Diversity of Virulence in *Phytophthora parasitica* on Tobacco, as Reflected by Nuclear RFLPs

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ABSTRACT

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A worldwide collection of *P. parasitica* isolates was investigated for the ability to infect tobacco and tomato, as related to elicitin production. Elicitin was produced by all nontobacco isolates, and nonproducing strains all were isolated from tobacco. In addition, producing strains were isolated from tobacco and coexisted with nonproducing (TE⁻) strains. Elicitin production generally was associated with low virulence on tobacco and frequent pathogenicity on tomato, whereas TE⁻ isolates generally

were highly virulent and specialized to tobacco. Analysis of both mitochondrial and nuclear DNA restriction fragment length polymorphisms indicated, for the first time, that black shank isolates can be distinguished from other *P. parasitica* isolates on the basis of genetic criteria. Our results suggest that severe black shank is caused by a limited number of TE⁻ strains that have been disseminated by clonal evolution. Mutations in the TE⁻ phenotype seem to have arisen independently in several genetic backgrounds and distinct geographic areas. The fortuitous absence of elicitin production has precluded population replacements in areas of intensive tobacco cultivation. Thus, monitoring the loss of elicitin production in developing tobacco areas should be considered in disease management.

The genus *Phytophthora* exhibits a wide array of host-pathogen interactions. Although all species of this genus are plant pathogens, their host specificity is extremely diverse, ranging from strict specialization, as for *P. idaei* on raspberry (17), to large host ranges, as for *P. cinnamomi*, which parasitizes more than 900 plant species (7). Within *P. parasitica* Dastur, both situations occur. This species exhibits an overall broad host range, but some isolates are specialized toward a single host, as is the case for tobacco (7). Infection of tobacco by *P. parasitica* causes black shank, by far the most destructive disease in this crop. Despite extensive work within the species *P. parasitica*, the taxonomic position of the isolates from tobacco is still unclear (7). Their artificial inoculation on some alternate hosts has been successful under certain conditions, but tobacco is their sole natural host plant (20). This preference led Tucker (26) to designate the black shank fungus as *P. parasitica* var. *nicotianae*, a designation that is still largely in use but that has come under sharp criticism (7). In fact, attempts to establish distinct subgroups within *P. parasitica* based on morphological (27) or other features (9,18,22) have failed. Moreover, examination of isozyme patterns (21) and restriction fragment length polymorphisms (RFLPs) of both nuclear and mitochondrial DNA (mtDNA) for a limited set of isolates (9) has revealed a low level of polymorphism in this species. For tobacco the situation is further confused by the large variability of virulence observed among *P. parasitica* isolates (20) and the occurrence of a disease (termed "brown stem rot" in eastern Africa) that is very similar to black shank but less severe and incited by nonspecialized isolates of *P. parasitica* (13). Therefore, the basis

of host specialization on tobacco still lacks molecular support and remains an open question.

One feature common to all *Phytophthora* species is secretion of elicitors (23). The 10-kDa proteins act as inducers of defense responses when applied on tobacco plants and protect the plants against subsequent infection by a compatible pathogen, including the black shank fungus (3). As an exception to the rule, some isolates lack elicitin production, and large-scale surveys have revealed that these cultures all belonged to *P. parasitica* and were exclusively black shank isolates (4,16). The hypothesis that the absence of elicitin secretion is a basis for pathogenicity to tobacco is tempting to consider, because elicitors are specifically active on *Nicotiana* spp. (24). However, the situation is not that simple, because other isolates from tobacco produce elicitin and are virulent on this plant, although to a lesser extent (4,16). Elicitin production, thus, adds to the diversity already recognized in this tobacco pathogen.

To investigate this diversity more precisely, a large collection of isolates from diseased tobacco in various areas of the world has been gathered. Isolates of *P. parasitica* from other host plants have been included for comparison. In a previous study, mtDNA polymorphism was low and related to the geographic origin rather than the host plant (18). In the current study, RFLPs of nuclear DNA were used to analyze the diversity in this collection, and we found that isolates that do not produce elicitors are not randomly distributed and do have particular pathogenic properties. These results provide the first indication that the concept of a tobacco forma specialis within *P. parasitica* may have a genetic basis.

MATERIALS AND METHODS

Fungal isolates and culture conditions. *P. parasitica* isolates were kept at the Institut National de la Recherche Agronomique (Antibes, France) culture collection and are listed in Table 1. They

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were obtained from various geographic areas with the help of COR-ESTA (Cooperation Center for Research Relative to Tobacco, Paris). Fungal cultures were grown on malt agar (1%:1% wt/vol) at 24°C in darkness. These isolates were kept in cryogenic storage.

Protein and nucleic acids analyses. Elicitin content evaluation, culture filtrate treatments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transfer, and immunodetection were carried out as previously described (19).

Total genomic DNA was extracted from 7-day-old cultures maintained on defined liquid glucose-asparagine medium (12), as described previously (22), except a RNase treatment was added prior to ethanol precipitation. Mitochondrial DNA was purified according to Hwang et al. (14). Mitochondrial haplotypes were determined as previously described (18).

Genomic DNA (5 µg) was digested with 25 units of restriction enzyme following the manufacturers' recommendations, and fragments were separated in a 1% agarose gel in 0.5× Tris-borate-EDTA buffer at 5 V cm⁻¹. Alternatively, 1× Tris-acetate-EDTA buffer was used in overnight electrophoresis at 3 V cm⁻¹. Blotting transfer, probe labeling, and hybridization were performed according to standard protocols (25). The final washings that followed the hybridizations were performed twice in 0.2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) and 0.1% SDS for 15 min at 65°C.

Nuclear probes were selected from a partial genomic library constructed as follows: genomic DNA from *P. parasitica* was par-

tially digested with *Bam*HI and *Hind*III, ligated into pBluescript SK⁻ vector (Stratagene, La Jolla, Ca), and used to transform *Escherichia coli* strain DH5α (Gibco BRL, Bethesda, MD). Each clone was tested for its relative abundance according to standard procedures (25).

Data analysis. The genetic relationships between mitochondrial haplotypes were assessed by the similarity coefficient of Nei and Li. A dendrogram was generated by the unweighted paired-group method, arithmetic mean (UPGMA) algorithm contained in the program package Phylip 3.57 (8). A second approach, which utilized the parsimony method, in which the presence or absence of individual restriction fragments of mtDNA was scored, also was undertaken. The most parsimonious trees were obtained by the branch and bound Dolpenny program included in the Phylip package (8). RFLP signals were qualitatively scored as 0 (absence) or 1 (presence). These data were treated by the factorial analysis of correspondence (FAC) method (2,11), using the PROC CORRESP procedure in the SAS program (SAS Institute, Cary, NC). This method consists of a weighted principal component analysis of a contingency table. It is used to find a low-dimension graphic representation of the association between rows (isolates) and columns (RFLP data) in a Euclidean space. Individuals and variables can be plotted separately or on the same graph. Each point partially contributes to axes determination.

In the current study, two individuals sharing close coordinates for one axis will be closely related based on RFLP signals con-

TABLE 1. Characteristics of the isolates used in this study

Isolate	Area	Country	Location	Year	Host	Mating- type	mtDNA ^a	Race	Stem invasion on tobacco ^b	Virulence on tomato ^c	Parasiticein production
Europe											
26	France	Alpes Maritimes	1971	Carnation	A2	A	...	14.5	0.8	+	
44	France	Corse	1972	Citrus	A1	A	...	3.5	0	+	
149	Spain	Unknown	1975	Tomato	A2	A	...	22	4	+	
384	Bulgaria	Blagoevgrad	1991	Tobacco	A2	A	0	100.5	0	—	
387	Bulgaria	Kurdjali	1992	Tobacco	A2	A	0	94	0	—	
385	Bulgaria	Gotse Delchev	1992	Tobacco	A2	A	0	70	0	+	
386	Bulgaria	Gotse Delchev	1992	Tobacco	A2	A	0	67.5	0.8	+	
329	Greece	Unknown	Unknown	Tobacco	A2	A	1	87	0	—	
330	Greece	Unknown	Unknown	Tobacco	A2	A	0	48	0	—	
332	Greece	Unknown	Unknown	Citrus	A2	A	...	11	0	+	
N. America											
181	United States	Unknown	Unknown	Tobacco	A2	A	1	84	0	—	
183	United States	Unknown	Unknown	Tobacco	A2	A	0	110	0	—	
301	United States	Unknown	Unknown	Tobacco	A1/A2 ^d	A	1	44	0	+	
369	United States	Kentucky	1986	Tobacco	A1	A	0	110	0	—	
370	United States	Kentucky	1991	Tobacco	A1/A2 ^d	A	1	100	0	—	
371	United States	Kentucky	1990	Tobacco	A2	A	0	105	0	—	
372	United States	Kentucky	1990	Tobacco	A1	A	1	105	0	—	
395	United States	Georgia	1990	Tobacco	A1	A	1	106	0	—	
396	United States	Georgia	1989	Tobacco	A1	A	0	101.5	0	—	
397	United States	Georgia	1990	Tobacco	A1	A	0	107.5	0	—	
398	United States	Georgia	1990	Tobacco	A1	A	0	106	0	—	
399	United States	Georgia	1991	Tobacco	A1	A	0	105	0	—	
400	United States	Georgia	1991	Tobacco	A1	A	1	111	0	—	
401	United States	Georgia	1991	Tobacco	A1	A	1	112	0	—	
402	United States	Georgia	1991	Tobacco	A2	A	1	106	0	—	
403	United States	Georgia	1991	Tobacco	A1	A	1	107	0	—	
404	United States	Georgia	1991	Tobacco	A1	A	0	104.5	0	—	
428	United States	North Carolina	1990	Tobacco	A1	A	0	96	0	—	
429	United States	North Carolina	1993	Tobacco	A1	A	0	118.5	0	—	
430	United States	North Carolina	1993	Tobacco	A2	D'''	0	98	0	—	
431	United States	North Carolina	1993	Tobacco	A2	D'''	0	83	0	—	
432	United States	North Carolina	1993	Tobacco	A2	A	0	28	0.8	+	
C. America and W. Indies											
324	Cuba	Pinar del Rio	1985	Tobacco	A1	A	0	98	0	—	
325	Cuba	La Habana	1984	Tobacco	A1	A	0	91	0	—	
326	Cuba	La Habana	1987	Tobacco	A1	A	0	95	0	—	

(continued on next page)

^a Mitochondrial groups were obtained as described by Lacourt et al. (18).

^b Length of brown lesions in millimeters.

^c Notation of symptoms is described in text.

^d Oospore production was observed after pairing with both A1 and A2 isolates but not in pure culture.

tributing to the determination of that axis. In a preliminary analysis, four isolates (370, 371, 372, and 395) introduced a distortion in the overall representation because they had unique additional RFLP bands. FAC was repeated, with these four isolates included as additional individuals. Variables other than nuclear RFLPs (mitochondrial haplotype, tobacco origin, and elicitor production) were introduced as additional variables that did not affect the results of FAC. This analysis was limited to the first five axes, resulting in an assessment of 55.4% of the total variability. RFLP data and nuclear probes are available upon request.

Pathogenicity assays on tobacco. *Stem inoculations.* Stem inoculations of 2-month-old tobacco plants (*N. tabacum* cv. Sam-sun) were performed as described previously (4). Briefly, two mycelial plugs were applied to the cut surfaces of decapitated stems. Invasion was estimated as the length of the brown lesion produced by each isolate. The mean of two independent measures was used as the final notation. The measures obtained for the same isolate always ranged within a 10-mm interval.

Root inoculations. Rooted plantlets of *N. tabacum* cv. Xanthine were propagated in vitro by monthly transfers of nodal cuttings on the following solid medium: inorganic salts of Murashige and Skoog medium, with NH_4NO_3 reduced to 800 mg liter⁻¹, myo-inositol at 100 mg liter⁻¹, vitamins (calcium pantothenate, thiamin HCl, nicotinic acid, pyridoxine, and cysteine HCl at 1 mg liter⁻¹ each), sucrose at 15 g liter⁻¹, and agar at 7 g liter⁻¹, pH 5.8. To assay pathogenicity, 25-mm-long terminal cuttings (the apical bud

and two leaves) were transferred to plastic bridges on the same medium without agar and rooted for 1 month. Inoculation was performed by replacing the growth medium with 20 ml of sterile water to which 100 zoospores had been added. The inoculation of each isolate was repeated three times. The plants were kept in a growth chamber at 23°C, with 16 h daily illumination at 40 mM·m⁻²·s⁻¹, and observed each day for 2 weeks.

Inoculation of tomato plants. Ten-day-old plants (*Lycopersicon esculentum* cv. Marmande) were removed from the soil, and their roots were carefully washed. The root tips were cut, and five plantlets were placed on water-soaked Whatman No. 1 paper (Whatman, Maidstone, England, U.K.), that covered half a petri dish (10 cm in diameter). A mycelium plug (1 cm diameter) was placed on the cut extremity of the root system of each plantlet. For each experiment, a positive control was made with the very aggressive tomato isolate 149. Two negative controls also were included: five plantlets were treated as previously described, except they were not inoculated, and five plantlets were inoculated by isolate 183, which is not pathogenic on tomato. A notation ranging from 0 to 4 was attributed to each plantlet, depending on the symptoms observed after 1 week (i.e., 0 = no symptoms; 1 = stem brown but not rotted; 2 = half of the stem rotted; 3 = entire stem and part of the leaves rotted; and 4 = plant dead). The average of five repeats was calculated as a final notation for each isolate.

Race determination. Two tobacco isogenic lines (46-8 and 46-10) were used to characterize races 0 and 1 among tobacco isolates.

TABLE 1. (continued from preceding page)

Isolate	Area Country	Location	Year	Host	Mating- type	mtDNA ^a	Race	Stem invasion on tobacco ^b	Virulence on tomato ^c	Parasiticein production
347	Cuba	Unknown	Unknown	Tobacco	A1	A	0	106	0	—
348	Cuba	Unknown	Unknown	Tobacco	A1	A	0	96	0	—
118	Cuba	Unknown	Unknown	Citrus	A1	E'	...	10	0	+
362	Cuba	Unknown	Unknown	Cocoa	A2	E''	...	23	0	+
364	Cuba	Unknown	Unknown	Cocoa	A2	E''	...	31.5	0	+
254	Martinique	Unknown	1983	Eggplant	A1	A	...	30	1.2	+
S. America										
413	Colombia	Valle del Cauca	1993	Tobacco	A2	A	1	119	0	—
408	Argentina	Jujuy	1993	Tobacco	A2	D'''	0	82.5	0	+
409	Argentina	Jujuy	1993	Tobacco	A2	D'''	0	99	0.6	+
410	Argentina	Jujuy	1993	Tobacco	A2	D'''	0	96	1.2	+
411	Argentina	Jujuy	1993	Tobacco	A2	D'''	0	87.5	1	+
Africa										
115	Congo	Unknown	Unknown	Citrus	A1	A	...	10.5	0	+
376	Zimbabwe	Mutepatepa	1985	Tobacco	A1	A	0	20	0	+
377	Zimbabwe	Muurwi District	1991	Tobacco	A1	A	0	26	4	+
378	Zimbabwe	Mutepatepa	1991	Tobacco	A1	A	0	87	2.2	+
379	Zimbabwe	Karoi District	1992	Tobacco	A1	A	nt	27	2.4	+
380	Zimbabwe	Karoi District	1992	Tobacco	A1	A	nt	37	2.2	+
381	Zimbabwe	Karoi District	1992	Tobacco	A1	A	0	32	4	+
373	Transvaal	Alkmaar	1992	Tobacco	A2	D'''	1	107	0	—
374	Transvaal	Heidelberg	1992	Tobacco	A2	D'''	1	120	0	—
375	Transvaal	Rustenburg	1992	Tobacco	A2	D'''	1	110	0	—
S.E. Asia										
391	Bali	Unknown	1990	Coconut	A2	A	...	12.5	0	+
388	Korea	Unknown	1992	Tobacco	A2	D'	0	108	0	—
365	Japan	Tokushima	1991	Tobacco	A2	D'	0	49	0	—
366	Japan	Tochigi	1991	Tobacco	A2	D'	0	93	0	—
367	Japan	Miyagi	1991	Tobacco	A2	D'	0	110	0	—
Australia										
405		New South Wales	Unknown	Tobacco	A2	B	0	76	1.2	—
383		Queensland	1988	Tobacco	A2	B	0	96	0	—
310		Queensland	1983	Tobacco	A1	D'	0	39	2.6	+
316		Queensland	1983	Tobacco	A2	C	0	42	2.2	+
317		Queensland	1986	Tobacco	A1	C	0	43	3	+
382		Queensland	1988	Tobacco	A2	B	0	60	0	+
414		South Perth	1983	<i>Chamaelaucium</i> sp.	A2	D'	...	25	0	+
415		Eneabba	1990	<i>Banksia leptophylla</i>	A2	D'	...	37	4	+
416		Perth	1991	<i>Hibiscus</i> sp.	A2	A	...	28	4	+
417		Perth	1991	<i>Banksia attenuata</i>	A2	D'	...	33	1.6	+
418		Perth	1992	<i>Banksia brownii</i>	A2	D'	...	1	2.4	+
419		Perth	1992	<i>Chamaelaucium</i> sp.	A2	D'	...	62.5	3.2	+

Line 46-8 harbored the major resistance gene *R1* derived from *N. plumbaginifolia* and *N. longiflora*. Race 1 isolates infected both lines, whereas race 0 isolates infected only line 46-10.

RESULTS

Elicitin production in culture filtrates. From the complete collection, a set of 73 isolates (57 from tobacco and 16 from other hosts) with geographic origins spanning all continents was selected (Table 1). Elicitin production in vitro was estimated by SDS-PAGE of culture filtrates (data not shown). Of the 73 isolates, 34 produced a protein migrating as parasiticein (the elicitin produced by *P. parasitica*) and serologically related to elicitins (Table 1). Parasiticein was produced by all nontobacco isolates (NTE⁺) and also by 18 tobacco isolates. This latter group of isolates has already been described from Australia (4) and Europe and Africa (18). The current study confirmed that the classification of tobacco isolates producing (TE⁺) and not producing (TE⁻) elicitin applies worldwide. Moreover, we observed that TE⁺ and TE⁻ isolates coexist in at least three geographic areas (Queensland, Australia; Bulgaria; and North Carolina, U.S.).

Pathogenicity on tobacco and tomato. The ability of *P. parasitica* isolates to infect tobacco was investigated by a decapitated stem-inoculation assay that allows fast screening of a large number of isolates that previously was especially discriminant (4). Using this technique, isolate virulence, as measured by the length of the stem lesion after 7 days, varied from 1 to 120 mm (Fig. 1). The distribution of the data was bimodal: 28 isolates had values less than 60 mm, 41 had values greater than 80 mm, and only 4 had values between 60 and 80 mm. This test, however, could be criticized based on the fact that the application of the mycelium on the fresh wound of a decapitated stem gives an initial advantage to the fungus. To test the validity of the stem assay, four isolates of the low-virulence group (44, 316, 365, and 382, with lesion lengths ranging between 3 and 60 mm) and seven isolates of the high-virulence group (408, 329, 366, 383, 384, 388, and 413, with lesion lengths longer than 80 mm) were inoculated in a more natural way, by placing zoospores on the unwounded root system of tobacco plantlets. With the former group, mycelium developed around the roots, but there were no observable symptoms after

2 weeks, whereas the latter group caused conspicuous symptoms and killed the plantlets. This result indicates that in the stem assay values less than 60 mm corresponded to isolates non- or weakly pathogenic on tobacco, whereas values greater than 80 mm corresponded to strongly pathogenic isolates. Most tobacco isolates, but not all, proved strongly pathogenic in this test. Isolate 419 from *Chamaelaucium* was exceptional, causing 62.5-mm-long lesions on tobacco stems.

The collection also was tested for pathogenicity on tomato, a plant that is a common host of many *P. parasitica* isolates (7). After root inoculation of young plants, 13 isolates proved highly virulent on tomato (notation of 2 or higher), 9 were weakly pathogenic, and 51 were not pathogenic (Fig. 1). No isolate was highly virulent on both tobacco and tomato, but many combined high virulence on one host plant with low virulence on the other plant, indicating that strict host specificity is not a general feature of tobacco isolates.

A remarkable result that emerged from the pathogenicity diagram (Fig. 1) was the distinct distribution of isolates according to elicitin production. In the elicitin-producing (E⁺) group, independent of host origin, two-thirds of the isolates could infect tomato, and 13 of 34 had high virulence. In contrast, only one TE⁻ isolate (405) caused symptoms on tomato and only to a very limited extent. Conversely, on tobacco high pathogenicity was the rule for TE⁻ isolates (all but two) and was the exception for E⁺ isolates, with the exception represented by the four tobacco isolates from Argentina (408 to 411) and by one isolate from Zimbabwe (378). Apart from these exceptions, elicitin-producing isolates that were collected on diseased tobacco plants statistically had the same range of pathogenic behavior as those from other host plants, whereas tobacco isolates lacking elicitin production were typically highly virulent on tobacco and nonpathogenic on tomato.

DNA polymorphism within *P. parasitica*. *Mitochondrial DNA polymorphism.* A previous analysis of the mtDNA of 87 *P. parasitica* isolates resulted in the distinction of 8 closely related haplotypes (18). Among these, one haplotype (A) was largely pre-

Fig. 1. Pathogenicity of the *Phytophthora parasitica* isolate collection toward tobacco and tomato. Aggressiveness toward tomato was estimated based on a notation scale of 0 to 4: 0 = no symptoms and 4 = death of plant. Isolates were classified as NTE⁺ (▲), TE⁺ (●), and TE⁻ (○), respectively (described in text).

Fig. 2. Most parsimonious tree of mitochondrial DNA polymorphism. Confidence limits of branches (in percents along the branches) were created by bootstrap analysis with 1,000 trials.

dominant and distributed over all continents, except Australia. No apparent correlation was found between haplotypes and host origin (18). The current study, which focused more on black shank, dealt with 73 isolates: several nontobacco isolates were excluded, and 16 new ones were introduced: 10 from tobacco and 6 from various Australian crops. The determination of their mitochondrial haplotypes did not reveal any new RFLP groups. With the incorporation of these additional data, the relationship between haplotypes and geographic origins (18) could be reassessed. Haplotype A remained prevalent and, in general, was typical of the Northern Hemisphere. Haplotype A was found once in Australia in a nontobacco isolate but not in South Africa or Argentina, where the D''' type prevailed. It also was only slightly represented in Southeastern Asia, where the D' type predominated. As previously observed, the minor haplotypes, B and C, appeared to be indigenous to Australia, because they were found only in Queensland and New South Wales. In contrast, when the distribution of haplotypes was compared with the pathogenicity data, a lack of correlation was revealed.

Phylogenetic relationships between the mtDNA haplotypes were investigated, using both cladistic and phenetic approaches (8). From the parsimony analysis (cladistic method), 12 trees were obtained; all of which were very similar (Fig. 2). All 12 trees separated haplotype A from 2 clusters composed of B, E', and E'' and C, D', and D'', respectively. The only differences between the trees involved changes in the relative positions of the haplotypes within each cluster. The phenograms generated using the UPGMA algorithm (phenetic method), which was available in the Phylip package (8), presented similar topologies (data not shown). Once again, the relative positions of haplotypes C, D', and D'' were uncertain and not validated by a subsequent bootstrap analysis (data not shown). Nevertheless, regardless of the algorithm used haplotype A was always distinct from the two clusters.

Nuclear DNA polymorphism. Nuclear DNA polymorphism was investigated with 25 DNA clones of 0.5 to 3 kb, which were randomly selected from a partial *P. parasitica* DNA library as hybridization probes, and 7 restriction enzymes. The most appropriate combinations were selected based on a small subset of tobacco and nontobacco isolates. Comparative hybridization to total DNA, purified mtDNA, and purified nuclear DNA led to the exclusion of two probes that strongly hybridized with mtDNA. Five probes, corresponding to highly repetitive DNA, produced complex patterns with irregular ladders that could not be used efficiently for RFLP studies. Several other probes produced faint signals due to low levels. Finally, eight probes were used to analyze the whole collection. Five of the probes revealed 1 to 3 bands, two probes revealed 6 to 7 bands, and the probe revealed up to 17 informative bands among the whole population. The RFLP generated by this latter probe is shown in Figure 3. Each probe was used in combination with a single restriction enzyme, leading to eight probe-enzyme combinations. From these combinations, 42 fragments were scored, 32 of which were polymorphic. The reliability of the patterns was verified, as well as their stability when tested on the DNA of distinct monozospore cultures from several isolates (data not shown). Because we did not have any information about the allelic state of each band scored, we could not rule out the supposition that some markers are linked, so we decided to analyze these RFLP data by a descriptive method, such as FAC (2,11). The pertinence of this method for polymorphism studies has been established previously (6). FAC also allows the inclusion of additional variables (such as host origin, mitochondrial haplotype, and elicitor production) that are not taken into account to describe the population but can be plotted on the same graph as the individuals.

The distribution of the 73 isolates in the factorial planes [1,2] and [3,4] is presented in Figure 4. Each of the first five axes explains a limited part of the total variability: 16.2, 11.1, 10.0, 9.1 and 9.0%, respectively. This result agrees with the high homoge-

neity previously observed in *P. parasitica* (9,21,22). Furthermore, the representation is not biased by any particular marker, because RFLP markers from different random DNA fragments contributed to the determination of each axis.

Some isolates had identical multicharacter patterns and, therefore, shared the same coordinates. In all instances, they also belonged to a single mitochondrial haplotype; however, they often differed in geographic origin, mating-type, or physiological race (for tobacco isolates). This led to a reduction in the number of cases of possible repeated isolation of the same individual in a given area. For instance, the 13 isolates that merged at a single point near -0.4 on axis 1 were collected from tobacco in Greece, Bulgaria, Colombia, Cuba, and in three U.S. states in different years. Nevertheless, as a test the FAC was repeated, using the different genotypes rather than the isolates as individuals, and we found that the general distribution of the population was not modified (data not shown).

Additional variables were included in the analysis: elicitor production, mtDNA haplotype A, and tobacco as host of origin. Elicitor production was strongly and positively correlated with axis 1, whereas the two latter variables were negatively correlated with the same axis (Fig. 4). These variables have no correlation with the other axes. Essentially, these correlations are due to a group of 28 isolates (including the 13 cited above) that contribute to 50% of the variability explained by axis 1 and represent the most conspicuous structure within an otherwise apparently homogeneous population. This group is composed solely of TE⁻ cultures that, with one exception (365), belong to haplotype A. If we take into account the results of the pathogenicity tests, we find that these 27 A/TE⁻ cultures also have in common the fact that they are nonpathogenic on tomato and highly virulent on tobacco (101 ± 13 mm). Throughout the rest of the population, NTE⁺, TE⁺, and TE⁻ isolates of various haplotypes appeared to be intermixed. Nevertheless, another small group of five TE⁻ isolates (373, 374, 375, 430, and 431, collected in Transvaal and North Carolina, U.S.) were identified. These isolates belonged to haplotype D''' and clustered at approximately +0.4 near axis 4 and contributed to 10% of the variability explained by this axis. Although they are genetically different from the A/TE⁻ group described above, they have the same pathogenic properties.

In contrast to the observations based on mtDNA polymorphism, isolates from the same geographic area generally were not clustered on the basis of nuclear DNA RFLPs. In Cuba for instance, cocoa isolates of haplotype E'' (362 and 364) were very distant from A/TE⁻ isolates (324, 325, and 326). In North Carolina, there coexisted three isolates belonging to the large A/TE⁻ group described above, two D'''/TE⁻ isolates (431, 430), and one A/TE⁺ (432), which are scattered along axis 1. The same was true for the six TE⁺ isolates from Zimbabwe. The four isolates from Argentina (408 to 411) appeared to be an exception to this rule, because they belonged to a clearly distinct group in the factorial plane [3,4]. Previously they were exceptionally virulent on tobacco (91 ± 7 mm) for the TE⁺ isolates.

Fig. 3. Nuclear polymorphisms based on probe C1 DNA hybridization patterns among various *Phytophthora parasitica* isolates (indicated along top). Total DNA (5 µg) was digested by *Hind*III, transferred, and hybridized under stringent conditions (described in text). Size standards (phage λ DNA digested with *Hind*III) are indicated in kilobases to the left.

DISCUSSION

This study provides some new insights into the diversity of *P. parasitica* isolates responsible for disease on tobacco. Pathogenicity tests confirmed that a large variation in virulence exists on tobacco, as has been known for a long time (20), and revealed that there may be potential for infection of tomato. A major finding of this study is that the ability or inability to produce elicitor is a feature that delineates the population into two groups that have markedly different pathogenic properties (Fig. 1). E⁺ isolates typically are weakly to moderately virulent on tobacco and are fre-

quently pathogenic on tomato. Within the limits of the pathogenicity tests used here, the distribution of these properties does not seem to be statistically different between cultures isolated from tobacco (TE⁺) or other host plants (NTE⁺). TE⁺ isolates, thus, appear as polyphagous *P. parasitica* isolates that include tobacco in their host range. In contrast, tobacco isolates lacking elicitor production (TE⁻) are characterized by high virulence on tobacco and the absence of pathogenicity on tomato. If we refer to classical definitions in tobacco pathology, the TE⁻ isolates exhibit the characteristics (virulence and host specificity) recognized as belonging to the black shank fungus, whereas TE⁺ isolates generally conform

Fig. 4. Distribution of 73 *Phytophthora parasitica* isolates analyzed in the current study according to the factorial analysis of correspondence method. Both factorial planes [1,2] and [3,4] are represented. ▲ = NTE⁺; ● = TE⁺; and ○ = TE⁻ isolates. Isolates that belong to mitochondrial DNA (mtDNA) group A are indicated in italics. Additional variables are plotted on the graph: ◇ = tobacco as the host; + = mtDNA haplotype A; and * = elicitor production

to the description of the polyphagous strains that cause tobacco brown stem rot in Rhodesia (13). In the current survey, isolates from Zimbabwe (formerly Rhodesia) were all TE⁺, but this type of isolate also was present in Australia, Bulgaria, the United States, and Argentina. Moreover, the TE⁺ Argentinean isolates (and also one from Zimbabwe) exhibited exceptionally high virulence on tobacco compared to some TE⁻ isolates. Because we have found that all E⁺ isolates secrete similar amounts of elicitor in vitro (data not shown), this observation raises some questions about the role of elicitors as avirulence factors in *P. parasitica*-tobacco interactions. Investigation will be undertaken to determine whether in these isolates elicitor gene expression might be down-regulated in planta, as was recently reported in the *P. infestans*-potato interaction (15).

We examined nuclear DNA RFLPs to study the genetic variability of an extensive isolate collection at a molecular level. To our knowledge, no similar study has been performed previously on such a large sample of *P. parasitica* isolates. Results were analyzed by the FAC method, which appeared especially suitable in this case. Because of the diploid nature of the organism and the lack of information about the Mendelian segregation of the molecular markers considered, analyses normally performed in other systems could not be used in this analysis. The allelic status of the RFLP markers is difficult to determine, because oospores obtained in *P. parasitica* crossings, in general, have a low germination level that precludes correct estimation of the segregation. Moreover single-copy probes did not reveal polymorphisms. The validity of FAC compared to other clustering methods, such as Nei's genetic distance and UPGMA representation, was assessed recently and indicated that similar groupings were obtained with these methods (5). This validation, together with the previous population analysis (6), confirms that the structure obtained in the current study is relevant and indicates that FAC could be a useful alternative in population studies that involve diploid organisms such as *Phytophthora*.

In this analysis (Fig. 4), the total variability generated by 32 polymorphic fragments was almost equally distributed among several axes. This result, together with the fact that many probe-enzyme combinations did not reveal polymorphism, confirms the results of previous studies that indicated *P. parasitica* has an overall lower intraspecific diversity than other *Phytophthora* species (9,21). Compared to the seven haplotypes defined on the basis of mtDNA polymorphism (18), nuclear DNA RFLP analysis revealed new information about the relationships among isolates. The most prominent result was that one subgroup, represented by 27 isolates in this study, can be distinguished within *P. parasitica*. In addition to their phenetic similarity, based on nuclear DNA RFLPs, these isolates also share a number of other characteristics. In particular, these isolates belong to haplotype A, and they lack elicitor production, come from tobacco, and exhibit high virulence on tobacco. This group encompasses most of the black shank isolates from the United States and probably represents the typical *P. parasitica* var. *nicotianae* cultures intensively studied there. To our knowledge, this is the first indication that the distinction of these cultures has a genetic basis beyond pathogenicity on tobacco.

This finding supports the previous proposal by Apple (1) that black shank fungus strains arise from rare mutation events in *P. parasitica* that are followed by dissemination. The low variability within the cluster and its position outside the bulk of the other isolates suggests it has evolved from a clonal origin under genetic isolation. Isolation could result from host specificity and the ability of such highly virulent isolates to displace less virulent types (20). Population replacement after migration has been well documented in *P. infestans* (10). Historically, the black shank epidemic in the United States originated in southern Georgia in 1915, invaded Florida, and spread to Tennessee and Kentucky during the next 20 years or so: the first observation of this disease in Puerto

Rico was in 1924 (20). If this reflects the diffusion of a single strain, it may have originated in Central America. This assumption is consistent with the results obtained in the current study, because the Cuban and Colombian isolates occur within the same cluster. Included within this group are some Greek and Bulgarian cultures, which are more likely to be the consequence of a long-distance transportation event from Central America to the Balkans (where the disease was not recorded until 1930), rather than of local adaptation.

The absence of elicitor production could be only one feature among several distinguishing this black shank strain. However, it appears that the 12 other TE⁻ isolates (365 to 388 along axis 1) scattered among the sample display the same peculiar pathogenic properties. For instance, five highly virulent cultures that lacked elicitor production and were collected from tobacco in the United States and Transvaal can be recognized as another cluster. Their position in the factorial planes and different haplotypes precludes the conclusion that they were derived from the same origin as the first group. This observation suggests that the absence of elicitor has a direct connection to the pathogenic behavior of the fungus. Furthermore, information obtained when gathering the culture collection showed that the TE⁻ isolates all came from areas with a long history of tobacco cultivation and severe black shank problems, whereas the TE⁺ isolates came from regions of recent and moderate disease outbreaks. The following scheme may be suggested as a working hypothesis.

A mutation suppressing elicitor production, in one step, would significantly affect fungus biology (16): its general fitness could be reduced (although the elicitor function is unknown, its high conservation suggests it plays an effective role for the fungus), but its specific pathogenicity on tobacco would be increased. The mutated clone would survive and become prevalent in conditions of dominant and repeated tobacco cultivation. These rare mutational events may have occurred several times independently in different genetic contexts within *P. parasitica* and distinct black shank strains.

Two inferences may be derived from this working hypothesis. First, the mechanism leading to the loss of elicitor production would be different in isolates from distinct clusters. Hybridization with elicitor probes did not reveal any pattern specific to E⁻ cultures (data not shown), and investigations are now in progress to unravel the molecular events involved. Second, intensive monitoring of the fungal population in areas where tobacco is exposed to TE⁺ isolates may reveal the emergence of a TE⁻ strain. In fact, such an event may have recently happened in Queensland, Australia. Isolates 382 and 383, both collected in 1988, are phenetically very similar and belong to the rare mtDNA haplotype B. Isolate 382 produces parasiticein and is moderately virulent on tobacco (60-mm-long lesion in the stem test; no infection in the root inoculation test), whereas isolate 383 lacks elicitor, was 96 mm long in the stem test, and killed plantlets in the root test. If our scheme is correct, a 383-type strain may become prevalent in the region, and the severity of the disease will increase accordingly. To predict disease evolution, similar monitoring could be recommended in regions such as Zimbabwe, where no TE⁻ isolates have been recorded. However, attention also should be given to the ability of the fungus to increase its virulence on tobacco without losing elicitor production, as exemplified by the Argentinean isolates used in this study.

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