BACTERIAL CITRUS CANKER: MOLECULAR ASPECTS OF A COMPATIBLE PLANT-MICROBE INTERACTION

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To Souad, Kamal, Aziz, Mouma, Mami, Nemat, and ma petite Shemsi
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# TABLE OF CONTENTS

| ACKNOWLEDGMENTS ................................................................. | iv |
| LIST OF TABLES .................................................................................. | viii |
| LIST OF FIGURES ............................................................................... | ix |
| ABSTRACT ......................................................................................... | xi |

## CHAPTER

1. **A 37 KB PLASMID FROM A SOUTH AMERICAN CITRUS CANKER STRAIN CARRIES A TYPE IV SECRETION SYSTEM ESSENTIAL FOR SELF-MOBILIZATION** .......................................................... 1

   Introduction................................................................................... 1

   Materials and Methods ..................................................................... 5

   Bacterial Strains, Plasmids and Culture Media .................................................. 5

   Marker Integration Mutagenesis............................................................... 5

   Plasmid Conjugal Transfer Techniques....................................................... 6

   Recombinant DNA Techniques ............................................................... 7

   Plant Inoculations ................................................................................ 7

   Results...................................................................................................... 7

   The Type IV Secretion System Found on pXcB is Required for Self-Mobilization .......................................................... 7

   Involvement of the TFSS of pXcB in Pathogenicity of Xca B69 ........................ 9

   Discussion.................................................................................................. 10

2. **IDENTIFICATION OF CITRUS GENES SPECIFICALLY RESPONSIVE TO PATHOGENICITY GENE pthB OF Xanthomonas citri pv. aurantifolii** ............................................ 23

   Introduction....................................................................................... 23

   Material and Methods ............................................................................. 26

   Plant and Microbial Material ................................................................. 26

   Bacterial Counts .................................................................................... 27

   Microscopy ............................................................................................. 27

   Differential Display-Reverse Transcriptase PCR ............................................ 28

   Suppressive Subtractive Hybridization (SSH) Library Construction .................. 28

   Northern Blots ...................................................................................... 29

   Reverse Northern Blots .......................................................................... 29
Statistical Analysis ........................................................................................................30
Results ...........................................................................................................................31
Macroscopic Disease Phenotype of Citrus Leaves Inoculated with X. c. aurantifolii B69 and Its Mutant Derivative BIM2 Lacking the Pathogenicity Gene pthB ..................................................................................................................31
PthB-Dependent Transcriptional Reprogramming Induced upon Infection with Xca ........................................................................................................................................32
Construction of Two Libraries Enriched in pthB Responsive cDNAs........33
Transcript Analyses of CCRs .....................................................................................33
Identity of cDNAs Identified as Up-Regulated by the Presence of pthB in X. citri Genome ........................................................................................................34
Identity of cDNAs Identified as Up-Regulated by X citri Lacking pthB ............35
Northern Blot Analysis of Representative CCRs .....................................................36
Microscopic Phenotype of B69 and BIM2 Inoculated Leaves .........................36
Discussion ....................................................................................................................38
PthB Induces Cell Division and Cell Expansion in Citrus Leaves .......................39
PthB Induces the Expression of Cell Wall Remodeling Enzymes .......................40
Enod8 and SAH7/LAT52 are a Link Between Canker Symptoms Development and Nodule Organogenesis and Pollen Tube Growth Respectively ..........42
PthB Induces Up-Regulation of a Tonoplast Aquaporin .....................................44
PthB Induces Up-Regulation of Two Components Involved in Vesicle Trafficking .................................................................44
Hormone Pathways are Possibly Involved in Canker Symptoms Development ........................................................................................................45
Conclusions and Future Prospects .........................................................................47

3 CHANGES IN SUMO CONJUGATION ARE ASSOCIATED WITH CITRUS CANKER DISEASE ........................................................................................................66

Introduction ..............................................................................................................66
Materials and Methods ............................................................................................69
Plant Inoculations .....................................................................................................69
Bacterial Strains and Culture Media .......................................................................70
Marker Integration Mutagenesis ............................................................................70
Bioinformatics ..........................................................................................................71
Protein Extraction and Western Blotting ...............................................................71
Results .......................................................................................................................72
SUMO Conjugation Profiles are Altered in X. citri-Infected Leaves .................72
SUMO Conjugation Profiles in Infected Leaves are Partially PthB Dependent .73
SUMO De-Conjugation Observed at 7 days Following Infection with B69 and BIM2 is Dependent on a Functional Type III SecretionSystem ........74
Discussion ................................................................................................................74
APPENDIX

A  LIST OF PLASMID AND STRAINS .................................................................83
B  NORTHERN BLOT ANALYSIS OF CCRS ......................................................85
LIST OF REFERENCES .......................................................................................86
BIOGRAPHICAL SKETCH ..................................................................................91
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>List of putative CCR identified by DD-PCR.</td>
<td>49</td>
</tr>
<tr>
<td>2-2</td>
<td>List of CCRs confirmed by reverse northern blot analysis.</td>
<td>50</td>
</tr>
<tr>
<td>A-1</td>
<td>List of strains and plasmids used in this study</td>
<td>83</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Organization of the type four secretion system ((\text{virB} \text{ operon})) found on pXcB compared to other described TFS systems.</td>
<td>13</td>
</tr>
<tr>
<td>1-2</td>
<td>Hybridization profiles of DNA from B69 integrative mutants interrupted in (\text{virB}4) of B69 (\text{virB}) clusters.</td>
<td>14</td>
</tr>
<tr>
<td>1-3</td>
<td>(\text{EcoRI}) and (\text{BamHI}) restriction digest profiles of plasmid pB13.1 and plasmid pB13.2, derivatives of pXcBo and pXcB, respectively, and integrated in gene (\text{virB}4).</td>
<td>15</td>
</tr>
<tr>
<td>1-4</td>
<td>PCR profiles using primers AB65 and AB66 specific of plasmid pXcB.</td>
<td>16</td>
</tr>
<tr>
<td>1-5</td>
<td>Self-mobilization of pXcB derivatives is dependent on a type IV secretion system.</td>
<td>17</td>
</tr>
<tr>
<td>1-6</td>
<td>Construction of suicide vector pBY17.1.</td>
<td>18</td>
</tr>
<tr>
<td>1-7</td>
<td>Scheme of FLP recombinase-mediated marker eviction.</td>
<td>19</td>
</tr>
<tr>
<td>1-8</td>
<td>PCR confirmation of suicide plasmid pBY17.1 integration in gene (\text{virB}4).</td>
<td>20</td>
</tr>
<tr>
<td>1-9</td>
<td>CR confirmation of Flp-mediated eviction of pBY17.1.</td>
<td>21</td>
</tr>
<tr>
<td>1-10</td>
<td>Pathogenicity phenotype of primary and secondary exconjugants disrupted in (\text{virB}4).</td>
<td>22</td>
</tr>
<tr>
<td>2-2</td>
<td>Late B69 and BIM2 phenotypes. (A) BIM2 inoculated leaves 30 dpi and (B) B69 inoculated leaves 30 dpi.</td>
<td>53</td>
</tr>
<tr>
<td>2-3</td>
<td>Quantification of bacterial population two days post inoculation with B69 and BIM2. (cfu: colony forming unit), Exp1: experiment 1, Exp2: experiment 2).</td>
<td>54</td>
</tr>
<tr>
<td>2-4</td>
<td>Diagram of PCR-Select cDNA subtraction.</td>
<td>55</td>
</tr>
<tr>
<td>2-5</td>
<td>Distribution of potential citrus canker responsive genes.</td>
<td>56</td>
</tr>
<tr>
<td>2-6</td>
<td>Distribution and origin of the clones stamped on the nitrocellulose membranes used in reverse northern blot analysis.</td>
<td>57</td>
</tr>
<tr>
<td>2-7</td>
<td>Cluster analysis of genes differentially regulated by PthB.</td>
<td>58</td>
</tr>
</tbody>
</table>
2-8 Northern blot analysis of CCR genes found differentially regulated by reverse northern blot analysis.

2-9 Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB).

2-10 Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB).

2-11 Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB) at 14 dpi.

2-13 Quantification of leaf thickening and cell division during B69 and BIM2 infection on Duncan grapefruit leaves.

2-13 Microscopic symptoms of rapidly developing canker.

3-1 Alignment of grapefruit SUMO (partial sequence) with (PopSUMO1, gi:23997054, and AtSUMO1, At4g26840).

3-2 SUMO profiles of B69- and mock-challenged grapefruit leaves.

3-3 SUMO de-conjugation occurs 7 days after infection.

3-4 Split leaf inoculation of Xanthomonas citri pv. aurantifolii (B69) and derivative BIM2 mutant.

3-5 B69 mutant derivative B23.5 lacks a functional Type III secretion system.

3-6 SUMO de-conjugation at 7 dpi requires a functional TTSS.

B-1 Northern blot analysis of CCR genes not found differentially regulated by reverse northern blot.
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BACTERIAL CITRUS CANKER: MOLECULAR ASPECTS OF A COMPATIBLE PLANT-MICROBE INTERACTION

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Canker is an important disease affecting citrus worldwide. It is caused by two phylogenetically distinct groups of strains of *Xanthomonas citri* (Xc), with all citrus cultivars being susceptible to at least one Xc strain. It is known that canker-causing xanthomonads carry at least one pathogenicity gene of the *pthA* (of Asiatic *X. citri* pv citri) gene family, which is required for causing canker on citrus. However little is known of the host molecular events leading to canker. Our goal was to understand host molecular mechanisms underlying disease development, and identify bacterial components related to phylogeny or pathogenicity of canker-causing xanthomonads.

First we identified on plasmid pXcB of the South American strain *X. citri* pv aurantifolii B69, a pathogenicity island composed of previously identified pathogenicity gene *pthB* and a type IV secretion system (TFSS). This TFSS was shown to be required for self-mobilization of pXcB, which led us to propose that natural horizontal transfer of a *pth* host-specific pathogenicity gene may account for the two phylogenetically distinct
groups of strains, (the Asiatic and the South American group of strains), causing canker symptoms on citrus.

Second, we investigated plant responses to PthB using differential display PCR and suppressive subtractive hybridization techniques. We identified forty-nine genes that were differentially regulated when RNA expression profiles of leaves inoculated with Xca B69 were compared to those of leaves inoculated with a B69 mutant carrying a disrupted \textit{pthB}. Among these were genes predicted to be involved in cell expansion, protein modification, biotic/abiotic stress responses and cell-wall metabolism.

Finally, we focused on one canker-responsive gene with strong similarity to the small ubiquitin like modifier (SUMO) from \textit{Arabidopsis}. Analysis of B69 mutant strains lacking PthB or the type III secretion system (TTSS) component, HrpG, revealed PthB-dependent and TTSS dependent/PthB–independent changes in SUMO conjugation profiles after infection with B69.

The genes and cellular processes that we identified reflect the molecular events leading to disease development. They contribute to the general aim of understanding the mechanisms underlying the variety of diseases caused by compatible interactions between xanthomonads and their host plants.
CHAPTER 1
A 37 KB PLASMID FROM A SOUTH AMERICAN CITRUS CANKER STRAIN CARRIES A TYPE IV SECRETION SYSTEM ESSENTIAL FOR SELF-MOBILIZATION

Introduction

The genus Xanthomonas is comprised of strains that exhibit a high level of host-specificity; over 125 different pathogenic variants (pathovars) of X. campestris have been described that differ primarily in host range (Bergey, 1994). Host specificity in Xanthomonas can be due to gene-for-gene interactions involving avirulence genes that act in a negative fashion to limit host range (Keen, 1990; Gabriel, 1999; Leach and White, 1996), but also can be due to positive acting factors that condition host range in a host-specific manner. For example, pthN, avrb6 of X. campestris pv. malvacearum (Yang and Gabriel, 1996), opsX of X. campestris pv. citrumelo (Kingsley et al, 1993) and pthA of X. citri pv. citri (Swarup et al., 1991 and 1992) act as positive effectors of host range. Interestingly, although a clonal population structure is observed among strains within many pathovars (Gabriel et al, 1988), some pathovars are comprised of phylogenetically distinct groups that have an identical host range and cause identical disease symptoms. Examples include 1) common bean blight, caused by two groups of strains (X. phaseoli and X. campestris pv. phaseoli var. fuscans) that are only 20% related by DNA-DNA hybridization (Hildebrand et al., 1990); 2) bacterial spot of tomato and pepper, caused by two major groups of strains within X. campestris pv. vesicatoria (Jones et al., 2000) that are less than 50% related by DNA-DNA hybridization (Stall et al., 1994), and 3) citrus canker disease, caused by two groups of strains that are only 62 -63% related by DNA-
DNA hybridization (Egel et al., 1991). Strains with 70% or greater DNA-DNA relatedness are usually defined as single species (Wayne et al., 1987). The question arises however, as to how phylogenetically diverse strains can cause identical diseases on an identical range of hosts.

To date, all pathogenic xanthomonads examined require *hrp* genes (reviewed by Alfano & Collmer, 1996, 1997; He, 1998; Cornelis & VanGijsegem, 2000) to cause disease. These genes encode a type III secretion machine that is close contact-dependent (Marenda et al., 1998) and used to inject highly adapted effector proteins into both host and nonhost cells (Silhavy, 1997; Kubori et al., 1998; and Jin and He, 2001). These effector proteins elicit the diverse programmed phenotypes of the plant hypersensitive response (HR) and various pathogenicity responses. The *hrp* (hypersensitive response and pathogenicity) injection system is thus appropriately named, and it is also highly indiscriminate, injecting whatever effector proteins are available, even some from animal pathogens (Anderson et al., 1999 and Rossier et al., 1999). If identical *hrp* effectors are available within two phylogenetically distinct xanthomonads, they can cause the same disease symptoms, provided both strains are compatible (able to multiply in the host) and both carry functional *hrp* systems. For example, *pthA* was transferred from *X. citri* to *X. campestris* pv. citromelo and converted the latter strain from a leaf-spotting strain to a strain with ability to cause citrus canker disease (Swarup et al., 1991). PthA appears to be an effector protein that is critical for citrus disease symptoms and is likely injected by *X. citri* into citrus cells, causing hyperplastic cankers (Duan et al, 1999).

Citrus canker disease is caused by two phylogenetically distinct and clonal groups of *Xanthomonas* strains; each group contains subgroups that are distinguished on the
basis of host range (Brunings and Gabriel, 2003). The first phylogenetically distinct
group is the Asiatic group, named *Xanthomonas citri* pv citri ex Hasse (syn = *X.
campestris* pv. citri Dye pathotype A and *X. axonopodis* pv. citri Vauterin, *Xca-A*). The
second phylogenetically distinct group is the S. American group, named *X. citri* pv.
aurantifolii Gabriel (syn = *X. campestris* pv. citri Dye and *X. axonopodis* pv. aurantifolii
Vauterin, *Xca-B*). Both groups cause identical citrus canker disease symptoms - circular,
water soaked raised lesions, that become dark and thick as canker progresses (Graham et
al., 2004; Stall and Civerolo, 1991; Gottwald et al, 2002; Brunings and Gabriel, 2003).
Significantly, *pthA* or homologues are present in every *Xanthomonas* strain tested that
causes citrus canker disease, and have not been found present in xanthomonads isolated
from citrus that do not cause canker (Gabriel, 1999; Cubero and Graham, 2002). Prior to
this work, two *pthA* homologues, named *pthB*, and *pthB0* were found on two separate
plasmids (pXcB and pXcB0, respectively) of a S. American canker strain (B69). Plasmid
pXcB carrying the functional homologue *pthB*, was then found to be readily cured from
B69 (Yuan and Gabriel unpublished, and Brunings, A.M., 2004 M.S. thesis University of
Florida). Readily cured plasmids are often mobilizable by conjugation. Since Asia is
considered to be the center of origin of citrus canker disease, and since Asiatic canker
strains are more widespread in S. America than S. American canker strains, it was of
interest to determine if pXcB could transfer horizontally. pXcB was found to horizontally
transfer *in-vitro* and *in planta* (Yuan and Gabriel unpublished) from the S. American
strain B69 to the Asiatic strain B21.1 lacking a functional *pthA*, restoring its capacity to
cause canker (Yuan and Gabriel unpublished). Presence of the type III effector *pthB* on a
self-mobilizing plasmid might explain the creation of the entire S. American group of canker strains, and why they are phylogenetically distinct from the Asiatic group.

pXcB was fully sequenced (NC_005240, gi32347275), and besides gene *pthB*, a complete Type IV secretion system (TFSS) was also found on the plasmid (Brunings, A.M., 2004 M.S. Thesis, University of Florida). TFSS are defined on the basis of homologies between the *A. tumefaciens* T-DNA transfer system, the conjugal transfer system Tra, and the *Bordetella pertussis* toxin exporter, Ptl (Winams et al., 1996 and Christie, 1997). Most members of the TFSS family function primarily to mobilize DNA, either from bacteria to bacteria (bacterial conjugation system) or from bacteria to eukaryotic cells (*Agrobacterium* oncogenic T-DNA transfer system) (Burns, 1999). In addition, several bacterial pathogens utilize conjugation machines to export effector molecules during infection. Such systems are said to be Type IV “adapted” conjugation or secretion systems, for their involvement in pathogenicity. Many non-plant pathogens such as *Bordetella pertussis*, *Legionella pneumophila*, *Brucella* spp. and *Helicobacter pylori* use a type IV “adapted” conjugation system to secrete effector proteins to the extracellular milieu or the cell cytosol (Burns, 1999; Christie, 1996; Christie and Vogel, 2000). Type IV systems are composed of products with homology to the *Agrobacterium virB* operon (Vogel, 2000). Sequence similarity analysis revealed that the Type IV secretion system of pXcB encodes twelve open reading frames, ten of which contained high sequence similarities to genes of previously described *virB* operons as well as similar relative positions within the cluster (Brunings, A.M., 2004 M.S. Thesis, University of Florida).
In order to investigate whether the TFSS found on pXcB is involved in self-mobilization of pXcB, a plasmid derivative lacking a functional TFSS was generated in this study and tested for its ability to self-mobilize in vitro. In addition, a B69 derivative lacking the TFSS was generated in a non-polar fashion to address whether this system was required for pathogenicity of B69. It was found that the TFSS of pXcB was required for self-mobilization of the plasmid. However, pathogenicity tests involving TFSS insertional mutants were inconclusive, and it remains unknown whether this secretion system is involved in pathogenicity of *X. citri* pv. aurantifolii.

**Materials and Methods**

**Bacterial Strains, Plasmids and Culture Media**

Bacterial strains and plasmids used in this study are listed in Table 1. *Xanthomonas* spp. were cultured in PYGM medium at 30°C (De Feyter et al., 1990). *Escherichia coli* were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). Antibiotics were used at the following concentrations (in µg/mL): Chloramphenicol (Cm), 35; Kanamycin (Kn) 12.5 or 25 (when used to grow *Xanthomonas* or *E. coli* respectively); Spectinomycin (Sp) 35 and Streptomycin (St) 35.

**Marker Integration Mutagenesis**

Gene-specific knockout mutations of *Xanthomonas* were created by triparental matings. An *E. coli* DH5α strain carrying an internal fragment of the target open reading frame (ORF) cloned in suicide vector pUFR004 was used as donor. A DH5α strain carrying pRK2013 was used as the helper. A single crossover in the exconjugates results in duplication of the internal fragment at the integration site, and also results in interrupting the target gene with the vector. To disrupt *virB4*, a PCR-generated, 270 bp
internal fragment of \textit{virB4} (virB4270) was cloned in pGEM–T Easy and recloned in pUFR004 creating pBY13.

**Plasmid Conjugal Transfer Techniques**

Plasmid transfer by triparental mating from \textit{E. coli} strains HB101 or DH5\(\alpha\) to various \textit{Xanthomonas} strains, using helper strain pRK2013 were performed essentially as described in De Feyter and Gabriel (1991). For plasmid transmission experiments on artificial media, overnight cultures of \textit{E. coli} strains grown without antibiotics were mixed with 50X concentrated overnight, mid-log phase cultures of \textit{Xanthomonas} strains, grown without antibiotics. Drops (10 \(\mu\)l each) of recipient donor and helper cells were placed on PYGM agar medium one after the other and without antibiotics. In each case excess liquid was allowed to absorb into the plate before addition of the next cell type. The mating plates were incubated at 30\(^\circ\)C overnight, and the spots were then streaked on PYGM selection medium supplemented with the appropriate antibiotics.

In \textit{Xanthomonas} to \textit{E. coli} matings, B69 carrying pB13.2 (pBY13 integrated in \textit{virB4} of pXcB) or B69 carrying pB13.1 (pBY13 integrated in \textit{virB4} of pXcB0) were used as donor strains (in independent matings) with DH5\(\alpha\) as the recipient strain. After selection against \textit{Xanthomonas} on MacConkey agar (DIFCO laboratories, Detroit MI, USA) with 35 \(\mu\)g/mL chloramphenicol, DH5\(\alpha\) exconjugants were screened for the presence of pBY13.2 or pBY13.1 by DNA mini-prep analysis. In \textit{E. coli} to \textit{E. coli} matings, DH5\(\alpha\)/pBY13.2, DH5\(\alpha\)/pBY13.1 and DH5\(\alpha\)/pBIM2 (pYY40.10 integrated in \textit{pthB}) were used as donor stains in independent matings with HB101 as recipient.

For frequency of transfer assays from one \textit{E. coli} strain to another, donor and recipient strains were grown overnight at 37 \(^\circ\)C to an O.D. \textit{600nm} of 0.5. Twenty
microliters of each culture were combined in a 1.5 ml Eppendorf tube containing 160 µl of LB and grown overnight at 37 °C. Cells were then resuspended in 1 ml of LB, pelleted and then serially diluted on medium containing chloramphenicol and streptomycin to select for HB101 transconjugants. All conjugation experiments were performed at least twice with duplicate samples in each experiment, and the numbers were averaged.

Recombinant DNA Techniques

Plasmid and total DNA were prepared from Xanthomonas as described by Gabriel and De Feyter (1992). E. coli plasmid preparation, restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation, and random priming reactions were performed using standard techniques (Sambrook et al., 1989). Southern hybridization was performed using nylon membranes as described by Lazo and Gabriel (1987).

Plant Inoculations

All citrus plants (Citrus paradisi ‘Duncan’, grapefruit) were grown under greenhouse conditions. Plant inoculations involving all citrus canker strains were carried out under quarantine at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Bacterial cells were harvested from log phase cultures by centrifugation (5,000 x g, 10 min.), washed once and resuspended in sterile tap water or distilled water saturated with calcium carbonate to 10^8 cfu/mL. Inoculations were performed by pressure-infiltration into the abaxial leaf surface of the plants. Experimental inoculations were repeated at least three times.

Results

The Type IV Secretion System Found on pXcB is Required for Self-Mobilization

Gene virB4 of the TFSS cluster of pXcB was chosen as target for marker insertional mutagenesis (Figure 1-1). For that, a 270 bp integral fragment of virB4
(virB4270) was cloned in pUFR004 (pBY13) and used in triparental matings to generate virB4 insertion mutants. Southern blots were used to verify integration events in the resulting transconjugants. These results demonstrate the existence of two copies of virB4 in the B69 strain (using virB4270 as probe Figure 1-2). One copy was carried by pXcB (as determined by sequencing) and was absent in the cured strain B69.4 [Rifamycin resistant strain cured of plasmid pXcB but carrying plasmid pXcB0, Yuan and Gabriel, unpublished (Lane 3)]. A second putative copy, carried by pXcB0, was maintained in B69.4 (Lane 3). Marker insertion resulted in two categories of exconjugants. Exconjugant strain B13.1 appeared to carry an interruption of the putative virB4 of pXcB0 (virB40) (Lane 7), while exconjugants B13.2, B13.4 and B13.5 appeared to carry interruptions of the virB4 gene of pXcB (Lanes 4, 5 and 6).

Plasmids pB13.1 and pB13.2 of strains B13.1 and B13.2 (marker interruptions in the virB4 homologues found on pXcB0 and pXcB, respectively) were further analyzed for their ability to transfer to E. coli. Matings with and without the helper strain resulted in DH5α exconjugants carrying plasmids that were chloramphenicol resistant, indicating that both plasmids were still mobilizing. Restriction enzyme digests of plasmid DNA extracted from the Xanthomonas (B13.2) to the E. coli exconjugant (DH5α/pB13.2), corresponded to the expected profile of pXcB integrated with pBY13 (Figure 1-3). Restriction enzyme digests of plasmid DNA extracted from DH5α/pB13.1 did not corresponded to the profile expected for a pXcB insertional derivative. Therefore, p13.1 is a derivative of a second native plasmid of B69, smaller in size than pXcB and inserted in a putative virB4 copy. These results were confirmed by PCR using primers specific to pXcB. As shown in Figure 1-4, when pB13.2 was used as template with pXcB specific
primers AB65/AB66 a 2014 bp band was obtained, while non specific bands were
obtained when pB13.1 was used as template

The ability of pB13.1 and pB13.2 to self-mobilize was then analyzed by
performing matings from DH5α to E. coli HB101. Using DH5α/pBIM2, and
DH5α/pBIM6 [pBIM6 is a derivative of pXcB where pUFR004 was inserted in a non-
ORF region, (Yuan and Gabriel, unpublished)] as a control, transfer of pBIM2 and
pBIM6 from DH5α to HB101 was found not to require the presence of a helper strain
and the transfer frequency was 7x10^{-03} and 6.6x10^{-05} per donor, respectively. By contrast,
E. coli to E. coli transconjugants harboring pB13.1 or pB13.2 were only recovered when
matings were performed in the presence of a helper strain (Figure 1-5). These results
indicated that the self-mobilization capacity of pXcB depended on the presence of an
intact virB cluster.

Involvement of the TFSS of pXcB in Pathogenicity of Xca B69

Non-polar knock out mutants of virB4 were generated using marker insertion
followed by FLP recombinase mediated marker eviction. Plasmid pBY17.1 was
generated so that a virB4 homology region was flanked by two FRT recognition sites
(See Figure 1-6 for illustration). After marker integration of suicide vector pBY17.2 into
primary transconjugants, the FLP recombinase plasmid pJR4, was used to evict the
marker, and generated non-polar secondary transconjugants (See Figure 1-7 for
illustration).

Several primary transconjugants (before FLP-mediated eviction of marker) (Figure
1-8) as well as secondary transconjugants (after FLP mediated eviction of marker) were
tested for integration events in a virB4 homologue using PCR. Bacterial cells directly
from the selection plates were used as template for PCR (Figure 1-9). PCR positive colonies were then grown in liquid culture and tested for pathogenicity on citrus. In all cases, primary exconjugants showed a decrease in pathogenicity while, unexpectedly, secondary exconjugants lost their potential to trigger canker disease on citrus (Figure 1-10). When the secondary exconjugants used in pathogenicity assays were tested by PCR for presence of pXcB it was found that the plasmid and therefore gene \textit{pthB} were lost upon culturing.

**Discussion**

The putative TFSS of pXcB (Brunings A.M., M.S. Thesis, University of Florida and Brunings and Gabriel, 2003) was functionally investigated to determine its involvement in plasmid transfer as well as in pathogenicity of B69. To investigate the role of this TFSS in plasmid transfer, gene \textit{virB4} was marker-interrupted and by consequence the whole system rendered dysfunctional. Self-mobilization experiments revealed that pXcB relied on a functional TFSS to self–mobilize. In the process a second putative \textit{virB4} homologue was identified on a second plasmid of B69, pXcB0. pB13.1, carrying a single insertion in \textit{virB40} of pXcB0 and pB13.2, carrying a single insertion in \textit{virB4} of pXcB were each able to mobilize from B13.1 and B13.2, respectively, to DH5\(\alpha\) in biparental matings (without helper strain), indicating that the two putative \textit{virB} systems co-existing in B69 might be compensatory.

The characterization of pXcB as a self-mobilizing plasmid carrying a TFSS and gene \textit{pthB} suggests that the canker causing and phylogenetically distinct South American strains may have arisen from horizontal gene transfer of an “ancestral” \textit{pthA} member. This horizontal transfer likely would have occurred from an Asiatic \textit{Xanthomonas citri}
strain to a compatible TTS system-carrying xanthomonad residing on the same host. B69 was indeed shown to carry a functional TTS system required for pathogenicity (see Chapter 3). The type IV secretion system together with \textit{pthB} on pXcB of S. American \textit{Xanthomonas citri} strains can therefore be considered an “auto-mobile” pathogenicity island (Hacker et al., 1997), capable of spreading among compatible bacteria by horizontal gene transfer.

Since pXcB from the South American strain is smaller, yet very similar to pXAC64 from the Asiatic strain, pXcB could be a deletion derivative of pXAC64 (Brunings and Gabriel 2003). However, while many genes on pXcB were found to be similar to genes on pXAC64, there were differences significant enough to conclude that a simple deletion cannot account for pXcB. More likely, several independent events were probably responsible for its divergence away from pXAC64.

Horizontal gene transfer is proposed to be a major mechanism explaining rapid genetic diversification in bacteria (Falcow, 1996; Syvanen and Kado, 1998; Lawrence and Roth, 1999). It has been proposed to explain the apparent enigma of why pathogens carry dispensable avirulence genes (Yang and Gabriel, 1996 and Gabriel, 1999). For example, \textit{avrBs3} of \textit{Xanthomonas campestris} pv. vesicatoria was found on a mobilizing plasmid carrying copper resistance, and therefore wide horizontal transfer of \textit{avrBs3} to \textit{X. campestris} pathovars may be due to coincidental linkage with copper resistance (Stall et al, 1986, Yang and Gabriel, 1996).

The TFSS of pXcB was also analyzed for its involvement in pathogenicity. Primary exconjugants carrying a marker integration in \textit{virB4} showed a decrease in pathogenicity while non-polar secondary exconjugants, resulting from marker eviction of the suicide
plasmid, lost all pathogenicity. This was then found to be possibly due to a loss of pXcB upon curing of secondary transconjugant strains. Another explanation is the presence of a large insertion vector in the native plasmid decreasing the copy number in the population. Further examination of the TFSS is necessary to access its role in pathogenicity if any.
Figure 1-1: Organization of the type four secretion system (virB operon) found on pXcB compared to other described TFS systems. ORF106 shows no similarity to any virB cluster gene of *Agrobacterium tumefaciens* and is shown as an insertion.
Figure 1-2: Hybridization profiles of DNA from B69 integrative mutants interrupted in virB4 of B69 virB clusters. Total DNA was digested with HindIII and probed with a $^{32}$P-labelled 270 bp internal fragment of virB4. The same fragment was used as a homology region for integration of suicide vector pBY13. B13.2, B13.4 and B13.5 were marker integrated in virB4 of pXcB, and B13.1 was marker integrated in virB4 of pXcB0. Hind III digestion results in splitting one restriction fragment harboring the targeted region into two hybridizing fragments. Therefore there are two bands hybridizing to the virB4$_{270}$ probe in the wild type strains, while there are three bands in the insertional strains. The only band hybridizing to the virB4$_{270}$ in the B69.4 lane corresponds to a putative virB4 copy present on a second native plasmid of B69. Indeed pXcB was lost upon curing in B69.4 and therefore one hybridizing band is lost.
Figure 1-3: EcoRI and BamHI restriction digest profiles of plasmid pB13.1 and plasmid pB13.2, derivatives of pXcB0 and pXcB, respectively, and integrated in gene virB4.
Figure 1-4: PCR profiles using primers AB65 and AB66 specific of plasmid pXcB. Plasmid DNA isolated from DH5α/pB13.1, DH5α/pB13.2, and total DNA isolated from B69 and B69.4 were used as templates. [AB65: CAG CCG CAA GTG TCT CAG GTC; AB66:GGC AAG AAA CCG TCC GAG TA (Tm 56°C)]. When B69.4 and pB13.1 DNA are used as template in the PCR reaction non-specific bands of low intensity are the resulting products. When B69 and pB13.2 (both derivatives of plasmid pXcB) are used as template, a specific band of 2014 bp is the resulting product of the PCR reaction. ( ): Amplification fragment specific to pXcB when AB65/AB66 primers are used.
Figure 1-5: Self-mobilization of pXcB derivatives is dependent on a type IV secretion system. (A) Mobilization of pXcB derivative, pBIM2 and pB13.2 and pXcB0 derivative pB13.1 from *E. coli* DH5α to *E. coli* HB101. Matings were carried with and without helper strain carrying plasmid pRK2013, and HB101 transconjugants were selected on LB supplemented with streptomycin and chloramphenicol. Each selection plate was separated in two sections. Results of matings with helper strain are shown on the left section, and results of matings without helper are shown on the right. Matings: (a) DH5α/pB13.1 with HB101; (b) DH5α/pB13.2 with HB101; (c) DH5α/pBIM2 with HB101. (B) Frequency of transfer of pXcB derivatives, pB13.2, pBIM2, pBIM6 and pXcB0 derivative, pB13.1 from *E. coli* DH5α into *E. coli* HB101.
Figure 1-6: Construction of suicide vector pBY17.1. PCR was used to amplify an internal
\textit{virB4} fragment using primers BY13 (gatcaggatctatgcgcctcgttgaggt) and
BY14 (cggtccgtcagtcagtcagagctctgaccaggtagtgcagga). \textit{Rsr}III and \textit{Bcl}I
restriction sites were incorporated in the primer sequences respectively in the
forward and reverse primer. The \textit{Rsr}III-\textit{Bcl}I fragment was used as the driver
for homologous recombination and was cloned between FLP sites in
pUFR012 (pUFR004 derivative carrying kanamycin resistance). FLP sites
were obtained by PCR using plasmid pKD4 (gi:15554332) (1.5 Kb fragment).
Primers FRTKn F (gaattcgctgcttcgaagttcctatac) and FRTKn R
(aagcttatcctccttagttcaattcc) carried an
\textit{Eco}RI and a \textit{Hin}dIII site for subcloning
from pGEMT-ez (Promega) into pUFR012.
Figure 1-7: Scheme of FLP recombinase-mediated marker eviction. Suicide plasmid pBY17.1 is marker integrated in gene *virB4* via homologous recombination, generating a *virB4* disruption. The light blue box represents the homology region targeted for recombination, and is found duplicated after insertion of the suicide plasmid pBY17.1 in pXcB. After transformation of the B69 derivative carrying pXcB::pBY17.1 (primary transconjugant) with plasmid pJR4 carrying a FLP recombinase gene, pBY17.1 is evicted (secondary transconjugants). pJR4 [derived from pFLP (gi:1245114) (Ready and Gabriel, unpublished)] is cured by culturing secondary transconjugants on PYGM supplemented with 5% sucrose. VirB4F and bes03 and bes04 are *virB4* specific primers and their locations are shown by arrows. 22mer and M13R are primers specific to the polylinker region of suicide vector pBY17.1 and are used to verify integration and eviction events. FRT sites recognized the FLP recombinase are symbolized by yellow circles and flank the internal fragment with homology to *virB4* cloned in pBY17.1. The green boxes symbolize DNA stretches carried over during sub-cloning steps.
Figure 1-8: PCR confirmation of suicide plasmid pBY17.1 integration in gene \textit{virB4}. 22 mer (gtttcncagtgaagcgac) and M13R (agcgagagac) are primer specific to the polylinker of pBY17.1. \textit{bes03} (catctttgatctgtcggtt) \textit{bes03DS}, \textit{bes04} (catgctgtgagcatcttt) and \textit{virB4} F (ggattagcaccagttaacgtgcc) are gene specific primers. Lanes 1, 4, and 7, B69 bacterial cells were used as template source for the PCR. Lanes 2, 5 and 8, B18.12 (primary transformant with pBY17.1 inserted in \textit{virB4}) bacterial cells were used as template source for the PCR. Lanes 3, 6 and 9, B18.15 (primary transformant with pBY17.1 inserted in \textit{virB4}) bacterial cells were used as template source for the PCR. Primer combinations used in each lane are indicated in the figure. PCR bands resulting from using \textit{virB4}-based primers in combination with suicide vector based primers are specific to an insertion in the targeted region and should not appear when the wild type strain is used. The light blue box represents the homology region targeted for recombination, and is found duplicated after insertion of the suicide plasmid pBY17.1 in pXcB. FRT sites recognized the FLP recombinase are symbolized by yellow circles and flank the internal fragment with homology to \textit{virB4} cloned in pBY17.1. The green boxes symbolize DNA stretches carried over during sub-cloning steps.
Figure 1-9: PCR confirmation of FLP-mediated eviction of pBY17.1. Genes specific primers (bes03, bes04, virB4F and vector based primers were used in appropriate combinations. B69 and B18.12 (primary transconjugant with pBY17.1 inserted in virB4) were used as negative and positive control for the suicide vector integration respectively. B18.12-1 is the secondary transformant resulting from suicide vector eviction from primary transconjugant B18.12. Bacterial cells from selection plates were used as template source for PCR. The expected size of each PCR band is indicated in the figure. The light blue box represents the homology region targeted for recombination, and is found duplicated after insertion of the suicide plasmid pBY17.1 in pXcB. FRT sites recognized the FLP recombinase are symbolized by yellow circles and flank the internal fragment with homology to virB4 cloned in pBY17.1. The green boxes symbolize DNA stretches carried over during sub-cloning steps.
Figure 1-10: Pathogenicity phenotype of primary and secondary exconjugants disrupted in virB4. B18.12; primary exconjugant (virB4::pBY17.1). B18.12-1 (secondary exconjugants, after eviction of pBY17.1) Picture on the left was taken 7 days post inoculation. The two pictures on the right were taken 15 days after inoculation. Note the delay phenotype of primary transconjugant B18.12, and the total loss of pathogenicity of transconjugant B18.12-1.
CHAPTER 2
IDENTIFICATION OF CITRUS GENES SPECIFICALLY RESPONSIVE TO PATHOGENICITY GENE \textit{pthB} OF \textit{Xanthomonas citri} \textit{pv. aurantifolii}

\textbf{Introduction}

Many studies on plant-pathogen interactions have dealt with incompatible interactions using model plant systems (for example see Malek et al., 2000). Emphasis has been on dissecting signaling pathways of resistance mechanisms, with few studies considering signaling pathways resulting in diseases of crop plants (Kazan et al., 2001). Therefore, the molecular events at the origin of disease induction by microbial effectors of pathogens remain obscure.

Many Gram-negative, phytopathogenic bacteria rely on a Type III secretion system (TTSS) to deliver effector proteins into the plant cells (He et al., 2004). Inactivation of the TTSS of bacterial species that utilize such a system results in loss of pathogenesis indicating that the proteins (named type III effectors) delivered by the TTSS are required for bacterial virulence (Rohmer et al., 2004). Most type III effectors identified to date were originally discovered and characterized by their avirulence function (Avr), while only few are recognized pathogenicity factors [PthA from \textit{X. citri} (Swarup at al., 1991), \textit{AvrB6} from \textit{X. campestris} \textit{pv. malvacearum} (Yang et al., 1996), \textit{AvrXa7} from \textit{X. oryzae} (Bai et al., 2000) and DspA from \textit{Erwinia amylovora} (Gaudriault et al., 1997)]. A limited number of type III effectors have been assigned proven or putative biochemical function (Collmer et al., 2000; Rohmer et al, 2000; Chang et al., 2004) and for a subset of these (principally avirulence effectors), a plant protein or cellular process has been identified as
a possible target for pathogenesis (Rohmer et al, 2004 and Chang et al. 2004). In two cases, a bacterial effector-triggered plant phenotype has been shown to be required for pathogenesis. In the case of pathogenicity factor DspA, a member of the *P. syringae* AvrE family, its induction of reactive oxygen species release by the host cell has been shown to be required for successful colonization (Venisse et al., 2003). While in the case of pathogenicity factor PthA, a member of the *Xanthomonas* AvrBs3/PthA family, its induction of cell division and/or cell expansion is required for pathogenesis (Swarup et al., 1991)

In this study the compatible interaction between citrus and *Xanthomonas citri* (*X. citri* pv. aurantifolii syn. *X. axonopodis* pv. aurantifolii) was examined. Probably originating in Southeast Asia, citrus canker has now spread to most citrus producing areas of the world and causes severe economical losses (Civerolo, 1994). All canker strains induce similar disease phenotypes, including water soaked lesions, formation of large hyperplastic erumpent pustules (cankers) on all aerial plant parts, and rupture of the epidermis with accompanying cell death (Swarup et al., 1991 and Duan et al., 1999).

Specific members of the *avrBs3/pthA* gene family are required by strains of *Xanthomonas citri* to cause cankers on citrus (Swarup and Gabriel, 1989; Swarup et al., 1990, Swarup et al., 1991). Members of the *avrBs3/pthA* gene family are found in many xanthomonads (Gabriel, 1999; Vivian and Arnold 2000), and all citrus canker strains examined carry multiple members of the gene family (Gabriel, 1999). All *Xanthomonas avrBs3/pthA* members described to date are 90-97% identical in DNA sequence and are characterized by 1) a series of 12.5-25.5 almost identical 34 amino acid repeats in the center of the protein that determines host specificity, pathogenicity and/or avirulence

Sequence and functional analysis of members of the avrBs3/pthA gene family showed that these proteins are type III effectors, acting in the plant nucleus potentially as transcriptional regulators (Yang and Gabriel, 1995, Zhu et al., 1998, Zhu et al., 1999, Yang et al., 2000, Szurek et al., 2001). When the pathogenicity gene pthA from X.citri was transiently expressed in susceptible plant cells (by Agrobacterium infection or particle bombardment delivery), it elicited canker-like pustules, indicating that pthA alone was sufficient to trigger canker symptoms (Duan et al., 1999). Unlike pthA and its active homologues in other X. citri pv. citri and X. citri pv. aurantifolii strains, avrBs3 is not required for pathogenicity of X. c. pv. vesicatoria (Bonas, 1989). However, it was found to induce a subtle hypertrophy in the mesophyll of leaves inoculated with slow-growing strains of X. c. vesicatoria, concomitant with the up-regulation of 13 plant genes (Marois et al., 2002). Taken together these results indicate that members of this gene family are able to induce transcriptional reprogramming in both susceptible and resistant plant cells.

In this study, the citrus canker system was used to probe the functions of pthB, another member of the avrBs3/pthA gene family, that is isofunctional with pthA in eliciting host-specific symptoms. A comparative analysis of gene expression in citrus leaves inoculated with the wild type X. c. aurantifolii strain B69 (carrying pthB) and an Xca mutant derivative carrying a defective (marker interrupted) pthB was performed.
Methodological approaches in this analysis included differential display-reverse transcriptase PCR (Liang and Pardee, 1992), suppressive subtraction hybridization, and microscopy. Forty-six clones of citrus canker responsive genes belonging to several broad categories of cellular functions were identified as being specifically regulated by \(pthB\). These categories included genes identified to be involved in cell wall loosening and growth, water homeostasis and vesicle trafficking. In addition evidence is presented for the involvement of hormone signaling in canker disease development.

**Material and Methods**

**Plant and Microbial Material**

Bacterial strains and plasmids used in this study are listed in Appendix A. B69 and BIM2 were grown on PYGM (De Feyter et al. 1990) supplemented with 35 mg/L spectinomycin and 35 mg/L spectinomycin plus 35 mg/L Chloramphenicol. All citrus plants (*Citrus paradisi* ‘Duncan’, grapefruit) were grown under greenhouse conditions. Plant inoculations involving all citrus canker strains were carried out under quarantine at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Bacterial cells were harvested from log phase cultures by centrifugation (5,000xg, 10 min.), washed (1X) and resuspended in sterile tap water or distilled water saturated with calcium carbonate to an OD\(_{600\text{nm}}\) of 0.6-0.7, unless stated otherwise. Inoculations were performed by pressure-infiltration into the abaxial leaf surface of the plants. Experimental inoculations were repeated at least three times.

For differential display-reverse transcriptase PCR (DD-PCR) experiments and construction of the suppressive subtractive libraries (SSH), inoculations were performed following a split leaf model. Strain B69 was inoculated on one side of the mid-vein; while BIM2 was inoculated on the opposite side of the mid-vein, in order to control for
leaf to leaf variations. Tissue was harvested 0, 2 or 7 days post inoculation (dpi) depending on the experiment.

**Bacterial Counts**

B69 and BIM2 bacterial cells were normalized to an OD$_{600}$ of 0.7 and infiltrated as described previously. At 0 and 2 dpi, a total of 9 discs (0.28 mm in diameter) from 3 leaves (3 discs per leaves) were harvested for each treatment and ground in 1ml of tap water. After serial dilution, the bacterial populations of wild type strain B69 and mutant strain BIM2 were counted. Bacterial cell count determinations represent the average of three replicate experiments.

**Microscopy**

Fresh, tender and half-expanded leaves were inoculated with a high inoculum of B69 or BIM2. At 0, 2, 7 and 14 dpi, leaf samples of an area of approximately 6 mm$^2$ were harvested and fixed in 2% glutaraldehyde in phosphate buffer saline (PBS) for 48 hr at 4°C. They were then washed three times for 15 min each and fixed in 1% buffered osmium tetroxide overnight at 4°C. This was followed by one wash in PBS for 10 mi and by two washes in distilled water. A stepwise dehydration was conducted after these washes using ethanol (25%, 50%, 75%, 95% and 100%) for 10 min each step, followed by three washes in acetone for 15 min each. Samples were then infiltrated at room temperature in 30% acetone/EMbed (Electron microscopy sciences, Pennsylvania) for 1 hr, followed by 50% acetone/EMbed for 1 hr and 70% acetone/EMbed for 2 hr. Samples were subsequently incubated in 100% EMbed overnight at room temperature to complete the infiltration and polymerized in fresh 100% EMbed in a 75°C oven overnight.
Differential Display-Reverse Transcriptase PCR

Two and seven days after inoculation, leaf tissue was harvested, pooled and frozen in liquid nitrogen for total RNA extraction as described (Chang et al., 1993). Potential canker responsive (CCR) cDNAs were cloned as fragments by differential display-reverse transcriptase PCR (DD-PCR) of mRNA using 48 primer combinations (Liang and Pardee, 1992) with the RNAimage kit from Genhunter (Nashville, TN, USA).

Suppressive Subtractive Hybridization (SSH) Library Construction

For polyA mRNA isolation, leaves were frozen in liquid nitrogen and stored at -80°C until extraction. PolyA mRNA was isolated from leaves using the FastTrack mRNA isolation kit (Invitrogen) according to the manufacturer’s protocol. SSH was constructed using a cDNA subtraction kit (Clontech PCR-Select, Palo Alto, CA). For construction of the forward subtraction library (FS), the tester was chosen to be the pool of mRNA isolated from B69 inoculated leaves at 2 dpi while the driver was chosen to be the pool of mRNA isolated from BIM2 inoculated leaves, and therefore, the FS was enriched in transcripts up-regulated by \( pthB \). For the reverse subtraction library (RS), transcripts isolated from BIM2 inoculated leaves (2 dpi) were used as tester, and therefore, while the driver was chosen to be the pool of mRNA isolated from B69 inoculated leaves. the RS library was enriched in transcripts up-regulated in the absence of \( pthB \).

Potential differentially regulated clones were sent for sequencing to the Interdisciplinary Center for Biotechnology Research (ICBR) core at the University of Florida. Putative functions were assigned based on annotation derived by BLAST analysis.
**Northern Blots**

For RNA sample preparation, NorthernMax Formaldehyde Load Dye was used as recommended by the manufacturer (Abion Austin, TX) with 5-10 µg of RNA. Samples were loaded on a denaturing formaldehyde agarose gel (1%) and electrophoresis was conducted at 5 V/cm. RNA was blotted on GeneScreen Plus hybridization transfer membrane (NemTM Life Science Products, MA) using 20X SSC as transfer buffer. Hybridization and washes were done as recommended by the manufacturer (UltaHyb, Ambion Austin, TX). Probes were made with DECA primeTMII (random priming), (Ambion Austin, TX) as recommended.

**Reverse Northern Blots**

For reverse northern blots, cDNAs identified by DD-PCR or SSH were amplified using vector primers and purified using Qiaquick columns in plate format (Qiagen, Valencia CA). Membrane arrays were made essentially as described by Desprez et al., (1998). cDNAs were arrayed onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ) using a 96-pin colony replicator (V&P Scientific, San Diego CA). Six replicate arrays were generated and used to analyze transcript abundance of a subset of potential canker responsive genes or CCRs. Each cDNA was spotted in two locations, and several cDNAs were represented by more than one clone. Three replicate membranes for each treatment (B69 or BIM2 infection) were used in hybridization experiments (total of six membranes or 3 pairs). Each membrane was probed with radiolabelled cDNA synthesized from RNA isolated from one of three split leaf-experiments conducted, 2 dpi. Each membrane pair was one of three biological replications. Signal intensities were statistically compared after normalization.
For probe preparation, first strand cDNA probes were prepared from 10 µg of total RNA by reverse transcription using MMLV-RT (Gibco-BRL, Gaithersburg MD) in the presence of \(^{32}\text{P}-\text{dCTP}\). Unincorporated nucleotides were separated from first strand cDNA using Sephadex G-50 columns (Amersham Pharmacia Biotech, Ithaca NY) and quantified using a liquid scintillation counter (Beckman Coulter, Fullerton CA). Pre-hybridization, hybridization and low and high stringency washes were carried out at 65°C. Membranes were exposed to phosphorimager screen for visualization. Spot intensities (called volumes) on the membrane arrays were quantified using a BioRad Molecular Imager FX run with the associated Quantity One software (Bio-Rad Laboratories, Inc. Hercules, CA). Data were imported into Microsoft Excel (Microsoft Corp., Redmond, WA, USA) for further analysis.

**Statistical Analysis**

A mixed model analysis (SAS Proc Mixed) was run on the log base 2 transformed (normalization) local background adjusted volumes. cDNAs that did not exhibit a mean value greater than 120 from either treatment were not included in the analysis. The linear model used included replication (three biological replications), treatment (B69 treated or BIM2 treated) and gene (CCRs or Citrus Canker responsive clones). Least square means for the treatment by gene interaction were saved and used to form by-gene contrasts between treatments. Significance of these contrasts was controlled for an experiment-wide alpha level.
Results

Macroscopic Disease Phenotype of Citrus Leaves Inoculated with X. c. aurantifolii B69 and Its Mutant Derivative BIM2 Lacking the Pathogenicity Gene pthB

*Xanthomonas* strains B69 (wt) and its nonpathogenic mutant derivative BIM2, carrying a marker integration in gene *pthB* (*pthB*:pUFR004), were inoculated at high levels (OD = 0.7) on tender half-expanded leaves of new flushes of Duncan grapefruit and the corresponding induced disease phenotype analyzed. At day two post-inoculation, no symptoms were visible and no macroscopic differences were observed among leaves inoculated with tap water, B69 or BIM2. By seven days post-inoculation, leaves that were mock inoculated showed no symptoms, while leaves inoculated with the wild type strain B69 showed a whitish canker phenotype, typical of South American canker disease. On the abaxial side of the leaf, the entire inoculated area became raised, with a soft, velvet-like appearance, while a few individualized pustules appeared at the margins of inoculated areas. Pustules possibly corresponded to areas where bacteria were infiltrated at low density (Figure 2-1, A and B). On the adaxial side of the leaf, no raising was apparent; instead some yellowing developed. This rapid symptom development is typically observed when a high inoculum is used on fresh, young expanding leaves.

By contrast, at 7 dpi, no major symptoms were visible on leaves inoculated with BIM2. Limited raising of the epidermis occurred at the margins of some inoculation zones, with development of minimal pustule-like structures reminiscent of those seen in canker (Figure 2-1, C and D). These symptoms were not observed in mock-inoculated leaves. BIM2 inoculated leaves ultimately displayed attenuated canker phenotypes after thirty days (Figure 2-2). This is possibly due to the weak canker-inducing activity of *pthB0*, the second *pthA* homologue found in the B strain, B69.
PthB-Dependent Transcriptional Reprogramming Induced upon Infection with Xca

A small scale DD-PCR was conducted to compare transcript levels of leaves inoculated with B69 to those of leaves inoculated with BIM2 at two and seven dpi. To maximize the homogeneity and the intensity of the response, B69 and BIM2 were inoculated at high levels (OD$_{600}$ of 0.7). In order to minimize leaf-to-leaf variation, a split-leaf inoculation strategy was used. An average of fifteen leaves (from three trees) were inoculated with B69 on one side of the mid-vein and with BIM2 on the other side. Two and seven days after inoculation, half-leaves were harvested, pooled into “B69 treated” or “BIM2 treated” samples and RNA extracted from both samples. Since B69 (carrying $pthB$ and $pthB0$) differs from BIM2 (carrying $pthB::pUFR004$ and $pthB0$) only by the presence of a single effector, PthB, differentially regulated transcripts (named citrus canker responsive or CCRs) were PthB responsive. Transcripts identified by DD-PCR appeared differentially regulated as early as two days post-inoculation despite a complete lack of symptoms. Twenty cDNAs were identified by DD PCR (Table 2-1), including six with homology to biotic or abiotic stress response genes (CCR20.2 to PR-1 proteins and CCR9.5, CCR15.1 to PR-5 proteins, CCR2.2, CCR17.2 to peroxidases and CCR12.1 to catalases). One cDNA, CCR6.4 displayed homology to cell wall remodeling enzymes of the cellulase family. CCR25.1 was homologous to the small ubiquitin like modifier SUMO.

To remove the possibility that potential changes in transcript level were due to differences in the number of bacteria present in B69 inoculated leaves compared to BIM2 inoculated leaves, both bacterial populations were monitored at 0 and 2 dpi. B69 and BIM2 bacterial populations were found to be comparable with almost no growth observed during the first two days post-inoculation (Figure 2-3). Bacterial growth at 2 dpi
will occur if bacteria are inoculated at lower initial levels (OD$_{600}$ of 0.3-0.4) (data not shown). However, when inoculated at lower levels, growth of BIM2 is very poor (data not shown and discussed later).

**Construction of Two Libraries Enriched in *pthB* Responsive cDNAs**

Following the same split leaf scheme as for the DD-PCR experiment, forward and reverse libraries were constructed by suppressive subtraction hybridization (see Figure 2-4 for illustration of the methodology), extending the collection of putative CCRs. The forward subtraction library (FS) was constructed to be enriched in transcripts up-regulated by PthB while the reverse subtraction library was constructed to be enriched in transcripts up-regulated in the absence of PthB (see Materials and Methods for design of the SSH).

Approximately 500 clones were sequenced and annotated using homology based searches. Figure 2-5 illustrates the distribution of CCRs for each of the FS and RS libraries according to their putative function. Categories representing genes of unknown function (8%) and genes involved in cell growth and division (10%) were found more frequently in the forward library (up-regulated in the presence of *pthB*) compared to the reverse library (1% and 2% respectively), while genes in the category representing abiotic and biotic stress responses were found more frequently in the reverse library (15% vs 6% in the FS).

**Transcript Analyses of CCRs**

cDNAs from each of the forward (131) and reverse (161) libraries, as well as 20 clones identified by DD PCR (total of 312 cDNAs) (Figure 2-6) were chosen for reverse northern-blot analysis. CCRs homologous to genes of known function were preferentially selected. Six replicate arrays were generated as described in materials and methods, and
used to analyze transcript abundance of a subset of potential CCRs. Three membranes per treatment were probed with radiolabelled cDNA synthesized from RNA isolated from three split leaf-experiments, 2dpi with B69 and BIM2 (three biological replicates). For each experiment, inoculated leaves were sampled from new and older flushes, were half to fully expanded and were all tender (minimal cuticle). Signal intensities were statistically compared after normalization as described in material and methods. Forty-six clones were identified as differentially regulated at \( p < 0.05 \) (Figure 2-7). Only fifteen out of forty-six clones were found up-regulated in the absence of PthB, while the remaining thirty-two were found up-regulated by PthB. Ratios of transcript abundance were calculated for each cDNA. Ratios ranged from -3.5 to +34.5 (- sign indicating over-expression of the gene in the absence of PthB and + sign indicating an up-regulation in the presence of PthB) (Table 2-2).

Identity of cDNAs Identified as Up-Regulated by the Presence of \( \text{pthB} \) in \( X. \text{citri} \) Genome

Of the forty-six clones identified as differentially regulated, all but four clones showed significant (e-value >2e-03) matches with sequences in available databases (Table 2-2). Thirty CCRs out of forty-six were found up-regulated by the presence of \( \text{pthB} \) in the bacterial genome \( i.e. \) up-regulated in B69 infected leaves compared to BIM2 infected leaves. These are listed in Table 2-2.

Cell growth. Twelve clones were highly similar to genes involved in cell growth (cell wall loosening and expansion): CCR339 was similar to cellulases; CCR1511, CCR113 were similar to expansins; CCR889 was similar to mannanendo-1,4-beta mannosidases; CCR571 and CCR1453 were similar to pectate lyases and CCR313 was similar to tonoplast aquaporins (TIP3). Another clone of interest, CCR575, had homology
to the early nodulin gene Enod8 (predicted cell wall localized esterase). An additional
gene represented by CCR 109, CCR959 and CCR501 had homology to a secreted cell-
wall-associated pollen-specific allergen of the ole e 1 family (SAH7).

**Giberellic acid pathway.** Two CCRs had homology to the GAST1 (GA
responsive genes of unknown function) family of genes.

**Vesicle trafficking.** Several clones had homology to proteins involved in vesicle
trafficking. For example, CCR673 had homology to a small GTPase of the Rab family
(RAB8B, Vernoud et al. 2003), and CCR1258 had homology to the beta COP protein of
the COPI complex.

**Unknown function.** Another eight clones found up-regulated had either no
significant homology to any sequences in available databases or had sequence homology
to genes of unknown function.

**Identity of cDNAs Identified as Up-Regulated by X citri Lacking pthB**

Sixteen CCRs out of forty-six were found up-regulated by *X. citri* lacking *pthB*
*i.e.* up-regulated in BIM2 infected leaves as compared to B69 inoculated leaves. These
are listed in Table 2-2.

**Cell growth.** CCR243, was the only BIM2 up-regulated gene involved in cell
wall metabolism. CCR243 is homologous to caffeic acid methyl transferases and is
involved in phenylpropanoid metabolism.

**GA pathway.** CCR 237 was homologous to cytP450 ent-keuren oxidase and
CCR105 was homologous to another cytP450 (possibly ent-kautenoic acid oxidase).

**Protein modification and stability.** For example, CCR409 had homology to
RD21a, a drought responsive cysteine proteinase, and CCR915 had homology to the
small ubiquitin modifiers (SUMO).
Transport. CCR1339 and CCR1435 were homologous to a mitochondrial import inner membrane translocase and a monosaccharide-H+ symporter, respectively.

Unknown function. Another four clones found-up regulated in BIM2 infected leaves had either no significant homology to any sequences in available databases or had sequence homology to genes of unknown function.

Northern Blot Analysis of Representative CCRs

Expression of several candidate CCR genes identified by reverse northern blot analysis was evaluated by northern blot analysis. Leaf tissue from split-leaf inoculations using B69 and BIM2 were harvested and processed for RNA extraction. Several labeled cDNA fragments were used to probe RNA blots (Figure 2-8). As in reverse northern blot analysis, clones corresponding to expansin, cellulase, SAH7/LAT52, GAST1, Enod8 and pectate lyase showed high levels of induction.

Microscopic Phenotype of B69 and BIM2 Inoculated Leaves

In order to characterize the microscopic phenotype of B69 and BIM2 infected leaves, leaf discs mock inoculated and infected with BIM2 or B69 were harvested and processed for light microscopy analysis (Figure 2-9, 2-10, 2-11 and 2-12). Leaves were pooled as fast-responding to canker when disease symptoms were fully developed by seven dpi (see figure 2-1, A and B). Leaves were pooled as slow-responding to canker when disease symptoms were fully developed by 12 to 14 dpi.

Slow-responding leaves. At 2 dpi B69, BIM2 and mock inoculated leaves looked identical at both the macroscopic and the microscopic level. At 7 dpi, while mock and BIM2 inoculated leaves showed no phenotypic signs at both the microscopic or macroscopic level (data not shown and Figure 2-9, A), the first signs of canker became visible on B69 inoculated leaves, i.e. regions of darker green color around the veins and
slight swelling. At the microscopic level, B69 leaves showed high levels of cell division occurring across all the inoculated area (Figure 2-9, compare B, C to A). Intense cell expansion and cell division phase resulted in complete filling of the air spaces of the spongy mesophyll in B69 infected leaves (Figure 2-9, compare B, C to A). The number of mesophyll cells from the abaxial to the adaxial epidermis more than doubled compared to the day 0 control or day 7 BIM2 inoculated leaves, while some cells almost tripled in size (Figure 2-10, compare A, B and D to C, and Figure 2-12). At later stages (14 dpi), increased raising of the epidermis and whitish coloration with soft or velvety appearance were observed at the macroscopic level. These phenotypes coincided with a phase of increased cell expansion (data not shown and Figure 2-11, compare A, B to C and D). While areas of cell division were still visible, a significant subset of cells became much larger and the leaf dramatically thickened (twice that of the control leaf, see Figure 2-12). A critical preliminary conclusion from these analyses indicated that the earliest visible canker phenotype was mainly due to cell division, with a moderate cell expansion, while late onset phenotypes were due to scattered but dramatic increases in cell expansion.

**Fast-responding leaves.** Macroscopic analysis indicated that cell expansion was the primary phenotype with very little cell division occurring (Figure 2-13, compare B, C and D to A). Furthermore, several areas of cell lysis, were visible immediately under the abaxial epidermis.

**Bacterial growth in B69 and BIM2 infected leaves.** Canker visible symptoms (cell division, cell expansion and resulting cell death) appeared necessary for B69 growth as very few bacteria were visible in BIM2 infected tissue 14 dpi while numerous pockets
of bacteria were seen in B69 infected tissue (Figure 2-11, compare B to C and D and data not shown).

**Discussion**

In this study, we have used macroscopic and microscopic phenotypic analysis in combination with targeted gene discovery techniques to understand how the pathogenicity factor *pthB*, of *X. c. aurantifolii* belonging to the *avrBs3/pthA* gene family elicits host-specific citrus canker symptoms in a compatible plant microbe interaction. The nonpathogenic mutant BIM2, lacking *pthB* was used in combination with the wild type strain, Xca B69, to study the specific effects of PthB on the plant cell transcriptome. A split-leaf inoculation experimental design was used to minimize leaf-to leaf variations in gene expression. In addition, bacterial cells were inoculated with high inoculum to: (1) ensure near saturation of infection sites, (2) maximize the synchronicity of the host response, and (3) artificially normalize the levels of bacterial populations (wild type and mutant) present during early infection stages of the plant leaves (up to 2dpi). In order to obtain a collection of genes potentially responsive to PthB, two complementary techniques, DD-PCR, and forward and reverse SSH, were used to enrich for: (1) transcript up-regulated when PthB is secreted in plant cells by *X citri* and (2) those up-regulated in the absence of a functional PthB. Transcript analysis of a subset of 312 clones was conducted using reverse northern blot technique. Statistical analysis was used to identify a list of forty-nine PthB responsive genes and differential regulation for a subset of these was verified by northern blot analysis. Northern blot analysis was also conducted on several CCR that did not show differential regulation by reverse northern blot analysis (Appendix B). Several of these showed differential regulation when northern analysis was used suggesting a better sensitivity than with reverse northern
analysis. This implies that the subtraction libraries contain additional CCR that need identification.

**PthB Induces Cell Division and Cell Expansion in Citrus Leaves**

When inoculated on citrus leaves, *Xanthomonas citri* pv. *aurantifolii* was able to cause cell division and cell expansion, consistent with previous reports on *pthA*-induced phenotypes (Duan et al. 1999). Quantification of the three visible phenotypes of canker *i.e.* cell division, cell expansion and the resulting thickening of the leaves was difficult due to (1) the heterogeneity of the cells in the spongy mesophyll and (2) the heterogeneity in distribution of the abundant air filled spaces in citrus leaf tissue. Therefore, as first approximation of the phenotype, quantification measurements were performed on areas where cellular activity was the most dramatic (areas of intense cell expansion, cell cycle activity and thicker leaf areas). Analysis of PthB induced symptoms over time revealed that the earliest visible phenotype associated with canker was cell division in the infected spongy mesophyll, whereas heterogeneous but massive cell expansion was observed at later stages of the infection. Interestingly, when canker developed rapidly, *i.e.* advanced canker symptoms at 7 dpi versus 12 to 14 dpi, symptoms of cell division were found to be reduced compared to slower developing canker. In addition, cell expansion was the major phenotype, primarily affecting mesophyll cells directly under the abaxial epidermis layer. This supports the hypothesis that the primary cellular mechanism affected by PthB alteration of the plant cell transcriptome is the integrity of the cell wall and the induction of cell expansion. In turn, cell division could either be: (1) a consequence of modification associated with cell expansion (*e.g.* changes in cell volume) and (2) due to a second and distinct effect of PthB. However, because cell expansion constituted a major phenotype in both rapid and slow developing canker,
induction of cell expansion may be the primary consequence of *pthB* functions in the plant cell. Furthermore, a specific set of genes with homology to genes involved in cell growth were identified as responsive to PthB.

**PthB Induces the Expression of Cell Wall Remodeling Enzymes**

In order to understand PthB-induced phenotypes on citrus leaves, we have identified a set of forty-six genes (CCRs) specifically regulated by the presence of this effector in the plant cell. Consistent with the PthB-induced morphological phenotypes, several CCRs were homologous to genes involved in plant cell wall modifications.

**Expansins.** Among these PthB-up-regulated plant genes, two were homologous to α-expansins. The role of the expansin gene family in wall loosening (polymer creep) and cell expansion has been widely documented (Cosgrove, 2000). Expansins are extracellular proteins that facilitate cell wall expansion probably by altering hydrogen bonds between hemicellulosic wall components and cellulose microfibrils (Cosgrove, 1998). These can act alone to induce cell wall extension *in vitro*, however, *in vivo* they act with a suite of enzymes capable of restructuring the plant cell wall (Cosgrove, 1998). Consistent with this, several CCRs homologous to genes associated with cell wall remodeling were also identified.

**Pectate lyases.** Among CCRs associated with cell wall remodeling, CCR571 and CCR1453 were similar to pectate lyases (PLs). These enzymes are involved in hydrolysis of wall polymers, via cleavage of de-esterfied pectin, thereby facilitating cell expansion (Carpita and Gibeaut, 1993 and Domingo et al., 1998). Although the role of bacterial secreted PLs in cell wall degradation is well known (Collmer and Keen, 1998), the role of endogenous plant PLs in development has not been extensively examined. In pollen,
plant PLs are thought to initiate the loosening of the cell wall enabling the emergence and growth of the pollen tube (Cosgrove et al., 1997). PLs also mediate cell wall breakdown in the style’s transmitting tissue, allowing penetration of the pollen (Taniguchi et al., 1995, Wu et al., 1996). Thus, induction of plant PLs by PthB can help account for aspects of the disease phenotype.

**Cellulases.** Another PthB up-regulated CCR was homologous to the cellulase family, another class of cell wall remodeling enzymes. Cellulases catalyze the cleavage of internal 1,4 β linkages of cellulose and are involved in several aspects of plant development involving cell wall modifications, including abscission, fruit softening and cell expansion (Lewis and Koehler, 1979, and Fisher and Bennet, 1991). Relevant to PthB induced phenotypes, it has been shown that constitutive expression of a poplar cellulase in *A. thaliana* led to a significant increase in cell size (Park et al., 2003).

**Beta-endo-mannanase.** In addition to CCRs homologous to expansins, PL, and cellulases, a fourth type of cell wall remodeling enzyme, a mannan endo-1,4-βD mannosidase (endo-beta-mannanase) was also identified as up-regulated by PthB. This enzyme catalyzes the hydrolysis of 1-4-βD mannosidic linkages in mannans, galactomannans, glucomannans and galactoglucoomannans (Matheson and McCleary, 1985 and Matheson, 1986) and has been implicated in cell wall weakening during anther and pollen development (Filichkin et al., 2004) and in seed ripening where it is involved in mobilization of the mannan-containing cell walls of the tomato seed endosperm (Mo and Bewley, 2003).

**Caffeic acid methyl transferase.** Only one gene involved in cell wall metabolism was down regulated by PthB, CCR243. This clone was homologous a caffeic acid methyl
transferase (COMT), belonging to the phenylpropanoid pathway that leads to lignin biosynthesis. Its expression has been shown to be regulated by biotic and abiotic elicitors including infection by avirulent and virulent bacteria (Toquin et al., 2003). It is possible that down-regulation of this enzyme relates to down-regulation of defense responses by down-regulation of lignin deposition. This event could occur due to alterations in the lignin content or composition. COMT down-regulation is in accord with the cell expansion induced by PthB since mature walls lack acid-induced extension (Cosgrove, 1989). It is also interesting that fully expanded mature leaves are more resistant to canker, whereas young leaves (one half to two-third expanded) are the most sensitive ones (Graham et al., 2004). This is consistent with the hypothesis that PthB targets the cell wall, inducing cell expansion ultimately resulting in disease progression.

A synthesis of our results indicates that type III effector PthB triggers the up-regulation of an array of proteins whose combined activities induce cell wall loosening and cell expansion. The roles of expansins, PLs and cellulases in cell wall loosening have been shown to be complementary in other systems (Cosgrove et al., 1998; Carpita and Gibeault; 1993, Domingo et al., 1998, Inouhe and Nevins, 1991).

**Enod8 and SAH7/LAT52 are a Link Between Canker Symptoms Development and Nodule Organogenesis and Pollen Tube Growth Respectively**

Two additional classes of CCRs (CCR575 and CCR109, 959 and 501) identified as up-regulated by PthB also support the theory that this effector targets cellular growth. The first one, CCR575, was homologous to *Enod8*, an early nodulin gene associated with the development of rhizobial nodule structures prior to nitrogen-fixation (Dickstein et al., 1988, 1993). *Enod8* has sequence similarity to exopolygalacturonase and lanatoside 15’-O-acetylesterase (Pringle and Dickstein, 2003). Intriguingly, the up-regulation of *Enod8*
in response to \textit{X. citri} and \textit{Rhizobium} suggests some common steps between nodule formation and canker pustule formation. This is also supported by the fact that both infections trigger cellular reprogramming events that lead to cellular growth. The function of Enod8 is unknown, but \textit{in-vitro} characterization and sequence analysis predict that it is a cell wall localized esterase with acetylated oligo- or polysaccharides as substrates (Pringle and Dickstein, 2004). Thus the enzymatic activity of Enod8, its cell wall localization and involvement in both nodule and canker pustule formation point to its involvement in modification of cell wall components during cellular growth.

The second class of CCRs reinforcing the hypothesis that the cell wall is the target of PthB, displayed homology to \textit{SAH7} and \textit{LAT52} genes encoding for members of the ole I family of proteins. Originally identified as pollen allergens, members of this family have also been found expressed in other tissues (e.g. \textit{SAH7} in leaves). A recent study of one homologue, \textit{LAT52} (tomato), indicates that these genes may be involved in controlling hydration and pollen tube growth (Tang et al., 2002). \textit{LAT52} interaction with the pollen receptor kinase LePRK2 (LRR kinase) led to the hypothesis that binding of \textit{LAT52} initiates a signal transduction pathway required for pollen germination and pollen tube growth (Tang et al., 2002 and Johnson and Preuss, 2003). The up-regulation of a \textit{LAT52}-like gene in canker might, therefore, be part of a signaling pathway leading to cell growth (the phenotype of both canker and pollen tube). Interestingly, pollen tube growth, which occurs by tip extension, involves expansion and deposition of cell wall precursors at the growing tip and requires the concerted action of endo-beta-mannanase, expansins and pectate lyases (Marin-Rodriguez et al., 2002, Cosgrove, 1998 and Filichkin et al., 2004), also found up-regulated during canker symptoms development.
PthB Induces Up-Regulation of a Tonoplast Aquaporin

CCR313, identified as up-regulated by PthB, displayed sequence similarity to a tonoplast aquaporin of the TIPs family (Maurel, 1997 and 2002, and Hill et al., 2004). Besides cell wall loosening, expansion requires extensive solute and water uptake resulting in the formation of a prominent vacuolar compartment. This maintains the turgor pressure that drives cell expansion (Veytsman and Cosgrove, 1998). Expansion is thought to require high hydrolic permeability of the tonoplast in order to support water entry into the vacuole, and tonoplast aquaporins (TIPs) play a critical role in this process (Ludevid et al., 1992; Chaumont et al., 1998). TIPs are enriched in zones of cell expansion (Tyerman et al. 2002) as well as in zones of active cell division where their up-regulation is linked to vacuole biogenesis (Marty, 1997). Whether the identified tonoplast aquaporin is indeed a marker for cell division and/or is actively involved in driving the cellular expansion is unknown.

PthB Induces Up-Regulation of Two Components Involved in Vesicle Trafficking

Cell expansion and cell division both require deposition of new wall components into the extending cell walls (Veytsman and Cosgrove, 1998) or into the cell plate of dividing cells (Staehlin and Hepler, 1996 and Samuels et al., 1995). This may be achieved via secretory processes involving vesicle trafficking. However, most genes identified here suggest that in response to canker, cell walls are mainly extended without the building of new cell wall components. This would imply that walls become thinner as cells expand. This indeed has been observed at late stages of canker (Figure 2-10 compare A, B to C and D). Although plant cell walls generally appear not to become thinner as they extend (Veytsman and Cosgrove, 1998), expansion without new cell wall deposition could be at the origin of the cell lysis observed in advanced canker stages.
Several CCRs identified as up-regulated by PthB are involved in vesicle trafficking. Among these, CCR673 and CCR1258 have homology to RAB8B and beta COP respectively. RAB8B is a member of the small GTPase gene family. The yeast homologue of Rab8 (also named RABE see Vernoud et al., 2003) regulates membrane trafficking to the daughter cell bud site (Salminen and Novick, 1987 and Goud et al., 1988). Interestingly, in tomato, members of this subfamily appear to be targeted by the Pseudomonas avirulence factor, AvrPto. This implies that in susceptible plants, AvrPto may interfere with membrane trafficking pathways (Bogdanove and Martin, 2000). It has been suggested that RAB8B might be involved in polarized secretion of antimicrobial compounds (Bogdanove and Martin, 2000).

In mammals, beta COP belongs to a large complex that coats COPI vesicles (Kreis et al. 1995). COPI vesicles transport membrane proteins and soluble molecules in a retrograde, and possibly anterograde, direction through mammalian Golgi stacks (Nickel and Wieland, 1997 and Harter, 1999). In plants little is known about COPI vesicles. Recent evidence suggests that COPI-like vesicles are functional in plant secretion and localize mainly to the Golgi apparatus as well as to the cell plate of dividing cells (Couchy et al, 2003).

**Hormone Pathways are Possibly Involved in Canker Symptoms Development**

Triggering of cell expansion as well as induction of expansins and pectate lyases constitutes a common point between the effect of *pthB* on citrus (this study) and that of the avirulence effector *avrBs3* on susceptible pepper plants (Marois et al., 2002). Cell expansion induction by both effectors share similar features; however, several plant auxin-induced proteins of the SAUR family were found up-regulated by *avrBs3* (Marois et al. 2002). Several clones identified as *pthB* responsive are regulated by auxin in other
systems. These include the expansins (Catala et al., 2000, Civello et al., 1999, Hutchison et al., 1999) and the pectate lyases (Domingo et al., 1998). In the pepper model, one of two identified α-expansins was found up-regulated by exogenous application of auxin; whereas a second expansin as well as a pectate lyase were not (Marois et al., 2002). These data suggest that an auxin-independent pathway might operate under certain conditions leading to cell expansion. In addition to a possible role of auxin in canker disease, there is evidence for the involvement of the gibberellic acid signaling pathway in the plant response to \( pthB \). CCRs with homology to ent-kaurenoic acid oxidase and possibly to ent-kaurene oxidase (KO) (of GA biosynthetic pathway) (Oszewski et al., 2002) and two clones with homology to the GAST1 family (GA induced genes) were identified. Interestingly the GAST1 homologues were up-regulated by \( pthB \); whereas the putative KO and KAO were down-regulated. This may be explained by feed-back regulation of KAO and KO expression by GA. However, feedback regulation of several enzymes of the GAs biosynthetic pathway has been described in other systems, it has not been reported to occur in the case of KAO and KO (Olszewski et al., 2002).

GA is known to regulate TIPs (Phillips and Huttly, 1994, Ozga et al., 2002), expansins (Oka et al., 2001, Vogler et al., 2003, Lee and Kende, 2002, Chen and Bradford, 2000), GAST1-like genes (Kotilainen et al., 1999 and Aubert et al., 1998), endo-beta-mannanase (Dutta et al., 1997, Yamaguchi e al., 2001) and cellulases (Litts et al., 1990). Therefore, \( PthB \) may act on regulatory steps upstream of GA biosynthesis. The involvement of GA does not preclude that auxin is also involved since the latter is able to regulate the production of the bioactive \( GA_1 \) in elongating shoots (Ross et al., 2000).
Indeed, these two hormones are known to, in concert, promote cellular division and elongation (Cleland, 2001 and Davies, 1995).

**Conclusions and Future Prospects**

The tight relationship between cell division and cell expansion makes it difficult to address the question of whether cell expansion or cell division are the cellular pathways that are altered as a downstream consequence of PthB regulating the plant cell transcriptome. However, the following results presented here support the hypothesis that cell wall loosening and expansion is the major plant cellular mechanism targeted by PthB: 1) cell expansion occurs whether canker symptoms develop rapidly or slowly, 2) genes involved in cell expansion have been identified as responsive to PthB, 3) cell expansion is triggered by one another member of the \textit{avrBs3/pthA} gene family and 4) PthB responsive genes are involved in cell growth.

Microscopic analysis of leaves showing a slow canker symptom development indicated that cell division is the major visible phenotype in initial infection stages, while cell expansion remains at a moderate level. During the late infection stage however, cells dramatically expanded leading to areas of cell lysis. It is possible that PthB induces cell expansion and cell division by targeting several distinct cellular mechanisms. Another hypothesis is that PthB targets cell expansion by altering cell wall composition (loosening). This in-turn leads to a cell autonomous response that mainly involves the triggering of cell division in the early stages and massive cell wall loosening and expansion in later stages. The concentration of bacteria surrounding infected cells and, therefore, the concentration of PthB protein secreted into the plant cells as well as the physiological state of the infected tissue (for example immature expanding leaves will readily expand) would modulate this response. When the concentration of PthB is low,
moderate expansion and the subsequent change in cell volume would lead to cell
division, while in later stages, elevated concentrations of PthB would lead to gross cell
expansion and cell lysis (Figure 2-14).

The relationship between cell expansion and cell division in plant growth and
development remains controversial. Whether growth starts by an increase in cell size,
triggering division, or whether division occurs first followed by restoration of the original
cell size (Foard, 1971 and Cleland, 2001) is mainly unknown. Studies on leaf primordial
(LP) initiation may begin to resolve this issue. Initially, since the first visible sign of a
new LP is a periclinal division in the L1 or L2 layer of the shoot apical meristem, it was
suggested that division occurs first (Steeves and Sussex, 1989). However, recent evidence
indicates that cell enlargement is the first step in LP initiation since LPs can be induced
by adding expansins either by microinjection of by up-regulation of expansin transcripts
a similar pattern where cells expand first and then divide in response to expansion.

The canker phenotype is necessary for optimal growth and dispersal of X. citri
(Swarup et al., 1991 and this study); therefore, induction of cell division and or expansion
are key steps in canker disease development and, unlike AvrBs3 for Xcv, PthA/B confer a
benefit to X. citri strains carrying it. The PTHA/B family of pathogenicity effectors may
prove to be a valuable tool in dissecting the molecular events surrounding microbe-
induced diseases since they are required for pathogenesis and can induce canker
symptoms alone. Finally, an understanding of the mechanisms by which PthB induces
canker phenotypes could help unravel the intricate relationship between cell division and
cell expansion that occurs in plant development.
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Table 2-2: List of CCRs confirmed by reverse northern blot analysis.

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*: A positive ratio indicates up-regulation in B69 infected tissue compared to BIM2 infected tissue. A negative ration indicates up-regulation in BIM2 infected tissue compared to B69 infected tissue.
Figure 2-1: Phenotype of B69 and BIM2 infections on grapefruit leaves. BIM2 lacks PthB and induces formation of very small pustule like structures, reminiscent of canker pustules, at the edges of some inoculated areas. (A), (C) are BIM2 (pUFR004::pthB) inoculations on grapefruit leaves and (B), (D) are wt B69 inoculations. Pictures were taken 7 days post inoculation.
Figure 2-2: Late B69 and BIM2 phenotypes. (A) BIM2 inoculated leaves 30 dpi and (B) B69 inoculated leaves 30 dpi. Note the much attenuated phenotype of BIM2 infected leaves.
Figure 2-3: Quantification of bacterial population two days post inoculation with B69 and BIM2. (cfu: colony forming unit), Exp1: experiment 1, Exp2: experiment 2).
Figure 2-4: Diagram of PCR-Select cDNA subtraction. Type e molecules are formed only if the sequence is up-regulated in the tester cDNA. Solid lines represent the Rsa I-digested tester or driver cDNA. Solid boxes represent the outer part of the Adaptor 1 and 2R longer strands and corresponding PCR primer 1 sequence. Green boxes represent the inner part of Adaptor 1 and the corresponding Nested PCR primer 1 sequence; red boxes represent the inner part of Adaptor 2R and the corresponding Nested PCR primer 2R sequence.
Figure 2-5: Distribution of potential citrus canker responsive genes.
Figure 2-6: Distribution and origin of the clones stamped on the nitrocellulose membranes used in reverse northern blot analysis.
Figure 2-7: Cluster analysis of genes differentially regulated by PthB. In green are genes down-regulated by PthB, and in red are genes up-regulated by PthB.
Figure 2-8: Northern blot analysis of CCR genes found differentially regulated by reverse northern blot analysis. rRNA was used as control for loading.
Figure 2-9: Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB). 7dpi BIM2 infected leaves, A; 7dpi B69 infected leaves, B, C. By In canker-infected tissue, by 7 dpi, air spaces of the spongy mesophyll are almost inexistent. These spaces are replaced by new divided cells as well as by cell of larger size, resulting in thickening of the leaves.
Figure 2-10: Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB). 7dpi BIM2 infected leaves, C; 7dpi B69 infected leaves, A, B, D. At 40X magnification, pockets of bacterial cells are visible surrounding mesophyll cells of B69 infected tissue while almost no bacteria is present in BIM2 infected tissue. Also not the areas of cell lysis in B69 infected tissue.
Figure 2-11: Microscopic phenotype of leaves inoculated with B69 (wt) and BIM2 (nonpathogenic mutant lacking PthB) at 14 dpi. A, B: BIM2, and C, D:B69 infected leaves. Note high levels of bacteria in B69 infected leaves compared to BIM2 infected leaves, as well as possible wall thinning of cells in B69 infected tissue.
Figure 2-13: Quantification of leaf thickening and cell division during B69 and BIM2 infection on Duncan grapefruit leaves. These measurements were taken on “slow canker-developing” leaves, i.e. leaves showing high rate of cell division when inoculated with B69. The number of cells from abaxial epidermis to adaxial epidermis was calculated by counting the number of cells that a virtual line perpendicular to the epidermal layers would cross. Ten lanes were used in the analysis and the number shown are averages.
Figure 2-13: Microscopic symptoms of rapidly developing canker. 14dpi BIM2 infected leaves, A; 14dpi B69 infected leaves, B, C, D. Note the highly enlarged cells, the large areas of cell lysis and the absence of high rate of cell division in B69 infected tissue.
Figure 2-14: Possible model for PthB effects on susceptible citrus cell showing parallel pathways activating cell division and expansion.
CHAPTER 3
CHANGES IN SUMO CONJUGATION ARE ASSOCIATED WITH CITRUS CANKER DISEASE

Introduction

Citrus canker is an important disease of citrus worldwide (Civerolo, E., 1984). It is caused by several pathovars of *Xanthomonas citri*, which differ mainly in their host range (Shubert et al, 2001, Verniere et al, 1998). Canker infections cause defoliation, fruit blemishes, premature fruit drop and tree decline, resulting in severe economical losses (Shubert et al, 2001). Considerable international regulatory efforts are implemented to prevent the spreading of the already quarantined pathogen, with negative effects on national and international trade of citrus (Timmer et al, 1996; Shubert et al, 2002).

Canker symptoms are characterized by erumpent corky lesions that can affect all aerial parts of citrus trees (Shubert et al, 2002). Microscopy studies showed that canker lesions result from hyperplasia (cell division) and hypertrophy (cell expansion) in the spongy mesophyll tissue, where the bacteria contact plant cells (Swarup et al, 1991; Duan et al, 1999 and Chapter 2). Ultimately, this intense increase in cellular growth ruptures the epidermis and causes necrosis. The rupture of the epidermis is thought to be crucial for bacterial dissemination and spread of the disease (Graham and Gottwald, 1991; Duan et al, 1999).

A crucial step towards understanding citrus canker disease was the identification of a pathogenicity gene, *pthA*, required by *X. citri* pv. citri to cause canker on citrus (Swarup et al., 1991). Since then, all canker-causing strains have been shown to carry at least two
members of the \textit{pthA} gene family, with one copy sufficient for most or all pathogenicity (Yang and Gabriel, 1995; Al-Saadi and Gabriel unpublished). \textit{pthA}, found in \textit{X. citri} pv. \textit{citri} (\textit{Xcc}) of the Asiatic group of strains, and \textit{pthB}, found in \textit{X. citri} pv. \textit{aurantifolii} B69 (\textit{Xca}) of the South American group, have been shown to be interchangeable in their ability to elicit canker (Yuan and Gabriel, unpublished). As for \textit{pthA} of \textit{Xcc}, \textit{pthB} of \textit{Xca} was also shown to be required for pathogenicity on citrus (Yuan and Gabriel, unpublished, and Chapter 2), and therefore, the B69 derivative mutant strain BIM2 lacking \textit{pthB} does not elicit the typical macroscopic symptoms associated with canker disease (Chapter 2). When transferred to other xanthomonads carrying a functional type III secretion system (TTSS), or transiently expressed in leaf cells, \textit{pthA} was found to induce cell division, cell expansion, and rupture of the epidermis - the three most prevalent canker symptoms (Swarup et al, 1991 and 1992; Duan et al, 1999). It was therefore concluded that \textit{pthA} alone was able to cause canker-like symptoms and that its delivery into the plant cell relies on a functional TTSS.

Members of the \textit{pthA} gene family are also found in non-canker causing strains of \textit{Xanthomonas}. Examples of genes belonging to this gene family include \textit{avrBs3} and \textit{avrBs3-2} of \textit{Xanthomonas campestris} pv. \textit{vesicatoria} (Bonas et al, 1989, and Bonas et al, 1993), \textit{avrXa10} and \textit{avrXa7} of \textit{Xanthomonas oryzae} pv. \textit{oryzae} (Hopkins et al, 1992); along with \textit{avrB4}, \textit{avrb6}, and \textit{avrb7} of \textit{Xanthomonas campestris} pv. \textit{malvacearum} (De Feyter and Gabriel, 1991 and 1993). Proteins encoded by members of this gene family are 90 to 97% similar and are characterized by several structural features essential for their function in avirulence and/or pathogenicity. Such features include 1) nearly identical 102-bp tandem repeats in their center, 2) C-terminal nuclear localization signals (NLS),

Little is known about how canker disease is initiated in planta. In order to understand the molecular mechanism underlying canker, a differential display PCR experiment was conducted to identify plant genes potentially responsive to canker (Chapter 2). At two days post inoculation (dpi), transcripts were compared between leaves inoculated with B69 and leaves inoculated with BIM2 (B69 derivative carrying a non-functional pthB). One clone was related to AtSUMO1 from Arabidopsis. SUMO belongs to the ubiquitin family of proteins that are conjugated to target proteins; however; its functions are distinct from those of ubiquitin.

SUMO conjugation has been shown to be an important regulatory step in processes such as protein stability, subcellular localization, and response to various stresses. SUMOylation is carried out in a ATP-dependant reaction cascade similar to the E1-E2-E3 reactions responsible for ubiquitin conjugation (Melchior F., 2000; Kim et al, 2002; Kurepa et al, 2003). In addition, SUMO modification has been shown to be important for cell cycle progression in yeast. Specifically, temperature-sensitive mutants lacking a functional SUMO conjugation pathway have been shown to arrest at the G2/M transition (Seufert et al, 1995; Johnson and Gupta, 2001). Such work is of interest, as Xanthomonas citri infection triggers division of mesophyll cells contacted by the bacteria.

Recent work has shown that strains of the phytopathogenic bacterium Xanthomonas campestris pv. vesicatoria encode at least two type III effectors with demonstrated SUMO protease activity (Hotson et al, 2003; Roden et al, 2004). Though
loss of these SUMO protease-like effectors did not affect pathogenicity on susceptible plants, it raises the possibility that the plant SUMO conjugation pathway could be targeted during infection by *X. c. vesicatoria* (Hotson et al, 2003; Roden et al, 2004).

This study indicates that: 1) changes in plant protein SUMOylation profiles occurred after host infection by *Xanthomonas citri pv. aurantifolii*, 2) these changes in SUMOylation profiles were of two types, gene *pthB*-dependent and independent, and 3) these changes in SUMOylation profiles did not occur following challenge with a non-pathogenic mutant strain lacking a TTSS. Together, these data indicate that the TTSS of *Xca* delivers one or more effectors that directly, or indirectly, de-conjugate SUMO from host proteins *in vivo*.

**Materials and Methods**

**Plant Inoculations**

All inoculations were done with needle-less syringes on the abaxial surface of the leaf. Plants (*Citrus paradisi* ‘Duncan’ grapefruit) were grown under greenhouse conditions. Inoculations involving strains B69 and its derivatives were carried out in BL-3P level containment (refer to Federal Register Vol.52 no 154, 1987) at the Division of Plant industry, Florida Department of Agriculture, Gainesville, FL. For inoculation, bacterial suspensions were standardized in sterile 10mM CaCO₃ (mock) to an optical density of 0.5 and pressure-infiltrated. For phenotypic observation, inoculations were repeated at least three times. For protein extraction, a split leaf inoculation scheme was followed to normalize differences due to physiological state of inoculated tissue. For each combination of treatments (i.e. mock/B69 and mock/BIM2), one treatment was inoculated on the right side of the mid-vein and the other strain on the left side of the
mid-vein. For each split-leaf experiment three trees were used, with an average of 10 leaves inoculated per tree (approximately 5 leaves per treatment combination).

**Bacterial Strains and Culture Media**

Bacterial strains and plasmids used in this study are listed in Table 1 Appendix A. All *Xanthomonas* strains were cultured in PYGM medium at 30°C (De Feyter et al. 1990). *Escherichia coli* were grown on Luria-Bertani (LB) medium (Sambrook et al., 1990). For culture on solid media, agar was added at 15 g/L. Antibiotics were used at the following concentrations: Spectinomycin (Sp), 35 mg/L; Kanamycin (Kn), 12.5 mg/L; Chloramphenicol (Cm), 35 mg/L; Gentomycin (Gt), 3 mg/L.

**Marker Integration Mutagenesis**

*hrpG* gene knock-out mutation was generated by triparental matings (as described in Chapter 1). Briefly, a 550 bp internal fragment of *hrpG* was cloned in the suicide vector pUFR012 [derivative of pUFR004 carrying kanamycin resistance (Gabriel laboratory, unpublished)] creating pBY23. Transconjugants resulting from *E. coli* DH5α/pBY23, DH5α/pRK2013 (helper plasmid) and B69 matings were selected on spectinomycin to select against *E. coli* and chloramphenicol and kanamycin to select for plasmid insertion events. Putative transconjugants were purified to a single colony, and Southern hybridization was used to confirm the integration of suicide vector pBY23 in *hrpG*.

For complementation purposes, a *HindIII* to *KpnI* fragment was cloned out of plasmid pXG8 (REF) and recloned in pUFR053 (Yuan and Gabriel, unpublished) creating pBY24. DH5α/pBY24 was used in triparental matings to create B23.5/pBY24 (B23.5c and B23.5c1). Putative exconjugants were purified to a single colony, and
Southern hybridization was used to confirm the presence of the complementation plasmid. Total DNA extractions were performed as described in Gabriel and De Feyter (1992). Southern hybridizations were performed as described by Lazo and Gabriel (1987).

**Bioinformatics**

Alignments and box shading were carried out using Clustal W (http://clustalw.genome.jp).

**Protein Extraction and Western Blotting**

Citrus leaf tissue was harvested at 0, 2 or 7 days post inoculation (dpi), depending on the experiment, and ground to a fine powder in liquid nitrogen. Soluble proteins were extracted in two volumes of extraction buffer (50mM Tris, pH = 8.0, 300mM sucrose, 2mM EDTA, 0.3% DIECA, 10mM N-ethylmaleimide, 1µg/µl pepstatin, 1µg/µl leupeptin, and 7.5% w/v PVPP). Extracts were vortexed and briefly sonicated, then clarified by two rounds of centrifugation at 16,000 x g for 10 min at 4°C. Soluble proteins were quantified by the BCA assay (Pierce Biotechnology, Rockford, IL).

Proteins were separated by polyacrylamide electrophoresis on a 15% Tris-Tricine gel, and transferred to PVDF membrane (Millipore, Bedford, MA). For immunoblot analysis, membranes were probed with 1:2,500 immunopurified polyclonal PopSUMO1 (gi:23997054) antiserum (Cocalico, Reamstown, PA) diluted in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.4 mM K2HPO4, 10.1 mM Na2HPO4, pH 7.4) containing 0.1% Tween 20 (T-PBS) with 1% v/v goat serum (Sigma, St. Louis, MO). The antibodies were raised against purified PopSUMO1, which also contained an additional N-terminal hexahistidine tag generated by PCR (Reed, J., Master’s Thesis University of Florida, 2005). For secondary antibody, the membranes were probed with 1:25,000 horseradish
peroxidase conjugated donkey anti-rabbit secondary antibodies (Amersham, Buckinghamshire, England) diluted in 1X T-PBS. Chemilluminescence was carried out according to the manufacturer’s instructions using the ECL plus (+) kit (Amersham). Following chemilluminescence, each membrane was rinsed in 1X T-PBS and stained with Coomassie R250 as a loading control.

Results

SUMO Conjugation Profiles are Altered in *X. citri*-Infected Leaves

The grapefruit partial cDNA, CCR915 was identified by differential display as being canker responsive. Following reverse northern blot analysis, CCR915 which shows homology to SUMO, was found up-regulated in leaves inoculated with BIM2 (lacking *pthB*) compared to leaves inoculated with B69 (wt) (Chapter 2). To determine if shifts in SUMO transcript abundance reflected regulation at the protein levels, a split-leaf experiment was conducted in which Duncan grapefruit leaves were mock inoculated on one side of the mid-vein, and *Xanthomonas citri* pv. aurantifolii strain B69 was inoculated on the other side. Soluble extracts taken from canker or mock –inoculated leaves were probed for CitSUMO and CitSUMO-conjugated proteins using PopSUMO1 antibodies. The grapefruit sequence was highly similar to poplar SUMO isoform PopSUMO1 (gi:23997054) (Figure 3-1) and as expected, the grapefruit SUMO and its protein conjugates cross-reacted with antibodies raised against PopSUMO1. Using anti PopSUMO1, it was found that at two days post inoculation, the profile of SUMO conjugation is noticeably altered (Figure 3-2). The amounts of free CitSUMO and high molecular weight CitSUMO conjugated proteins were higher in B69-infiltrated leaves as compared to mock-infiltrated leaves.
SUMO Conjugation Profiles in Infected Leaves are Partially PthB Dependent

To determine if SUMOylation patterns were associated with disease symptom development, a split leaf inoculation experiment was conducted and the effects of three separate treatments examined over time. Split-leaves were mock infiltrated, or inoculated with wild type strain B69, or the non-pathogenic mutant strain BIM2, which lacks *pthB*. At 0, 2, and 7 dpi, half-leaves were harvested and soluble proteins examined by western blot analysis.

SUMO profiles of leaves inoculated with B69 were compared to those of leaves inoculated with mutant BIM2 at two dpi. There were no changes in the abundance of free CitSUMO or SUMOylated proteins in BIM2 inoculated leaves (Figure 3-3, lane 4 and 5). The expected changes were seen in leaves inoculated with B69, *i.e.* an increase in the amount of free SUMO and SUMO-conjugated proteins (Figure 3-3, lane 7 and 8).

SUMO profiles at 7 days post inoculation revealed that the majority of the high molecular weight conjugates seen at 2 dpi in canker infected leaves were lost (Figure 3-3, lane 8 and 9). Interestingly, this loss of high molecular weight conjugates was also observed in leaves inoculated with non-pathogenic mutant strain BIM2. Whether the identities of SUMOylated proteins in canker infected leaves are similar to the ones in BIM2 infected leaves is unknown; however, in both cases, SUMO de-conjugation occurred 7 dpi. These findings suggest that the SUMO de-conjugation observed at 7 dpi, in both BIM2- and B69-inoculated leaves is PthB-independent and is also independent of the development of the macromolecular disease symptom of canker (Figure 3-4). Conversely, the increase in the amounts of free SUMO and SUMO-conjugated proteins seen at 2 dpi with B69 are PthB-dependent.
SUMO De-Conjugation Observed at 7 days Following Infection with B69 and BIM2 is Dependent on a Functional Type III Secretion System

To determine if the SUMO de-conjugation observed at day 7 post inoculation in both B69- and BIM2-inoculated leaves is dependent on a functional type III secretion system, a *hrpG* integrative mutant, B23.5, was generated. B23.5 was no longer pathogenic on citrus, and the *hrpG* phenotype was complemented after transformation of B23.5 with pUFR057::Xcv*hrpG* (Figure 3-5).

There was no SUMO de-conjugation at day 7 following inoculation with B23.5 (Figure 3-6), indicating that SUMO de-conjugation relies on a functional TTSS. In addition, B23.5 inoculation stimulated accumulation of a 45kDa SUMO conjugate. A SUMOylated product of similar size was observed in leaves inoculated with B69 and BIM2, but did not accumulate (Figure 3-3).

**Discussion**

A great deal of effort has been directed towards investigating the mechanisms by which plants mount defense responses towards pathogenic bacteria. Most studied cases involve incompatible plant microbe interactions that lead to the classical hypersensitive response or HR (Malek et al., 2000; Kazan et al, 2001). However, far less effort has been invested in trying to elucidate the mechanisms by which a specific pathogen, or a group of pathogens elicit a particular disease with specific sets of morphological and molecular symptoms. In an effort to understand the processes by which different pathovars of *Xanthomonas citri* trigger canker symptoms, a canker responsive gene with sequence similarity to the *SUMO* gene family was identified by differential display PCR.

The SUMO conjugation pathway in canker disease was investigated using a split-leaf inoculation experiment to normalize for leaf-to-leaf variations. It was found that at 2
dpi, *X. citri* pv. aurantifolii infection induces an increase in free CitSUMO and an increase in the number of high molecular weight SUMOylated proteins. These changes were not observed in mock-inoculated leaves. SUMO conjugation in plants and other systems has been shown to be up-regulated by various instances of biotic and abiotic stresses (Kurepa et. al., 2003, Lois et. al., 2003 and O’Donnell et. al., 2003).

In order to test if changes in SUMO conjugation observed were specific to *X. citri* pv. aurantifolii infection, two mutant strains unable to cause canker on citrus were used in this study, BIM2 (interrupted in pathogenicity gene *pthB*) and B23.5 (interrupted in the TTSS regulatory gene, *hrpG*). Disruption of *hrpG* was previously shown to disable the type III secretion system in *Xanthomonas* (Wengelnik et al. 1996). Using split leaf inoculations, it was shown that in BIM2 inoculated leaves, at 2 dpi, there were no changes in the amount of free SUMO and SUMOylated high molecular weight proteins. Thus, the increase in free SUMO and in the number of SUMOylated proteins is likely to be a PthB-specific plant response rather than a general stress response. A large number of SUMO targets identified in other organisms are cell-cycle related (Melchior, 2000). It has been shown in yeast (*Saccharomyces cerevisiae*) that temperature-sensitive mutants corresponding to SUMO and the enzymes involved in its conjugation pathway arrest the cell cycle at the G2/M transition, therefore, showing a critical role for SUMO in cell cycle progression (Johnson and Gupta, 2001). It is possible that the observed up-regulation of SUMOylated proteins and free SUMO reflects activation of the plant cell cycle by *X. c. pv. aurantifolii* in the early stages of infection. Remarkably, this increase in free SUMO and in the amount of high molecular weight SUMOylated proteins is lost 7 dpi, potentially indicating a transition to a second disease phase. The deconjugation
phenotype observed at 7 dpi with B69 is also observable at 7 dpi with BIM2, and therefore, the triggering factor of de-conjugation is probably independent of PthB.

The possibility that another effector could be the trigger of the de-conjugation observed at 7 dpi came from the finding that the TTSS mutant B23.5, did not induce de-conjugation. Therefore it is possible that another type three effector, beside PthB is responsible for the de-conjugation observed at day 7. Alternatively, it is possible that the second PthA homologue, PthB0 (not required for canker, Chapter 2), found in B69 and BIM2 is also able to trigger the de-conjugation observed 7dpi.

It has been proposed that the abundance of SUMO proteases in *X. campestris* pv. vesicatoria could reflect an important role of theses effectors in *Xcv* pathogenesis (Hotson et al. 2003 and Roden et al 2004). However, none of the identified proteases have been implicated in disease and are, in fact, dispensable. Given the critical role of SUMO conjugation in cell cycle processes (Melchior, 2000), and the lack of apparent SUMO proteases encoded by another canker causing strain *X. citri* pv. citri it is possible that the late de-conjugation phenotype is not directly triggered by a type III effector of a protease nature, but rather that a type III effector(s) acts to induce endogenous citrus SUMO protease(s) leading to the de-conjugation observed in late stages of canker infection.

Both hypotheses are not mutually exclusive and characterization of additional *X. citri* effectors as well as citrus proteins SUMOylated in response to canker are required to better characterize the involvement of SUMOylation in the infection process of canker causing xanthomonads.
<table>
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<tr>
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<th>popSUMO1</th>
<th>GfSUMO/CCR915</th>
<th>AtSUMO1</th>
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<td>MSEATGQPQEBDKWPDQGSSAHINLKVQGDGNEVFVFRIRSKRSTQQLKLMNAYCDRQVEIN</td>
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<td>GfSUMO/CCR915</td>
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</tr>
<tr>
<td>AtSUMO1</td>
<td>SIAFLFDQRLRAEGTFDELDMDGEIDAMHLQGTGGAVKASDYA</td>
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<td>56</td>
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Figure 3-1: Alignment of grapefruit SUMO (partial sequence) with (PopSUMO1, gi:23997054, and AtSUMO1, At4g26840).
Figure 3-2: SUMO profiles of B69- and mock-challenged grapefruit leaves. 10μg of crude protein from day 2 of the split leaf experiment was separated by electrophoresis, blotted to PVDF and (A) probed with purified PopSUMO1 antisera. Lane 1, Mock treated leaf; lane 2, B69 inoculated leaf; lane 3, 2 ng purified recombinant PopSUMO1. ([]) high molecular weight SUMOylated proteins. (→): un-conjugated SUMO. (B) The membrane was stained with Coomassie R250 as a loading control (Shown is the small subunit of Rubisco).
Figure 3-3: SUMO de-conjugation occurs 7 days after infection. Leaves were inoculated with Mock, BIM2, and B69 strains. 7.5 µg of crude protein from 0, 2, and 7 dpi from each treatment of the split leaf experiment was separated by electrophoresis, blotted to PVDF and (Upper panel) probed with purified PopSUMO1 antiser. ([[]]): high molecular weight SUMOylated proteins. (→): un-conjugated SUMO. (Lower panel) The membrane was stained with Coomassie R250 as a loading control (Shown is the small subunit of Rubisco).
Figure 3-4: Split leaf inoculation of *Xanthomonas citri* pv. aurantifolii (B69) and derivative BIM2 mutant. Duncan grapefruit leaf 7 dpi with B69 (shown on the left side of the mid-vein and BIM2 (shown on the right side of the mid-vein). (A) adaxial side and (B) abaxial side of the leaf. Note the whitish canker characteristic of the Xca strain and yellowing associated with the day 7 post inoculation canker phenotype. (C) Advanced B69 canker phenotype.
Figure 3-5: B69 mutant derivative B23.5 lacks a functional Type III secretion system. (A) Southern blot hybridization profiles contrast B69, B23.5 and B23.5c (B23.5/hrpG). DNA was digested with HindIII and probed with the same internal fragment of hrpG used as homology region for marker interruption. (B) B69 and B23.5c inoculation on Duncan grapefruit. hrpG complemented the hrp⁻ phenotype of B23.5
Figure 3-6: SUMO de-conjugation at 7 dpi requires a functional TTSS. Leaves were inoculated with B23.5 and B69 strains. 7.5 µg of crude protein from 0, 2, and 7 dpi from each split leaf treatment was separated by electrophoresis, blotted to PVDF and (A) probed with purified PopSUMO1 antisera. ([[]): high molecular weight SUMOylated proteins. (→): un-conjugated SUMO. (*) novel 70kDa protein unique to B23.5 7 dpi leaves. Equal amounts of protein was loaded in each lane.
## APPENDIX A
### LIST OF PLASMIDS AND STRAINS

Table A-1: List of strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<td><strong>Escherichia coli</strong></td>
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<td>DH5α</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;, endA1, hsdR17(α&lt;sup&gt;+&lt;/sup&gt;m&lt;sub&gt;K&lt;/sub&gt;'), supE44, thi-1, recA1, gyrA, relA1, ϕ80lacZΔM15, Δ(lacZYA-argF)U169</td>
<td>Gibco BRL, Gaithesburg, MD</td>
</tr>
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<td>HB101</td>
<td>supE44, hsdS20(α&lt;sup&gt;+&lt;/sup&gt;m&lt;sub&gt;K&lt;/sub&gt;'), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Boyer and Roulland-Dussoix</td>
</tr>
<tr>
<td>ED8767</td>
<td>supE44, supF58, hsdS3(α&lt;sup&gt;+&lt;/sup&gt;m&lt;sub&gt;K&lt;/sub&gt;'), recA56, galK2, galT22, metB1</td>
<td>Murray et al. 1977</td>
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<td><strong>Xanthomonas</strong></td>
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<td>3213&lt;sup&gt;T&lt;/sup&gt;</td>
<td>X. citri pv. citri A</td>
<td>Gabriel et al. 1989</td>
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<td>3213Sp</td>
<td>X. citri pv. citri A, Sp&lt;sup&gt;R&lt;/sup&gt; derivative of 3213</td>
<td>Swarup et al., 1991</td>
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<td>B21.1</td>
<td>pthA::Tn5-gusA, marker exchanged mutant of 3213Sp, Sp&lt;sup&gt;R&lt;/sup&gt;Kn&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Swarup et al., 1991</td>
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<td>B69</td>
<td>X. axonopodis pv. aurantifolii 69, ATCC, B form citrus canker type strain</td>
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<td>B69Sp</td>
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<td>BIM2</td>
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<td>BIM6</td>
<td>Marker integration mutant of B69Sp, Cm&lt;sup&gt;R&lt;/sup&gt; integrated upstream of pthB, Sp&lt;sup&gt;R&lt;/sup&gt;Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>VirB4::Cm&lt;sup&gt;R&lt;/sup&gt;, marker integration mutant of B69Sp, Sp&lt;sup&gt;R&lt;/sup&gt;Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>Strains or plasmids</td>
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<td>pRK2013</td>
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<td>Figurski and Helinski, 1979</td>
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<td>pUFR004</td>
<td>ColE1, Mob&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;, lacZα&lt;sup&gt;−&lt;/sup&gt;</td>
<td>De Feyter et al, 1990</td>
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<td>pUFR012</td>
<td>Derivative of pUFR004 with Kn resistance. ColE1, Mob&lt;sup&gt;+&lt;/sup&gt;, Kn&lt;sup&gt;R&lt;/sup&gt;Cm&lt;sup&gt;R&lt;/sup&gt;, lacZα&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>pBY13</td>
<td>270 bp fragment of <em>virB4</em> cloned in pUFR004, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pB13.1</td>
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<td>pXcB0</td>
<td>Natural plasmid of B69 carrying <em>pthB0</em></td>
<td>Unpublished</td>
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<td>pBIM2</td>
<td><em>pthB</em>:Cm&lt;sup&gt;R&lt;/sup&gt;(pYY40.10) of pXcB, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pBIM6</td>
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<td>B23.5c</td>
<td>B23.5/pBY23c</td>
<td>This study</td>
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**Figure B-1:** Northern blot analysis of CCR genes not found differentially regulated by reverse northern blot
LIST OF REFERENCES


Yang, Y., Yuan, Q. and Gabriel, D.W. 1996. Watersoaking function(s) of XcmH1005 are redundantly encoded by members of the Xanthomonas avr/pti gene family. Mol. Plant-Microbe Interact. 9:105-113.
BIOGRAPHICAL SKETCH

Basma El Yacoubi was born on October 12 1973, in Meknès, Morocco. She obtained her D.E.U.G and License in cell biology and physiology from University Joseph Fourier in Grenoble, France; and her Maitrise cell biology and physiology from Paris 7 University in Paris, France. In the summer of 1996, she joined the University of Florida, and attended the English Language Institute during fall 1996. In spring 1997, she began her graduate studies, and obtained a Master of Science degree from the Department of Environmental Horticulture. In fall 1999, she joined the Plant Molecular and Cellular Biology program, working on her Ph.D. in the department of Plant Pathology.