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Induction of mutants of Fusarium oxysporum f. sp. lycopersici with altered virulence

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Abstract

Mutants of *Fusarium oxysporum* f. sp. *lycopersici* were obtained by UV irradiation. The mutants of race 1 and race 2 caused disease symptoms on plants with resistance genes against the corresponding wild type strains. Mutants of race 1 of the pathogen were stable, whereas mutants of race 2 lost the ability to cause disease symptoms in plants carrying the I-2 resistance gene, after prolonged maintenance on potato dextrose agar. Mutants of race 1 resembled race 2 in pathogenicity and they were vegetatively compatible with race 2, but no longer with race 1. These results suggest that the isolated strains with an altered virulence pattern have mutations in loci involved in avirulence.

Introduction

Populations of *Fusarium oxysporum* Schlecht. are differentiated in formae speciales and races based on differences in pathogenicity to various hosts and specificity to cultivars of the host. Race-cultivar specificity of many plant-pathogen interactions can be described by a gene-for-gene model (Crute, 1985). This model implies that a product of an avirulence gene of the pathogen is recognized by a product of a resistance gene of the plant, resulting in an incompatible interaction. Races of a pathogen without a functional avirulence gene cause a susceptible reaction, because they are not recognized by the host.

In Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* a gene-for-gene relation could exist. The resistance of the plant is controlled by dominant genes: the I-1 gene against race 1 of the fungus and the I-2 gene against race 2. Because classical genetic studies are not possible with this anamorphic fungus, the molecular basis of virulence and avirulence is difficult to investigate.

Induction of mutations from avirulence to virulence can give evidence for the existence of a gene-for-gene relation in this plant-pathogen interaction. Such mutations from avirulence to virulence have been reported for several plant pathogens. Bouhot (1981) observed new races of *F. oxysporum* f. sp. *melonis* after mutagenic treatments. A race 4 of *Cladosporium fulvum* was produced with N-methyl-N-nitro-N- nitrosoguanidine treatment of race 0 (Higgens et al., 1987). Mutants with increased virulence were induced in *Erysiphe graminis* f. sp. *tritici* (Gabriel et al., 1982). Generally, in these studies alteration from avirulence to virulence could be related to the loss of a functional avirulence gene product.

Strains of *F. oxysporum* f. sp. *lycopersici* with a mutation from avirulence to virulence have not yet been described. Only Sidhu and Webster (1979) isolated auxotrophic mutants of *F. oxysporum* f. sp. *lycopersici* among which the arginine-and adenine-requiring ones showed a slightly increased virulence. Most of these mutants, however, showed decreased virulence.

In this study conidial suspensions of race 1 and 2 of *F. oxysporum* f. sp. *lycopersici* were treated with UV irradiation. In order to obtain evidence for the existence of a gene-for-gene relation, mutant strains with altered or increased virulence were isolated and partially characterized.

Materials and methods

Tomato plants and pathogen. Tomato plants (cultivar Moneymaker) of the near-isogenic lines GCR161 (resistant to race 1 of *F. oxysporum* f. sp. *lycopersici*), C295 (resistant to race 1 and 2 of this fungus) and C32 (susceptible to both races), were grown in a glasshouse at 22-24 °C. Seeds of the tomato lines were obtained from the Glasshouse Crops Research Institute, Littlehampton (U.K.).

F. oxysporum f. sp. *lycopersici* race 1 (WCS 801) and race 2 (WCS 862) were maintained on potato dextrose agar (PDA). Conidial suspensions were obtained by culturing the fungus in Czapek Dox medium (Oxoid CM95) on a reciprocal shaker for 7 days at 25 °C. Mycelial fragments were removed by filtering through sterile glass-wool. After washing in sterile water, the conidial suspension was adjusted to the desired concentration.

Induction and isolation of mutants. Mutants of race 1 and race 2 of the fungus were obtained after treatments with UV irradiation. Ten ml of a conidial suspension (5×10^6 conidia/ml) was irradiated in a 9 cm Petri dish with a UV lamp (Philips TUV 30W) at 50 cm height for 0-4 minutes. Survival percentages were determined on PDA. The virulence of surviving colonies was tested using tomato plants. Three-week-old plants were uprooted, roots were immersed in a conidial suspension (1×10^7 conidia/ ml) of the mutants for 10 minutes and plants were repotted. Disease symptoms were assessed using an ordinal disease index: 0: healthy, 1: epinasty of some leaves, 2: wilting of some leaves, 3: yellowing and necrosis of some leaves, wilting of all leaves, 4: yellowing and necrosis of most leaves, some leaves fallen, 5: plant dead.

Vegetative compatibility of mutants. Mutants causing disease symptoms in plants resistant to the wild types were isolated from plants by placing slices of the stem on PDA supplemented with streptomycin (0.02%) and penicillin (0.01%). *F. oxysporum* colonies growing out of the slices were grown on minimal medium agar amended with 1.5% $KCLO_3$ and 0.16% L-asparagine to isolate spontaneous chlorate-tolerant mutants (Puhalla, 1985). These mutants were screened for auxotrophy for organic nitrogen sources (which frequently accompanies tolerance to chlorate) by transferring them to minimal medium. The nitrate non-utilizing (*nit*) mutants were used to determine vegetative compatibility between wild type races and mutants with altered virulence. *Nit* mutants were paired on minimal medium. The pairings were incubated at room temperature and after 7-14 days scored for complementation, indicated by the development of dense aerial mycelium where the two *nit* mutants came in contact.

Results

Four minutes UV-irradiation resulted in a survival of 0.01 % of race 1 conidia, and of 5% of those of race 2. Conidia surviving irradiation of 0.5, 1 and 1.5 minutes (survival of 10-80% for race 1, 36-90% for race 2) were tested on plants. Most colonies growing from irradiated conidia appeared to be white. After one cycle of subculture, however, most of them regained their original red color. Twenty-four colonies of race 1 were tested on susceptible plants (C32) and plants resistant to race 1 (GCR161). Eleven of these colonies were virulent on the susceptible as well as on the resistant line (Table 1). Twenty-one colonies of race 2 were tested. Five of them were virulent on resistant plants (C295) (Table 2).

After 3 and 8 months the virulence of the isolated mutants was assessed again. Inoculation with mutants of race 1 again resulted in disease symptoms in both the susceptible (C32) and resistant (GCR161) plants, with the exception of M1-16. When race 1 mutants were tested on plants resistant to both race 1 and 2 of *F. oxysporum* f. sp. *lycopersici* (C295), no disease symptoms developed, with the exception of M1-10

Table 1. Number of plants of the near-isogenic tomato lines C32 and GCR161 with disease symptoms of index 0-5, 2 weeks after inoculation with mutants (M1) or the wild type of *F. oxysporum* f. sp. *lycopersici* race 1. Line C32 is susceptible, and line GCR161 resistant to race 1.

Fungus	Plant	Disease index					
		0	1	2	3	4	5
M1-2	C32					3	2
	GCR161				4	4	
M1-8	C32				1	1	3
	GCR161					2	6
M1-9	C32				1	1	3
	GCR161				4	2	2
M1-10	C32					4	1
	GCR161				5	3	
M1-11	C32					4	1
	GCR161		1		5	2	
M1-12	C32				2	3	
	GCR161		2		4	2	
M1-16	C32				1	4	
	GCR161			1	6	1	
M1-18	C32				2	3	
	GCR161				5	3	
M1-21	C32					1	4
	GCR161			1	4	1	2
M1-22	C32					3	2
	GCR161			3	5		
M1-24	C32			1	4		
	GCR161	3		2			
race 1	C32					2	3
	GCR161	8					

Table 2. Numbers of plants of the near-isogenic tomato lines C32 and C295 with disease symptoms of index 0-5, 5 weeks after inoculation with mutants (M2) or the wild type of *F. oxysporum* f. sp. *lycopersici* race 2. Line C32 is susceptible and line C295 resistant to race 1 and 2.

Fungus	Plant	Disease index					
		0	1	2	3	4	5
M2-9	C32					2	3
	C295	2		1	2		
M2-13	C32		3	2			
	C295		3	2			
M2-14	C32		1		2		2
	C295						5
M2-15	C32				2	2	1
	C295	3		1	1		
M2-21	C32		1		1		3
	C295	2	2	1			
race 2	C32			3	1		1
	C295	5					

which caused disease symptoms in both types of plants (data not shown). Three months after isolation, the race 2 mutants had all lost the ability to cause disease symptoms in resistant plants (C295).

The mutants of race 1 and race 2 were maintained on PDA at 4 °C and subcultured every 3 months. Growth decreased gradually and several strains had stopped growing altogether after 6 month (race 2) and 14 months (race 1) of subculture. Lyophilization proved a better alternative for long-term storage of the mutants. Cultures, regrown from freeze-dried conidia, consistently induced disease symptoms in resistant as well as in susceptible plants (data not shown). In a second mutagenesis experiment additional mutants with an altered virulence pattern were isolated. After testing 16 strains from each race, one mutant strain could be isolated from each race. Some other race 2 mutants showed increased aggressiveness on susceptible plants and on plants resistant to race 1.

Vegetative compatibility characteristics of eight race 1 mutants were determined. Of each mutant, a number of complementary nitrate non-utilizing (*nit*) mutants were induced on chlorate-containing minimal medium. These *nit* mutants were paired with various *nit* mutants of the wild types of race 1 and race 2 of *F. oxysporum* f. sp. *lycopersici*. The wild types of race 1 and 2 did not form heterokaryons with each other. Race 1 and race 2 were self-compatible (Table 3). The three *nit* mutants of race 1 wild type were identified by Baayen and Kleijn (1989): *nit* mutant a was identified at *nit* M (mutation at locus that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity), *nit* mutant b was identified at *nit* 3 (mutation at a nitrate-assimilation pathway-specific regulatory locus) and *nit* mutant c was identified at *nit* 1 (mutation at a nitrate reductase structural locus) (Correll et al., 1987). All the mutant strains tested were able to form heterokaryons with one or more *nit*

Table 3. Heterokaryon formation between various *nit* mutants of wild types of race 1 and 2 of *F. oxysporum* f. sp. *lycopersici*; + development of aerial mycelium, − no complementation.

		Race 1 [1)]			Race 2				
		a	b	c	a	b	c	d	e
race 1	a	−	+	+	−	−	−	−	−
	b		−	−	−	−	−	−	−
	c			−	−	−	−	−	−
race 2	a				−	−	+	+	−
	b					−	−	+	−
	c						−	+	+
	d							−	+
	e								−

[1)] a: *nit* M, b: *nit* 3, c: *nit* 1.

Table 4. Complementation between *nit* mutants of race 1 and 2 of *F. oxysporum* f. sp. *lycopersici* and *nit* mutants of the virulence mutants (M1) of race 1 of this fungus; + development of aerial mycelium, − no complementation. *Nit* mutants from single strains, followed by different letters, are different from each other.

		nit mutants race 1			*nit* mutants race 2				
		a	b	c	a	b	c	d	e
M1-2	a	−	−	−	−	−	−	+	−
	b	−	−	−	+	−	−	+	−
M1-9	a	−	−	−	−	−	−	+	−
M1-10	b	−	−	−	+	−	−	+	+
M1-11	a	−	−	−	+	+	−	+	+
	b	−	−	−	−	−	−	+	−
	c	−	−	−	−	−	+	+	−
M1-12	a	−	−	−	−	−	+	+	−
	b	−	−	−	+	+	+	+	+
	c	−	−	−	−	−	−	+	−
M1-16	a	−	−	−	+	+	−	+	+
M1-18	a	−	−	−	−	−	+	+	−
	b	−	−	−	−	−	−	+	−
M1-21	a	−	−	−	+	−	+	+	+
	b	−	−	−	−	−	−	+	−
	c	−	−	−	+	−	−	+	+

mutants of *F. oxysporum* f. sp. *lycopersici* race 2. No heterokaryosis was observed between these strains and *nit* mutants of *F. oxysporum* f. sp. *lycopersici* race 1 (Table 4).

Discussion

UV irradiation of conidial suspensions of race 1 and 2 of *F. oxysporum* f. sp. *lycopersici* induced mutants with an altered virulence pattern towards plants with the corresponding genes for resistance or induce mutants with an increased virulence to susceptible plants. Mutants of race 1 resembled race 2 in various ways. They did cause disease symptoms in plants resistant to race 1, but not in plants resistant to race 1 and 2. They were vegetatively compatible with race 2 and they did not have the ability to form heterokaryons with race 1 any more. These results can be explained by a model in which the loss of a functional product of an avirulence gene results in a failure to recognize the product of the resistance gene of the plant. This mutation seems to be related to heterokaryon formation. The absence of mutants with only an altered compatibility pattern (unaltered in virulence) can give more evidence that compatibility genes and avirulence genes are closely linked. One mutant of race 1 (M1-10) even caused symptoms on plants with both resistant genes, suggesting the loss of functional products of both avirulence genes.

The reduced fitness of these strains may explain the rare occurrence of this kind of mutants in nature. Resistance to race 1 provided by the I-1 gene completely controlled wilt in tomato for 20 years before race 2 became a problem (Beckman, 1987). Heale (1989) proposed a model for the appearance of a new race, in which apart from a mutation at the avirulence locus several mutations at loci determining non-specific interactions are necessary for the development of a new highly virulent race. This model indeed would explain the problems when cultivating the mutants on agar media (reduced fitness) and the long term stability of the I-1 gene. The model would also explain the results obtained with race 2 mutants. Here too, apparently fitness of the hypervirulent mutants was affected in a way that resulted in a loss of hypervirulence if the mutants were kept on agar media for a prolonged period. In contrast to the race 1 mutants, a race 2 mutant (M14, Table 2) nevertheless showed a full breakdown of the resistance of tomato line C295, suggesting that a reduced fitness of this mutant on potato dextrose agar did not necessarily affect the behaviour of the mutant *in planta*.

For a further analysis of the genetic basis of the relationship between *F. oxysporum* f. sp. *lycopersici* and the tomato plant, the molecular background of the products of both the avirulence genes of the pathogen and the corresponding resistance genes of the plant need to be elucidated. Evidence proving that single genes are involved in the recognition events in race-specific resistance was already obtained for several plant-bacterium combinations by the molecular analysis of mutants with altered virulence patterns. Kearny et al. (1988) showed that transposon mutation in the avirulence locus avrBs1 of *Xanthomonas campestris* pv. *vesicatoria*, the cause of spot disease in pepper, resulted in the failure of the recognition process and therefore in virulence. Also in other bacteria, genes for avirulence already have been cloned (Gabriel et al., 1986; Staskawicz et al., 1984). The only example of a fungal avirulence gene cloned is the cDNA of gene avr9 of *Cladosporium fulvum*, the causal agent of tomato leaf mold (Van Kan et al., 1991).

Similar experiments should be extended to other plant pathogenic fungi. Transformation systems for fungal pathogens are being developed (Dickman, 1988; Oliver et al., 1987; Rodriguez and Yoder, 1987). With the aid of transformation systems for

Fusarium oxysporum (Kistler and Benny, 1988; Langin et al., 1990) the existence of avirulence genes and their products could be investigated in *F. oxysporum* f. sp. *lycopersici*. In this way molecular evidence can be gained for a gene-for-gene relation in the interaction between *F. oxysporum* f. sp. *lycopersici* and tomato plants. Our study already provides some important evidence for the existence of such a relationship.

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