Characterization of resistance genes to *Cladosporium fulvum* on the short arm of chromosome 1 of tomato

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Stellingen

1. Het onderscheiden van vier tot acht verschillende *Cf* genen in nakomelingen van één ouder op basis van minimale verschillen in mate van resistentie getuigt van een onderschatting van de invloed van de genetische achtergrond.
   Kanwar et al (1980) Rep. Tomato Gen. Coop. 30:20-23
   dit proefschrift

2. Het gebruik van het begrip virulentiefactor voor de avirulentiefactor ECP2 leidt tot verwarring en derhalve dient in dit geval aggressiviteitsfactor of pathogeniciteitsfactor te worden gebruikt.
   Laugé et al. (1998) Proc. Natl. Acad. Sci. USA 95:9014-9018
   dit proefschrift

3. *Eco*RI/*Mse*I AFLP merkers vertonen een sterke clustering in gebieden van het tomatengenoom die overeenkomen met de posities van het heterochromatine.
   dit proefschrift

4. Het *Hcr9* cluster Milky Way is niet het enige *Hcr9* cluster op chromosoom 1 dat functionele *Cf* genen bevat.
   dit proefschrift

5. De nomenclatuur voor *Cf* genen moet grondig worden herzien.
   dit proefschrift

6. Er zijn minimaal 85 verschillende *Hcr9* sequenties in het genus *Lycopersicon*.
   dit proefschrift

7. Het gebruik van het woord fenotype daar waar genotype wordt bedoeld geeft aan dat genetici een nuttige bijdrage kunnen leveren aan fytopathologisch onderzoek.
   Krahl and Randle (1999) HortSci 34:690-692

8. Het afdrukken van twee identieke blots pleit voor het inschakelen van statistici om te bepalen welk gedeelte van een experiment herhalingen behoeft.
   Vallélial-Bindschedler et al. (1998) Mol Plant-Microbe Interact. 11:702-705

9. Rekeningrijden zal het aantal files evenveel terugdringen als eertijds het kwartje van Kok heeft gedaan.

10. Het zogenaamde jobhoppen creëert extra banen.

11. Als op de verpakking van voedsel vermeld moet worden dat er gewerkt is met ingrediënten op basis van genetisch gemodificeerde organismen, zou ook vermeld moeten worden welke gewasbeschermingsmiddelen in de teelt van het betreffende gewas zijn gebruikt.

12. De angst voor het milleniumprobleem is de angst voor ons eigen kunnen.

Stellingen behorende bij het proefschrift: Characterization of resistance genes to *Cladosporium fulvum* on the short arm of chromosome 1 of tomato, door Jair Haanstra, in het openbaar te verdedigen op dinsdag 4 januari 2000, te Wageningen.

Jair Haanstra

Characterization of resistance genes to *Cladosporium fulvum* on the short arm of chromosome 1 of tomato

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 4 januari 2000
des namiddags te vier uur in de Aula

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Aan mijn ouders
Voor Jantine

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Chapter 1

General Introduction

Supplying the ever-growing world population with enough food is and will be one of the biggest challenges facing mankind. Moreover, the increase of food production should go together with sustainable production methods. At present, approximately 25% of all production is lost due to pests and pathogens (Agrios, 1997). The protection of cultivated crops against pests and pathogens still relies greatly on the availability of chemicals, which are considered to be of serious threat to natural ecosystems. Exploitation of defense mechanisms of plants offers a great opportunity in reaching for a high as well as environmentally safe production of food. There are several natural mechanisms which plants use to protect themselves against pests and pathogens. Mostly these mechanisms are divided into two categories, i.e. quantitative and qualitative resistance, which can occur simultaneously in plant-pathogen systems.

1 Resistance mechanisms

1.1 Qualitative disease resistance

Qualitative resistance is characterized by an absolute protection of the crop against pathogens or pests. This resistance is nearly always associated with a hypersensitive reaction (HR), resulting in local cell death at the site of attempted infection. This type of resistance is generally monogenic and race specific. It is explained by a gene-for-gene relationship: each plant resistance gene matches a specific avirulence gene of the pathogen (Flor, 1942). This model can also explain the often-observed non-durability, since a single mutation in an avirulence gene can result in virulence on a host carrying the corresponding resistance gene. Over the last years, a great number of plant resistance genes (Table 1.1; Hammond-Kosack and Jones, 1997) as well as several avirulence genes (Laugé and De Wit, 1998) involved in the hypersensitive reaction of several plant-pathogen interactions have been isolated.

Several mechanisms other than the hypersensitive reaction are known, by which the product of a single gene is sufficient against pathogen attack. The first disease resistance gene ever cloned from plants was *Hm1*, a gene from maize conferring resistance to the fungal pathogen

Cochliobolus carbonum. The fungus produces the HC toxin, which is required for pathogenicity and *Hm1* encodes the HC toxin reductase, which inactivates the HC toxin (Johal and Briggs, 1992). This system in which susceptibility to a disease is accompanied by sensitivity to toxins produced by the pathogen may occur more frequently, like in the relationship between *Alternaria alternata* f.sp. *lycopersici* and its host, tomato (Van der Biezen et al., 1996).

Table 1.1 Resistance genes cloned, the plant species from which they were isolated, features, pathogen to which the gene confers resistance and references.

Resistance gene cloned	Plant species isolated from	Features[1]	Pathogen to which resistance is conferred	Reference(s)
Pto	Tomato	PK	*Pseudomonas syringae* pv *tomato*	Martin et al., 1993
Prf	Tomato	LRR,NBS,LZ	*Pseudomonas syringae* pv *tomato*	Salmeron et al., 1996
RPS2	*Arabidopsis*	LRR,NBS,LZ	*Pseudomonas syringae* pv *tomato*	Bent et al., 1994
RPS5	*Arabidopsis*	LRR,NBS,LZ	*Pseudomonas syringae* pv *phaseolicola*	Warren et al., 1998
RPM1	*Arabidopsis*	LRR,NBS,LZ	*Pseudomonas syringae* pv *glycinea*	Grant et al., 1995
RPP8	*Arabidopsis*	LRR,NBS,LZ	*Peronospora parasitica*	McDowell et al., 1998
I2	Tomato	LRR,NBS,LZ	*Fusarium oxysporum* f.sp. *lycopersici*	Simons et al., 1998
Mi-1	Tomato	LRR,NBS,LZ	*Meloidogyne incognita, Macrosiphum euphorbiae*	Milligan et al., 1998 Vos et al., 1998
L6	Flax	LRR,NBS,TIR	*Melampsora lini*	Lawrence et al., 1995
M	Flax	LRR,NBS,TIR	*Melampsora lini*	Anderson et al., 1997
N	Tobacco	LRR,NBS,TIR	Tobacco Mosaic Virus	Whitham et al., 1994
RPS4	*Arabidopsis*	LRR,NBS,TIR	*Pseudomonas syringae* pv *pisi*	in: Aarts et al., 1998b
RPP1/10/14	*Arabidopsis*	LRR,NBS,TIR	*Peronospora parasitica*	Botella et al., 1998
RPP5	*Arabidopsis*	LRR,NBS,TIR	*Peronospora parasitica*	Parker et al., 1997
Dm3	Lettuce	LRR,NBS	*Bremia lactucae*	Meyers et al., 1998
Xa1	Rice	LRR,NBS	*Xanthomonas oryzae* pv *oryzae*	Yoshimura et al., 1998
Xa21	Rice	LRR,TM,PK	*Xanthomonas oryzae* pv *oryzae*	Song et al., 1995
Cf-2	Tomato	LRR,TM	*Cladosporium fulvum*	Dixon et al.,1996
Cf-4	Tomato	LRR,TM	*Cladosporium fulvum*	Thomas et al., 1997
Cf-4A	Tomato	LRR,TM	*Cladosporium fulvum*	Takken et al., 1998
Cf-5	Tomato	LRR,TM	*Cladosporium fulvum*	Dixon et al., 1998
Cf-9	Tomato	LRR,TM	*Cladosporium fulvum*	Jones et al., 1994
Hs1$^{pro\text{-}1}$	Sugar beet	LRR,TM	*Heterodera schachtii*	Cai et al., 1997
Hm1	Maize	O: toxin reductase	*Cochliobolus carbonum*	Johall and Briggs, 1992
Mlo	Barley	O: ???	*Erysiphe graminis* f.sp. *hordei*	Büschges et al., 1997

[1] PK = Protein Kinase, LRR = Leucine Rich Repeat, NBS = Nucleotide Binding Site, LZ = Leucine Zipper, TIR = Toll/Interleukin Receptor, TM = TransMembrane, O = Other

In several plants species, mutants have been identified that show resistance as well as spontaneous formation of lesions (Büschges et al., 1997 and references therein). So far, the *Mlo* gene from barley is the only gene that has been isolated from this class. Mutation-induced recessive alleles of this gene confer a broad spectrum resistance to *Erysiphe graminis* f.sp. *hordei*, the causal agent of powdery mildew. The wild-type *Mlo* gene encodes for a predicted protein of 60 kDa with six putative membrane-spanning helices and a putative nuclear

localization motif (Büschges et al., 1997). Recessive alleles of *Mlo* show dead cell leaf lesions even in axenically grown seedlings (Wolter et al., 1993) and therefore it is hypothesized that Mlo is a negative regulator of cell death (Büschges et al., 1997). Furthermore, Büschges et al. (1997) conclude that (partial) inactivation of the Mlo protein primes the responsiveness for the onset of several defense functions.

1.2 Quantitative disease resistance

Quantitative resistance is characterized by a continuous variation in the level of resistance, which is thought to be conferred by many genes. The mechanism of resistance is different from qualitative resistance and avirulence genes are unknown. Generally, it is assumed that this quantitative resistance is durable, for many genes in both the plant and pathogen play a role in this interaction and a change from avirulence to virulence in the pathogen may not easily occur. However, the fact that many genes control this resistance and the quantitative character of resistance hampers the breeding for this type of resistance greatly. With the advent of molecular markers, many quantitative trait loci (QTLs) governing different traits have been mapped (Young, 1996). For example, using AFLP markers on several barley RIL populations, Qi et al. (1998b) mapped several QTLs involved in partial resistance against barley leaf rust (*Rphq*s). The map positions of these QTLs do not coincide with those of the race-specific resistance genes against barley leaf rust (*Rph* genes). Therefore, the advent of marker assisted selection, will be of great benefit to breeding programs that focus on quantitative resistance.

2 Structure and function of plant HR genes

2.1 Resistance genes leading to HR

As this thesis is focussed on the gene-for-gene interaction between tomato and *Cladosporium fulvum*, most attention is paid here to resistance genes involved in the hypersensitive reaction (HR). Based upon the predicted amino acid sequences, plant resistance genes share several features and can be classified into a limited number of categories (Table 1.1; Figure 1.1). One structural motif shared by almost all resistance genes in this class is the leucine-rich repeat (LRR), which is found to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994; Leckie et al., 1999).

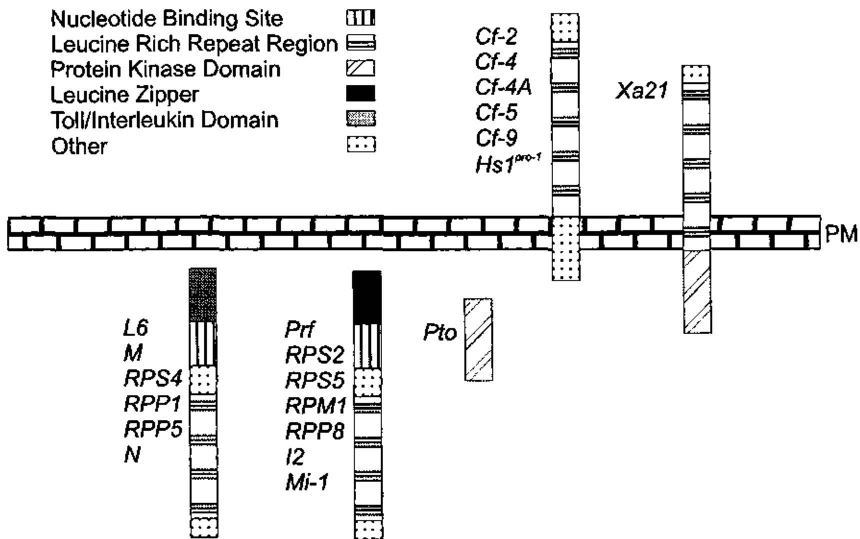

Figure 1.1 Major protein motifs shared between the predicted proteins of hitherto cloned HR genes. The plasma membrane is indicated with PM.

The second plant resistance gene cloned was *Pto*, which confers resistance to *Pseudomonas syringae* pv *tomato* strains harboring the *AvrPto* gene. *Pto* encodes a protein kinase and is (thought to be) cytoplasmic. Kinases are active in signal transduction pathways and therefore it is hypothesized that the *Pto* gene product is active in a signal transduction cascade, leading to HR. Another gene involved in this plant-pathogen interaction is *Prf*. *Prf* is required for *Pto* activity and encodes a protein, which is member of a class that contains leucine-rich repeat (LRR) and nucleotide binding site (NBS) domains and a leucine zipper (LZ) region. Resistance genes that belong to the same class as *Prf* are the *Arabidopsis RPS2*, *RPS5*, *RPM1* and *RPP8* genes and the tomato resistance genes *I2* and *Mi-1*. Interestingly, *Mi-1* confers resistance not only to the root-knot nematode *Meloidogyne incognita*, but also to the potato aphid *Macrosiphum euphorbiae* (Vos et al., 1998). Also *RPM1* determines dual specificity, by conferring resistance to the bacterial pathogen *Pseudomonas syringae* expressing either *avrRpm1* or *avrB* (Grant et al., 1995). A very similar class is formed by the resistance genes that encode proteins also with LRR and NBS domains, but with an N-terminal region showing homology to cytoplasmic domains of the *Drosophila* Toll protein and the mammalian interleukin-1 receptor (TIR) instead of a LZ region. Members of this class are the flax *L6* and *M* genes, the *RPS4*, *RPP1* and *RPP5* genes from *Arabidopsis* and the *N* gene from tobacco. A

resistance gene from lettuce, *Dm3*, has been isolated, which contains LRR and NBS domains, but no TIR or LZ regions and therefore forms a separate class, together with the *Xa1* gene of rice. Although all classes containing a NBS domain are thought to be cytoplasmic, by epitope tagging Boyes et al. (1998) found that RPM1 is likely to be a peripheral membrane protein, residing at the cytoplasmic face of the plasma membrane. A class that so far consists of a single gene, is formed by *Xa21* from rice, which encodes LRR and kinase domains, separated by a transmembrane (TM) domain. The last class is formed by the tomato genes *Cf-2*, *Cf-4*, *Cf-4A*, *Cf-5* and *Cf-9*, conferring resistance to *C. fulvum* and $Hs1^{pro\text{-}1}$ from sugar beet conferring resistance to the beet cyst nematode *Heterodera schachtii* Schmidt. The common features these genes encode are LRR and TM domains, without an obvious cytoplasmic domain.

2.2 Other genes required for HR

By screening for loss of pathogen resistance, several mutagenized plants have been identified that have mutations in putative signal transduction genes. Mostly, these mutations only partially reduce the resistance. In tomato, mutations in the *Prf* gene abolish the *Pto*/*AvrPto* mediated resistance response and *rcr-1* and *rcr-2* are weak suppressors of the *Cf-9* mediated resistance (Hammond-Kosack et al., 1994). Another mutation, *rcr-3* affects *Cf-2* mediated resistance, but not the resistance conferred by *Cf-5* nor *Cf-9* (Jones et al., 1999). In barley, two mutants, *rar-1* and *rar-2* have been identified that disrupt the resistance to powdery mildew mediated by the *Mla-12* gene (Jørgensen 1996). So far, the mutants in barley and tomato have not been analyzed for their effect on resistance conferred by *R* genes involved in other plant-pathogen interactions.

In *Arabidopsis*, several mutants have been found that affect the resistance conferred by more than one *R* gene. Recently, Warren et al. (1999) identified five mutations in three genes of *Arabidopsis* with different effects on *R* gene mediated resistance. The mutations in *PBS1* seemed only to affect resistance mediated by *RPS5*, whereas the mutation in *PBS2* affected the resistance conferred by *RPS5* and *RPM1*, as well as some *RPP* genes. The mutation in *PBS3* severely affected resistance to avirulent as well as virulent *P. syringae* strains and affected *RPP* gene mediated resistance to a lesser extent. This suggests that these mutations are involved in different steps of the signal transduction pathway that are shared by specific plant-pathogen interactions. Furthermore, Warren et al. (1998) studied *rps5-1*, which has a mutation

in the LRR region of RPS5 that not only affects the *RPS5* function, but also (partially) suppresses the resistance genes *RPS2*, *RPP4* and *RPP9* and possibly *RPM1*. The function of *RPP4* is only partially restored upon transformation of this *RPS5* mutant with a functional *RPS5* gene. Warren et al. (1998) hypothesized that the *rps5-1* encoded protein might specifically bind to a protein, which is common to several resistance gene pathways, and in that way prevents the function of these resistance genes.

The recently cloned gene *EDS1* has proven to be required for the resistance mediated by the TIR-NBS-LRR proteins RPS4, RPP1 and RPP5, whereas the resistance conferred by the LZ-NBS-LRR proteins RPS2, RPS5 and RPM1 is dependent on a functional *NDR1* gene (Aarts et al., 1998b). The RPP8 (a LZ-NBS-LRR protein) mediated resistance is weakly dependent on EDS1, and some cross-utilization seems to be possible (Aarts et al., 1998b). *NDR1* encodes a novel protein with two potential transmembrane domains (Century et al., 1997). EDS1 encodes a protein that possesses three appropriately spaced sequences that show significant similarity to the consensus motifs comprising a lipase catalytic site (Falk et al., 1999).
In summary, a complex of proteins may be responsible for binding the avirulence gene product and onset of the signal cascade leading to HR. Some of these proteins, like EDS1 and NDR1 are employed in several plant-pathogen interactions, while the *R* genes are *Avr* gene(s) specific. The *R* genes share a lot of homologies, while too few genes that are required for resistance have been isolated, to be able to generalize about their structural features.

2.3 Interaction with avirulence genes

The interaction between resistance and avirulence gene products is still unknown. It has been hypothesized that the product of an avirulence gene directly interacts with the LRR region in the protein encoded by the corresponding resistance gene, which triggers a pathway, that eventually leads to a localized cell death. Indeed, using the yeast two-hybrid system, avrPto showed a direct interaction with Pto from tomato, although this does not imply that this interaction also occurs *in vivo* (Tang et al., 1996). At the other hand, Kooman-Gersmann et al. (1996) have shown that plasma membranes of tomato genotypes with and without the resistance gene *Cf-9* have a high-affinity binding site for the fungal elicitor AVR9 of *Cladosporium fulvum*. A possibility might be that the AVR9 protein binds to a complex of proteins, and that only upon binding in the presence of the *Cf-9* gene product the signal transduction cascade is triggered. Indeed, there is accumulating evidence that complexes of

proteins are involved in binding of the avirulence determinants. In *Arabidopsis*, attempts to show a direct interaction between the avirulence gene products of *avrB* or *avrRpm1* and the product of the corresponding resistance gene *RPM1*, using the yeast two-hybrid system were not successful. However, the N-terminus of RPM1 was found to bind to the carboxyterminal cytoplasmic domain of a novel protein (Boyes et al., 1998). While this C-terminus showed no homology to other proteins, it contained six to seven putative transmembrane segments at the N-terminus.

3 Genetic and molecular organization of plant HR genes

Studies using classical genetics have shown that resistance genes often reside at complex loci. By classical genetics, 13 alleles of the *L* locus have been identified in flax, which confer resistance to the flax rust pathogen *Melampsora lini* (Islam and Shepherd, 1991). These data have more recently been confirmed by Southern hybridization experiments, where only one hybridizing band was detected (Ellis et al., 1995). In contrast to the *L* locus, the complex *M* locus of flax, conferring also resistance to *M. lini*, consists of non-allelic resistance genes, of which seven specificities have been determined (Ellis et al., 1995). Southern hybridization experiments of the *M* locus detected about 15 homologs. The homologs at this locus resemble those at the *L* locus (Anderson et al., 1997).

In addition, loci that contain genes conferring resistance to different pathogens are very common. In *Arabidopsis*, complex loci have been identified that contain functional resistance genes against different pathogens. PCR and hybridization experiments showed that several resistance gene homologs reside at these loci (Aarts et al., 1998a; Speulman et al., 1998). Also in tomato several complex loci have been identified by classical genetics. A locus on the short arm of chromosome 9 contains two genes conferring resistance against tobacco mosaic virus (TMV), which have been designated *Tm-2* and $Tm\text{-}2^2$ (Young and Tanksley, 1989). Another complex locus is located on chromosome 6 and harbors two genes conferring resistance against *Cladosporium fulvum* (*Cf-2* and *Cf-5*), one against *Meloidogyne incognita* (*Mi-1*) that also confers resistance to aphids and one against tomato yellow leaf curl virus (*TY-1*; Dickinson et al., 1993; Zamir et al., 1994). The complex locus on the short arm of chromosome 1 will be discussed in § 5.

4 Mechanisms responsible for polymorphism at HR loci

A prerequisite for plants to compete in the gene-for-gene arms race is that novel resistance specificities are developed in time. By now, it is evident that *R* loci are highly polymorphic (Parniske et al., 1997; McDowell et al., 1998; Meyers et al., 1998). Several mechanisms have been found to be involved in generating variation at *R* loci:

Gene conversion is the mutation from one allele to another and is caused by correction of mispaired nucleotides in heteroduplex DNA to either wild-type or mutant pairs. This mechanism is thought to be important in gaining polymorphism under conditions of weak selection and the rate is faster than point mutation rates (Parham and Ohta, 1996).

Unequal crossing-over caused by asymmetric pairing and recombination is a mechanism that has been frequently found in plant resistance gene families (Sudupak et al., 1993, Anderson et al., 1997, Parniske et al., 1997, McDowell et al., 1998). For *RPP8*, an allele was found that arose from an unequal crossing-over between linked, non-allelic genes (McDowell et al., 1998). If this recombination occurs between the same genes or alleles, this mechanism is often referred to as **intragenic** or **intra-allelic recombination**

Intragenic recombination between gene family members has been observed for the *Xa21* gene family in rice (Song et al., 1997) and the *Cf-4*/*Cf-9* gene family in tomato (Parniske et al., 1997; Thomas et al., 1997). Intra-allelic recombination has been observed at the *M* and *RPP5* loci of flax and *Arabidopsis*, respectively, where recombinant alleles were identified that arose from an ectopic recombination between LRRs resulting in the expansion or contraction of the number of LRRs (Anderson et al., 1997; Parker et al., 1997). Finally, **interlocus recombination** has been observed on the short arm of chromosome 1 of tomato. Three clusters of *Hcr9*s (homolog of *C. fulvum* resistance gene *Cf-9*) have been observed on the short arm of chromosome 1, which can be divided into two subclasses. One cluster harbors a single *Hcr9* that has the features of the other subclass of *Hcr9* loci (Parniske and Jones, 1999).

Gene duplication is necessary to obtain closely related sequences promoting the asymmetric pairing and unequal crossing-over (Sudupak et al., 1993).

Nonhomologous DNA end joining is a mechanism which is predominantly employed by plants to repair double-strand breaks (Gorbunova and Levy, 1997). Nonhomologous end joining often occurs at short repeats and results in the insertion of filler DNA, which are short patches of DNA from adjacent regions (Gorbunova and Levy, 1997). This mechanism has been proposed to be involved in repair after a deletion of RPS5 (Henk et al., 1999).

Purifying and **diversifying selection**, which is selection against and in favor of, respectively, certain amino acid substitutions, is expressed as the ratio between K_a and K_s, K_a being the number of non-synonymous nucleotide substitutions and K_s the number of synonymous substitutions. If K_a:K_s <1, sequences are under purifying selection. If K_a:K_s >1, sequences are under diversifying selection. Mostly, amino acid substitutions are deleterious to structure and function of proteins and purifying selection is necessary. However, sometimes there is a selective advantage for amino acid diversity and regions are under diversifying selection (Michelmore and Meyers, 1998). Diversifying selection has been found for the LRR subdomain that is predicted to form a ß strand/ß turn structure in the *Cf-4/Cf-9*, *Xa21* and *RPP8* family members (Parniske et al., 1997;Song et al., 1997; McDowell, 1998).

The phenomena described above all have been found to be involved in generating sequence diversity of *R* loci. The birth-and-death process has been proposed to explain the evolution of the vertebrate major histocompatibility complex (MHC) and immunoglobulin clusters and involves the expansion and contraction of clusters by unequal crossing-over and evolution of individual genes by diversifying selection (Nei et al., 1997). Since molecular evolution of resistance gene clusters appears to be driven by the same mechanisms that generate diversity in the MHC and immunoglobulin clusters, the birth-and-death model has also been proposed for *R* loci (Michelmore and Meyers, 1998). An important feature of this model is that although unequal crossing-over occurs, the rate of unequal crossing-over is too low to homogenize sequences. Variation of individual genes is predominantly generated by interallelic recombination and gene conversion. Recombination between paralogs is rare. This is consistent with the observations that orthologs show more sequence similarity than paralogs (Parniske et al., 1997). There will be selection in favor of genes with increased efficiency and for multiple specificities. Rare unequal crossing-over events cause duplication and deletion of (blocks of) genes. Recent duplications are subject to further duplication and deletion, because of relatively frequent unequal crossing-overs. Rapid divergence of intergenic regions reduces this frequency and homologs become fixed in the haplotype. Finally, duplicated genes tend to diverge again. Two major questions remain to be unanswered.
Firstly, why do *R* gene homologs without any known function still have an intact open reading frame? Is there some kind of molecular mechanism to maintain a complete open reading frame or is this simply maintained by positive selection? Secondly, why do *R* genes of the same cluster mediate resistance to the same pathogen, while this does not depend on the

structure of the R gene (McDowell et al., 1998)? The only exception detected so far is the *Mi* gene, which mediates resistance to two distinct pathogens, the root-knot nematode *Meloidogyne incognita* and the potato aphid *Macrosiphum euphorbiae*. The answers to these questions will provide us with essential information on how plants keep up in the gene-for-gene arms race with pathogens.

5 The *L. esculentum-Cladosporium fulvum* interaction: a gene-for-gene model system

The subject of this thesis is the interaction between tomato and the fungal pathogen *Cladosporium fulvum*. Like most crops which are grown as monocultures, tomato suffers from many pests and diseases, one of them, which has caused many problems on greenhouse tomatoes since the 1920s, is *C. fulvum*, the causal agent of leaf mold. *C. fulvum* is specialized on tomato and enters the plant through open stomata. Subsequently, the fungus grows intercellularly and eventually sporulates, which can be observed predominantly at the lower side of the leaf. The interaction between tomato and *C. fulvum* complies with the gene-for-gene relationship (Van den Ackerveken and De Wit, 1994). If a plant resistance gene matches a fungal avirulence gene, the plant is resistant and fungal growth is arrested, which is characterized by a hypersensitive response (HR). If either the plant *R* gene or the fungal *Avr* genes are absent, the plant is susceptible. Over the years, several resistance genes, conferring resistance to *C. fulvum* have been identified in wild *Lycopersicon* species and introgressed into cultivated tomato.

The availability of both near-isogenic lines of tomato carrying different resistance genes, as well as different races of *C. fulvum* that overcome resistance genes have facilitated extensive research on the gene-for-gene relationship of this system. The race-specific elicitors AVR4 and AVR9 have been isolated from intercellular washing fluids of compatible interactions and the corresponding fungal avirulence genes *Avr4* and *Avr9* have been cloned (Joosten et al., 1994; Van Kan et al., 1991). In a similar way, the extracellular proteins ECP1 and ECP2 have been purified and their corresponding genes, *Ecp1* and *Ecp2*, have been isolated (Van den Ackerveken et al., 1993). These fungal proteins have been shown to be factors involved in pathogenicity (Laugé et al., 1997), as well as elicitors (Laugé et al., 1998a; Laugé et al., 1999). The avirulence genes that have been isolated are small and contain many cysteines. Fungal races that are able to circumvent *Cf-4* mediated resistance, have point mutations in the *Avr4* gene, resulting in unstable gene products (Joosten et al., 1994; Joosten et al., 1997). At the

other hand, races that overcome *Cf-9* mediated resistance completely lack the *Avr9* gene (Honée et al., 1994). Since the *Ecp* genes encode pathogenicity factors that can be recognized by specific *Cf* genes, it has been speculated that these *Cf* genes might be more durable (Laugé et al., 1998a). Over the last few years, the plant resistance genes *Cf-2*, *Cf-4*, *Cf-4A*, *Cf-5* and *Cf-9*, conferring resistance to AVR2, AVR4, AVR4A, AVR5 and AVR9 respectively, have been isolated (see §2.1; Table 1.1). *Cf-2* and *Cf-5* map to a complex locus on tomato chromosome 6 (Dickinson et al., 1993), whereas *Cf-4*, *Cf-4A* and *Cf-9* map to a complex locus on chromosome 1 (Balint-Kurti et al., 1994). Molecular data revealed a cluster of five homologs at both the *Cf-4* and the *Cf-9* loci (Parniske et al., 1997). Recombinations between these clusters may occur at very low frequencies (Thomas et al., 1997). At the *Cf-4* locus, two homologs, *Cf-4 (=Hcr9-4D)* and *Cf-4A (=Hcr9-4E)*, encode a functional resistance gene (Takken et al., 1998). Within short genetic distance of this so-called 'Milky Way' cluster, other clusters of *Cf* homologs, designated 'Northern Lights and 'Southern Cross', have been identified with no known function (Parniske et al., 1999; Parniske and Jones, 1999). The mechanisms that are involved in the development of these clusters have been discussed in §4.

Outline of this thesis

At the start of the research described in this thesis, it was known that several clusters of *Cf* homologs exist on the short arm of tomato chromosome 1. Also, literature mentioned the possibility of other functional resistance genes mapping to this region (e.g. Kanwar et al., 1980a,b). A strategy was chosen to identify new *Cf* genes on the short arm of chromosome 1 to increase our knowledge about the genetic and molecular organization of *Cf* homologs on the short arm of chromosome 1, as well as to study structural differences between functional and non-functional *Cf* homologs. Although many molecular markers for this region on chromosome 1 were already available, more molecular markers were expected to be convenient. The AFLP technique, which was just developed at that time, was used to generate a high-density molecular marker map of tomato, which could also be exploited in other tomato research at our laboratory. In Chapter 2 the resulting map is presented, consisting of 1175 AFLP markers and covering a genetic distance of 1482 cM. The strong clustering of *Eco*RI/*Mse*I AFLP markers observed around the centromeric regions is discussed.

To find previously uncharacterized *Cf* genes on the short arm of chromosome 1, we have tested 66 *Lycopersicon* accessions, which were reported to confer resistance to *C. fulvum*, for

the presence of a resistance gene closely linked or allelic to *Cf-4*. To be certain that only resistance genes with uncharacterized specificities would be identified, the accessions were prescreened for the presence of *Cf-4* and *Cf-9*. A relatively large proportion of the lines specifically recognized AVR4 and appeared to harbor a *Cf-4* gene, which is described in Chapter 4. In Chapter 3, the identification of several additional *Cf* genes on the short arm of chromosome 1 is described. This eventually resulted in the mapping of *Cf-ECP5* and *Cf-ECP2*. *Cf-ECP5* confers resistance to *C. fulvum* through recognition of the fungal protein ECP5 from *C. fulvum* (Chapter 3). *Cf-ECP2* confers resistance to *C. fulvum* through recognition of the fungal protein ECP2, encoded by the *Ecp2* gene, which is necessary for full pathogenicity of *C. fulvum* on tomato (Chapter 5). At the *Cf-ECP5* locus, several *Hcr9*s were identified that are candidates for this resistance gene (Chapter 6). Finally, the resulting current knowledge on the organization of *Cf* genes on the short arm of chromosome 1 is discussed in Chapter 7.

Chapter 2

An integrated high density RFLP-AFLP map based on two *Lycopersicon esculentum* x *L. pennellii* F_2 populations

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Abstract

Two independent F_2 populations of *Lycopersicon esculentum* x *L. pennellii* which have previously been investigated in RFLP mapping studies were used for construction of a highly saturated integrated AFLP map. This map spanned 1482 cM and contained 67 RFLP markers, 1078 AFLP markers obtained with 22 *Eco*RI+*Mse*I primer combinations and 97 AFLP markers obtained with five *Pst*I+*Mse*I primer combinations, 231 AFLP markers being common to both populations. The *Eco*RI+*Mse*I AFLP markers were not evenly distributed over the chromosomes. Around the centromeric region, 848 *Eco*RI+*Mse*I AFLP markers were clustered and covered a genetic distance of 199 cM, corresponding to one *Eco*RI+*Mse*I AFLP marker per 0.23 cM; on the distal parts 1283 cM were covered by 230 *Eco*RI+*Mse*I AFLP markers, corresponding to one marker per 5.6 cM. The *PstI*/*Mse*I AFLP markers showed a more even distribution with 16 *PstI*/*Mse*I AFLP markers covering a genetic distance of 199 cM around the centromeric regions and 81 *PstI*/*Mse*I AFLP markers covering a genetic distance of 1283 cM on the more distal parts, corresponding to one marker per 12 and 16 cM respectively. In both populations a large number of loci showed significant skewed segregation, but only chromosome 10 loci showed skewness that was similar for both populations. This ultra-dense molecular marker map provides good perspectives for genetic and breeding purposes and map-based cloning.

Introduction

The development of molecular linkage maps for most cultivated crop species has made possible the application of a variety of new techniques in plant breeding, for example QTL mapping (reviewed by Young 1996), marker-assisted breeding (Tanksley et al. 1996) and map-based cloning (reviewed by Young 1995). Most of these linkage maps were generated using Restriction Fragment Length Polymorphisms (RFLPs) (Tanksley et al. 1992; Kleinhofs et al. 1993; Hauge et al. 1993). RFLP markers are locus-specific and co-dominant and, therefore, very informative. However, generating RFLP data is time and labour-consuming and requires a relatively large amount of DNA. In addition, Random Amplified Polymorphic DNA (RAPD) markers have been used to construct genetic linkage maps (Giese et al. 1994; Uphoff and Wricke 1995). This PCR-based technique is fast, easy to perform and requires only small amounts of DNA. The disadvantages of RAPDs are poor reproducibility (Penner et al. 1993), dominant inheritance and population specificity. Most other marker techniques (microsatellite, CAPS) make use of PCR with specific primers that are designed from known sequences. The advantage is the ease of the technique, however, sequence information is required to design the specific primers which limits the usefulness of the application.

A relatively new technique, that does not require *a priori* sequence information, is the AFLP technique, which is very efficient and combines the advantages of PCR with high reproducibility and locus specificity among populations of RFLP markers (Zabeau and Vos, 1993; Vos et al. 1995; Qi et al. 1998a). AFLP maps have been constructed for potato (Van Eck et al. 1995), barley (Becker et al. 1995; Waugh et al. 1997; Qi et al. 1998a), sugar beet (Schondelmaier et al. 1996), soybean (Keim et al. 1997), petunia (Gerats et al. 1995) and rice (Maheswaran et al. 1997), enabling the mapping of QTLs in these crops. For tomato (*Lycopersicon esculentum*), several inter- and intraspecific maps have been generated using RFLP markers (Paterson et al. 1991; Van Ooijen et al. 1994; Lindhout et al. 1994; Goldman et al. 1995; Maliepaard et al. 1995), which were originally mapped on an F_2 population of a cross between *Lycopersicon esculentum* and *L. pennellii* (Tanksley et al. 1992). The aim of the present study is the integration of AFLP markers in the RFLP map of tomato. To this end two populations were used. One population consisted of 67 F_2 plants from the interspecific cross *L. esculentum* cv. VF36-*Tm2a* x *L. pennellii* LA716. This population has been used to construct a map containing 1030 RFLP markers (Tanksley et al. 1992). The resulting map has

been used for several mapping studies (Paterson et al. 1988; Mutschler et al. 1996) as well as for the construction of a backcross inbred line (BIL) population (Eshed and Zamir 1994; Paran et al. 1995). The second population was an F_2 of the same interspecific cross *L. esculentum* cv. Allround x *L. pennelli* LA716 (Odinot et al. 1992) that has also previously been used in several mapping studies (Van der Beek et al. 1994; Arens et al. 1995; Van Tuinen et al. 1997). This map consists of 65 RFLP markers, which were selected by the criterion of even distribution over the tomato genome at an average distance of 25 cM, based on the first map (Tanksley et al. 1992).

Here, we describe the generation of an integrated genetic map containing 1175 AFLP markers by using two independent F_2 populations of *L. esculentum* x *L. pennellii.*

Materials and Methods

Plant material and DNA isolation

One population of 67 F_2 plants (the 'Cornell population') was derived from a cross between *L. esculentum* cv. VF36-*Tm2a* and *L. pennellii* LA716 (Tanksley et al. 1992). The DNA isolation procedure has been described (Tanksley et al. 1992). For the AFLP analysis, which was carried out at Keygene, DNA was available for 42 plants. Another F_2 population of 84 plants (the 'CPRO population') was derived from a cross between *L. esculentum* cv. Allround and *L. pennellii* LA716 (Odinot et al. 1992). DNA was extracted from frozen leaves according to the method developed by the group of S.D. Tanksley with some minor modifications as described by Van der Beek et al. (1992). DNA of 80 plants was available for AFLP analysis, which was carried out at the Department of Plant Breeding of the Wageningen Agricultural University.

RFLP analysis and mapping

RFLP analysis and mapping on the Cornell population has been previously reported (Tanksley et al. 1992). RFLP analysis of the CPRO population was carried out as described by Van der Beek et al. (1992) with RFLP probes developed and mapped by Tanksley et al. (1992). Mapping of RFLP markers was performed using the computer program JoinMap (Stam, 1993) and the resulting map has been described (Arens et al. 1995). The present map contains 13 more RFLP markers.

Table 2.1 List of primers and adapters

Primers/adapters		Sequences*
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
M00 (universal primer)		GATGAGTCCTGAGTAA
*Mse*I+1-primer	M02	M00+C
*Mse*I+3-primers	M47	M00+CAA
	M48	M00+CAC
	M49	M00+CAG
	M50	M00+CAT
	M54	M00+CCT
	M58	M00+CGT
	M59	M00+CTA
	M60	M00+CTC
	M61	M00+CTG
	M62	M00+CTT
*Eco*RI adapter		5'- CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
E00 (universal primer)		GACTGCGTACCAATTC
*Eco*RI+1-primer	E01	E00+A
*Eco*RI+3-primers	E32	E00+AAC
	E33	E00+AAG
	E35	E00+ACA
	E38	E00+ACT
	E39	E00+AGA
*Pst*I adapter		5'-CTCGTAGACTGCGTACATGCA-3' 3'-CATCTGACGCATGT-5'
P00 (universal primer)		GACTGCGTACATGCAG
*Pst*I+2-primers	P11	P00+AA
	P14	P00+AT

*: DNA sequences are always from a 5' to 3' orientation unless otherwise indicated.

The AFLP protocol

The AFLP procedure as described by Vos et al. (1995) was applied to the Cornell population, except for the *Eco*RI adapter, which was used without the biotin label. Restriction enzymes, adapters and primers used are listed in Table 2.1. The following primer combinations were used: E32+M47, E32+M48, E32+M49, E32+M50, E32+M59, E32+M60, E32+M61, E32+M62, E33+M47, E33+M49, E35+M47, E35+M48, E35+M49, E35+M50, E35+M59, E35+M60, E35+M61, E35+M62, E38+M61, E38+M62 and E39+M50.

For the AFLP analysis of the CPRO population, the protocol described by Qi and Lindhout (1997) was used. The restriction enzymes, adapters and primers used are listed in Table 2.1. The following primer combinations were used: E32+M47, E32+M48, E32+M49, E32+M50,

E32+M61, E35+M47, E35+M48, E35+M50, E35+M58, E35+M59, E35+M62, E39+M50, P11+M50, P11+M54, P14+M49, P14+M50, and P14+M60. Primer combinations were selected as most informative from a previous study by Vos et al. (data not shown).

AFLP data analysis

Segregating AFLP markers in the mapping population were designated according to the primer combination used, the parent species from which they were derived and the estimated fragment size (see Fig. 1). Images of AFLP markers of the Cornell population were analysed and co-dominantly scored using the AFLP Image Analysis Software, which has been developed for internal use by Keygene N.V. For the CPRO population, markers were scored visually and co-dominantly. For both populations, bands occurring with an ambiguous intensity were scored as dominant. The frequency of dominant scored bands was higher in the CPRO population than in the Cornell population.

Map construction

For both the Cornell population and the CPRO population, the computer program JoinMap 2.0 (Stam, 1993; Stam and Van Ooijen 1996) was used to construct an RFLP-AFLP map. For the CPRO population, fixed order files consisting of RFLP markers ordered according to the map described by Tanksley et al. (1992) were used. Fixed files consisting of AFLP markers common to both populations and at distances of about 15 cM were used to construct the integrated map of both populations. For calculating map distances, Kosambi's mapping function was used (Kosambi, 1944) and a recombination threshold value of 0.49. The LOD threshold value for the CPRO map construction was 0.01 and for the Cornell map construction 0.1. For the integrated map the LOD threshold value for mapping was also set at 0.01.

Distorted segregation

The datasets of both populations were analysed for the occurrence of distorted segregation. Theoretically, in both F_2 populations the segregation of a marker into the three possible genotype classes *ee*, *ep* and *pp* (homozygous *L. esculentum*, heterozygous and homozygous *L. pennellii* respectively) should equal 1:2:1. The module JMSLA32 of JoinMap 2.0 (Stam, 1993; Stam and Van Ooijen 1996) was used to test this hypothesis. This module calculates the probability that the observed ratio differs from the expected 1:2:1 ratio. Since dominant

scoring results in 3:1 segregation, which is less informative, only markers which were predominantly scored as co-dominant were used for this analysis.

Results

AFLP markers in two *L. esculentum x L. pennellii* F_2 populations

In order to construct a reliable AFLP map, AFLP markers were evaluated in two independent *L. esculentum x L. pennellii* F_2 populations, which have previously been used in RFLP mapping studies. Tanksley et al. (1992) have mapped over 1000 RFLP markers in the Cornell population. By analysing 21 *Eco*RI+*Mse* I primer combinations in this population, 909 AFLP markers were scored, of which 433 were *L. esculentum* specific and 476 *L. pennellii* specific (Table 2.2). The average number of informative markers per primer combination was 43, ranging from 27 (E32+M61) to 61 (E32+M59).

As a skeleton map for the CPRO population, 65 RFLP markers were analysed at intervals of 25 cM, based on the map of Tanksley et al. (1992). By using 12 *Eco*RI+*Mse*I primer combinations and 5 *Pst*I+*Mse*I primer combinations, 642 AFLP markers were scored in the CPRO population, of which 303 were *L. esculentum*-specific and 339 *L. pennellii*-specific (Table 2.3). The actual number of polymorphic bands on a gel was higher, however close migration of bands of nearly identical size prevented a reliable scoring of the bands. The average number of informative markers per *Eco*RI+*Mse*I primer combination was 42, ranging from 25 (E32+M48) to 60 (E32+M50), compared to 27 markers identified per *Pst*I+*Mse*I primer combination that ranged from 23 (P11+M50) to 36 (P14+M50).

The average total number of bands per *Eco*RI+*Mse*I primer combination was 111, compared to 93 per *Pst*I+*Mse*I primer combination. The *Eco*RI+*Mse*I primer combinations had an average polymorphism rate of 77% compared to 63% of the *Pst*I+*Mse*I primer combinations, which is significantly lower ($P<0.01$).

Most AFLP markers were identical in both populations. However, because not all AFLP markers were evaluated in both populations, a total number of 228 AFLP markers were common to both populations.

Table 2.2 Number of amplification products and polymorphisms for 10 of the 21 primer combinations used for the Cornell population.

Primer Combin.	total # bands	# *L. esc.* specific	polym. rate (%)	#polym. scored
E32+M47	141	45	67	36
E32+M48	98	30	67	28
E32+M49	90	28	70	36
E32+M61	93	27	70	27
E35+M47	173	53	66	35
E35+M48	118	47	81	54
E35+M50	131	42	63	36
E35+M59	133	47	77	47
E35+M62	136	50	77	45
E39+M50	136	57	79	56
Average	125	43	71	40

Table 2.3 Number of amplification products and polymorphisms per primer combination used for the CPRO population.

Primer Combin.	total # bands	# *L.esc* specific	polym. rate (%)	#polym. Scored
E32+M47	130	42	68	49
E32+M48	103	34	77	25
E32+M49	83	27	76	33
E32+M50	136	37	70	60
E32+M61	78	31	77	44
E35+M47	155	52	77	39
E35+M48	100	36	82	40
E35+M50	132	51	75	34
E35+M58	86	33	79	33
E35+M59	119	41	78	56
E35+M62	106	42	84	39
E39+M50	110	45	82	53
Average	112	39	77	42
P11+M50	91	27	65	23
P11+M54	95	25	65	27
P14+M49	79	18	51	25
P14+M50	110	37	72	36
P14+M60	90	21	57	25
Average	93	26	63	27

<u>Map construction</u>

After scoring the AFLP markers, separate maps of both populations were constructed. To assign markers to chromosomes, RFLP markers were used at an average distance of 25 cM, based on the RFLP map constructed by Tanksley et al. (1992). Markers were placed in one linkage group if they were at least linked to another marker in this group with a LOD value of 2.5 to 3.5. For map construction of the CPRO population, AFLP markers were selected with

the following quality criterion: if a marker shows linkage to other markers with less than 5, 10 or 20% recombination the corresponding LOD values for linkage should be greater than 10, 5 and 1, respectively. If this criterion was not met for three or more times, such a marker was considered as not accurately scored and hence removed from the dataset. In addition, of marker groups that showed identical segregation, only one of these markers was taken as representative and the other markers were not used in further analysis. However, these markers were later positioned on the same locus as the representative marker.

The maps of both populations showed a high similarity (not shown). Map positions of markers were generally similar and only three AFLP markers mapped on different chromosomes (indicated in Figure 2.1). Consequently, an integrated map could be generated using a fixed order consisting of skeleton AFLP markers at strategic chromosome loci based on the most unambiguously mapped markers in both populations (Figure 2.1). The resulting total map length was 1482 cM, with no gaps bigger than 21 cM.

Clustering of *Eco*RI+*Mse*I AFLP markers

Preferably, to obtain a genetic map with the smallest possible intervals between markers, these markers should be evenly distributed over the genome. However, often the distribution is not random and markers are clustered in certain regions on the chromosome (Tanksley et al., 1992). A very clear clustering of *Eco*RI+*Mse*I AFLP markers was observed on all chromosomes (Figure 2.1). The vast majority of the *Eco*RI+*Mse*I markers (848) mapped in clusters (represented by closed bars on the chromosomes in Figure 2.1) which together cover 199 cM, while 230 *Eco*RI+*Mse*I markers were distributed over the remaining 1283 cM. The positions of these clusters, as determined by using RFLP markers with known map position as landmarks, were in the centromere regions (Tanksley et al., 1992). A similar clustering was not observed for *Pst*I+*Mse*I AFLP markers.

Distorted segregation

In F_2 populations, segregation of a single co-dominant locus should result in a 1:2:1 ratio of the three possible genotypes *ee*:*ep*:*pp* (homozygous *L. esculentum*, heterozygous and homozygous *L. pennelli* respectively). However, in both populations several chromosome regions showed distorted segregation, although the location of distorted segregation could differ (Figure 2.2 A,B). For the Cornell population skewness was observed in regions of chromosomes 2, 4, 7, 8,

10, 11 and 12, while the CPRO population showed skewness for regions of chromosomes 9, 10 and 12. This distorted segregation was observed for both RFLP and AFLP markers. For most of the markers that showed a skewed distribution, the *L. pennellii* allele was overrepresented. The most skewed marker of the CPRO population was TG230 on chromosome 10. None of the plants was *ee* for this marker locus and the allele frequencies of *e* and *p* were 28% and 72%, respectively. This region on chromosome 10 was also the most distorted in the Cornell population with allele frequencies of 23% (*e*) and 77% (*p*) for TG230 (Figure 2.2B).

Discussion

Map construction

Using a limited number of only 27 primer combinations, we were able to construct highly saturated AFLP maps of two interspecific populations. Both individual maps were very similar in marker order as well as in map distances, facilitating the integration of both maps. Clearly, this indicates that AFLP markers are reliable, reproducible and locus specific. Waugh et al. (1997) showed that 78 out of 81 co-migrating AFLP markers segregating in more than one population, mapped to similar loci in three different barley populations, while Qi et al. (1998a) found that all 38 co-migrating AFLP markers, mapped to the same position on two barley maps.

The integrated map presented in this paper was 1482 cM in length, which is considerably longer than the map of 1276 cM, presented by Tanksley et al. (1992). A major reason for this increase is due to more distal markers, adding approximately 110 cM to the total map length.

Chromosome 1

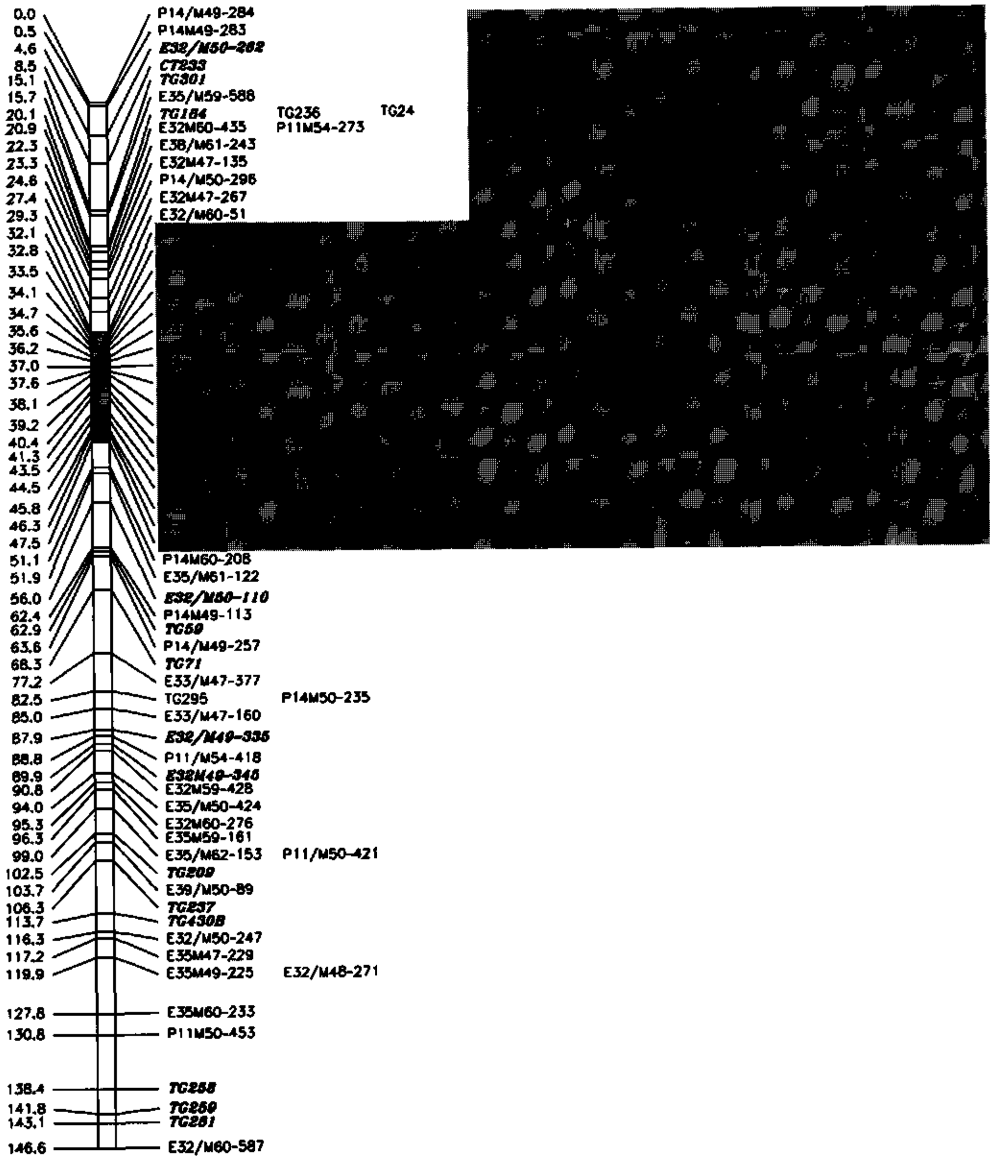


Fig. 2.1 See page 33 for legend

Chromosome 2

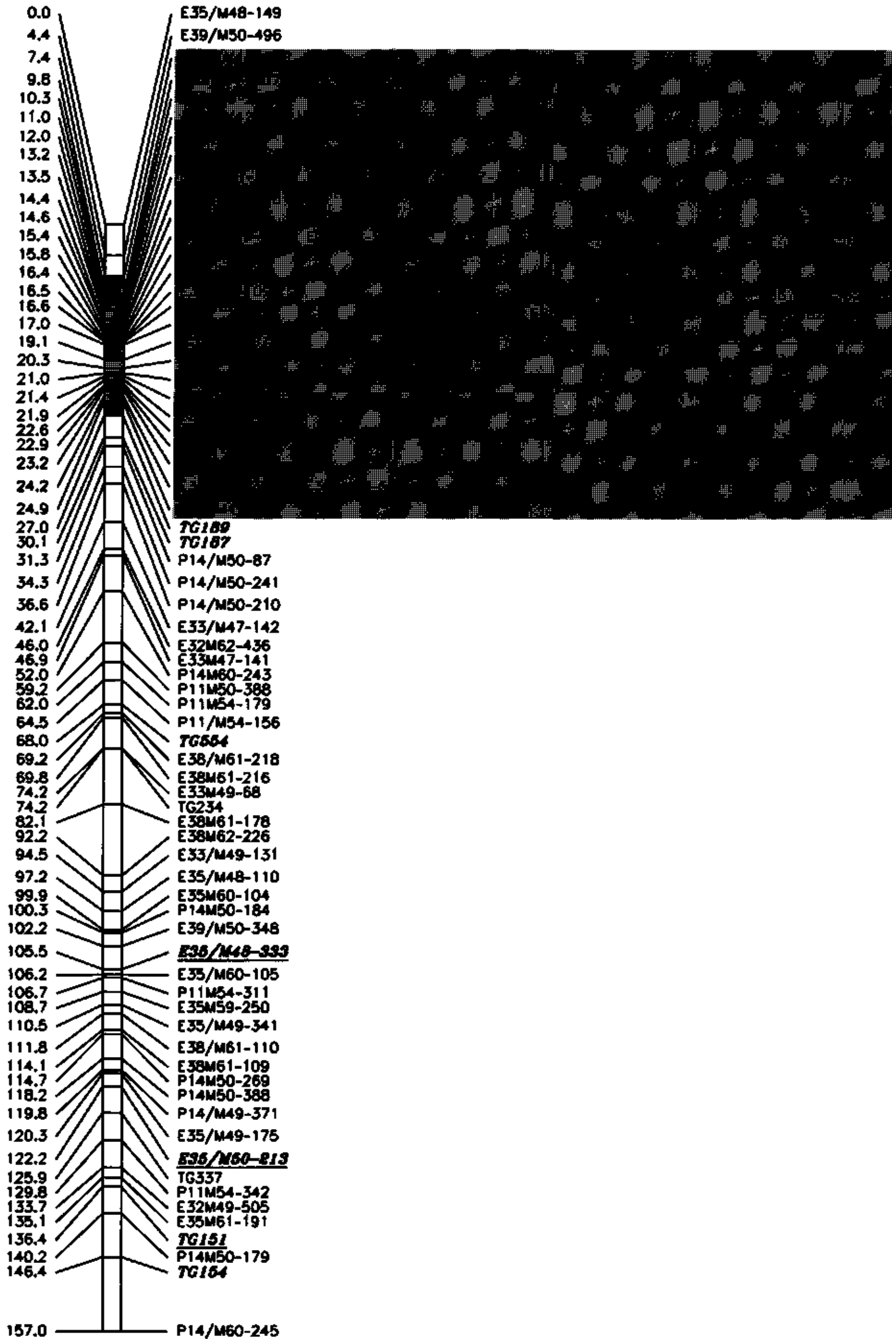


Fig. 2.1 See page 33 for legend

Chromosome 3

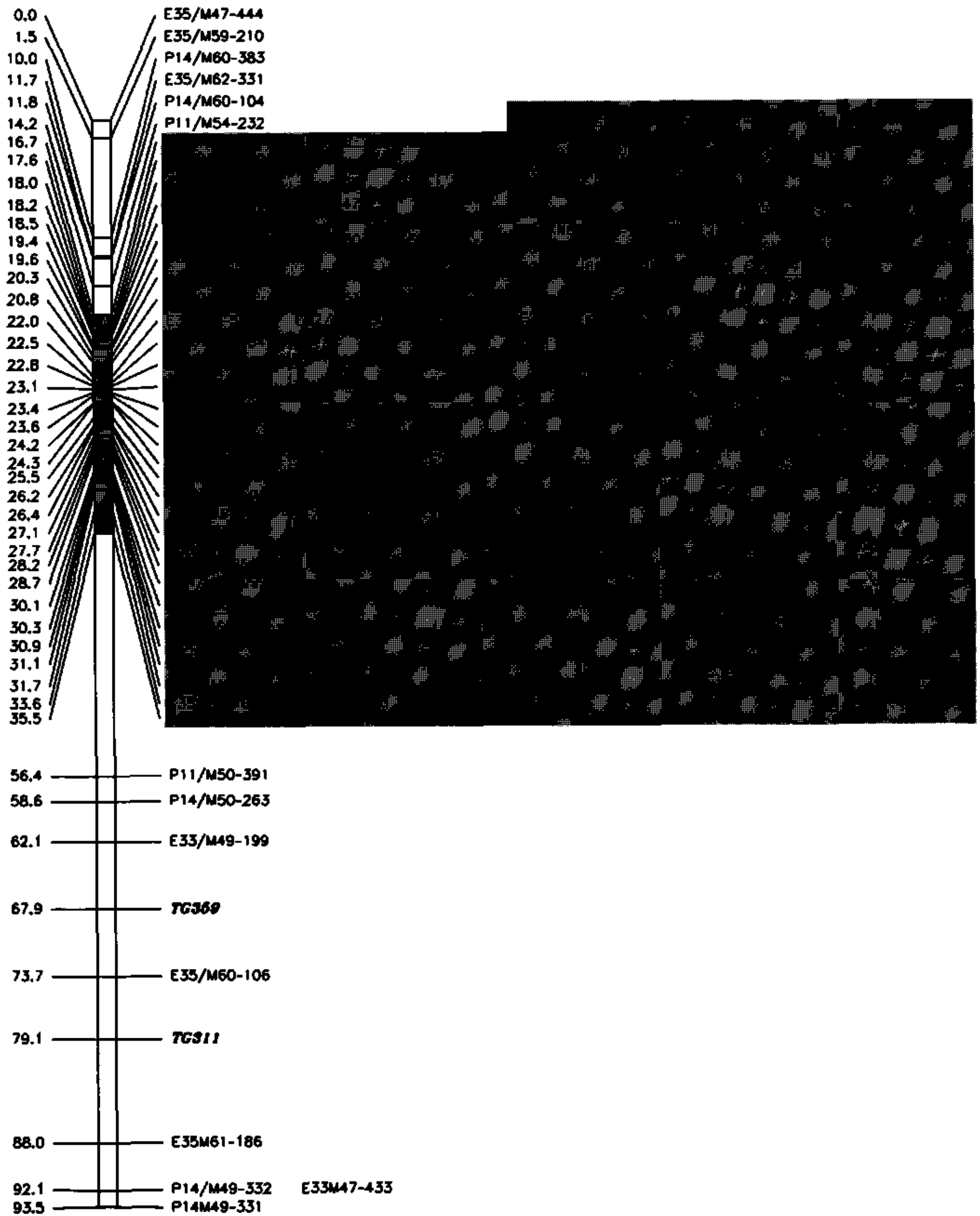


Fig. 2.1 See page 33 for legend

Chromosome 4

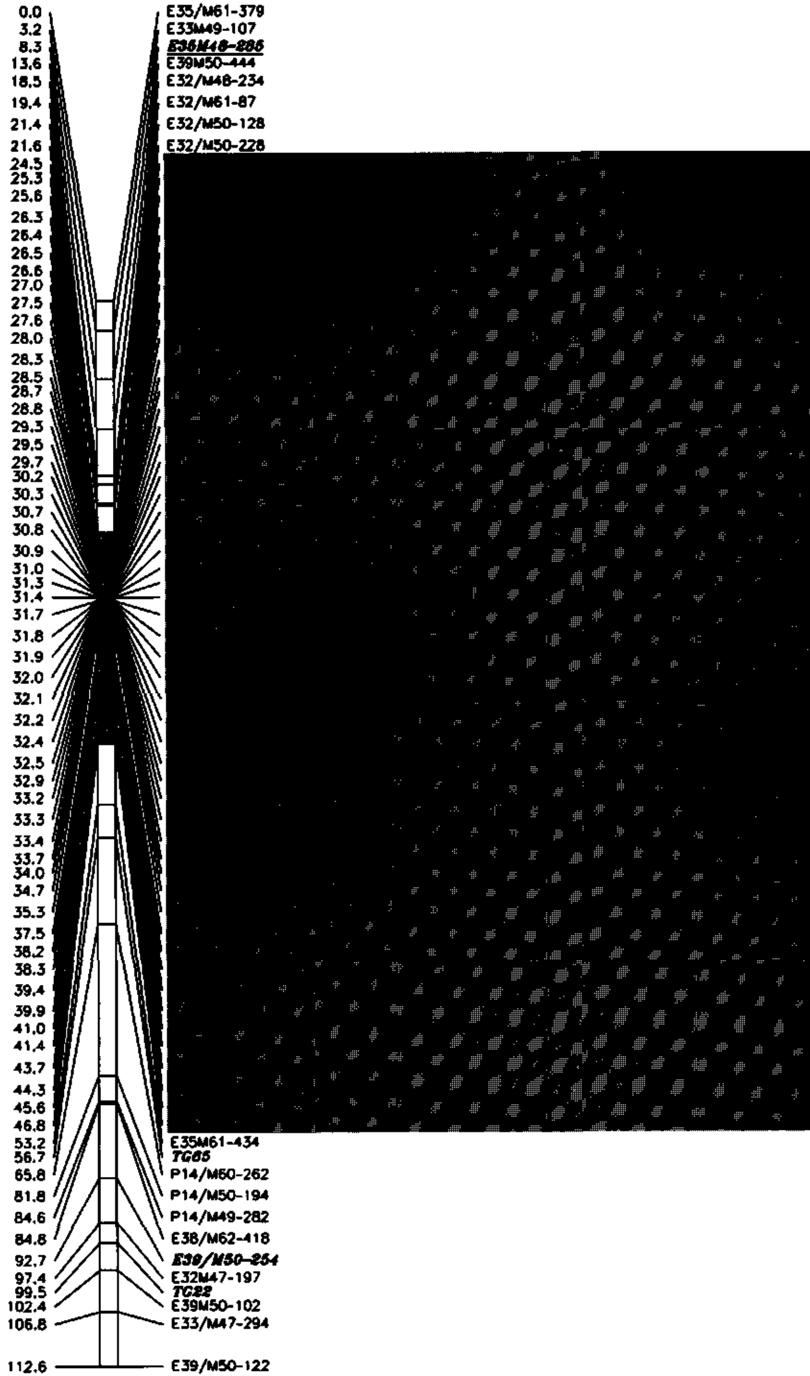


Fig. 2.1 See page 33 for legend

Chromosome 5

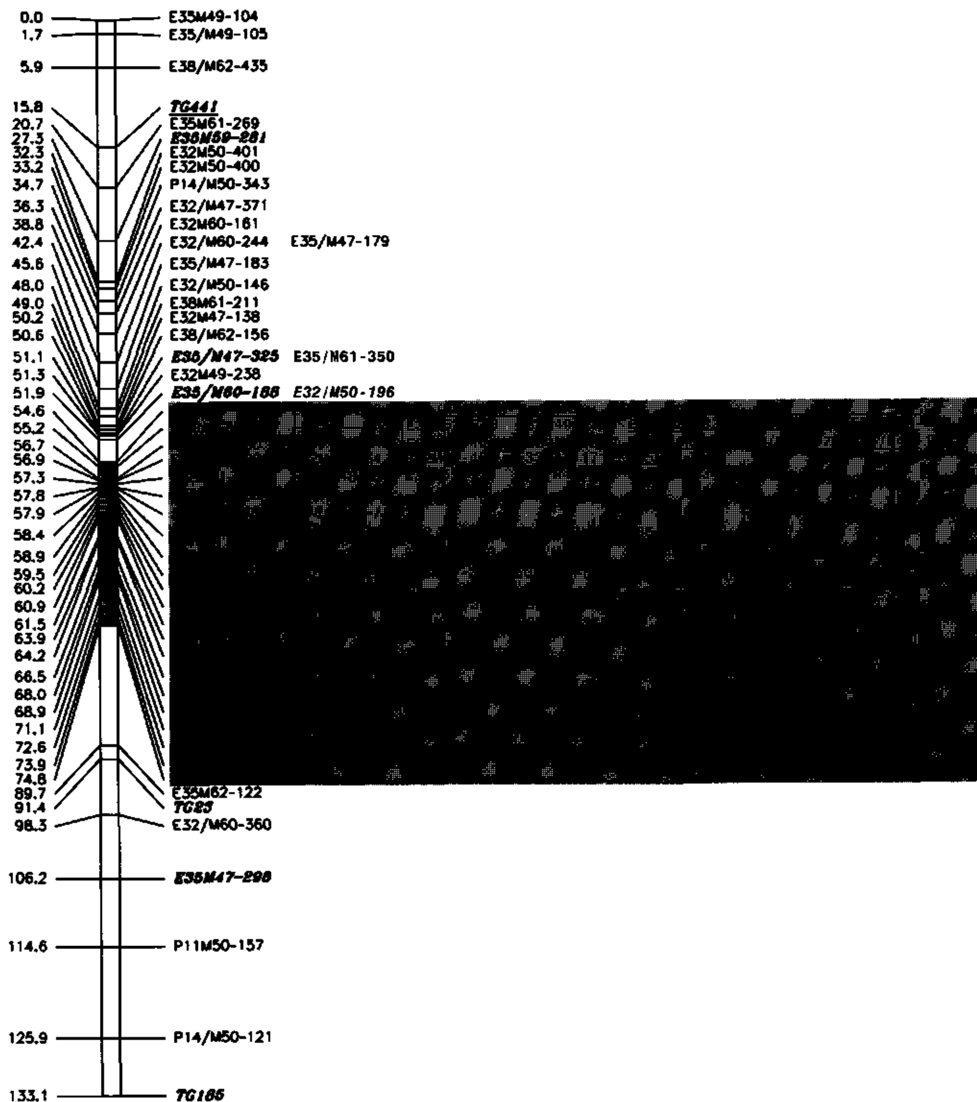


Fig. 2.1 See page 33 for legend

Chromosome 6

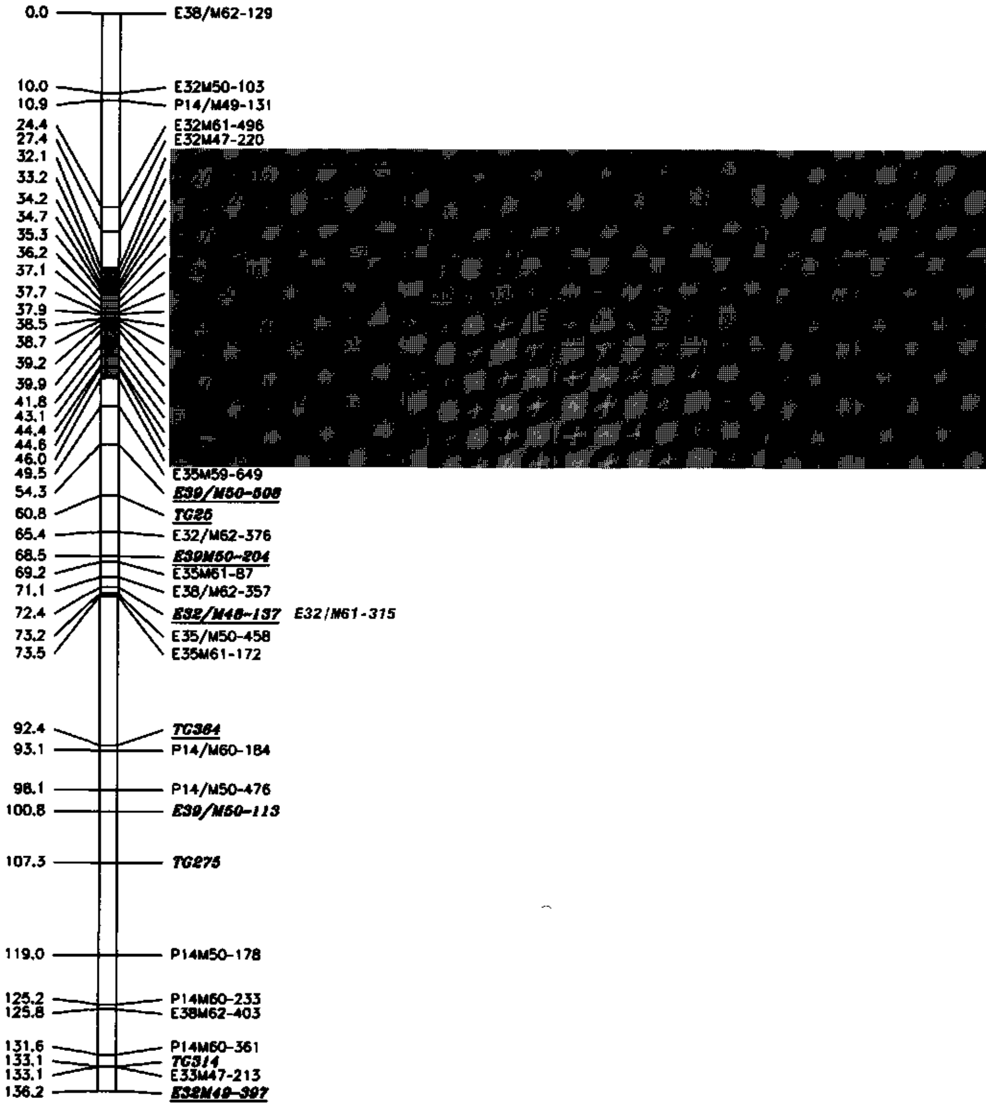


Fig. 2.1 See page 33 for legend

Chromosome 7

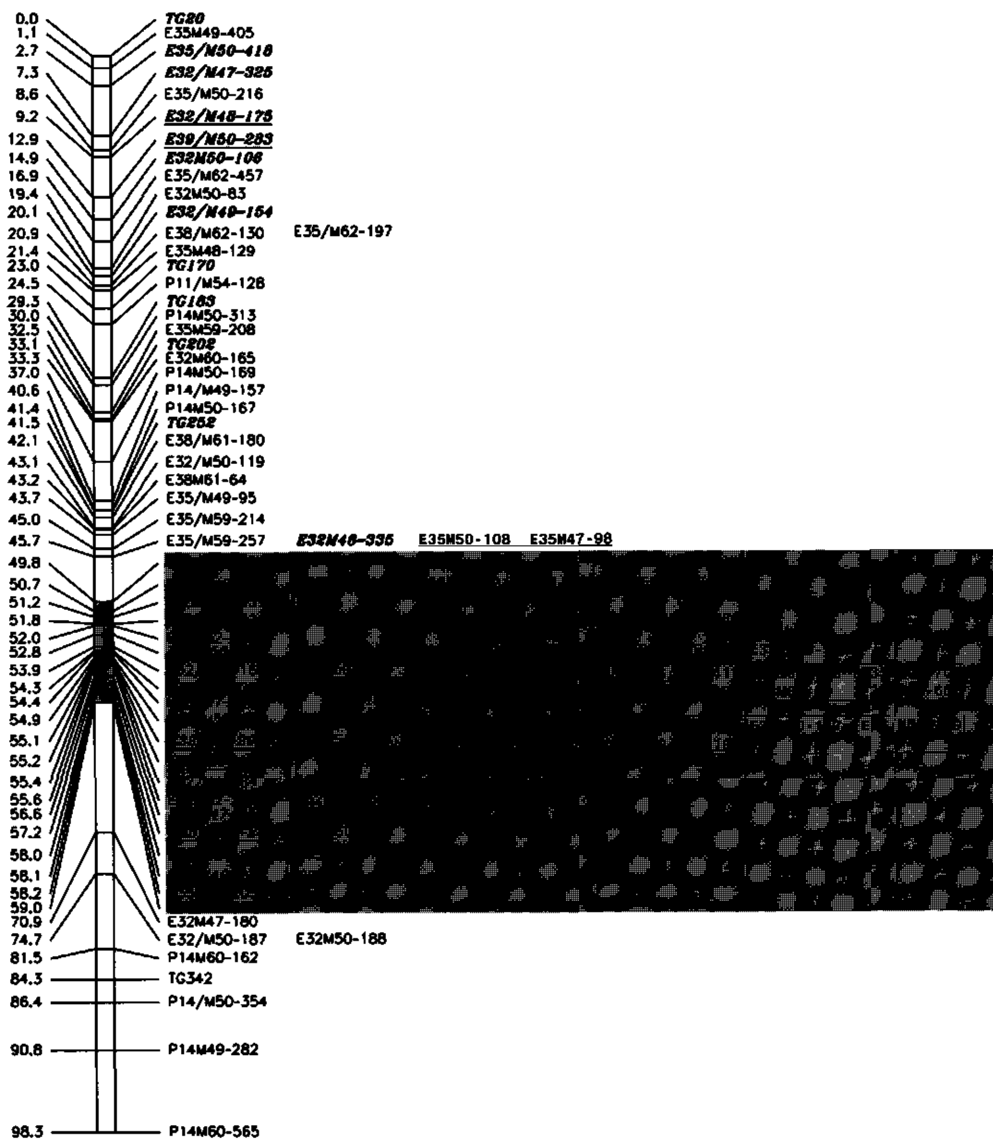


Fig. 2.1 See page 33 for legend

Chromosome 8

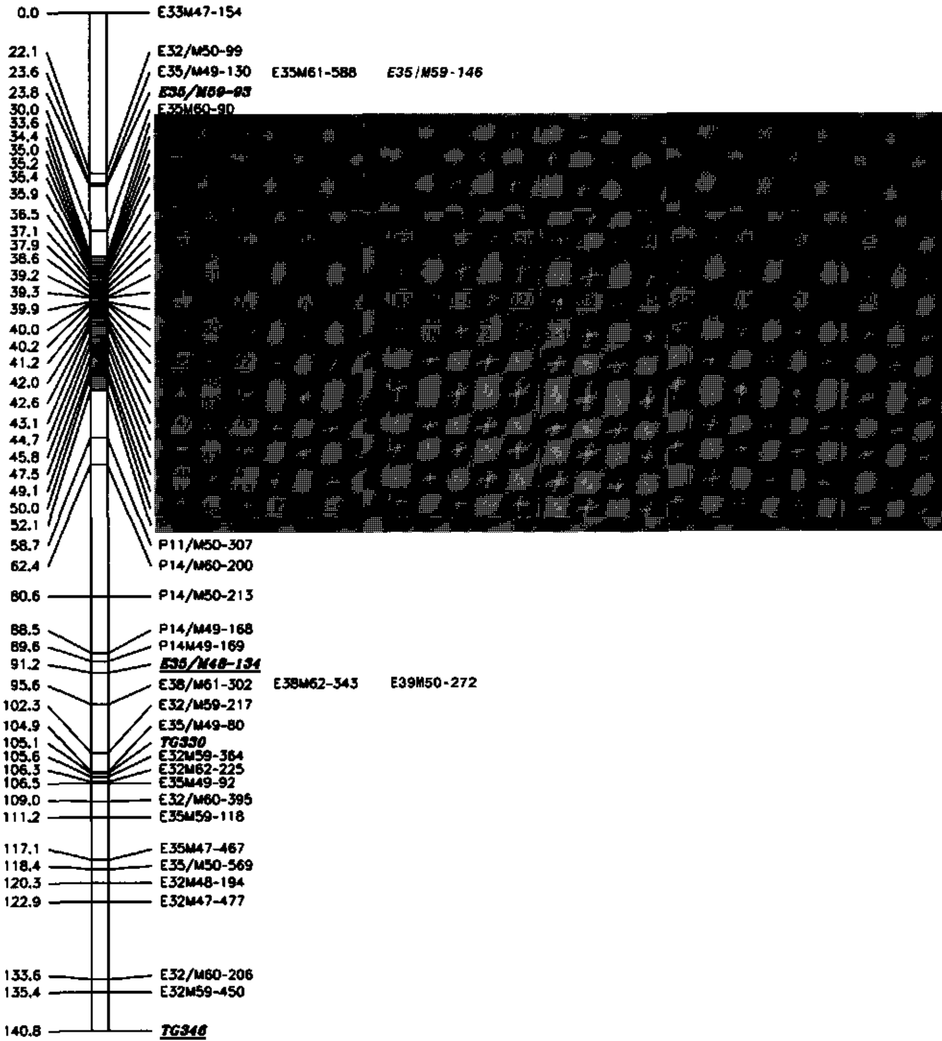


Fig. 2.1 See page 33 for legend

Chromosome 9

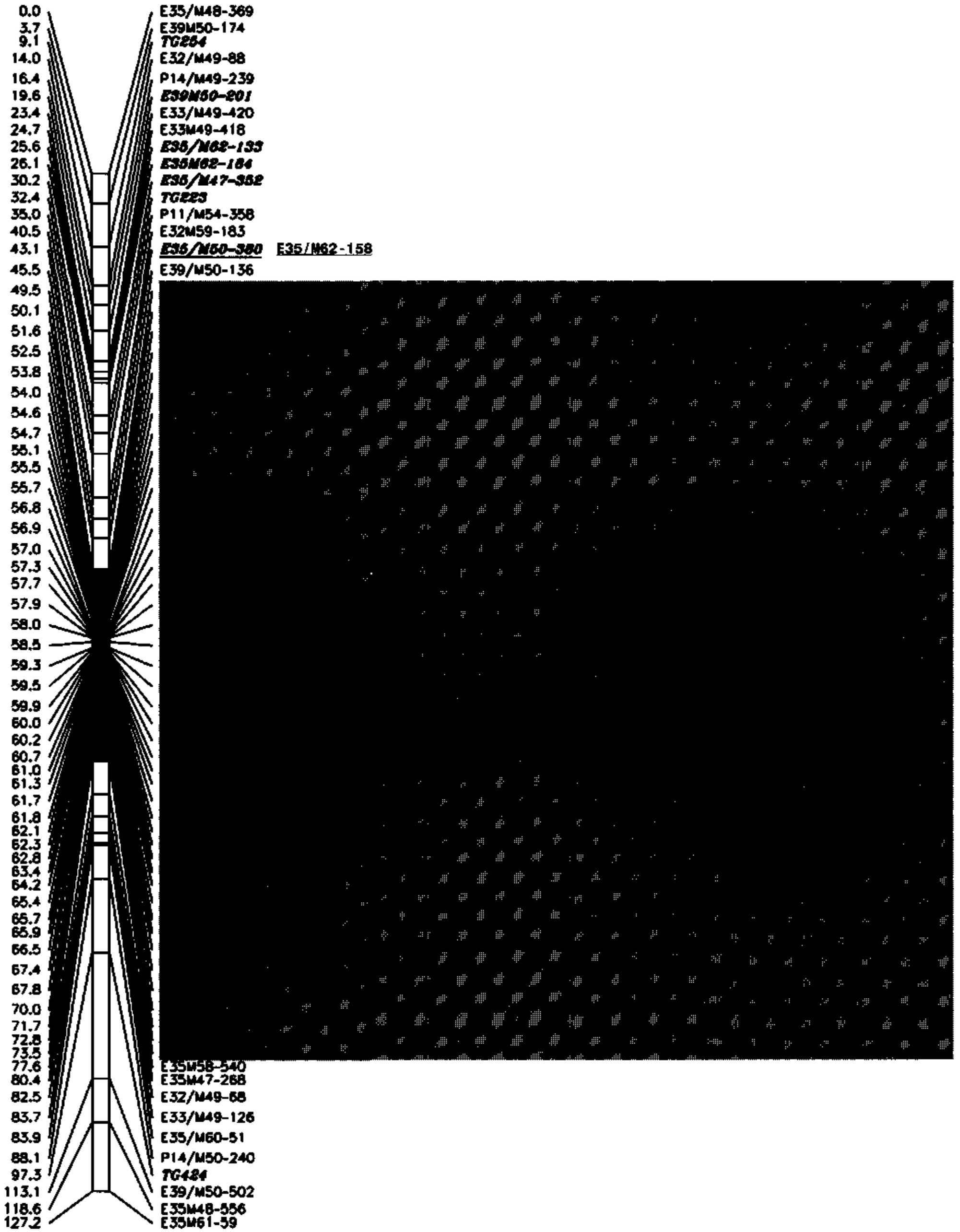


Fig. 2.1 See page 33 for legend

Chromosome 10

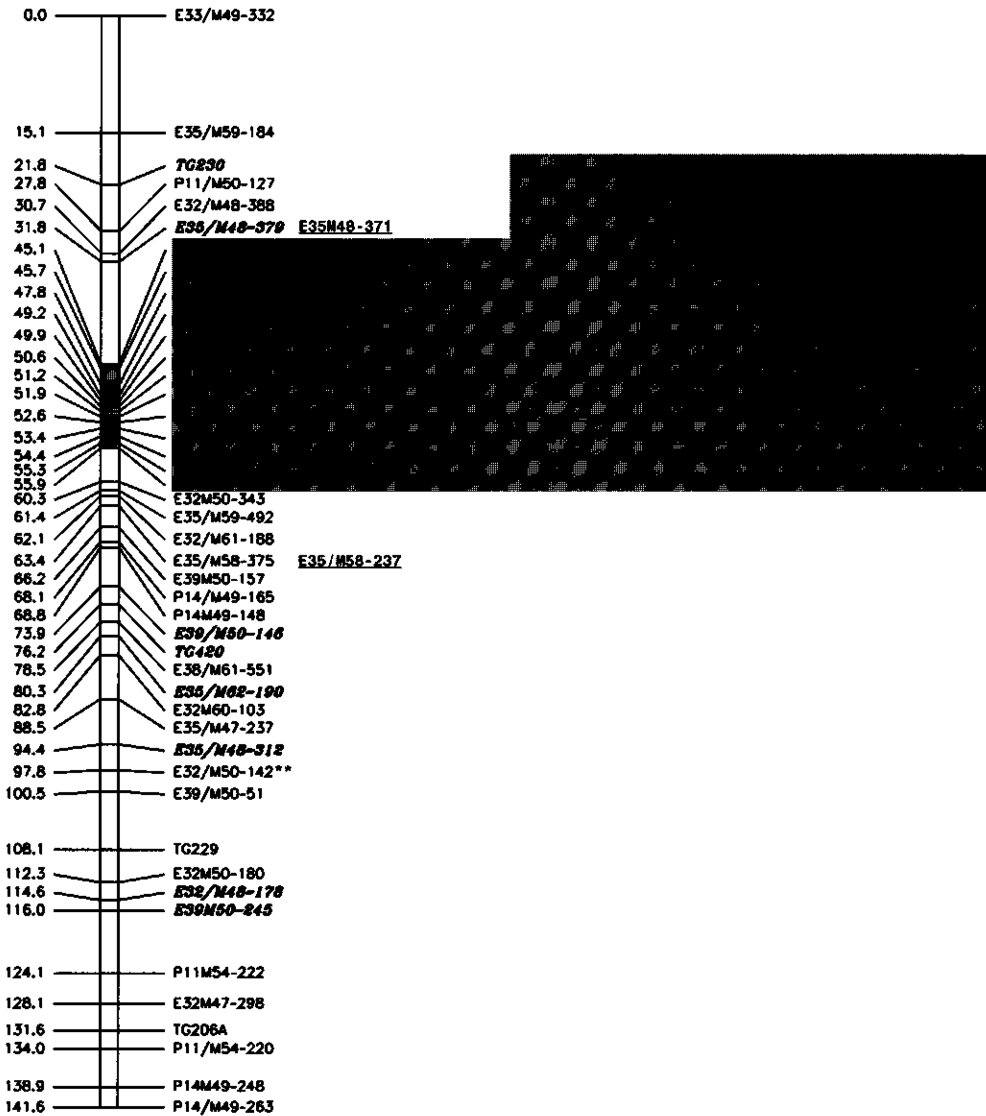


Fig. 2.1 See page 33 for legend

Chromosome 11

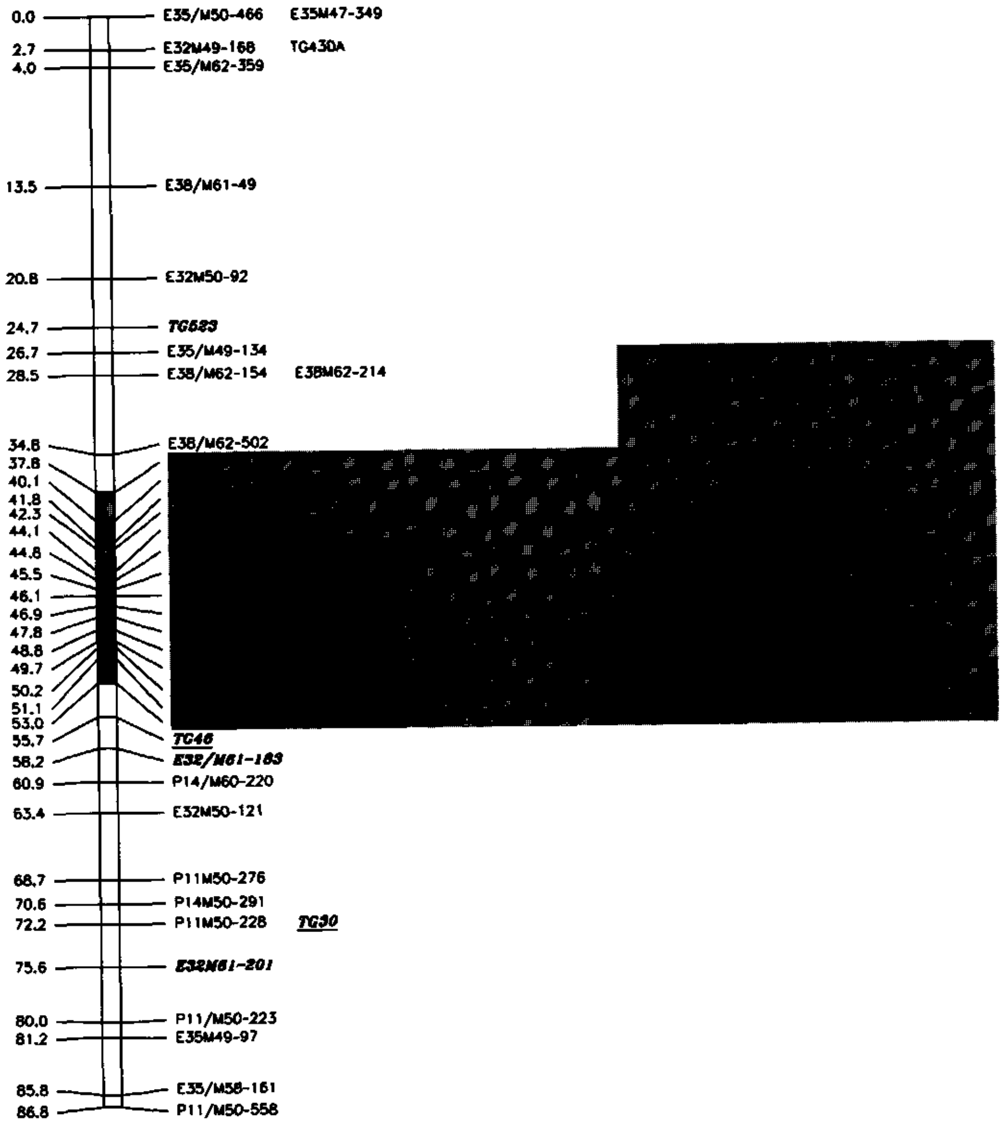


Fig. 2.1 See page 33 for legend

Chromosome 12

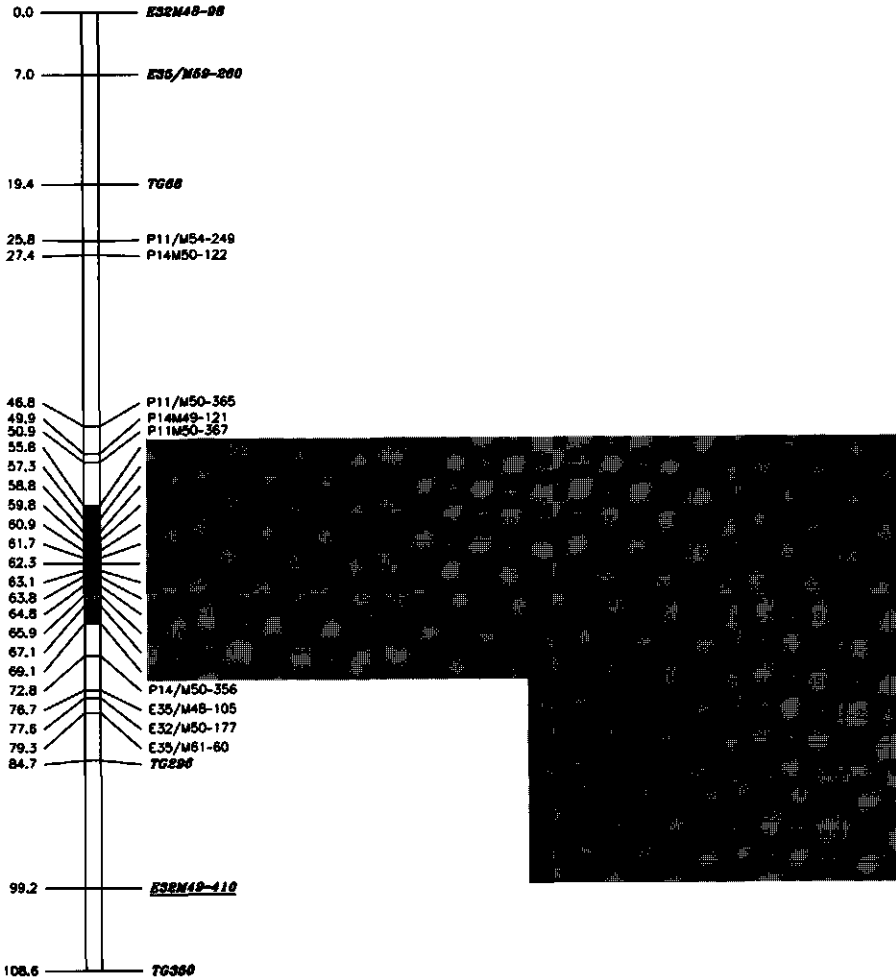


Figure 2.1 An integrated map based on two interspecific *L. esculentum* x *L. pennellii* F_2 populations. Markers that were scored in both populations are printed boldface in italics. Underlined markers represent markers of the CPRO population with no recombination with other markers of the CPRO population. Markers that were scored but not mapped in the CPRO population and were mapped in the Cornell population are printed boldface in italics and underlined. Three markers were scored in both population but were mapped in different linkage groups. These markers are indicated with ', * and **. The solid bars within the chromosomes represent the clusters. When six or more markers mapped to identical loci, these loci where chosen as part of a cluster. If marker loci were separated by 2.5 cM or more, the cluster ceased.

Furthermore, chromosomes 5 and 10 were each 30 cM longer at the most distal RFLP markers on both chromosome ends, compared to the map presented by Tanksley et al. (1992). Part of the increase in map length may be due to errors in the scoring of markers. Lincoln and Lander (1992) described that an error rate of 1% increases the map length by a factor of two for markers with low average spacing (1 cM). Since the majority of the markers in the present map are clustered with an average spacing of less than 1 cM, it is remarkable that the total map length did not increase by several hundred cM. This suggests that most markers were accurately positioned.

Clustering of markers

By constructing an AFLP map, it was found that the vast majority of *Eco*RI+*Mse*I markers occurred in clusters. The positions of the clusters in the present map are presumably the heterochromatic regions around the centromere (Tanksley et al., 1992). A similar strong clustering of *Eco*RI+*Mse*I markers around the putative centromere has also been reported in barley (Qi et al., 1998a) and maize (Vuylsteke et al., 1997). In addition, in soybean, sugar beet and potato, clustering of *Eco*RI+*Mse*I AFLP markers has been observed, although it was not known whether the clusters were in the centromeric regions (Keim et al., 1997; Schondelmaier et al., 1996; van Eck et al., 1995). By determining the frequency and distribution of recombination nodules on tomato synaptonemal complexes, Sherman and Stack (1995) observed a much lower frequency of recombination nodules in heterochromatin compared to euchromatin. We found 848 of the 1078 *Eco*RI+*Mse*I AFLP markers in clusters, which equals 78.7% of the mapped markers. This corresponds remarkably well with the percentage of DNA present in the heterochromatic regions, which is 77% according to Peterson et al. (1996) who studied pachytene chromosomes of *L. esculentum*. This suggests that clustering of *Eco*RI+*Mse*I markers is due to suppression of recombination in the heterochromatic regions near the centromere rather than to a non-random distribution of markers on the chromosomes.

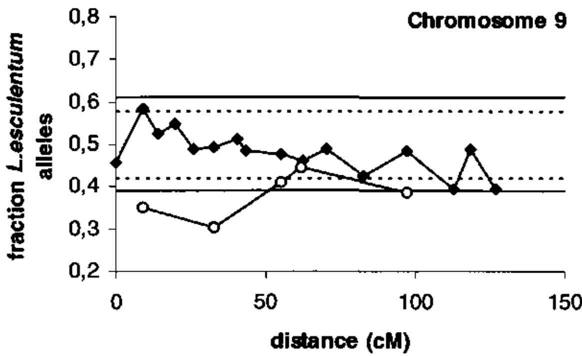
It was expected that the methylation sensitive restriction enzyme *Pst*I would recognise restriction sites in non-methylated euchromatin but not in the methylated heterochromatin (Gruenbaum et al., 1981). Consequently, fewer *Pst*I+*Mse*I markers should be obtained, but these markers should be located in the more distal parts of the chromosomes. To avoid the reduction in the number of markers, only two selective bases were used at the 3' end of the *Pst*I primer. Indeed, *Pst*I+*Mse*I markers were not clustered like *Eco*RI+*Mse*I markers; only 16

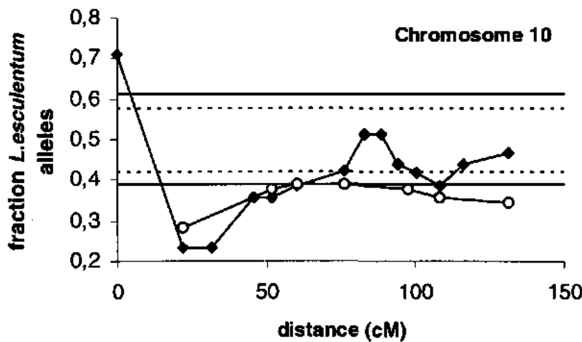
*Pst*I+*Mse*I AFLP markers mapped in the clusters, which span 199 cM, while 81 *Pst*I+*Mse*I AFLP markers mapped to the remaining 1283 cM. In agreement, using only *Pst*I+ *Mse*I AFLP markers in a doubled haploid rice population, Maheswaran et al. (1997) showed a random distribution of AFLP markers on the genetic map. These differences between *Eco*RI+*Mse*I and *Pst*I+*Mse*I AFLP markers have implications for the choice of restriction enzyme in different studies. In most genetic studies, especially QTL mapping, it is desirable to have markers evenly distributed over the genetic map. Consequently, *Pst*I+*Mse*I AFLP markers are more suitable for these purposes. However, it has to be stated that methylation varies between genotypes and during plant development, and this can influence marker analysis and the predictive value of such markers within the germplasm. For map based cloning, the choice of AFLP markers is dependent upon the position of the gene of interest. For example, the tomato gene *Cf-9*, conferring resistance to *Cladosporium fulvum*, is located in the euchromatin and consequently *Pst*I+*Mse*I AFLP markers were preferred to fine-position this gene (Thomas et al., 1995). On the other hand, the tomato *Mi* gene, conferring resistance to *Meloidogyne incognita*, is located in the heterochromatin and *Eco*RI+*Mse*I markers were preferred to clone this gene (Kaloshian et al., 1998).

Distorted segregation

Distorted segregation has been observed in both populations. However, it is remarkable that not all regions that were skewed showed this distorted segregation in both populations, since they are derived of similar crosses. For most of the skewed markers, prevalence for the *L. pennellii* allele was found, although some regions with minor distortion towards the *L. esculentum* allele were also found. A similar distortion in favour of *L. pennellii* alleles was observed before (DeVicente and Tanksley, 1993). The most skewed region in both populations was around the RFLP marker TG230 on chromosome 10. There are most likely hypotheses explaining this skewness. The first hypothesis is selection against zygotes of the *ee* genotype, resulting in an expected segregation of *ee:ep:pp* of 0:2:1. The second model is selection against pollen with an *e* allele, which would result in a segregation of *ee:ep:pp* of 0:1:1. The ratio *ee:ep:pp* for the most extreme marker TG230 of 2:25:35 for the Cornell population and 0:44:34 for the CPRO population more resembles a 0:1:1 rather than a 0:2:1 segregation, which indicates that pollen selection is more likely than zygote abortion of the homozygous *L. esculentum* genotype.

A





B

Figure 2.2A, B The frequency of alleles originating from *L. esculentum* per marker locus of the Cornell population (—◆—), and the CPRO population (—O—). Panel A refers to chromosome 9 and panel B to chromosome 10 respectively. For the Cornell population, values between 0.39 and 0.61 (as indicated by the horizontal lines) do not differ significantly from 0.5 ($P<0.05$). For the CPRO population, values between 0.42 and 0.58 (as indicated by the dashed horizontal lines) do not differ significantly from 0.5 ($P<0.05$).

In conclusion, AFLP provides a rapid way to construct reliable integrated genetic linkage maps. Especially when special considerations with respect to the choice of restriction enzymes are made, this technique enables both researchers as well as breeders to use AFLP markers for a variety of purposes, such as gene and QTL mapping, map-based cloning and marker assisted breeding.

Acknowledgements

We thank Dr. Anne Frary for editorial assistance.

Chapter 3

Mapping strategy for resistance genes against *Cladosporium fulvum* on the short arm of chromosome 1 of tomato: *Cf-ECP5* near the *Hcr9* Milky Way cluster

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Abstract

In the past, numerous *Lycopersicon* accessions have been described that harbor resistance genes to *Cladosporium fulvum* (*Cf* genes). Several *Cf* genes have been isolated, like *Cf-4*, *Cf-4A* and *Cf-9*, which are present on the short arm of chromosome 1 and *Cf-2* and *Cf-5*, which reside on chromosome 6. To identify *Cf* genes linked to the *Hcr9* cluster "Milky Way" on the short arm of chromosome 1, 66 resistant *Lycopersicon* accessions were test-crossed to the near isogenic line Moneymaker-Cf4 and the F_1s were crossed to the susceptible tomato cultivar Moneymaker. Putative linkage between an unknown *Cf* gene and *Cf-4* was concluded in small scale allelic tests from an under-representation of susceptible genotypes in the progenies of 24 plants after inoculation with race 0 of *C. fulvum*. In this way, of the 21 resistant lines tested, 10 harbored a *Cf* gene that was linked to the *Hcr9* Milky Way cluster. Moreover, one of the lines harboring a *Cf* gene closely linked to *Cf-4* specifically recognizes the extracellular protein ECP5 of *C. fulvum* and was designated *Cf-ECP5*. Using a testcross population of 338 plants, *Cf-ECP5* was mapped more accurately at 4 cM proximal to the *Hcr9* Milky Way locus. This report shows that the method of small-scale allelic tests provides a useful tool to rapidly screen for *Cf* genes on the short arm of chromosome 1. Further analysis of these *Cf* genes will elucidate the complex genetic organization of *Cf* genes on chromosome 1 of tomato.

Introduction

There is a long history in breeding for resistance to *Cladosporium fulvum* in tomato (*Lycopersicon esculentum* Mill.). A number of years after the introduction of a *Cf* resistance gene in tomato cultivars, the resistance often rendered ineffective due to changes in the virulence pattern of the fungus. Subsequently, other *Cf* genes from wild *Lycopersicon* species were introduced into tomato cultivars. In this way, several resistance genes have been introduced in tomato cultivars and intensive studies have been performed to find novel *Cf* genes (Kerr et al., 1971; Kanwar et al., 1980a,b). Since also many different races of *C. fulvum* have been characterized (Lindhout et al., 1989), the interaction between tomato and *C. fulvum* has become one of the best-studied plant-pathogen interactions that fits the gene-for-gene relationship (Joosten and De Wit, 1999). The resistance genes *Cf-2* (Dixon et al., 1996), *Cf-4* (Thomas et al., 1997), *Cf-4A* (Takken et al., 1998), *Cf-5* (Dixon et al., 1998) and *Cf-9* (Jones et al., 1994) have been isolated, as well as the avirulence genes *Avr4* (Joosten et al., 1994) and *Avr9* (Van Kan et al., 1991). The isolation of these genes has been an important step in elucidating the mechanism that underlies molecular recognition between resistance gene products and avirulence gene products leading to a hypersensitive response (HR). Numerous breeding lines are still available, harboring yet uncharacterized *Cf* genes (Boukema, 1981; Kanwar et al., 1980a,b; Laterrot, 1986; Stamova and Yordanov, 1978 a,b).

Several resistance genes have been isolated, but an issue that remains to be expounded is the mechanism by which new resistance specificities have developed. Most of the hitherto mapped *Cf* resistance genes are located on either the short arm of chromosome 1 (*Cf-4*, *Cf-4A*, *Cf-9*), or on chromosome 6 (*Cf-2* and *Cf-5*). The short arm of chromosome 1 contains at least three clusters of *Cf* homologues (designated as *Hcr9* loci). However, only one cluster, designated "Milky Way" was known to harbor functional *Cf* genes (Parniske et al., 1999). Also in other plant-pathosystems, clusters of resistance genes or resistance gene homologues exist. These clusters can be very different in size and disease specificities (Michelmore and Meyers, 1998). For example, in flax several loci conferring resistance to *Melampsora lini* have been identified (Anderson et al., 1997). The *L* locus contains only a single gene, of which 13 alleles are known, while the *M* locus comprises of a tandem array of approximately 15 homologous sequences (Anderson et al., 1997). For the development of tandem array loci, gene duplication events are presumed to have occurred. In tomato, complete *Hcr9* loci might have been

duplicated, because the sequences flanking these loci are very similar (Parniske et al., 1999). However, in between these loci unique DNA sequences have been identified, which is not expected when these genes would have originated from large duplication events (Parniske et al., 1999). The birth-and-death model presented by Michelmore and Meyers (1998) proposes that unequal crossing-over events are responsible for these duplication events. A high amount of sequence similarity in the intergenic regions may provoke new unequal crossing-over events. This is consistent with the findings of Parniske et al. (1997) who found that in a *Cf-4/Cf-9* heterozygote unequal crossing-overs occurred in the intergenic regions with high sequence similarity rather than within genes, thus preventing homogenization of *Cf* genes. Although this might explain the variation of *Hcr9s* at one locus, it does not explain the presence of multiple *Hcr9* loci. The presence of open reading frames of *Hcr9* genes outside the Milky Way cluster suggests that there may be functional *Cf* genes in these *Hcr9* clusters (Parniske et al., 1999). The isolation of resistance genes from other accessions might better clarify the development of new genes with other specificities in perspective of the birth-and-death model. In principle, these genes might be present in all *Solanaceae*, since all solanaceous species tested harbor *Cf* homologues (Kooman-Gersmann et al., 1996). Moreover, Laugé et al. (1999) showed recognition of isolated extracellular proteins (ECPs) of *C. fulvum* in *Nicotiana paniculata*, indicating the presence of functional *Cf* genes in these species. However, since *C. fulvum* is only pathogenic on tomato, only in *Lycopersicon* species these *Cf* genes may have a function in the resistance to this fungus. Alternatively, AVRs and ECPs may be conserved in other plant pathogenic fungi.

Recently, we have mapped a resistance gene, *Cf-ECP2*, conferring resistance to *C. fulvum* through recognition of the extracellular protein ECP2. The mapping of *Cf-ECP2* on chromosome 1, between the Milky Way and Southern Cross clusters (Chapter 5) presented additional evidence for the presence of functional *Cf* genes on chromosome 1 that are not part of the Milky Way cluster. In order to study the genetic variation and organization of *Cf* genes on the short arm of tomato chromosome 1 we describe the results of a screening of 66 *Lycopersicon* accessions for *Cf* resistance genes on the short arm of chromosome 1. The variation in map position and specificity of the *Cf* genes is discussed with respect to the development of new resistance specificities.

Materials and Methods

Plant Material and Disease Tests

Sixty-six *Lycopersicon* accessions with reported *Cf* resistance were collected from various gene banks (Table 3.1). Seeds, kindly donated by the Center for Genetic Resources Netherlands, Wageningen, The Netherlands (CGN) and the Center for Plant Breeding and Reproduction Research, Wageningen, The Netherlands (CPRO), originate from pioneer work of I.W. Boukema and seeds, kindly donated by the Plant Genetic Resources Canada, Ottawa, Canada (PGRC), result from the pioneer work of Dr. E.A. Kerr. Seeds of several *C. fulvum*-resistant accessions, as reported in the SOLGENES Internet database, were provided by the C.M. Rick Tomato Genetic Resources Center, UC Davis, USA (TGRC). To confirm *Cf* resistance, accessions were tested for resistance to *Cladosporium fulvum* race 0, a race that contains all known avirulence genes, by inoculation with a suspension of spores in the two-leafs stage as described previously (Chapter 5). Accessions were crossed to the near-isogenic line Moneymaker-Cf4 (MM-Cf4). Subsequently, the F_1 was crossed to the susceptible tomato cultivar Moneymaker (MM). These crosses are referred to as 'testcrosses' and their progenies as 'testcross progenies'. The *L. esculentum* line G1.1161 was found to show a specific HR upon exposure to the fungal extracellular protein ECP5 (Laugé et al., 1999) and was designated "CfECP5". F_3 lines of 231 F_2 plants, derived from the cross MM x CfECP5 were obtained for segregation tests of *Cf-ECP5* resistance.

PVX assays

All *Lycopersicon* accessions were tested for the presence of *Cf-4* or *Cf-9* by inoculation of plants at the two-leafs stage with recombinant potato virus X (PVX) expressing the *Avr4* gene (PVX::*Avr4*; Joosten et al., 1997) or the *Avr9* gene (PVX::*Avr9*; Hammond-Kosack et al., 1995). Fourteen days after inoculation, plants were scored visually in two classes: necrotic (HR, indicating presence of *Cf-4* or *Cf-9*) or mosaic (no HR, indicating absence of *Cf-4* or *Cf-9*). Twenty to 40 plants of each of the 231 F_3 lines of the cross MM x CfECP5, were tested for the segregation of *Cf-ECP5* by inoculation of seedlings with recombinant PVX carrying the *Ecp5* gene (Laugé et al., 1999), similar to the other PVX inoculation assays.

Linkage analysis between *Cf* genes

To study the linkage between an unidentified *Cf* gene and *Cf-4*, the testcross progenies were inoculated with *C. fulvum* race 0 as described above. The genetic distance between these *Cf* genes was estimated from the frequency of susceptible recombinants. After the evaluation of the disease tests with race 0, all diseased leaves of the testcross progeny MM x (MM-Cf4 x CfECP5) were removed and plants were grown for 10-14 days under conditions with a low relative humidity to prevent further growth of *C. fulvum*. Subsequently, this testcross population was tested for resistance to race 2.4.5 as described for race 0. Also, cuttings of two months old plants of this testcross population were assayed with PVX::*Avr4* as described above.

DNA isolation, CAPS analysis and genetic linkage analysis

DNA was isolated according to the protocol described by Van der Beek et al. (1992). DNA of the parents CfECP5, MM and MM-Cf4 was used to screen for cleaved amplified polymorphic sequence (CAPS) markers on the short arm of chromosome 1 as described in Chapter 5 (Table 3.2). The testcross progeny MM x (MM-Cf4 x CfECP5) and the F_2 progeny of 233 plants of MM x CfECP5 were tested for segregation of these CAPS markers. An integrated genetic map was calculated using the computer package JoinMap 2.0 (Stam and Van Ooijen, 1996).

Results

Cf resistance in parents

In the past, several researchers have searched for novel sources of *Cf* resistance. They developed the breeding material that we obtained from gene banks and used for the research described here. Most *Lycopersicon* accessions obtained were tested for resistance to *C. fulvum* race 0. All tested accessions were confirmed to be resistant (Table 3.1). The accessions *L. esculentum* G1.1145 and *L. esculentum* LA2443, harboring the resistance gene *Cf-1*, showed less sporulation than the MM control plants, and were considered as incomplete resistant.

Presence of *Cf-4* and *Cf-9* in the *Lycopersicon* accessions

The genes *Cf-4* and *Cf-9* have been frequently used in breeding programs and numerous tomato lines harboring these genes are available. In addition, *Cf-4* and *Cf-9* have been mapped on chromosome 1 and have been isolated (Thomas et al., 1997; Jones et al., 1994). In order to avoid confusion of putative novel *Cf* genes with *Cf-4* and *Cf-9* we have tested the *Lycopersicon* accessions for the presence of *Cf-4* and *Cf-9* using PVX::*Avr4* and PVX::*Avr9* assays respectively. Upon inoculation with PVX::*Avr4*, 17 genotypes exhibited necrosis (Chapter 4). Upon inoculation with PVX::*Avr9*, only MM-Cf9 (*L. esculentum* G1.1154) and *L. pimpinellifolium* PI126947 (CGN15808) showed necrosis (Table 3.1). Consequently, genotypes responding with necrosis to either of the two recombinant PVX strains were not tested for linkage to *Cf-4* or *Cf-9*.

Test for linkage of putative *Cf* genes to the *Cf-4*/*Cf-9* cluster

A number of randomly chosen resistant accessions which did show necrosis upon inoculation with PVX::*Avr4* or PVX::*Avr9*, were tested for carrying a *Cf* gene allelic to *Cf-4* (Table 3.1). In most trials, 24 plants per testcross population were tested for segregation of resistance to *C. fulvum* race 0. If two or less showed disease symptoms, we concluded that the uncharacterized *Cf* gene was probably linked to the *Hcr9* Milky Way cluster ($P<0.06$). From the 21 accessions tested, we identified ten accessions with a *Cf* gene that was putatively linked to the *Hcr9* Milky Way cluster (Table 3.1). To confirm linkage, we tested another 181 plants of the testcross population of *L. esculentum* LA3049, a randomly chosen testcross population that did not contain any susceptible plant out of eighteen initially tested. Only five were susceptible to *C. fulvum* race 0 (Table 3.1). Assuming the presence of a single *Cf* gene in *L. esculentum*

LA3049, this gene is expected to be at a distance of 5.5 cM from the *Hcr9* Milky Way cluster. It is not known from which accession the resistance in LA3049 originated, but the *Cf* resistance is present in a Moneymaker background (R. Chetelat, pers. comm.).

Table 3.1 Accessions which have been tested for resistance to *C. fulvum* race 0, for necrotic response to AVR9 using PVX::*Avr9* and for segregation of resistance to *C. fulvum* race 0 in testcross progenies.

Nr.	Accession[1]	Database[2]	Designation of resistance[3]	race 0[4]	PVX::*Avr9*[5]	Segregation[6]	Conclusion[7]
1	*L.esc* G1.1145	CPRO	*Cf-1*	IR	-	Not tested	
2	*L.esc* G1.1146	CPRO	*Cf-2*	R	-	8/24	unlinked
3	*L.esc* G1.1147	CPRO	*Cf-3*	R	-	Not tested	
4	*L.esc* G1.1148	CPRO	*Cf-4*	R	-	Not tested	
5	*L.esc* G1.1149	CPRO	*Cf-5*	R	-	Not tested	
6	*L.esc* G1.1150	CPRO	*Cf-?*	R	-	Not tested	
7	*L.esc* G1.1151	CPRO	*Cf-?*	R	-	4/24	unlinked
8	*L.esc* G1.1152	CPRO	*Cf-?*	R	-	Not tested	
9	*L.esc* G1.1153	CPRO	*Cf-? (Cf-ECP3)*	R	-	0/24	linked/allelic
10	*L.esc* G1.1154	CPRO	*Cf-9*	R	+	Not tested	
11	*L.esc* G1.1155	CPRO	F_4BC_5	R	-	5/24	unlinked
12	*L.esc* G1.1156	CPRO	F_4BC_5	R	-	Not tested	
13	*L.esc* G1.1157	CPRO	F_3BC_5	R	-	Not tested	
14	*L.esc* G1.1158	CPRO	F_3BC_5	R	-	7/24	unlinked
15	*L.esc* G1.1159	CPRO	F_3BC_5	R	-	3/24 + 5/24	unlinked
16	*L.esc* G1.1160	CPRO	F_3BC_5	R	-	Not tested	
17	*L.esc* G1.1161	CPRO	F_3BC_5 *(Cf-ECP5)*	R	-	1/24 + 2/339	linked
18	*L.esc* G1.1162	CPRO	F_3BC_5	R	-	Not tested	
19	*L.esc* G1.1163	CPRO	F_3BC_5	R	-	2/24	linked
20	*L.esc* G1.1164	CPRO	F_3BC_5	R	-	8/24	unlinked
21	*L.esc* G1.1165	CPRO	F_3BC_5	R	-	Not tested	
22	*L.esc* G1.1653	CPRO	*Cf-11*	R	-	Not tested	
23	*L.esc* G1.1656	CPRO	*Cf-6*	R	-	9/24	unlinked
24	*L.esc* 881271	CPRO	*Cf-8*	R	-	Not tested	
25	*L.esc* LA2443	TGRC	*Cf-1*	IR	NT	Not tested	
26	*L.esc* LA2446	TGRC	*Cf-4*	R	-	Not tested	
27	*L.esc* LA3045	TGRC	*Cf-4*	R	-	Not tested	
28	*L.esc* LA3047	TGRC	*Cf-9*	R	NT	Not tested	
29	*L.esc* LA3048	TGRC	*Cf-?*	R	-	Not tested	
30	*L.esc* LA3049	TGRC	*Cf-?*	R	-	0/18 + 5/181	linked
31	*L.esc* LA3050	TGRC	*Cf-?*	R	-	Not tested	
32	*L.esc* LA3051	TGRC	*Cf-?*	R	-	Not tested	
33	*L.esc* LA3266	TGRC	*Cf-?*	R	-	Not tested	
34	*L.esc* LA3267	TGRC	*Cf-?*	R	-	Not tested	
35	*L.esc* LA3271	TGRC	*Cf-?(Cf-ECP3)*	R	-	Not tested	
36	*L.esc* LA3272	TGRC	*Cf-?*	R	-	Not tested	
37	*L.esc* LA3431	TGRC	*Cf-?*	R	-	Not tested	
38	*L.pim* CGN14354	CGN	*Cf-?*	R	-	1/24	linked
39	*L.esc* CGN15397	CGN	*Cf-4*	R	-	Not tested	
40	*L.pim* CGN14353	CGN	*Cf-?*	R	-	Not tested	
41	*L.pim* CGN15529	CGN	*Cf-?*	R	-	Not tested	
42	*L.pim* CGN15808	CGN	*Cf-ECP2*	R	+	Not tested	
43	*L.pim* CGN15814	CGN	*Cf-9?*	R	-	0/24	linked/allelic

(Continued on next page)

44	*L.min* CGN15815	CGN	*Cf-?*	R	-	Not tested	
45	*L.esc* CGN15839	CGN	*Cf-6*	R	-	Not tested	
46	*L.esc* CGN15840	CGN	*Cf-15*	R	-	7/18	unlinked
47	*L.esc* CN0078	PGRC	*Cf-1,Cf-2,Cf-3,Cf-4*	NT	-	Not tested	
48	*L.esc* CN0335	PGRC	*Cf-1,Cf-2,Cf-4*	NT	-	Not tested	
49	*L.esc* CN0354	PGRC	*Cf-2,Cf-4*	NT	-	Not tested	
50	*L.esc* CN0355	PGRC	*Cf-1,Cf-2,Cf-4*	NT	-	Not tested	
51	*L.esc* CN0356	PGRC	*Cf-1,Cf-2,Cf-3,Cf-4*	NT	-	Not tested	
52	*L.esc* CN0697	PGRC	*Cf-2,Cf-4*	NT	-	Not tested	
53	*L.esc* CN0698	PGRC	*Cf-1,Cf-2,Cf-3,Cf-4*	NT	-	Not tested	
54	*L.esc* CN1711	PGRC	*Cf-23 (Cf-ECP2)*	NT	-	Not tested	
55	*L.esc* CN2002	PGRC	*Cf-8*	NT	-	Not tested	
56	*L.esc* CN2418	PGRC	*Cf-12*	NT	-	Not tested	
57	*L.esc* CN6762	PGRC	*Cf-18 (Cf-ECP2)*	NT	-	0/24	linked/allelic
58	*L.esc* CN6763	PGRC	*Cf-20 (Cf-ECP2)*	NT	-	2/24	linked
59	*L.esc* CN6764	PGRC	*Cf-21*	NT	-	7/24	unlinked
60	*L.esc* CN6765	PGRC	*Cf-22*	NT	-	2/24	linked
61	*L.esc* CN6766	PGRC	*Cf-13*	NT	-	Not tested	
62	*L.esc* CN6767	PGRC	*Cf-14*	NT	-	2/24	linked
63	*L.esc* CN6768	PGRC	*Cf-16*	NT	-	7/24	unlinked
64	*L.esc* CN6770	PGRC	*Cf-24 (Cf-ECP2)*	NT	-	Not tested	
65	*L.esc* CN7494	PGRC	*Cf-17*	NT	-	6/24	unlinked
66	*L.esc* IVT771891	CPRO	*Cf-?*	NT	-	Not tested	

[1] *L.esc = Lycopersicon esculentum*; *L.pim = L. pimpinellifolium*; *L.min = L. minutum*

[2] CGN = Center for Genetic Resources Netherlands; TGRC = Tomato Genetic Resource Center, UC Davis, USA.; PGRC = Plant Genetic Resources Canada, Ottawa, Canada; CPRO = Center for Plant Breeding and Reproduction Research, Wageningen, The Netherlands

[3] *Cf* genes as they have been previously designated in literature, *Cf-?* = uncharacterized *Cf* resistance, F_4BC_5 and F_3BC_5 indicate that the resistance in those lines is obtained from resistant wild *Lycopersicon* species after backcrossing to the susceptible tomato cultivar Moneymaker, followed by selfing and selection for homozygous resistant plants.

[4] IR = incomplete resistance to *C. fulvum* race 0; R = resistance to *C. fulvum* race 0; NT = not tested with *C. fulvum* race 0

[5] + = necrosis upon infection by PVX::*Avr9*; - = mosaic upon infection by PVX::*Avr9*; NT = not tested with PVX::*Avr9*

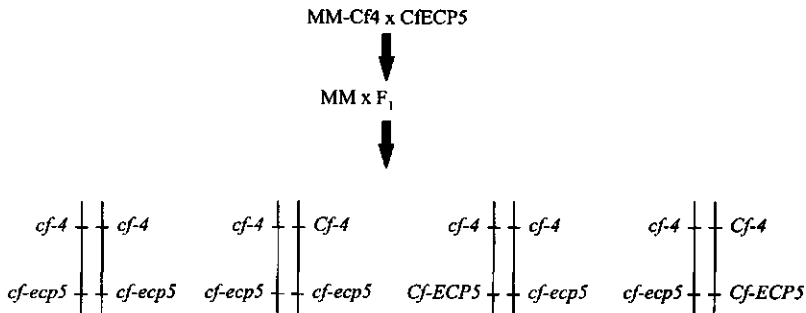
[6] The number in front of the dash (/) indicates the number of susceptible plants per testcross population upon inoculation with *C. fulvum* race 0. The number behind the dash (/) indicates the number of plants tested per progeny.

[7] Linked and allelic indicates that the resistance gene in those lines is linked, respectively allelic to *Cf-4*. Unlinked indicates that the resistance in those lines is not linked to *Cf-4*.

Test for linkage between *Cf-ECP5* and *Cf-4*

The *L. esculentum* accession G1.1161 (CfECP5) was an F_3BC_5 of MM with *L. pimpinellifolium* CGN15529 (Boukema, pers. comm.) and showed a specific HR to the extracellular protein ECP5 from *C. fulvum* (Laugé et al., 1999). This *Cf* gene has been designated *Cf-ECP5*. Initially, a testcross of CfECP5 with MM-Cf4 only showed one susceptible plant out of 24 tested (Table 3.1). A larger population of 338 offspring of the same testcross was evaluated for segregation of resistance to *C. fulvum* race 0. Only two plants appeared to be susceptible, confirming close linkage of *Cf-ECP5* with *Cf-4*. In addition, all offspring were inoculated with race 2.4.5 to identify plants harboring only *Cf-4*, as this race is able to overcome *Cf-4*, but not *Cf-ECP5*. Finally, PVX::*Avr4* assays were used to discriminate

plants harboring only *Cf-ECP5* from the plants harboring both *Cf-4* and *Cf-ECP5* (Figure 3.1). The segregation into the genotypes *cf-4cf-4/cf-ecp5cf-ecp5*, *Cf-4cf-4/cf-ecp5cf-ecp5*, *cf-4cf-4/Cf-ECP5cf-ecp5* and *Cf-4cf-4/Cf-ECP5cf-ecp5* was 2:158:167:11. This gives a distance of 3.8 ± 1.0 cM between *Cf-4* and *Cf-ECP5*.



Response to *C. fulvum* and PVX inoculations:

C. fulvum race 0	S	R	R	R
C. fulvum race 2.4.5	S	S	R	R
PVX::*Avr4*	M	N	M	N
# observed	2	158	167	11

Figure 3.1 Construction and analysis of a testcross population of CfECP5. R indicates resistant and S susceptible to the two fungal races indicated. M indicates mild mosaic symptoms and N systemic necrosis upon inoculation with PVX::*Avr4*. The number of plants per genotype class are indicated by '# observed'.

CAPS analysis and mapping of *Cf-ECP5*

To further identify the map position of *Cf-ECP5*, the parent genotypes MM, MM-Cf4 and CfECP5 were screened for CAPS markers on the short arm of chromosome 1 (Table 3.2). Out of 325 primer- restriction enzyme combinations tested, we detected 10 polymorphisms, representing 6 of the 12 marker loci tested (Table 3.2). Furthermore, to confirm the results of the disease tests on the testcross population, the PCR product of Cf4f3xCf4r3 was digested with *Eco*RI (Table 3.2). This CAPS gives a *Cf-4* specific band of 842 bp that is absent in MM and MM-Cf9 (F. Takken pers. comm.) and is therefore diagnostic for the presence of *Cf-4* in the testcross progeny. However, CfECP5 gave a slightly larger band of approximately 870 bp. The *Cf-4* and CfECP5 specific bands could not be separated on an agarose gel and were therefore not useful for screening of the testcross population. The CfECP5 specific band was mapped using the F_2 population of MM x CfECP5. Of the testcross population, 295 plants were screened with the CAPS marker CP46-*Hinf*I, and 50 plants with TG236-*Ssp*I, CT116-

Table 3.2 Primer sequences, lengths of amplified PCR products and restriction enzymes revealing CAPS markers between *L. esculentum* cv. Moneymaker (MM), Moneymaker-Cf4 (MM-Cf4) and CfECP5.

Locus	Primer sequence	PCR product size (bp)	Enzymes detecting polymorphism	Total number of enzymes tested*
CT233	see Bonnema et al. (1997)	1650	-	43/43
CT2	F: AAG CCT CTA ATC AAG AAA ATG G R:TTC AGT GCA ATA ATA ATG AGG G	490	*MseI*[1,2]	20/11
TG301	see Bonnema et al. (1997)	800	*AluI*[1,2],*RsaI*[1,2]	41/41
CP46	see Bonnema et al. (1997)	1000	*DdeI*[3], *HinfI*[1,2], *MboII*[2,3]	14/7
FT33	F: AGA AGG ATA AAG CTC AAC ATC GG R: AAG GAA CAT CTG TGG TTC GC	1100	-	13/13
TG236	see Bonnema et al. (1997)	1000	*SspI*[2,3]	32/26
CT268	F: ATG AAA ATG CTC AAA TGT TGT TG R: CTT GGA TCT TCT GGA TTC TAC TAC C	300	-	4/4
CT116	see Bonnema et al. (1997)	1700	*HhaI*[2,3],*MseI*[2,3]	24/24
TG67	see Bonnema et al. (1997)	1000	-	42/28
TG184	see Bonnema et al. (1997)	1155	-	17/17
CT209	see Bonnema et al. (1997)	600	-	33/33
TG51	see Bonnema et al. (1997)	1500	*HinfI*[3]	42/39
Cf-4	F3: TTT CAT GCT ATA TGT CTT TCT C R3: AAT TGG TCC TTC AAG ATG GTT A	1000	*EcoRI*[1,2,3]	1/1

[1]= Polymorphism between MM and MM-Cf4, [2]= polymorphism between CfECP5 and MM-Cf4 and [3]= polymorphism between MM and CfECP5. *Numbers before the dash (/) indicate the number of enzymes tested for MM and CfECP5 only and number behind the dash (/) indicate the number of enzymes tested on all three genotypes.

HhaI and TG51-*HinfI*. The F_2 population of 233 plants derived from the cross MM x CfECP5 was evaluated for the segregation of the CAPS markers Cf4f3r3-*EcoRI*, TG236-*SspI*, CT116-*HhaI* and TG51-*HinfI*. The genotype of the *Cf-ECP5* locus was determined by testing the F_3 lines from the F_2 population of MM x CfECP5 for the segregation of *Cf-ECP5* using PVX::*Ecp5* inoculation assays. From the 231 lines tested, 166 lines showed clear segregation for presence or absence of necrosis. It was often impossible to determine whether the non-necrotic plants exhibited mild mosaic symptoms (indicating a successful PVX infection and consequently absence of *Cf-ECP5*) or whether they escaped from infection by PVX. Therefore, these lines were scored as dominant for the *Cf-ECP5* locus. In the remaining 65 F_3 lines, no necrosis was observed in any plant. Consequently, the corresponding F_2 plants were classified as homozygous *cf-ecp5 cf-ecp5*. Maps of the testcross population and the F_2 were constructed

(Figure 3.2). The dominant marker Cf4f3r3-*Eco*RI cosegregated with *Cf-ECP5* in the F_2 population. *Cf-ECP5* maps proximal to the *Hcr9* Milky Way cluster, but distal to CT116, which is cosegregating with *Cf-ECP2* (Chapter 5).

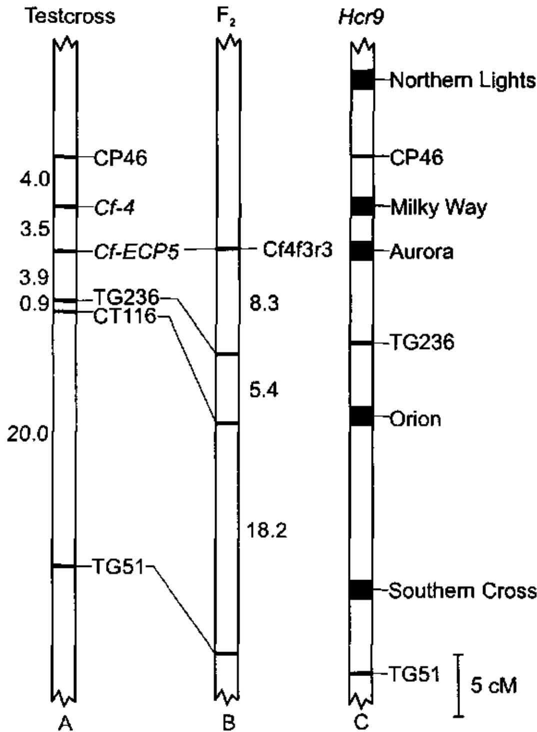


Figure 3.2 Genetic maps of the short arm of chromosome 1 around *Cf-ECP5*. Map A is based on the progeny of the cross MM x (MM-Cf4 x CfECP5), map B on the F_2 population of the cross MM x CfECP5. Map distances (in cM) have been calculated using the software package JoinMap 2.0 (Stam and Van Ooijen, 1996). Map C shows the presence of all *Hcr9* loci so far identified on the short arm of chromosome 1. The location of Northern Lights and Southern Cross were retrieved from Parniske et al. (1999), the location of Orion from Chapter 5 and the locations of Milky Way and Aurora resulted from the present study. Distances between markers are obtained from Bonnema et al. (1997).

Discussion

Cf resistance genes on the short arm of chromosome 1

In the present study we have tested 21 lines for the presence of *Cf* genes that are linked to the *Hcr9* Milky Way cluster on the short arm of chromosome 1 of tomato. We have identified 10 accessions with such putative *Cf* genes, four of which showed a specific response to three different extracellular proteins (ECPs) of *Cladosporium fulvum*: two accessions responded to ECP2, one to ECP3 and one to ECP5 (Table 3.1). For the other six accessions the interaction with specific avirulence factors is still obscure, but as they do not respond to the known avirulence genes, at least one additional resistance gene is present in these lines. In conclusion, at least four unknown *Cf* genes have been characterized by map position and specificity. By using molecular marker analyses, *Cf-ECP2* and *Cf-ECP5* were shown to be closely linked to the *Hcr9* Milky Way cluster. Most likely, the remaining seven other accessions also harbor *Cf* genes near or at the *Hcr9* Milky Way cluster.

Most *Cf* genes that have been investigated in this study originate from *L. pimpinellifolium*. It is likely that even more *Cf* genes are present in the other *Lycopersicon* species, as these species show more genetic variation than *L. pimpinellifolium* (Miller and Tanksley, 1990). In the past, breeders and researchers preferred to study *L. pimpinellifolium* as this species is most closely related to *L. esculentum* and hence is the easiest exploitable species to transfer *Cf* genes to the cultivated tomato. An example of a *Cf* gene in another wild species is the *Cf-4* gene, which probably originates from *L. hirsutum* (Kerr and Bailey, 1964; Thomas et al., 1997). Also *Cf* genes originating from *L. pennellii,* (Stamova et al., 1985) *L. peruvianum* (Kerr and Bailey, 1964), *L. chilense* (Stamova and Yordanov, 1978a) and *L. cheesmanii* (Stamova and Yordanov, 1978b) have been reported. In addition, other solanaceous species have been reported to harbor *Cf* homologues (Kooman-Gersmann et al., 1996) or can specifically recognize ECPs (Laugé et al., 1999). The characterization of *Cf* gene clusters in other tomato and solanaceous species will be of great significance to extend our knowledge on the variation in specificity and structure of *Cf* resistance genes.

Mapping of *Cf-ECP5*

One of the accessions (CfECP5) harbored a *Cf* gene on the short arm of chromosome 1 that responded to exposure to the *C. fulvum* extracellular protein ECP5, which has been isolated by Laugé et al. (1999). This gene that was designated *Cf-ECP5*, was mapped using different strategies. Firstly, a large testcross population was evaluated for resistance to the *C. fulvum* races 0 and 2.4.5 and for reaction to PVX::*Avr4* to determine the segregation and hence the linkage of *Cf-4* and *Cf-ECP5*. Secondly, an analysis with chromosome 1-specific CAPS markers was performed to more accurately determine the genetic distance between these *Cf* genes and marker loci on the short arm of chromosome 1. Thirdly, F_3 lines of a cross between MM and CfECP5 were inoculated with PVX::*Ecp5* to determine the genotype of the *Cf-ECP5* locus in the F_2 plants. This assay also provided additional information on the distance between *Cf-ECP5* and several marker loci on the short arm of chromosome 1. There were differences between the map that was based upon the testcross progeny and the map based on the F_2 population. Whereas the distance between CT116 and TG236 was 1 cM in the tescross progeny, this distance was 6 cM in the F_2 population. We have previously mapped *Cf-ECP2*, using a similar F_2 population. Like *Cf-ECP5*, *Cf-ECP2* also originates from a wild *L. pimpinellifolium* species. In the latter F_2 population , the distance between CT116 and TG236 was 4.5 cM (Chapter 5). This closer resembles the distance between CT116 and TG236 observed in the F_2 mapping population, than that observed in the testcross population of CfECP5. However, the distance between CT116 and TG51 in the F_2 and tescross population with *Cf-ECP5* was 18.1 and 20.0 cM respectively, which is more similar. Previously, it has been shown that recombination frequencies are decreased around the TG236 locus in a cross between *L. hirsutum* (the supposed donor of *Cf-4*) and *L. esculentum*, relative to recombination frequencies in a cross between *L. peruvianum* and *L. esculentum*. The introgression segment of *Cf-4* in MM extends over TG236 but not over TG51 (Parniske et al., 1999). If *L. pimpinellifolium* (the donor of *Cf-ECP5*) resembles *L. esculentum*, and therefore shows normal recombination in crosses with *L. esculentum* and decreased recombination frequencies around TG236 in crosses with *L. hirsutum*, the decreased recombination around TG236 in crosses with *L. hirsutum* may explain the variation in recombination differences between both mapping populations as well as between different markers within one mapping population.

In conclusion, we have mapped *Cf-ECP5* on the short arm of chromosome 1, between the *Hcr9* Milky Way cluster and CT116. The CAPS marker Cf4f3r3-*Eco*RI, which is based upon

sequence homology to *Cf-4*, cosegregated with *Cf-ECP5*, indicating the presence of an *Hcr9* at this locus. Previously, we have mapped *Cf-ECP2*, which is at an *Hcr9* locus that is closely linked to CT116 (Chapter 5). This locus has been designated "Orion". Since no *Hcr9* clusters have been mapped between Milky Way and Orion before, we have designated the *Hcr9* locus containing *Cf-ECP5* "Aurora".

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Chapter 4

Dispersion of *Cf-4* in *Lycopersicon* germplasm

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Submitted to Heredity

Abstract

In the past, numerous *Cf* genes have been reported in tomato (*Lycopersicon esculentum* Mill.) that confer resistance against leaf mold (*Cladosporium fulvum* Cke.). We are interested in genetic variation at *Cf* loci. Therefore, previously uncharacterized *Cf* genes were further analyzed. Recognition of the AVR4 elicitor, DNA gel blot analysis, PCR analysis and sequencing of part of the *Cf-4* locus showed that a large proportion of the accessions tested harbored the *Cf-4* resistance gene. We concluded that despite differences in nomenclature, all these accessions harbor the same *Cf-4* locus, probably introgressed from the same donor. The origin of the *Cf-4* locus and the reasons for discrepancies with earlier reports is discussed.

Introduction

The interaction between tomato (*Lycopersicon esculentum* Mill.) and its fungal pathogen *Cladosporium fulvum* Cke., causing leaf mold, complies with the gene-for-gene relationship (Van den Ackerveken and De Wit, 1994). Breeding for resistance against this fungal pathogen dates back to the early 1930s when the first gene, *Cf-1*, was identified in a cultivated tomato line (Langford, 1937). Resistance conferred by *Cf-1* was rendered ineffective by adaptation of the pathogen within a few years, which prompted breeders to introduce another gene (*Cf-2*) which was also rapidly rendered ineffective. The introduction of new *Cf* genes and the occurrence of new races made continuous breeding for resistance inevitable. This effort resulted in the introgression of numerous *Cf* genes in tomato cultivars. Often, one *Lycopersicon* accession was used as the donor of several different *Cf* genes (Kanwar et al., 1980a,b), while for other *Cf* genes, e.g. *Cf-11*, the original donor could not be determined (Kerr et al., 1980). As a consequence, there is much confusion about the designation of *Cf* genes. For example, Cf-*4* was thought to originate from *L. hirsutum* 3833 (Kerr and Bailey, 1964 ;Thomas et al., 1997), but *L. peruvianum* and *L. pimpinellifolium* were also reported to harbor a gene that conferred resistance to a similar spectrum of *C. fulvum* races (Kerr and Bailey, 1964). Moreover, it was found that the genotype supposed to harbor *Cf-8* contained a *Cf* resistance gene, which conferred resistance to a similar spectrum of C. fulvum races as *Cf-4* (Gerlagh et al., 1989). Meanwhile, the resistance genes *Cf-4* and *Cf-9* and their corresponding *Avr* genes have been isolated (Thomas et al., 1997; Jones et al., 1994; Joosten et al., 1994; Van den Ackerveken et al., 1992). *Cf-4* and *Cf-9* reside in a cluster of *Cf* homologues on the short arm of chromosome 1, designated 'Milky Way' (Parniske et al., 1997). The *Cf-4* cluster harbors two functional resistance genes, *Cf-4* and *Hcr9-4E* (*Cf-4A* according to Takken et al., 1998) Moreover, other clusters of *Cf* homologues have been identified on the short arm of chromosome 1 (Parniske et al., 1999). We are interested in characterizing additional *Cf* genes on the short arm of chromosome 1. One challenging question is raised by the reports that *Cf-4* is present in several different *Lycopersicon* species (Kerr and Bailey, 1964). Either *Cf-4* originates from an ancient common ancestor and was present before these species diverged, or that *Cf-4* evolved independently in different *Lycopersicon* species. If these species harbor different *Cf-4* alleles that all recognize AVR4, the sequence variation in these alleles could provide information about sequences that are necessary for AVR4 recognition. We have screened 64 accessions reported to contain *Cf* resistance for the presence of *Cf-4* by infection

with transgenic PVX, expressing the *Avr4* gene. In this report we present the results of the screening with PVX::*Avr4* and the subsequent analysis of the *Cf-4* locus in accessions harboring a functional *Cf-4* gene. The results are discussed in the scope of breeding history, nomenclature and the origin of *Cf-4*.

Materials and Methods

Plant material and PVX assays

We screened 64 out of 66 available *Lycopersicon* genotypes with reported *Cf* resistance for the presence of a functional *Cf-4* gene using PVX::*Avr4* assays (Chapter 3). The *L. esculentum* accessions LA2443 and LA3047 were not tested. Accessions which showed systemic necrosis upon infection with PVX::*Avr4* are listed in Table 4.1. We considered the genotypes *L. esculentum* G1.1148 and LA3045 as controls, since they have been reported as being Moneymaker-Cf4 and the *Cf-4* gene was isolated from this genotype (Thomas et al., 1997). The genotype *L. minutum* CGN15815 was not further analyzed since two week old seedlings were too small to determine the reaction of this accession to PVX::*Avr4* unambiguously.

DNA isolation and Southern analysis

Gel blot analysis using genomic DNA isolated according to the protocol described by Van der Beek et al. (1992) was performed as described by Thomas et al. (1997).

DNA sequence analysis

A 1500 bp fragment was amplified by PCR using the forward primer GAG TTC AAG TCC AAA ACA TTA AGT A and the reverse primer TTT TAT CTG CAT CCC AAG CAA CTG, which amplified the 3' part of the *Cf-4* gene as well as part of the untranslated 3' region (Figure 4.1). The PCR product was cloned into the pGEM-T Easy vector (Promega,Leiden, The Netherlands). The cloned fragments were prepared for sequence analysis using the primers T7 and SP6 in a reaction using the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, La Jolla, CA). Sequence products were run on an automated sequencer (model 373A; Applied Biosystems) and aligned using the software module Megalign (DNAStar Inc.) for sequence comparison. Each clone was sequenced twice.

Results

Presence of a functional *Cf-4* gene

Of the 64 lines tested 17 exhibited systemic necrosis upon infection with PVX::*Avr4* (Table 4.1). Surprisingly, the accession *L. esculentum* CN0697, reported to harbor *Cf-4* from *L. peruvianum* (Bailey and Kerr, 1964), showed no systemic necrosis upon PVX::*Avr4* infection. Of these 17 accessions, ten have been reported to harbor *Cf-4* (nrs 1-4 and 9-14 from Table 4.1). In addition, three *L. esculentum* accessions with unidentified resistance reacted to PVX::*Avr4*, indicating that these accessions also harbor an active *Cf-4* gene. As expected, both accessions reported to harbor the gene *Cf-8*, as well as the accessions reported to harbor the genes *Cf-11* and *Cf-13* respectively, exhibited systemic necrosis upon infection with PVX::*Avr4*, indicating a functional *Cf-4* gene. Previously it has been reported that *Cf-8* is indistinguishable from *Cf-4* (Gerlagh et al., 1989). On the other hand, *Cf-11* previously showed a differential interaction upon inoculation with different races of *C. fulvum*, and was resistant to race 2.4.5 (Lindhout et al., 1989).

Table 4.1 Accessions that showed necrosis upon infection with PVX::*Avr4* with their common names and reported origin of *Cf* resistance.

Nr	*L. esculentum* accession nr.	Common name	Reported origin of *Cf* resistance
1	G1.1148	Moneymaker-Cf4	*L. hirsutum*[1]
2	LA3045	Moneymaker-Cf4	*L. hirsutum*[1]
3	LA2446	Purdue 135	*L. hirsutum*[1]
4	CGN15397	Purdue 135	*L. hirsutum*[1]
5	881271	*Cf-8*	*L. pimpinellifolium* PI124161[2]
6	CN2002	*Cf-8*	*L. pimpinellifolium* PI124161[2]
7	G1.1653	*Cf-11*	unknown[3]
8	CN6766	*Cf-13*	*L. pimpinellifolium* PI211839[4]
9	CN0078	Vantage	*L. peruvianum*[1]
10	CN0354	V501	*L. hirsutum* var *glabratum*[1]
11	CN0355	V542	*L. peruvianum*[1]
12	CN0335	V543	*L. peruvianum*[1]
13	CN0356	V545	*L. pimpinellifolium*[1]
14	CN0698	V593	*L. peruvianum*[1]
15	LA3051	unknown	unknown
16	LA3266	unknown	unknown
17	LA3267	unknown	unknown

[1]Bailey and Kerr (1964)
[2]Kanwar et al. (1980a); PI124161 is the donor of *Cf-7* and *Cf-10*.
[3]*Cf-11* appeared as an aberrant plant in the line Massachusetts No. 2 (Kerr and Patrick, 1977)
[4]Kanwar et al. (1980b); PI211839 is the donor of *Cf-6*, *Cf-12* and also *Cf-14*.

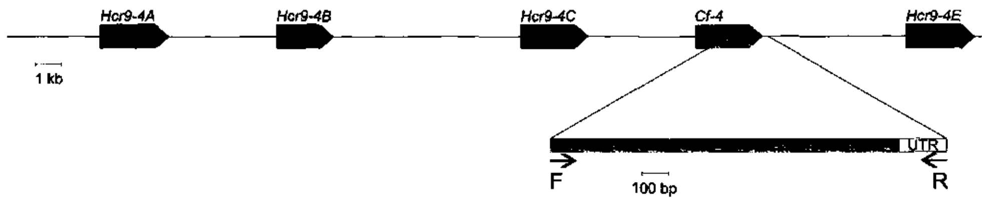

Figure 4.1 Position of the primers used for sequencing the 3' of *Cf-4* and 200 bp of the 3' untranslated region (UTR) in the tomato genotypes 3-17 of Table 4.1.

Southern analysis of the *Cf-4* locus

The accessions that exhibited necrosis upon infection with PVX::*Avr4* were reported to harbor *Cf* genes originating from various donors (Table 4.1), as well as several lines with multiple *Cf* genes (Bailey and Kerr, 1964). In order to examine the presence of *Cf* homologues in these accessions, we performed Southern analysis using the 5' end of *Cf-9* (Jones et al., 1994) as a probe on *Bgl*II digested genomic DNA (Figure 4.2). All lines do not only harbor a *Cf-9* hybridizing band with exactly the same mobility as the *Cf-4* gene, but also a band at the same position as the *Hcr9-4E* gene, which is located downstream of *Cf-4* (Thomas et al., 1997). These results imply that not only *Cf-4*, but also *Hcr9-4E* is present in these lines. Except for the *L. esculentum* accessions CN0354 (V501) and CN6766 (*Cf-13*), which contain two and one additional hybridizing bands, respectively (indicated with * in Figure 4.2), the hybridization patterns of lines containing a functional *Cf-4* gene were identical.

Partial sequence analysis of *Cf-4*

The *Cf-4* genes from some accessions were reported to originate from other *Lycopersicon* species than *L. hirsutum*, like *L. peruvianum* and *L. pimpinellifolium* (Table 4.1). There might be a possibility that *Cf-4* was present in an ancestor of these *Lycopersicon* species. If so, most likely small sequence changes would have occurred in course of time. Also, if *Cf-4* would have evolved independently in these *Lycopersicon* species, sequence differences would be likely. In order to investigate the possibility that slightly different genes confer recognition to AVR4, a part of the 3' end of *Cf-4*, as well as approximately 200 bp of the untranslated 3' region were sequenced (Figure 4.1). Of each of the genotypes listed in Table 4.1, except the Moneymaker-*Cf-4* lines, two clones were sequenced. All sequences were identical to the reported sequence of *Cf-4* (Parniske et al., 1997; Thomas et al., 1997).

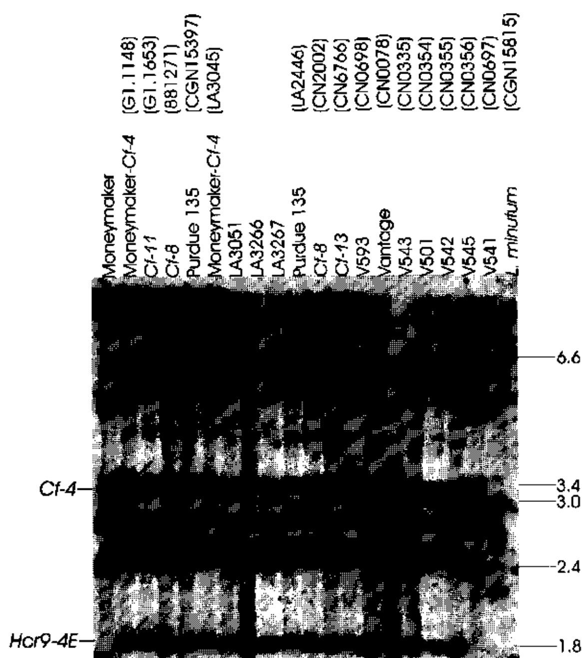

Figure 4.2 Southern analysis of tomato genotypes with the 5' fragment of *Cf-9*. Names of tomato genotypes are above the lanes and their numbers are as described in Table 4.1 and Materials and Methods. The location of the *Cf-4* and *Hcr9-4E* genes are indicated by arrows.

Discussion

Dispersion of *Cf-4*

We have identified and further investigated 17 *Lycopersicon* accessions that exhibited systemic necrosis upon infection with PVX::*Avr4*. Several of these lines were reported to have a different breeding history with respect to their reported *Cf* donor (Bailey and Kerr, 1964). Therefore, we have asked the question: Was *Cf-4* already present in a common ancestor of these *Lycopersicon* species or did a functional *Cf-4* develop independently in these species? To answer this question we have analyzed the *Cf-4* locus by Southern hybridization as well as determined the DNA sequence of part of the *Cf-4* gene. There were no differences in the sequences obtained. The lack of differences in the 3' end of *Cf-4* and the 3' untranslated region

is in contrast with both the hypothesis that *Cf-4* originates from a common ancestor as well as the hypothesis of independent evolution of a functional *Cf-4* gene in different *Lycopersicon* accessions. These results point to a common donor of *Cf-4* in all the breeding lines tested, which is, of course, a much more recent event. Certainly, all the lines with a reported *Cf* gene result from the work performed at dr. Kerr's laboratory. Therefore, most likely *Cf-4* was present in a common parent of these lines and that *Cf-4* was crossed into these breeding lines unintentionally, since no matching *Avr* gene was characterized at that time. Indeed, it has been mentioned that lines containing *Cf-4* have been used for generating a number of tomato breeding lines (Kanwar and Kerr, pers. comm.). Also the Southern analyses support this hypothesis, for all genotypes that show a systemic HR upon infection with PVX::*Avr4* not only have an identical band compared to the *Cf-4* band, but also a band comigrating with *Hcr9-4E,* which is at the same locus and several other hybridizing bands which are characteristic of *Hcr9*s at the *Cf-4* locus (Thomas et al., 1997).

It has been shown previously that *Cf-8* is indistinguishable from *Cf-4* since there is no differential interaction and the *Cf* resistance is allelic to *Cf-4* (Gerlagh et al., 1989). Here, we have presented molecular evidence to support these results. On the other hand, *Cf-11* shows a differential interaction, although all fungal races that overcome *Cf-11* are also able to overcome *Cf-4* (Lindhout et al., 1989). Therefore, *Cf-11* probably has an additional *Cf* gene, which was not detected by Southern hybridization and which is therefore probably not located at the *Cf-4* locus. The line reported to harbor *Cf-13* at least harbors *Cf-4*. It is possible that this line, like *Cf-11* harbors another *Cf* gene. To resolve this possibility, disease tests have to be performed to compare *Cf-4* and *Cf-13* with as many different races as possible.

This report shows that an identical *Cf-4* gene is present in many breeding lines. *Cf-4* is probably originally introgressed from *L. hirsutum* and subsequently the resulting line has been used to generate these breeding lines. As a result, we propose to change the name of *Cf-8* into *Cf-4* and add *Cf-4* to the lines *Cf-11* and *Cf-13.*

Acknowledgements

We thank Yuan Yinan for technical assistance.

Chapter 5

The *Cf-ECP2* gene is linked to, but not part of the *Cf-4/Cf-9* cluster on the short arm of chromosome 1 of tomato

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Abstract

A gene has been identified in tomato, which confers resistance to *Cladosporium fulvum* through recognition of the pathogenicity factor ECP2. Segregation analysis of F_2 and F_3 populations showed monogenic dominant inheritance, similar to previously reported *Cf* resistances. The gene has been designated *Cf-ECP2*. Using several mapping populations, *Cf-ECP2* has been accurately mapped on chromosome 1, 7.7 cM proximal to TG236 and 6.0 cM distal to TG184. Although *Cf-ECP2* is linked to *Cf-4*, it is not located on the *Hcr9* cluster 'Milky Way'. Therefore, *Cf-ECP2* is the first functional *Cf* homologue on chromosome 1, which does not belong to this *Hcr9* cluster. No recombination event between *Cf-ECP2* and CT116 has been observed in three populations tested representing 282 individuals. Given the low physical distance per cM around CT116 reported previously and the high probability of *Cf-ECP2* to be a *Hcr9*, this facilitates cloning of *Cf-ECP2* using *Cf-9*, as well as CT116.

Introduction

The relationship between the cultivated tomato (*Lycopersicon esculentum*) and *Cladosporium fulvum* Cke., the causal agent of leaf mould, is one of the best studied plant-pathosystems (Van den Ackerveken and De Wit, 1994). Since the 1930s many genes for resistance to *C. fulvum* have been identified in wild tomato species and subsequently introgressed into the cultivated tomato (Stevens and Rick, 1988). However, soon after the introduction of resistant cultivars, new races of *C. fulvum* have appeared, that rendered the resistance ineffective (Bailey and Kerr 1964; Laterrot et al. 1985; Laterrot 1986). This phenomenon of non-durable resistance has been observed in many plant-pathogen relationships. The gene-for-gene hypothesis (Flor, 1942) explained the non-durability of plant-pathogen interactions. The tomato - *C. fulvum* interaction proved to be an ideal model system to study the gene-for-gene interaction. The plant resistance genes *Cf-4* (Thomas et al. 1997) and *Cf-9* (Jones et al. 1994) have been isolated as well as the corresponding fungal avirulence genes *Avr4* (Joosten et al. 1994) and *Avr9* (Van den Ackerveken et al. 1992) respectively. Fungal races that were able to circumvent the *Cf-4* resistance harbored mutant *Avr4* alleles (Joosten et al. 1994) that differed only by a single base-pair from the wild-type *Avr4* allele, while fungal races which were able to colonize plants carrying the *Cf-9* resistance gene lacked the *Avr9* gene completely Van Kan et al., 1991).

Another *C. fulvum* gene, *Ecp2*, that plays an important role in the pathogenicity, has been isolated (Van den Ackerveken et al. 1993; Laugé et al. 1997). Gene replacement studies showed that strains lacking *Ecp2* poorly colonized the leaf and produced only few conidia (Laugé et al. 1997). It is expected that *Cf* genes conferring resistance upon recognition of pathogenicity factors might give rise to a more durable resistance, because an isolate with an altered corresponding *Avr* gene may lose its pathogenicity. In a survey of 21 *Lycopersicon* accessions four lines were identified that showed a specific HR upon inoculation with a recombinant potato virus X derivative, expressing *Ecp2* (Laugé et al. 1998a). These lines all originated from one *L. pimpinellifolium* accession (PI126947), which also showed specific necrosis to ECP2 (Laugé et al. 1998a). Boukema (1980) provided evidence that this *L. pimpinellifolium* accession contained a resistance gene allelic to *Cf-9*. Most *Cf* genes mapped so far are closely linked or allelic and map either on chromosome 1 or chromosome 6 (Balint-Kurti et al. 1994, Dickinson et al. 1993). Molecular analyses demonstrated the presence of

three clusters that contain *Cf* homologues on the short arm of chromosome 1, hereafter referred to as *Hcr9* clusters (Parniske et al. 1999). The functional *Cf* genes of chromosome 1 that have been cloned so far, *Cf-4, Cf-4A* and *Cf-9* (Thomas et al. 1997;Takken et al. 1998; Jones et al. 1994) reside in one of these three *Hcr9* clusters, designated the Milky Way cluster (Parniske et al. 1999). It was hypothesized that *Cf-ECP2* could be located in one of the three clusters on the short arm of chromosome 1. This report describes the accurate mapping of *Cf-ECP2* on the short arm of chromosome 1 of tomato between two of the three previously reported *Hcr9* clusters, Milky Way and Southern Cross, respectively .

Materials and Methods

Plant material

From a collection of *C. fulvum* resistant genotypes, the four breeding lines Ontario 7518 (Cf18), Ontario 7520 (Cf20), Ontario 7523 (Cf23) and Ontario 7819 (Cf24) (Kanwar et al. 1980b) as well as their wild ancestor *L. pimpinellifolium* PI126947 showed systemic HR upon infection with a potato virus X (PVX) strain expressing the *Ecp2* gene (PVX::*Ecp2*; Laugé et al. 1998a). Three of these lines, Cf18, Cf20, and Cf24, were crossed to the near-isogenic line Moneymaker-Cf4 (MM-Cf4). Subsequently, the F_1 plants were crossed to the susceptible tomato cultivar Moneymaker (MM). The progenies of these testcrosses were designated "TCf18", "TCf20" and "TCf24", respectively.
An F_2 population of 106 plants from a cross of MM x Ontario 7518 ('F_2Cf18') was selfed, rendering an F_3, designated 'F_3Cf18'.

C. fulvum resistance test

A strain of *C. fulvum* race 0 was obtained from the IPO-DLO (Wageningen, The Netherlands). The fungus was grown on potato dextrose agar (Duchefa, Haarlem, The Netherlands) at 22 °C and sporulated abundantly in about 14 days. Twenty-four plants of each of the populations TCf18 and TCf20, as well as MM and MM-Cf4 control plants, were inoculated and scored as described by Lindhout et al. (1989) with some minor modifications. A relative humidity of 100% was maintained for 24 hrs by using a humidifier. Plants at the two-true-leaf stage were inoculated twice, with a two-day interval.

PVX::*Avr4*, PVX::*Avr9* and PVX::*Ecp2* assays

Plants of the breeding lines Cf18, Cf20, Cf23, Cf24 and their ancestor *L. pimpinellifolium* PI126947 were inoculated at the two-leaf stage with PVX expressing the *Avr4* gene (PVX::*Avr4*; Joosten et al. 1997) as well as PVX::*Avr9* (Hammond-Kosack et al. 1995). Two weeks after inoculation the plants were scored visually in two classes: necrotic (HR) or mosaic (non-HR).

From 79 TCf18 plants as well as from 108 TCf24 plants, two cuttings per plant were taken for further analysis. After fourteen days one of these plants was inoculated with PVX::*Avr4* and the other with PVX::*Ecp2* (Laugé et al. 1998a). Two weeks after inoculation the plants were scored as described above for the PVX::*Avr4* and PVX::*Avr9* tests.

Cuttings of 106 F_2Cf18 plants and at least ten F_3Cf18 plants per F_2 parent were inoculated with PVX::*Ecp2* and analyzed as described above. If upon infection with PVX::*Ecp2* an F_3 line did not contain any plant exhibiting necrosis, but only mild mosaic symptoms, we classified the F_2 parent as homozygous *cf-ecp2*. If an F_3 line contained plants exhibiting necrosis, as well as plants exhibiting only mosaic symptoms, we classified the corresponding F_2 parent as heterozygous (*Cf-ECP2/cf-ecp2*). If all plants of an F_3 line exhibited necrosis we classified the line as homozygous *Cf-ECP2*.

DNA isolation, CAPS and genetic linkage analysis

DNA was isolated from frozen leaf tissue according to a protocol described by Van der Beek et al. (1992). DNA of the parents Moneymaker, Cf18, Cf24 and *L. pimpinellifolium* PI126947 was used to screen for Cleaved Amplified Polymorphic Sequences (CAPS) markers. PCR primers for all CAPS markers on the short arm of chromosome 1 were kindly provided by G. Bonnema (Bonnema et al. 1997, Table 5.2), with the exception of the FT33 PCR primers that were kindly provided by F. Takken (Free University, Amsterdam, The Netherlands). A PCR was carried out in a total volume of 50 µl which contained 50 ng DNA, 50 ng of each primer, 100 µM dNTPs, 1x *Taq* buffer (Amersham, UK) and 0.2 U *Taq* polymerase (Amersham, UK). PCR products of the parents were digested with several restriction enzymes (Table 5.2) to identify which primer pair-restriction enzyme combinations generated CAPS markers. Marker analysis was performed on DNA of pooled leaves of the 106 lines of F_3Cf18. Recombination frequencies and LOD values were calculated using the mapping software package JoinMap 2.0

(Stam and Van Ooijen, 1996). The segregation of the CAPS marker CT116-*Hha*I was evaluated for the populations TCf18 and TCf24, which were also screened with PVX::*Avr4* and PVX::*Ecp2*.

Southern hybridization

*Bgl*II digested DNA of pooled leaves of 10 plants of 26 lines of the F_3Cf18 population was fractionated on a 0.8 % agarose gel and blotted onto a Hybond-N+ membrane. Filters were hybridized with the ^{32}P-labeled 753 bp *Xho*I-*Sst*I 5' fragment of the *Cf-9* gene (probe 1: Jones et al. 1994) at 65°C. Blots were washed at 65°C, twice in 2x SSPE, 0.1% SDS, once in 1xSSPE, 0.1% SDS and finally once in 0.2xSSPE, 0.1% SDS.

Results

Presence of *Cf-4* and *Cf-9*

Recently, the lines Cf18, Cf20, Cf23, Cf24 and their ancestor *L. pimpinellifolium* PI126947 were shown to harbor *Cf-ECP2* (earlier designated line #1-line#4 and ancestor, respectively; Laugé et al. 1998a). In an earlier study, Boukema (1980) did not detect any susceptible recombinants in 100 plants of a testcross population derived from a cross between *L. pimpinellifolium* PI126915 (the original donor line of *Cf-9*) and *L. pimpinellifolium* PI126947. This suggested that *L. pimpinellifolium* PI126947 carries either *Cf-9*, or a *Cf* gene which is very closely linked or allelic to *Cf-9*, or both. To test for the presence of *Cf-4* or *Cf-9*, all parents exhibiting necrosis upon inoculation with PVX::*Ecp2* were inoculated with PVX::*Avr4* and PVX::*Avr9* (results not shown). None of the parents Cf18, Cf20, Cf23, Cf24, neither their ancestor *L. pimpinellifolium* PI126947, showed necrosis upon inoculation with PVX::*Avr4*. Upon inoculation with PVX::*Avr9*, only *L. pimpinellifolium* PI126947 exhibited necrosis. This indicates the presence of at least two resistance genes, *Cf-9* and *Cf-ECP2*, in this *L. pimpinellifolium* accession, but that the breeding lines Cf18, Cf20, Cf23 and Cf24, which are derived from this accession only contain *Cf-ECP2*.

Inheritance of *Cf-ECP2*

We hypothesized that *Cf-ECP2*, like all reported *Cf* resistances that show HR, inherits as a monogenic dominant trait. To test this hypothesis we evaluated a population of 106 F_2 plants

from a cross (MM x Cf18) for the segregation of *Cf-ECP2*. Eighty-one plants showed necrosis and 18 plants mild mosaic symptoms. The remaining seven plants could not be scored unambiguously. This segregation is not different from 3:1 (P>0.1) and indicates a monogenic dominant inheritance.

To discriminate between the homozygous and heterozygous *Cf-ECP2* plants, each F_2 plant was selfed and F_3 progenies were collected and evaluated for *Cf-ECP2* segregation. The number of homozygous *cf-ecp2*, heterozygous, and homozygous *Cf-ECP2* plants were 21, 64 and 21 respectively, corresponding to a monogenic inheritance (P>0.1). This substantiated the hypothesis of one dominant gene *Cf-ECP2* for resistance to *C. fulvum* conferred by HR-associated recognition of ECP2.

Linkage tests between *Cf-ECP2* and *Cf-4*

To investigate the possibility that *Cf-ECP2* was linked to the Milky Way cluster we crossed two *Cf-ECP2* harboring lines (Cf18 and Cf20) to the tomato cultivar MM-Cf4, and the resulting F_1 to the susceptible cultivar MM. For both populations 24 plants were evaluated for susceptibility or resistance to *C. fulvum* race 0. In case no linkage between *Cf-ECP2* and *Cf-4* would exist, a 3:1 segregation ratio between resistant and susceptible plants is expected in these populations. In case of linkage, relatively more resistant plants are expected. Three weeks after inoculation with *C. fulvum* race 0, none out of 24 plants of TCf18 were sporulating and for TCf20 two out of 24 plants sporulated, indicating genetic linkage between *Cf-ECP2* and *Cf-4* (P<0.005 and <0.1 resp.).

In a second experiment larger testcross populations of TCf18 and a TCf24 were inoculated with PVX::*Ecp2* and with PVX::*Avr4* to identify the presence of the *Cf-ECP2* and *Cf-4* genes, respectively. The plants were divided into four classes: necrotic to both PVX strains, only necrotic to PVX::*Ecp2*, only necrotic to PVX::*Avr4* or necrotic to none of the strains (Table 5.1). In case of no linkage the segregation for the four classes should be 1:1:1:1. However, for both the TCf18 as well as the TCf24 population this segregation deviated significantly from 1:1:1:1 (P<0.001), indicating linkage between *Cf-ECP2* and *Cf-4*. The recombination percentages between these two *Cf* genes in the two populations were 13 and 10, respectively, corresponding to a genetic distance of 11.3 ± 1.8 cM.

Table 5.1 Segregation of *Cf-ECP2* and *Cf-4* within two testcross populations using PVX::*Ecp2* and PVX::*Avr4* respectively.

population	No *Cf* genes[a]	*Cf-ECP2*[b]	*Cf-4*[c]	Both *Cf* genes[d]	Missing[e]	χ^{2f}
TCf18	5	36	32	5	1	43.5
TCf24	7	47	49	4	1	67.8

[a] No *Cf* genes indicates that the plants of one genotype did not react necrotic to PVX::*Ecp2* nor to PVX::*Avr4*.
[b] *Cf-ECP2* indicates that only plants inoculated with PVX::*Ecp2* exhibited necrotic symptoms.
[c] *Cf-4* indicates that only plants inoculated with PVX::*Avr4* exhibited necrotic symptoms.
[d] Both *Cf* genes indicates that plants inoculated with PVX::*Ecp2* as well as plants inoculated with PVX::*Avr4* both exhibited necrotic symptoms.
[e] Missing indicates plants that escaped from PVX infection.
[f] The populations were tested for a 1:1:1:1 segregation using a χ^2 test, indicating that the *Cf-ECP2* and *Cf-4* genes are unlinked. The threshold value (P<0,001) was 16.3.

Assignment of *Cf-ECP2* to the short arm of chromosome 1

In order to map *Cf-ECP2* more accurately, we screened the susceptible tomato cultivar Moneymaker and lines harboring *Cf-ECP2* for chromosome 1-specific CAPS markers. Out of 366 primer-enzyme combinations tested, we detected twelve polymorphisms, representing seven from the twelve loci tested (Table 5.2). Based on these CAPS markers the size of the introgression fragment of *Cf-ECP2* in the Cf18 and Cf24 lines was determined (Figure 5.1). The Cf24 line showed the smallest introgression fragment of less than 26 cM, covering only the CT116 and TG184 loci.

For mapping of *Cf-ECP2*, the F_2Cf18 population was evaluated for the CAPS markers FT33-*Taq*I, TG236-*Ssp*I, TG184-*Bcl*I and CT116-*Hha*I and for HR-response to PVX::*Ecp2*. Linkage analysis showed that no recombination between *Cf-ECP2* and CT116-*Hha*I was detected in this population (Figure 5.2). To generate an accurate map around *Cf-ECP2*, 74 TCf18 and 102 TCf24 plants were tested for segregation of PVX::*Avr4*, PVX::*Ecp2* and CT116-*Hha*I. Again, CT116-*Hha*I cosegregated completely with *Cf-ECP2* (Figure 5.3).

Southern analysis

As all reported *Cf* genes on the short arm of chromosome 1 are homologous to *Cf-9* (*Hcr9s*), RFLP analysis with a *Cf-9* probe was performed to detect *Hcr9*s in the lines carrying *Cf-ECP2*: *L. pimpinellifolium* PI126947, Cf18, Cf23 and Cf24 (data not shown). As expected from the results obtained with PVX::*Avr9*, *L. pimpinellifolium* PI126947 showed the 6.7 kb *Bgl*II fragment corresponding to *Cf-9* (Jones et al. 1994). This fragment was not present in Cf18, Cf23 and Cf24. These three breeding lines show one extra *Bgl*II fragment of ~7.5 kb that was

not detected in the near isogenic lines MM, MM-Cf4 and MM-Cf9. In the F_3Cf18 population, this *Bgl*II fragment of ~7.5 kb cosegregated with the presence of *Cf-ECP2* (Figure 5.4).

Table 5.2 Loci, primer sequences, lengths of PCR products and restriction enzymes revealing polymorphisms between *L. esculentum* cv. Moneymaker (MM) and different *Cf-ECP2* lines.

Locus	Primer sequence	PCR product length (bp)	Enzymes detecting polymorphisms	Total number of restriction enzymes tested
CT233	see Bonnema et al. (1997)	1650	*Taq*I[3], *Dde*I[3], *Hae*III[3], *Hind*III[3] *Nco*I[3]	43
CT2	F: AAG CCT CTA ATC AAG AAA ATG G R:TTC AGT GCA ATA ATA ATG AGG G	490	*Hpa*II[1]	35
TG301	see Bonnema et al. (1997)	800	-	42
CP46	see Bonnema et al. (1997)	1000	-	30
FT33	F: AGA AGG ATA AAG CTC AAC ATC GG R: AAG GAA CAT CTG TGG TTC GC	1100	*Taq*I[1,2]	14
TG236	see Bonnema et al. (1997)	1000	*Ssp*I[1,2]	42
CT268	F: ATG AAA ATG CTC AAA TGT TGT TG R: CTT GGA TCT TCT GGA TTC TAC TAC C	300	-	4
CT116	see Bonnema et al. (1997)	1700	*Hha*I[1,2,3], *Hinf*I[1,2,3]	25
TG67	see Bonnema et al. (1997)	1000	-	42
TG184	see Bonnema et al. (1997)	1155	*Bcl*I[1,2,3]	18
CT209	see Bonnema et al. (1997)	600	-	26
TG51	see Bonnema et al. (1997)	1500	*Hinf*I[1]	45

[1]= Polymorphism between MM and *L. pimpinellifolium* PI126947, [2]= polymorphism between MM and Cf18 and [3]= polymorphism between MM and Cf24. F: forward primer, R: reverse primer.

Discussion

Classical breeding for resistance to *Cladosporium fulvum* in tomato has resulted in many resistant cultivars, of which some contain genes with known specificity and some with unknown specificity (Van der Beek et al. 1992). After screening with the fungal protein ECP2, we found that the breeding lines Cf18, Cf20, Cf23 and Cf24, which were reported to contain different *Cf* genes (Kanwar et al. 1980b) all contain one resistance gene responding to ECP2. Consequently, this gene was designated *Cf-ECP2* (Laugé et al. 1998a). In contrast with their identical specificity these *Cf* lines have been designated with different symbols, notably Cf18,

Cf20, Cf23 and Cf24 (Kanwar et al. 1980b), based on phenotypic differences in the resistance reaction to *C. fulvum*. Similarly, Gerlagh et al. (1989) showed that *Cf-4* and *Cf-8* are undistinguishable with respect to their interaction with *C. fulvum* races. These results indicate that phenotypic differences in the resistance reaction are not conclusive for the designation of a new *Cf* gene. Hence, for the correct designation of a new *Cf* gene, it is essential either to find races of *C. fulvum* that differentiate between this new *Cf* gene and all previous described *Cf* genes or to characterize it by its specific interaction with a new avirulence gene like *Ecp2*. Based on these results and considerations we propose not to use the designations Cf18, Cf20, Cf23 and Cf24 anymore, but to replace them all by *Cf-ECP2*.

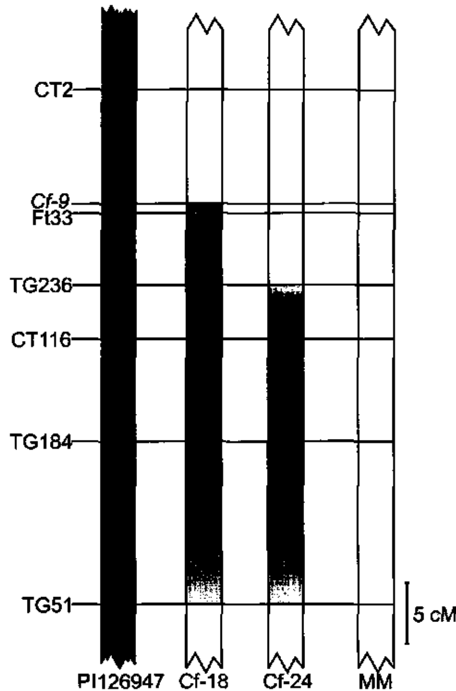

Figure 5.1 Size of the introgression fragments in lines harboring *Cf-ECP2* on the short arm of chromosome 1 in the breeding lines Cf18 and Cf24, based upon the CAPS between the donor genotype, *L. pimpinellifolium* PI126947 (PI126947) and the susceptible tomato cultivar Moneymaker (MM; see also Table 5.2). CAPS loci are depicted at the left. Distances between loci are estimated based on the present F_2 population (FT33, TG236, CT116 and TG184) and other tomato maps (CT2: Tanksley et al. 1992 and TG51: Bonnema et al. 1997). Since *Cf-4* and *Cf-9* are nearly allelic (Thomas et al. 1997), the position of *Cf-9* is the same as the position of *Cf-4* calculated from our experiments (see text). The white bar indicates the Moneymaker (MM) and the black bar the *L. pimpinellifolium* PI126947 genotype. The positions of recombination events are represented by a transition of black to white or vice versa. One cM equivalent is indicated as a vertical bar on the right.

Cf-ECP2 might give longer lasting resistance than other known *Cf* genes that have been used in breeding programs and have rapidly lost their effectiveness, as the *Cf-ECP2* gene recognizes the fungal protein ECP2, which is essential for pathogenesis (Laugé et al. 1998a).

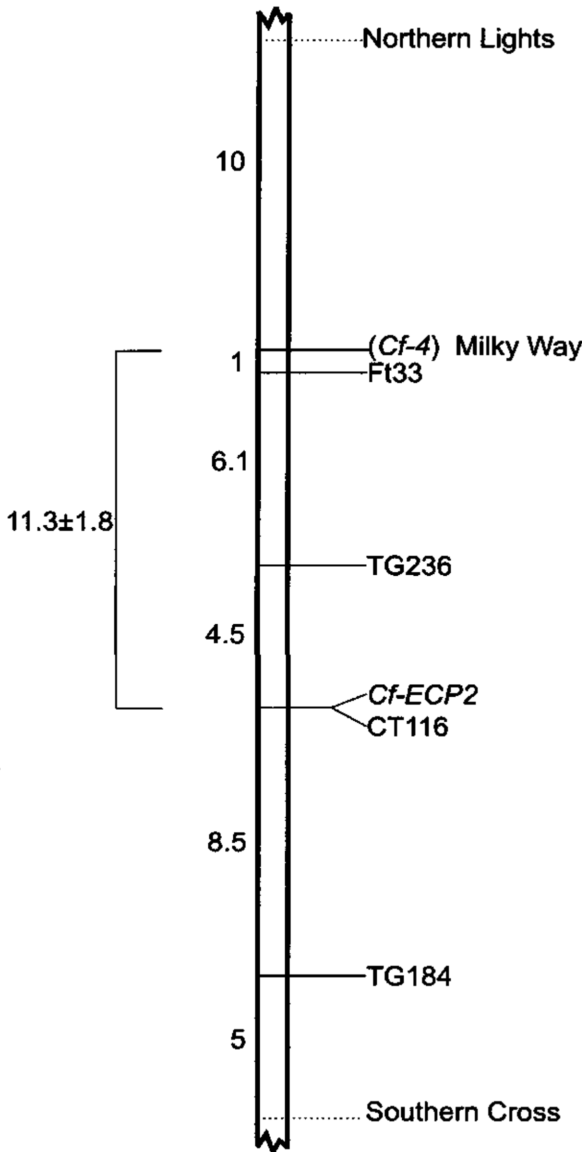

Figure 5.2 Integrated genetic map of the short arm of chromosome 1 around *Cf-ECP2*. Recombination percentages of CAPS markers and *Cf-ECP2* are based upon segregation analysis of F_2Cf18 and F_3Cf18. The position of *Cf-4* is only estimated relative to *Cf-ECP2* and based upon segregation analysis of 79 TCf18 and 108 TCf24 plants (see text). The map distances (in cM) are indicated on the left.

Using PVX::*Avr9*, it was shown that the original donor of *Cf-ECP2*, *L. pimpinellifolium* PI126947, harbors not only *Cf-ECP2*, but also a gene for the recognition of AVR9. Jones et al. (1994) have isolated *Cf-9*, which originates from another *L. pimpinellifolium* accession, PI126915 (Balint-Kurti et al. 1994; Tigchelaar, 1984). Since Boukema (1980) did not find susceptible recombinants in the offspring from the testcross between the *L. pimpinellifolium* accessions PI126915 and PI126947, there is at least one gene closely linked, allelic or in common in these two accessions. PI126915 only harbors one dominant *Cf* gene (Boukema, 1980), which is *Cf-9* (Tigchelaar, 1984). Since *Cf-4* and *Cf-9* are nearly allelic (Thomas et al. 1997), and the distance between *Cf-ECP2* and *Cf-4* is 11.3±1.8 cM, *Cf-ECP2* is not the resistance gene which is closely linked or allelic to the *Cf-9* gene from PI126915. Therefore, the second resistance gene present in PI126947, which recognizes AVR9, is closely linked or allelic to the *Cf-9* locus of PI126915. The fact that both lines are *L. pimpinellifolium* accessions suggests that these *Cf-9* genes are either identical or originate from a common ancestor. If they are derived from a common ancestor, it will be interesting to isolate and sequence the *Cf-9* gene from PI126947 to see whether mutations are allowed without loss of AVR9 recognition.

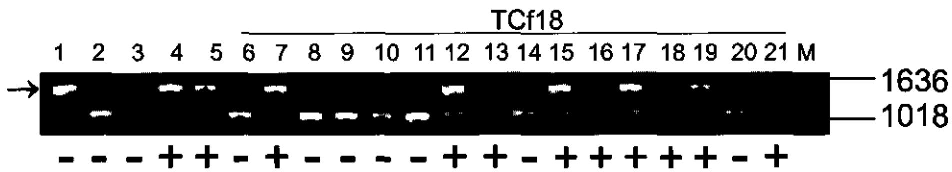

Figure 5.3 PCR products corresponding to the CT116 locus of 16 individuals of the TCf18 population and the parental lines after digestion with *Hha*I and electrophoresis in a 0.8 % agarose gel. Lane 1 contains the undigested PCR product of Moneymaker (MM), lanes 2 to 21the *HhaI* digested PCR product of MM (lane 2), MM-Cf4 (lane 3), Cf18 (lane 4), *L. pimpinellifolium* PI126947 (lane 5) and 16 individuals of the TCf18 population (lanes 6-21), respectively. Lane 22 contains the 1 kb ladder (Life Technologies) as size marker. A '+' below the lane indicates necrosis upon inoculation with PVX::*Ecp2*, while a '-' indicates mosaic symptoms upon inoculation with PVX::*Ecp2*. The product of app. 1550 bp from the Cf18 parent, indicated by the arrow, cosegregated with *Cf-ECP2*.

The previously reported *Cf* genes, which are located on the short arm of chromosome 1, *Cf-4* and *Cf-9*, are present in the 'Milky Way' cluster (Parniske et al. 1999). As concluded from the testcross analysis, *Cf-ECP2* is linked to *Cf-4* at a distance of 11.3±1.8 cM. This indicates that *Cf-ECP2* is not part of the Milky Way cluster. Subsequently, *Cf-ECP2* was mapped accurately by using CAPS markers discriminating between *L. pimpinellifolium* PI126947 and Moneymaker (Table 5.2). By using these CAPS markers and the PVX::*Avr9* test, we determined the length of the introgression fragments in the breeding lines Cf18 and Cf24, which are derived from *L. pimpinellifolium* PI126947. Cf24 has the smallest introgressed region and, like Cf18, does not contain the Milky Way cluster of the *L. pimpinellifolium* donor. The size of the introgression fragments is less than 33 cM in Cf18 and less than 26 cM in Cf24, which suggests a limited linkage drag (Young and Tanksley, 1989). From the length of the smallest introgression fragment, which is present in Cf24, it can be concluded that *Cf-ECP2* is located between TG236 and TG51. Indeed, *Cf-ECP2* was mapped at the locus CT116, which is between TG236 and TG184 (Figure 5.2). In most populations studied, these markers reside in a cluster of markers, referred to as 'the TG236 cluster' (Tanksley et al. 1992; Balint-Kurti et al. 1994). However, by using an F_2 population derived from an interspecific cross between *L. esculentum* and *L. peruvianum*, Bonnema et al. (1997) showed that the markers present in the TG236 cluster span a region of approximately 15 cM with a distance of 12.6 cM between TG236 and TG184. In the present F_2 population we found a similar distance of 13.7 cM between TG236 and TG184. *Cf-ECP2* cosegregated completely with CT116 in all populations tested (n=282). Parniske et al. (1999) showed the presence of at least one *Hcr9* cluster proximal to the TG236 cluster, designated 'Southern Cross'. However, no *Hcr9* clusters cosegregating with the TG236 cluster (containing CT116) were reported. Consequently, *Cf-ECP2* is located on a yet unidentified *Cf* locus and is the first functional *Cf* gene that is reported to be located on the short arm of chromosome 1, without being part of the 'Milky Way' cluster. Moreover, Southern analysis showed a *Cf-9* hybridizing band of ~7.5 kb, cosegregating with *Cf-ECP2*. Therefore, it is likely that *Cf-ECP2* is homologous to *Cf-9* (*Hcr9*) as all other *Cf* genes on the short arm of chromosome 1 reported so far. We propose to designate this *Hcr9* locus 'Orion'.

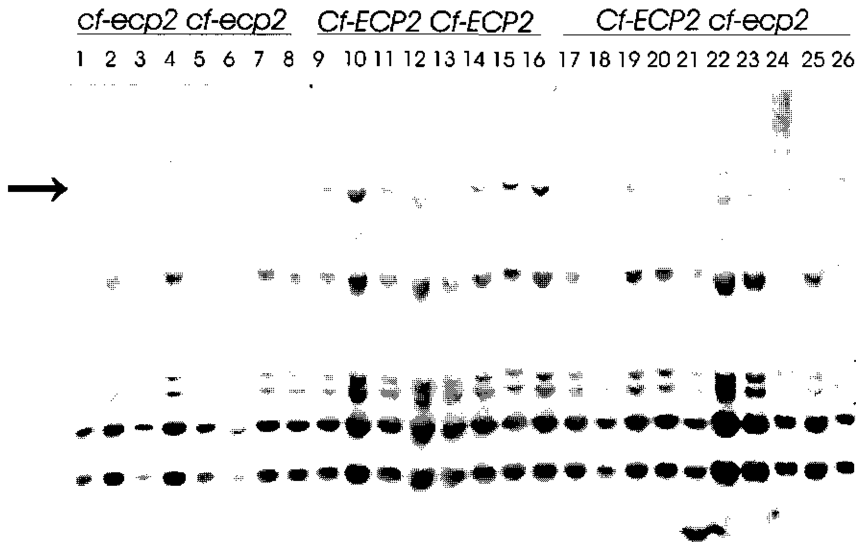

Figure 5.4 Cosegregation of a ~7.5 kb *Cf-9* hybridizing *Bgl*II fragment with *Cf-ECP2*. Filters were prepared with blotted, *BglII* digested DNA of eight F_3Cf18 families originating from homozygous (*cf-ecp2 cf-ecp2*) F_2 parents (lanes 1-8), eight F_3Cf18 families from homozygous (*Cf-ECP2 f-ECP2*) F_2 parents (lanes 9-16) and ten F_3Cf18 families from heterozygous (*Cf-ECP2 cf-ecp2*) F_2 parents (lanes 17-26). The filter was hybridized with the 753 bp *Xho*I-*Sst*I fragment of *Cf-9* (probe 1: Jones et al. 1994). The location of the cosegregating band of ~7.5 kb is indicated by an arrow.

Although no other *Hcr9s* cosegregating with *Cf-ECP2* have been observed it can not be excluded that other *Hcr9s* might be present at Orion, but could have escaped detection, when these *Hcr9s* are not polymorphic to *Hcr9s* from other clusters after *Bgl*II digestion. Similarly, the ~7.5 kb *Bgl*II fragment does not necessarily correspond to *Cf-ECP2*.

Parniske et al. (1999) suggested that new *Cf* clusters might develop by chromosomal duplication or retroposition events. The finding of *Cf-ECP2* at a position where no *Cf* homologues have been reported before, might give us new insights in molecular processes leading to the development of new resistance specificities. If the wild ancestor of *Cf-ECP2*, *L. pimpinellifolium* PI126947, also harbors the *Cf-9* gene of PI126915, the isolation and the genetic organization of *Cf-ECP2* will reveal how these *Cf* genes and *Cf* clusters have been

evolving. Bonnema et al. (1997) showed in an F_2 population derived from a cross between *L. esculentum* and *L. peruvianum* LA2157, that around the CT116 locus 1cM corresponds to a physical distance of maximally 52 kb. Since genetic distances are similar in the F_2 population that we have used, and *Cf-ECP2* is likely to be a *Hcr9*, the mapping of *Cf-ECP2* to CT116 will facilitate a cloning approach using *Cf-9* as well as CT116 to isolate this resistance gene.

Chapter 6

The *Hcr9* cluster Aurora of tomato, conferring resistance to *Cladosporium fulvum* Cke. through recognition of the extracellular protein ECP5, harbors several *Cf* homologs

J.P.W. Haanstra and P. Lindhout

Abstract

Previously, we have reported the mapping of the *Hcr9* cluster Aurora on the short arm of chromosome 1 of tomato, which harbors a functional resistance gene against *Cladosporium fulvum* through recognition of the fungal extracellular protein ECP5. One of the markers used in this mapping study was a PCR product, which was obtained with primers based upon the sequence of *Cf-4*, followed by digestion with *Eco*RI. This marker cosegregated with the recognition of ECP5. This product has been cloned into a plasmid vector and subsequently nine clones have been (partially) sequenced. The sequences of these nine clones were compared to each other and to the previously sequenced *Hcr9s*. From these comparisons we conclude that there are at least four different *Hcr9s* present within the Aurora cluster, which all are most similar to *Hcr9*-MW0.

Introduction

The interaction between the fungal pathogen *Cladosporium fulvum* and its host tomato is characterized by the gene-for-gene relationship (Van den Ackerveken and De Wit, 1994). For every plant resistance gene (*Cf* gene), there is a matching avirulence gene (*Avr* gene) in the fungus. Recognition of the *Avr* gene product by the *Cf* gene product results in a hypersensitive response, preventing fungal growth and, consequently, resulting in resistance of the plant. From the tomato - *C. fulvum* pathosystem, several *Avr* genes as well as several *Cf* genes have been isolated (van den Ackerveken et al., 1992; Joosten et al., 1994; van den Ackerveken et al., 1993; Jones et al., 1994; Thomas et al., 1997; Takken et al., 1998; Dixon et al., 1996; Dixon et al., 1998). *Cf* genes reside in clusters on chromosome 1 and chromosome 6 on the genome of tomato (Parniske et al., 1997; Dixon et al., 1998). These so called *Hcr9* clusters consist of functional resistance genes, as well as genes with a hitherto unknown function and pseudogenes (Parniske et al., 1997; Parniske et al., 1999). The number of *Hcr9s* per cluster may vary between one and five. On the short arm of chromosome 1 of tomato, so far five of these *Hcr9* clusters have been mapped (Parniske et al., 1999; Chapters 3, 5). The 'Aurora' cluster harbors *Cf-ECP5*, a not yet cloned *Cf* gene conferring resistance to *C. fulvum* through recognition of the extracellular protein ECP5 (Chapter 3). The recognition of ECP5 cosegregated with a molecular marker, Cf4f3r3-*Eco*RI, which was obtained by *Eco*RI cleavage of a PCR product, obtained with primers based upon the sequence of *Cf-4*. Since this product is based upon sequence homology to *Cf-4* and it cosegregates with *Cf-ECP5*, it is a candidate for (part of) *Cf-ECP5*. Therefore, we have cloned and sequenced the Cf4f3r3-*Eco*RI product. Here we describe the comparison of the sequence of nine clones and discuss the results in the perspective of the number of *Hcr9*s present at the Aurora cluster.

Materials and Methods

Cloning and sequencing of Cf4f3r3-*Eco*RI

DNA of *Cf-ECP5* was used in a PCR with the primers Cf4f3 (for primers and adaptor sequences see Table 6.1) and Cf4r3. This resulted in a product of around 980 bp (Chapter 3). The PCR product was cleaved using *Eco*RI, resulting in a Cf4f3r3-*Eco*RI digestion product of around 870 bp. Since this product contained one blunt end and one sticky end, we ligated an

*Eco*RI adapter, which we use in our AFLP system. Therefore, the digested PCR product was sliced out of the gel and purified using the QIAEX II Gel Extraction Kit (Qiagen). Concentrations and conditions of the ligation are as described in Chapter 2.

Table 6.1 List of primers and adapters

Primers/adapters	Sequences*
*Eco*RI adapter	5'- CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
E00	GACTGCGTACCAATTC
Cf4f3	TTT CAT GCT ATA TGT CTT TCT C
Cf4r3	AAT TGG TCC TTC AAG ATG GTT A

*: DNA sequences are always from 5' to 3' orientation unless otherwise indicated.

The 10x diluted ligation product was used for two different PCR reactions, one with the E00 primer and the Cf4f3 primer and the other with the E00 primer and the Cf4r3 primer. Both reactions were performed in duplo, using an annealing temperature of 54°C. After PCR, only the reaction mixtures with Cf4r3 gave a product, which was of the expected size of ~890 bp.

The PCR product was cloned into the pGEM-T Easy vector (Promega,Leiden, The Netherlands). PCR fragments of nine clones were prepared for sequence analysis using the primer T7 in a reaction using the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, La Jolla, CA). Sequence products were run on an automated sequencer (model 373A; Applied Biosystems) and aligned using the software module Megalign (DNAStar Inc.). Three of the nine clones were sequenced using the SP6 primer in the system described above. From the sequence alignment, sequence pair distances were calculated. For each clone, differences to clone 8 (base-pairs 1 to 611) and clone 3 (base-pairs 612 onwards) were counted and divided by the sequence length.

Results

The marker Cf4f3r3-*Eco*RI, which is obtained after PCR with primers based upon the sequence of *Cf-4* and which cosegregates with *Cf-ECP5* was cloned. After ligation of an Eco adapter to the purified Cf4f3r3-*Eco*RI product followed by PCR using the E00 primer in conjunction with either the Cf4f3 or Cf4r3 primer, only the combination with Cf4r3 resulted in a product. This product was of the expected size of ~890 bp, indicating that the *Eco*RI site in the Cf4f3r3 PCR product is at approximately 100 bp from the 5' end. Nine inserts were sequenced, three fully and six partially using the T7 primer only. Strikingly, all sequences obtained with the T7 primer corresponded to the 5' end of the insert, indicating that the insert specifically ligated to the vector in this orientation. Sequences were aligned (Figure 6.1) and sequence pair distances were calculated. We considered that sequence pair distances smaller than 0.7 percent are due to sequence misreadings, since clones were only sequenced once. Based upon this assumption, the nine clones could be classified into four groups. Three groups (I, II and IV) consist of only one individual clone (1, 2 and 4) and one group (III) consists of six clones (3, 5, 6, 7, 8 and 9). Sequences of all clones were compared to all known *Hcr9* sequences. The *Hcr9* present at the Milky Way cluster of *L. esculentum* was the closest relative of all clones. The amino acid sequence was predicted from comparison to other *Hcr9s*. Clones 1, 3, 6 and 8 encode an intact ORF. Clone 4 has a T to A base-pair change on position 58 and consequently a stop codon is encoded. The rest of the sequence encodes an amino acid sequence of near identity to the other clones. Clones 2, 5, 7 and 9 have out-of-frame mutations, which cause stop codons in the amino acid sequence. In the original reading frame, the amino acid sequence of all these clones was nearly identical to those of other clones, without any additional stop codon.

Amino acid sequences of clone 1, representing group I and of clone 3, representing group III were compared to amino acids of other *Hcr9s* (Figure 6.2). Strikingly, the amino acid sequence in the predicted ß-strand/ß-turn motif, which is indicated with "xxLxLxx" in Figure 6.2, is variable at positions where other *Hcr9s* are variable and conserved in stretches where other *Hcr9s* have conserved amino acids (Parniske et al., 1997), except for a few amino acids, which are marked by a "*". This would indicate that the locus from which these clones were derived encodes a protein with a similar structure as other *Hcr9* proteins. The predicted amino acid sequence of these clones was compared to the predicted amino acid sequence of all

```
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 1
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 2
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 3
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 4
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 5
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATTTT    Clone 6
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 7
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 8
1    GACTGCGTACCAATTCAAGAACATGTTTACCGTTAATCCT    Clone 9

41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 1
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 2
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 3
41   AATGCTTCTGATTATTGATACGACTATACAGGCGTAGAGA    Clone 4
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 5
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 6
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 7
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 8
41   AATGCTTCTGATTATTGTTACGACTATACAGACCAAAGGA    Clone 9

81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 1
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 2
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 3
81   TTCAGTCATATCCAAGAACTCTTTCCTGGAACATGAGCAC    Clone 4
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 5
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 6
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 7
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 8
81   TGCAGTCATATCCAAGAACTCTTTTCTGGAACAAGAGTAC    Clone 9

121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 1
121  AGATTGTTGCTCATGGGATGGCATTCATCGTGACGAGACG    Clone 2
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 3
121  GGACTGCTGCTCATGGGATGGCGTTAATTGTGACGAGACG    Clone 4
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 5
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 6
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 7
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 8
121  AGATTGTTGCTCATGGGATGGCATTCATTGTGACGAGACG    Clone 9

161  ACAGGACAAGTGATTGCGCTTGACCTCTGTTGTAGCAAAC    Clone 1
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGCGCAGCCAAC    Clone 2
161  ACAGGACAAGTAGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 3
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 4
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 5
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 6
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 7
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 8
161  ACAGGACAAGTGGTTGAGCTTGATCTCCGGTGCAGCCAAC    Clone 9
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Fig. 6.1 See page 83 for legend

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201 TTCGAGGCAAGTTTCATACCAATAGTAGCCTCTTTCAACT    Clone 1
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 2
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 3
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 4
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 5
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 6
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 7
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 8
201 TTCAAGGCAAGTTTCATTCCAATAGTAGCCTCTTTCAACT    Clone 9

241 CTCCAATCTAAAAAGGCTTGATTTGTCGAATAATAACTTC    Clone 1
241 CTCCAATCTAAAAAGGCTTGATTTGTCTTTTAATGATTTC    Clone 2
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 3
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 4
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 5
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 6
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 7
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 8
241 CTCCAATCTCAAAAGGCTTGATCTGTCGAATAATAACTTC    Clone 9

281 ACTGGATCGCTCATTTCACCTAAATTTGGTGAGTTTTCAA    Clone 1
281 ACTGGATCGCCCATTTCACCTAAATTTGGTGAGTTTTCAG    Clone 2
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 3
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 4
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 5
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 6
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 7
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 8
281 ATTGGATCGCTCATTTCACCTAAATTTGGTGAATTTTCAG    Clone 9

321 ATTTGACGCATCTTGTTTTGTCTGATTC-AAGTTTTACAG    Clone 1
321 ATTTGACGCATCTCGATTTGTCGCATTCTA-GTTTTACAG    Clone 2
321 ATTTGACGCATCTCGATTTGTCGGATTCTA-GTTTTACGG    Clone 3
321 ATTTGACGCATCTCGATTTGTCGGATTCTA-GTTTTACGG    Clone 4
321 ATTTGACGCATCTCGATTTGTCGGGTTCTA-GTTTTACGG    Clone 5
321 ATTTGACGCATCTCGATTTGTCGGATTCTA-GTTTT        Clone 6
321 ATTTGACGCATCTCGATTTGTCGGATTCTAAGTTTTACGG    Clone 7
321 ATTTGACGCATCTCGATTTGTCGGATTCTA-GTTTTACGG    Clone 8
321 ATTTGATGCATCTCGATTTGTCGGATTCTA-GTTTTACGG    Clone 9

360 GTCTTTATCCCTTTTGAAATCTCTCACCTTTCTAAACTACA   Clone 1
360 GTCTAATCCCTTTTGAAATCTCTCACCTTTCTAAACTGCA    Clone 2
360 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 3
360 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 4
360 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 5
355                                             Clone 6
361 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 7
360 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 8
360 GTGTAATCCCTTCTGAAATCTCTCATCTTTCTAAACTACA    Clone 9
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Fig. 6.1 See page 83 for legend

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400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 1
400 CGTTCTTCGTATCCGTGG-CAATATAAACTTAGTCTTGT-   Clone 2
400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 3
400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 4
400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 5
355                                            Clone 6
401 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 7
400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 8
400 CGTTCTTCGTATCCGTGATCCAAATGAGCTTAGTCTAGGG   Clone 9

440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTGACCCAAT   Clone 1
338 CCTCACAATTTTGAACTGCTCCTTAAGAACTTGCCCCAAT   Clone 2
440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTGACCCAAT   Clone 3
440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTGACCCAAT   Clone 4
440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTG-CCCAAT   Clone 5
355                                            Clone 6
441 CCTGACAATTTTGAACTGCTCCTTAAGAACT            Clone 7
440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTGACCCAAT   Clone 8
440 CCTCACAATTTTGAACTGCTCCTTAAGAACTTG-CCCAAT   Clone 9

480 TAAGAGATCTCCACCTTGAATCTATCAACATCTCTTCCAC   Clone 1
478 TAAGAGACCTCCAACTTGAATCTATCAACATCTCTTCCAC   Clone 2
480 TAAGAGATCTCCACCTTGAATCTATCAACATCTCTTCCAC   Clone 3
480 TAAGAGATCTCCACCTTGAATCTATCAACATCTCTTCCAC   Clone 4
479 TAAGAGATCTCCACCTTGAATCTATCAACATCTCTTCCAC   Clone 5
355                                            Clone 6
471                                            Clone 7
480 TAAGAGATCTCCACCTTGAATCTATCAACATCTCTTCCAC   Clone 8
479 TAAGAGAGCTCCACCTTGAATCTGTCAACATCTCTTCCAC   Clone 9

520 AGTTCCATCAAATTTCTCTTCTCATTT-AACGAATCTACT   Clone 1
518 AGTTCCATCAAATTTCTCTTCTCATTT-AACGAATCTACG   Clone 2
520 AGTTCCATCAAATTTCTCTTCTCATTT-AACGAATCTACT   Clone 3
520 AGCTCCAT                                   Clone 4
519 AGTTCCATCAAATTTCTCTTCTCATTTTAACGAATCTACT   Clone 5
355                                            Clone 6
471                                            Clone 7
520 AGTTCCATCAAATTTCTCTTCTCATTT-AACGAATCTACT   Clone 8
519 AGTTCCATCAAATTTCTCTTCTCATTT-AACGAATCTACT   Clone 9

559 TCTTCCATTCACAAAGTTACATGGGATATTGACCGAAAGA   Clone 1
557 GCTTCCATTCACAGAGTTACGTGGGATATTGCCCGAAAGA   Clone 2
559 TCTTCCATTCACAAAGTTACATGGGATATTGACCGAAAGA   Clone 3
527                                            Clone 4
559 TCTT                                       Clone 5
355                                            Clone 6
471                                            Clone 7
559 TCTTCCATTCACAAAGTTACATGGGATATTGACCGAAAGA   Clone 8
558 TCTTCCATTCACAAAGTTACATGGGATATTGACC         Clone 9
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Fig. 6.1 See page 83 for legend

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599 GTTTTTCACCTTTCCAACTTAGAATTTCTCGATTTAACAT   Clone 1
597 TTCTTCCACCTTTCCAACTTAGAATCCCTTGATTTATTCAT   Clone 2
599 GTTTTTCACCTTTCCGACTTAGAATTTCTCGATTTAACAT   Clone 3
527                                            Clone 4
562                                            Clone 5
355                                            Clone 6
471                                            Clone 7
599 GTTTTTCACCTTT                              Clone 8
591                                            Clone 9

639 ATAATCCCCAGCTCACAGTTAGGTTTCCCACAACCAAATG   Clone 1
637 TCAATCCCCAGCTCACGGTTAGGTTTCCCACAACCAAATG   Clone 2
639 ATAATCCCCAGCTCACAGTTAGGTTTCCCACAACCAAATG   Clone 3
527                                            Clone 4
562                                            Clone 5
355                                            Clone 6
471                                            Clone 7
611                                            Clone 8
591                                            Clone 9

679 GAATAGCAGTGCATCACTCATCAAGTTATATCTCTATAAT   Clone 1
677 GAATAGCAGTGCATCACTCGTGAATTTATATCTCGCTAGT   Clone 2
679 GAATAGCAGTGCATCACTCATCAAGTTATATCTCTATAAT   Clone 3
527                                            Clone 4
562                                            Clone 5
355                                            Clone 6
471                                            Clone 7
611                                            Clone 8
591                                            Clone 9

719 GTGAATTTTACTGGTACGTTACCTGAATCGTTTAGCTATC   Clone 1
717 GTGAATATTGCTGATAGGATACCTGAATCATTTAGCCATC   Clone 2
719 GTGAATTTTACTGGTACGTTACCTGAATCGTTTAGCTACC   Clone 3
527                                            Clone 4
562                                            Clone 5
355                                            Clone 6
471                                            Clone 7
611                                            Clone 8
591                                            Clone 9

759 TAACTGCACTTCATAAGATGGACATGGGTTATACTAATCT   Clone 1
757 TGACTGCACTTCATGAGTTGTACATGGGTCGTTCTAATCT   Clone 2
759 TAACTGCACTTCATAAGCTGGACATGGGTTATACTAATCT   Clone 3
527                                            Clone 4
562                                            Clone 5
355                                            Clone 6
471                                            Clone 7
611                                            Clone 8
591                                            Clone 9
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Fig. 6.1 See page 83 for legend

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799 GTCAGGGCCCATTCCTAAACCTCTATGGAATCTCACCAGC    Clone 1
797 GTCAGGGCATTATTCCTAAACCTCTATGGAATCTCACCAAC    Clone 2
799 GTCAGGGCCCATTCCTAAACCTCTATGGAATCTCACCAGC    Clone 3
527                                             Clone 4
562                                             Clone 5
355                                             Clone 6
471                                             Clone 7
611                                             Clone 8
591                                             Clone 9

839 ATAGAGATTTTGTACCTTGATTATAACCATCTTGAAGGAC    Clone 1
837 ATAGAGAGTTTGTTCCTTGATGATAACCATCTTGAAG-AC    Clone 2
839 ATAGAGATTTTGTACCTTGACTATAACCATCTTGAAGGAC    Clone 3
527                                             Clone 4
562                                             Clone 5
355                                             Clone 6
471                                             Clone 7
611                                             Clone 8
591                                             Clone 9

879 CAATT                                       Clone 1
876 CAATT                                       Clone 2
879 CAATT                                       Clone 3
527                                             Clone 4
562                                             Clone 5
355                                             Clone 6
471                                             Clone 7
611                                             Clone 8
591                                             Clone 9
```

Figure 6.1 Alignment of (partial) DNA sequences of nine Cf4f3r3-*Eco*RI clones. Boxes indicate base-pairs differing from clone 8 (base-pairs 1 to 611) and clone 3 (base-pairs 612 onwards).

known *Hcr9s* using Megalign. None of the predicted *Hcr9* proteins closely resembles either clone 1 nor 3 (data not shown).

Discussion

At least four *Hcr9*s are present at the Aurora cluster

A product cosegregating with *Cf-ECP5* resistance was identified using PCR primers based upon the sequence of *Cf-4*. This product was cloned and nine clones were (partially) sequenced. Based on the sequence, the nine clones were divided into four groups, each representing a *Cf* homolog. DNA sequences were compared to publically available *Hcr9* sequences and all clones mostly resembled the *Hcr9* present at the Milky Way cluster of the susceptible *L. esculentum* cv. Moneymaker. Since four groups have been found with sequence pair distances ranging from 3.0-8.5 percent, it may be concluded that at least four *Hcr9s* are present at the Aurora cluster, since all the clones of the four groups are derived from the same band which was cosegregating with *Cf-ECP5*. There might even be more *Hcr9s* present at the Aurora cluster, when the PCR primers used do not fit those extra *Hcr9s* or if they do not have an internal *Eco*RI site. Also sequences representing new groups might have been found if more clones would have been sequenced. The number of *Hcr9s* which are usually present at a cluster ranges from 1 to 5 (Parniske et al., 1997; Parniske et al., 1999). In this respect, the number of *Hcr9s* identified at the Aurora cluster is not surprising. However, since the Aurora cluster from the breeding line containing *Cf-ECP5*, like the Milky Way cluster from Moneymaker-*Cf-9* and the Orion cluster of the breeding lines containing *Cf-ECP2* are all derived from (different) *L. pimpinellifolium* accessions, the variation in *Hcr9s* in this species is very great indeed. Considering that other *Lycopersicon* species also contain functional *Hcr9*s, the total variation in *Hcr9s* within the *Lycopersicon* genus must be excessively high. Miller and Tanksley (1990) studied the genetic variation in the genus *Lycopersicon* by RFLP analysis. They found that the self-compatible species *L. pimpinellifolium* accounted for 6.9% of novel RFLP alleles, whereas the self-incompatible species *L. hirsutum*, *L. pennellii* and *L. peruvianum* together contain 75% of novel RFLP alleles. So far, five *Cf* genes have been identified that originate from *L. pimpinellifolium*: *Cf-9*, *Cf-ECP2*, *Cf-ECP3*, *Cf-ECP5* and a *Cf* gene with unknown specificity (Chapter 3). If the three self-incompatible *Lycopersicon* species show over 10 times the number of novel RFLP alleles as compared to *L. pimpinellifolium*, this suggests that in these three species at least 50 different *Cf* genes are present. Although a lot of *Hcr9s* already have been sequenced and models for evolution have been proposed (Parniske et al., 1997; Parniske et al., 1999), sequencing more *Hcr9s*, especially from the other *Lycopersicon* species and determining their specificity would provide us with valuable

information on structure-function relationships of *Cf* genes and with data to support or modify the models proposed.

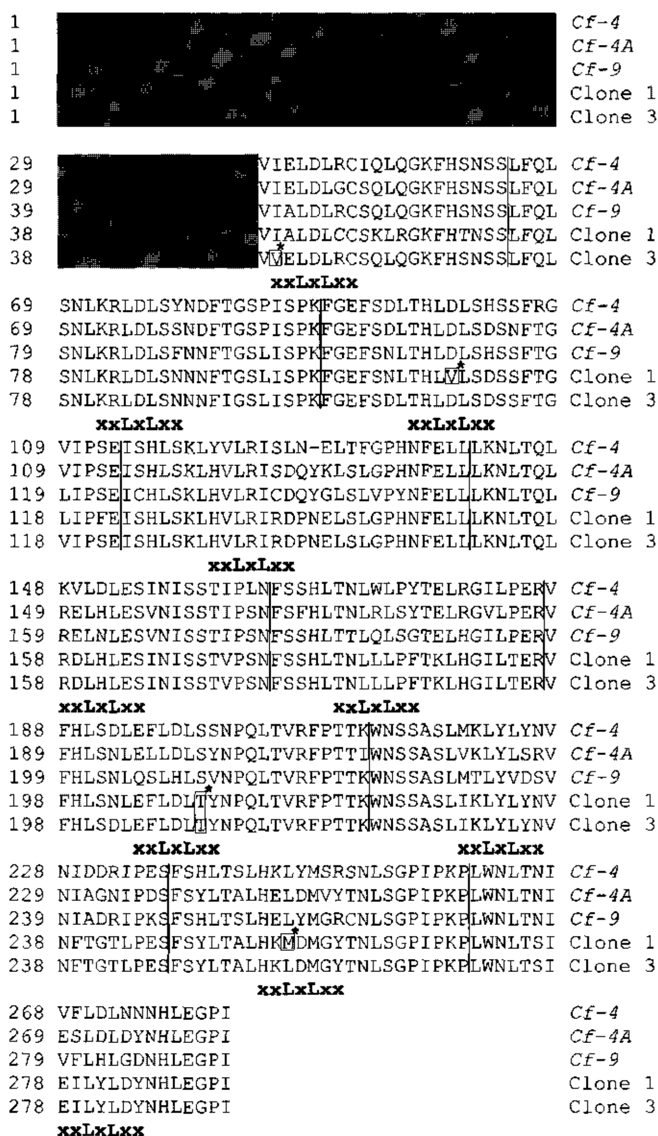

Figure 6.2 Alignment of amino acid sequences of clones 1 and 3 with part of the amino acid sequence of *Cf-4*, *Cf-4A* and *Cf-9*. Domain and LRR structure are according to Parniske et al. (1997). The part corresponding to domain B is shaded. The different LRRs of domain C are separated by vertical lines. The predicted ß-strand/ß-turn structural motif of the LRRs is indicated by the position of "xxLxLxx" below the sequence alignment. Boxes with a " * " indicate amino acid changes at positions that are usually conserved (according to Parniske et al., 1997).

Sequence divergence at the Aurora cluster

Based upon the DNA sequence, the nine clones could be divided into four groups. Members of group II and IV do not have intact open reading frames. However, if the original reading frame was maintained, the amino acid sequences were nearly identical to those of the other *Cf* genes. The pseudogenes at the Northern Lights and Southern Cross clusters, identified by Parniske et al. (1999) showed greater differences in their sequence compared to other Hcr9s, than the one base-pair differences, present in clones 2,4,5,7 and 9. It is more likely that these differences are due to errors in sequencing, since they also occur on different sites in three out of the six clones of group III. Group I has an intact open reading frame which encodes an amino acid sequence, comparable to other *Hcr9* proteins. The amino acid sequence starts in the B-domain and ends in the tenth LRR of the C-domain (Jones et al., 1994). Group III encodes a stretch of amino acids that is very similar to group I. As mentioned before, differences in the ß-strand/ß-turn motif of these two groups compared to other *Hcr9s* are generally at the hypervariable regions that have been observed previously (Parniske et al., 1997). If no more than the four *Hcr9s* reside at the Aurora cluster, it is likely that either of these two groups represent the functional *Cf-ECP5* gene. Clone 1 has a leucine to methionine change at position 259 compared to other *Cf* genes (Figure 6.2). This shift is not present in clone 3 and might alter the xxLxLxx motif of LRR9. Clone 3 does not have such an amino acid change and might therefore represent the functional *Cf-ECP5* gene.

All four sequences at the Aurora cluster more resemble the *Hcr9* present at the Milky Way cluster of *L. esculentum* than any other previously sequenced *Hcr9*. This suggests a different evolutionary pathway for the Aurora cluster of *L. pimpinellifolium* than for the Milky Way clusters of *L. hirsutum* (containing *Cf-4* and *Cf-4A*) and *L. pimpinellifolium* (containing *Cf-9*), involving several duplication events. Since clone 1 and 3 share high sequence homology, they might have arisen from a recent duplication. Isolation and sequencing of the Aurora cluster should provide additional insights in the development of *Hcr9* clusters.

Chapter 7

General Discussion

Variation in genetic maps

Genetic maps have been generated from segregating populations based on the frequencies of recombination between markers that determine the genetic distances. These frequencies and, consequently, the genetic distances, may vary between populations (Chapters 3 and 5). For instance, the order of the markers that are present at the so-called 'TG236 cluster', could not be resolved in several F_2 populations of crosses between *L. pennellii* LA716 and different *L. esculentum* genotypes (Tanksley et al., 1992; Chapter 2). In contrast, an F_2 of a cross between *L. esculentum* cv. Solentos and *L. peruvianum* LA 2157 revealed remarkably high recombination frequencies in this region (Bonnema et al., 1997). The distances between these markers in the F_2 populations of CfECP2 and CfECP5 with Moneymaker were similar to those of the above mentioned F_2 between *L. esculentum* and *L. peruvianum* (Figure 7.1). Only small variations occurred, which are negligible as compared to those in F_2 populations of crosses between *L. pennellii* and *L. esculentum*. Since *Cf-ECP2* and *Cf-ECP5* were introgressed from *L. pimpinellifolium* (Chapters 5 and 3 respectively), this indicates that the inversions or small deletions at the TG236 region between *L. pennelli* and *L. esculentum*, which were suggested by Bonnema et al. (1997), are not present between *L. esculentum* and *L. pimpinellifolium*.

Variation of *Hcr9* loci

Several *Cf* genes have been mapped on the short arm of chromosome 1. Three of these genes, *Cf-4*, *Cf-4A* and *Cf-9* have been isolated and share high sequence homology (Thomas et al., 1997; Takken et al., 1998; Jones et al., 1994). These genes are part of a cluster (designated "Milky Way") of homologous sequences (designated "*Hcr9*s"). In addition, two other clusters harboring *Hcr9*s which encode proteins with unknown recognition specificities and pseudogenes have been identified, designated "Northern Lights" and "Southern Cross" (Parniske et al., 1999). So far, no function for the *Hcr9*s at the Northern Lights and Southern Cross clusters have been identified.

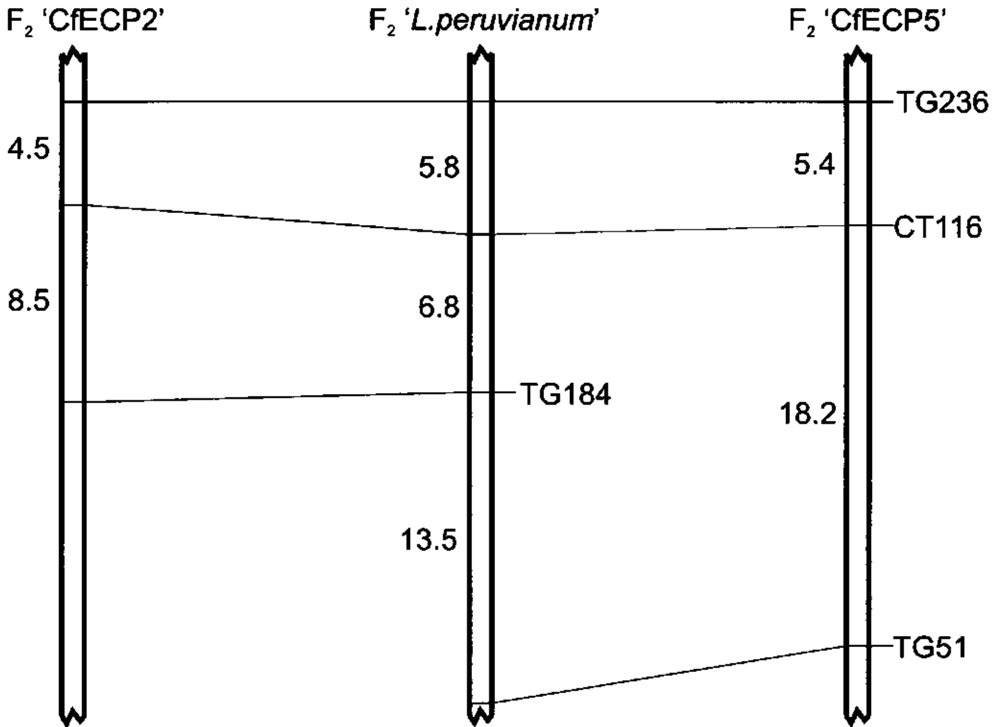

Figure 7.1 Comparison between maps based on three different F_2 populations. F_2'CfECP2' is based upon the F_2 population of a cross between *L. esculentum* cv. Moneymaker and CfECP2 (Chapter 5), F_2 '*L. peruvianum*' is based upon the F_2 population of a cross between *L. esculentum* cv. Solentos and *L. peruvianum* LA2157 (Bonnema et al., 1997) and F_2 'CfECP5' is based upon the F_2 population between *L. esculentum* cv. Moneymaker and CfECP5 (Chapter 3). The distances between markers within a mapping population are indicated in cM.

The research presented in this thesis has identified several new *Cf* genes that are located at the short arm of chromosome 1 (Chapter 3). Two of these genes have been studied further, *Cf-ECP2* and *Cf-ECP5* that confer resistance through recognition of the extracellular fungal proteins ECP2 and ECP5, respectively. ECP2 is necessary for *C. fulvum* to be fully pathogenic on tomato (Laugé et al., 1998a; Chapter 5), but the function of ECP5 is still unknown. Interestingly, these two *Cf-ECP* genes do not belong to the Milky Way cluster, which harbors the previously isolated *Cf* genes (Parniske et al., 1999), but reside on different loci that are probably also part of *Hcr9* clusters. The putative cluster that comprises *Cf-ECP2* was designated "Orion" and the putative cluster harboring *Cf-ECP5* was designated "Aurora". The fact that these *Cf* genes do not reside on previously identified *Hcr9* clusters, raises the question

on the number of *Hcr9* clusters present on the short arm of chromosome 1 in the genus *Lycopersicon*. Since *Cf-9*, *Cf-ECP2* and *Cf-ECP5* were all introgressed from different *L. pimpinellifolium* species, even more *Hcr9* clusters might be identified in other *Lycopersicon* species, though *Cf-4* and *Cf-4A*, which were introgressed from a single *L. hirsutum* accession, do reside in the Milky Way cluster that also comprises *Cf-9* from *L. pimpinellifolium*.

Previously, several *Cf* genes have been reported that have different specificities (Kanwar et al., 1980a,b). In this study, some of these genes were shown to be identical (Chapters 3 and 5). Since their specificities were also determined unambiguously using the PVX expression system and appeared identical, these genes have been renamed according to the elicitor or to the AVR protein which they recognize (Table 7.1). This has surely contributed to a much better description and characterization of *Cf* genes. As far as we can conclude now, 21 different *Cf* genes can be classified (Table 7.1).

The total number of *Cf* genes and *Hcr9s* should also be high. Considering the four *Cf* genes yet identified from *L. pimpinellifolium* (*Cf-9*, *Cf-ECP2*, *Cf-ECP3* and *Cf-ECP5*) and the relatively high variation among and between some other *Lycopersicon* species (Miller and Tanksley, 1990), one can expect at least another 45 *Cf* genes in *L. chilense*, *L. hirsutum*, *L. pennellii* and *L. peruvianum* (Chapter 6). *Cf-4* and *Cf-4A* originate from one *L. hirsutum* accession (Thomas et al., 1997; Takken et al., 1998). There are also reports of *Cf* genes from other *Lycopersicon* species (Chapter 3).

The number of *Hcr9*s varies per *Hcr9* locus and per genotype. The Northern Lights cluster contains four to five homologs, the Milky Way cluster one to five and the Southern Cross cluster two *Hcr9*s, depending on the genotype (Parniske et al., 1999). Most likely, the Aurora cluster of CfECP5 harbors at least four homologs (Chapter 6) and the *Cf-ECP2* gene is a member of the Orion cluster that probably harbors two homologs (M. De Kock, pers. comm.). The Aurora cluster as well as the Orion cluster have not been identified in cv. Moneymaker (Parniske et al., 1999), although it cannot be excluded that these clusters escaped the detection in case they were not polymorphic in the populations used for mapping *Hcr9*s. However, a YAC contig has been constructed that covers the Orion locus, which is around the molecular

marker CT116 (Bonnema et al., 1997) and no *Hcr9* was detected on these YACs (G. Bonnema, pers. comm.), confirming the absence of the Orion cluster in *L. esculentum.*

Based on the results obtained over the last few years, a rough estimation can be made on the total number of *Hcr9*s present in the *Lycopersicon* genus. A number of observations and considerations should be taken into account for this estimation. First of all, in tomato, at least five *Hcr9* clusters exist. These five clusters each contain one to five *Hcr9*s per genotype. Different genotypes harbor different *Hcr9*s at the same cluster. So far, no identical *Hcr9*s have been found at the same cluster (paralogs) between accessions, or between clusters (orthologs). Assuming an equal probability of non-identity between paralogs and orthologs, the minimum number of *Hcr9*s can be estimated using the formula:

$$P = \frac{N!}{(N-n)!\, N^n}$$

with N = total number of unique *Hcr9*s,

n = sample size, and

P = probability that n *Hcr9*s, sampled with replacementfrom N, are unique.

Using 22 as sample size (Thomas et al., 1997; Parniske et al., 1997; Parniske et al., 1999; Chapter 6) one obtains that for P to be equal to 0.05, N equals 85.

The *Hcr9*s with known function, all confer resistance to *C. fulvum* by specific recognition of extracellular fungal proteins (AVRs and ECPs). *C. fulvum* does not form specialized feeding structures and is confined to the apoplast (Van den Ackerveken and De Wit, 1994), whereas other fungal plant pathogens enter the cell. Since the *Cf* genes form a distinct structural class of *R* genes, this might indicate a relation between the structure of these genes and the site of pathogen attack. The only example of a Cf-like gene that is not involved in an interaction in the apoplast, is the $Hs1^{pro\text{-}1}$ gene that confers resistance to the sugar beet nematode *Heterodera schachtii* (Cai et al., 1997), which forms specific feeding structures. This gene belongs to the same sctructural class of R genes as the *Hcr9*s, although it contains only eight imperfect LRRs, whereas the *Hcr9* and *Hcr2* genes encode 25-38 LRRs (Dixon et al., 1998). It cannot be excluded that even more *Hcr*s confer resistance to pathogens, other than *C. fulvum*, but these have not yet been identified. Another possible function of *Hcr9*s is the involvement in other,

developmental, processes. In *Arabidopsis*, the *CLAVATA1* (*CLV1*) and *CLAVATA2* (*CLV2*) genes function to promote differentiation at the shoot meristem (Clark et al., 1999). *CLV1* encodes a receptor-like kinase (Clark et al., 1997) and shares structural homology to the protein encoded by *Xa21*, conferring resistance to *Xanthomonas oryzae* pv *oryzae* in rice. *CLV2* encodes a protein with extracellular LRRs and a very short cytoplasmic domain, like the proteins encoded by the *Cf* resistance genes (Clark et al, 1999). CLV1 and CLV2 are part of the same protein complex. Therefore, some *Hcr9*s, which strongly resemble CLV2 structure, might also be part of protein complexes, involved in other signaling pathways.

Durability of *Cf* genes and breeding perspectives

Cladosporium fulvum is the causal agent of leaf mold in tomato. Over the years, the tomato – *C. fulvum* pathosystem, that complies with the gene-for-gene relationship, has been the subject of extensive research (Van den Ackerveken and De Wit, 1994). Over the years, different *Cf* genes have been introduced in commercial tomato varieties to control *C. fulvum*. Mostly, resistance rendered ineffective within a few years by the occurrence of new strains of *C. fulvum*. *Cf-9* has been successful in giving resistance for a longer period, although fungal races exist that circumvent *Cf-9* mediated resistance (Van Kan et al., 1991). So far, these races have not become endemic. The durability of the *Cf* genes may be dependent on characteristics of both the *Hcr9* cluster as well as the corresponding *Avr* genes. For example, the *Cf-9* introgression fragment in MM-Cf9 harbors additional resistance genes (Laugé et al., 1998b), that may have contributed to a higher durability of the resistance, as only mutations in all avirulence genes that match the functional *Cf* homologues on the *Cf-9* cluster render a previous avirulent strain virulent on tomato lines harboring the *Cf-9* locus. However, in a similar situation, the *Cf-4* cluster comprising at least two functional *Cf* genes (*Cf-4* and *Cf-4A*) was not durable.

Races that are compatible on tomato lines carrying *Cf-9* lack the *Avr9* gene completely (Van Kan et al., 1991), while races that circumvent *Cf-4* mediated resistance generally harbored an *Avr4* allele that only differed by a single base-pair from the wild-type allele that results in unstable proteins (Joosten et al., 1994; Joosten et al., 1997). While no evident role in pathogenicity has yet been observed for AVR4 and AVR9 (Laugé and De Wit, 1998), another

fungal protein, ECP2, is necessary for full pathogenicity of *C. fulvum* on tomato (Laugé et al., 1997) and therefore cannot be lost by the fungus. Consequently, it is possible that *Cf-ECP2* mediated resistance may be more durable than resistance conferred by other *Cf* genes that match with fungal avirulence genes without an obvious function, like AVR4 and AVR9 (Laugé et al., 1998a). However, a single base-pair change in *Avr4* can already be sufficient for *C. fulvum* to avoid recognition and become virulent on tomato lines harboring *Cf-4* (Joosten et al., 1994; Joosten et al., 1997). Therefore, a single base-pair change in *Ecp2* may be also sufficient to circumvent resistance if different domains of ECP2 are essential for either pathogenicity or elicitor function. Then, mutations may occur that only affect the elicitor function but not the pathogenicity. Consequently, *Cf-ECP2* mediated resistance is not necessarily more durable.

It is difficult to combine two closely linked *Cf* genes by using traditional breeding methods. When no races are available that discriminate between the different *Cf* genes, this is even impossible. Nowadays, *Cf* genes can be combined in one genotype or cultivar by the use of molecular markers, as long as these genes do not belong to the same *Hcr9* cluster (*Cf-4* and *Cf-9*). The ability to screen for fungal proteins that act as elicitors, without using a set of differential races, also contributes to more effective ways of breeding for resistance against *C. fulvum*, even more if the genes encoding these putative elicitors are expressed in the PVX expression system (Laugé, 1999; Chapters 3, 4 and 5). Over 20 *Cf* genes are already available and many more resistance genes are expected in other *Lycopersicon* species (Table 7.1; Chapter 6). Expression of putative elicitor genes in PVX will provide rapid screening methods for yet unidentified *Cf – Avr* gene combinations in the tomato - *C. fulvum* pathosystem. After identification of new *Cf* genes, they can be rapidly exploited using marker assisted breeding.

Table 7.1 A reclassification of *Cf* genes.

Former name	Proposed name	Evidence for uniqueness[A]	Reference
Cf-1	*Cf-1*	1	Kerr et al., 1971
Cf-2	*Cf-2*	1,3	Lindhout et al., 1989; Dixon et al., 1996
Cf-3	*Cf-3*	1	Kerr et al., 1971
Cf-4	*Cf-4*	1,2,3	Lindhout et al., 1989; Thomas et al., 1997; Chapter 4
Cf-5	*Cf-5*	1,3	Lindhout et al., 1989; Dixon et al., 1998
Cf-6	*Cf-6*	1	Lindhout et al., 1989
Cf-7	*Cf-7*	1	Laterrot, 1980
Cf-8	*Cf-4*	1,2	Chapter 4
Cf-9	*Cf-9*	1,2,3	Lindhout et al., 1989; Jones et al., 1993
Cf-10	*Cf-10*	1	Laterrot, 1980
Cf-11	*Cf-4 Cf-11*	1,2	Lindhout et al., 1989; Chapter 4
Cf-12	*Cf-12*	0	Kanwar et al., 1980b
Cf-13	*Cf-4* + ?	2	Chapter 4
Cf-14	*Cf-14*	0	Kanwar et al., 1980b
Cf-15	*Cf-15*	0	Kanwar et al., 1980b
Cf-16	*Cf-16*	0	Kanwar et al., 1980b
Cf-17	*Cf-17*	0	Kanwar et al., 1980b
Cf-18	*Cf-ECP2*	2	Chapter 5
Cf-19	*Cf-19*	0	Kanwar et al., 1980b
Cf-20	*Cf-ECP2*	2	Chapter 5
Cf-21	*Cf-21*	0	Kanwar et al., 1980b
Cf-22	*Cf-22*	0	Kanwar et al., 1980b
Cf-23	*Cf-ECP2*	2	Chapter 5
Cf-24	*Cf-ECP2*	2	Chapter 5
	Cf-ECP3	2	Chapter 3
	Cf-ECP5	2	Chapter 3

[A] Evidence for uniqueness is based upon: 0 = only resistance to *C. fulvum*; 1 = a differential set of *C. fulvum* races; 2 = specific necrosis to a *C. fulvum* elicitor; 3 = isolation of the *Cf* gene

Research perspectives of the tomato - leaf mold interaction

By identifying ECP2 and ECP5 and their corresponding *Cf* genes, the tomato - *C. fulvum* interaction is the plant-pathogen interaction for which most gene-for-gene combinations have been identified. Genetic stocks are available for different types of analyses. For the future, this provides a good potential for further studies on the variation in number and structure of *Hcr9* clusters, as well as individual *Cf* genes and *Hcr9*s in the genus *Lycopersicon*. Firstly, the molecular evolution of *Hcr9*s can be studied by studying different *Cf* genes and their clusters, as well as clusters of homologues of which no function has been identified yet. Parniske and co-workers (Parnike et al., 1997; Parniske and Jones, 1999; Parniske et al., 1999), already have sequenced the *Hcr9* cluster Milky Way of three tomato genotypes, the Northern Lights cluster of two genotypes and the Southern Cross *Hcr9* cluster of one genotype. By studying other clusters, like Aurora and Orion, and by studying *Hcr9* clusters from other *Lycopersicon*

species, additional sequence information might provide new insights in the variation of this system. Secondly, by exchange of different domains, information will become available on the function of different parts of the *Cf* gene products. A better understanding of the nature of recognition, might give way to engineering of resistance genes with new specificities. The PVX expression system is a powerful aid to test functionality of the different *Cf* constructs. Finally, the defense signalling pathway of *Cf* genes can be studied. By mutation analysis, four alleles of one locus, named *rcr-3* (for required for *Cladosporium* resistance), have been identified that affect *Cf-2* mediated resistance, but not *Cf-9* nor *Cf-5* mediated resistance (Jones et al., 1999). When more mutants will become available that are affected in the signal transduction pathway leading to resistance, the availability of many *R* genes in tomato enable the determination of specificity of these mutations and hence the parts of the signal transduction pathway that are specific to a pathogen or elicitor or those that are more generally used.

As stated above, there is a large number of different *Hcr9*s. An interesting question is by which mechanism these sequences are maintained through the population. A similar high frequency of polymorphism occur in major histocompatibility complex (MHC) genes that are involved in defense mechanisms in animals. For the MHC, the force behind the high polymorphism rate could be heterozygote advantage or overdominance (Nei et al., 1997). However, since tomato, as well as *L. pimpinellifolium* are inbreeders, this mechanism is inadequate for these species. Another hypothesis is the frequency-dependent selection, in which there is only a limited selection pressure for the pathogen to overcome MHC alleles that are present in populations in low frequencies (Parham and Ohta, 1996). For the tomato – leaf mold system, this means that if a high number of *Cf* genes would be present in low frequencies in nature, the selection pressure for *C. fulvum* to overcome these individual *Cf* genes, would be low. In contrast, in modern cropping practice, tomato is grown in monoculture, hence *Cf* genes are present in high frequencies and, consequently, selection pressure is high and resistance genes are rendered ineffective and not durable.

To reiterate, investigations of the variation of *Hcr9*s in the genus *Lycopersicon*, will result in more knowledge of the processes that are involved in the generation of new *R* gene

specificities. The large reservoir of *Hcr9*s with hitherto unknown function present in this genus represents a challenge to present and future scientists. Eventually, this knowledge can result in better understanding the potential durability of *R* genes and in this way will lead to crops with resistance that is more durable.

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Summary

Plant breeders generally use qualitative resistance that is associated with a hypersensitive reaction (HR) to obtain cultivars that are resistant to pathogens and pests. The genetics of this resistance is based on the gene-for-gene relationship, which involves the product of a plant resistance gene and the product of an avirulence gene of the pathogen occurs. The interaction between leaf mold (*Cladosporium fulvum*) and its solely host, tomato (*Lycopersicon esculentum*), complies with this model. In the last few years, the isolation of several avirulence (*Avr*) and resistance (*R*) genes have contributed to an increase in our knowledge on this interaction. Several resistance genes to *C. fulvum* (*Cf* genes) have been isolated from tomato. These *Cf* genes are located on two different clusters on the tomato genome, which contain not only functional *Cf* genes, but also several homologs with yet unknown function.. The short arm of chromosome 1 harbors one of these clusters, designated "Milky Way", comprising functional *Cf* genes (*Cf-4, Cf-4A, Cf-9*). Moreover, two other clusters are located on the short arm of chromosome 1, designated "Northern Lights" and "Southern Cross", which only harbor homologs (*Hcr9*s), but no functional *Cf* genes. Also, there are several reports about the presence of other *Cf* genes on the short arm of chromosome 1.

To increase our knowledge on the genetic and molecular organization of *Cf* genes on the short arm of chromosome 1, an experimental approach was chosen to identify *Cf* genes with novel specificities that map on the short arm of chromosome 1. To saturate the tomato genome with molecular markers, an integrated high-density AFLP-RFLP map was constructed using two different *L. esculentum* x *L. pennellii* F_2 mapping populations. Although 1175 AFLP markers were mapped on the tomato genome, covering 1482 cM, only nine AFLP markers were detected between the RFLP markers CT233 and TG51, which mark a 23.6 cM interval, comprising several *Hcr9* clusters, on the short arm of chromosome 1. This relatively low number of markers is due to the clustering of most *Eco*RI/*Mse*I AFLP markers around the centromeres (Chapter 2).

Testcross populations of 66 *C. fulvum* resistant *Lycopersicon* accessions were obtained by crossing these accessions with the near isogenic line Moneymaker-Cf4 and subsequent crossing of the F_1 with the susceptible tomato cultivar Moneymaker (Chapter 3). Using disease resistance tests with *C. fulvum* race 0 on only 24 plants of these testcross populations, susceptible plants were identified. An under-representation of susceptible plants identified *Cf* resistance linked to *Cf-4*, and hence location of the unknown resistance on the short arm of chromosome 1. Out of the 21 resistant accessions that have been tested in this way, ten showed a *Cf-4* linked *Cf* gene. Among these ten accessions, one accession specifically recognized the extracellular protein ECP5 of *C. fulvum* and the corresponding gene was designated *Cf-ECP5*. This gene was more accurately mapped using a testcross population of 338 plants and an F_2 population of a cross between Moneymaker and CfECP5, consisting of 233 individuals. *Cf-ECP5* mapped 4 cM proximal to the *Hcr9* locus Milky Way and the corresponding *Cf* locus was designated Aurora. An amplification product that cosegregated completely with the *Cf-ECP5* gene, was cloned and nine clones were sequenced (Chapter 6). These nine clones could be classified into four groups, indicating that the Aurora locus comprises several *Hcr9*s.

Of the 66 resistant *Lycopersicon* accessions mentioned above, 64 have been screened for the presence of *Cf-4* and/or *Cf-9*, using PVX::*Avr4* and PVX::*Avr9*, respectively. A relatively large proportion of these accessions all harbored the functional genes *Cf-4* and *Cf-4A* (Chapter 4). Sequence analysis of the 3' end of *Cf-4* and part of the 3' untranslated region of *Cf-4* showed no differences from the previously published *Cf-4* sequences, hence these lines contain an introgression fragment with identical *Cf-4* and *Cf-4A* genes. Since several of these lines were previously designated with different *Cf* digits, a change in nomenclature is proposed.

Five out of the 66 accessions studied, showed an HR upon specific recognition of ECP2 and therefore harbor the corresponding resistance gene *Cf-ECP2* (Chapter 5). Using two different testcross populations and one F_2 population from a cross between Moneymaker and CfECP2, representing in total 282 individuals, *Cf-ECP2* was accurately mapped. *Cf-ECP2* cosegregates with the molecular marker CT116, which is located proximal to the Milky Way and Aurora clusters, but distal to the Southern Cross locus. Southern hybridization, using *Cf-9* as a probe,

showed a hybridizing band of ~7.5 kb cosegregating with *Cf-ECP2*, indicating that *Cf-ECP2* is a member of a previously unidentified *Hcr9* locus, that has been designated Orion.

Studies in Chapters 3, 5 and 6 show that functional *Cf* genes can be located on several different *Hcr9* loci on the short arm of chromosome 1 and that these *Hcr9* loci are highly polymorphic.

Samenvatting

In het algemeen gebruiken plantenveredelaars kwalitatieve resistentie, die is gebaseerd op een overgevoeligheidsreactie (HR), om rassen te verkrijgen die resistent zijn tegen ziekten en plagen. De genetica van deze resistentie berust op de gen-om-gen relatie, die afhankelijk is van het product van een resistentiegen in de plant en het product van een avirulentiegen in het pathogeen. De interactie tussen bladvlekkenziekte (*Cladosporium fulvum*) en zijn enige gastheer tomaat (*Lycopersicon esculentum*) is in overeenstemming met dit model. De laatste jaren zijn verschillende avirulentie- (*Avr*) en resistentiegenen (*R*) geïsoleerd, wat een grote bijdrage aan de kennis over deze interactie heeft geleverd. Van tomaat zijn verschillende resistentiegenen voor *C. fulvum* (*Cf* genen) geïsoleerd. Deze *Cf* genen liggen op twee verschillende clusters op het tomatengenoom, die naast functionele *Cf* genen ook meerdere homologen bevatten met nog onbekende functie. De korte arm van chromosoom 1 herbergt een van deze clusters, genaamd "Milky Way", met functionele *Cf* genen (*Cf-4*, *Cf-4A*, *Cf-9*). Daarnaast zijn er twee andere clusters op de korte arm van chromosoom 1, genaamd "Northern Lights" en "Southern Cross", die slechts homologen (*Hcr9*s) bevatten, maar geen functionele *Cf* genen. Ook zijn er diverse publicaties die melding maken van andere *Cf* genen op de korte arm van chromosoom 1.

Om meer te begrijpen van de genetische en moleculaire organisatie van *Cf* genen op de korte arm van chromosoom 1, is in het kader van dit promotieonderzoek een experimentele benadering gekozen om *Cf* genen met nieuwe specificiteiten te vinden op de korte arm van chromosoom 1. Om het tomatengenoom te verzadigen met moleculaire merkers, is een zeer dichte geïntegreerde RFLP-AFLP kaart gemaakt op basis van twee verschillende *L. esculentum* x *L. pennellii* F_2 populaties. Ondanks de kartering van 1175 AFLP merkers op deze kaart, die 1482 cM beslaat, werden er slechts negen AFLP merkers geïdentificeerd tussen de RFLP merkers CT233 en TG51, die een 23.6 cM interval markeren, waarin verschillende *Hcr9* clusters liggen op de korte arm van chromosoom 1. Dit relatief kleine aantal merkers is veroorzaakt doordat de meeste *Eco*RI/*Mse*I AFLP merkers geclusterd zijn rond de centromeren (Hoofdstuk 2).

Er zijn teskruisingspopulaties gemaakt van 66 *C. fulvum* resistente herkomsten door deze te kruisen met de bijna isogene tomatenlijn Moneymaker-Cf4 en de F_1 vervolgens te kruisen met de vatbare cultivar Moneymaker (Hoofdstuk 3). Om vatbare planten aan te tonen, zijn ziektetoetsen met *C. fulvum* fysio 0 uitgevoerd met slechts 24 planten van deze testkruisingspopulaties. Weinig vatbare planten duiden op koppeling van de *Cf* resistentie met *Cf-4* en daarmee positionering van de onbekende resistentie op chromosoom 1. Van de 21 resistente herkomsten die op deze manier zijn getoetst, vertoonden tien een *Cf-4* gekoppeld *Cf* gen. Eén van deze tien herkomsten herkende specifiek het extracellulaire eiwit ECP5 van *C. fulvum* en het corresponderende *Cf* gen werd *Cf-ECP5* genoemd. Dit gen is nauwkeuriger gekarteerd met behulp van een testkruisingspopulatie van 338 planten en een F_2 populatie van een kruising tussen Moneymaker en CfECP5, bestaande uit 233 individuen. *Cf-ECP5* werd vier cM proximaal van het *Hcr9* locus Milky Way gekarteerd en het corresponderende locus werd Aurora genoemd. Een amplificatieproduct dat absoluut gekoppeld was aan het *Cf-ECP5* gen, werd gekloneerd en van negen individuele klonen werd de DNA sequentie bepaald (Hoofdstuk 6). Deze negen klonen konden in vier groepen worden geclassificeerd, wat duidt op de aanwezigheid van meerdere *Hcr9*s op het Aurora locus.

Van de 66 bovengenoemde resistente *Lycopersicon* herkomsten zijn 64 getoetst op de aanwezigheid van *Cf-4* en/of *Cf-9* door middel van PVX::*Avr4*, respectievelijk PVX::*Avr9* inoculaties. Een onevenredig groot deel van deze herkomsten bevatten allemaal de functionele genen *Cf-4* en *Cf-4A* (Hoofdstuk 4). Sequentie analyse van het 3' uiteinde van *Cf-4* en een deel van het niet-coderende deel 3' van *Cf-4* vertoonden geen verschillen ten opzichte van de reeds gepubliceerde sequenties van *Cf-4*, dus bevat het introgressiesegment in deze herkomsten een identiek *Cf-4* en *Cf-4A* gen. Omdat verschillende van deze herkomsten in het verleden met een andere *Cf* nummer zijn aangeduid, is nu een wijziging in de nomenclatuur van *Cf* genen voorgesteld.

Vijf van de 66 bestudeerde herkomsten vertoonden HR door een specifieke herkenning van het extracellulaire schimmeleiwit ECP2 en bevatten dus het corresponderende resistentiegen *Cf-ECP2* (Hoofdstuk 5). Met behulp van twee testkruisingspopulaties en één F_2 populatie van een kruising tussen Moneymaker en CfECP2, welke in totaal 282 individuen vertegenwoordigden,

werd *Cf-ECP2* nauwkeurig gekarteerd. *Cf-ECP2* is absoluut gekoppeld aan de moleculaire merker CT116 en ligt proximaal van de *Hcr9* clusters Milky Way en Aurora, maar distaal ten opzichte van het Southern Cross locus. Het gebruik van *Cf-9* als een probe in een Southern hybridisatie toonde een hybridiserende band van circa 7.5 kb aan dat uitsplitste met *Cf-ECP2*, wat erop duidde dat *Cf-ECP2* deel uitmaakt van een tot nu toe onbekend *Hcr9* locus, dat Orion is genoemd.

Het onderzoek dat is beschreven in de Hoofdstukken 3, 5 en 6 laten zien dat *Cf* genen zich op verschillende *Hcr9* loci op de korte arm van chromosoom 1 kunnen bevinden en dat deze *Hcr9* loci veel polymorphisme vertonen.

Nawoord

Het nawoord is een gedeelte van het proefschrift dat door zijn titel altijd een beetje apart staat. Toch is het vaak één van de meest gelezen gedeeltes, wat het belang van wetenschap iets relativeert. De afgelopen vier jaar heb ik met enorm veel plezier op de vakgroep Plantenveredeling dit promotieonderzoek uitgevoerd. Dit is in de eerste plaats te danken aan de groep mensen die bij het onderzoek zijn betrokken en die ik dus wil bedanken.

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Curriculum vitae

Op 3 juli 1971 werd in Emmeloord, gemeente Noordoostpolder, een gezonde jongen geboren die naar de naam Jair Haanstra ging luisteren. Na 9 jaar inwoner van Tollebeek in dezelfde gemeente te zijn geweest, verhuisde hij met zijn ouders van het nieuwe naar het oude land en vestigde zich voor de volgende 9 jaar in Dordrecht, alwaar hij in 1989 het VWO diploma behaalde aan het Christelijk Lyceum in die plaats. Datzelfde jaar werd Wageningen zijn woonplaats om aldaar aan de toenmalige Landbouwuniversiteit Plantenziektekunde te gaan studeren, waarbij afstudeervakken werden afgelegd bij Agronomie, Fytopathologie en Moleculaire Biologie. De praktijktijd voor Fytopathologie werd doorgebracht bij de Biotechnology Unit van het Department of Agriculture van Victoria in Melbourne, Australië. In 1995 werd deze studie succesvol afgesloten en werd hij aangesteld als Assistent In Opleiding (AIO) bij de vakgroep Plantenveredeling op een project dat werd gefinancierd door de Associatie van Biotechnologische Onderzoekscholen in Nederland (ABON) wat leidde tot dit proefschrift. In die tijd ging hij ook weer in Dordrecht wonen met zijn vrouw Jantine. Vanaf 1 oktober 1999 is hij bij Rijk Zwaan werkzaam in de paprika- en auberginveredeling.