# GENETIC CHARACTERIZATION OF PLANT-PATHOGEN INTERACTIONS BETWEEN *Xanthomonas campestris* pv. vesicatoria AND TOMATO (*Lycopersicon esculentum* L.)

By
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To my wonderful wife Juliana
and my families in Costa Rica
and Brazil

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Bacterial spot caused by *Xanthomonas campestris* pv. vesicatoria (Xcv) is one of the most important diseases of tomato (*Lycopersicon esculentum*). A new source of resistance to tomato race 3 of this pathogen was found in the wild species *L. pennellii*. Genetic segregation of the resistance was determined with an $F_2$ progeny of 245 plants derived from a cross between the tomato line *L. esculentum* Hawaii 7998, susceptible to this race, and the resistant parent *L. pennellii* LA716. Monogenic segregation of this resistance was confirmed by a goodness of fit test ($\chi^2_{3:1}$= 2.287; P=0.13). A collection of 50 *L. pennellii*-chromosome segment introgression, that covered the whole genome, were screened by inoculation with Xcv tomato race 3 in order to identify the genomic localization of the R gene. The resistance gene (*Xv4*) was located on chromosome 3. Linkage analysis of the

resistance with neighboring RFLP and CAPS markers indicated the following gene order: TG599- 9.3 cM- Xv4- 11.1 cM- TG134. The resistance gene X*v4* maps to an approximate 22 cM interval defined on the centromeric side by the RFLP markers TG599 at 9.3 cM and on the telomeric side by TG134 at 11.1 cM. The role and characterization of the corresponding avirulence gene, *avrXv4*, are also discussed.

A different gene-for-gene system (*avrXv3-Xv3*) controlling the resistance to this pathogen in tomato was previously described. In order to elucidate the possible role of *avrXv3* in eliciting the hypersensitive reaction (HR) on *Xv3* genotypes, a collection of mutated *avrXv3* were generated by PCR-mediated deletion mutagenesis of putative domains inferred from the hydrophobicity analysis of the predicted protein, and the modification of the termini of the protein by the addition of 6 histidine residues. All constructs were screened for their ability to elicit HR using transconjugants of a virulent strain of Xcv and *Agrobacterium*-mediated transient expression. Since preliminary data suggested a possible involvement of AvrXv3 in transcription activation, the mutants and wild type proteins were assessed for their ability to activate transcription in yeast. The results suggest that AvrXv3 protein had transcription activation activity in yeast. Whether or not this activity is associated with the ability to elicit the HR in tomato was not determined conclusively. Modifications of the termini of this protein seemed to block secretion of the AvrXv3 protein into the host cell.

# CHAPTER 1
# INTRODUCTION

Vegetable production is one of the most important and profitable agricultural activities in the state of Florida. Among those vegetables produced, tomato (*Lycopersicon esculentum* L.) provides the highest revenue to the state. According to the Florida Agricultural Statistics, the total value of tomato production during the 1994-95 season was about $461,369 million with approximately 49,000 acres harvested.

Tomato is the target of many infectious diseases that cause severe yield losses. Among them, bacterial spot of tomato, incited by *Xanthomonas campestris* pv. vesicatoria (Xcv), is one of the most important diseases, especially when weather conditions are suitable for its development (Pohronezny and Volin, 1983). This particular disease also affects peppers (*Capsicum* spp.) and has been reported throughout the world wherever tomatoes and peppers are grown (Stall, 1995a). Bacterial spot is primarily characterized by the occurrence of greasy-appearing, water-soaked, circular lesions on leaves, stems, and fruits. These lesions vary in size and shape, and normally develop into necrotic spots. As a final consequence, leaf abscission in pepper plants or necrosis of tomato leaflets may occur (Stall, 1995b).

Bacterial spot disease, which is very difficult to manage, causes reduced plant growth, fruit yield, and quality (Sahin and Miller, 1996). Despite many efforts to control this disease, not a single method has been completely effective. The efficacy of chemical

control with copper compounds and streptomycin has been marginal. The rise of resistant strains of Xcv to both of these chemicals is also responsible for reduced control (Stall and Thayer, 1962; Stall *et al.*, 1986; Bender *et al.*, 1990; Ritchie and Dittapongpitch, 1991; Sahin and Miller, 1996). Thus, management of bacterial spot relies essentially on exclusion of the disease by using pathogen-free seeds and seedlings, sanitation, and resistant varieties (Sahin and Miller, 1996). The inability to control the disease with cultural practices and/or antibacterial agents leaves resistance as one of the most important alternatives for controlling this disease.

In the 1980's, a research team established a program to search for useful sources of resistance in the hope of contributing in the fight against bacterial spot in Florida (Scott and Jones, 1986). Early efforts to find high levels of resistance in tomato to Xcv were unsuccessful. Instead, measurable levels of resistance or foliar tolerance were found in almost all lines or PI accessions screened (Stall, 1995a). It was not until the discovery of the tomato genotype Hawaii 7998 (H7998) that a high level of resistance to Florida strains was identified (Jones and Scott, 1986). However, segregation analysis of $F_2$ populations indicated that the inheritance of this resistance was somewhat complex, and possibly determined by multiple genes (Jones and Scott, 1986; Wang *et al.*, 1994). Yu *et al.* (1995) identified three different genetic loci in H7998 that appeared to act independently and to have an additive effect on this resistance.

With respect to the variability of the pathogen, three different groups have been found among Xcv strains. The XcvT group includes strains that are pathogenic on tomato, the XcvP group includes strains that are pathogenic on pepper, and the XcvTP

group includes strains that are pathogenic on both plant species (Minsavage *et al.*, 1990). Among XcvT strains, three races have been identified so far based on their reaction on three tomato cultigens: H7998, H7981, and Bonny Best (Stall, 1995b; Jones *et al.*, 1998b). Thus, those strains that showed incompatibility only with H7998 were designated as tomato race 1 (T1) (Wang *et al.*, 1994). Strains that caused hypersensitive reaction only on H7981 were designated as tomato race 3 (T3), while strains unable to elicit HR on any of the three cultigens were designated as tomato race 2 (T2) (Wang *et al.*, 1994; Jones *et al.*, 1995).

Stall *et al.* (1994) and Bouzar *et al.* (1994b) thoroughly characterized the three races. As a result, T1 strains were classified into *X. campestris* pv. vesicatoria group A. Strains in this group have a 32-35 kD protein band, and exhibit negative or weak amylolytic and pectolytic activity (Bouzar *et al.*, 1994a). On the other hand, T2 strains are in *X. campestris* pv. vesicatoria group B, have a 25-27 kD protein band, and exhibit strong amylolytic and pectolytic activity (Bouzar *et al.*, 1994a). A proposal has recently been made to reclassify group A and B into different species, *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas vesicatoria*, respectively, on the basis of DNA-DNA homology (Vauterin *et al.*, 1995). Since this proposal has not been generally accepted by the scientific community, the old nomenclature is used throughout this work.

In 1991, following an outbreak of bacterial spot in Florida, a new race capable of overcoming the resistance in H7998 was found and characterized (Jones *et al.*, 1995). Despite its strong amylolytic and pectolytic activity characteristic of group B, T3 strains are different from T2 strains in their ability to cause hypersensitive reaction in several

genotypes such as H7981 and two plant introductions of *L. pimpinellifolium* PI128216 and PI126932 (Jones *et al.*, 1995; Scott *et al.*, 1995; Jones *et al.*, 1998b). In addition, T3 strains could be considered pathogenically and physiologically as group C differing from group A and B strains. Although group C strains had unique DNA restriction profiles, DNA:DNA hybridization data suggests that group C strains are related to group A strains, and they might even be a subspecies of group A strains (Jones *et al.*, 1998b). Perhaps the most striking feature of this third race is its competitive nature in the presence of T1 strains. Jones *et al.* (1998a) found that T3 predominated over T1 strains both in the field and under controlled conditions. This enhanced fitness or aggressiveness shown by T3 strains might be associated with its antagonistic activity towards T1 strains (Jones *et al.*, 1998a). Tudor-Nelson *et al.* (1995) determined that T3 strains produced more than one bacteriocin-like substance active against T1 strains *in vitro*.

The first report of a high level of resistance to Xcv race T3 came when Scott *et al.* (1995) found resistance to T3 strains in *L. pimpinellifolium* PI 128216 and PI 126932, and *L. esculentum* cultigen Hawaii 7981 (H7981). This resistance is inherited as a single incompletely dominant gene (*Xv3*), and appears to be controlled by the same gene in all three lines (Scott *et al.*, 1995; Minsavage *et al.*, 1996).

The primary objective of this work was to study and characterize certain aspects involved in plant-pathogen interactions between Xcv race T3 and tomato that lead to the elicitation of the hypersensitive reaction and consequently confer resistance to the host. The scope of this work included examining different sources of resistance to Xcv in tomato, attempting to identify the genomic location(s) of the novel gene(s) responsible

for controlling resistance to Xcv race T3, and the analysis of the AvrXv3 protein in order to identify domains involved in HR and pathogen recognition.

# CHAPTER 2
# LITERATURE REVIEW

Evolution has provided plant pathogens with a significant number of mechanisms to enhance their pathogenic potential and to ensure their survival. Likewise, plants have developed an equally diverse set of countermeasures to avoid their own demise. Throughout time, this co-evolution between host and pathogen has given form to what is defined today as plant-pathogen interactions.

Since the early stages of plant pathology, natural resistance to plant pathogens has been considered a desirable trait for selection of crop plants. The first attempts to study resistance in plants focused on finding new sources of resistance throughout the world. Those findings were then used to establish breeding programs in order to introduce resistance into commercial varieties. Recently, the focus of this type of research has been partially shifted towards the exploration of the molecular basis of resistance, and ultimately to the improvement of the ability to genetically engineer durable resistance into commercial crops. Perhaps the first scientist to begin this exploration was H.H. Flor. who, in the 1940's, proposed the gene-for-gene model to explain the inheritance of plant disease resistance and pathogen virulence (Flor, 1971). Since then, a great deal of knowledge on the mechanisms controlling plant disease resistance has been accumulated. The purpose of this review is to summarize the most relevant information regarding plant disease resistance and the role of bacterial plant pathogens in eliciting such responses.

## Diversity Among Plant-Pathogen Interactions

In order to understand the underlying mechanisms involved in plant disease resistance, it is important to understand the level of diversity that prevails in the world of plant-pathogen interactions. Although plant pathogenic microbes are a relatively small group of organisms in the context of nature's diversity, this singular group exhibits a great deal of variation when it comes to the type of interaction that they have with their hosts. For instance, some plant pathogenic microbes have evolved diverse mechanisms to colonize and kill plant tissue in order to survive. These so-called necrotrophic organisms normally do so by attacking with enzymes or toxins that weaken and kill host cells. In contrast, other groups such as biotrophs and hemibiotrophs evolved different strategies to keep their host cells alive while they grow and reproduce (Hammond-Kosack and Jones, 1997).

Host plants have also evolved a variety of responses to pathogen attack. Thus, plants with constitutive resistance may have one or more preformed barriers that passively prevent pathogens from causing disease (Osbourn, 1996). As opposed to these preformed barriers, other plants need the presence of the pathogen in order to trigger the resistance response. In most cases, this response seems to be displayed by particular genotypes of the host towards particular races of the pathogen (host-specific resistance). Well-known examples of these active mechanisms are the systemic acquired resistance (SAR) and the hypersensitive response (HR) (Yang *et al.*, 1997).

On the other hand, the concept of constitutive susceptibility has also been proposed to explain the interaction between pathogens that produce host-specific toxins and their

hosts. In this particular case, susceptibility factors seem to be preformed (toxin receptors) while resistance relies only on the absence of those factors (recessive trait) (Yoder, 1980).

Regarding the genetic basis of compatibility in plant-pathogen interactions, two well understood scenarios could be expected. First of all, toxin-dependent compatibility encompasses those interactions where pathogenicity is a dominant trait in the pathogen (toxin production) while susceptibility is recessive in the host (detoxification factor absent) (Hammond-Kosack and Jones, 1997). Host plants expressing toxin-insensitive targets display a variation of this interaction. In this case, resistance is also a recessive trait (Levings *et al.*, 1995). Finally, the second type of compatibility system involves two dominant traits. In the gene-for-gene system, an interaction occurs between a pathogen expressing an avirulence gene product and a host expressing the appropriate resistance gene product. As a result, this interaction will lead to cell death and a limited spreading of the pathogen (Bent, 1996).

More recently, the exploration of the molecular basis of the resistance-avirulence incompatibility system has led scientists to follow two basic research avenues. These are the identification and cloning of avirulence (*avr*) and resistance (*R*) genes, and the characterization of cellular responses after recognition of the pathogen. These two lines of research have contributed significantly to the understanding of the possible interactions involved between plant pathogens and their host, and have also helped scientists to start putting together the pieces of this puzzle.

## Avirulence in Plant Pathogenic Bacteria

When Flor proposed the gene-for-gene hypothesis, the existence of *avr* genes in bacteria was only suggested by the specific interactions observed between pathogen strains and their hosts. However, since the advent of molecular biology, more than 30 *avr* genes from bacteria have been cloned and characterized (Leach and White, 1996). The first of them was *avrA*, cloned from the race 6 of the soybean pathogen *Pseudomonas syringae* pv. glycinea (Staskawicz *et al.*, 1984).

Early observations on the specificity of the interaction between *avr* genes and host resistance genes suggested the existence of at least two groups of *avr* genes (Leach and White, 1996). The first group included those gene products involved only in race-specific interactions. Thus, for instance, when *avrA* is transferred to other races of *Pseudomonas syringae* pv. glycinea, it confers the ability to elicit HR on soybean cultivars with the *Rpg2* gene for resistance (Staskawicz *et al.*, 1984). A second group of *avr* genes referred to as heterologous genes exhibited a broader range of interactions. These genes are able to confer the ability to elicit HR in a host-specific manner when transferred to other pathovars that have a different host range. So far, about ten of these genes have been identified and characterized (Leach and White, 1996).

The structural organization and location of *avr* genes are also variable. The vast majority is monocistronic, that is, one single open reading frame (ORF) is responsible for their activity. However, avirulence activity of other genes such as *avrE* and *avrPphD* has been shown to require two ORF's (Wood *et al.*, 1994; Lorang and Keen, 1995).

Avirulence genes can be either plasmid-borne or chromosomal (Minsavage *et al.*, 1990; Leach and White, 1996). Leach and White (1996) suggested that this variability might indicate a possible association between the mobility of *avr* genes and the introduction of genetic variation in the evolution of host-pathogen interactions. For example, the sequence and location of *avrB* represents a very particular case since sequence analysis suggested that this *avr* gene does not reflect the GC content of individuals belonging to the *P. syringae* group. Therefore, it has been suggested that *avrB* is probably derived from outside this group of bacteria (Tamaki *et al.*, 1988). More recently, Kim *et al.* (1998) reported that *avrA*, *avrB*, *avrC*, *avrPphC*, *avrRpm1*, and *avrPpiA1* are bordered by sequences similar to those of transposable elements of Gram-negative bacteria, while *avrPto* was found to be associated with a DNA region homologous to a bacteriophage sequence. They also suggested that the association of avirulence genes with transposable elements and bacteriophage sequences, along with the presence of several of these genes on plasmids, supports the idea of horizontal transfer and frequent exchange of avirulence genes among bacterial pathogens (Kim *et al.*, 1998). Along the same line, *avrD* homologues have been found widely distributed among Pseudomonads and the soft rot bacteria, *Erwinia carotovora* (Hanekamp *et al.*, 1997). Hanekamp *et al.* (1997) also found that DNA linked to *avrD* showed evidence of class II transpositions and contained a novel IS3-related insertion sequence. Besides, short sequences linked to *avrD* were similar to pathogenicity genes from a variety of unrelated pathogens. These data led them to conclude that this *avr* gene must have a conserved

function beyond virulence, and that it may have been transferred horizontally among species (Hanekamp *et al.*, 1997).

Even though *avr* predicted products do not exhibit any similarity to known functional domains, sequence similarities among *avr* genes and among heterologous genes have been noted at both the protein and DNA level. Among them, *avrB* and *avrC* are known to have 42% of amino acid sequence identity (Tamaki *et al.*, 1991), while *avrBs1* and *avrA* have been shown to share about 47% of their sequence at the carboxy-terminal region (Ronald and Staskawicz, 1988). Perhaps the most striking case is that of *avrRxv* which shows a remarkable similarity to *yopJ* gene from *Yersinia pseudotuberculosis* (Leach and White, 1996).

Despite the few cases of similarity found among *avr* genes, one group seems to have recently emerged. The *avrBs3* family of *avr* genes represents a unique case within this particular group. The type member of this family is the *avrBs3* gene, first isolated from *Xanthomonas campestris* pv. vesicatoria race P1 (Bonas *et al.*, 1989). Since then, many other members of this gene family have been found to be limited to several pathovars of the genus *Xanthomonas*. Among them, *avrBs3-2* was cloned from *X. campestris* pv. vesicatoria (Bonas *et al.*, 1993), *pthA* from *X. citri* (Swarup *et al.*, 1992), *avrXa10* from *X. oryzae* pv. oryzae (Hopkins *et al.*, 1992), and *avrB4*, *avrb7*, *avrBIn*, *avrB102*, and *avrB6* from *X. campestris* pv. malvacearum (De Feyter *et al.*, 1993; Yang *et al.*, 1994; Yang *et al.*, 1996). All members of this gene family exhibit 90 to 97% amino acid sequence identity, and multiple copies of related homologues have also been found within the same pathovars; however, not all copies appeared to have avirulence activity

(Leach and White, 1996). Another common feature is that all members have a common central domain composed of a series of directly repeated sequences of about 102 bp (34 aa) (Bonas *et al.*, 1989; Canteros *et al.*, 1991; Hopkins *et al.*, 1992; Swarup *et al.*, 1992; Bonas *et al.*, 1993; De Feyter *et al.*, 1993). The number of copies of these repeated sequences generally varies among members of the family from 13.5 to 17.5, and is thought to be involved in *avr* gene specificity (Bonas and Van den Ackerveken, 1997). Despite the conserved nature of this domain, differences in sequence can occur within this region, but they normally are concentrated within a variable two-codon region (Leach and White, 1996).

The function of *avr* gene products has always been one of the most puzzling aspects of this subject. With the exception of *avrD*, *avrBs2*, and *avrXa10*, no tangible evidence exists to reveal the exact function of the remaining *avr* genes in the context of plant-pathogen interactions. Yucel *et al.* (1994) determined that the *avrD* gene cloned from *P. syringae* pv. tomato conferred avirulence to *P. syringae* pv. glycinea by enzymatically directing the production of several secondary metabolic compounds. These compounds, called syringolides, are responsible for eliciting a hypersensitive reaction (HR) in soybean plants carrying the *Rpg4* disease resistance gene. Similarly, Swords *et al.* (1996) suggested that *avrBs2* may have enzymatic function due to its similarity with *Agrobacterium tumefaciens* agrocinopine synthase. Kearney and Staskawicz (1990) indicated that *avrBs2* had a dual role, delivering the avirulence signal and promoting pathogen virulence. In contrast, the highly acidic carboxy-terminal domain of the protein

encoded by *avrXa10* has been found to have transcription activation activity (Zhu *et al.*, 1998).

Although conclusive data regarding the function of *avr* gene products is not available, several characteristics of these proteins and the mechanism involved in triggering HR could be used to formulate plausible hypothesis to explain the function of *avr*-encoded proteins. First of all, the majority of Avr proteins are hydrophilic in nature and lack signal peptide sequences that could indicate secretion. Secondly, none of the proteins encoded by bacterial *avr* genes induced hypersensitive reaction (HR) when injected in the intercellular space of leaves of plants with the complementary *R* genes (Alfano and Collmer, 1996; Leach and White, 1996). Finally, the involvement of *avr* products in activities other than avirulence, such as pathogen fitness, has also been demonstrated by Ritter and Dangl (1995) and Swords *et al.* (1996), who showed that mutations of *avrRpm1* and *avrBs2* affected fitness of *P. syringae* pv. maculicola and *X. campestris* pv. vesicatoria, respectively. As a result, the first possible scenario may involve *avr* gene products limited to the bacterial cytoplasm as opposed to being membrane-bound or secreted proteins. Consequently, these proteins may have an indirect function in eliciting HR ruling out a possible recognition of Avr proteins in the plant intercellular spaces (Leach and White, 1996; Bonas and Van den Ackerveken, 1997). Secondly, a novel secretion system may enable the transport of these proteins without the requirement of signal peptide sequences.

Although the exact function of *avr* gene products is unknown, the highly specific *avr-R* gene interaction suggests some type of cellular recognition by resistant plants. This

receptor-ligand interaction model might explain how the defense-signaling pathway is activated after recognition of the pathogen. An argument against this model suggested that if the function of *avr* genes is only associated with recognition, the lack of selective advantage of such model could be enough for bacterial evolution to eliminate such genes (Bonas and Van den Ackerveken, 1997). However, recent reports have indicated that *Agrobacterium*-mediated transient expression of several *avr* genes as well as their permanent expression in transgenic plants were enough to cause the elicitation of HR in resistant plants. These findings support the receptor-ligand model, and suggest that the presence of the Avr proteins inside the host cells may be required for the elicitation of the HR (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996; Bonas and Van den Ackerveken, 1997; Parker and Coleman, 1997; De Feyter *et al.*, 1998). As a result of these observations, it is thought that a conserved bacterial delivery system capable of introducing Avr proteins into plant cells must exist among plant pathogenic bacteria.

Early studies on virulence mechanisms of important Gram-negative mammalian pathogens confirmed the existence of a novel secretion system now known as type III protein secretion pathway (Hueck, 1998). Proteins secreted using this mechanism lack both the cleavable N-terminal signal peptide characteristic of *sec*-dependent secretion pathways (type II and type IV), and the C-terminal signal peptide associated to proteins secreted by the *sec*-independent type I pathway (Hueck, 1998). Instead, it is thought that at least some type III-secreted proteins possess the secretion signal in the 5’ region of the mRNA, which encodes the secreted protein (Anderson and Schneewind, 1997).

Pathogens such as *Yersinia* spp., *Salmonella typhimurium*, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Clamidia* spp. exhibit a specialized cluster of genes involved in assembling the type III secretion system (Hueck, 1998). Early work on avirulence and pathogenicity of bacterial plant pathogens led scientists to the discovery of a cluster of genes, known as hypersensitivity and pathogenicity genes (*hrp*), involved somehow in regulating both susceptible and incompatible interactions (Bonas, 1994). Sequence similarity of at least nine *hrp* genes with components of the type III secretion pathway described in mammalian pathogens first indicated the possibility that *hrp* genes could be involved in the secretion of a broad range of virulence factors, elicitors, and perhaps even Avr proteins. These conserved *hrp* genes are now called *hrc* genes. Furthermore, the lack of distinctive signal peptides in Avr proteins and their critical dependence on *hrp* activity are important evidences to support such hypothesis (Bogdanove *et al.*, 1996; Baker *et al.*, 1997; Bonas and Van den Ackerveken, 1997). The secretion of only non-specific elicitors such as PopA from *Ralstonia solanacearum* (Arlat *et al.*, 1994), and harpins from *Pseudomonas* (He *et al.*, 1993) and *Erwinia* (Wei *et al.*, 1992; Bauer *et al*., 1995) has been confirmed to be mediated by Hrp proteins. Likewise, a type III secretion system associated with cultivar-specific nodulation has also been reported in *Rhizobium* spp. (Hueck, 1998).

Regarding Avr proteins, Bonas and Van den Ackerveken (1997) suggested that cell-to-cell contact between the bacterium and the host might be required to secrete these proteins directly into the host cell. This hypothesis is based on the fact that upon cell contact utilizing the type III secretion pathway, *Yersinia* is able to secrete several Yop

proteins into the host cell (Bonas and Van den Ackerveken, 1997). Evidence for *hrp*-dependent secretion of Avr proteins has recently been presented for *avrB* and *avrPto* (Hyun-Han *et al.*, 1998). When the *hrp* cluster from the host-promiscuous *E. chrysanthemi* was introduced into the non-plant pathogenic bacterium *Escherichia coli*, it allowed *E. coli* to secret AvrB and AvrPto in culture, and induced hypersensitive reaction in inoculated plants carrying the appropriate resistance gene (Hyun-Han *et al.*, 1998).

## Disease Resistance Genes

Vertical or monogenic resistance follows a gene-for-gene interaction as originally demonstrated by Flor (1971). Incompatibility between the host and the pathogen is the result of the interaction between a dominant resistance gene (*R*) in the plant and a dominant avirulence gene in the pathogen. The activation of several signal transduction pathways, and the initiation of the hypersensitive reaction characterize the initial recognition event. The hypersensitive reaction occurs following a rapid oxidative burst and localized cell death. Activation of the antioxidant defense mechanisms in the cells surrounding the developing lesion characterizes this defense response (Lamb and Dixon, 1997). The neighboring cells surrounding the lesion could also synthesize anti-microbial phytoalexins, several pathogenesis-related proteins (PR), and cell wall fortifications (Dixon, 1986; Bowles, 1990; Dixon and Lamb, 1990).

The nature of the *R-avr* gene interactions has led scientists to predict several properties that resistance gene products may exhibit. First, it is thought that R proteins could be expressed in healthy unchallenged plants in preparation for the attack. Secondly, they may be able to recognize *avr*-gene-dependent ligands. The third feature of R

proteins implies a rapid evolution of specificity to cope with the fast changing pathogens (Hammond-Kosack and Jones, 1997).

So far, 14 genes involved in conferring resistance in different plant species have been cloned and characterized. Five genes (i.e., *N* gene from tobacco, *L6* and *M* genes from flax, *Cf-9* from tomato, and *Hm1* from maize) were cloned using transposon tagging, whereas 8 genes (i.e., *RPS2* and *RPM1* from *Arabidopsis*, *Xa21* from rice, $I_2C$, *Pto*, *Cf-4*, *Cf-5*, and *Cf-2* from tomato) were cloned by map-based or positional cloning (Johal and Briggs, 1992; Martin *et al.*, 1993; Bent *et al.*, 1994; Jones *et al.*, 1994; Dinesh-Kumar *et al.*, 1995; Lawrence *et al.*, 1995; Song *et al.*, 1995; Dixon *et al.*, 1996; Anderson *et al.*, 1997; Ori *et al.*, 1997; Thomas *et al.*, 1997).

Plant disease resistance genes can be grouped into five distinct classes on the basis of their predicted structural motifs and their possible location in the plant cell (Hammond-Kosack and Jones, 1997). The first of these classes is involved with detoxification of plant pathogenic toxins and includes a single gene isolated from maize that confers resistance to Race 1 strains of *Cochliobolus carbonum* (Johal and Briggs, 1992). The function of this particular gene, known as *Hm1*, is independent from the presence or absence of any pathogen *avr* gene product, and it encodes a NADPH-dependent detoxifying enzyme known as HC-toxin reductase (Johal and Briggs, 1992; Bent, 1996).

The second class of *R* genes includes proteins associated with the cytoplasmic membrane and may be involved in signal transduction. It has a unique member cloned from tomato and designated *Pto* due to its specific interaction with *avrPto* from *Pseudomonas syringae* pv. tomato (Martin *et al.*, 1993). Sequence analysis of this gene

suggests that it encodes a serine/threonine kinase capable of autophosphorylation (Loh and Martin, 1995). This motif may be involved in signal transduction; however, no other motifs with recognition capabilities such as leucine rich repeats (LRR) or nucleotide binding sites (NBS) were found in its sequence. Nevertheless, direct interaction between *Pto* and AvrPto was later confirmed by using the yeast 2-hybrid system (Tang *et al.*, 1996; Hammond-Kosack and Jones, 1997). More recently, two more genes were found forming a clustered family of genes along with *Pto*. First of all, the *fen* gene is known to confer sensitivity to the insecticide fenthion and seems to encode another serine/threonine kinase exhibiting 80% identity to the Pto protein (Martin *et al.*, 1994). Despite this similarity, Fen has been confirmed to be unable to interact with the *avrPto*-encoded protein, and consequently incapable of eliciting the resistance response (Tang *et al.*, 1996; Hammond-Kosack and Jones, 1997). The second of these genes, *Prf*, is located about 24 kb from *Pto* and encodes a large protein with a leucine zipper (LZ), a NBS, and LRR of the 23-amino acid type (Salmeron *et al.*, 1994). Mutations in the *Prf* gene rendered tomato plants susceptible to *P. syringae* pv. tomato carrying the *avrPto* and insensitive to fenthion (Martin *et al.*, 1994). Hammond-Kosack and Jones (1997) hypothesized that due to the fact that both Pto and Prf are required for resistance, LRR-containing proteins and kinases could be components of the same signaling pathway. More recently, Oldroyd and Staskawicz (1998) demonstrated that overexpression of Prf in tomato plants led to enhanced resistance to several normally virulent bacterial and viral pathogens, and an increased sensitivity to fenthion. They also noted that the constitutive levels of salicylic acid and pathogenesis-related proteins in these transgenic plants were

comparable to those in plants induced for systemic acquired resistance (SAR). Therefore, overexpression of Prf could be used to activate SAR in a pathogen-independent manner leading to enhance broad-spectrum resistance (Oldroyd and Staskawicz, 1998). Finally, using Pto as a bait in an interaction hunt with the yeast 2-hybrid system, one more gene encoding a serine/threonine protein kinase was found to be a substrate of phosphorylation by Pto and autophosphorylation (Zhou *et al.*, 1997). This gene, named *Pti1*, is thought to be part of the kinase cascade specific for Pto-AvrPto signaling (Bent, 1996). Since then, other Pto-interacting proteins known as Pti4, Pti5, and Pti6 have been identified (Bent, 1996). These proteins exhibit a remarkable similarity to ethylene-responsive element binding proteins from tobacco, which function as transcription factors and bind PR-box DNA sequences located within the promoter region of many pathogenesis-related (PR) proteins. Experimental evidence supporting the binding to PR-boxes has been obtained only for Pti5 and Pti6 (Bent, 1996).

The third class includes those *R* genes predicted to encode cytoplasmic proteins (Hammond-Kosack and Jones, 1997). Seven resistance genes have been included in this class so far. Among them, *RPS2* and *RPM1* were isolated from *Arabidopsis thaliana* and confer resistance to different strains of *P. syringae* carrying *avrRpt2*, and *avrB* or *avrRpm1*, respectively (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). *RPP5*, cloned from *Arabidopsis*, belongs to this class and confers resistance to *Peronospora parasitica* (Hammond-Kosack and Jones, 1997). In addition, several other *R* genes (e.g., *N* gene in tobacco that confers resistance to tobacco mosaic virus, the *M* and *L6* genes from flax that confer resistance to the rust fungus *Melampsora lini*, and the

*$I_2C$* family of genes involved in conferring resistance to *Fusarium oxysporum* f.sp. *lycopersici* in tomato) have also been included in this class (Whitham *et al.*, 1994; Lawrence *et al.*, 1995; Anderson *et al.*, 1997; Ori *et al.*, 1997). All members of this class exhibit at least three conserved motifs relating to structural organization. Thus, a NBS region is found at their amino termini, LRR at the carboxyl termini, and an internal hydrophobic domain (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995; Hammond-Kosack and Jones, 1997). In addition, two sub-groups can be made within this class on the basis of the presence of a variable motif immediately upstream of the NBS (Hammond-Kosack and Jones, 1997). The $I_2C$ family, RPS2, and RPM have a LZ whereas M, L6, N, and RPP5 exhibit an amino terminal TIR (Toll/Interleukin-1 resistance) domain with homology to the cytoplasmic domain of *Drosophila* Toll protein and the mammalian interleukin-1 receptor (IL-R) protein (Anderson *et al.*, 1997; Hammond-Kosack and Jones, 1997; Ori *et al.*, 1997). In *Arabidopsis*, mutational analysis has revealed the existence of at least two loci involved in regulating race-specific disease resistance (Century *et al.*, 1995; Glazebrook *et al.*, 1996; Parker *et al.*, 1996). Aarts *et al.* (1998) have presented evidence for the existence of two distinct signaling pathways based on the differential requirements for *EDS1* (enhance disease susceptibility) and *NDR1* (nonrace-specific disease resistance) by several resistance genes. Thus, *RPP2*, *RRP4*, *RPP5*, *RPP21*, and *RPS4* conferring resistance to *P. parasitica* and *Pseudomonas* spp. carrying the *avrRps4* gene are only dependent on *EDS1*, while *RPS2*, *RPM1*, and *RPS5* rely uniquely on *NDR1* (Aarts *et al.*, 1998).

The fourth class of *R* genes encodes proteins associated with the cytoplasmic membrane and comprises four resistance genes isolated from tomato that specify resistance towards different isolates of *Cladosporium fulvum*, causal agent of leaf mold of tomato (Hammond-Kosack and Jones, 1997). These gene products, designated Cf-2, Cf-4, Cf-5, and Cf-9, are characterized by the presence of an extra-cytoplasmic LRR, a single membrane spanning region, and a short cytoplasmic carboxyl terminus (Jones *et al.*, 1994; Dixon *et al.*, 1996; Hammond-Kosack and Jones, 1997; Thomas *et al.*, 1997). The proteins encoded by these genes seem to belong to the same family of proteins since they all share a similar overall structure (Hammond-Kosack and Jones, 1997).

Finally, the last class includes a single member, *Xa21*, isolated from rice. The gene confers resistance to over 30 strains of *Xanthomonas oryzae* pv. oryzae, causal agent of the leaf blight disease of rice (Song *et al.*, 1995). Sequence analysis of the predicted protein indicated the presence of a putative signal peptide, an extracytoplasmic LRR with several glycosylation sites, a single membrane spanning domain, and a cytoplasmic serine/threonine kinase domain (Song *et al.*, 1995).

The presence of common structural features among *R* genes with different specificity suggests the existence of a conserved pathway used by plants to trigger defense responses (Bent, 1996). Thus, while LRR, LZ, and TIR domains may be involved in protein-protein interactions and/or pathogen recognition, serine/threonine kinase domains could be directly involved in signal transduction (Bent, 1996; Hammond-Kosack and Jones, 1997). For those *R* genes without kinase activity, the presence of NBS domains suggest that they

could activate other kinases or G-proteins, which in turn could initiate the downstream signaling (Hammond-Kosack and Jones, 1997).

## Molecular Markers for Genetic Mapping

The characterization of genes involved in conferring disease resistance in plants is often accomplished by using molecular markers (Tanksley *et al.*, 1995). Several different techniques have been used to generate molecular markers in order to locate resistance genes in plant genomes.

RFLP (restriction fragment length polymorphism) markers were perhaps one of the first types of molecular markers developed for analysis of genomes from different organisms. Their use is based on the principle that polymorphism in restriction fragment lengths between two individuals could be detected on DNA blots using labeled probes that hybridize to a single target sequence in the genome (Bolstein *et al.*, 1980). Although this technique has been used in a variety of situations, the major applications of RFLP's have been for the selection of markers for mapping and the analysis of genetic diversity in populations. RFLP markers are co-dominant, allowing the detection and characterization of multiple alleles at a given RFLP locus among individuals in a population. Several types of polymorphism can be detected, including single base substitutions, insertions, and deletions (Rafalski *et al.*, 1996). Later on, the advent of the polymerase chain reaction (PCR) opened the possibility of merging this powerful technique with traditional RFLP analysis. Thus, by sequencing the termini of RFLP probes and designing specific primers for PCR, dominant or co-dominant markers known

as cleaved amplified polymorphic sequence (CAPS) markers could be obtained (Konieczny and Ausubel, 1993).

The random amplified polymorphic DNA (RAPD) technique is based on the use of single, short, synthetic oligonucleotide primers of arbitrary sequence for the amplification of randomly distributed segments of genomic DNA (Welsh and McClelland, 1990; Willians *et al.*, 1990). The resulting polymorphic profiles are a consequence of mutations or rearrangements at the oligonucleotide primer binding sites in the genome. The presence or absence of one or more amplification products can distinguish differences between individuals. This technique has been used extensively for fingerprinting and DNA mapping (Rafalski *et al.*, 1996). Sequence characterized amplified regions (SCAR) are PCR-based markers obtained when single bands from RAPD profiles are cloned, sequenced, and specific PCR primers are designed for their amplification (Paran and Michelmore, 1993).

Simple sequence repeat regions (SSR**)** or microsatellite repeats are stretches of tandemly repeated mono-, di-, tri-, tetra-, penta-, or hexanucleotide motifs. They are widely used as molecular markers due to their length variation, their abundance, and their random distribution throughout eukaryotic genomes. Polymorphism is obtained by amplifications of individual SSR loci using specific primers for a unique flanking DNA sequence. Since the number of tandem repeats varies from one SSR locus to another, amplified SSR loci show high levels of polymorphism (Rafalski *et al.*, 1996).

AFLP (amplified fragment length polymorphism) markers have been developed on the basis of the selective amplification of restriction fragments from total digested

genomic DNA (Vos *et al.,* 1995). The suitability of the AFLP technique to identify markers relies on the fact that most AFLP fragments correspond to unique positions on the genome, so they can be exploited as landmarks in genetic and physical maps. AFLP-based methods have been used in constructing high density maps of genomes or genome parts, detecting corresponding genomic clones in libraries, and fingerprinting of cloned DNA segments like cosmids, P1 clones, bacterial artificial chromosomes (BAC), or yeast artificial chromosomes (YAC) (Vos *et al.*, 1995).

A different approach that has been recently used to generate DNA-based markers for mapping purposes is called DAF or DNA amplification fingerprints (Prabhu and Gresshoff, 1994; Jiang and Gresshoff, 1997). It was originally developed to create fingerprints from PCR products and whole genomes, to establish genetic relationships between plant taxa at the interspecific and intraspecific level, and to identify closely related fungal isolates and plant species (Caetano-Anollés *et al.,* 1991). This technique is based on the use of short arbitrary oligonucleotide primers to generate amplification products that are separated on polyacrylamide gels and then stained with silver (Caetano-Anollés and Gresshoff, 1996).

# CHAPTER 3
# GENOMIC LOCALIZATION OF A SINGLE LOCUS CONTROLLING RESISTANCE TO *Xanthomonas campestris* pv. vesicatoria RACE T3 IN TOMATO

Two sources of resistance to *Xanthomonas campestris* pv. vesicatoria (Xcv) have been reported previously, one each to races T1 and T3 (Jones and Scott, 1986; Scott *et al.*, 1995). More recently, analysis of a $F_2$ progeny from the cross between the *Lycopersicon esculentun* cultigen H7981 (*Xv3*), resistant to T3 strains, and the wild relative *L. pennellii* indicated the presence of a novel resistance gene against T3 strains in the latter genotype (Astua-Monge and Stall, unpublished data).

In tomato, DNA-based markers have been extensively used to characterize the inheritance and genomic localization of several resistance genes (Martin *et al.*, 1993; Jones *et al.*, 1994; Yu *et al.*, 1995; Dixon *et al.*, 1996; Anderson *et al.*, 1997; Ori *et al.*, 1997; Thomas *et al.*, 1997; Moreau *et al.*, 1998). In addition, the existence of a saturated linkage map of tomato (Tanksley *et al.*, 1992) and the availability of introgression lines between *L. esculentum* and *L. pennellii* (Eshed and Zamir, 1994) make it possible to genetically characterize possible sources of resistance from wild relatives.

The main objective of this research was to characterize the novel source of resistance to Xcv race T3 in tomato derived from its wild relative *Lycopersicon pennellii*. The genomic localization of the gene responsible for conferring this resistance was also

attempted by using CAPS and RFLP markers to analyze a collection of introgression lines between *L. esculentum* and *L. pennellii*.

## Materials and Methods

### Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in the Appendix. Strains of Xcv were grown overnight at 28 °C on nutrient agar plates (Becton Dickinson, Cockesysville, MD) or in nutrient broth for plant inoculations. *Escherichia coli* strains were grown overnight at 37 °C on Luria-Bertani (LB) medium (Maniatis *et al.*, 1982). All bacterial strains were stored at room temperature in sterile tap water or at –70 °C in nutrient broth containing 30% glycerol.

### Plant Material, Inoculum Production, and Plant Inoculations

$F_1$ and $F_2$ populations, provided by Robert E. Stall, University of Florida, were derived from an interspecific cross between *Lycopersicon pennellii* LA 716 and *Lycopersicon esculentum* accession Hawaii 7998, a susceptible tomato cultigen. Roger Chetelat (Curator, Tomato Genetics Resource Center) kindly provided the population of 50 introgression lines generated as described by Eshed and Zamir (1994).

*Xanthomonas* strains used for inoculations were grown in nutrient broth for 20 h at 28 °C with shaking. Bacterial cells were pelleted by centrifugation at 1500 g for 15 min, and resuspended in sterile tap water. The concentration was adjusted to an $A_{600} = 0.3$ with a spectrophotometer (Spectronic 20, Baush & Lomb, Inc.). This reading represents approximately 2-5 x $10^8$ colony forming units (cfu)/ml. Leaves were infiltrated with the

bacterial suspension as described by Hibberd *et al.* (1987). When whole plants were inoculated, the bacterial suspension was prepared as described above, but diluted to $10^6$ cfu/ml in a solution containing 250 μg/ml of Silwet L77 (Osi Specialties Inc., Danbury, CT), an organosilicon surfactant. Inoculations were carried out by dipping the foliage for 15 s in the suspension. Each treatment was replicated three times and the experiment was also repeated 3 times unless otherwise indicated.

Two hundred and forty-five $F_2$ plants, their parental lines, and 50 introgression lines were grown in the greenhouse for four to five weeks at temperatures ranging from 25 to 35 °C. Inoculated plants were moved to a growth room kept at constant temperature of 22 °C and 16 h light period. Assessments for hypersensitivity were carried out 24 and 36 h after inoculations. Plants exhibiting confluent necrosis (HR) within this period of time were scored as resistant to bacterial spot. When dip inoculation was carried out, assessments were performed 4-5 days after inoculations. A visual scale, designed for these experiments, was used with scores ranging from 0 (no symptoms) to 11 (100% leaf area affected by bacterial spot). Plants with scores of five or below were considered resistant.

DNA Extraction and Hybridization Analysis

Plant genomic DNA was isolated from leaf tissue of 104 $F_2$ plants and progenitors as described by Prince *et al.* (1997). For Southern hybridization, genomic DNA was digested with *EcoRV* according to the conditions established by the manufacturer (Promega, Madison, WI), and DNA fragments were resolved by agarose gel electrophoresis (Sambrook *et al.*, 1989) and transferred to a Nytran membrane

(Schleicher & Schuell, Keene, NH) as described by Southern (1975). The RFLP probes, provided by Dr. Steve Tanksley of Cornell University, were labeled with digoxigenin-11 dUTP (Boehringer Mannheim, Indianapolis, IN) by PCR as described by Lanzillo (1990) and probed against the DNA immobilized on the Nytran membrane. Hybridization signals were developed with CSPD (disodium 3-(4-methoxyspiro)1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3, $3.1.1^{3.7}$] decan-4-yl-phenyl phosphate) chemiluminescense substrate according to the conditions established by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

Construction of Cleaved Amplified Polymorphic Sequence (CAPS) Markers and Identification of Recombinants between the Resistance Gene and Selected Markers

The termini of the RFLP probes used in this study were sequenced at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center of Biotechnology Research (ICBR). Oligonucleotides specific for the amplification of each RFLP probe were synthesized at the ICBR DNA Synthesis Laboratory, University of Florida, Gainesville. In the case of the marker NBS3, two degenerate oligonucleotide primers were used. These primers were designed based on the amino acid sequences of two highly conserved motifs of the nucleotide binding site (NBS) in tobacco *N* and *Arabidopsis RPS2* genes (Yu *et al.,* 1996). The profiles obtained from the amplification of the parental lines and the introgression lines LA3488 and LA3523 were compared. All primer pairs were used to screen a subset of 104 $F_2$ plants and the parental lines. DNA for PCR amplifications was extracted as described before for the hybridization assays.

Polymerase chain reaction was carried out using a DNA automated thermocycler PTC-100 equipped with a hot bonnet (M. J. Research, Watertown, MA), and with *Taq* DNA polymerase (Promega, Madison, WI). Unless otherwise indicated, each 25-μl PCR reaction contained 1x amplification buffer (from the manufacturer), 100 μM of each dNTP, 17.5 μM of each primer, 1.5 mM of $MgCl_2$, 1.25 U of *Taq* DNA polymerase, and 100 ng of template DNA. Generally, the template DNA was initially denatured at 95 °C for 3 min followed by 30 PCR cycles. For most of the primer pairs, each cycle consisted of 30 s of denaturation at 95 °C, 30 s of annealing at 50 °C, and 1 min. of extension at 72 °C. For the final cycle, the extension step was extended to 5 min. Oligonucleotide sequences used in this study are shown in Table 3-1.

Aliquots of 20 μl of the PCR products were mixed with 1 μl of tracking dye and added to wells of a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) as described by Sambrook *et al.* (1989). Agarose electrophoresis was performed at 5 V/cm for 1.5 h, then stained with 0.5 μg/ml of ethidium bromide in water, visualized on a UV transilluminator and photographed with Polaroid type 55 film (Polaroid Corp., Cambridge, MA).

Restriction Endonuclease Digestion of PCR Products

Amplified DNA fragments obtained with oligonucleotides designed from probes TG284a and TG599 were digested with *HindIII* and *HindIII/EcorRI*, respectively, according to the conditions established by the manufacturer (Promega, Madison, WI). Restriction fragments were resolved by agarose gel electrophoresis in 4% agarose gels

Table 3-1. Sequence of specific oligonucleotides used for PCR-amplification of genomic DNA from *L. esculentum*, *L. pennellii*, introgression lines, and $F_2$ population. Modifications to the general conditions described before are also indicated.

| Marker | Chromosome | Oligonucleotide Sequences[1] | Modified Conditions |
|---|---|---|---|
| TG50c | 3 | FP 5'TGGAACATGTGTCGACCTTT 3'<br>RP 5'TATGTCCACCTCCAAAACCT 3' | 2.5 mM $MgCl_2$ |
| TG377 | 3 | FP 5'TTGGCCCTTTCTTACTCTCT 3'<br>RP 5'CGGGTTGATTCTTAATGTACG 3' | - |
| TG284a | 3 | FP 5'TGACTCCGTTGAAACAATTTA 3'<br>RP 5'AACTGTGGGCTTGTCTTTTG 3' | - |
| TG457 | 3 | FP 5'AGGCCAGGTGACTTTATTAGG 3'<br>RP 5'TTTGTGTGTTGGTTTCCCCT 3' | Annealing temp. 53 °C |
| TG599 | 3 | FP 5'TGTTGATCCTTGCTTGCTGT 3'<br>RP 5'TTGTATGGTGCAACTTCCC 3' | - |
| NBS3 | 3 | FP 5'YCTAGTTGTRAYDATDAYYYTRC3'<br>RP 5'GGAATGGGNGGNGTNGGNAARAC3'<br>Y= C/T; R= A/G; D= A/G/T | Annealing temp. 55 °C and 2.5 mM $MgCl_2$ |

[1] FP= forward primer, RP= reverse primer

(3% NuSieve and 1% Seakem GTG [FMC BioProducts, Rockland, ME]) in TAE buffer at 8 V/cm. The gel was stained with 0.5 μg/ml ehidium bromide in water for 30 min, destained in 1 mM $MgSO_4$ for 30 min, visualized by UV transillumination and photographed as described above.

Genetic Analysis

Linkage and segregation analyses were performed with the software package MapMaker/Exp 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992). Statistical analysis was conducted with the Statistical Analysis System (SAS Institute, SAS Circle, Box 8000, Cary, NC).

## Results

### Analysis of the Segregating Population

The development of confluent necrosis in *L. pennellii* LA716 24 to 36 h after inoculation with T3 strains of Xcv indicated the existence of HR-related resistance in this wild species (Figure 3-1, A). To construct a population segregating for this novel resistance gene, the cultigen Hawaii 7998 (susceptible to T3 strains) was crossed with *L. pennellii* LA716. A subset of 16 $F_1$ plants was screened for resistance by inoculations with the T3 strains Xcv 91-118 and Xcv 97-2. By 24 to 36 h after inoculation, all the $F_1$ plants produced a hypersensitive reaction in the infiltrated areas (Figure 3-1, B). When 245 $F_2$ plants were screened with the same two strains for resistance, 79% (194) of them developed hypersensitive responses, whereas only 21% (51) remained asymptomatic 24-36 hours after inoculation. Analysis of the segregation ratio indicated a good fit to the 3:1 Mendelian segregation ($\chi^2$=2.287; P=0.13).

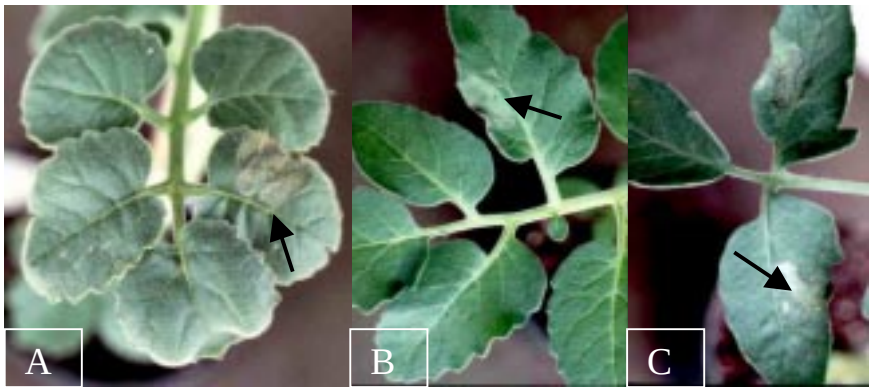


Figure 3-1. Confluent necrosis in tomato plants 24 to 36 hours after inoculation with the Xcv T3 strain 91-118. (A) *L. pennellii*, (B) an $F_1$ individual, and (C) the introgression line LA3488. Arrows point at the characteristic necrosis.

In order to test whether this resistance was the same as that described for *L. pimpinellifolium* PI 128216 and PI 126932, and *L. esculentum* cultigen Hawaii 7981, resistant plants were inoculated with the marker-exchange mutant strain M24 of Xcv 91-118 (T3) and the complemented mutant carrying the *avrXv3* gene. The M24 mutant is unable to cause HR on H7981 or *L. pimpinellifolium* PI 128216 and PI 126932 (Minsavage *et al.*, unpublished data). When these strains were inoculated into parental lines, $F_1$ and $F_2$ plants, and resistant introgression lines, both strains exhibited the same ability to elicit the hypersensitive response described before (Table 3-2).

Table 3-2. Response of different genotypes of tomato to strains of *Xanthomonas campestris* pv. vesicatoria[1]

| Strain | *L. esculentum* H7998 | *L. pennellii* LA716 | *L. esculentum* 216 | *L. esculentum* Fla. 7060 |
|---|---|---|---|---|
| XcvT3M24 ($HR^-$) | Sus | HR | Sus | Sus |
| XcvT3M24+ *avrXv3* ($HR^+$) | Sus | HR | HR | Sus |
| Xcv-T3wt | Sus | HR | HR | Sus |

[1] Sus, susceptible response; HR, resistant response

Analysis of Introgression Lines Indicates a Chromosome-3 Location

When a collection of 50 introgression lines was screened for resistance to T3 strains, only two lines, LA3488 and LA3489, developed confluent necrosis characteristic of the hypersensitive reaction which was previously observed in *L. pennellii*, $F_1$, and $F_2$ populations (Figure 3-1, C). In order to confirm this phenotype under more natural conditions using strain Xcv 97-2, dip inoculations of all introgression lines were carried out. As expected, LA3488 and LA3489 exhibited the lowest levels of infection scoring 4

and 5, respectively (data not shown). Both LA3488 and LA3489 carry overlapping fragments from chromosome 3 of *L. pennellii* (Eshed and Zamir, 1994).

Identification of Markers Linked to the Resistance Locus

Two loci (TG50c and TG134) located at each end of the chromosomal fragment carried by the introgression line LA3488 were chosen for the initial linkage analysis via RFLP. Genomic clones for four neighboring loci were converted into PCR-based markers by first sequencing the termini of the clones, and then designing primers suitable for PCR amplification. Of these, TG457 produced a *L. esculentum* amplicon that behaved as dominant locus (Figure 3-2). Primers for TG377 yielded allelic amplicons that differed in size, whereas those for TG284a and TG599 were digested with *HindIII* and *HindIII*/*EcoRI*, respectively, in order to distinguish the two alleles (Figure 3-2). Comparison of profiles obtained with NBS degenerated primers among parental and introgression lines indicated the presence of a 565 bp fragment unique to *L. pennellii* and LA3488, and absent in *L. esculentum* H7998 and LA3523 profiles (Figure 3-3). This unique fragment was designated NBS3.

The observed segregation ratios for the markers were a good fit to the 3:1 ratio. The results of the goodness of fit tests were as follows: TG599 ($\chi^2$=3.63), TG377 ($\chi^2$=1.14), TG134 ($\chi^2$=1.20), TG457 ($\chi^2$=0.08), and TG284a ($\chi^2$=2.56). Unlike the other markers, NBS3 ($\chi^2$=4.9) slightly deviated from the 3:1 ratio, while marker TG50c exhibited a segregation that strongly deviates from the Mendelian 3:1 ratio ($\chi^2$= 8.60). Therefore, TG50c was not used for further analysis.

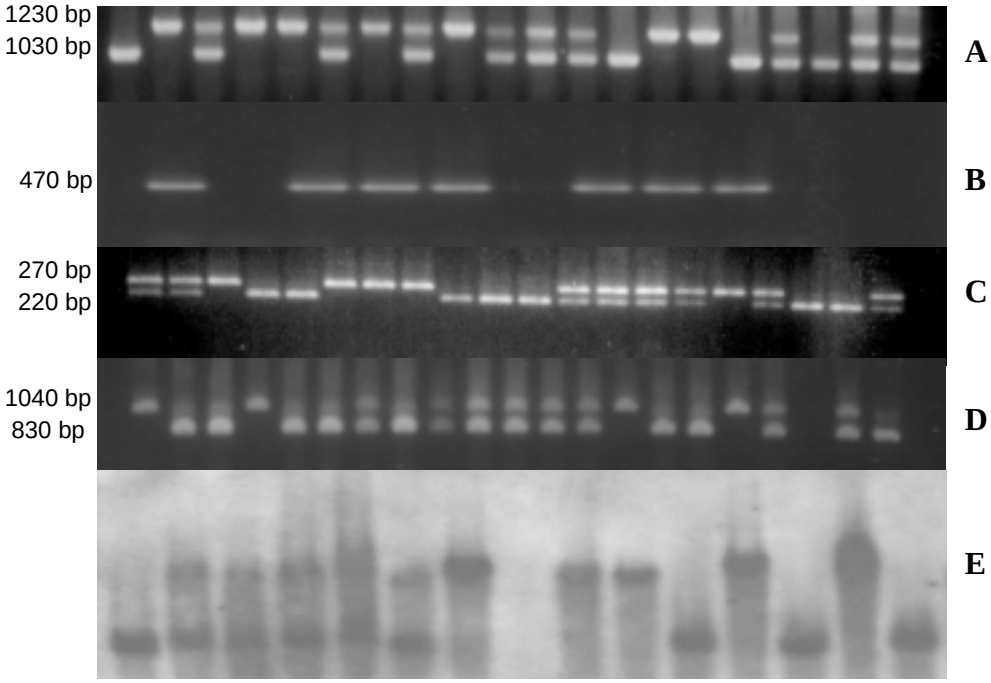


Figure 3-2. Examples of polymorphism obtained with PCR-based markers (C, D) and RFLP markers (E) used to screen the $F_2$ progeny. The approximate size of each band is also indicated. (A) TG377, co-dominant; (B) TG457, dominant; (C) TG599, digested with *HindIII/EcoRI*, co-dominant; (D) TG284a, digested with *HindIII*, co-dominant; and (E) TG134, co-dominant marker.

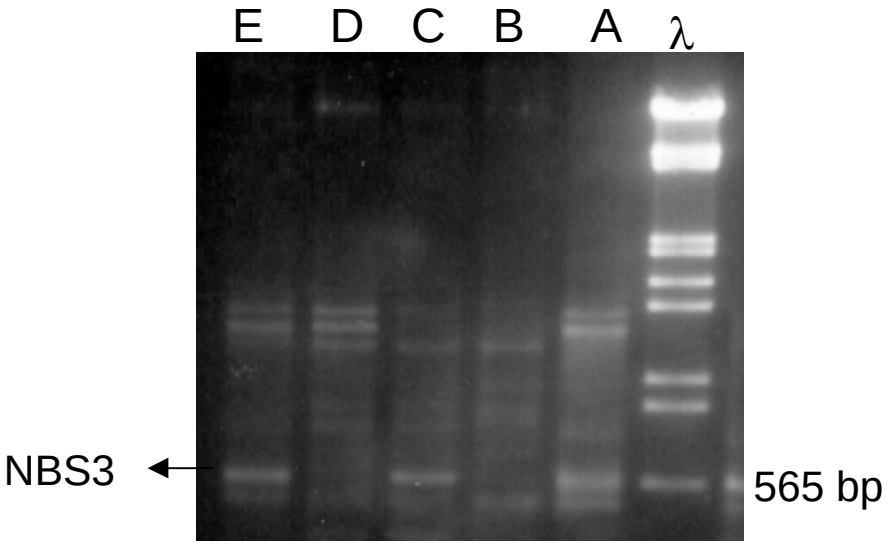


Figure 3-3. Amplification profiles obtained with degenerated oligonucleotide primers designed based on the amino acid sequences of two highly conserved motifs of the nucleotide binding site (NBS) in tobacco *N* and *Arabidopsis RPS2* genes (Yu *et al.*,1996) (Table 1). (λ) Lambda DNA digested with *EcoRI/HindIII*,(A) *L. pennellii*, (B) *L. esculentum* H7998, (C) introgression line LA 3488, (D) introgression line LA 3523, and (E) resistant $F_2$ individual.

Construction of a Genetic Map Around the Resistance Gene

Linkage analysis of RFLP, CAPS markers, and NBS3 indicates that the new resistance gene designated *Xv4* is linked to all markers tested (LOD 3.0, max. distance 50.0). The data were analyzed with MapMaker (Lander *et al.*, 1987) and are summarized in Figure 3-4. As expected, LA3488 and LA3489 share the same region of chromosome 3. Chromosome location and gene order were similar to those reported by Tanksley *et al*. (1992).

## Discussion

This study indicates the existence of resistance to T3 strains in the wild tomato relative *L. pennellii*. Segregation analysis of an $F_2$ population obtained from the interspecific cross between *L. esculentum* and *L. pennellii* indicates that a single dominant gene controls this novel resistance. The observed segregation ratio is a good fit to the 3:1 ratio expected for a character controlled by a single gene. The dominance of this character seems to be complete since $F_1$ plants showed HR responses similar in intensity and speed of development as *L. pennellii*.

Introgression lines have been previously used to locate qualitative and quantitative trait loci in interspecific crosses of *L. esculentum* and *L. pennellii* (Eshed and Zamir, 1995; Eshed *et al.*, 1996; McNally and Mutschler, 1997; Moreau *et al.*, 1998). In the present study, screening of the 50 introgression lines suggested that the gene controlling resistance to T3 strains is located in the lower arm of chromosome 3.

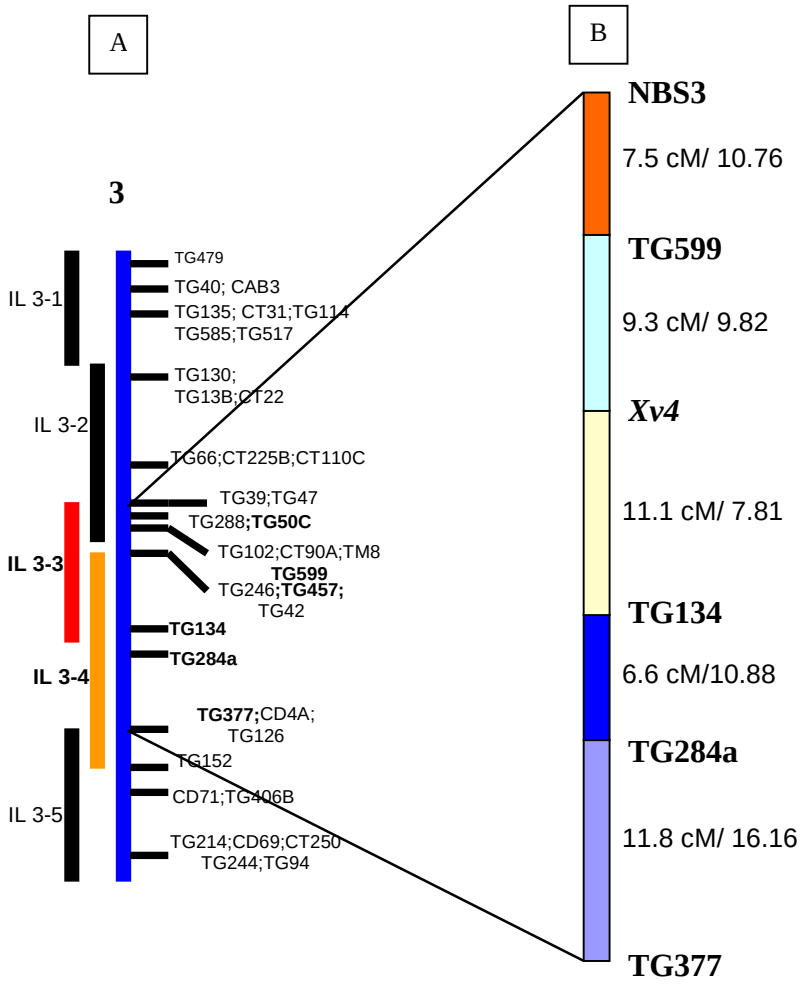


Figure 3-4. Comparative map locations of CAPS and RFLP markers. (A) Marker order as determined by Tanksley *et al.* (1992). Introgression fragments from the chromosome 3 of *L. pennellii* in different lines were mapped by Eshed and Zamir (1994) and are shown superimposed on the map. IL3-3= LA3488, IL3-4= LA3489. (B) Marker order around the resistance gene *Xv4* as determined by MapMaker. All markers shown are CAPS except for the RFLP probe TG134. Distance between markers/LOD score is also indicated.

Linkage analysis with RFLP probes and CAPS markers suggested that at least four of those markers are linked to the resistance gene in chromosome 3. Analysis of the data indicated that the *Xv4* locus is located between TG599 and TG134 (Figure 3-4).

The mapping of *Xv4* was carried out with the long-term goal of cloning this gene by chromosome landing (Tanksley *et al.*, 1995). The next step would be to generate a high-density map around this gene with the assistance of a large number of markers and recombination events. Through the use of bulked segregant analysis (Michelmore *et al.*, 1991) and AFLP (Vos *et al.*, 1995) and/or DAF (Caetano-Anollés *et al.*, 1991), markers closely linked to *Xv4* might be generated and used to identify individual BAC or YAC clones carrying that region of the genome.

Two lines of evidence suggest that this resistance is different from that described before in *L. esculentum* cultigen H7981 and two plant introductions of *L. pimpinellifolium*, PI 128216 and PI 126932 (Scott *et al.*, 1995; Minsavage *et al.*, 1996). First of all, knocking out the activity of *avrXv3* in Xcv proved to be completely independent from the ability of T3 strains to elicit HR on *L. pennellii* or its progeny carrying the newly discovered resistance gene. Secondly, when plants bearing the *Xv3* were challenged with Xcv strains carrying the putative *avr* gene, no HR was observed. Therefore, we can conclude that this incompatible interaction involves two previously undescribed genes. We propose *avrXv4* and *Xv4* as the symbols for these genes.

# CHAPTER 4
# *avrXv4*: A NEW AVIRULENCE GENE RESPONSIBLE FOR THE HYPERSENSITIVE REACTION IN THE WILD RELATIVE OF TOMATO *Lycopersicon pennellii*

Cloning and characterization of avirulence genes from different plant pathogenic bacteria have yielded important evidence as to how hosts and pathogens carry out their interactions (Leach and White, 1996). Several *avr* genes have been cloned and characterized previously from different strains of *Xanthomonas campestris* pv. vesicatoria (Xcv). Among them, *avrBs1*, *avrBs2*, *avrBs3*, and *avrBsP* were cloned from Xcv strains pathogenic to pepper, whereas *avrRxv*, *avrBsT*, and *avrXv3* were cloned from Xcv strains pathogenic to tomato (Ronald and Staskawicz, 1988; Bonas *et al.*, 1989; Minsavage *et al.*, 1990; Whalen *et al.*, 1993; Canteros *et al.*, 1995; Minsavage *et al.*, 1996).

Based on the assumption that a gene-for-gene interaction is involved, the main purpose of this work was to clone and characterize the putative avirulence gene(s) from *X. campestris* pv. vesicatoria race T3 involved in specifying resistance in the wild tomato relative *L. pennellii* which carries the resistance gene *Xv4*, characterized in Chapter 3.

## Materials and Methods

### Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study are listed in the Appendix. Strains of Xcv and *E. coli* were grown as described in Chapter 3. Plasmids were intro-

duced into *E. coli* by transformation (Maniatis *et al.*, 1982) and mobilized into Xcv strains by conjugation using pRK2073 as the helper plasmid in triparental matings (Figurski and Helinski, 1979; Ditta *et al.*, 1980). Triparental matings were made at 28 °C on plates of NYG agar (Daniels *et al.*, 1984). Antibiotics were added to the medium at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 25 or 50 μg/ml; rifamycin SV, 100 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 12.5 μg/ml.

Plant Material and Plant Inoculations

$F_1$ seeds from the cross between *L. esculentum* and *L. pennellii* were planted in Plugmix (W. R. Grace & Co., Cambridge, MA). After two weeks, the emerged seedlings were transferred to Metromix 300 (W. R. Grace & Co.) in 10 cm plastic pots. Seedlings were grown in the greenhouse at temperatures ranging from 25 to 35 °C (night/day). Tomato plants were grown for four to five weeks and then the main stem was removed above the fully expanded sixth true leaves. Plants were inoculated approximately 7 days after topping and transferred to a growth room kept at a constant temperature of 22 °C with a daily 16 h photoperiod.

Xcv strains for plant inoculations were grown as described in Chapter 3. For population dynamics studies, bacterial suspensions were diluted to a concentration of 2-5 x $10^5$ cfu/ml in sterile tap water. Infiltration of tomato leaves and electrolyte leakage measurements were carried out as previously described by Hibberd *et al.* (1987). Unless otherwise indicated, all experiments were arranged as a completely randomized design with three replications. All experiments were repeated twice. For electrolyte leakage and growth curve experiments, statistical analysis was conducted using the ANOVA proce-

dure of the Statistical Analysis System (SAS Institute, SAS Circle, Box 8000, Cary, NC) using the area under the curve as the variable for analysis.

Molecular Genetics Techniques

Standard molecular techniques were used for the extraction of genomic and plasmid DNA, restriction endonuclease digestions of DNA, and cloning procedures (Maniatis *et al.*, 1982; Ausubel *et al.*, 1992). Enzymes for restriction digestion and ligation reactions were purchased from Promega (Madison, WI) and used following the manufacturer's protocol.

A total genomic DNA library of Xcv strain 91-118 (tomato race 3) was constructed in the cosmid vector pLAFR3 as previously described (Minsavage *et al.*, 1990). Individual clones were mobilized into Xcv strain ME-90$^{rif}$ by triparental mating (Daniels *et al.*, 1984) and transconjugants were inoculated by leaf infiltration into the $F_1$ (*L. esculentum x L. pennellii*) tomato plants to screen for elicitation of a hypersensitive response.

Transposon Mutagenesis

For transposon mutagenesis, the plasmid pLAFR3 carrying the target insert was transformed into the polyA-dependent *E. coli* strain DH5α containing the transposon-carrying plasmid pHoKmGus and the plasmid pSShe carrying the transposase gene (Stachel *et al.*, 1985). Transformants were selected on media containing chloramphenicol, tetracycline, and kanamycin. After overnight growth, pLAFR3 carrying mutated inserts was isolated and transformed into the polyA-independent *E. coli* strain C2110. Two hundred individual transformants, selected on media containing nalidixic acid, kanamycin, and tetracycline, were mobilized into the virulent Xcv strain ME-90$^{rif}$ by triparental mat-

ing, and screened for their inability to elicit HR in $F_1$ plants obtained from the cross between *L. esculentum* and *L. pennellii*.

## Results

### Resistance of *Lycopersicon pennellii* to Xcv T3

Tomato race 3 strains of Xcv are able to elicit a hypersensitive response (HR) in leaves of *L. pennellii* LA716 (Figure 4-1). Phenotypic responses of parental lines and $F_1$ populations are summarized in Table 4-1. Unlike H7998, LA716 and $F_1$ plants inoculated with Xcv T3 strains exhibited the characteristic confluent necrosis about 24-36 h after infiltration at temperatures ranging between 22 to 25 °C. On the other hand, LA716 and $F_1$ plants inoculated with Xcv ME-90 remained free of symptoms for up to 48-60 h after infiltration. When the growth of Xcv strains in leaves of tomato was examined, the wild type strain 91-118 of Xcv T3 showed an increase in population size of about 100-fold by 4 days after infiltration but afterwards the growth curve leveled off (Figure 4-2). In contrast, the population of the virulent strain of Xcv ME-90 kept increasing up to 10000-fold by the sixth day after infiltration (Figure 4-2). Statistical analysis of the area under the curve indicated that strains carrying the putative *avr* gene exhibited an overall growth significantly different from that of those strains that did not carry it.

Regarding the speed and degree of cell damage caused by strains of Xcv (Figure 4-3), no significant differences were observed between Xcv T3 and ME-90 12 h after infiltration. However, in the following 48 h, electrolyte leakage increased significantly in

Figure 4-1. Confluent necrosis in a leaf of *Lycopersicon pennellii* LA216, 24 to 36 hours after inoculated with the Xcv T3 strain 91-118. Arrow points at the characteristic necrosis.

Table 4-1. Response of different tomato genotypes to strains of *Xanthomonas campestris* pv. vesicatoria[1]

| Xcv strain | *L. pennellii* LA716 | *L. esculentum* Hawaii 7998 | $F_1$ (*L. pennellii* LA716 x *L. esculentum* Hawaii 7998) |
|---|---|---|---|
| ME-90 | Sus | HR | Sus[2] |
| Xcv T3 91-118 | HR | Sus | HR |
| Xcv-60 [3] | HR | Sus | HR |
| Xcv-60::33[4] | Sus | Sus | Sus |

[1] Sus, susceptible response; HR, resistant response.[2] The HR response in $F_1$ plants to ME-90 is delayed, so it was considered susceptible.[3] Xcv60 = ME-90 carrying the cosmid clone pXcvT3-60.
[4] cosmid clone pXcvT3-60 carrying a transposon insertion.

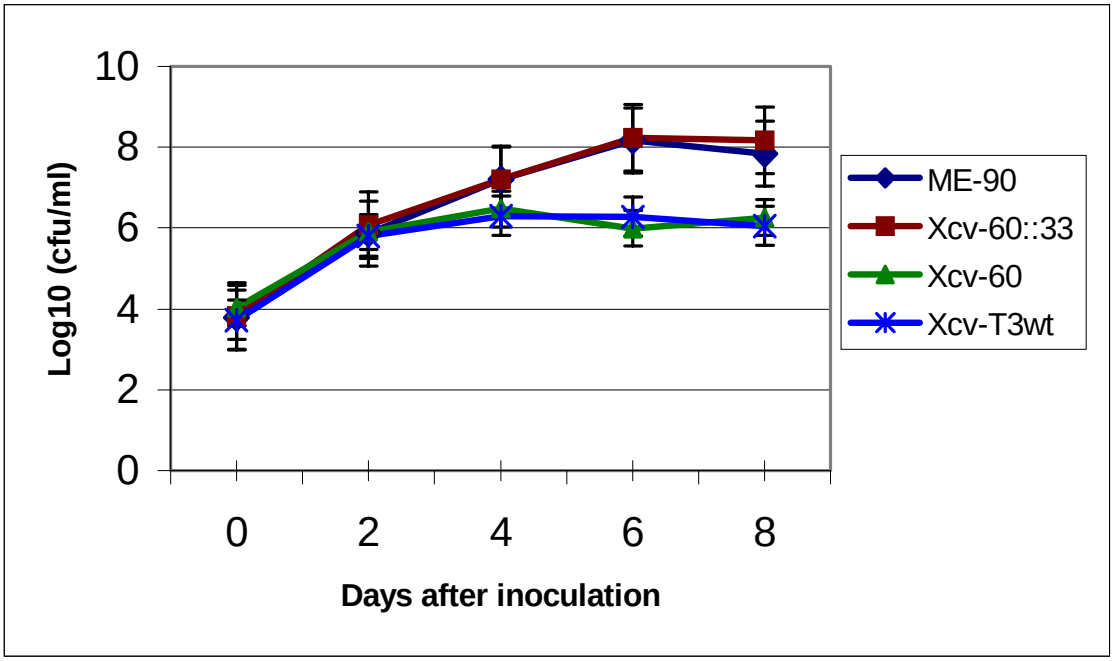


Figure 4-2. Time course of growth of *Xanthomonas campestris* pv. vesicatoria tomato races and transconjugants in $F_1$ plants obtained from the cross *L. pennellii* LA716 X *L. esculentum* H7998. (ME-90) wild type virulent strain; (Xcv-60::33) ME-90 carrying the mutant cosmid clone pXcv-60::33; (Xcv-60) ME-90 carrying the intact cosmid clone pXcv-60; and (Xcv-T3wt) wild type strain 91-118 of Xcv race T3. Bars indicate standard errors.

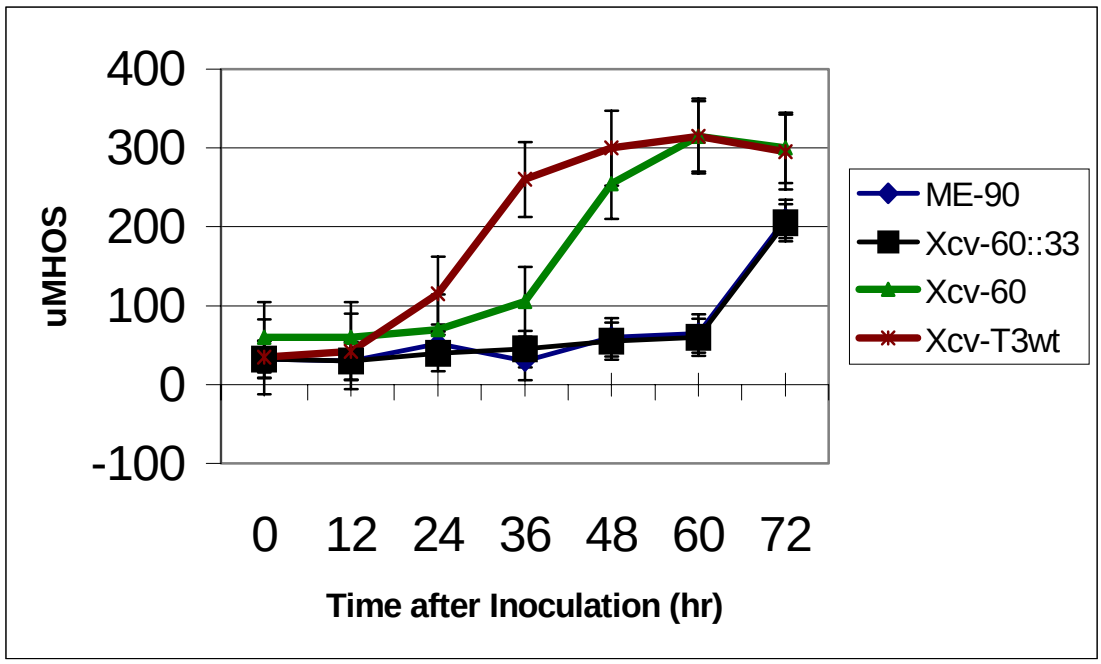


Figure 4-3. Time course of electrolyte leakage from leaves of resistant $F_1$ plants obtained from the cross *L. pennellii* LA716 X *L. esculentum* H7998 infiltrated with strains and transconjugants of *Xanthomonas campestris* pv. vesicatoria. (ME-90) wild type virulent strain; (Xcv-60::33) ME-90 carrying the mutant cosmid clone pXcv-60::33; (Xcv-60) ME-90 carrying the intact cosmid clone pXcv-60; and (Xcv-T3wt) wild type strain 91-118 of Xcv race T3. Bars indicate standard errors.

tissue infiltrated with Xcv T3 while that caused by ME-90 remained almost unchanged. During the final 12 h, cell damage caused by ME-90 started to increase while that caused by the wild type Xcv T3 leveled off (Figure 4-3). Statistical analysis of the area under the curve indicated that there were significant differences in the degree and speed of the damage caused by strains carrying the putative *avr* as compare to those strains that did not have the gene.

Cloning of the Avirulence Gene *avrXv4*

A total of 600 cosmid clones from a genomic DNA library of Xcv tomato race 3 strain 91-118 were mobilized into the Xcv strain ME-90 (virulent on *L. pennellii*) by conjugation and the resulting transconjugants were inoculated onto leaves of resistant tomato plants. One clone, pXcvT3-60, carried by the strain ME-90 induced resistance on $F_1$ plants obtained from the cross between *L. esculentum* and *L. pennellii* (Table 4-1). The cosmid clone pXcvT3-60 contained a 29 kb fragment of Xcv DNA, as determined by restriction endonuclease digestion (data not shown).

When leaves of LA716 and $F_1$ plants were infiltrated with the Xcv strain ME-90 carrying pXcvT3-60, the population growth exhibited a similar trend to that described before for the wild type Xcv T3 (Figure 4-2). Similarly, cell damage caused by the same transconjugant was identical to that caused by the wild type T3 (Figure 4-3).

Tn3-*gusA* Mutagenesis

The cosmid clone pXcvT3-60 containing the *avrXv4* was mutagenized by transposon insertion using Tn3-*gusA*. Three out of 200 transconjugants carrying transposon deriva-

tives were unable to elicit HR when inoculated into $F_1$ (*L. esculentum x L. pennellii*) tomato plants.

Growth curves of Xcv ME-90 carrying the mutant clone pXcvT3-60::33 indicated that insertion mutations in *avrXv4* prevent the negative effect on growth that the intact form of the gene had on the virulent strain ME-90 (Figure 4-2). Similarly, the speed at which cell damage occurred in resistant plants was drastically reduced when Xcv ME-90 carrying the mutant pXcv-60::33 was used for inoculations (Figure 4-3).

## Discussion

A new gene-for-gene model has been found involving T3 strains of Xcv and the resistant host *L. pennellii*. Mobilization of a genomic library into a virulent strain of Xcv was carried out with the purpose of finding a clone carrying the putative *avr* gene. One single cosmid clone was able to convert the virulent strain into a fully avirulent one. As shown before for other *avr* genes (Minsavage *et al.*, 1990; Whalen *et al.*, 1993), the incorporation of heterologous *avr* genes into virulent strains of the pathogen modify their host specificity. The resistant tomato plants used in the screening carried the resistance gene *Xv4* from *L. pennellii*, so the putative *avr* gene was designated *avrXv4*.

A series of experiments were performed in order to further substantiate that the isolated clone carried *avrXv4*. The experiments included comparing the effect of the wild type and mutated genes on the speed and degree of damage caused to the plant, and the growth rate *in planta* of different transconjugants. In one experiment, the presence of an active avirulence gene in the virulent strain resulted in a lower growth rate of the bacte-

rium in the resistant plant. This limited growth was most likely due the onset of the HR as has also been reported for other Xcv strains carrying *avr* genes such as *avrRxv* (Whalen *et al.*, 1993). In contrast, when the mutated form of the avirulence gene was present in the virulent strain, an HR was not observed and the growth rate of the transconjugant was not reduced resembling that of the virulent strain.

Klement (1982) determined that electrolyte leakage is a measure of membrane disruption in a plant undergoing an HR. When electrolyte leakage was measured, the speed and degree of damage caused by the transconjugants carrying the intact gene and the wild type T3 strain were equally high because the incompatible interaction between *avrXv4* and *Xv4* rapidly lead to cell death. Also, the onset of the electrolyte leakage induced by avirulent strains correlated with the onset of the visible HR (24-36 hours). This finding agrees with observations made from other gene-for-gene interactions involving other races of Xcv (Minsavage *et al.*, 1990; Whalen *et al.*, 1993). Conversely, the speed and amount of damage caused by the wild type virulent strain and the transconjugant carrying the mutated form were significantly lower which is characteristic of compatible interactions. These results strongly indicate that *avrXv4* was the only *avr* gene restricting growth of the strain ME-90 and inducing the HR in resistant $F_1$ plants.

# CHAPTER 5
# FUNCTIONAL DOMAINS OF *avrXv3* AND THEIR ROLE IN ELICITING THE HYPERSENSITIVE REACTION IN TOMATO (*Lycopersicon esculentum* L.)

A thorough understanding of plant-pathogen interactions is vital for the development of new and environmentally friendly strategies to control plant diseases. In plant-pathogen interactions that fit the gene-for-gene model, determining the role of *avr* gene products is essential to understand how plants defend themselves from their attackers. However, with very few exceptions, little is known about how *avr* genes function.

Bacterial spot caused by *Xanthomonas campestris* pv. vesicatoria (Xcv) is a serious disease on tomato and pepper. Minsavage *et al.* (1996) reported cloning and characterization of an avirulence gene from the race T3 of Xcv that is responsible for the elicitation of a hypersensitive reaction in one genotype of *Lycopersicon esculentum* and two plant introductions of *L. pimpinellifolium*. The gene was designated as *avrXv3* and encodes one of the smallest peptides found among bacterial *avr* gene products (Minsavage *et al.*, 1996).

The main objective of this research was to study the functional domains of the AvrXv3 protein and its possible involvement in eliciting the hypersensitive reaction in tomato.

## Materials and Methods

### Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in the Appendix. Strains of Xcv *and E. coli* were grown as described in Chapter 3.

### Plant Material, Inoculum Production, and Avirulence Activity Assays

Plants of the tomato near-isogenic lines 216 and Fla. 7060, resistant and susceptible to Xcv tomato race 3, respectively, were grown for four to five weeks and then the main stem was removed above the fully-expanded sixth true leaf. Plants were inoculated approximately seven days after topping and transferred to a growth room kept at a constant temperature of 25 °C with a 16 h photoperiod.

Inoculations with Xcv strains were carried out as described in Chapter 3. Unless otherwise indicated, inoculation experiments were replicated three times.

### Hydrophobicity Chart and Sequence Homology of *AvrXv3*

The analysis of the distribution of hydrophobic and hydrophilic residues throughout the AvrXv3 protein was carried out as described by Shaw (1995). Regions showing clustering of amino acids with similar hydrophobic properties were considered as targets for mutation (Figure 5-1).

A search for homology of the nucleotide sequence of *avrXv3* and the amino acid sequence of the predicted AvrXv3 were carried out on the World Wide Web using the Blast 2.0 algorithm (Altschul *et al.*, 1997) and DARWIN (Data Analysis and Retrieval with Indexed Nucleotide/peptide sequences).

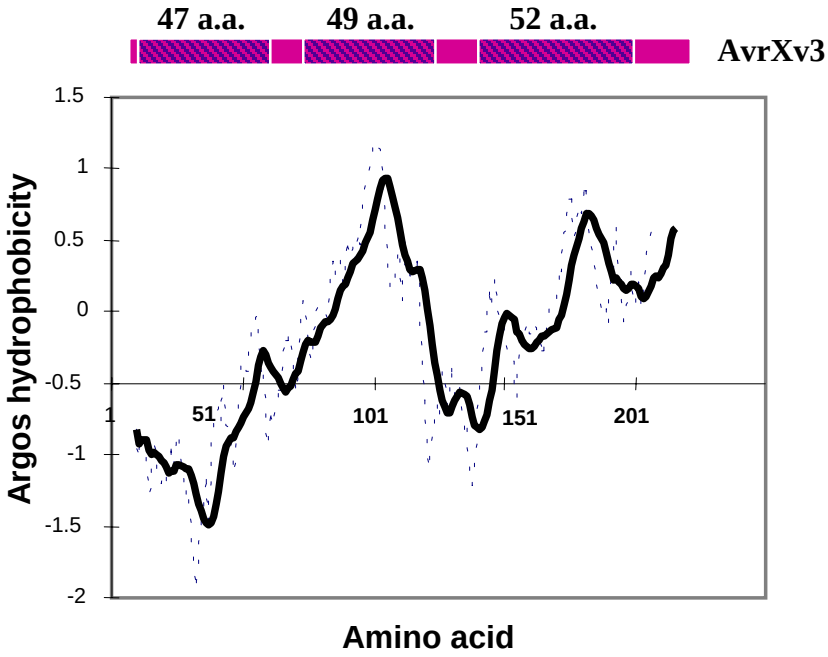


Figure 5-1. Distribution of hydrophobic residues in the predicted protein of *avrXv3*. Argos hydrophobicity values were calculated as described by Shaw (1995). Solid line depicts the average of the hydrophobicity values for 20 amino acid residues. Dashed line represents the average of 10 amino acid residues.

Mutagenesis of *avrXv3*

PCR-based deletion mutagenesis was performed on the clone pLAFR119ΔPst to create in-frame deletions of about 50 amino acids in three different sites along the sequence of this gene. This clone carries the entire open reading frame of *avrXv3* and its original promoter region. First, pLAFR119ΔPst was digested with *HindIII* and *EcoRI* and the fragment containing the promoter region and the ORF of a*vrXv3* was transferred to pBluescript KS, resulting in pBS:T3ΔPst. The targets for deletion were chosen by identifying putative domains defined by the distribution of hydrophobicity residues in the predicted protein (Figure 5-1). A set of 8 oligonucleotide primers were synthesized at the ICBR DNA Synthesis Laboratory, University of Florida, Gainesville, in order to make the in-frame deletions of the three putative domains (Table 5-1). As shown in Figure 5-2, separated PCR reactions were carried out using pBS:T3ΔPst as template and the following primer combinations: P1/P2, P3/P8, P1/P4, P5/P8, P1/P6, and P7/P8. In order to ease the process of screening for the right construct, unique restriction sites for the endonucleases *XhoI*, *AvaI*, and *KpnI* were engineered as silent mutations in primers P3, P5, and P7, respectively (Table 5-1). Polymerase chain reaction was carried out as described in Chapter 3. The annealing temperature use for all primers was 60 °C. Oligonucleotide sequences used in this study are shown in Table 5-1.

Subsequently, PCR products were diluted 100-fold and an aliquot of 2 μl of each product was combined as follows: P1/P2 + P3/P8, P1/P4 + P5/P8, and P1/P6 + P7/P8. The mixtures were used as templates for a second PCR reaction using the primers P1/P8 and the same conditions described above. The resulting modified constructs were de-

Table 5-1. Sequence of specific oligonucleotides used for PCR-based deletion mutagenesis of *avrXv3*. Restriction sites added by each oligonucleotide are also indicated.

| ID | Oligonucleotide Sequence | Restriction Enzyme Site added |
|---|---|---|
| P1 | 5'GCGCGCAATTAACCCTCACTAAAG3' | - |
| P2 | 5'GTAACGATTGATACTACTTGTCATGG3' | - |
| P3 | 5'GACAAGTAGTATCAATCGTTCGCTCGAGTGGAGCAGGTCG3' | *XhoI* |
| P4 | 5'AACGCCCTTGATCGGCTTATTTCG3' | - |
| P5 | 5'ATAAGCCGATCAAGGGCGTTGTTATGCCCGAGAATCGC3' | *AvaI* |
| P6 | 5'TTTAGCGGCATACCCCTGCGAACG3' | - |
| P7 | 5'CGCAGGGGTATGCCGCTAAAGAAAAGGGTACCGTAAGG3' | *KpnI* |
| P8 | 5'CG CGCGTAATACGACTCACTATAG3' | - |

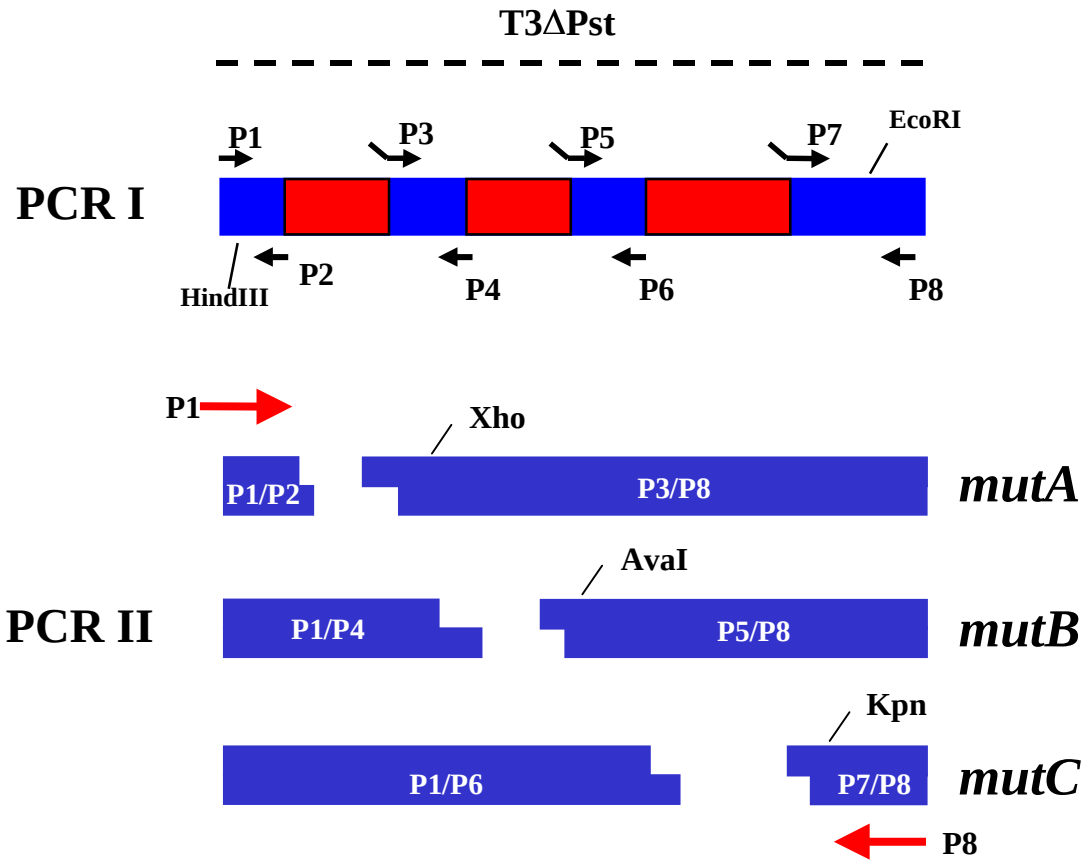


Figure 5-2. Diagram of the procedure followed for PCR-based deletion muatagenesis. For PCR I, individual reactions were carried out using pBS:T3ΔPst as template and the following primer combinations: P1/P2, P3/P8, P1/P4, P5/P8, P1/P6, and P7/P8. For PCRII, products from PCRI were diluted 100-fold and an aliquot of 2 µl of each product was combined as follows: P1/P2 + P3/P8, P1/P4 + P5/P8, and P1/P6 + P7/P8. The mixtures were used as templates for a second PCR reaction using the primers P1/P8. Unique restriction sites for the endonucleases *XhoI*, *AvaI*, and *KpnI* were engineered as silent mutations in primers P3, P5, and P7, respectively

signated as *mutA*, *mutB*, and *mutC*. These mutants were digested with *HindIII* and *EcoRI* and cloned into pLAFR6.

In order to determine the effect of possible changes in the three-dimensional structure of the AvrXv3 on its activity, the termini of the predicted protein were also modified by the addition of six histidine residues. First, the ORF of *avrXv3* was isolated by PCR using the primers RST88 (5'CCGCTCGAGCTACTTAACGAGATTTGTTAC3') and RST89 (5'CCGCTCGAGATGACAAGTAGTATCAATC3') which add *XhoI* restriction sites. The PCR product was cloned into pET15b, in frame with a histidine tag at the 5' terminus of the ORF. The resulting construct was designated pET15b:HisT3. Secondly, primers P1 and RST88b (5'CCGCTCGAGCTTCTTAACGAGATTTGTTAC3'), which eliminate the stop codon of the gene, were used to isolate the ORF of *avrXv3* and its original promoter from pBS:T3ΔPst. The resulting fragment was digested with *HindIII* and *XhoI* and cloned into pET22b, in frame with a histidine tag at the 3' end of the ORF. This construct was designated pET22b:T3His. Polymerase chain reaction was carried out as described in Chapter 3. For both primer pairs, each cycle consisted of 30 s of denaturation at 95 °C, 30 s of annealing at 56 °C, and 1 min of extension at 72 °C. For the final cycle, the extension step was prolonged to 5 min.

All mutants were sequenced at the ICBR sequencing facility (University of Florida, Gainesville, FL) using the Applied Biosystems model 373 system (Applied Biosystems, Foster City, CA).

Avirulence Activity in Xcv Background

The constructs pLAFR6:*mutA*, pLAFR6:*mutB*, and pLAFR6:*mutC* were introduced into Xcv strain ME-90 by triparental mating (Daniels *et al.*, 1984) and inoculated into the tomato near-isogenic lines 216 and Fla. 7060 as described above. Regarding T3His, pET22b:T3His was digested with *HindIII* and *PstI* and cloned into pLAFR3. HisT3, on the other hand, was cloned into a previously constructed plasmid, pLAFR3:Pt3, under control of the original promoter region of *avrXv3*. The resulting constructs, pLAFR3:T3His and pLAFR3Pt3:HisT3, were introduced into Xcv strain ME-90 by tri-parental mating (Daniels *et al.*, 1984) and inoculated into the tomato near-isogenic lines 216 and Fla. 7060 as described above.

*Agrobacterium*-mediated transient expression

The binary vector pMD-1, kindly provided by Dr. B. Staskawicz, was used for all *Agrobacterium*-mediated transient expression assays. The ORF's of *avrXv3*, *mutA*, *mutB*, and *mutC* were isolated by PCR using the primers RST89b (5'CCGTCTAGAATGACA AGTAGTATCAATC3') and RST88c (5'CCGGGATCCCTTCTTAACGAGATTTGTT AC3'), which add the restriction sites *XbaI* upstream of the start codon, and *BamHI* downstream of the stop codon, respectively. Following digestion with the appropriate en-zyme combination, constructs were cloned into pMD-1. The ORF of *avrXv3* was also cloned into the binary vector pO4541 that lacks the 35S promoter. HisT3 was isolated from pET15b:HisT3 by PCR using the primers HIST3-F (5'CCGGAATTCAT GGGCAGCAGCCATCAT3') and RST88c, which add *EcoRI* and *BamHI* sites, respec-tively. T3His was isolated from pET22b:T3His by PCR using the primers RST89c

(5'CCGGAATTCATGACAAGTAGTATCAATC3') and T3HIS-R (5'GCTGGATCCA GTTATTGCTCAGCGG3'), which add *EcoRI* and *BamHI* sites, respectively. Following digestion with the appropriate enzyme combination, constructs were cloned into pMD-1. All constructs were maintained in *E. coli* and transferred to *A. tumefaciens* by triparental mating (Daniels *et al.*, 1984).

The *Agrobacterium tumefaciens* strain C58C1 containing the Ti-plasmid pGV2260 (Deblaere *et al.*, 1985) and individual transconjugants were grown overnight at 28 °C in YEB medium (Kapila *et al.*, 1997) amended with 10 mM N-morpholino-ethanesulfonic acid (MES) (Sigma) pH 5.6, 20 μM acetosyringone (Sigma), and the appropriate antibiotics. After overnight growth, the concentration was adjusted to an $A_{600}$ = 0.5-0.6, bacteria were pelleted, washed with MMA medium pH 5.6 (Murashige and Skoog's medium (Gibco BRL) amended with 10 mM MES, sucrose 20 g/l, and 200 μM acetosyringone, and resuspended in the same medium to a final concentration of $A_{600}$ = 0.05. Bacterial suspensions were kept at 25 °C for 1 h and then used for infiltration. Fully expanded leaves of the tomato near-isogenic lines Fla. 7060 and 216 were infiltrated with the bacterial suspension as described by Hibberd *et al.* (1987). After infiltration, plants were incubated at 22 °C and continuous light for 48 h or until symptoms developed. Unless otherwise indicated, inoculations were replicated three times.

Transcription Activation Activity

The yeast strain EGY48 (*Saccharomyces cerevisae*) carrying the plasmid pMW106, which contains the *LacZ* gene under control of LexA-regulated promoters, was used for testing transcription activation activity. The vector pEG202 containing the LexA DNA

binding domain sequence under control of the constitutive yeast ADH1 promoter and a polylinker region at the C terminus was used for generating fusions with the wild type and modified AvrXv3 proteins. The ORF of all mutant genes was isolated by PCR using the following primer combinations: *mutB* and *mutC* with RST89c/RST88, *mutA* with RST89c/RST88c, *HisT3* with HIST3-F/RST88c, and *T3His* with RST89c/T3HIS-R. The plasmids pMW106 and pEG202 were maintained in yeast by selection for uracil and histidine auxotrophy, respectively, and in *E. coli* by selection for resistance to ampicillin and kanamycin, respectively. The plasmid pSH17-4 carrying the LexA fused to Gal4p activation domain was used as a positive control for transcription activation, and pRFHM1 carrying the non-activating fusion between LexA and the *Drosophila* protein Bicoid was used as negative control for transcription activation.

Expression of the expected fusion proteins in yeast was determined by standard Western blot analysis (Ausubel *et al.*, 1992) using a polyclonal antibody raised against the LexA DNA-binding domain (kindly provided by Dr. E. Golemis). The membranes were reacted for 1 h at 25 °C with primary Anti-LexA polyclonal antiserum followed by 1 h incubation with the anti-rabbit IgG-alkaline phosphatase conjugate (Sigma). Bound antibody was detected using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate tablets (Boehringer Mannheim Biochemicals). Methods for yeast manipulations were as described by Golemis and Brent (1997). β-galactosidase activity assays were performed as described by Clontech Laboratories. Dr. Roger Brent, Department of Molecular Biology, Massachusetts General Hospital, kindly provided all plasmids and yeast strains used in this study. Unless otherwise indicated, all

experiments were arranged in a completely randomized design with three replications. All experiments were repeated twice. Statistical analysis was conducted with the Statistical Analysis System (SAS Institute, SAS Circle, Box 8000, Cary, NC).

## Results

### Sequence Analysis of *avrXv3*

A sequence homology search of *avrXv3* and its predicted protein was carried out with the computer program Gapped BLAST 2.0. The output of the search did not yield any significant homology to any known gene or protein. Subsequent analysis using the database DARWIN yielded three proteins with homology scores greater than 84%. The three best matches included the human transcription factor Sp4 (98%), the human DNA repair protein RAD52 homolog (89%), and the SCD2 protein from *Schizosaccharomyces pombe* (84%). The region of homology between Sp4, RAD52, and AvrXv3 is located near the N-terminus of AvrXv3, while the homologous region with the SCD2 protein seems to expand most of the middle portion of AvrXv3.

Analysis of the distribution of hydrophobic residues indicated clustering of amino acids with similar hydrophobic properties (Figure 5-1). The regions expanding the three most prominent peaks in the chart were chosen as targets for deletion.

### Mutagenesis of *avrXv3*

PCR-based deletion mutagenesis yielded three different mutant proteins lacking 47 aa (MutA), 49 aa (MutB), and 52 aa (MutC) at the N-terminus, middle portion, and C-terminus of the AvrXv3, respectively. Addition of histidine tags at the C- and N- termi-

nus of the AvrXv3 protein yielded two larger proteins of about 27-kD. The sequence of all mutants was confirmed by sequence analysis.

Avirulence Activity of Xcv Carrying Modified *avrXv3* Constructs

In order to test the avirulence activity of the mutated proteins, all constructs were cloned into the wide-host range plasmid pLAFR6, and introduced into the virulent strain of Xcv ME-90. Despite the fact that all modified genes were under control of their natural promoter, none of the constructs was able to confer this strain the ability to elicit the hypersensitive reaction in the resistant tomato genotype 216 (data not shown).

Cell Death Induced by AvrXv3 When Expressed Inside the Plant Cell

The use of *Agrobacterium tumefaciens* for the expression of *avrXv3* inside plant cells led to hypersensitive reaction in the resistant tomato cultigen 216 but not in the susceptible genotype Fla. 7060 48 h after inoculations. The confluent necrosis observed in the resistant genotype as a result of transient expression resembles the HR reaction induced by race T3 of Xcv expressing *avrXv3*. Furthermore, confluent necrosis in the resistant cultigen 216 only occurred when the 35S promoter controlled the expression of *avrXv3*. *Agrobacterium tumefaciens* carrying the empty binary vector pMD-1 did not induce the development of any symptoms on either Fla. 7060 or 216 (Figure 5-3). When mutated constructs of *avrXv3* were introduced into the plant cells by *Agrobacterium*-mediated transient expression, only HisT3 and T3His were able to elicit the development of confluent necrosis (data not shown).

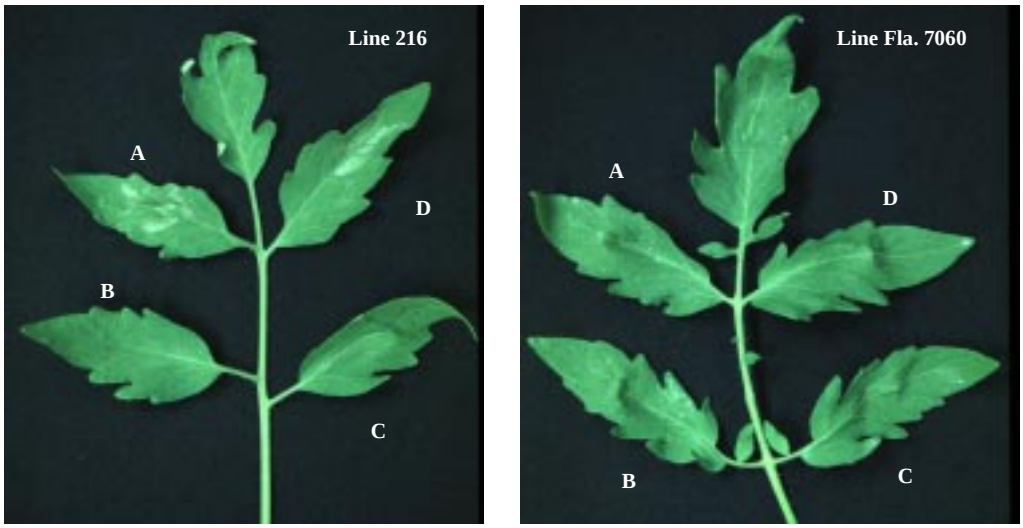


Figure 5-3. *Agrobacterium*-mediated transient expression of *avrXv3* constructs in the two near-isogenic tomato lines 216 and Fla. 7060. (A) *avrXv3* in pMD-1 under control of the 35S promoter, (B) *avrXv3* in pO4541 without the 35S promoter, (C) *A. tumefaciens* strain C58C1 carrying pMD-1, and (D) *X. campestris* pv. vesicatoria strain 91-118 (T3).

Transcription Activation Activity

Wild type and the mutated forms of *avrXv3* were expressed in the yeast strain EGY48 as fusion proteins with the LexA DNA binding domain. As shown in Figure 5-4, all constructs expressed a protein of the expected size.

The transcription activation activity of each mutant and the wild type AvrXv3 were determined by indirect measurement of the activity of β-galactosidase in the presence of the substrate o-nitrophenyl β-D-galactopyranose (ONPG). As shown in Figure 5-5, the wild type AvrXv3 exhibited significant transcription activation activity as compared to the negative control the homeodomain of the *Drosophila* protein Bicoid, and the plasmid pEG202 without insert.

Regarding the mutant proteins, deletion of the putative domains located at the N-terminus and middle portion of the AvrXv3 protein did not alter its transcription activation activity. However, the deletion of 59 aa in MutC at the C-terminus of the protein seemed to cause a total shut down of the transcription activation activity of AvrXv3. The addition of histidine tags to either end of the protein did not significantly modify its activity.

## Discussion

The experiments conducted for this research confirmed the ability of the *avrXv3*-encoded protein to elicit the HR only in the resistant host. *Agrobacterium*-mediated transient expression of *avrXv3* indicated that the gene product must be present inside the host cell in order to trigger the resistant response. These results agree with what has already shown for several other *avr* proteins (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*,

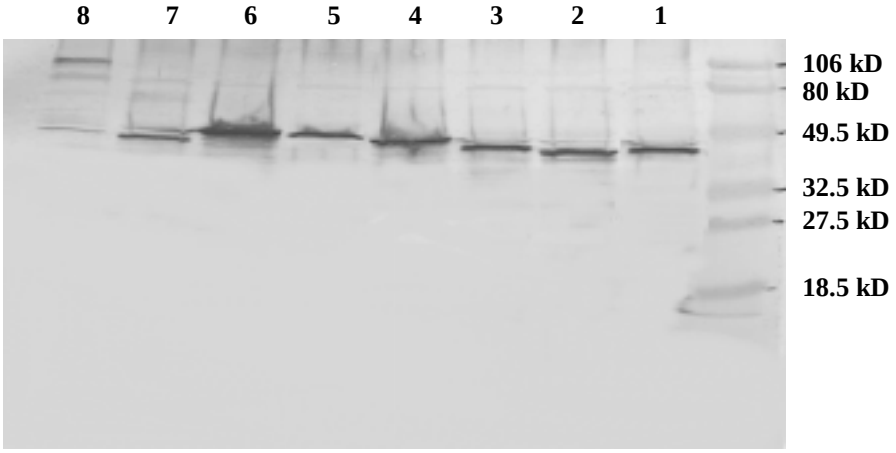


Figure 5-4. Western blot showing the expression of mutated and wild type AvrXv3 protein in the yeast strain EGY48. All proteins were expressed as fusions with the DNA binding domain of the LexA protein. (1) LexA-MUTA, (2) LexA-MUTB, (3) LexA-MUTC, (4) LexA-AvrXv3 wild type, (5) LexA-HisT3, (6) LexA-T3His, (7) LexA-Homeodomain of Bicoid, (8) LexA-Galp4 activation domain.

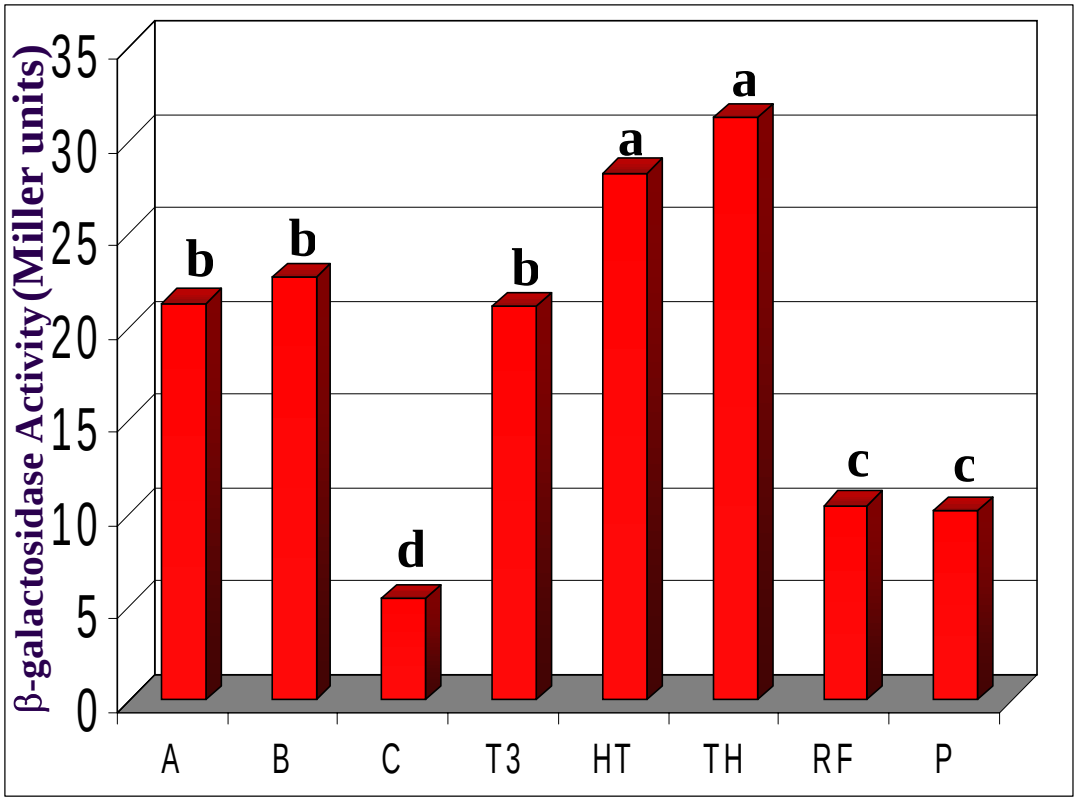


Figure 5-5. Transcription activation of different mutants and wild type AVRXv3 expressed as LexA fusion in the yeast strain EGY48. (A) MutA, (B) MutB, (C) MutC, (T3) AvrXv3 wild type, (HT) HisT3, (TH) T3His, (RF) Homeodomain of Bicoid, negative control, and (P) plasmid pEG202 without any insert.

1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996; Bonas and Van den Ackerveken, 1997; Parker and Coleman, 1997; de Feyter *et al.*, 1998).

The results of the transcription activation experiments demonstrated that AvrXv3 has transcription activation activity in yeast, and that this activities are similar to those presented by Zhu *et al.* (1998) for the AvrXa10 protein from *Xanthomonas oryzae* pv. oryzae. Further evidence to support the transcription activation activity of AvrXv3 was found by sequence comparisons using DARWIN. Based on these results, AvrXv3 may have similarity to the human transcription factor Sp4.

Many eukaryotic transcription activators have a modular structure with at least two functional domains, one that directs binding to specific DNA sequences and one that activates transcription (Hope and Struhl, 1986). These two domains seem to act independently from one another, and can be exchanged among transcription factors without loosing activity (Brent and Ptashne, 1985). Experiments conducted to map the position of the putative domains involved in transcription activation in yeast and the avirulence activity in tomato indicated that while the C-terminus of AvrXv3 seem to encode an active transcription activation domain, the entire protein is required for normal avirulence activity in the resistant host. Even though these results may indicate a lack of correlation between these two traits, the inability of MutA and MutB to cause HR in tomato may be explained by the disruption of a potential DNA-binding or protein-binding domain. In yeast, this effect was not detectable since the LexA DNA-binding domain complemented that mutation.

Addition of histidine tags at both termini of the AvrXv3 protein did not alter its ability to elicit the HR in tomato by transient expression or activate transcription in yeast. However, when both constructs were introduced into a virulent strain of Xcv, the resulting transconjugants were unable to elicit HR in the resistant host. These results may indicate that modifications of the termini could be interfering with the secretion of AvrXv3 by either modifying an unknown signal for secretion recognized by the Hrp system, or by altering the three-dimensional structure of the protein needed for transport.

# CHAPTER 6
# CONCLUSIONS

The analysis of an $F_2$ population obtained from the cross between *Lycopersicon pennellii* cultigen LA716 and *Lycopersicon esculentum* Hawaii 7998 confirmed that the former genotype is a new source of resistance to T3 strains of *Xanthomonas campestris* pv. vesicatoria (Xcv). Segregation ratios of this trait suggested that the resistance found in *L. pennellii* is controlled by a single gene. Inoculation of $F_2$ plants with a strain of Xcv T3 carrying an inactive form of *avrXv3* indicated that this resistance gene is different from that found in *L. esculentum* H7981 or *L. pimpinellifolium* PI 128216 and PI 126932. This new resistance gene was designated *Xv4*.

Screening of introgression lines and linkage analysis with CAPS and RFLP markers indicated that the resistance gene *Xv4* maps to an approximately 21.9 cM interval defined on the centromeric side by TG599 at 9.3 cM and, on the telomeric side by TG134 at 11.1 cM. High-density mapping should be carried out in this region of the genome in order to obtain more closely linked markers useful for chromosome walking or chromosome landing.

Screening of a genomic library obtained from the strain 91-118 of Xcv yielded a single clone able to confer to a virulent strain of Xcv the ability to elicit a hypersensitive reaction in plants carrying the *Xv4* gene for resistance. Comparison of the growth rates *in*

*planta*, and the speed/degree of damage between wild type and transconjugants of Xcv strains carrying the putative *avr* gene confirmed that a newly discovered avirulence gene was responsible for eliciting HR in *L. pennellii*. Sequence analysis of this gene and homology searches should be carried out in order to determine its possible role in eliciting the HR and/or similarities to other known genes. The designation *avrXv4* is proposed for this gene. Since *avrXv4-Xv4* is the second gene-for-gene system described for the race T3 and tomato, future breeding programs focused on transferring the two different resistant genes into commercial cultigens of tomato will ensure a better chance of achieving durable resistance in the field against the race T3 of Xcv.

In order to explore the role of *avr* genes in incompatible interactions, the *avrXv3* gene was used in a series of experiments designed to examine some of the features that could be involved in eliciting the HR in tomato. First of all, *Agrobacterium*-mediated transient expression confirmed the direct role of *avrXv3* in eliciting the HR in tomato. Furthermore, these results suggested that this *avr* gene product must be present inside the host cell in order for the plant to trigger the defense response.

Mutational analysis of *avrXv3* and transcription activation assays in yeast revealed that this Avr protein possesses transcription activation activity, and that the putative domain responsible for that activity might be located near the C-terminus of the protein. In addition, the remaining deleted sites of the protein that were examined by deletion analysis might be involved in binding DNA, or another protein that binds DNA, so that the assembly of the transcription factor would be completed. Finally, the addition of histidine

residues to the termini of the protein seemed to disrupt the secretion of the AvrXv3 protein, perhaps by modifying the secretion signal or by changing the structural conformation of the protein. Further studies should be carried out in order to determine if the transcription activation domain found at the C-terminus of the AvrXv3 is active in plants, and if there is any region of the protein involved in protein-DNA or protein-protein interactions.

APPENDIX
BACTERIAL STRAINS, YEAST STRAINS, AND PLASMIDS USED IN THIS STUDY

Table A-1. Bacterial strains and plasmids used in this work

| Designation | Relevant characteristics and use | Reference or source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | *SupE44 lacU169 (f80 lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1, Nxr* | Bethesda Research Laboratories |
| C2110 | $Nal^r$ $polA^-$ | Stachel *et al*., 1985 |
| *Xanthomonas campestris* pv. vesicatoria | | |
| ME-90 | Pepper race 3, 87-3 carrying Tn5, $Rif^r$ $Km^r$ | |
| 91-118 | Tomato race 3, $Rif^r$, $avrXv3^+$ | This study |
| 97-2 | Tomato race 3, $Rif^r$, $avrXv3^+$ | This study |
| M24 | Tomato race 3, $Rif^r$ $Tet^r$ $Km^r$, $avrXv3^-$, Marker-exchange mutant | G. Minsavage[1] |
| *Saccharomyces cerevisiae* | | |
| EGY48 | MATα, *trp1, his3, ura3, 6ops-LEU2* | R. Brent[2] |
| *Agrobacterium tumefaciens* | | |
| C58C1 | carrying pGV2260, $Rif^r$ | B. Staskawicz[3] |
| Plasmids | | |
| pLAFR3 | $Tc^r$ r/x $^+$RK2 replicon | B. Staskawicz |
| pLAFR6 | $Tc^r$ r/x $^+$RK2 replicon | B. Staskawicz |
| pLAFR3T3p | $Tc^r$ r/x $^+$RK2 replicon, carrying *avrXv3* promoter region | This study |
| pBluescript II KS +/- | Phagemid sequencing vector, $Ap^r$ | Stratagene |
| pET15-b | His tag expression vector, $Ap^r$ | Novagene |
| pET22-b | His tag expression vector, $Ap^r$ | Novagene |

Table A-1-- *Continued*

| | | |
|---|---|---|
| pHoKmGus | $Km^r$, $Ap^r$, *tnpA*$^-$, Tn3-*gusA* fusion | B. Staskawicz |
| pSShe | $Cm^r$ *tnpA* | Stachel et al., 1985 |
| pMD-1 | Binary vector, 35S, $Km^r$ | B. Staskawicz |
| pEG202 | LexA-fusion, *HIS3*, 2μ, $Ap^r$ | R. Brent |
| pMW106 | *URA3*, 2μ, 8ops-*lacZ*, $Km^r$ | R. Brent |
| pRFHM1 | *HIS3*, Homeodomain of Bicoid, 2μ, $Ap^r$ | R. Brent |
| pSH17-4 | *HIS3*, LexA-Gal4p, 2μ, $Ap^r$ | R. Brent |
| pXcvT3-60 | $Tc^r$ cosmid clone, *avrXv4*$^+$ | This study |
| pXcvT3-60::33 | *avrXv4* Tn3-*gusA* derivative | This study |

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[3] Department of Plant Biology, University of California, Berkeley, CA

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## BIOGRAPHICAL SKETCH

Gustavo Astúa-Monge was born on July 8, 1967, to Miguel Astúa and María de los Angeles Monge in San José, Costa Rica. He received a degree of Bachiller en Ingeniería Agronómica from the Universidad de Costa Rica in 1990 and graduated as Licenciado en Ingeniería Agronómica in 1991 at the same institution. From 1991 to 1993, he worked as a teaching assistant and junior research scientist at the Plant Pathology Laboratory of the University of Costa Rica. In 1993, he obtained a fellowship from LASPAU/FULBRIGHT and came to the United States to pursue a Master of Science degree in plant pathology at the University of Florida, which was completed in 1995. Gustavo was granted an assistantship to continue his graduate studies towards a Doctor of Philosophy degree in plant pathology at the University of Florida. Upon completion of his Ph.D. degree, Gustavo will be joining Dr. Eduardo Vallejos' program as a postdoctoral fellow.