Parasexual fusion in Fusarium oxysporum; Biological and molecular characterization of avirulence recombinants
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CHAPTER 1

Introduction

Fungi are eukaryotic, carbon-heterotrophic microorganisms that have successfully occupied most natural habits. Most fungi live saprophytically by degrading dead organic material. Only a small minority (<10%) of the known fungal species (~100,000) is able to colonize living plants, and even a smaller fraction is capable of causing disease (Knogge, 1996). Although infection of plants and subsequent disease development are relatively rare, severe crop yield losses have made phytopathogenic fungi a serious economic factor, attracting the attention of farmers, plant breeders and scientists. By research on plant-pathogen interactions the knowledge about plant diseases is increased and, at the same time, novel methods, equipment and materials are being developed through which plant diseases can be avoided or controlled. As a model for the interplay between plants and pathogens, various research groups are studying the interaction between the fungal pathogen *Fusarium oxysporum* and its hosts. Within this fungal species the genetic variability is high, which is striking considering the fact that *F. oxysporum* is an imperfect fungus lacking sexual reproduction. Very likely 'parasexuality' plays an important role in diversification. In this chapter the genetic variation of *F. oxysporum* and the parasexual cycle will be discussed. Furthermore, genetic aspects of plant-pathogen interactions in general and more specifically the tomato-*F. oxysporum* interaction will be discussed.

GENETIC VARIATION WITHIN THE SPECIES *FUSARIUM OXYSPORUM*

Vegetative incompatibility

*Fusarium oxysporum* Schlechtend.: Fr. belongs to the Deuteromycetes (Fungi Imperfecti) and is classified in the section Elegans within the genus *Fusarium* (Snyder and Hansen, 1940). In spite of the asexual nature of *F. oxysporum*, phylogenetic evidence indicates a close relationship to taxa in the section Liseola with perfect forms in the *Gibberella*
Chapter 1

*fujikuroi* complex (Burns et al., 1991; O'Donnell et al., 1998; Mes, 1999). *F. oxysporum* is an anamorphic species including both pathogenic and non-pathogenic isolates, which are found in soils throughout the world. Given this widespread occurrence, it is generally assumed that pathogenic forms are derived from originally non-pathogenic antecedents (Gordon and Martyn, 1997). The pathogenic members cause root and crown rot through maceration of tissue or wilting due to colonization of the xylem vessels (MacHardy and Beckman, 1981; Mes, 1999). The host range of *F. oxysporum* is very wide since pathogenic variants are able to infect over 100 plant species. Individual isolates, however, have the ability to cause disease in a limited number of plant species only. Based on their host range, isolates of *F. oxysporum* are grouped into more than 80 formae speciales (Armstrong and Armstrong, 1981). Forma specialis *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen infects only tomato (*Lycopersicon esculentum*) and is the causal agent of Fusarium wilt disease in this crop. Its host range is restricted to *Lycopersicon* species.

Within many formae speciales several genetically isolated subpopulations or vegetative compatibility groups (VCGs) are found (Fig. 1A). Isolates are assigned to VCGs based on their ability to form heterokaryons. Since vegetative compatibility is based on *vic* loci, new VCGs can arise by mutations in a single *vic* locus. This means that VCGs can be genetically closely related. Molecular analysis has shown that VCGs within a forma specialis are not necessarily more closely related to each other than to VCGs from other formae speciales (Mes, 1999; Correll, 1991; Gordon and Martyn, 1997). As illustrated in Figure 1A, two related VCGs from different formae speciales might have originated from one genetic source. Within a monophyletic taxon, evolution has allowed the development of different host specificities (Fig. 1A). Alternatively, two VCGs within one forma specialis might have evolved from different genetic sources. Within such a polyphyletic taxon, which would describe the situation for the forma specialis *lycopersici*, the ability to cause disease in one particular host (tomato) has evolved independently (Fig. 1B).

Within VCGs subdivision into physiological races is possible (Fig. 1C). Different races of the pathogen are distinguished based on the (in)ability to infect particular cultivars known to carry one or more genes for resistance (Gordon and Martyn, 1997). For some formae speciales VCGs correlate with races, but for most formae speciales they do not (Fig. 1C). Since the same races have been identified within different VCGs, a parallel evolution for these races has been suggested (Kistler and Momol, 1990; Correll, 1991; Mes, 1999). For *F. oxysporum* f. sp. *lycopersici* three different races, named in order of discovery: race 1, 2 and 3, are known. A worldwide collection of over 100 *F. oxysporum* f. sp. *lycopersici* isolates representing all three races was analyzed and grouped with regard to vegetative compatibility (Elias and Schneider, 1991). One major (VCG0030), three minor (VCG0031, VCG0032 and VCG0033) and many single-member VCGs were
The identification of many formae speciales and races of *F. oxysporum* and the existence of several independently evolved VCGs is indicative for the extended genetic variability observed within this species. The *F. oxysporum* genome is observed to be very flexible. Extensive karyotypic variation is observed even for genetically related isolates within a VCG (Kim et al., 1993; Boehm et al., 1994; Migheli et al., 1995; Mes, 1999; Chapter 2). Estimations based on karyotype analysis revealed *F. oxysporum* genome size variation between 18.1 and 51.5 Mb (megabases) whereas the number of observed chromosomes varies between 7 to approximately 15, ranging in size from 0.6 Mb to at least 6.7 Mb (Migheli et al., 1993; Mes, 1999). For *F. oxysporum* f. sp. *lycopersici* the genome size is estimated to be between 41-51.5 Mb. Electrophoretic karyotyping allowed the identification of nine, ten and twelve chromosome bands for the three VCG0030 derived wild type isolates, Fol004, Fol007 and Fol029, respectively (Chapter 2 and 4). This is probably an underestimation of the real number of chromosomes, since intense bands might contain two or more chromosomes of approximately equal size.

For species that lack sexuality (e.g. *F. oxysporum*), an inverse correlation between the frequency of meiosis, and the extent of intra-specific karyotypic variation and genome flexibility has been proposed (Kistler and Miao, 1992). In the absence of meiosis, three
mechanisms contribute to the occurrence of karyotypic variation and genome flexibility: mutagenesis, chromosome rearrangements and mitotic recombination processes. Movement of mobile DNA elements has been proposed to be the main cause for mutations and chromosome rearrangements and thus karyotypic variation. Excision of transposable elements followed by variable religation can introduce inversions, deletions and chromosome breakage. So far nine families of transposable elements have been characterized in isolates of *F. oxysporum*. These include the transposon families *Fot1, Fot2, Impala, Hop* (reviewed in Daboussi and Langin, 1994), Tfo1 (Okuda et al., 1998) and the retrotransposons *Foret1 and Skippy* (Julien et al., 1992; Anaya and Roncero, 1995). Furthermore, retrotransposon-like repeated and interspersed sequences *Palm* and *Foxy* have been identified (Mouyna et al., 1996; Daboussi and Langin, 1994; Mes et al., 2000a). In addition to the excision and reintegration of transposable elements, the occurrence of genetic duplications in *F. oxysporum* has been indicated as a potential cause for intra-specific differences in genome size and chromosome number between isolates (Davière et al., 2001). Genetic duplications (e.g. copies of transposons and repeated sequences and/or duplicated unique sequences) can serve as substrates for ectopic recombination events causing chromosome rearrangements.

**The parasexual cycle**

Besides the occurrence of mutations and chromosome rearrangements, mitotic recombination might also explain the widespread morphological and physiological variation observed among and within imperfect fungal species (Weber and Arnold, 1992). Mitotic recombination may function as an alternative for sexual recombination. Pontecorvo (1956) introduced the term parasexuality, which has been generally accepted to describe mitotic recombination in asexual organisms. The parasexual cycle includes three phases: heterokaryon formation, karyogamy and haploidization (Fig. 2). The first phase is heterokaryon formation by which two genetically different haploid cells or hyphae fuse. Heterokaryons are generally stable but can spontaneously revert to monokaryons that continue their vegetative life cycle. The second phase is the occurrence of karyogamy: fusion of nuclei resulting in a heterozygous diploid. The chance for karyogamy to occur is rare, approximately $1 \times 10^{-6}$ as described for *Aspergillus niger* (Swart et al., 2001) and $1 \times 10^{-8}$ to $2 \times 10^{-8}$ for *Penicillium chrysogenum* (Weber and Arnold, 1992). In these species the diploid stage is relatively stable. Diploids show fairly normal growth and many individual diploid spores can be harvested from one fusion event (Klaas Swart, Laboratory of Genetics, Wageningen University, personal communication). During the diploid phase and the subsequent haploidization process, mitotic cross-overs can occur between homologous chromosomes. The non-disjunctual haploidization process reduces the diploid nucleus via aneuploid stages to a stable haploid stage. This process can be induced
by e.g. benomyl for all individual diploid spores separately. The haploidization process may take several mitotic generations and results in haploid recombinant colonies releasing a single type of spores.

Figure 2: The parasexual cycle including the three different stages: heterokaryon formation, karyogamy and haploidization. Both hyphal anastomosis (A) and protoplast fusion (B) can induce the parasexual cycle.

In natural situations, parasexual recombinants originate from hyphal anastomosis. Anastomosis is the process in which hyphae fuse, yielding in a network of interconnected hyphae or mycelium (Glass et al., 2000). Different fungal strains are capable of undergoing hyphal fusion with each other to form a heterokaryon. However, heterokaryon formation between two genetically different strains is impossible if they differ in one or more \textit{vic} loci (vegetative incompatibility; also called \textit{het} for \textit{heterokaryon incompatibility}). Vegetative incompatibility occurs in many fungi. When vegetative incompatibility is significant and many \textit{vic} loci are present, heterokaryon formation is likely to be rare and parasexuality unimportant for the generation of genetic diversity. When the converse is true, for example...
during the initial colonization of a new area by presumably one single haploid strain, vegetative incompatibility will be insignificant and mutations are expected to be the most important source of genetic variation. Hence, as mutants arise, parasexuality might bring about genetic exchange. Genetic diversity as a result of parasexual recombination would increase to a point where new combinations or mutations in *vic* loci lead to vegetative incompatibility again (Carlile et al., 2001).

Because anastomosis is limited to vegetatively compatible strains, incompatible strains representing different races, different formae speciales or even different species cannot be crossed. Protoplast fusion provides an efficient alternative for both genetic analysis and strain development through mitotic recombination in all kinds of species, regardless the occurrence of parasexuality by natural means (Anné, 1983). The first description of successful fusion of fungal protoplasts was reported by Ferenczy et al. (1974). Application of the fusogenic chemical polyethylene glycol (PEG) resulted in an improvement of the fusion efficiency of fungal protoplasts (Anné and Peberdy, 1975; Ferenczy et al., 1975). Despite its toxicity, PEG has become the most commonly used fusogen (Peberdy, 1987). The ability to produce heterokaryons and recombinants at high frequency provided geneticists the opportunity to carry out genetic analysis in asexual species. Interspecies hybridization also allows examination of chromosome homology between species and investigation of taxonomical relationships (Anné, 1983). Furthermore, interspecies protoplast fusion shows great potential in combining properties in order to improve industrial strains. Hence, many reports on intra- and interspecific parasexual crosses by means of protoplast fusion have been published for filamentous fungi (Table 1).

Protoplast generation involves the treatment of hyphae with cell wall degrading enzymes in an osmostabilising medium. Protoplasts are separated from non-digested hyphae by filtration, although not all hyphal fragments can be removed. Subsequently, protoplasts of different strains are combined and treated with PEG to induce fusion. The optimum conditions for PEG-mediated fusion have been shown to be fairly consistent for all fungal species: PEG of molecular weight 4,000 to 6,000 at a concentration of 25-40%, the presence of Ca\(^{2+}\) ions (10-100 mM CaCl\(_2\)) and pH in the range 7-9 are the essential requirements (Peberdy, 1987). During and after exposure to the fusogen, the protoplasts are subject to dehydration, shrinkage and distortion. Aggregates of an indeterminate number of protoplasts are formed. These may consist of protoplasts of the individual parent strain or a mixture of both strains. The latter are parasexual recombinants and should be recovered by the application of selection strategies. In most of the previous studies fusion products were selected through nutritional complementation of auxotrophic mutants. This selection method has been shown to be very accurate and useful for classical genetics (Anné, 1983).
Table 1: Fungal species for which a complete parasexual cycle has been described.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>References</th>
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<tbody>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Molnar et al., 1985a, 1985b and 1990</td>
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<td></td>
<td>Sidhu and Webster, 1978</td>
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<tr>
<td></td>
<td>Madhosingh, 1992 and 1994</td>
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<tr>
<td><em>Trichoderma</em> spp.</td>
<td>Toyoma et al., 1984</td>
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<td></td>
<td>Manczinger and Ferenczy, 1985</td>
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<td></td>
<td>Furlaneto and Pizzirani-Kleiner, 1992</td>
</tr>
<tr>
<td><em>Nectria haematococca</em></td>
<td>Daboussi and Gerlinger, 1992</td>
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<td></td>
<td>Bradshaw et al., 1983</td>
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<tr>
<td></td>
<td>Swart et al., 2001</td>
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<tr>
<td><em>Penicillium</em> spp.</td>
<td>Anné and Eyssen, 1978</td>
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<td></td>
<td>Durand et al., 1993</td>
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<tr>
<td><em>Beauveria</em> spp.</td>
<td>Couteuadier et al., 1996</td>
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<tr>
<td></td>
<td>Viaud et al., 1998</td>
</tr>
<tr>
<td><em>Pseudocercosporella herpotrichoides</em></td>
<td>Hocart et al., 1993</td>
</tr>
<tr>
<td><em>Dictyostelium discoideum</em></td>
<td>Gingold and Ashworth, 1974</td>
</tr>
<tr>
<td><em>Cladosporium fulvum</em></td>
<td>Talbot et al., 1988</td>
</tr>
<tr>
<td></td>
<td>Arnaud and Oliver, 1993</td>
</tr>
<tr>
<td><em>Cephalosporium acremonium</em></td>
<td>Hamlyn et al., 1985</td>
</tr>
<tr>
<td><em>Pyricularia oryzae</em></td>
<td>Genovesi and Magill, 1976</td>
</tr>
<tr>
<td><em>Verticillium</em> spp.</td>
<td>Typas, 1983</td>
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<tr>
<td></td>
<td>O’Garro and Clarkson, 1992</td>
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</table>

However, the use of auxotrophs in parasexual fusion has several disadvantages. 1) It requires a time-consuming selection procedure; 2) Mutants reverse with a frequency of $10^{-6}$-10^{-7} which is comparable to the chance for karyogamy; 3) Virulence behaviour of auxotrophic mutants (parental strains) is difficult or impossible to determine since these strains grow poorly *in planta*. In addition, loss of virulence has been described for prototrophic fusion products probably resulting from induction of additional mutations in the auxotrophic parents (Roebroeck, 2000); 4) For industrial strain improvement the use of auxotrophs is not suitable because the introduction of auxotrophic markers often impairs metabolite production. As an alternative for the use of auxotrophic mutants, antibiotic resistance genes can be introduced by transformation, and used as dominant selection markers in protoplast fusion experiments (Talbot et al., 1988; Arnaud and Oliver, 1993; Durand et al., 1993; Roebroeck, 2000).
Following protoplast fusion with auxotrophic parental strains, the selection of prototrophic heterokaryons is performed on minimal regeneration medium. When dominant drug resistance markers are used, doubly resistant fusion products are selected on medium containing both antibiotics. Differences in growth and stability of heterokaryons and differences in the ability to produce diploid progeny have been observed for various species. Heterokaryons of some well studied species such as *Penicillium* spp. and *Aspergillus* spp. are easily obtained (Kevei and Peberdy, 1979 and 1984). On further incubation heterozygous diploid mycelium and spores could be obtained. Recombinant sectors can develop within a diploid indicative for haploidization. When diploid colonies are cultivated on complete medium with benomyl, the haploidization process is enhanced and haploid recombinants arise with high frequency (Anné, 1983; Durand et al., 1993; Swart et al., 2001). For other species like *Cephalosporium acremonium* and *Fusarium oxysporum* the different phases of the parasexual cycle are more difficult to detect. Heterokaryons appear as slowly growing colonies indicative for nuclear imbalance during heterokaryosis. Diploid hybrids have never been observed in these species. Failure to isolate diploids might be due to a transientness of the diploid stage in the parasexual cycle, followed by rapid intrachromosomal recombination and non-disjunctual haploidization processes (Hamlyn and Ball, 1979; Molnar et al., 1990). Cells in which these processes have been completed might grow normally and form a fast growing sector, which can easily be distinguished from the heterokaryon phase. Spores from these fast growing sectors might represent stable haploid recombinants. Molecular analysis of these stable, haploid recombinants will teach us more about the genetic exchange which has taken place during the parasexual cycle.

**GENETIC ASPECTS OF PLANT-PATHOGEN INTERACTIONS**

**Gene-for-gene concept**

The outcome of the interaction between a specific race of a given pathogen and a host plant species depends on the genetic background of both partners. When the pathogen contains an avirulence (*Avr*) gene that corresponds to a resistance (*R*) gene in the plant, resistance is observed. This resistance is complete and depends fully on the presence of both the *Avr*-gene and the corresponding *R*-gene in the interaction. Flor (1942) was the first to recognize that monogenic, dominant avirulence genes in a pathogen, i.e. the fungus *Melampsora lini*, correspond to monogenic, dominant resistance genes in its host (flax). Presence of both genes is required for resistance. When one of them or both are absent, the pathogen is virulent and the plant develops disease symptoms. This concept, known as the gene-for-gene hypothesis, infers that *Avr*-genes are defined by corresponding *R*-genes and the other
way around: no $R$-gene without a corresponding $Avr$-gene. Since the hypothesis was put forward by Flor (1942), many other plant-pathogen interactions were found that supported the gene-for-gene relationship (Crute, 1985; Keen, 1990). From a biochemical/physiological point of view, the concept can be interpreted as a ligand-receptor model in which the $Avr$-gene encodes the ligand (or race-specific elicitor) that is recognized by a receptor encoded by the corresponding $R$-gene. Direct recognition triggers the resistance response. Alternatively, the race-specific elicitor may bind to a virulence target that is 'guarded' by the resistance gene product (Van der Biezen and Jones, 1998; Van der Hoorn et al., 2002; De Wit, 2002). According to this 'guard' interpretation of the gene-for-gene model, binding of the elicitor to the virulence target (implying a virulence function for the elicitor), is proposed to cause conformational changes, (de)phosphorylation and/or recruitment or release of additional factors by which the guarding resistance gene product triggers the defense responses in a timely fashion.

The above situation can best be described as absolute resistance: when a $R$-gene is present in the plant and the fungus carries the matching $Avr$-gene, the plant is resistant (Fig. 3A). However, when a pathogen's $Avr$-gene or its corresponding $R$-gene in the plant are missing from the interaction, disease symptoms can range from moderate to severe. This observation suggests that both the plant's general defense and fungal virulence factors play a role in the outcome of the interaction (Fig. 3B). Development of disease symptoms can be influenced by e.g. additional elicitors, plant-specific defense molecules, fungal-specific repressors of defense or fungal toxic compounds.

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**Figure 3A:** Plant resistance according to the gene-for-gene concept ($R+Avr$) **B:** The outcome of other interactions ($R+avr, r+Avr, r+avr$) is determined by the plant's general defense and fungal virulence factors.
Avirulence genes and gene products
To date, a variety of viral, bacterial and fungal avirulence genes have been cloned and characterized. Analysis of these genes has not yet identified the physiological role of the gene products. Based on DNA sequences, little similarity has been found among the various avirulence genes or to other sequences in the databases. In addition, no conserved peptide motifs have been found in the avirulence gene products (Leach and White, 1996). In Table 2 the fungal avirulence genes that have been cloned and characterized are listed. In general, fungal avirulence genes encode relatively small, mostly cysteine-rich precursor proteins which are either N- and/or C-terminally processed by fungal and/or plant proteases (Luderer and Joosten, 2001).

Table 2: Overview of the fungal avirulence genes and their matching resistance genes.

<table>
<thead>
<tr>
<th>avirulence gene/factor</th>
<th>pathogen</th>
<th>matching R gene</th>
<th>host</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvrPITA</td>
<td>Magnaporthe grisea</td>
<td>Pi-ta</td>
<td>Rice</td>
<td>Jia et al., 2000</td>
</tr>
<tr>
<td>Avr2</td>
<td>Cladosporium fulvum</td>
<td>Cf-2</td>
<td>Tomato</td>
<td>Luderer et al., 2002</td>
</tr>
<tr>
<td>Avr4</td>
<td>Cladosporium fulvum</td>
<td>Cf-4</td>
<td>Tomato</td>
<td>Joosten et al., 1994</td>
</tr>
<tr>
<td>Avr4E</td>
<td>Cladosporium fulvum</td>
<td>Her9-4E</td>
<td>Tomato</td>
<td>De Wit et al., 2002</td>
</tr>
<tr>
<td>Avr9</td>
<td>Cladosporium fulvum</td>
<td>Cf-9</td>
<td>Tomato</td>
<td>Van Kan et al., 1991</td>
</tr>
<tr>
<td>ECP1</td>
<td>Cladosporium fulvum</td>
<td>Cf-ECP1</td>
<td>Tomato</td>
<td>Laugé et al., 1997 and 2000</td>
</tr>
<tr>
<td>ECP2</td>
<td>Cladosporium fulvum</td>
<td>Cf-ECP2</td>
<td>Tomato</td>
<td>Laugé et al., 1997 and 2000</td>
</tr>
<tr>
<td>ECP3</td>
<td>Cladosporium fulvum</td>
<td>-</td>
<td>Tomato</td>
<td>Laugé et al., 2000</td>
</tr>
<tr>
<td>ECP4</td>
<td>Cladosporium fulvum</td>
<td>Cf-ECP4</td>
<td>Tomato</td>
<td>Laugé et al., 2000</td>
</tr>
<tr>
<td>ECP5</td>
<td>Cladosporium fulvum</td>
<td>-</td>
<td>Tomato</td>
<td>Laugé et al., 2000</td>
</tr>
<tr>
<td>NIP1</td>
<td>Rhynchosporium secalis</td>
<td>Rrs1</td>
<td>Barley</td>
<td>Rohe et al., 1995</td>
</tr>
</tbody>
</table>

Why do pathogens carry avirulence genes that prevent successful infection of a host that carries the matching resistance genes? One of the working hypotheses suggests that avirulence genes play a role in virulence. The role as virulence factor became apparent for some avirulence genes when they were inactivated or transferred to different microbes or after ectopic expression in host plants (Van 't Slot and Knogge, 2002). In the extensively
studied plant-pathogen interaction between tomato and *Cladosporium fulvum*, the *Avr9* gene was present and identical in all analyzed avirulent fungal strains but was absent in virulent strains (Van Kan et al., 1991). However, *Avr9* disruption mutants were not affected in their growth pattern in *vitro* or on Cf0 plants (Marmeisse et al., 1993), suggesting that the pathogen does not require the intact *AVR9* for full virulence. The absence of *AVR4* had no effect on fungal virulence either. In contrast to *AVR4* and *AVR9*, an effect on fungal virulence has been shown for two other extracellular proteins, *ECP1* and *ECP2* (Laugé et al., 1997; Van 't Slot and Knogge, 2002). Both *Ecp* genes are present in all tested fungal strains. The *Ecp1*-deficient strain was not affected in its invading and colonizing ability but conidiophore formation was drastically reduced whereas the *Ecp2*-deficient strain was inhibited in its development and hardly produced any conidia. In addition to this apparent role in fungal development, it has been postulated that *ECPs* are suppressors of host defense reactions (Laugé et al., 1997). Similar to the *ECPs* from *C. fulvum*, the avirulence factor *NIP1* from *Rhynchosporium secalis* has been suggested to participate in virulence because of its toxic activity. Disruption of the *Nip1* gene resulted in a weak reduction in symptom expression on susceptible plants (Van 't Slot and Knogge, 2002). The exact mode of action of the race-specific elicitors in avirulence and in virulence remains to be determined. The identification of more avirulence genes in various plant-pathogen interactions will help to understand more of their function.

**The interaction between *F. oxysporum* f. sp. *lycopersici* and tomato**

The life cycle of *F. oxysporum* f. sp. *lycopersici* includes a saprophytic and a parasitic phase. The fungus survives on plant debris as mycelium, microspores, macrospores and, especially in the cooler temperature regions, as chlamydospores (Agrios, 1997). The formation and disposition of the thick-walled chlamydospores which are very specific for the species, permits the fungus to persist in most soils for many years (Beekman, 1987; Mes, 1999). Spores are stimulated to germinate by plant exudates (Catska et al., 1975) and the developing hyphae colonize the root surface. Penetration of the tomato roots occurs through wounds or at points of lateral root formation since mature epidermis is absent at these points. This offers the fungus the possibility of a direct entrance into the vascular system. In the xylem vessels the fungus sporulates and spores are transported through the transpiration stream until their upward movement is stopped by sieve elements. In order to overcome the obstruction, spores germinate and the newly formed hyphae penetrate the sieve element. More spores will be produced in the next vessel. The mycelium also advances laterally into the adjacent vessels, penetrating them through the pits. As a response, tomato actively tries to restrict the pathogen to the site of infection to prevent spreading throughout the plant. After perception of the fungus, the plant reacts with a complex mixture of defense responses. Callose-containing apposition layers and papillae
are formed within the xylem parenchyma cells to prevent lateral spreading of the fungus (Beckman, 1987; Mes, 1999). Vascular blockage is accomplished by deposition of tyloses and by the formation of pectic gels and gums to avoid systemic spread. Accumulation of hyphal cell wall degrading enzymes like β-1,3 glucanases and chitinases, pathogenesis-related (PR) proteins, phytoalexins like rishitin and plant growth regulators like IAA and ethylene has been observed. All these responses have been observed in both resistant and susceptible tomato plants. The difference between a resistant and a susceptible interaction is proposed to be determined by the speed and extent of the defense responses. A resistant plant will succeed in localizing the fungus to the site of infection and will therefore survive. A susceptible plant, however, is not capable to stop fungal spreading. The evermore increasing vascular blockage will result in wilting of the leaves and eventually in the suicidal death of the plant, allowing the fungus to complete its life cycle.

Since monogenic dominant race-specific resistance genes in tomato have been found against *F. oxysporum* f. sp. *lycopersici*, the gene-for-gene concept was adapted as working hypothesis for this interaction. Resistance to the three races of *F. oxysporum* f. sp. *lycopersici* (I and I-1 to race 1, I-2 to race 2 and I-3 to race 3) has been identified in several *Lycopersicon* accessions (reviewed in Mes, 1999). The I-2 resistance gene, which confers resistance to race 2 isolates, has been cloned and characterized (Simons et al., 1998). The gene is located on chromosome 11 within a cluster of seven homologues. The I-2 protein contains three structural domains: a N-terminal coiled coil domain, a central NB-ARC domain and a C-terminal leucine rich repeat region (LRR). The I-2 protein is proposed to be cytoplasmatic as no membrane spanning domains were predicted. Expression studies of the I-2 protein revealed vascular-specific expression in xylem parenchyma cells, premature xylem vessels and rays cells of young roots. In stems of older plants I-2 was also expressed in endodermis, cambial zone and phloem (Mes et al., 2000b). The expression of I-2 in the cells directly involved in the resistance response suggested an important role for this protein in the recognition of the pathogen. Because nothing is known about the race-specific elicitors, we can only speculate about the mechanisms by which recognition will occur. For instance, it is unknown whether *F. oxysporum* f. sp. *lycopersici* is capable of injecting race-specific elicitors into the plant applying a *hrp*-like mechanism as described for bacterial systems (Leach and White, 1996), in order to allow direct recognition by the cytoplasmic I-2 receptor. Alternatively, a membrane bound I-2 interactor or a receptor complex might directly be involved in elicitor recognition. In order to answer any of these questions the avirulence genes need to be cloned and characterized.
OUTLINE OF THE THESIS

To study the interaction between tomato and *Fusarium oxysporum* f. sp. *lycopersici* on a molecular level, the cloning of the plant resistance genes encoding species-specific receptors, as well as the corresponding avirulence genes of the pathogen encoding the race-specific elicitors are a prerequisite. This thesis describes the work done in a collaborative STW project (ABI55.3754) aimed to identify, isolate and characterize the tomato resistance genes *l*, *l-1* and/or *l-3* and the corresponding avirulence genes *A1* and/or *A3* of *F. oxysporum* f. sp. *lycopersici*. The study described in this thesis concentrated on the fungal site of the interaction. To identify fungal avirulence genes different approaches can be envisaged:

*Cloning by complementation.*

Avirulence genes of pathogenic bacteria have been cloned by complementation (Dangle, 1994). Due to the large genome size and the low transformation efficiency, a complementation strategy is not feasible for *F. oxysporum* (Mes, 1999). For example: assuming the genome size to be 50 Mb and an average insert size to be 25 kb per cosmide clone, 2,000 clones are needed to cover the entire genome just once. When a cosmid library is constructed with a genome coverage of five times, 10,000 individual transformants should be tested for complementation. Since the transformation efficiency is approximately one transformant per 2x10⁶ protoplasts, depending on the plasmid and the size of the insert, the production of 10,000 transformants alone would take at least five years. The *Avr2* gene of *Cladosporium fulvum* has been recently cloned employing a functional screen based on the hypersensitive (HR)-inducing activity of the encoded protein (Takken et al., 2000; Luderer et al., 2002). Unfortunately, a HR has never been observed macroscopically for *F. oxysporum* and, therefore, this approach is not applicable for this pathogen.

*Transposon tagging, insertional mutagenesis.*

Many transposable elements have been described in *F. oxysporum* (Daboussi, 1997) but a transposon-tagging strategy was not effective so far (Brown and Holden, 1998).

*Product-based cloning.*

Avirulence genes of the fungal pathogens *Cladosporium fulvum* (Van Kan et al., 1991; Joosten et al., 1994; Laugé and De Wit, 1998) and *Rhynchosporium secalis* (Rohe et al., 1995) have been successfully cloned by a product-based cloning strategy. Since avirulence gene products of *F. oxysporum* f. sp. *lycopersici* are not known and are most likely present in infected plant tissues in amounts too low to be isolated, a product-based cloning strategy for the isolation of avirulence genes was not feasible at the time of the start of this project. Recently, Rep et al. (2002) have shown that infection-specific up-regulated proteins derived from both plant and fungus can be identified in the xylem sap of infected plants.
Therefore, attempts to isolate avirulence gene products from xylem sap of infected susceptible plants seems to be very promising.

**Map-based cloning.**

A random deletion mutagenesis approach using gamma-irradiation to generate a *F. oxysporum* f. sp. *lycopersici* Avr1-2 mutant appeared to be very inefficient. Furthermore, no markers could be found that allowed mapping of the mutated gene (Mes et al., 1999). Genetic mapping of avirulence genes (Van der Lee et al., 2001; MacGregor et al., 2002) and subsequent map-based cloning has been successful in *Magnaporthe grisea* (Valent, 1997; Orbach et al., 2000). Although for *F. oxysporum* no sexual stage has ever been observed, a map-based cloning strategy could be feasible when crosses between different races can be forced.

In this thesis the forced crosses between different races of *F. oxysporum* f. sp. *lycopersici* in order to generate recombinants for a map-based cloning strategy are described. Parasexual crosses by means of protoplast fusion were performed between race 1 isolate Fol004 (putative genotype *A1a2a3*) and race 3 isolate Fol029 (putative genotype *a1a2A3*) to generate avirulence recombinants. The identification and characterization of 32 recombinants (fusion products) are described in Chapter 2. Among the 32 fusion products two avirulence recombinants were identified containing both avirulence gene *A1* and *A3*. Karyotype analysis revealed the enormous flexibility of the *F. oxysporum* genome. Evidence for the occurrence of chromosome rearrangements including mitotic recombination was postulated. However, no direct correlation between karyotype and avirulence phenotype could be identified.

In order to search for a correlation between a molecular marker and the avirulence phenotype, extensive molecular analysis was performed. The use of a newly developed molecular marker technique, an applied AFLP technique based on the retroposon *Foxy* (*Foxy*-AFLP), enormously increased our possibilities to study the 32 recombinants on a molecular level. Polymorphism rates between the genetically closely related isolates Fol004 and Fol029 were increased from approximately 2% found with standard AFLP to approximately 50% using *Foxy*-AFLP (Mes et al., 2000a; Chapter 3). Based on 83 randomly segregating *Foxy* markers the first genetic map of *F. oxysporum* was generated. This genetic map provides anchor points for mapping any gene of interest. We were able to find one *Foxy* marker that completely co-segregates with the presence of *A1* (Chapter 3). Although the presence of both *A1* and the linked *Foxy* marker shows a biased segregation pattern, this molecular linkage seems to be very promising and might lead to identification of avirulence gene 1.

In addition to parasexual crosses involving race 1 and race 3 isolates, fusions between race 1 isolate Fol004 and race 2 isolate Fol007 (putative genotype *a1A2A3*) have
been performed. Most fascinating was the discovery of a near-isogenic *F. oxysporum* f. sp. *lycopersici* strain with a novel combination of avirulence characteristics that originated from this cross (Chapter 4). The presumed genotype of this new strain (F1-27), near-isogenic to its Fol007 parent, is *a1A2a3*. Foxy-AFLP analysis revealed eleven new Foxy insertions in F1-27. Segregation of both avirulence and Foxy-AFLP markers was observed in a backcross population from parasexual crosses between F1-27 and its parent Fol007. One marker was found to co-segregate with the presence of the *a3*-allele. The combination of a near-isogenic strain together with co-segregating markers provide excellent tools in search for avirulence gene 3.

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Chapter I


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