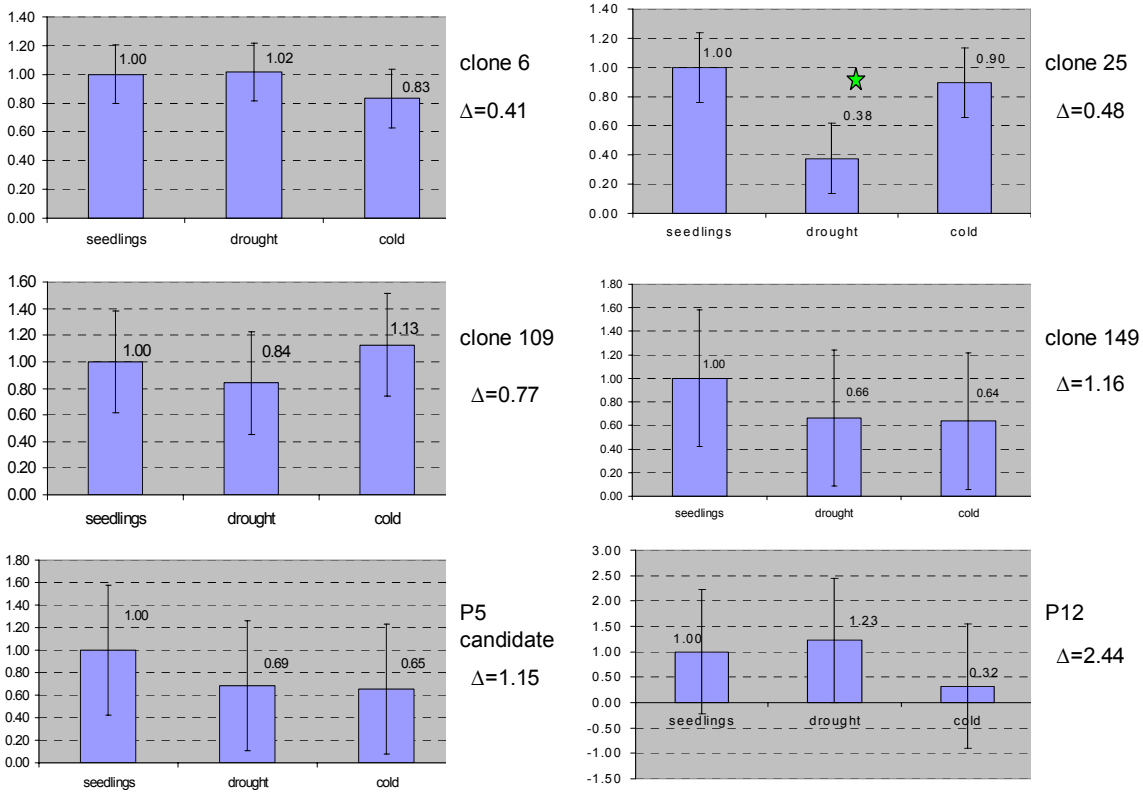


Figure 5-5. Contrasts comparing the effect of cold and drought treatments on the relative transcriptional level of the clone 109 and other clones. The Tuckey test was employed and an observed significant difference was highlighted with a star. The bars represent the minimum significant difference for the contrast (Δ values, for $p<0.05$) of each clone.

under drought stress. Another experiment using adult trees under controlled conditions may address this question. Similar reasoning may be considered for the other genes as well. A definitive answer about the effect of major stresses would need adult trees under

controlled conditions. Therefore, the responsive genes to citrus blight, and apparently not affected by drought and cold stresses, seem to be at this point, only the clone 109, the P5 candidate and the P12.

The Potential Effect of Redundancy and Gene Families

The selected clone 38 represents a citrus chitinase gene. Two genes (chitinase class I, accession AB081944.1; and chitinase class II , accession Z70032.1) were matched to the 235 bases of the clone 38 with the same e-value of 1e-87. The homology was seen towards the 3'end of the subjected sequences (Figure 4-8, chapter 4). Chitinases are enzymes that catalyze hydrolysis of chitin polymers, acting against plant intruders by destroying chitin-containing cell walls. The difference between the class I and the class II is based in the presence (I) or absence (II) of a N-terminal chitin binding domain (source: http://us.expasy.org/cgi-bin/prosite-search-ac?pdoc00620, last accessed June 1, 2004). Therefore, the clone 38 arrayed in the cDNA array membranes (chapter 4) could represent any of the two described genes.

The 22 aminoacids of the P5 protein (accession AAB46813) had 80% identity to the same chitinase class I, accession AB081944.1, with an e-value of 7e-05. The matched region was however in the conserved 5' end of the subjected sequence. The Taqman probe and primers were designed to span this region, covering 88 nucleotides long. The Figure 5-1 displays the homolog regions between the sequences of the reference chitinase, clone 38 and P5 candidate.

Therefore, it is not possible to discern precisely if the clone 38 and the P5 candidate represent the same gene, or not, within the chitinase family. In addition, the sequence for citrus is limited, but searching the genome of the model plant *Arabidopsis thaliana*, the reference chitinase class I, accession AB081944.1, had 15 matches in the protein

databank (source: TAIR, http://www.arabidopsis.org/, last accessed June 1, 2004), with e-values varying from 3e-7 to 4e-95. It is possible that this situation is similar for citrus, with a high number of redundant genes, imposing another level of difficulty in the evaluation of transcriptomes. The situation can be similar for P12, which had 17 matches in the protein database of TAIR, all expansins, with e-values from 2e-04 to 5e-30. There are two gama expansins with noted similarity to P12. Therefore, it is possible that more copies of homolog P12 genes are also present in citrus plants.

CHAPTER 6
IDENTIFICATION OF AN ETIOLOGICAL CONTRAST POTENTIALLY ASSOCIATED WITH THE CITRUS BLIGHT DISEASE

A crucial question in citrus blight research is the origin of the problem. Several causal agent candidates and other theories have been examined, but none has proved to be definitive for blight.

Each reported theory has been based on observed characteristics of the disease; however blight is a complex problem, and different views and perspectives are possible under different circumstances. For instances, the non-transmissibility of citrus blight by canopy tissues was observed long ago (Rhoads, 1936; and COHEN, 1968), but transmission by root grafting was later obtained (Tucker et al., 1984). Soils with higher pH and levels of Ca were associated with severe incidences of blight in Florida (Wutscher, 1989); however, blight does occur in acidic soils and examples are common, especially in the São Paulo citrus belt. Soil born pathogens were associated to blight (Nemec et al., 1982); but transmission of blight by soil replacement was not obtained (Timmer and Graham, 1992). *Xylella fastidiosa* was considered to be the causal agent of blight (Hopkins, 1988); but this xylem limited bacterium is naturally present in many vascular plants, including citrus. A higher incidence of blight was observed after the implementation of the nucellar programs in Florida and in São Paulo (Derrick and Timmer, 2000), probably reflecting the new endophytic balance in the nucellar plants but blight has also a high incidence on certain old line trees, and examples on ‘Olímpia’, and on other clones of the ‘Pera’ sweet orange that were not pre-immunized against citrus

tristeza virus (CTV) are commons in São Paulo. The theory about a molecular origin for blight suggested the involvement of defective signals transmitted from plant to plant (Carlos et al,2000), but no experimental evidence for that have been found. Strains of citrus tristeza virus (CTV) were reported to be associated with a potential variant of blight (named Rangpur lime decline) and to citrus sudden death diseases in Brazil (Derrick et al., 2003), but CTV is commonly present in stem-pitted sweet orange plants in São Paulo causing no major disturbances (Costa et al., 1954).

In spite of the merit and investigations of each theory, one characteristic initially proposed by Swingle and Webber (1896) seems difficult still to be denied: citrus blight apparently has an infectious nature and dissemination. In order to investigate a potential causal agent for citrus blight, qualitative and quantitative experiments were performed.

The First Screening of the Subtracted Libraries

When the subtracted libraries were made (chapter 3), during the summer of 2001, the following question regarding which genes were represented there was first addressed using a virtual northern blot experiment.

The differentially enriched cDNAs from the blighted minus healthy (B - H) library were cloned and grown in the *E. coli* vector and plated on LB medium. Ninety six clones were randomly selected for re-growing on two equally printed nylon membranes. After growth, the membranes were rinsed with extensive washes and hybridized overnight with P32 labeled probes. The probes were also made from the subtracted blighted (B – H) and healthy (H – B) enriched cDNA libraries, uncovering tentatively more abundant transcripts represented there. A B – H clone when revealed by the B – H probes and not by the H – B probes implied a larger presence of the transcript of that gene in the blighted samples that originated the library, from the affected trees. To the contrary, a B - H clone

when revealed by both types of probes (B – H and H – B) indicated similar amounts of the transcript in both libraries, and therefore, were of no interest. Virtual northern blots can be effective to reveal abundant transcripts in enriched libraries (Source: http://www.bdbiosciences.com/clontech/, last accessed January 15, 2004), which may include those of RNA based viruses, such as the majority of the plant viruses.

Figure 6-1 displays the results for the virtual northern blot experiment. Among others, the arrows indicate that the clones E8-13 and E8-14 were far more abundant in the B - H library.

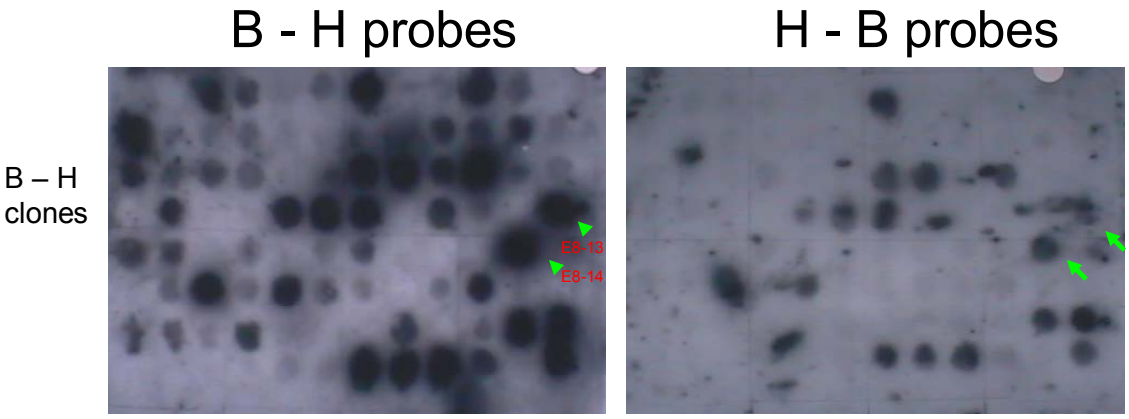


Figure 6-1. The virtual northern blot of the B - H clones. Each clone was printed in two membranes that were respectively probed with B - H and H - B probes labeled with P32. The subtracted libraries were made using the superficial roots of Rough lemon (*Citrus jambhiri* Lush) collected in the central Florida area, during the summer of 2001. The green arrows indicate the clones E8-13 and E8-14.

Sixteen clones were considered to be differentially transcribed in the B - H library and were selected for sequencing. Among the plant genes, possible up regulated ones included clones with similarities to metallothionein and several unknowns. However,

based on sequence homology, the clones E8-13 and E8-14 were not plant genes, but part of the 3'end of the P27 divergent citrus tristeza virus (CTV) coat protein gene. The clone E8-13 was 311 base pairs long and the E8-14 was revealed to be only a shorter and redundant version of the same gene, with 155 base pairs. Table 6-1 displays the first four nucleotide based homolog sequences found in all databases of Genbank for clone E8-13, using the Blast-N search analysis (Altschul et al., 1997). Similar results were found using the translated query searches.

Table 6-1. The clone E8-13 had homolog sequences matching different isolates of the citrus tristeza virus (CTV). The clone E8-13 is 311 base pair long and was found in the citrus blighted minus healthy (B-H) subtracted library made from superficial roots of Rough lemon (*Citrus jambhiri* Lush).

clone	Homolog gene	e-values
E8-13	>gi|1732493|gb|U56902.1|CTU56902 Citrus tristeza virus p346, 54-kDa RNA dependent RNA polymerase, p33, p6, p65, p61, p27, 25-kDa coat protein (CPG), p18, p13, p20, and p23 genes, complete cds	e-162
	>gi|11414863|dbj|AB046398.1| Citrus tristeza virus genomic RNA, complete genome, seedling yellows strain	e-147
	>gi|2098825|gb|AF001623.1|CTAF001623 Citrus tristeza virus, complete genome	e-136
	>gi|3550999|dbj|AB011189.1| Citrus tristeza virus genomic RNA for 27K protein and coat protein, partial cds, isolate KS3A2	2e-87

Looking at the homolog sequences, the first match, accession number U56902, was isolated in Israel and represents the coat protein gene of CTV (Mawassi et al., 1993). The second, accession AB046398, was a seedling yellows strain from Japan (Suastika et al.,

unpublished). The third, accession AF001623, was isolated in Texas causing severe symptoms in sweet orange (Yang et al., 1999). The fourth, accession AB011189 is the P27 gene also isolated from strains of Japan (Kano et al., unpublished).

Sequence information is limited for citrus, but to verify other potential origins for the clone E8-13, other databases were searched. Homolog sequences were not found in different plant databases. The first matched outcome in the blast searches (Altschul et al., 1997) using the non-mouse and non-human ESTs and the *viridiplantae* databases of the Genebank yielded respectively matched sequences with e-values of only 5.7 and 1.1. Using the Brazilian citrus EST database, the result was not different, with an e-value of only 0.18 for the first outcome. Therefore, the clone E8-13 was considered to be part of the P27 gene of the CTV genome. The highest matched sequence indicated similarities to an Israeli isolate.

In addition, both clones, E8-13 and E8-14, displayed polyadenylated 3'ends in their sequences. The question whether that was an artifact of the method used to build the cDNA libraries (Smart cDNA kit, source: http://www.bdbiosciences.com/clontech/, last accessed June 20, 2001), or an adaptation of the CTV to the molecular machine of citrus remains to be investigated.

The presence of CTV in citrus plants is not novel, and the solution for the Tristeza disease in sweet orange groves was reported long ago (Costa et al., 1954). However, the presence of CTV strains in roots of citrus plants affected by blight is intriguing. This result was similar to previous observations made by Derrick et al.(unpublished). It is also necessary to review that Rough lemon is susceptible to CTV and those findings alone may not add novelty to what is already known about CTV and citrus host interactions.

Other CTV Genes were also Found in both Subtracted Libraries

Later, around four hundred clones randomly selected from the blighted minus healthy (B-H) and 100 from the healthy minus blighted (H – B) enriched libraries were sequenced. Clones with sequence similarities to other CTV genes were relatively common, matching different isolates and strains. Redundancy was observed, and normally, more than one clone matched the same analyzed gene. The Table 6-2 displays examples of pieces of CTV genes found in both libraries.

Table 6-2. Other sequences with homology to CTV genes found in the blighted minus healthy (B-H) and healthy minus blighted (H-B) subtracted libraries.

library	CTV gene	e-value	clone
B – H	p23	e-153	M1H7
	p346RDRPol	6e-70	M2E2
	p33	3e-43	M3A1
H – B	p61	e-131	E8-28
	hsp90 p61	e-158	E8-21
	p65	0.000	E8-22

Quantitative Evaluation of the P27 Candidate Gene in the Blighted Trees

In order to evaluate the presence of the transcripts of the P27 candidate gene, reverse transcriptase quantitative real time PCR was run.

The chosen Taqman probe employed the ‘Fam6-5’sequence 3’-Tamra’ architecture and had an accepted PCR efficiency (Chapter 5). The absolute value for the slope of the linear function (y= -0.1008x +22.13), that described the log input amount of the total RNA (x) against the ΔCt (y) between the target and the normalizer 18S, was 0.1008. The tested range was a 2 fold dilution series from 3.9 to 1000 ng of total RNA. The

comparative ΔCt method was used to avoid the need of *in vitro* synthesis and purification of the P27 RNA for absolute quantifications. For calculation purposes, reactions that did not display amplifications were considered having a Ct value of 45 cycles. To minimize non-specific variations, two reactions per sample were run. The same healthy, mildly affected and fully blighted plants that were used to evaluate other genes (Chapter 5) were used here. They were Valencia sweet orange (*Citrus sinensis* L.. Osbeck cv. Valencia) on Carrizo citrange (*Citrus sinensis* L. Osb. *x Poncirus trifoliata* L.. Raf.) of around 10 years and from the central Florida area. Four groups of plants, or replicates, were evaluated.

The transcripts of the P27 candidate gene were present in far larger amounts in affected than in healthy trees (Figure 6-2). The fully blighted trees on the group B of plants still have around eight times more P27 transcripts than the healthy plants.

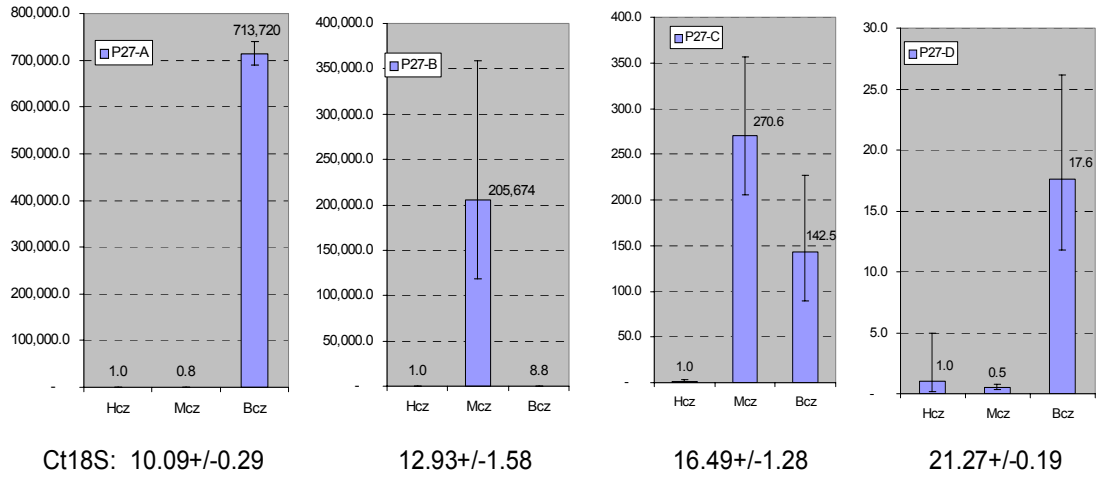


Figure 6-2. The transcripts of the p27 candidate gene were abundant in the roots of the affected trees, Carrizo citrange (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.). The Ct levels for the 18S were displayed below the graphics; columns of the blighted (B) and mildly affected (M) were compared to healthy (H) Carrizo (cz) trees.

It is worthy to note that *Poncirus trifoliata* and its hybrids are considered to be resistant to CTV (Deng et al., 2001).

The overall comparative amounts of the P27 transcripts indicated that CTV is present in feeder roots of Carrizo citrange affected by citrus blight. Further experiments can elucidate whether or not CTV causes or enhances citrus blight, or only grows better in roots of already debilitated plants. Eventual synergistic or antagonistic effect on the real causal agent of blight may also be investigated. Another possibility would be to consider the clone E8-13 as part of another virus, with sequence similarities to CTV. Another closterovirus would probably be the immediate suspect.

CHAPTER 7
CONCLUSIONS

Citrus blight has imposed consistent losses and changes to the citrus industry since its origin, in the late of the XIX century (Swingle and Webber, 1896). The molecular mechanisms involved in the plant responses are still unknown at this point.

Evidences for differentially transcribed genes, under different citrus blight incidences, were observed in this study. Three genes, represented by the clones studied in this work, had higher transcriptional level in blighted than in healthy trees. The level of response was dependent of the evaluated group of plants but patterns were observed. This study employed transcriptional assessment of citrus genes in feeder roots of healthy, mildly affected and fully blighted trees. Under these studied conditions, P12 had higher transcriptional level in mildly than in fully blighted trees. The chitinase(s) represented by the P5 candidate sequence and by the clone 38 had higher levels in fully blighted than in mildly or healthy trees. The clones 25 and 109 showed higher levels in fully blighted compared to healthy samples. Research is needed to reveal the function of the P12 gene and the genes represented by the other clones. Cloning of the true P5 gene and the genes represented by the other clones can also be another task for future efforts.

It is also possible that more clones from the cDNA array experiment are truly differentially transcribed under non and blighted plants, but more confirmation is needed. In addition, the created subtracted libraries can be a wealthy source of clones for further experiments. The suppressive subtraction method was an effective way to create enriched cDNA libraries.

The finding of transcripts of CTV genes in roots of a blight-susceptible-CTV-resistant rootstock immediately raises further etiological questions. Under this context, CTV can intuitively be associated with citrus blight. However, whether or not it has a synergistic or antagonistic effect on blight, it is not known yet. Another potential consequence of this finding is regarded to options for tolerant rootstocks to be used not only for blight but also against citrus tristeza disease. Carrizo citrange may fall in discredit, contributing to the already known need of research on citrus rootstocks.

Finally, it is possible to say that the end is certainly not near for blight. Research in roots seems the logical alternative to study the problem. In this way, it is hoped that the knowledge gained during this study can be of some use, helping to understand and control citrus blight.

APPENDIX
BLAST ANALYSIS BASED ON NUCLEOTIDE SEQUENCES OF EACH INDIVIDUAL CLONE

Table A-1. Blast analysis based on nucleotide sequences of each individual clone. The clones were obtained from the subtracted libraries (chapter 3) and used in the cDNA array experiment (chapter 4). Three databases were used for the search and the outcomes were highlighted in black (for the All Genebank with 1,367,736 sequences), blue (for the Brazilian citrus CCSM ESTs with 13,610 clusterized sequences) and green (for ESTs of the non-human non-mouse Genebank with 4,893,238 sequences). The clones 7 to 12 were obtained from other libraries of citrus leaf tissues, and all the others were from the subtracted libraries of root tissues, as described in chapter 3.

Clone	Highest match description/score/e-value	e-values	Organism	Function
5	>gi|806738|gb|U16304.1|CTU16304 Citrus tristeza virus complete genome	e-132	CTV	p61
6	>gi|1785673|emb|Y08501.1|MIATGENA A.thaliana mitochondrial genome, part A	0	Arabidopsis	mitochondrial
	Contig204	7e-11	Citrus	
7	>gi|167366|gb|L08199.1|COTPROXDS Gossypium hirsutum peroxidase mRNA, complete cds	6E-68	Gossypium	peroxidase
	Contig373	e-173	Citrus	
	1 - gb|BQ624415.1|BQ624415 USDA-FP_01506 Ridge pineapple sweet orange	e-171	Citrus	
8	>gi|6469118|emb|AJ275306.1|CAR275306 Cicer arietinum partial mRNA for mitochondrial phosphate	4E-26	Cicer	mitochondrial
	CSJE01-038D08.g	6e-88	Citrus	
	1 - gb|BQ623911.1|BQ623911 USDA-FP_00991 Ridge pineapple sweet orange	6e-83	Citrus	
9	>gi|18401634|ref|NM_112657.1| Arabidopsis thaliana chromosome 3 CHR3v07142002 genomic sequence	6E-43	Arabidopsis	unknown

	Contig1602	e-153	Citrus	
	1 - dbj|C22180.1|C22180 C22180 Miyagawa-wase satsuma mandarin orange	4e-84	Citrus	
10	cxContig94	e-146	Citrus	
	1 - gb|BQ623982.1|BQ623982 USDA-FP_01073 Ridge pineapple sweet orange	e-145	Citrus	
11	CSJE01-038D08.g	2e-94	Citrus	
	1 - gb|BQ623911.1|BQ623911 USDA-FP_00991 Ridge pineapple sweet orange	2e-84	Citrus	
12	Contig2919	8e-27	Citrus	
	1 - gb|BQ623455.1|BQ623455 USDA-FP_00546 Ridge pineapple sweet orange	3e-65	Citrus	
13	Poor matches			unknown
14	Poor matches			unknown
15	CSContig70	2e-20	Citrus	
16	cxAC01-022F03.g 52 7e-08	7e-08	Citrus	
	1 - dbj|C22172.1|C22172 C22172 Miyagawa-wase satsuma mandarin orange...	1e-13	Citrus	
17	Poor matches			unknown
18	Poor matches			unknown
19	Poor matches			unknown
20	Contig1687	7e-64	Citrus	
	1 - gb|BQ625123.1|BQ625123 USDA-FP_02214 Ridge pineapple sweet orange	9e-66	Citrus	
21	1 - gb|BQ624910.1|BQ624910 USDA-FP_02001 Ridge pineapple sweet orange	e-135	Citrus	
22	cxContig1377	6e-71	Citrus	

23	Poor matches			unknown
24	Poor matches			unknown
25	Contig416	1e-91	Citrus	
26	Poor matches			unknown
27	Poor matches			unknown
28	LPAC00-013G11.g	2e-06	Citrus	
29	Poor matches			unknown
30	Poor matches			unknown
31	Contig416	e-101	Citrus	
32	Poor matches		Human	unknown
33	Contig2752	1e-10	Citrus	
34	>gi|7228328|emb|Y18788.1|MSY18788 Medicago sativa mRNA for putative TFIIIA (or kruppel)-like zinc finger protein	2E-14	Medicago	Zn finger
	cxContig1077	6e-07	Citrus	
35	Contig2879	0.0	Citrus	
36	Contig2919	9e-65	Citrus	
37	Contig2879	3e-53	Citrus	
38	Contig1434	3e-95	Citrus	
39	Poor matches			unknown
40	Poor matches			unknown
41	Poor matches			unknown
42	>gi|1220143|emb|Z70032.1|CSACHIT2 C.sinensis mRNA for class II acidic chitinase	6E-79	Citrus	chitinase
	Contig1512	1e-81	Citrus	

43	>gi|1220143|emb|Z70032.1|CSACHIT2 C.sinensis mRNA for class II acidic chitinase	8E-40	Citrus	chitinase
	Contig1434	1e-43	Citrus	
44	Contig2879	2e-62	Citrus	
45	FCContig127	5e-56	Citrus	
46	Contig2919	2e-34	Citrus	
47	Contig2879	5e-63	Citrus	
48	Contig2879	6e-50	Citrus	
49	Poor matches			unknown
50	>gi|599725|emb|Z46824.1|CSLEA5PMB C.sinensis mRNA for Lea5 protein	2E-90	Citrus	LEA
	Contig3802	4e-86	Citrus	
51	No significant similarity found			unknown
52	Contig2879	e-100	Citrus	
53	Contig2919	2e-53	Citrus	
54	>gi|5917784|gb|AF184068.1|AF184068 Citrus limon vacuolar membrane ATPase subunit G (LVMA10) mRNA,	2E-28	Citrus	ATPase
	Contig2879	e-107	Citrus	
55	Contig308	e-121	Citrus	
56	Contig2879	e-130	Citrus	
57	>gi|20809305|gb|BC029618.1| Homo sapiens, glyceraldehyde-3-phosphate dehydrogenase, clone	e-128	Human	G3PDH
	Contig80	1e-04	Citrus	

58	>gi|5815312|gb|AF176034.1|AF176034 Coliphage phiX174 isolate Anc, complete genome	e-171	Coliphage	unknown
59	cxJE01-111G08.g	0.0	Citrus	
60	CXAC02-065D12.g	1e-42	Citrus	
61	cxJE01-085A10.g	0.0	Citrus	
62	>gi|21206806|gb|AY103728.1| Zea mays PCO142212 mRNA sequence	5E-19	Zea	unknown
	Contig1287	e-162	Citrus	
63	>gi|6653735|gb|AF209908.1|AF209908 Prunus dulcis unknown mRNA	3E-07	Prunus	unknown
	Contig16	e-110	Citrus	
64	>gi|1732493|gb|U56902.1|CTU56902 Citrus tristeza virus p346, 54-kDa RNA dependent RNA polymerase, p33, p6, p65, p61, p27, 25-kDa coat protein (CPG), p18, p13,p20, and p23 genes, complete cds, p20, and p23 genes, complete cds	e-162	CTV	p27
65	>gi|3308979|dbj|AB008100.1| Citrus unshiu mRNA for metallothionein-like protein, complete cds	e-138	Citrus	metallothionein
	Contig3121	e-142	Citrus	
66	Poor matches			unknown
67	>gi|806738|gb|U16304.1|CTU16304 Citrus tristeza virus complete genome	e-130	CTV	p25/p27
68	>gi|806738|gb|U16304.1|CTU16304 Citrus tristeza virus complete genome	e-160	CTV	hsp90 p61
69	>gi|4239714|emb|Y18420.1|CITV18420 Citrus tristeza virus complete genome, isolate T385	0	CTV	p65
70	>gi|806738|gb|U16304.1|CTU16304 Citrus tristeza virus complete genome	e-127	CTV	p65

75	Poor matches			unknown
77	cxContig629	8e-90	Citrus	
	1 - gb|BQ623985.1|BQ623985 USDA-FP_01076 Ridge pineapple sweet orange	3e-96	Citrus	
78	Poor matches			unknown
79	Poor matches			unknown
80	Poor matches			unknown
81	Poor matches			unknown
82	Poor matches			unknown
83	Poor matches			unknown
84	Poor matches			unknown
85	Poor matches			unknown
86	Poor matches			unknown
87	Poor matches			unknown
88	Poor matches			unknown
89	Contig2919	1e-31	Citrus	
90	Poor matches			unknown
91	Poor matches			unknown

92	Poor matches			unknown
93	Poor matches			unknown
94	Contig2919 1 - gb|BQ623148.1|BQ623148 USDA-FP_00239 Ridge pineapple sweet orange	2e-46 1e-77	Citrus Citrus	
95	>gi|6728952|gb|AC020576.2|T12C22 Sequence of BAC T12C22 from Arabidopsis thaliana chromosome 1, complete	1E-08	Arabidopsis	unknown
96	Poor matches			unknown
97	Poor matches			unknown
98	Poor matches			unknown
99	>gi|9087297|dbj|AP000397.1|AP000396S2 Beta vulgaris mitochondrial genomic DNA, complete sequence, section 2/2	e-125	Beta	unknown
100	>gi|9087297|dbj|AP000397.1|AP000396S2 Beta vulgaris mitochondrial genomic DNA, complete sequence, section 2/2	e-114	Beta	mitochondrial
101	Poor matches			unknown
102	Poor matches			unknown
103	Poor matches			unknown
104	Poor matches			unknown
105	Poor matches			unknown
106	Poor matches			unknown
107	Poor matches			unknown

108	Poor matches			unknown
109	>gi|28619247|gb|CB293790.1|CB293790 UCRCS01_06cg09_g1 Washington Navel orange cold acclimated flavedo & albedo cDNA library Citrus sinensis cDNA clone...8e-44	8e-44	Citrus	
110	Poor matches			unknown
111	Poor matches			unknown
112	Poor matches			unknown
113	Poor matches			unknown
114	Poor matches			unknown
115	1 - dbj|C95562.1|C95562 C95562 Citrus unshiu Miyagawa-wase maturatio... 331 2e-88	2e-88	Citrus	
117	Poor matches			unknown
118	Poor matches			unknown
119	Poor matches			unknown
120	Poor matches			unknown
121	Poor matches			unknown
122	Poor matches			unknown
123	Poor matches			unknown
124	Poor matches			unknown

125	Poor matches			unknown
131	Poor matches			unknown
132	Poor matches			unknown
134	Poor matches			unknown
135	Poor matches			unknown
136	FAAC01-035B05.g	1e-30	Citrus	
137	>gi|12249|emb|X03775.1|CHSOATP1 Spinach plastid genes atpI-H-F for ATP synthase CF(O) subunits IV,	6E-86	Spinach	ATP synthase
138	Poor matches			unknown
139	Poor matches			unknown
140	1 - gb|BQ623784.1|BQ623784 USDA-FP_00875 Ridge pineapple sweet orang... 220 6e-55	6e-55	Citrus	
141	>gi|6693795|gb|AF112970.1|AF112970 Daucus carota strain Imperator STS3A mitochondrial DNA segment 1 - gb|BE460852.1|BE460852 EST412271 tomato breaker fruit, TIGR Lyco...	3E-74 5e-68	Daucus Tomato	mitochondrial
142	1-gb|BM371429.2|BM371429 EBma08_SQ002_L04_R maternal, 28 DPA, no t...	9e-11		unknown
143	Poor matches			unknown
144	Poor matches			unknown
145	Poor matches			unknown

146	>gi|18857892|dbj|AB061306.1| Citrus jambhiri mitochondrial ACRS gene for ACR toxin-sensitivity	4E-48	Citrus	ACR toxin-sen.
	Contig4126	3e-16	Citrus	
	1 - gb|BM358396.1|BM358396 GA__Ea0008M10r Gossypium arboreum 7-10 dp... 351 2e-94	2e-94	Gossypium	
147	Poor matches			unknown
148	>gi|5688942|dbj|AB017426.1| Oryza sativa (japonica cultivar-group) mitochondrial gene for ribosomal protein L5, complete cds	2E-59	Oryza	mitochondrial
	1 - dbj|AV420567.1|AV420567 AV420567 Lotus japonicus young plants (t... 315 7e-84	7e-84	Lotus	
149	CXJE02-097F04.g	2e-54	Citrus	
150	CXJM02-089E08.g	2e-19	Citrus	
151	1 - gb|BM371381.2|BM371381 EBma08_SQ002_I05_R maternal, 28 DPA, no t... 86 1e-14	1e-14		
152	CXContig831	3e-92	Citrus	
153	Poor matches			unknown
154	Poor matches			unknown
155	1 - gb|BI180911.1|BI180911 TY3H09 hepatocellular carcinoma expressio... 90 1e-15	1e-15		
156	Poor matches			unknown
157	1 - gb|BI180911.1|BI180911 TY3H09 hepatocellular carcinoma expressio...	3e-14		
158	1 - gb|BM376133.1|BM376133 EBma01_SQ002_H07_R maternal, 4 DPA, no tr...	4e-12		

159	1 - gb|BM371429.2|BM371429 EBma08_SQ002_L04_R maternal, 28 DPA, no t...	7e-08		
160	Poor matches			unknown
161	1 - gb|T44610.1|T44610 7873 Lambda-PRL2 Arabidopsis thaliana cDNA cl... 359 1e-96	1e-96		unknown
162	1 - gb|BM371429.2|BM371429 EBma08_SQ002_L04_R maternal, 28 DPA, no t... 84 9e-14	9e-14		
163	1 - gb|BM371429.2|BM371429 EBma08_SQ002_L04_R maternal, 28 DPA, no t... 72 2e-10	2e-10		
164	>gi|12830831|gb|AF320906.1|AF320906 Citrus unshiu metallothionein-like protein (MT45) gene, complete cds	3E-90	Citrus	metallothionein
	Contig2243	2e-92	Citrus	
	1 - gb|BQ624047.1|BQ624047 USDA-FP_01138 Ridge pineapple sweet orang...	1e-86	Citrus	
165	1 - gb|BQ623549.1|BQ623549 USDA-FP_00640 Ridge pineapple sweet orang... 80 3e-13	3e-13	Citrus	unknown
166	1 - gb|BQ624621.1|BQ624621 USDA-FP_01712 Ridge pineapple sweet orange	1e-96	Citrus	
167	Poor matches			unknown
168	1 - emb|AL729032.1|AL729032 AL729032 Danio rerio embryonic inner ear... 58 3e-06	3e-06		
169	1 - gb|BQ414420.1|BQ414420 GA__Ed0086E05r Gossypium arboreum 7-10 dp... 60	2e-07		
170	Poor matches			unknown

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BIOGRAPHICAL SKETCH

Eduardo Fermino Carlos was born on February 27, 1967, in Votuporanga, State of São Paulo, Brazil. He was actively exposed to routine farming life and discovered a great interest in agriculture and general science. He graduated in 1990 in agronomic engineering at the University of Londrina (UEL), and started to work as consultant for citrus and other fruit growers. He received a master degree in 1996 at the State University of São Paulo (UNESP) working with molecular techniques in citrus, and a plant breeding specialist degree at the University of Wageningen (IAC) in 1998. He worked as scientific researcher for Fundecitrus from 1995 to 1998 and for Sylvio Moreira Citrus Center in 1998, before receiving a scholarship from CNPq, a Brazilian federal agency, to accomplish doctoral training abroad. This manuscript details his work in the Plant Molecular and Cellular Biology program at the University of Florida, under the supervision of Dr. Gloria Moore and Dr. Kenneth Derrick.