GENETIC DETERMINANTS OF HOST RANGE SPECIFICITY OF THE WELLINGTON STRAIN OF *Xanthomonas axonopodis* pv. *citri*

By

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To God my guide and fortitude

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A new strain of the citrus canker bacterium (*Xanthomonas axonopodis* pv. *citri*) was detected in Florida. It was significant in that it was primarily pathogenic on Key lime trees, but not on grapefruit trees. This strain has been designated as Xac-A^w. This strain has caused concern among regulators with regard to how to treat this bacterium in the eradication program. Of major concern was the stability of the bacterium in regard to host specificity of the strain; for example, could the strain mutate to attack grapefruit and other citrus plants. We investigated the frequency of the development of mutants, the transfer of genes by conjugation, and the presence of an avirulence gene to grapefruit in the genome. We never were able to find a mutant, either natural or induced, that changed host range. In conjugation studies, transfer of marker genes on the chromosome to and from the Xac-A^w strain by conjugation was not successful. In an attempt to locate possible avirulence genes, a genetic library of Xac-A^w DNA was made using the pLAFR vector and transformed into *Escherichia coli*. This library was transferred to strain 91-

118 of *X. perforans*, which is pathogenic to tomato, but causes a null reaction in grapefruit leaves. The transconjugants were screened for HR in grapefruit leaves. The inoculated leaves were observed for development of an HR in the infiltrated area. In the screening of the Xac-A^w genomic library we found an avirulence gene in the genome of Xac-A^w that interacts with grapefruit leaves to cause an HR. This is the first report of an avirulence gene in the genome of a citrus canker bacterium that interacts with a citrus species. This gene was designated as *avrGf1*, was sequenced, and then was characterized. The possibility of a second *avr* gene exists in the Xac-A^w strain, because the Xac-$A^w\Omega$ lacking a functional *avrGf1* did not cause exactly the same symptoms and develop the same population as the wild-type Xac-A strain when it was inoculated into grapefruit leaves.

CHAPTER 1
INTRODUCTION

Citrus production in Florida contributes greatly to the economy of the state. Production for the 2003-04 seasons totaled 16.4 million tons from 679,000 acres of trees. Florida accounted for 79 percent of the total U.S. citrus production, followed by California with 18 percent; and Texas and Arizona which accounted for the remaining 3 percent. The value of the 2003-04 U.S. citrus crop was up 4 percent from the previous season to $2.35 billion (packinghouse-door equivalent) as published by the USDA, Statistical Service (2004).

Citrus plants are susceptible to diseases caused by bacteria. One of these diseases, citrus canker, is caused by two pathovars of *Xanthomonas axonopodis*. Pathovars of this bacterium have been distinguished based on phenotypic reactions on different hosts, i.e., *Xanthomonas axonopodis* pv. *citri* (Xac) and *Xanthomonas axonopodis* pv. *aurantifoii* (Xaa). The most destructive bacterium is *Xanthomonas axonopodis* pv. *citri* which causes Asiatic citrus canker (Xac-A).

Asiatic citrus canker was discovered in North America in 1912 and was distributed throughout the Gulf states (Loucks, 1934; Dopson, 1964). Eradication of the disease began in 1915 and the disease was eradicated in 1947. This disease was rediscovered again in 1986 in Manatee County, Florida, and declared eradicated in 1994 (Schubert et al., 1996). A new focus of this disease appeared in Miami in 1995 (Gottwald et al., 1997a; Schubert et al., 2001), and a current eradication program exists. This disease was also successfully eradicated from the United States, South Africa, Australia, and New

Zealand, but a new focus of citrus canker appeared in Australia in 2004 (Hibberd, A., personal communication). An eradication program currently exists in Brazil. The disease is endemic to Argentina, Paraguay, and Uruguay. It is also endemic to all countries in eastern Asia.

The symptoms of the disease are erumpent lesions on fruit, foliage, and young stems of susceptible citrus cultivars (Schubert et al., 2001 and Gottwald et al., 2002a). The occurrence of citrus canker lesions on the fruit rind decreases the commercial quality and infected fruit is not accepted by the most important markets. The warm spring and summer conditions of Florida, together with rains with strong winds that occasionally occur, offer ideal conditions for the spread of Xac. Optimum temperatures for infection range between 20 and 30°C (Koizumi, 1985).

The introduction of the leafminer, *Phyllocnistis citrella* Stainton, in Florida in 1993, has increased citrus canker severity due to wounds caused by the insect that expose leaf mesophyll tissues to splashed inoculum, thus increasing the probability of ingress by Xac (Gottwald et al., 1997a). The leafminer adults, however, are not efficient vectors of the pathogen (Belasque et al., 2005).

Changes have recently occurred in the eradication protocols performed by the Division of Plant Industry (Gottwald et al., 2002b). The increased disease severity associated with the leafminer has resulted in increased areas of tree disposal around a focus of infection (Schubert et al., 2001 and Gottwald et al., 2002a). In addition, tornados and hurricanes during the summer months have caused dissemination of Xac-A much greater distances than previously thought possible. Legal questions about eradications, in

general, have hindered eradication (Gottwald et al., 2002b). Citrus canker has spread rapidly in Florida despite changes in eradication protocols (McEver, 2005).

Schubert et al. (2001) described at least two separate introductions of Xac-A in Florida since 1985. In one of the introductions a strain of Xac that is pathogenic to Key lime and alemow plants, but not to grapefruit and orange was found. This strain (Xac-A^w) was characterized by Sun et al. (2004) and found to be related genetically to Xac-A rather than Xaa-B or Xaa-C, which have more similar host ranges. Regulations and eradication protocols adopted for this strain were different from those adopted for Asiatic citrus canker. For the Xac-A^w only Key lime and alemow plants were destroyed. Because of the relationship of Xac-A^w to Xac-A, questions as to whether Xac-A^w could revert to an Xac-A strain exist. If this occurred, a new focus of Xac-A could occur with the eradication program that was adopted.

The purpose of this work was to investigate the stability of the Xac-A^w strain in terms of host specificity. The Xac-A^w strain causes a hypersensitive reaction in grapefruit. Such a reaction is usually the result of an avirulence gene interacting with a resistance gene in the host (Minsavage et al., 1990b). If resistance in grapefruit to the Xac-A^w strain is the result of the presence of a single gene in the bacterium, then the stability of the host specificity of the Xac-A^w may be low. The change to virulence in grapefruit of the Xac-A^w strain was studied by mutagenesis of the strain, by conjugations of strains, and by isolation of avirulence genes in the genome of Xac-A^w.

CHAPTER 2
LITERATURE REVIEW

Many reviews have been published on the citrus canker disease (Locks, 1934; Garnsey et al., 1979; Schoulties et al., 1987; Graham and Gottwald, 1991; Stall and Civerolo, 1991; Schubert et al., 2001 Gottwald et al., 2002a; Graham et al., 2004). An excellent early review is an unpublished manuscript that was written by K. W. Loucks in 1934. The review is located in the library of the Division of Plant Industry, Florida Department of Agriculture, Gainesville. It is a review of research on regulation and eradication of this disease from the appearance of citrus canker disease in Florida until eradication of citrus canker was declared in 1933. This manuscript contains a history and geographical distribution of citrus canker in the world, United States, and Florida. Hosts reported to be susceptible and the economic importance of citrus canker were included. A description of citrus canker symptoms of leaves, twigs, thorns, branches and fruits is given. The general appearance on an infected tree with citrus canker was described. The morphology, physiology, cultural characteristics and taxonomy of the causal organism at that time were described in this review. The viability and longevity of the pathogen were described; including describing the ability of the bacterium to enter the host tissue and remain quiescent until higher temperatures occurred. The means of dissemination, methods of infection, and the incubation period involved with the disease cycle were reviewed. The histology of the disease was described. Humidity and temperature relationships for disease development, as well the seasonal development of the host and disease, were included. Progress on the eradication and control of citrus canker and the

epidemiological situation at the time that this review was written were discussed. The last issue that Louck considered in his review was the future danger of the disease to citrus in Florida.

An analytical bibliography of research publications relating to citrus canker was annotated by Rossetti et al. (1981). This huge effort contains abstracts of scientific papers, reviews, extension reports and notes from 1912 though 1981. There are 1246 abstracts included from all over the world.

Before the reintroduction of citrus canker in Florida in 1985 there were concerns about the reintroduction of citrus canker after it was declared eradicated in 1933 from Florida and in 1947 from the USA (Stall and Seymour, 1983). Those concerns were based on importation of citrus fruits into the United States from Japan, where citrus canker is endemic; an epidemic of canker occurring in South America, and the interception of citrus canker at ports of entry. This article described the causal agent, the disease, the history of citrus canker, information about losses due to this disease, measures of control and finally the outlook about the real risks of the reintroduction of citrus canker in the USA.

A bacterial disease of citrus was observed in Florida in 1984. This disease was referred to as the nursery form of citrus canker, or sometimes as canker E (Schoulties et al., 1987). In this review they described the nursery form of the disease, diagnosis, symptoms, distribution, host range, eradication and regulatory program. The review also discussed the introduction of the Asiatic form of the disease in Florida in 1985 and the eradication policies regarding the reintroduction of Asiatic citrus canker. The article also

mentioned the costs of the eradications and regulatory programs which were $25 million from September 1984 to July 1986.

Stall and Civerolo (1991) described Asiatic canker and ongoing research with both types of diseases of citrus in Florida. They reviewed the history of the disease in Florida, and the efforts on eradication, the different forms of citrus canker, and the localization of citrus canker in the USA. Research related to regulation, and eradication problems included the comparisons between the citrus canker and the bacterial spot disease. Isozyme analyses, serological studies, and DNA analyses of different strains involved in the two diseases were included in the comparison of the strains.

Two recent reviews pertain mostly to the introduction of Asiatic citrus canker in Florida in 1995 (Schubert et al., 2001 and Gottwald et al., 2002a). These reviews provide a history of the disease in Florida and describe a citrus canker disease cycle. Both reviews describe the host range of the citrus canker disease, although Gottwald et al. (2002a) provide a more expansive list. Schubert et al. (2001) list three separate introductions of the Asiatic citrus canker from 1910 until now and describe a fourth introduction in 2000. The pathogen associated with the 2000 introduction has a limited host range and was designated as the Wellington strain. Both reviews treated the problems of regulation and eradication of citrus canker in Florida. Gottwald et al. (2002b) discuss the epidemiology of the disease in the urban setting which was not considered previously. Both reviews discuss the role of the Asian citrus leafminer (*Phyllocnistis citrella* Stainton) damage in the epidemiology of the disease.

From the above reviews it is apparent that different xanthomonads are pathogenic to various citrus species. Only those that cause a lesion that is erumpent or pustule-like in

the early stages of symptoms on leaves are now included in the citrus canker disease (Graham and Gottwald, 1990). These lesions become crateriform as they increase in size. The bacteria that do not cause such lesions are not presently classed as causing citrus canker, and this includes the xanthomonad that causes the citrus bacterial spot disease.

The nomenclature of the bacterial strains that cause citrus canker has evolved. Gabriel et al. (1989) proposed that *Xanthomonas citri* be restored for the name of the Xac-A group. They also proposed that the Xaa-B and Xaa-C strains be named *Xanthomonas campestris* pv. *aurantifolii.* Their primary evidence for this nomenclature was based on restriction fragment length polymorphism (RFLP) using DNA probes hybridizing to total DNA of the strains. The change in nomenclature based on this technique was criticized by Young et al. (1991) and Vauterin et al. (1990). Vauterin et al. (1991) studied the xanthomonads that cause the citrus canker disease using DNA-DNA hybridization and other techniques and concluded that they should be divided into two groups.

Vauterin et al. (1995) revised the nomenclature of the genus *Xanthomonas* and placed the bacteria that cause citrus canker into *X. axonopodis* that also included many other plant disease-causing xanthomonads. The A group was given pathovar status and named *X. axonopodis* pv. *citri.* The B and C strains were placed into the pathovar *aurantifolii* as was proposed by Gabriel et al. (1989) previously. This nomenclature seems to be the most accepted at present for the bacteria that cause the citrus canker disease.

Two groups of strains that are placed in *X. axonopodis* pv. *citri* cause citrus canker can be distinguished, based on DNA and other genetic aspects (Verniere, 1998). The first

group is the most important group and consists of *X. axonopodis* pv. *citri* strain A (Xac-A) that causes Asiatic citrus canker. Several subgroups have been identified. One subgroup is referred to as A^* because strains are genetically similar to the Xac-A strains (Hartung and Civerolo, 1989; Cubero and Graham, 2004), but have different host specificities and a few physiological differences from typical Xac-A strains (Sun et al. 2004). This subgroup of strains occurs in southeastern Asia, Iran and possibly India. Another subgroup of strains was recently found in Florida and is referred to as Xac-A^w strains (Sun et al., 2004). These strains are pathogenic on Key lime and alemow plants, but not on grapefruit or orange plants. The Xac-A^w strains are also closely related genetically to the Xac-A and Xac-A^* strains, but have some physiological differences from the latter strains.

Another group consists of strains that cause citrus canker and are significantly different genetically from the Xac-A strains.The members within this group are closely related genetically (Vauterin et al; in 1991). The strains that cause canker B and C, *X. axonopodis* pv. *aurantifolii,* are in this group. This group can be subdivided based on pathogenicity and a few physiological tests. Both subgroups are not pathogenic on grapefruit and oranges, but are pathogenic on Key lime. The B subgroup (Xaa-B) of strains is pathogenic on lemon trees also. After the introduction of Xac-A in Argentina in 1973 the B subgroup was not isolated from the field any more and is only present in pathogen collections (Nelly Canteros, personal communication). The Xaa-C strain was isolated in Brazil, but does not occur in nature at this time.

Some work has been done regarding the host specificities of the strains of bacteria that cause citrus canker. In Loucks review it was pointed out that some species of citrus

are more susceptible than others. Levels of resistance, or susceptibility, of the citrus species and cultivars were recorded during evaluations in the field. The extreme susceptibility of grapefruit to the Xac-A strains was attributed to the larger stomatal pores compared to the mandarins which are more resistant and have smaller stomatal pores (Mc Lean, 1921). This may be a factor, but Stall et al. (1982a) demonstrated that many more lesions developed in grapefruit leaves also after infiltration with a low level of inoculum. The infiltration technique of inoculation was used to evaluate a few cultivars of orange and grapefruit plants in the field. There was good correlation of disease and susceptibility in the field results. Viloria et al. (2004) used this technique to evaluate many citrus genotypes under greenhouse conditions. There seemed to be a continuum of resistance or susceptibility levels in the genotypes tested.

Hypersensitivity in citrus to Xac-A strains has not been reported. However, a hypersensitivity reaction was suggested as occurring after inoculations of grapefruit leaves with a strain of the canker C (Xaa-C) from Brazil (Stall et al., 1982b). This was suggested because a rapid necrosis occurred in young grapefruit leaves inoculated with high concentrations of cells (10^8 cfu/ ml). The rapid necrosis was compared to relatively slow necrosis in grapefruit caused by the Xac-A strain and a strain of canker B (Xaa-B). Further documentation of the hypersensitive reaction in grapefruit caused by the C strain has not been reported.

CHAPTER 3
EVIDENCE FOR HYPERSENSITIV REACTION IN XAC-A^{W} STRAIN IN GRAPEFRUIT

Introduction

Xanthomonas axonopodis pv. *citri* strain A^{w} (Xac-A^{w}) was compared with Xac-A (strain that causes Asiatic citrus canker) in Duncan grapefruit and Key lime (Sun et al., 2004). Even though rapid necrosis occurs when high concentrations of the Xac-A^{w} bacterium are infiltrated into grapefruit leaves, data for the development of a hypersensitive reaction (HR) in grapefruit were not reported. In this chapter we present experiments on electrolyte leakage and bacterial growth after inoculations with high concentrations of Xac-A and Xac-A^{w} strains into grapefruit leaves to determine if a typical HR occurs in grapefruit leaves.

Materials and Methods

Plants. Plants of grapefruit (*Citrus paradi* Macf.), cultivar Duncan, were kept in a quarantine greenhouse at the Division Plant Industry at 20º to 35º C and used in this experiment. Before inoculation the plants were pruned to stimulate new growth. Leaves on the newly developed shoots were inoculated ca. 10 days after new shoots began growth. The leaves chosen for inoculations were fully expanded, but soft to the touch, and not as fully green as mature leaves. By using this procedure all the inoculated leaves were in a similar developmental stage.

Bacterial strains and preparation of inoculum. Two Xac strains were used. Suspensions of strains Xac-A^{w} 12879 and Xac-A 40 (Corrientes, Argentina) were each

transferred to nutrient agar medium (NA) from culture stored at -80°C and isolated colonies were obtained. Several colonies of each strain were transferred to nutrient broth. After the cultures were shaken overnight, the bacterial suspensions were centrifuged and the cells were resuspended in sterile tap-water, and standardized to an absorbance of 0.3 at a light wavelength of 600 nm in a Spectronic 20 spectrophotomer. This optical density corresponds to a bacterial concentration of $5x10^8$ cfu /ml.

Electrolyte leakage. Leaves of grapefruit were inoculated with $5x10^8$ cfu/ml of Xac-A^w or Xac-A strains (15 leaves each). The inoculations were made by infiltrating leaves with 10 ml syringe and 27 g nettle (Klement, 1963). After 2 h and 2, 4, 6, and 8 days electrolyte leakage was measured from three leaves infiltrated with each strain. Electrolyte leakage was determined as an increase in electrical conductivity over a 2 h period of 3 ml of de-ionized water containing six 0.5 cm^2 leaf disks of each inoculated area (Cook and Stall, 1968; Hibberd et al., 1987). The mean of the three determinations was used as the conductivity at each time, but each determination was used to determine the experimental error.

Bacterial populations. The populations of the Xac-A^w and Xac-A strains in grapefruit leaves were determined in the same leaves and the same times as electrolyte leakage determinations. From each inoculated leaf 0.5 cm^2 of leaf area was taken and triturated in one ml of sterile tap-water, and after appropriate ten-fold dilutions, 50 µl were plated on NA medium. The colonies were counted 3 days later (Cook and Stall, 1968; Hibberd et al., 1987). Three replicates were included at each time period. The experiment with population and electrolyte leakage was repeated three times.

Results

The appearance of rapid necrosis in grapefruit leaves occurred only on young leaves of grapefruit plants (Figure 3-3). Therefore, the inoculation of leaves in a young stage of development is essential for consistent results. The inoculation of new growth that resulted from pruning of shoots of grapefruit plants gave consistent reactions.

Electrolyte leakage of grapefruit leaves. The conductivity of water containing leaf disks inoculated with the Xac-A^w strain was very high at day four compared to conductivity of water containing leaf disks inoculated with the Xac-A strain (Figure 3-1). Conductivity of water containing leaf disks inoculated with the Xac-A strain was highest at day eight. Thus, the Xac-A^w strain caused more rapid electrolyte leakage from grapefruit leaves than the Xac-A strain.

Growth of Xac-A^w and Xac-A strains. The populations of the Xac-A strain were significantly higher than the Xac-A^w strain in grapefruit leaves at day four and day six. The populations reached 10^9 cfu/cm^2 of leaf tissue with the Xac-A strain, but the populations of the Xac-A^w strain were slightly lower and reached only 10^8 cfu/cm^2 (Figure 3-2).

Discussion

The populations obtained in leaves inoculated with Xac-A^w were very similar to the results obtained by Sun et al. (2004). Growth was slower in leaves inoculated with the Xac-A^w strain than growth in leaves inoculated with Xac-A strain. In both studies the Xac-A^w populations were nearly ten-fold less than the Xac-A strain. Comparisons of growth after infiltration in Key lime leaves were not determined in this study, because we were only interested in determination of HR in grapefruit leaves of the Xac-A^w strain.

However, Sun et al. (2004) found no differences in growth of Xac-A and Xac-A^w in leaves of Key lime.

To determine if necrosis occurs more rapidly in leaves inoculated with strain Xac-A^w compared with Xac-A, electrolyte leakage was monitored in grapefruit leaves after infiltration with these strains. Leakage of electrolytes from citrus leaf tissue infected by Xac-A was determined previously (Goto, et al., 1979). In this work necrosis caused by Xac-A^w occurred more rapidly compared with Xac-A strain (Figure 3-1). Electrolyte leakage reflects the extent of necrosis in leaves. A rapid necrosis in plant tissue and lower populations in leaves compared to a susceptible reaction are indications of a HR. Therefore, it is reasonable to assume that Xac-A^w causes an HR in grapefruit leaves based on this work. HR is the result of an avirulence (*avr*) gene interacting with a resistance gene (Flor, 1955; Ellingboe, 1976; Klement, 1982; Staskawicz et al. 1984; Crute, 1985; Gabriel et al. 1986). In future work we will determine if an *avr* gene is involved in this reaction.

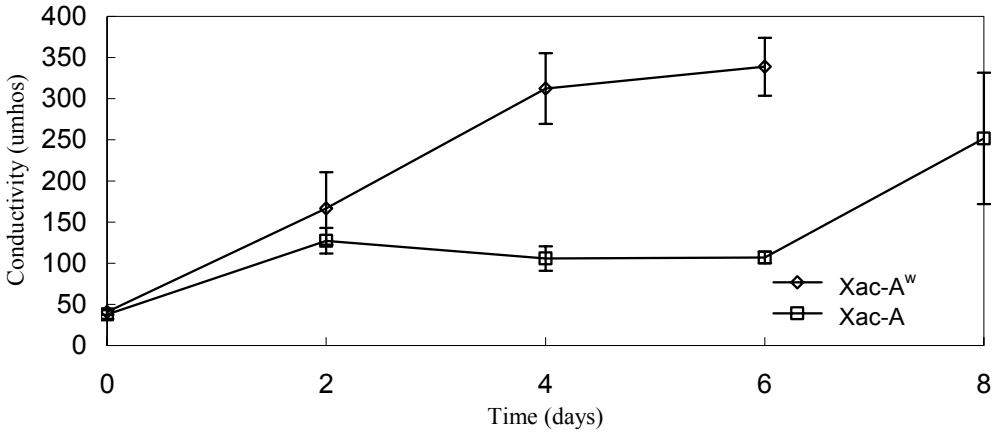

Figure 3-1. Electrolyte leakage in grapefruit leaves inoculated with Xac-A^w and Xac-A strains at a concentration of $5x10^8$ cells per milliliter at various times after inoculation. The leaves inoculated with Xac-A^w abscised 6 days after inoculation. Vertical bars represent standard error.

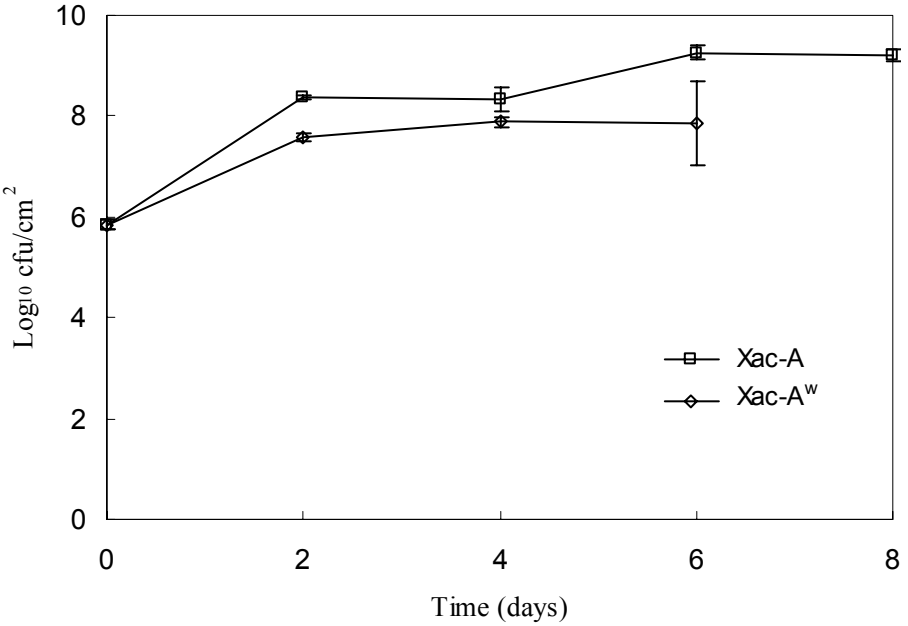

Figure 3-2. Internal bacterial populations in grapefruit leaves inoculated with Xac-A^w and Xac-A strains at various times after inoculation. The leaves inoculated with Xac-A^w abscised 6 days after inoculation. Vertical bars represent standard error.

Figure 3-3: Symptoms of Xac-A^{w} (left side of leaf) and Xac-A (right side of leaf) in old (left) and young leaves (right) of grapefruit on the abaxial face of the leaves (Four days after inoculation).

CHAPTER 4
CHANGE OF HOST RANGE BY MUTATION.

Introduction

Xanthomonas axonopodis pv. *citri* strain A^w (Xac-A^w) causes a hypersensitive response (HR) in grapefruit leaves. The HR is correlated with the gene-for-gene relationship in many plants. In such gene-for-gene interactions an avirulence (*avr*) gene in the pathogen interacts with a corresponding resistance (*R*) gene in the plant (Minsavage et al., 1990b). Mutation of an *avr* gene often leads to a susceptible reaction. Such mutations can occur in high frequency in some strains of *Xanthomonas* (Dahlbeck and Stall, 1979). The purpose of this chapter is to determine if a mutation in a postulated *avr* gene would change the host specificity of the Xac-A^w strain after treatment with a mutagen and if so, to determine the frequency of the mutation.

Materials and Methods

Inoculum. The Xac-A^w 12879 strain was used because it causes an HR in grapefruit leaves and a susceptible reaction in leaves of Key lime. The preparation of inoculum was the same as described in Chapter 3.

Mutation in vitro. The Xac-A^w cells were treated with N-methyl-nitro-N-nitrosoguanidine (NTG), which is a strong mutagen (Gerhardt et al., 1981). A stock solution of NTG containing 1 µg/ml in deionized water was made and 100 µl of stock NTG were added to 900 µl of a suspension of 5 x 10^8 cfu/ml of Xac-A^w cells in sterile tap-water. After 1 h at 28 C° the suspension was diluted serially ten-fold, and 200 µl of a 5 x 10^3 cfu/ml bacterial suspension were spread onto the surface of nutrient agar in Petri

plates. In previous work (unpublished) the NTG treatment killed ca. 50 % of bacterial cells. After 3 days the colonies were counted to determine the actual concentration of viable cells following treatment with NTG and to observe for development of mutant white colonies from the typical yellow colonies. In addition 200 µl of a suspension consisting of the 10^5 cfu/ ml were spread onto nutrient agar containing 200 µg/ml streptomycin sulfate to check for growth of colonies resistant to streptomycin.

About 30 young grapefruit leaves were completely infiltrated with a suspension consisting of 5 X 10^3 cfu/ml of mutagenized cells. At the same time ten leaves of Key lime were infiltrated with the same inoculum as a control. The leaves used in these experiments were at the same stage of development as described in Chapter 3. The technique used by Klement (1963) was used to infiltrate each leaf with inoculum. Lesions in both Key lime and grapefruit were counted and were calculated as lesions per cm^2 of inoculated leaf area (Table 4-3). The leaf area of tissue infiltrated was determined using the dot-counting method (Marshall, 1968). The mutagenesis experiment was repeated eleven times.

Results

Mutagenesis. The numbers of Xac-A^w cells remaining after NTG treatment ranged from 0.5 to 3.0 x 10^8 cfu/ml in the eleven experiments. White colonies developed in five of the experiments in which 10^3 cfu/ml were spread on NA. Streptomycin-resistant colonies formed in which 10^5 cfu/ml were spread on NA containing streptomycin in only one experiment (Table 4-1). No white colonies of Xac-A^w or streptomycin resistant colonies developed with non-treated suspensions. These results were evidence that NTG did cause mutations in the population of Xac-A^w cells used for inoculum.

Change of host range. The Xac-A^w strain caused fleck lesions in grapefruit leaves (Figure 4-1) and typical citrus canker lesions in Key lime (Figure 4-2). Each lesion was probably caused by a single cell of the bacterium, because of the low inoculum level used. Over the eleven experiments an average of 10.1 canker lesions per cm^2 occurred in Key lime and 2.7 fleck lesions per cm^2 occurred in grapefruit leaves. Assuming the intercellular spaces on grapefruit and Key lime leaves were similar, only 27 % of the Xac-A^w cells in grapefruit actually caused a visible fleck lesion.

The total area of grapefruit leaves infiltrated with inoculum in the eleven experiments was 20,827 cm^2 (Table 4-2). Using the estimate of 10.0 lesions/cm^2 based on what occurred in Key lime leaves (Table-4-3), approximately 2.0 x 10^5 cells of Xac-A^w were infiltrated into grapefruit leaves in the eleven experiments. Typical citrus canker lesions were never observed in grapefruit leaves receiving the mutagenized cells of Xac-A^w .

Discussion

Cells of Xac-A^w were treated with NTG, a powerful mutagenic agent, to increase the frequency of mutation. The number of white colonies and streptomycin resistant colonies after NTG treatment reflected the development of mutants in culture.

The frequency to streptomycin resistance in non-mutagenized cultures of *X. campestris* pv. *vesicatoria* was around 1.9 x 10^{-9} per cell per division (Dahlbeck and Stall, 1979). In this work, streptomycin resistant colonies occurred rarely after NTG treatment but did occur in one test and was determined to be approximately one per 10^5 cells. The frequency of mutation for white colonies was higher than for streptomycin resistance. At least seven genes are involved in the development of the yellow pigment (Poplawsky and Chun, 1997). Mutation in any of them will result in loss of pigmentation.

Thus, one would expect a higher frequency of white colonies than streptomycin resistant colonies after NTG treatment. As resistance to streptomycin is encoded by a single gene, the frequency of streptomycin resistance probably corresponds better to an *avr* gene mutation, if the change of the host range depends only on a single *avr* gene. Thus, one would not expect a change of host range of the Xac-A^w strain unless nearly 10^5 cfu were infiltrated into the leaves of grapefruit. We inoculated grapefruit leaves with 2×10^5 cells and, thus, one would have expected a single avirulence gene to be mutated and detected in our work. However, this did not occur. Therefore, possibly more than one gene is involved in the incompatibility of the grapefruit leaves to the Xac-A^w strain.

Table 4-1. Effect in vitro of NTG mutagenesis on development of white colonies and streptomycin resistant colonies of Xac-A^w strain

Expeiment no.	Yellow [a] colonies	White [b] colonies	Streptomycin [c] resistant colonies
	x 10^8 cfu	x 10^3	x 10^5
1	3.00	0.00	0.00
2	0.90	0.00	0.00
3	0.68	0.00	0.00
4	1.70	2.00	1.80
5	1.31	0.00	0.00
6	1.99	0.00	0.00
7	2.11	0.00	0.00
8	0.85	3.80	0.00
9	1.75	2.00	0.00
10	0.50	2.00	0.00
11	0.76	0.20	0.00
Average	1.41	0.91	0.16

[a] These values were based on the colonies that developed on nutrient agar after dilution to 10^3 cfu/ml. The values are based on three replicates per experiment.

[b] These values are number of colonies that developed on nutrient agar after dilution to 10^3 cfu/ml. A suspension of 200 µl were spread on the plate. Average of three replicates per experiment

[c] There values are number of colonies that developed on nutrient agar containing 200 µg of streptomycin per liter. A suspension of 200 µl of 10^5 cfu/ml were spread on the plate. Average of three replicates per experiment.

Table 4-2. Total area of inoculated Key lime and grapefruit leaves in all experiments

Experiment No	Key lime cm^2	Grapefruit cm^2
1	34.5	711.50
2	60.0	2122.5
3	40.6	1632.6
4	39.5	1136.5
5	379	1853.8
6	246	3115.0
7	32.8	1597.0
8	60.8	1653.3
9	25.6	1263.0
10	51.0	3093.8
11	56.0	2647.5
Total	1025.8	20826.5

Table 4-3. Number of lesions per cm^2 that were produced in Key lime and grapefruit leaves inoculated with 10^3 cfu/ml of Xac-A^w

	Numbers of lesion per cm^2	
Experiment No	Key lime [a]	Grapefruit[b]
1	12.5	4.4
2	7.1	1.7
3	3.5	0.9
4	19.6	5.2
5	4.8	2.4
6	11.5	1.1
7	18.2	1.7
8	1.3	0.5
9	15.3	0.4
10	4.5	2.4
11	12.2	8.4
Average	10.1	2.7

[a] Lesions were typical of citrus canker

[b] Lesions were sunken and very small (typical fleck lesion caused by HR)

Figure 4-1. Symptoms of *Xanthomonas axonopodis* pv. *citri* strain Xac-A^w in grapefruit leaf after infiltration with 10^3 cfu/ml bacterial concentration. Fleck lesions occur on upper half of leaf (30 days after inoculation).

Figure 4-2. Symptoms of *Xanthmonas axonopodis* pv. *citri* strain Xac-A^w in Key lime leaf after infiltration with 10^3 cfu/ml bacterial concentration. Typical pustule type lesions occur on upper half of leaf (21 days after inoculation).

CHAPTER 5
CHANGE OF HOST RANGE BY CONJUGATION

Introduction

More than one *avr* gene may be involved in the hypersensitive reaction (HR) by the Xac-A^w strain in grapefruit leaves. If that is true, the host specificity of the Xac-A^w strain would not change by simple mutation. However, the factor for host specificity of the Xac-A^w strain might cause the Xac-A strain to change after conjugation of the strains, because large amounts of DNA transfer during conjugation. Conjugation is a process in which the DNA of a donor bacterium moves to a recipient bacterium. Plasmid or chromosomal DNA can move to recipient cells in conjugation. Conjugation requires cell to cell contact and the presence of transfer genes in the DNA of the donor. Many avirulence genes involved in host and cultivar specificities (Leyns et al., 1984; Hayward, 1993), and the genes for resistance to copper (Stall et al., 1986) and streptomycin are located on plasmids (Minsavage et al., 1990a). However, some avirulence genes are located on the chromosome (Ronald and Staskawicz, 1988; Tamaki et al., 1988; 1988; Minsavage et al., 1990b; Jenner et al., 1991; Mansfield et al., 1994; Whalen et al., 1993; Lorang and Keen, 1995). Horizontal transfer of chromosomal genes among strains of *X. axonopodis* pv. *vesicatoria* was confirmed by Basim et al. (1999), but the frequency of transfer of plasmid DNA is usually higher than for chromosomal DNA.

Materials and Methods

Bacterial strains. All bacterial strains, relevant characteristics, and origin are listed in Table 5-1.

Change of host range by conjugation. To test for conjugation among strains, genetic markers were incorporated into both Xac-A^{w} and Xac-A so that each could be used as a donor or recipient. Some strains were marked with fluorescent proteins by G.V. Minsavage (Plant Pathology, Department of University of Florida) using the pMOD^{TM}-2<MCS> vector that carries 19-bp mosaic end sequence flanking restriction sites. The antibiotic cassette used in this experiment was excised from pKRP11 plasmid (Km^{R}) and pKRP13 (Sm^{R} Sp^{R}). A Km^{R} clone was marked with the EYFP gene with lacZ promoter from pEYFP and a clone that carried the Sm^{R} Sp^{R} was marked with the ECFP gene with the lacZ promoter from pECFP. The fluorescent-marked strains were obtained by electroporation using EZ::TN transposase enzyme and Biorad Gene Pulser (for more details contact G.V. Minsavage).

Strains with antibiotic resistance were selected after plating 200 µl of a culture containing 10^{8}-10^{9} cfu/ml onto nutrient agar containing the selective antibiotic. Colonies that developed on the selective medium were purified by single-colony selection on the antibiotic medium. Strains of Xac-A and Xac-A^{w} containing the GFP in the chromosome or non-pigmented strains obtained in Chapter 4 were sometimes used for selection of antibiotic resistance. Strains were stored at -80° C. The antibiotics used and final concentrations were: rifamicyn (80 µg/ml), nalidixic acid (50 µg/ml), kanamycin (50 µg/ml), streptomycin (50 µg/ml), and spectinomycin (50 µg/ml).

For conjugation on a solid medium, donor and recipient strains were grown in nutrient broth (NB) overnight on a rotary shaker at 28° C, after which 100 µl of culture was added to 900 µl of sterile tap-water and centrifuged. The pellets obtained were resuspended in 100 µl of sterile tap-water and donor and recipient strains were mixed in

different proportions (10 or 200 µl of donor was added to 100 µl of recipient). Then 10 µl of each mixture was plated on nutrient-yeast-glycerol agar (NYGA), (Daniels, et al., 1984). The same suspensions sometimes were inoculated into leaves of grapefruit or Key lime for conjugation. After 48 h each mixture was resuspended in 800 µl of sterile tap-water and 400 µl was transferred to each of two plates of an antibiotic selective medium.

For conjugation in liquid medium, bacteria were grown during shaking at 28° C overnight in 4 ml of NB, then donor and recipient strains were mixed, and incubated with very gentle shaking for 5 h. Afterward 200 µl of the mixture were plated on appropriate selective medium and incubated at 28°C for 2-3 days until conjugants developed. Sometimes colonies grew on media containing two antibiotics. To test whether the colonies were mutants or conjugants, cells were observed for the presence of GFP using a UV microscope was performed and PCR with the primers specific for GFP (G.V. insavage). Sometimes they were inoculated into grapefruit leaves for strain identification.

To determine if Xac-A^w and Xac-A could act as donors, or recipients in conjugation, experiments were designed for transfer of Cu resistance genes which were known to be transferred by conjugation (Basim et al, 1999). Two strains of *X. campestris* pv. *vesicatoria* (one contained Cu resistance on a plasmid, the other on a chromosome) were mated with Xac-A^w and Xac-A recipient strains and screened for transfer of copper-resistance genes (Cu^r). In addition, four Cu^r Xac-A strains (plasmid-borne Cu^r) from Argentina and a strain of *X. axonopodis* pv. *citrumelo* that was copper resistant were mated with an Xac-A copper sensitive (Cu^s) strain from Florida. The conjugations using only the strains from Florida were made on solid NYGA medium and the conjugations among strains from Argentina and Florida were made using liquid medium. Copper

resistance was determined by growth on nutrient agar supplemented with copper sulfate (200 μg/ml). The frequency of Cu^r transfer during conjugations was determined from dilution plating of bacteria after matings.

Plasmid DNA isolation. Plasmid DNA was extracted by a modification of the method of Kado and Liu (1981). Detection of plasmids was performed by electrophoresis of the plasmid extraction as described previously (Stall et al., 1986). Donors and recipients could be identified by plasmid profiles (Figure 5-1, Lanes 2 and 4).

Results

Selected Xac-A^w strains were mated with Xac-A strains in all combinations of the donor and recipient. In addition, the Xac-A^w strain was mated with itself and the A strain was also mated with itself. In these tests a marker gene on a chromosome was never transferred by conjugation. Putative transconjugants that grew on antibiotic media all were negative for transfer of chromosomal genes.

The copper resistance genes on a plasmid in Xcv 75-3 were transferred to Xac-A in vitro and in planta in all tests performed; however, these copper resistant genes were transferred to the Xac-A^w strain only one time, and that occurred in planta (Figure 5-1). No transfer to either Xac-A or Xac-A^w of the chromosomal Cu genes was observed.

Conjugal transfer of Cu^r genes from copper resistant Xac-A strain from Argentina to copper sensitive Xac-A strain from Florida also occurred (Figure 5-2). The copper resistance genes in Xac-A were found to be plasmid-borne (Figure 5-3). The frequency of transfer of the Cu^r plasmid (per donor cell) was variable depending on the donor and recipient combination (Table 5-2). The same variability of transfer frequency was observed when the conjugation was made with the Cu^r and Cu^s strains from Argentina

(Table 5-3). It is interesting that in this combination, the Xac-A 16 Cu^r strain was the best donor and had the highest frequency of transfer with the Xac-A 40 Cu^s strain. However, no transfer was observed with the Xac-A 45 Cu^r strain used as the donor and with any recipient strain (Table 5-3). When Xcv 75-3 Cu^r was used as a plasmid donor, the frequency of transfer of Cu^r genes to Xac-A Cu^s strains was very high (Table 5-4). When the copper resistant donor, *X. axonopodis* pv. *citrumelo* strain (2a, Cu^r), was mated with Xac-A 20, (Cu^s), the Cu plasmid was transferred from the donor to the Xac-A recipient strain (Lane 12 of the Figure 5-3).

Discussion

The marker genes developed in these experiments were probably inserted in the chromosome. The genes for yellow pigment had been determined to be in the chromosome (Poplawsky and Chun, 1997). The markers were developed to primarily see if chromosomal movement occurs in Xac-A and Xac-A^w by conjugation. The experiments were designed to have two markers in the donor strain, but only one marker was included in the recipient strain. The development of some colonies on selective media after mating was due to mutation for the donor selective marker in the recipient. The mutants could be detected by the lack of movement of the other chromosomal genes, such as the GFP or pigment genes.

Apparently chromosomal movement between strains of Xac is not a significant factor in change of host specificities in nature. However, conjugal movement of plasmids between strains of Xac does occur. Movement of plasmids among strains of Xac can be particularly significant for the movement of copper resistance genes between strains. Copper is used in the field to control citrus canker and the development of resistance among strains would hinder control. The first report of copper resistance in strains of Xac

was by Canteros et al. (2004). In the present work we found that the copper resistance genes in strains from Argentina were plasmid borne, which had not been demonstrated previously. Copper resistance among strains of Xac in Florida was not detected when over 100 random strains were screened for resistance. However, copper resistance was detected in a strain of *X. axonopodis* pv. *citrumelo* from Florida (R.E. Stall, personal communication) and the resistance was determined to be plasmid-borne and could be transferred by conjugation to an Xac-A Cu^s strain. Since *X. axonopodis* pv. *citrumelo* and Xac-A both cause disease of citrus it is expected that cell to cell contact of the two pathovars can occur in nature. If citrus canker were to become widespread in Florida and copper is used for control, it would be expected that copper resistance strains of Xac-A would be selected quickly in the field.

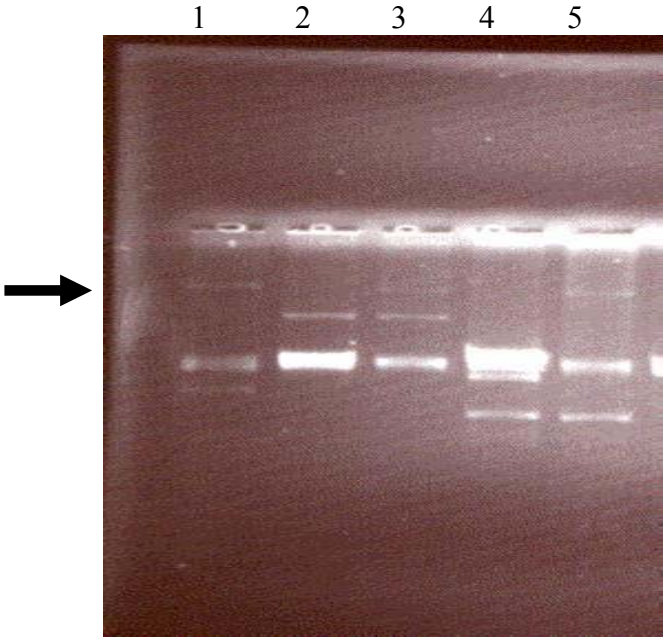

Figure 5-1. Agarose gel stained with ethidium bromide. Lanes contain plasmid extractions from different strains: 1= Xcv 75-3 (Cu^r), 2= Xac-A (1874, GFP^+, Cu^S), 3= Xcv 75-3 x Xac-A (conjugant), 4= Xac-A^w (GFP^+ Cu^S), and 5= Xcv x Xac-A^w (conjugant).Strains in lanes 1, 3, and 5 were resistant to copper and contained a large plasmid (arrow). The large plasmid was transferred by conjugation to copper sensitive strains.

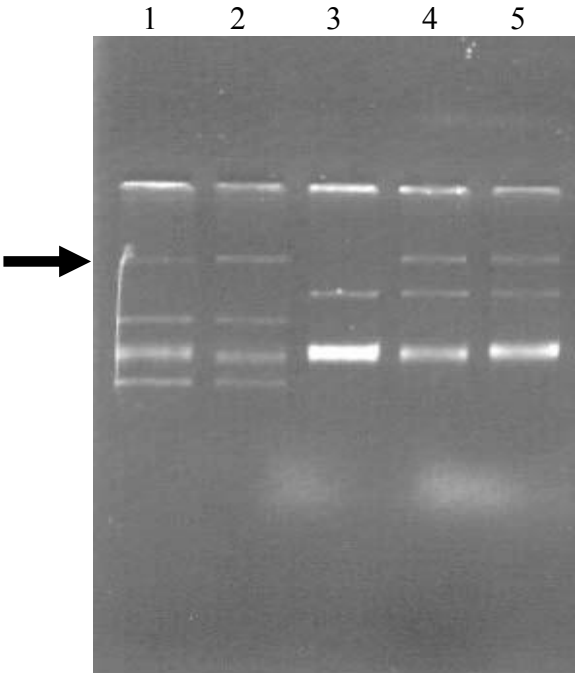

Figure 5-2. Agarose gel stained with ethidium bromide. Lanes contain plasmid extractions from different strains:1= Xac-A (16, Cu^r), 2= Xac-A (26, Cu^r), 3= Xac-A (1874, Cu^s), 4= Xac-A (16) x Xac-A (1874) conjugant, and 5= Xac-A (26) x Xac-A (1874) conjugant. Strains in lanes 1, 2, 4 and 5 were copper resistant and contained a large plasmid (arrow). The large plasmid from strains 16 and 26 were transferred to 1874 by conjugation.

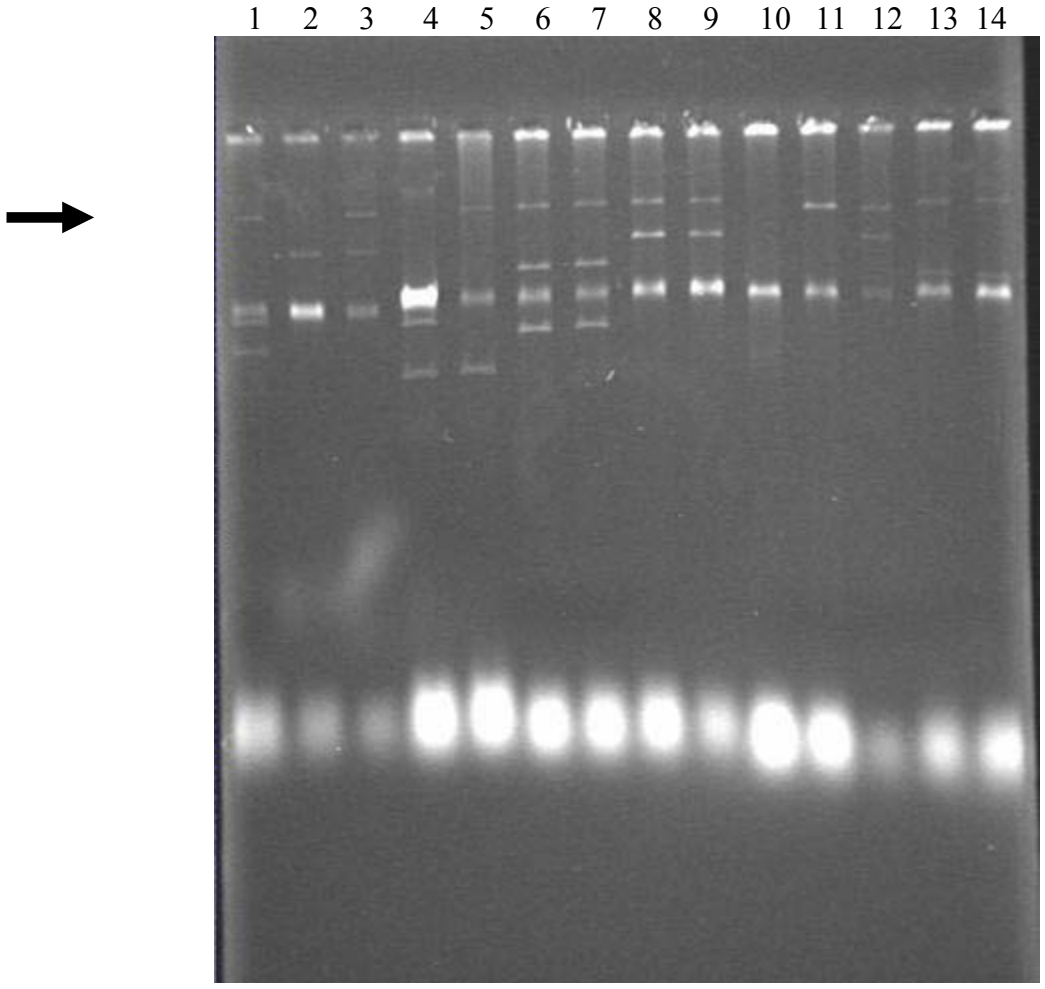

Figure 5-3. Agarose gel stained with ethidium bromide. Lanes contained plasmid extractions from different strains: 1= Xcv 75-3 (Cu^r) , 2= Xac-A 20 (1874, GFP^+, Cu^s), 3= Xcv 75-x Xac-A 20) conjugant, 4=Xac-A^w (GFP^+ Cu^s), 5=Xcv x Xac-A^w ,conjugant, 6=Xac-A (16 Cu^r), 7- Xac=A (26 Cu^r), 8= Xac-A 16 (Cu^r) x Xac-A 20 (Cu^s) conjugant , 9= Xac-A 26 x Xac-A 20, conjugant, 10= Xac-E (104, Cu^s), 11= Xac-E (2a, Cu^r),12= Xac-E (2a x Xac-A 20), conjugant, 13= Xac-E (1c Cu^r), 14= Xac-E (9 a Cu^r). Strains in lanes 1, 3, 5, 6, 7, 8, 9, 11, 12, 13 and 14 were copper resistant and contained a large plasmid (arrow).

Table 5-1. List of bacterial strain used in chapter 5

Designation	Relevant characteristics	Source
Xanthomonas axonopodis pv. *citri*		
Xac-A^{w} (12879)	GFP^{+}; Kan^{r}	Fl.
Xac-A 20 (1874	GFP^{+}; Strep^{r}; Rif^{r};White	Fl.
Xac-A 20 (1874	GFP^{-}; Rif^{r} ; Spec^{r}	Fl.
Xac-A^{w} (12879	GFP^{-}; Spec^{r}; Rif^{r}	Fl.
Xac-A 16 (Xc02-1443)	Cu^{r}	Ctes.
Xac-A 26 (Xc01-1394-1)	Cu^{r}	Ctes.
Xac-A 44 (Xcc03-1338-1-1)	Cu^{r}	Ctes.
Xac-A 45 (Xcc03-1639-1-4)	Cu^{r}	Ctes.
Xac-A 40 (Xcc03-1633-1)	Cu^{s}	Ctes.
Xac-A 40 (Xcc03-1634)	Cu^{s}	Ctes.
Xac-A 42 (Xcc03)	Cu^{s}	Ctes.
Xanthomonas axonopodis pv. *citrumelo*		
Xac-E (104)	Cu^{s}	Ctes.
Xac-E (2a)	Cu^{r}	Ctes.
Xac-E (1c)	Cu^{r}	Ctes.
Xac-E (9^{a})	Cu^{r}	Ctes.
Xanthomonas campestris pv. *vesicatoria*		
Xcv (75-3)	GFP^{-}; Cu^{r} (plasmid)	Fl.
Xcv (XVP26)	GFP^{-}; Cu^{r} (chromosomal)	Fl.

Xanthomonas axonopodis pv. *citri* (Xac), *Xanthomonas campestris* pv. *vesicatoria* (Xcv), Fl, Florida, provided by Robert Stall from DPI Gainesville Fl. collection and Ctes, Corrientes, provided by Nelly Canteros from INTA, Bella Vista, Corrientes, Argentina, Kanamycin resistant (Kan^{r}), Streptomicyn resistant (Strep^{r}) , Rifamycin resistant (Rif^{r}), Spectinomycin resistant (Spec^{r}), Copper resistance (Cu^{r}); Copper sensitive (Cu^{s}).

Table 5-2. Frequency of transfer of copper resistance genes among *Xanthomonas axonopodis* pv. *citri* (Xac-A) after conjugation in a liquid medium

Donor strain	Recipient strain	Range of frequency of transfer (per donor cell)[a]
Xac-A 16	Xac-A 20	3.9×10^{-6} to 1.25×10^{-5}
Xac-A 26	Xac-A 20	0 to 1.12×10^{-6}
Xac-A 44	Xac-A 20	0 to 8.66×10^{-6}
Xac-A 45	Xac-A 20	0 to 4×10^{-7}

[a] Based on four determinations per donor x recipient combinations.

Table 5-3.Frequency of transfer of copper resistance genes among strains of *Xanthomonas axonopodis* pv. *citri* (Xac-A) after conjugation in a liquid medium

Donor strain	Recipient strain	Range of frequency of transfer (per donor cell)[a]
Xac-A 16	Xac-A 40	0 to 2.4×10^{-4}
Xac-A 16	Xac-A 41	0 to 5×10^{-5}
Xac-A 16	Xac-A 42	0 to 1.82×10^{-9}
Xac-A 26	Xac-A 40	0
Xac-A 26	Xac-A 41	0 to 9.09×10^{-9}
Xac-A 26	Xac-A 42	0 to 1.3×10^{-5}
Xac-A 45	Xac-A 40	0
Xac-A 45	Xac-A 41	0
Xac-A 45	Xac-A 42	0

[a] Based on four tests of conjugation.

Table 5-4. Frequency of transfer of copper resistant genes from *Xanthomonas campestris* pv. *vesicatoria* (Xcv) to copper sensitive strains of *Xanthomonas axonopo*dis pv. *citri* by conjugation on a solid medium

Donor strain	Recipient strain	Range of frequency of transfer (per donor cell)[a]
Xcv 75-3	Xac-A 40	0 to 2×10^{-4}
Xcv 75-3	Xac-A 41	0 to 1.33×10^{-6}
Xcv 75-3	Xac-A 42	0 to 1.33×10^{-6}

[a] Based on four tests of conjugation.

CHAPTER 6
CLONING AN *AVR* GENE FROM THE XAC-A^{W} STRAIN

Introduction

Previously we found no evidence for a genetic basis for the host specificity of Xac-A^{w} strains compared to the Xac-A strains. In many cases, however, hypersensitivity of a strain in a particular host is the basis for and is the result of the interaction of an *avr* gene in the pathogen and a resistance gene in a host. The purpose of this chapter is to provide results of screening a DNA library of Xac-A^{w} for the presence of *avr* genes in Xac-A^{w} that cause a hypersensitivity in grapefruit leaves.

Materials and Methods

Bacterial strains and media. Strains used in this study are listed in Table 6-1. All strains were maintained at -80° C and subcultured, when needed, on nutrient agar (NA). Rifamycin-resistant strains were obtained by plating 500 µl of Xac-A^{w} strain 12879 at 10^{9} colony-forming units (cfu) /ml onto nutrient agar supplemented with 80 µg/ml of rifamycin. *Escherichia coli* (Ec) strains were cultured on Luria-Bertani medium (Maniatis et. al., 1982). Conjugations were performed on nutrient-yeast-glycerol agar (NYGA, Daniels et. al., 1984). The antibiotics and concentration used in media were: rifamycin, 75 µg/ml; tetracycline, 12.5 µg/ml; and kanamycin 5 µg/ml.

Growth of plants and inoculum preparation. Growth of plants and inoculum preparation were the same as described in Chapter 3.

Electrolyte leakage determinations. Electrolyte-leakage determinations were made in the same way as described in Chapter 3.

Bacterial growth curves. Bacterial growth curves were obtained in the same way as described in Chapter 3.

Recombinant DNA techniques. Techniques used for cosmid cloning, enzyme digestion, ligation, Southern transfer, plasmid alkaline lysis, and agarose gel electrophoresis were described by Maniatis et al. (1982).

A genomic library of Xac-A^w strain 12879 was constructed in the vector pLAFR3 as previously described (Metz et al., 2005) and supplied by G. V. Minsavage (Plant Pathology Dept., University of Florida). Individual clones were maintained in Ec DH5α (BRL) and maintained on Luria-Bertani medium. The helper plasmid pRK2013 in Ec HB 101 was used in conjugations involving triparental matings.

Individual clones of an Xac-A^w genomic library in Ec DH5α were conjugated into strain 91-118 of *X. perforans* (Jones et al., 2004). Transconjugants were infiltrated into a 1 cm^2 area of leaf tissue with a syringe and needle. The recipient bacterium is pathogenic to tomato, but causes a null reaction in grapefruit leaves. A clone that caused an HR reaction in both grapefruit and tomato leaves was detected by rapid necrosis in the infiltrated area of the leaf. Tomato leaves were inoculated as a control, because the Xac-A^w strain also elicits an HR in tomato leaves (Figure 6-1). Grapefruit leaves and tomato leaves were inoculated with ca. 300 transconjugants individually. Each clone contained approximately a 25-kb fragment of Xac-A^w DNA.

Subcloning. A clone, pL799-1 with HR activity in grapefruit, but not tomato was obtained and consisted of about a 25-kb insert of Xac-A^w DNA. This clone was subcloned to contain only DNA necessary for HR activity. In this subcloning, to identify the exact location of the gene in pL799-1, the transposon pHoGus was used to knock out

the gene responsible for HR activity by the procedure described by Huguet and Bonas, 1997. About 160 kanamycin resistant clones, which contained pHoGus inserts were screened for the lack of HR in grapefruit after they were transferred to *X. perforans*. Three clones were selected for lack of an HR and one clone (pL799-1) was selected from the three for further work. The clone pL799-1 was restricted with each of several enzymes to find a fragment that contained the Tn3 insert. A *Hin*dIII restriction fragment, contained the Tn3 insert and about 3.0 kb of DNA. This fragment was ligated into pBluescript II KS and labeled pBs3.0. A portion of the 3.0 kb insert in pBs3.0 was then sequenced using forward and reverse primers from the cloning vector. Custom oligonucleotide primers were designed to complete the sequencing of the intact *Hin*dIII fragment.

An open reading frame (ORF) was identified in the sequence of the intact *Hin*dIII fragment. However, the ORF was at the end of the fragment and was not complete. When the intact *Hin*dIII fragment was ligated into pLAFR6 (pL799-2) and conjugated into *X. perforans*, no HR occurred in grapefruit leaves. Then primers were selected for sequencing beyond the end of the *Hind*III fragment in the original clone to obtain the sequence of the complete ORF. Primers were selected from the sequence to amplify by PCR a 2.3-kb fragment containing the complete ORF, which was then ligated into pGEM®T Easy Vector (Promega, Madison, Wisconsin). The 2.3 kb insert was removed from pGEM®T Easy Vector with *Eco*RI enzyme and then ligated into the vector pUFR043, and designated pU799-3. The pUFR043 cosmid was used as a vector because DNA inserts in this vector could be conjugated into strains of Xac-A^{w}, whereas pLAFR

derivatives could not be transferred to strains of Xac-A^{w}. The pU799-3 in *X. perforans* caused an HR in grapefruit leaves. The open reading frame was labeled *avrGf1*.

Mutation of *avrGfl* in Xac-A^{w}. The *avrGf1* gene in Xac-A^{w} was mutated to investigate the role of the gene in the host specificity of the bacterium. Mutation in *avrGf1* in pGEM®T Easy Vector occurred by inserting an Omega cassette into the gene by the procedure described by Huguet et al., (1998). The inactive gene was exchanged into the Xac-A^{w} strain by using the suicide vector pOK1. Eventually an Xac-A^{w} strain with the mutated *avr* gene was obtained. This strain was labeled Xac-A^{w} Ω.

DNA sequence analysis. Sequencing of pBluescript clone pBs3.0 was initiated at the sequencing facility (University of Florida, Gainesville, Fl, USA) with the Applied Biosystems model 373 system (Foster City, CA, U.S.A.). To complete sequencing of both strands of DNA, custom primers were synthesized at the ICBR facility with an Applied Biosystem model 394 DNA synthesizer. The computer program SeqAid II version 3.81 was used to analyze nucleotide sequence data and predicted protein products of the 2.3 kb region that contained *avrGf1*. A search for nucleotide and amino acid sequence homology was conducted with the BLASTN and BLASTP 2.2.11 programs (Altschul et al. 1997).

PCR procedure. Based on DNA sequence analysis of the avirulence gene identified in Xac-A^{w} , custom primers were designed to amplify a fragment from the avirulence gene from DNA of xanthomonads that cause disease in citrus plants. The primers used were forward 5-‘CGCCGGTTTCTGTCCTGCACTTG-3’ and reverse 5’-GCCGCCTTTGCCATCGACCAG-3’. The final product was 199 bp. PCR reactions were performed in a thermocycler (M.J. Research, Watertown, MA, U.S.A.).

Southern hybridization. All hybridization experiments were performed on nitrocellulose membranes using the GENIUS nonradioactive DNA labeling and detection kit according to the manufacturers instructions (Boeheringer Mannheim Biochemicals, Indianapolis, IN, U.S.A). Genomic DNA extractions were made using the GenomicPrepTM Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Inc. 800 Centennial Avenue, PO Box 1327 Piscataway, NJ 08855, USA). Plasmid extraction from Xac-A and Xac-A^w strains was as described in Chapter 5.

Results

Selection of avirulence gene. Three clones were selected that caused rapid necrosis in grapefruit leaves but not in tomato leaves, and three other clones caused rapid necrosis in tomato leaves, but not in grapefruit. The first three clones were designated pL799-1, pL22, and pL622. All three clones were successfully transferred from *E. coli* by conjugation into a strain that causes Asiatic citrus canker (Xac-A) in grapefruit and Key lime. Only one of the three clones, pL799-1, caused an HR in grapefruit leaves when expressed in the Xac-A strain (Figure 6-2). The pL799-1 clone in the Xac-A strain did not cause an HR in Key lime leaves, which is typical of the host range of the wild Xac-A^w strain (Figure 6-3). This clone was further characterized in this work.

To obtain more information about the significance of the avirulence gene in the host range of Xac-A^w, primer sequences were obtained for amplification of a portion of avirulence genes by PCR from the sequence of the 2.3 fragment in pU799-3. DNA from three Xac-A strains and four Xac-A^w strains was extracted and tested for the presence of the avirulence gene by amplification of DNA with those primer sequences. Amplification of a product of the expected size occurred with all of the Xac-A^w strains, but no amplification of a fragment of the expected size occurred with any Xac-A strain. Using

Southern hybridization with the pU799-3 clone as a probe, the avirulence gene was determined to be present in the Xac-A^{w} strains but not in the Xac-A strains. In addition, the avirulence gene was not present in strains of the B and C groups of *X. axonopodis* pv. a*urantifolii*, or in strains of the Xac-A* strains of the Xac group, or in strains of *X. axonopodis* pv. *citrumelo* (Figure 6-8). The B strain and strains of *X. axonopodis* pv. *citrumelo* did not cause rapid necrosis in grapefruit leaves but the C strain did. Also, at least some of the Xac-A* strains also caused rapid necrosis in grapefruit leaves (Data not presented). Apparently, other avirulence genes may exist in those strains. The avirulence gene (*avrGf1*) hybridized with *Xanthomonas campestris* pv. *campestris* strain 8004 but the restriction pattern was different. Strain 8004 causes rapid necrosis in grapefruit leaves. The avirulence gene (*avrGf1*) was located in the chromosome because the plasmid DNA did not hybridize with the *avrGf1* probe (Figure 6-8).

Comparison of mutated Xac-A^{w} with the Xac-A and Xac-A^{w}. The strain Xac-A^{w} with the mutated avirulence gene was compared with the Xac-A and Xac-A^{w} strains in inoculation tests in grapefruit leaves. Visually, the symptoms caused by the mutant strain of Xac-A^{w} were more like the wild-type Xac-A strain than the wild-type Xac-A^{w} strain (Figure 6-4 and 6-5). However, the symptoms of the mutated strain were not identical to Xac-A strain. Nevertheless, inactivation of the avirulence gene in Xac-A^{w} did alter the disease reaction in grapefruit leaves.

Growth of the strains in grapefruit leaves was compared. In addition, electrolyte leakage from the leaves inoculated with the strains was determined to differentiate the time to necrosis after inoculations. In these experiments cultures, of Xac-A 40, Xac-A^{w} 12879, Xac-A 40 (pU799-3), Xac-A$^{w}\Omega$, and Xac-AwΩ (pU799-3) were compared.

Illustrations of the results are included in Figures 6-6 and 6-7. Growth of all strains was about equal for the first four days after inoculation. However, at day six the populations of the strains were different. At day ten the population of the Xac-A strain was the greatest. The population of the Xac-A^w strain was about 1.5 log units lower than the Xac-A strain. The Xac-$A^w\Omega$ strain reached a population intermediate between the Xac-A and the Xac-A^w . The strains with the pU799-3 clone had the lowest populations.

In electrolyte leakage determinations the Xac-A^w strain and the Xac-A strains with the clone pU799-3 began to increase at day four compared to that caused by Xac-A and Xac-$A^w\Omega$ strains. At day eight the strains with the avirulence gene had significantly greater electrolyte leakage than the strains that did not contain the avirulence gene. This data confirmed the visual observations of necrosis caused by those strains.

DNA sequencing analysis. Sequence analysis of the 2.3 kb fragment in pU799-3 was determined. A 1599 bp nucleotide open reading frame (ORF) was found that was sufficient for *avrGf1* activity. The complete sequence of the 2.3 kb, the *avrGf1* gene and the primers used to amplify the *avrGf1* are shown in the Figure 6-9. The upstream regions of this ORF do not contain a *hrp* box but do contain an imperfect PIP box TTCGT-N10-TTCGC 80 bp upstream of the start codon (Huguet and Bonas, 1997). The GenBank search identified significant homology with a gene in the genome of *Xanthomonas campestris* pv. *campestris* (Xcc) str. 8004 (84 % identical). When an alignment was done between the completely sequenced Xcc3600 gene and *avrGf1* using the clustal W (1.82) multiple sequence alignment 84.99 % identity was found (Figure 6-10).

Discussion

A genomic library of an Xac-A^w strain was successfully produced and incorporated into *E. coli* DH5α. For successful screening of the library for avirulence genes one would

normally transfer each clone into a strain that was pathogenic on the plant in question; in this case into a strain of Xac-A. However, in previous experiments the transfer of clones in pLAFR3 cosmid to strains of Xac-A did not occur at high frequency by triparental matings (G. V. Minsavage, Plant Pathology Dept., University of Florida, Gainesville). To circumvent this problem the Xac-A^w library was transferred from *E. coli* to strain 91-118 of *X. perforans* by triparental conjugations and nearly 100 % of the matings were successful. The *X. perforans* strain contains the *hrp* genes (Bonas et al., 1991) necessary for transfer of avirulence gene proteins into host cells, so it was thought that an avirulence gene in the genome of Xac-A^w to grapefruit could be found by this procedure. In fact, an avirulence gene in the Xac-A^w library was found. This procedure could possibly be used to locate other avirulence genes when transfer of clones to a pathogen occurs very infrequently during triparental matings with *E. coli.*

Three clones from the Xac-A^w library were obtained that produced an HR in grapefruit leaves when expressed in *X. perforans*. When the three clones were transferred to the Xac-A strain only one caused an HR in grapefruit leaves. The two clones that did not cause an HR probably have similar DNA sequences based on restriction enzyme digestion (data not given). One of the clones, pL22, is being investigated further to determine the reason that no HR occurred in grapefruit when expressed in the Xac-A strain.

The clone that was expressed in Xac-A and that caused an HR in grapefruit leaves did not cause an HR in leaves of Key lime. This is the same reaction as the Xac-A^w strain in the two hosts. The importance of the avirulence gene in determination of the host specificity of the Xac-A^w strain was further investigated by determining the presence of

the gene in other bacterial strains pathogenic to citrus. This gene was only found in Xac-A^w strains by PCR and Southern hybridization techniques. When the gene was mutated in an Xac-A^w strain the symptoms caused by the mutated strain were similar to those caused by the Xac-A strains. However, the symptoms were not quite the same. In addition the growth of the mutated strain in grapefruit leaves was significantly greater than the wild-type Xac-A^w strain but lower than the Xac-A strain. Therefore, the avirulence gene *avrGf1* seems to be important in delimiting the pathogenic specificity of the Xac-A^w strain.

An assumption could be made that another *avr* gene exists in the wild type of Xac-A^w that prevents the symptoms of the mutated strain to be equal to as the wild-type Xac-A strain and also prevents the populations of the two strains from being the same. There could be another *avr* gene in the genomic library that was not identified because we only screened 300 clones. More clones should be screened to search for another *avr* gene or host range limiting factor.

Clones causing an HR in tomato, but not in grapefruit were also found in the genomic library of Xac-A^w. Wild-type strains of Xac-A and Xac-A^w cause an HR in tomato, but not in leaves of tobacco or pepper (Figure 6-1). Very little was done with these clones in this work. It would be interesting to determine if those clones contain an avirulence gene that provides the differential reactions in the three plants.

Figure 6-1. Symptoms caused by Xac-A (left) and Xac-A^{w} (right) strains in tobacco, tomato, and pepper leaves.

Figure 6-2. Symptoms in grapefruit leaves after infiltration with the following bacterial suspensions: 1= Xac-A 40; 2= Xac-A 40 (pL799-1); 3= Xac-A 40 (pL22); 4= Xac-A 40 (pL622).

Figure 6 -3. Symptoms in Key lime leaves after infiltration with different bacterial suspensions: 1= Xac-A 40; 2= Xac-A 40 (pL799-1); 3= Xac-A 40 (pL22); 4= Xac-A 40 (pL622).

Figure 6-4. Symptoms in grapefruit following inoculation with Xac-A 40 strain (A), Xac-A^w 12879 strain (A^w) and Xac-A^w Ω (3).

Figure 6-5. Symptoms in grapefruit after inoculation by needle pricks with Xac-A^w strain 12879 (right top), Xac-A^w Ω (bottom left), and Xac-A 40 (bottom right).

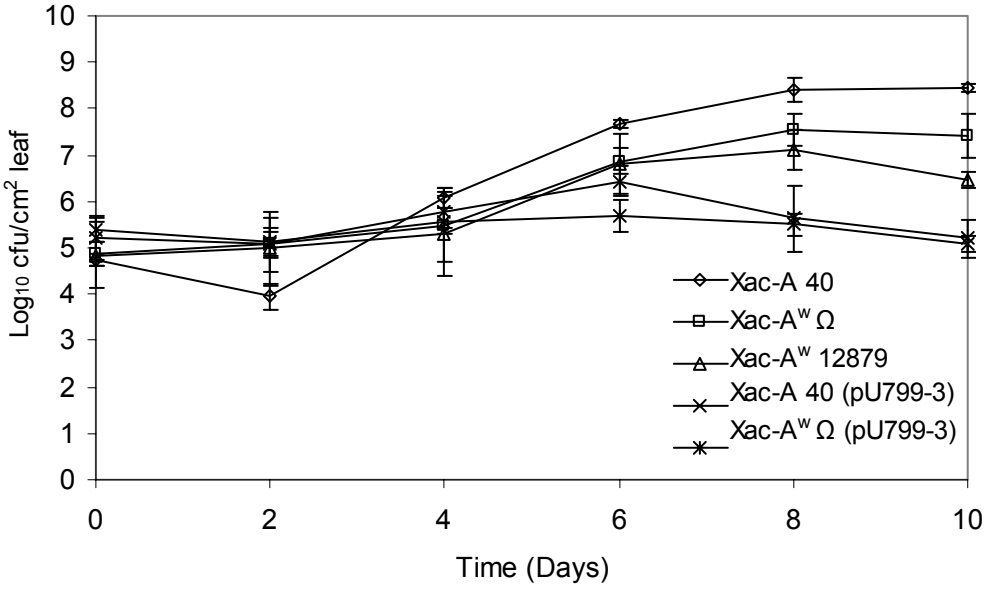

Figure 6-6. Bacterial populations in grapefruit leaves infiltrated with Xac-A 40; Xac-A^{w} Ω; Xac-A^{w} 12879;Xac-A 40 (pU799-3), Xac-A^{w} Ω (pU799-3) at various times after inoculation.

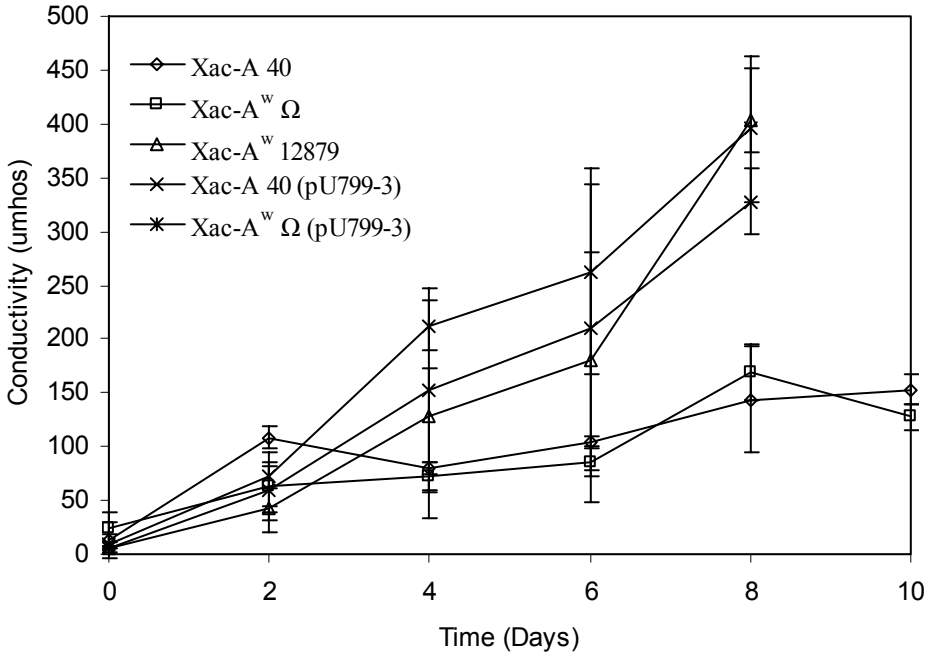

Figure 6-7. Electrolyte leakage in grapefruit leaves infiltrated with of Xac-A 40, Xac-A^{w}Ω, Xac-A^{w} 12879, Xac-A 40 (pU799-3), Xac-A^{w} Ω(pU799-3) at a concentration of 5x10^{8} cells (cfu/ml).

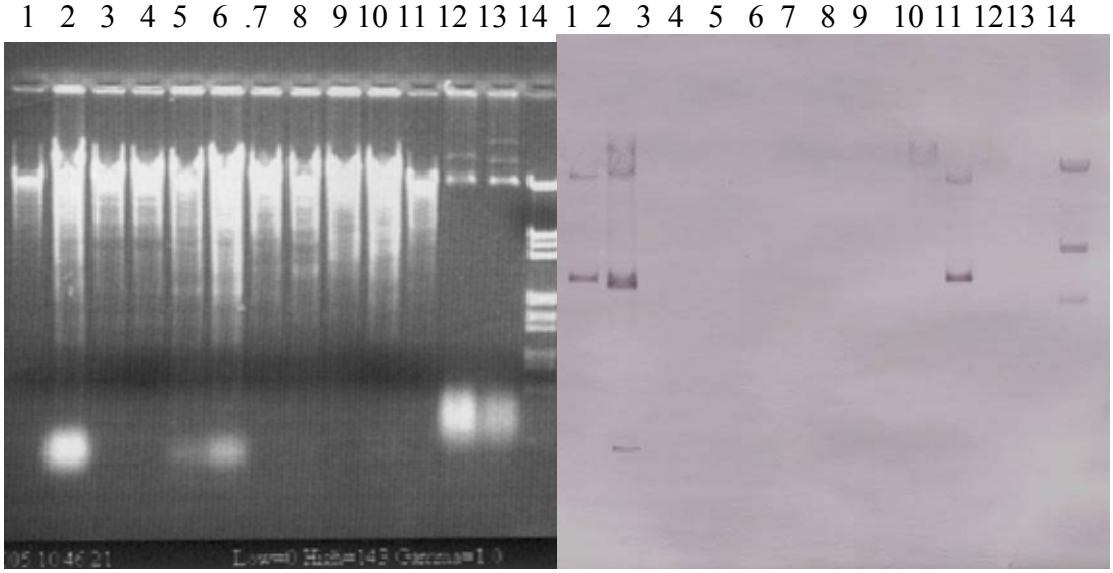

Figure 6-8. Hybridization of the subclone pU799-3 fragment in *avrGf*1 to total genomic DNA of *Xanthomonas* strains digested whith *Hind*III. Lanes 1= Xac-A^{w}; 2= Xac-A^{w} Ω; 3= Xac-A 40; 4= Xac-A 306; 5=Xac- A* 1574; 6=Xac-A* 1575; 7= Xaa-B; 8=Xaa-C; 9= Xac-E 1887; 10= Xcc 8004; 11= Xac-A^{w} 12879; 12= plasmid Xac-A^{w}; 13= Plasmid Xac-A 40; 14= λ Marker digested with *Hind*III. Ethidium bromide stained gel on the left. Southern blot of gel on the right.

GATCGGCGCCAGGAAGGGCCTGCCATGTCACAACCTGATTCATCTGTGCCAAGACCACCTCGCACCGCGT
GCGCGCATGGTGTACTCCGCGTCTCCAAAGAAAGCTGCATCGCCATCTTCCGATTCGGCTCAGTGTGTCTT
TTTTGCAAGCGTCTGATGGGGGTTGGAGTTATTCAAGCCTCCCGAGTCAACGTGATGCGGACACGCTTAC
ACCAAACCGAAATGCATCGGTCCGCCTGGTGCGATCGCCGAGCTTTCGCACAGCCACTCGTTTGTGACAT
CGCTTTTTCGCATTCGAGATGCAGACAGCTCAGGCCTTCAGGAAAAATAGGTCGTCAATCCGGTAGCAGT
GAAATACACGGCGTTATATGCACATTTACGTCTTGTCATCCCGATGGCTTGTCGCTCCGGATCGCTGGTAA
CTCACCACGGTCGATGAGTGCCGCCAGCCCAGGCACGCACAGTCAACACGGCCTCGCCGCGGACTGATA
GCAATCTTCCATGTGATACCCGCAGAGTGGATCGATATGTCGTCGGTAGCGCGCGGCCATGCTTTTCATA
GTCCCTATTCCATTCGTCCATCAGCACTTCGCCTAGTCAACAAGCTCCGATGGCTCCGAGCATGCATTCGG
CGGCGTCGCCGGTTTCTGTCCTGCACTTGAGAGATACATCC**ATGGCTCCGAGCATGCATTCGGCGGCG**
TCGCCGGTTTCTGTCCTGCACTTGAGAGATACATCCATGCGCACCAAAGCCCAACTCCCATTGACT
GCCATTCAACGGTTTCTTGCCCATGATGCAGCGTCAACGCAGGCCCCCTCTGCATCGGCATCCACA
TCGCTCCACAAAAATGAGACCGCAGGCTTGCTGGCAGCCTTGCCAGCGCGAAACGCCAGGCAAGG
AGCGCAGAGGAAGTCCGGCGAAAAAGAAGGCGCACGCCAAAACAACGGGGGCCGGGGCGGACAA
TGGGCATCTCGGGCGGCCAAGTACGCCCTGGGAATCGCCGGTGCTGGCTATGTTGCAGACAATTT
CGTTCTGTCCACGACATCGCTGGTCGATGGCAAAGGCGGCTTTACCAGTAATGATCGTTTGGATAA
AGCATGCGCAAAGGCCGAGACGTATTACGCCCGGTACCACAGTGCCACTGAGGATGAGCGTGCAT
CCCATAGCCGCCCCTTTGTACCGATCAGAACGTGCGGTTCCAACCAGTTCGCCACCATGACCGACT
ACCGCGCGGCGACCAAGGTCCATGTCGGTCATCTTTTCGACAGCCAAGCCGCGCGCGAATCGCTC
GTCACCAACCTCGCCTGCCTCAAGGGCGAGCGGATCAAGCAGGAGTGCATCATCAGGTATGCGCC
TGCGCAGGTGCCAGCGGATCCGGACCTAAGCAAGAGCGAGCTGTACGACAGGAAAAACAAGTACT
CGTTGGTTGGCATGCCCAACGCCCAAACCGGAGCAAGTGGATATACCTCACGCTCGATCACCCAGC
CCTTCATCAACCGCGGCATGGAGCATTTCAGACAGGCTTCACAGAGCGACAAGGCACTGTCCCTGA
GGCAGTGCATGCAATCGCTTGAACGGGCACTGCAGGACACTGACAAGCTTGGCAAGCAAGCACAG
CACGCCGCCGGCCAGGCAATCCTGAATTTCCGGCAGGTCTATGCGGCCGACGAGCATTGGGGTCA
CCCGGAGAAAGTCATCATGAAAACCCTGATCGCCAACGGACTGCTATCGCAAGAACAAACCGACA
GGATCGATGCGACCCTGATGTTCGAAGATCCCTCCATCAGTGTCCTGAAAAGAAATACCAGCATCG
CCGGACCGTTACTGCAAAAACTGGAAACGAAGATTCAGTCAAAACGTCTGCAGGATCAACCTGAAA
CGCTTGCAGACTTCATGGAAATGGCTAAGCAGAAGAACATGGAAGGCCTGCCGATCGCGCACTTC
AAACTCAACGCCGAAGGCACCGGTTTTGAAGACTGCTCTGGCCTGGGCGACTCGTTTACTAGTGCA
AATGCCGTTGCATGCATCAACCATGCACGCTTGATGAGCGGAGAGCCGCGCCTTTCGAAAGAGGA
TGTCGGTGTCGTGGTTGCCTGCCTCAATGCTGTATACGACGATGCCAGCAGCATCCGGCACTCGCT
TCATGAAATCGCACGCGGATGCTTCGTAGGTGCCGGCTATACAACGGAAGATGCCGATGCGTTCTA
TGAGCAGATTTGCAAGGATGCTGCACGGGCATTCTATGCAGGAAAGTCAATGACCAGCAGCGACT
AATCGCCCCCTTCCCACCTCATTGCGCTGACGCAACTTGGCGCCCGGTTGAGG

Figure 6-9. Nucleotide sequence of the 2.3 kb fragment of DNA from Xac-A^{w} containing *avrGf1* nucleotide sequencing (bold letters).The primer sequences used to amplify a fragment of avrGf1 by PCR are underlined. Imperfect PIP-box is shaded in gray.

```
seq_1           ----------------------------CTGATTTCCGTCCTGCACTCTAGGAGACCC
seq_2           ATGGCTCCGAGCATGCATTCGGCGGCGTCGCCGGTTTCTGTCCTGCACT-TGAGAGATAC
                                           * * **** ********** *  ****  *

seq_1           ATC-ATGCGAACAAAAACCTCACTGCCGTTGGCCACCGTCCAGCGGCTACTGACCCCTGG
seq_2           ATCCATGCGCACCAAAGCCCAACTCCCATTGACTGCCATTCAACGGTTTCTTGCCCATGA
                *** ***** ** *** **  *** ** *** *  ** * ** *** * **  *** **

seq_1           CACCTCCACCGGGTTATCCACCCCCGCGTCGGCGTCAGCCACTCCCTGTGCAGAAACGAC
seq_2           TGCAGCGTCAACGCAGGCCCCCTCTGCATCGGCATCCACATCGCTCCACAAAAATGAGAC
                  *  *  *   *    ** ** * ** ***** **  *  * * *     * *   ***

seq_1           GGCAGGCTTACTGGGAGCGTTGCCAACTCGAAAGAACAAGCAAAAACAGCAAAGCCAGCG
seq_2           CGCAGGCTTGCTGGCAGCCTTGCCAGCGCGAAACGCCAGGCAAGGAGCGCAGAG--GAAG
                 ******** **** *** ****** * *****   ** ****  *  *** **     *

seq_1           TCCACCCAATACGCAGGACGGTACACCAAAGAATGGCAGAGACCATGGCGGACAGTGGGC
seq_2           TCCGGCGAAAAAGAAGGC--GCACGCCAAA--ACAACGGGGGCCGGGGCGGACAATGGGC
                ***  * ** * * ***   * ** *****  *   * * * **  ******** *****

seq_1           AACACGAGCTGCCAAGTACGCTCTTGGCATTGCTGGCGCAGGCTATGTGGCAGACAACTT
seq_2           ATCTCGGGCGGCCAAGTACGCCCTGGGAATCGCCGGTGCTGGCTATGTTGCAGACAATTT
                * * ** ** ********** ** ** ** ** ** ** ******** ******** **

seq_1           CTTTCTTTCAACGACCTCGCTCCGCGACGGCAAGGCCGGATTTAGCAGCAATGATCGGCT
seq_2           CGTTCTGTCCACGACATCGCTGGTCGATGGCAAAGGCGGCTTTACCAGTAATGATCGTTT
                * **** ** ***** *****   *** ***** * *** **** *** ********  *

seq_1           TGAGAAAGCATGCGTGAAAGCGGAGAGCTATCACGCGCGGTATCACAGCGCTACCGAAGG
seq_2           GGATAAAGCATGCGCAAAGGCCGAGACGTATTACGCCCGGTACCACAGTGCCACTGAGGA
                 ** **********  ** ** ****  *** **** ***** ***** ** ** ** *

seq_1           AGAACGCGCATCGCACAGCCGTCCCTTCGTACCGATCAGAACGTGCGGGTCCAACCAGTT
seq_2           TGAGCGTGCATCCCATAGCCGCCCCTTTGTACCGATCAGAACGTGCGGTTCCAACCAGTT
                 ** ** ***** ** ***** ***** ******************** ***********

seq_1           CGCCACCATGAGCGACTACCGTGCGGCGACCAAGGTTCATATCGGCCACCTCTTCGACAG
seq_2           CGCCACCATGACCGACTACCGCGCGGCGACCAAGGTCCATGTCGGTCATCTTTTCGACAG
                *********** ********* ************** *** **** ** ** ********

seq_1           CCAGCACGCACGGCAATCGCTGCTCACCAACCTTGCCTGCCTCAAGGGCGAGCGCATCAG
seq_2           CCAAGCCGCGCGCGAATCGCTCGTCACCAACCTCGCCTGCCTCAAGGGCGAGCGGATCAA
                ***   *** **  *******  ********** ******************** ****

seq_1           GGACGAGTGCATTGCCCAGTACGCCCCTACGCATGTCCCGGCCAATCCGGACCTGAGTAG
seq_2           GCAGGAGTGCATCATCAGGTATGCGCCTGCGCAGGTGCCAGCGGATCCGGACCTAAGCAA
                * * ********   *  *** ** *** **** ** ** **  ********** ** *

seq_1           AAGCCCGCTCTACGAAACCAAGAACAAGTACTCTCTGACCGGCGTACCCAATGCTCAGAC
seq_2           GAGCGAGCTGTACGACAGGAAAAACAAGTACTCGTTGGTTGGCATGCCCAACGCCCAAAC
                 ***  *** ***** *  ** **********  **   *** * ***** ** ** **

seq_1           CGGTGCAAGCGGATATACCTCACGATCAATCACCCAGCCCTTCATCAATCGCGGCATGCA
seq_2           CGGAGCAAGTGGATATACCTCACGCTCGATCACCCAGCCCTTCATCAACCGCGGCATGGA
                *** ***** ************** ** ******************** ********* *
```

Figure 6-10. Alignment between the complete sequence of XCC3600 from *Xanthomonas campestris* pv. *campestris* and the *avrGf1* using the clustal W (1.82) multiple sequence alignment (seq_1 xc3600 and seq_2 *avrGF1*). The Xcc 3600 gene and *avrGf1* are 84.99 % similar.

```
seq_1           GCATTTCAAGCAGGATTCCCAGAGCGACAGAGCGTTGTCGCTCAAACAGTGCATGGAATT
seq_2           GCATTTCAGACAGGCTTCACAGAGCGACAAGGCACTGTCCCTGAGGCAGTGCATGCAATC
                ********  **** *** **********  **  **** ** *  ********* ***

seq_1           GCTTGAACGTACACTGGAGGGCGACGACAAACTTGGCAAGCAGGCACAACACGCTGCCGG
seq_2           GCTTGAACGGGCACTGCAGGACACTGACAAGCTTGGCAAGCAAGCACAGCACGCCGCCGG
                *********  ***** *** *   ***** *********** ***** ***** *****

seq_1           CCAAGCGATCCTGAATTTCCGTCAGGTGTATGCCGCCGACGAGCATTGGGGCCACCCCGA
seq_2           CCAGGCAATCCTGAATTTCCGGCAGGTCTATGCGGCCGACGAGCATTGGGGTCACCCGGA
                *** ** ************** ***** ***** ***************** ***** **

seq_1           AAAAGTCATCATGAAAACGCTGATCGCCAACGGGCTGCTATCGCAGGAGCAAACGGACAG
seq_2           GAAAGTCATCATGAAAACCCTGATCGCCAACGGACTGCTATCGCAAGAACAAACCGACAG
                 ***************** ************** *********** ** ***** *****

seq_1           GATCGATGCGACCCTGATGTTCGAAGATCCGTCCATCAGCGTATTGAAAAAAAACACCAG
seq_2           GATCGATGCGACCCTGATGTTCGAAGATCCCTCCATCAGTGTCCTGAAAAGAAATACCAG
                ***************************** ******** **  ****** *** *****
```

Figure 6-10---continued.

Table 6-1. List of bacterial strains used in chapter 6

Bacterium	Abbreviation	Original strain number	Origin
X. perforans			J.B Jones
91-118 (pL799-1)			This work
91-118 (PL799-2)			This work
91-118 (pL22)			This work
91-118 (pL622)			This work
Xa pv. citri	Xac-A 40	(Xcc03-1633-1)	Ctes.
Xac-A 40 (pL799-1)			This work
Xac-A 40 (PL799-2)			This work
Xac-A 40 (pL22)			This work
Xac-A 40 (pL622)			This work
Xa pv. citri	Xac-A^w	12879	DPI
Xac-A^w (pU799-3)			This work
Xac-$A^w\Omega$			This work
Xac-$A^w\Omega$(pU799-3)			This work
Xa pv. *citri*	Xac-A 306	2422	DPI
Xa pv.*citri*	Xac-A*	1974	DPI
Xa pv.*citri*	Xac-A*	1975	DPI
Xa pv.*aurantifolii*	Xaa-B	1622	DPI
Xa pv. *aurantifolii*	Xaa-C	5979	DPI
Xa pv.*citrumelo*	Xac-E	1887	DPI
Xc pv. *campestris*	Xcc	8004	DPI

Xanthomonas axonopodis pv. *citri* (Xac), *Xanthomonas campestris* pv. *vesicatoria* (Xcv), Fl, Florida, provided by Robert Stall from DPI Gainesville Fl. Collection and Ctes, Corrientes, provided by Nelly Canteros from INTA, Bella Vista, Corrientes, Argentina.

CHAPTER 7
DISCUSSION

Many pathogenically different strains of the causal agent of citrus canker (*Xanthomonas axonopodis* pv. *citri* and *X. axonopodis* pv. *aurantifolii)* have been described. These strains sometimes differ genetically and pathologically. The variation within the strains involved in citrus canker disease is not unusual. For example, the xanthomonads that cause the bacterial spot disease of tomato and pepper consist of many strains and some have significant genetic differences, resulting in different species of *Xanthomonas* (Jones et al., 2004).

The Xac-A^w strain that was characterized by Sun et al. (2004) was found to be a close relative of Xac-A even though they had pathogenic differences (Cubero and Graham, 2002). Contrary to the xanthomonads that cause the bacterial spot disease of pepper and tomato, there were no reports of a hypersensitive reaction (possible exception Xac-C strain) responsible for host range differences. The characterization of the HR caused by Xac-A^w in grapefruit leaves is new.

The Xac-A^w strains presented a problem for the eradication and regulation program of the Division of Plant Industry (DPI) for citrus canker (Sun et al., 2004). After careful consideration, DPI decided to destroy only Key lime and alemow plants in a 1900 ft radius, and not other citrus plants, near the focus of disease caused by the Xac-A^w strain. One of the considerations in establishing the program was the lack of knowledge of the stability of the pathogenicity of the Xac-A^w strain. To obtain information on this problem, we chose three avenues of research. We investigated the frequency of

development of mutants for host specificity of the Xac-A^{w} strain, the transfer of genes by conjugation, and the search of the genome of the Xac-A^{w} strain for an avirulence gene.

We never were able to find a mutant of the Xac-A^{w} strain that was pathogenic on grapefruit after treatment with the mutagen NTG. However, streptomycin and pigment production mutants of the Xac-A^{w} strain developed in vitro. The methods used to find mutants pathogenic to grapefruit were used previously with *X. campestris* pv. *vesicatoria* to find mutants for virulence on pepper plants with a plant resistance gene (Dahlbeck and Stall, 1979). Mutants for change of race 2 to race 1, which occurred very frequently in *X. campestris* pv. *vesicatoria*, was due to an insertion element that inactivated an avirulence gene (Kearney et al., 1990). In addition, inactivation of the *avrBs2* gene in *X. campestris* pv. *vesicatoria* by several ways makes the pathogen susceptible to plants with the *Bs2* gene for resistance. This has occurred frequently in the field (Gassmann et al., 2000).

Chromosomal genes were also transferred from donor to recipient in *X. campestris* pv. *vesicatoria* by conjugation (Basim, 1999). The *hrp* genes, which are involved in pathogenicity and hypersensitivity in bacteria, were transferred in that work. The *hrp* gene cluster contains ca. 25 kb of DNA. However, it is not known if the host specificity genes in Xac-A^{w} are in the chromosome or in a plasmid. We did determine that a plasmid can move by conjugation between strains of Xac and this was demonstrated by using copper (Cu) resistant strains. It was determined that Cu resistance genes occur on a plasmid in Xac-A from Argentina and in *X. axonopodis* pv. *citrumelo* strains from Florida. The Cu resistance genes from both pathogens were transferred to Cu sensitive strains. Copper resistance was not found in strains of Xac-A from Florida (R.E. Stall,

personal communication). Copper resistance was associated with transfer of a large plasmid.

The most important part of this work was that we found an avirulence gene, *avrGf1*, in the genome of Xac-A^{w} that interacts with grapefruit leaves to cause an HR. The *avr* gene was found to be located in the chromosome. This is the first report of an avirulence gene in the genome of the citrus canker bacterium that functions in citrus. The HR depends on a gene for resistance in grapefruit, based on the gene-for-gene hypothesis (Minsavage, 1990b). The evidence for a resistance gene in the grapefruit genome will be very difficult to obtain, because crossing between susceptible and resistant plants is usually required. Genetic analysis of characteristics in citrus is difficult (Novelli et al., 2000).

Identification of the Xac-A^{w} strain by the division of plant industry is currently done by inoculation of citrus plants. In addition, the Xac-A^{w} strain can be identified by serological means in an ELISA test (Sun et al., 2004). The primers selected in the avirulence gene (*avrGf1*) can also be used to identify the Xac-A^{w} strain by PCR. The avirulence gene was not found in other strains of xanthomonads pathogenic to citrus.

The regulation and eradication procedures of the Division of Plant Industry were probably correct for the Xac-A^{w} strain. The factors for host specificity of the Xac-A^{w} strain seem to be quite stable based on this work. Even if the single *avrGf1* gene is responsible for host specificity, inactivation of the gene in the Xac-A^{w} genome does not appear to be of a high frequency as occurs with *avr* genes in *X. campestris* pv. *vesicatoria* (Dahlbeck and Stall, 1979). One should note, however, that the frequency of inactivation

of *avrGf1* could change with the introduction of an insertion sequence (Kearney et al., 1990).

The stability of the host specificity of the Xac-A^w strain could be the result of the presence of a second *avr* gene in wild Xac-A^w. The fact that we did not obtain mutants that were pathogenic to grapefruit when the cells of Xac-A^w were treated with the mutagen NTG (Chapter 4) would support this. If only one gene was present, mutants should have been found. If two genes were present in Xac-A^w, each of which was important in susceptibility to grapefruit, one would not expect to find pathogenic mutants after treatment with a mutagen. Other evidence for another *avr* gene in Xac-A^w was that there were differences in populations and symptoms for the Xac-A strain with the mutant lacking a functional *avrGf1* in comparison with the wild-type Xac-A.

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BIOGRAPHICAL SKETCH

Myrian Rybak was born in Leandro N. Alem, Misiones, Republica Argentina, on June 29, 1967. She is the daughter of Veronica Kuyesen and Jose Rybak.

Myrian graduated from the Nacional University of the Northeast in Corrientes, Argentina, in 1992 with the Agricultural Engineer degree. In 1993 she began to work in the National Institute of Agricultural Technology (INTA). In April-May of 1995 Myrian was awarded a fellowship of Rotary International, Exchange Study Groups (IGE), Mexico. In 1998 she was awarded a fellowship to pursue graduate studies in the University of La Plata, La Plata, Buenos Aires, Argentina. She graduated with the master's degree in 2000.

In 2001 she was awarded a fellowship from INTA-FULBRIGHT to pursue graduate studies in the United States.

Myrian enrolled at the University of Florida in Fall 2001 with Dr. Jeffrey Jones as her adviser. She is currently a candidate for the degree of Doctor in Philosophy. At the end of her studies she will resume her position in INTA, Argentina.

Myrian was awarded the Francis Aloysius Wood Memorial Award in recognition of outstanding graduate student research in plant pathology at the College of Agricultural and Life Sciences, on March, 2005.

Myrian is a member of Argentinean Society of Horticulture (ASHAO), International Society of Citriculture (ISC), Professional Council of Agricultural Engineers (CPIA) and American Phytopathological Society (APS)