Loss of Production of the Elicitor Protein INF1 in the Clonal Lineage US-1 of *Phytophthora infestans*

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ABSTRACT

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The extracellular protein INF1 of *Phytophthora infestans* is a member of the elicitin family of protein elicitors known to induce a hypersensitive response on some solanaceous and cruciferous plants. The presence of INF1 elicitin in culture filtrates of 102 *P. infestans* isolates from 15 countries was examined. All tested isolates produced INF1 except five isolates collected in 1976 and 1977 from infected potatoes in East Germany (the former German Democratic Republic). Based on hybridization to the multi-locus DNA fingerprint probe RG57, all the INF1-nonproducing isolates were shown to belong to the clonal lineage US-1 that dominated world populations until the 1980s. Phylogenetic analysis of a set of European US-1 isolates using amplified fragment length polymorphism fingerprint data indicated that loss of INF1 production evolved independently in separate lineages within US-1. DNA and RNA blot hybridizations showed that INF1-nonproducing isolates still retain a copy of the *inf1* gene, whereas little *inf1* mRNA could be detected. Hypothetical interpretations of the evolution in a restricted geographic area of *P. infestans* lineages deficient in the production of a specific elicitor protein are discussed.

Additional keywords: avirulence, late blight of potato.

The basis of host specificity in interactions between plants and pathogens has long been a central theme in plant pathology research. In recent years, it became apparent that molecular signal exchange between pathogens and hosts determines the outcome of the interaction (4). Plant resistance to pathogens often involves induction of plant defense responses including localized cell death (hypersensitive response [HR]). These responses are triggered by pathogen molecules termed elicitors. Elicitor molecules, produced directly or indirectly by avirulence genes, are thought to interact with plant receptors encoded by resistance (*R*) genes, thereby eliciting a signal transduction cascade that leads to activation of defense responses and HR. One consequence of this model is that races of the pathogen mutated in their elicitor or avirulence genes can arise to escape defense responses and become virulent on particular host genotypes (11,31,39). This process of race evolution is thought to contribute to the lack of durability of *R*-gene mediated resistance noted in agronomic systems (8,39).

Phytophthora infestans, a heterothallic oomycete plant pathogen, causes late blight, the most economically important disease of potato worldwide (2). Based on DNA and isozyme fingerprint analyses, it was suggested that before the 1980s a single clonal lineage of the A1 mating type, termed US-1, dominated most populations of *P. infestans* worldwide (18). US-1 isolates are thought to have propagated by asexual reproduction from *P. infestans* strains introduced in the 1840s from Mexico to North America and later to Europe and the rest of the world (17,18). However, recent migration events, probably from Mexico, to Europe and North America of populations of *P. infestans* that include aggressive and A2 mating type strains led to the establishment of a sexual cycle and the displace-ment of US-1 populations (12,14–16). Particularly devastating epidemics caused by the migrant strains were recently observed as late blight reemerged as a serious threat to potato production worldwide (2,14,15).

The molecular basis of host specificity of *P. infestans* is poorly understood (25). To date, no late blight resistance gene of *Solanum* spp. or race-specific avirulence gene of *P. infestans* has been isolated. However, in recent years, a family of extracellular protein elicitors termed elicitins has been identified, and evidence has accumulated for a role of these molecules in delimiting the host range of *Phytophthora* spp. (21,28,48). Elicitins are highly conserved 10-kDa proteins that are secreted by all tested *Phytophthora* and *Pythium* spp. (22,29,34). Elicitins induce defense responses including HR on a restricted number of plants, specifically *Nicotiana* spp. in the Solanaceae family (6,30). Responsive plants, such as *Nicotiana* spp., are thought to display higher levels of resistance to elicitin-producing *Phytophthora* isolates than to elicitin-deficient ones. Direct experimental support of this hypothesis has recently been achieved. *P. infestans* strains deficient in the production of the major secreted elicitin INF1 were engineered by transformation with antisense *inf1* gene constructs. In contrast to the wild-type and control transformants, these *inf1* silenced transformants-induced disease lesions when inoculated on *N. benthamiana*, suggesting that INF1 elicitin is an avirulence factor (28).

In a recent study, we examined the potential role of INF1 as an elicitor in interactions between *P. infestans* and *Solanum* spp. (27). Eighteen lines, representing eight *Solanum* spp. with various levels of resistance to *P. infestans*, were found not to respond to an *Escherichia coli*-expressed INF1. Therefore, within the tested set of *Solanum* spp., resistance to *P. infestans* does not appear to be mediated by a defense response elicited by INF1. However, considering the extraordinary diversity noted in the genus *Solanum* and the extensive period of association between several *Solanum* spp. and *P. infestans* (41,47), response to INF1 may occur in yet unidentified *Solanum* genotypes.

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In this study, we examine the variation in production of extracellular INF1 by *P. infestans*. A large set of isolates was examined, and five isolates collected in 1976 and 1977 from infected potatoes in East Germany (the former German Democratic Republic) were found to be deficient in INF1 production. We used amplified fragment length polymorphism (AFLP) DNA fingerprinting to reconstruct the evolution of the loss of INF1 production in *P. infestans* populations. The occurrence of the *inf1* gene and *inf1* mRNA was also examined in representative isolates. Hypothetical interpretations of the evolution of *P. infestans* lineages deficient in the production of the protein elicitor are discussed.

MATERIALS AND METHODS

***Phytophthora* isolates and culture conditions.** The various *P. infestans* isolates used in this study are listed in Table 1, along with their geographic origin, mating type, original host, and RG57 DNA fingerprint pattern. The 1976 and 1977 isolates from East Germany are probably identical to those described by Dagget et al. (9,10,36), because they were obtained from similar sources. Strains were routinely cultured on rye agar medium supplemented with 2% sucrose (7). For INF1 elicitin production, culture filtrates were harvested following growth for 3 to 4 weeks at 18°C in still cultures in the synthetic medium described by Kamoun et al. (29).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Culture filtrates were subjected to Tris-tricine SDS-PAGE as described elsewhere (37,38). Following electrophoresis, gels were silver-stained following the method of Merril et al. (33).

DNA manipulations. Total DNA of *P. infestans* was isolated from mycelium grown in liquid culture as previously described (35).

TABLE 1. Production of INF1 elicitin by *Phytophthora infestans* isolates from 15 countries in five continents[a]

Country	Accession #	Year	Mating type	Host	Fingerprint pattern[b]	Genotype[c]	INF1[d]
Africa							
Rwanda	RWA02	…	A1	Potato	1110101001001101011111011	Other	+
Rwanda	RWA04	1985	A1	Potato	1011101011001101000110011	US-1	+
Rwanda	RWA08	1984	A1	Potato	1110111011001101011111011	Other	+
Uganda	UG-3	1996	…	Tomato	…	…	+
Uganda	UG-7	1996	…	Tomato	…	…	+
Uganda	UG-8	1996	…	Tomato	…	…	+
Asia							
Philippines	PH9001	1990	A1	…	1011101011001101000110011	US-1	+
Philippines	PH9002	1990	A1	…	1011101011001101000110011	US-1	+
Philippines	PH9055	1990	A1	…	1011101011001101000110011	US-1	+
Europe							
Denmark	DE9061	1990	…	Potato	…	…	+
Denmark	DE9062	1990	…	Potato	…	…	+
Denmark	DE9063	1990	…	Potato	…	…	+
Germany (East)	DDR7601	1976	A1	Potato	1011101011001101000110011	US-1	+
Germany (East)	DDR7602	1976	A1	Potato	1011101011001101000110011	US-1	−
Germany (East)	DDR7603	1976	A1	Potato	1011101011001101000110011	US-1	−
Germany (East)	DDR7604	1976	A1	Potato	1011101011001101000110011	US-1	−
Germany (East)	DDR7605	1976	A1	Potato	1011101011001101000110011	US-1	−
Germany (East)	DDR7702	1977	A1	Potato	1011101011001101000110011	US-1	−
Germany (East)	DDR7704	1977	A1	Potato	1011101011001101000110011	US-1	+
Germany (East)	DDR8001	1980	A1	Potato	1000100001011101000110011	Other	+
Germany (East)	GE900068	1990	A1	Potato	…	…	+
Germany (East)	GE900071	1990	A1	Potato	…	…	+
Germany (East)	GE900079	1990	A1	Potato	…	…	+
Germany (East)	GE900083	1990	A1	Potato	…	…	+
Germany (East)	GE900089	1990	A1	Potato	…	…	+
Germany (East)	GE900090	1990	A1	Potato	…	…	+
Germany (East)	GE900091	1990	A1	Potato	…	…	+
Germany (East)	GE900093	1990	A1	Potato	…	…	+
Germany (East)	GE900098	1990	A1	Potato	…	…	+
Germany (West)	GER7401	1974	A1	Potato	1011101011001101000110011	US-1	+
Germany (West)	GER8451	1984	A1	Potato	1011101011001101000110011	US-1	+
Germany (West)	GER8501	1985	A1	Potato	1011101011001101000110011	US-1	+
Germany (West)	GER8601	1986	A1	Potato	1011101011001101000110011	US-1	+
Germany (West)	GER8603	1986	A1	Potato	1011101011001101000110011	US-1	+
Italy	IT8001	1980	A1	Tomato	1011101011001101000110011	US-1	+
Netherlands	66006	1966	A1	Potato	1011101011001101000110011	US-1	+
Netherlands	68308	1968	A1	Potato	1011101011001101000110011	US-1	+
Netherlands	70001	1970	A1	Potato	1011101011001101000110011	US-1	+
Netherlands	74001	1974	A1	Potato	1011101011001101000110011	US-1	+
Netherlands	80029	1980	A1	Potato	010–10000100110100010101011	Other	+
Netherlands	81197	1981	A2	Potato	110–101000001101011111011	Other	+
Netherlands	84215	1984	A1	Potato	111–11101100110001110011	Other	+
Netherlands	85005	1985	A1	Potato	101–11100100110000011011	Other	+
Netherlands	86004	1986	A1	Potato	111–11111001101011110011	Other	+
Netherlands	86063	1986	A2	Tomato	100–101000001101011111011	Other	+
Netherlands	86061	1986	A2	Tomato	100–101000001101011111011	Other	+
Netherlands	87030	1987	A2	Potato	111–101001001100011111011	Other	+
					(*continued on the next page*)		

[a] … = not available.

[b] DNA fingerprint pattern refers to bands 1 to 25 hybridizing to probe RG57 as defined by Goodwin et al. (19). 0 = no band, 1 = a band, and – = undetermined. Note that band 4 could not be determined unambiguously for some isolates and was, therefore, excluded from the cluster analysis shown in Figure 1.

[c] Genotype designations are as indicated in Goodwin et al. (18,20).

[d] + refers to the production of INF1 and – to the absence of INF1 in culture filtrate as determined by SDS-PAGE. Examples are shown in Figure 4C.

Alkaline DNA transfer to Hybond N+ (Amersham, Arlington Heights, IL) and Southern hybridizations were performed at 65°C as described elsewhere (3,37). Filters were washed at 55°C in 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate). Note that the size of the *P. infestans Bam*HI fragment containing the *inf1* gene was more accurately determined to 2.3 kilobase (kb) and not 3.2 kb as previously reported (27).

RNA manipulations. Total RNA from *P. infestans* was isolated using the guanidine hydrochloride extraction method (32). For RNA blot analyses, 10 to 15 mg of total RNA was denatured at 50°C in 1 M glyoxal, 50% dimethyl sulfoxide (DMSO), and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N+ membranes (3,37). Hybridizations were conducted at 65°C in 0.5 M sodium phosphate buffer, 7% SDS, and 1 mM EDTA. Filters were washed at 55°C in 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate).

DNA and RNA blot hybridization probes. Gel-purified DNA fragments containing the full *inf1* cDNA insert from pFB7 (27) or the *actA* gene from pSTA31 (42) were used as probes and radiolabelled with α-^{32}P-dATP using a random primer labeling kit (Gibco BRL, Bethesda, MD).

Restriction fragment length polymorphism (RFLP) and AFLP DNA fingerprinting. RG57 DNA fingerprinting patterns (bands 1 to 25) were compiled from several published sources and databases from our laboratory and the laboratory of W. E. Fry (Cornell University). RG57 band 4 was omitted from the analysis, because it was scored differently by independent laboratories. AFLP fingerprints were generated as described by Vos et al. (46) using experimental conditions that generate highly informative fingerprints of *P. infestans* (44). Briefly, total DNA was digested with the restriction enzymes *Eco*RI and *Mse*I, after which adapters were ligated to the DNA fragments. The adapter fitting the *Eco*RI restriction

TABLE 1. (*continued from preceding page*)

Country	Accession #	Year	Mating type	Host	Fingerprint pattern[b]	Genotype[c]	INF1[d]
Europe (*continued*)							
Netherlands	88069	1988	A1	Tomato	…	…	+
Netherlands	88074	1988	A1	Tomato	101–101001001100111110011	Other	+
Netherlands	88133	1988	A2	Potato	101–101101001100011111011	Other	+
Netherlands	88034	1988	A2	Tomato	100–100100001101011111011	Other	+
Netherlands	89038	1989	A2	Potato	…	…	+
Netherlands	89148	1989	A1	Potato	111–101001001100111111011	Other	+
Netherlands	90210	1990	A1	Potato	…	…	+
Netherlands	90209	1990	A1	Potato	110–100001001101000111011	Other	+
Netherlands	90128	1990	A2	Potato	100–100011001100000110011	Other	+
Netherlands	91014	1991	A1	Potato	101–111001001101011111011	Other	+
Netherlands	91011	1991	A2	Potato	101–101001001101011111001	Other	+
Netherlands	91001	1991	A2	Potato	101–101100001101100110011	Other	+
Netherlands	91002	1991	A2	Potato	101–101111001101000110011	Other	+
Netherlands	95001	1995	…	Potato	…	…	+
Netherlands	95002	1995	…	Potato	…	…	+
Netherlands	95003	1995	…	Potato	…	…	+
Netherlands	95004	1995	…	Potato	…	…	+
Netherlands	95005	1995	…	Potato	…	…	+
Netherlands	95006	1995	…	Potato	…	…	+
Netherlands	95007	1995	…	Potato	…	…	+
Netherlands	95008	1995	…	Potato	…	…	+
Netherlands	95009	1995	…	Potato	…	…	+
Netherlands	95010	1995	…	Potato	…	…	+
Poland	POL8402	1984	…	Potato	…	…	+
Poland	PO850020	1985	A1	Potato	…	…	+
Poland	PO860019	1986	A1	Potato	…	…	+
Poland	PO860021	1986	A1	Potato	…	…	+
Poland	PO880041	1988	A1	Potato	…	…	+
Poland	PO880052	1988	A1	Potato	…	…	+
Romania	ROM1	…	…	…	…	…	+
Spain	SP9665	1996	…	Potato	…	…	+
Spain	SP9669	1996	…	Potato	…	…	+
Spain	SP9670	1996	…	Potato	…	…	+
Spain	SP9671	1996	…	Potato	…	…	+
Spain	SP9696	1996	…	Potato	…	…	+
Spain	SP9698	1996	…	Potato	…	…	+
Spain	SP9699	1996	…	Potato	…	…	+
Spain	SP9600	1996	…	Potato	…	…	+
United Kingdom	UK7818	1978	A1	Potato	101110101100110100011 0011	US-1	+
United Kingdom	UK7225	1972	A1	Potato	1011101011001101000110011	US-1	+
North America							
United States	USA-128	1982	A1	Potato	1011101011001101000110011	US-1	+
United States	BIN-16	1992	A1	…	1011111001001100010110011	US-6	+
United States	W193-13	1993	A1	Potato	1011101011001101000110011	US-1	+
United States	ME93-2A	1993	A2	Potato	1001100001001101000110111	US-8	+
United States	US940478	1994	A1	…	…	…	+
United States	BG-8	…	A2	…	1001100001001101010110011	US-7	+
Mexico	TV545	…	A1	Potato	1000100001001101011110001	Other	+
Mexico	TV580	…	A1	Potato	1010101101001101000110111	Other	+
Mexico	TV618	…	A2	Potato	1000100001001101000110011	Other	+
South America							
Peru	PE807	1985	A1	Potato	1011101011001101000110011	US-1	+
Peru	PE821	1984	A1	Potato	101–101011001101000111011	Other	+
Unknown	46210	1946	…	…	…	…	+
Unknown	51366	1951	A1	Potato	101–101000001101000110011	Other	+

site was 5′-CTCGTAGACTGCGTACC/CTGACGCATGGTTAA-5′, and the adapter fitting the *Mse*I restriction site was 5′-GACG-ATGAGTCCTGAG/TACTCAGGACTCAT-5′. A subset of the restriction fragments was amplified in two subsequent polymerase chain reaction (PCR) amplification steps. In the first PCR, primers were used that are homologous to the adapter sequence, the restriction site, and one additional selective base at the 3′ end (5′-GACTG-CGTACCAATTC*A* for the *Eco*RI site and 5′-ATGAGTCCTGAG-TAA*C* for the *Mse*I site). In the second PCR, both the *Eco*RI and the *Mse*I primers contained two selective bases. The *Eco*RI restriction site primer was 5′-GACTGCGTACCAATTC*AC*, and the *Mse*I restriction site primers were 5′-ATGAGTCCTGAGTAA*CA*, -*CC*, -*CG*, -*CT*. In the second PCR, the *Eco*RI primer was labeled at the 5′ end with ^{33}P and, after electrophoresis on 4% denaturing polyacrylamide gels, PCR fragments were detected by autoradiography. More experimental details can be found in Vos et al. (46) and van der Lee et al. (44).

Phenetic and phylogenetic analyses. A matrix of simple genetic distance based on RFLP data was constructed using the software NTSYS-pc 1.7 (F. J. Rohlf, Exeter Software, New York). The matrix was used to generate an unweighted pair group method with arithmetic means (UPGMA) phenogram using the PHYLIP 3.5c software package (J. Felsenstein, University of Washington, Seattle). Maximum-parsimony analysis of AFLP fingerprinting data was conducted using the PAUP 3.1 (phylogenetic analysis using parsimony) software (D. L. Swofford, Illinois Natural History Survey, Champaign). Heuristic search using 1,000 bootstrap replicates was conducted with branch swapping by tree bisection-reconnection (TBR).

RESULTS

Most *P. infestans* isolates produce INF1. We surveyed 102 isolates of *P. infestans* from 15 countries and five continents for INF1 elicitin production by PAGE of crude culture filtrates. The production of INF1 was almost ubiquitous in *P. infestans*; PAGE of culture filtrates from 97 isolates (95%) showed a 10-kDa band that comigrates with authentic elicitins (Table 1). These INF1 producers include both A1 and A2 mating type isolates from potato and tomato, suggesting no correlation between INF1 production and mating type or host of origin. In contrast to these 97 isolates, PAGE of culture filtrates from the remaining five isolates consistently failed to reveal a 10-kDa band, suggesting that these *P. infestans* isolates do not secrete INF1.

Absence of INF1 production is restricted to East German populations of the US-1 genotype. Phenetic analysis, using UPGMA clustering calculated from RFLP data obtained previously with the multilocus DNA fingerprint probe RG57, was conducted with a set of 55 isolates tested for INF1 production. As shown in Figure 1, the resulting phenogram indicates that a diverse range of *P. infestans* genotypes was tested in this study. Despite this diversity, absence of INF1 production was noted only in isolates with the US-1 genotype. Interestingly, even though 26 *P. infestans* US-1 isolates from nine countries were tested, all five isolates that fail to produce INF1 (DDR7602, DDR7603, DDR7604, DDR7605, and DDR7702) were collected in East German potato fields in 1976 and 1977 (Table 1). These results suggest that INF1 nonproducing mutant(s) arose within the US-1 clonal populations of East Germany. Furthermore, isolates of diverse genotypes collected in the eastern part of Germany in 1980 and 1990 produce normal levels of INF1, suggesting that the INF1-nonproducing populations were displaced in the 1980s by new INF1-producing migrant strains.

Independent loss of production of INF1 in the *P. infestans* clonal lineage US-1. To identify sublineages within the US-1 clonal lineage, we used AFLP DNA fingerprinting to generate additional molecular markers. A set of 18 European US-1 isolates, including the five INF1 deficient isolates and two 1990 German isolates of different genotypes, were tested. Similar to RFLP analyses, *P. infestans* isolates of the US-1 genotype showed distinct AFLP patterns and can be easily distinguished from isolates of different genotypes (Fig. 2). To determine the number of evolutionary events that led to loss of INF1 production within the US-1 clonal lineage, a total of 146 polymorphic AFLP bands were scored for all 20 isolates. Phylogenetic analysis was conducted using the maximum-parsimony software PAUP 3.1. Of the 146 examined bands, 94 were informative in the parsimony analysis and 49 were polymorphic among the 18 US-1 isolates. A total of 1,000 bootstrap replications were conducted to determine the significance of the branches obtained in the shortest tree (Fig. 3). Within the US-1 lineage, three significant branches (>50%) were identified. One contains isolates from Germany and the Netherlands. The other two contain isolates from Germany and the United Kingdom, respectively. Additionally, four isolates from Germany, the Netherlands, and Italy did not significantly associate with any of the three branches. Interestingly, the five INF1-nonproducing isolates were split over at least three branches.
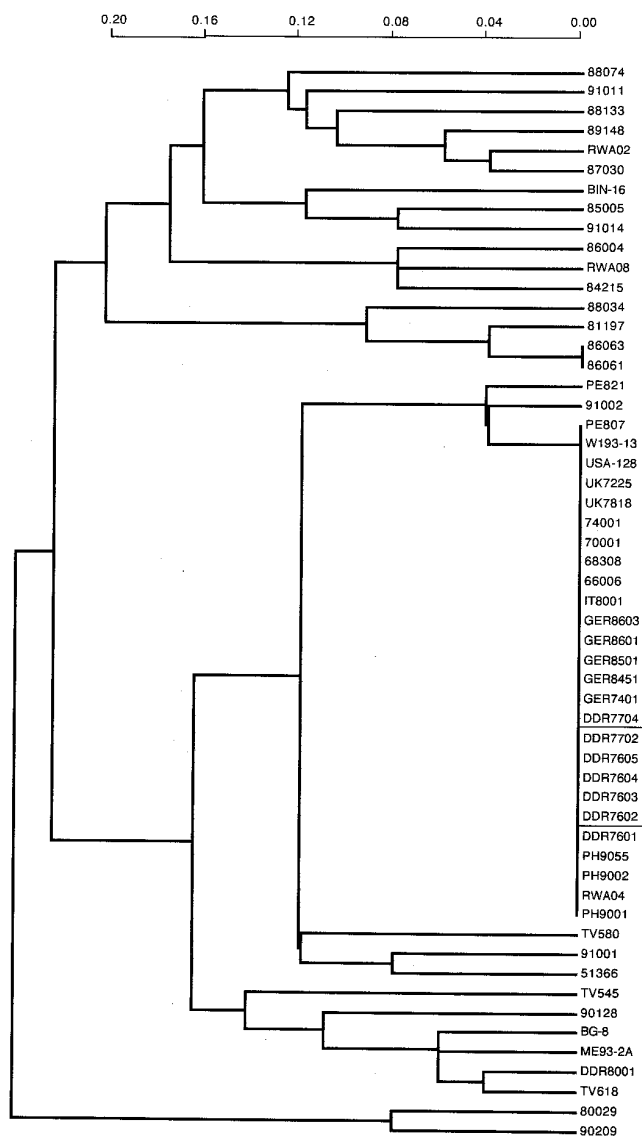

Fig. 1. Phenetic tree of 55 *Phytophthora infestans* isolates used in this study based on RG-57 restriction fragment length polymorphism fingerprint markers. The tree was generated using unweighted pair group method with arithmetic means clustering of a simple genetic distance matrix. The five East German isolates that do not produce INF1 are shown in a box. The scale represents genetic similarity distance.

This suggests that several mutations leading to loss of INF1 production occurred independently.

INF1-nonproducing isolates still retain the *inf1* gene, but have little *inf1* mRNA. We previously determined that in three *P. infestans* isolates with different genotypes the *inf1* gene is localized in a conserved 2.3-kb *Bam*HI fragment (27). To determine whether the isolates deficient in INF1 production still retain *inf1* sequences, *Bam*HI-digested total DNA from a representative set of 16 isolates of *P. infestans* was hybridized with a probe from the *inf1* gene. A single hybridizing *Bam*HI band of 2.3-kb was observed in all examined isolates including the INF1-nonproducing isolates DDR7602 and DDR7702 (Fig. 4A and C). These results suggest that the *Bam*HI fragment carrying the *inf1* gene is highly conserved in *P. infestans* and that no gross alteration of the *inf1* gene occurred in isolates deficient in INF1 production. To determine whether INF1 deficiency correlates with the absence of *inf1* mRNA, total RNA was isolated from the same set of isolates and hybridized with the *inf1* probe. All examined INF1-producing isolates showed significant levels of *inf1* mRNA, but little or no *inf1* mRNA was detected in the nonproducing isolates DDR7602 and DDR7702 (Fig. 4B and C). Control hybridizations with a probe of the constitutively expressed *actA* gene showed that all lanes contained similar amounts of total RNA (Fig. 4B). Note that in the experiment shown in Figure 4, isolates GER8501 and GE900083 show lower levels of *inf1* mRNA and INF1 protein than other producer isolates (Fig. 4B and C, lanes 6 and 15). However, in independent replicate experiments, both isolates produced higher levels of INF1 comparable to other INF1-producing isolates. In addition, repeated culturing of isolate DDR7602 revealed that trace amounts of *inf1* mRNA and INF1 protein may occasionally be detected (Fig. 4B, lane 2).

Absence of INF1 production is not associated with the inability to form sporangia in vitro. Ritch and Daggett (36) reported that isolates from East Germany collected in the 1970s fail to sporulate when grown in vitro. We also noted that some of the INF1-nonproducing isolates, which are probably identical to Ritch and Daggett isolates, frequently fail to form sporangia when cultured on rye-sucrose medium. This raised the possibility that the lack of INF1 production noted in this study for some East German isolates was correlated to the lack of sporulation. To test this hypothesis, we carefully analyzed sporulation of the INF1-nonproducer DDR7602. This isolate is generally nonsporulating, but it occasionally forms sporulating sectors when grown on rye-sucrose medium. Following four sequential subcultures of mycelium from both sporulating and nonsporulating sectors, cultures stable for either phenotype were obtained. PAGE of culture filtrates from both sporulating and nonsporulating derivatives of DDR7602 failed to show a 10-kDa band (data not shown), suggesting that deficiency in INF1 production by DDR7602 is not correlated to its inability to produce sporangia.

DISCUSSION

The extracellular elicitins of *Phytophthora* act as avirulence factors that restrict the host range of the pathogen by triggering defense responses in sensitive plants (28,48). In this paper, we investigate variation in the production of INF1, the major secreted elicitin of *P. infestans*. Isolates from 15 countries in five continents representing a wide array of genetic diversity were tested for production of INF1 (Table 1, Fig. 1). Similar to other *Phytophthora* spp. (29), most *P. infestans* isolates (95%) produce large amounts of elicitin when cultured in vitro. No correlation between INF1 production and mating type or host of origin (potato or tomato)
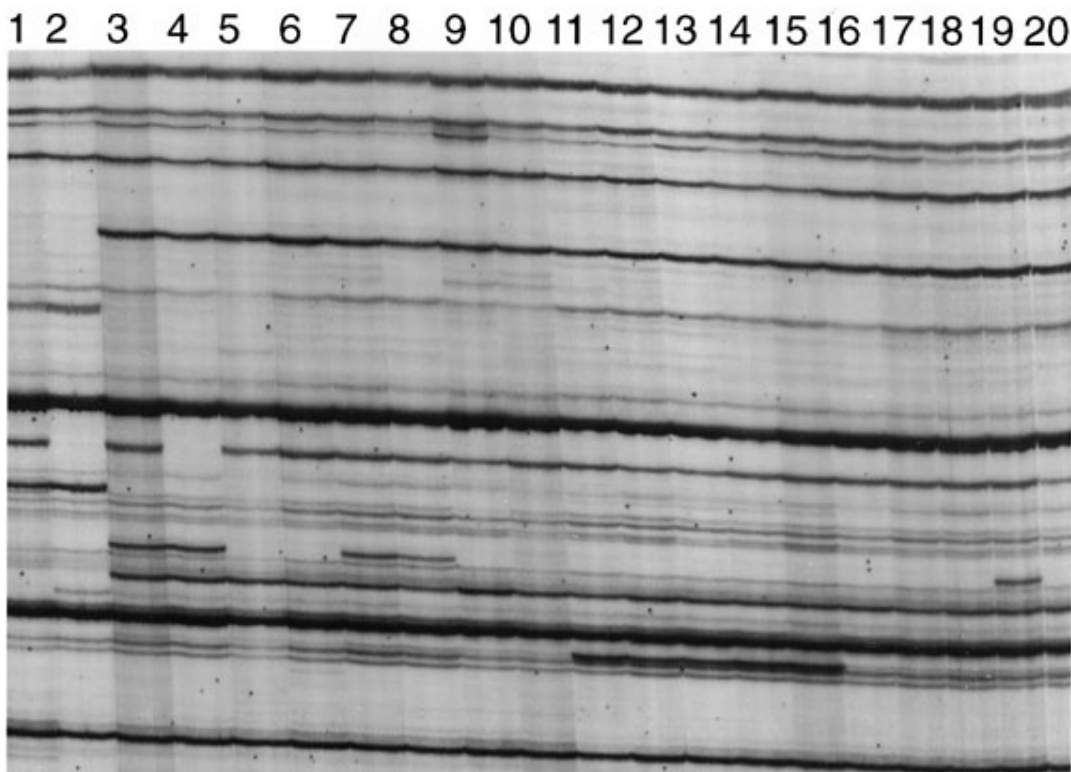

Fig. 2. Section of autoradiograph showing amplified fragment length polymorphism (AFLP) DNA fingerprints of *Phytophthora infestans* isolates as follows: lane 1, GE900089; lane 2, GE900083; lane 3, UK7818; lane 4, UK7225; lane 5, GER8501; lane 6, GER8451; lane 7, NL68308; lane 8, NL66006; lane 9, GER8601; lane 10, GER7401; lane 11, DDR7605; lane 12, DDR7604; lane 13, DDR7603; lane 14, DDR7602; lane 15, DDR7601; lane 16, GER8603; lane 17, IT8001; lane 18, NL74001; lane 19, NL70001; and lane 20, DDR7702. The AFLP DNA fingerprints shown here were generated with the primer combination GACTGCGTACCAATTC*CAA*, a primer fitting the *Eco*RI adapter and three selective bases, and ATGAGTCCTGAGTAA*CG*, a primer fitting the *Mse*I adapter and two selective bases. Approximately 20% of the total length of the lanes is shown.

was noted. The absence of INF1 production was noted only in 5 out of 102 *P. infestans* isolates, DDR7602, DDR7603, DDR7604, DDR7605, and DDR7702. All these isolates were collected in 1976 to 1977 from potato fields in East Germany. Since multiple isolates of similar origin fail to produce INF1, it is unlikely that this trait arose in the lab as a result of a rare mutation. Additionally, all other isolates tested including those collected 50 years ago produce normal levels of INF1, making it unlikely that continuous in vitro culturing frequently gives rise to INF1 nonproducers. Therefore, we assume that the absence of INF1 production noted in vitro reflects a frequently occurring phenotype (71%) among East German isolates of *P. infestans* from the mid 1970s.

Of the isolates tested in this study for production of INF1, 26 bear a DNA fingerprint pattern diagnostic of US-1, the clonal lineage that dominated the fields in Europe until the 1980s (12,18). All five INF1 nonproducers identified belong to this clonal lineage. Even though US-1 isolates from nine countries in five continents were tested, all nonproducers originate from a restricted geographic area, East Germany. Interestingly, US-1 isolates from neighboring countries such as West Germany (Federal Republic of Germany) and Poland produce normal levels of INF1.

Since *P. infestans* isolates of the US-1 genotype have propagated clonally by asexual reproduction, it is possible to reconstruct the phylogeny of these isolates given enough polymorphic molecular markers. To determine whether all five INF1 nonproducers arose from one common mutant ancestor or whether loss of INF1 production occurred repeatedly in *P. infestans*, we conducted a phylogene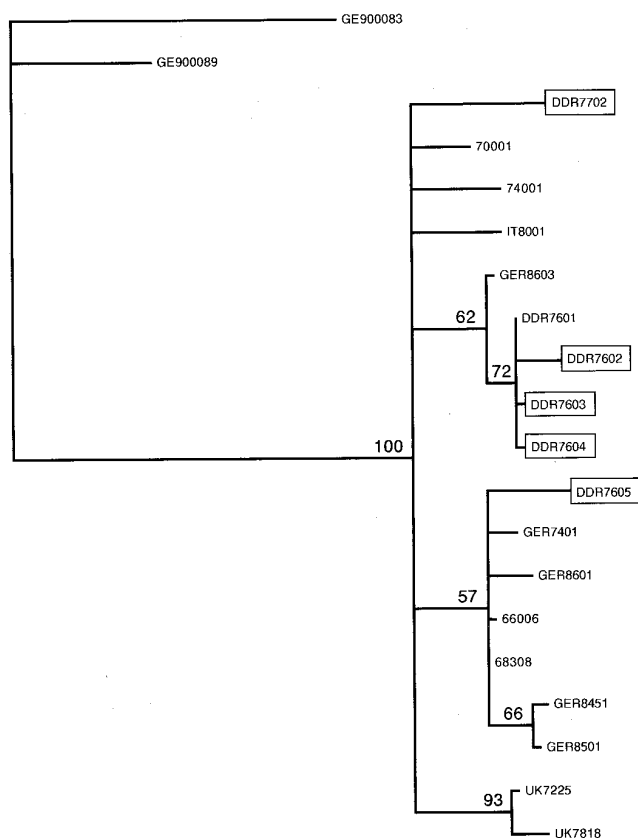tic analysis on a set of 18 European US-1 isolates using markers generated by AFLP DNA fingerprinting (Figs. 2 and 3). A phylogenetic tree was generated using parsimony analysis, and 14 of the tested isolates were found to belong to three statistically significant branches within the US-1 lineage. Interestingly, the INF1-nonproducing isolates were split over at least three branches of the tree, suggesting that loss of INF1 production occurred on multiple occasions within the US-1 lineage. Such a pattern of rapid evolution is reminiscent of the evolution of virulence and pathogenicity in *Phytophthora* spp. (13,17,20). However, alternative interpretations of the phylogenetic reconstruction of the European US-1 isolates are possible. For example, a high rate of mutation at the *inf1* locus could have resulted in reversion to the INF1-producing phenotype in strains closely related to the nonproducers. In addition, it can not be excluded that atypical genetic mechanisms such as mitotic recombination or somatic fusion between different isolates may have led to aberrant changes in INF1 production. However, as long as the exact nature of the mutated INF1 phenotype in East German isolates is unknown, we assume the simplest interpretation of the phylogenetic tree. We favor the hypothesis that loss of INF1 production occurred independently in the three different sublineages and that the absence of INF1 production may have contributed some fitness gain to East German US-1 populations of *P. infestans*. A consequence related to this hypothesis is that INF1 nonproducers might be more virulent than INF1 producers on particular plant genotypes cultivated in East Germany in the 1970s. Selective pressure would have resulted in increase in frequency of INF1-nonproducing *P. infestans* isolates. We plan to test this by examining a large set of potato lines cultivated in East Germany in the 1970s for response to the INF1 elicitor and resistance to INF1-producing and -nonproducing strains.

Modern analyses of population genetics of *Phytophthora* using variation in isozymes and RFLP markers resulted in great insight into the population structures and macro-evolution of several species (12,13,16,18,20,49). However, the levels of analysis obtained using these markers remain limited, particularly in populations showing little polymorphism. We have previously exploited the AFLP DNA fingerprinting technique to generate the first high-resolution genetic map of *P. infestans* (44). In this study, we demonstrated that this technique could also be useful in achieving fine levels of genetic analysis of *P. infestans* populations. We identified a total of 49 polymorphic AFLP bands in a set of 18 US-1 isolates of *P. infestans* with no known isozyme or RFLP polymorphisms. With the advent of such a powerful fingerprinting technique, routine high-resolution analyses can now be achieved in population genetic studies of *Phytophthora*.

Virulent races of fungal and bacterial pathogens are generally defined by their failure to express particular avirulence genes (11, 31,39). In several cases, this phenotype is associated with the absence of DNA sequences homologous to the avirulence gene in the virulent strain (43,45). Alternatively, nonfunctional recessive alleles of the avirulence gene may occur in the virulent strain (23,24). In *P. parasitica*, which secretes the elicitin PARA1, virulent, PARA1-nonproducing strains retain elicitin genes, but in one isolate, no PARA1 mRNA was detected (26). Similarly, in the two *P. infestans* INF1-nonproducing strains examined in this study, *inf1* gene sequences were present, but little or no *inf1* mRNA was detected (Fig. 4). Genetic analyses and transformation of tagged *inf1* genes in the various backgrounds will help determine whether the inability to produce INF1 in *P. infestans* results from *cis*-acting mutations in *inf1* or from *trans*-acting mutations in genes encoding regulatory proteins.

Apart from their ability to elicit defense responses in plants, the intrinsic role of elicitins in the biology of *Phytophthora* remains unknown. Based on their abundant secretion and primary amino acid structure, elicitins have been proposed to function as structural proteins (40,48). Expression studies of the *inf1* gene of *P. infestans* revealed that the highest levels of *inf1* mRNA occurred in heavily sporulating mycelium, whether in vitro or in planta (27). These

Fig. 3. Strict consensus tree of 18 European *Phytophthora infestans* isolates of the US-1 genotype inferred by bootstrap analysis of 146 amplified fragment length polymorphism fingerprint markers. Significant bootstrap values (above 50%) from 1,000 replications are indicated at the nodes. Two 1990 isolates from the eastern part of Germany, GE900083 and GE 900089, were used as outgroups. The five East German isolates that do not produce INF1 are shown in boxes.

observations, along with the trace amounts of *inf1* mRNA and INF1 protein that were occasionally detected in DDR7602, raised the possibility that the lack of INF1 production in East German isolates is correlated to the inability to produce sporangia noted in some of these isolates. However, stable cultures derived from sporulating sectors of the INF1-nonproducer isolate DDR7602 still did not secrete INF1, indicating that loss of INF1 production is not related to the morphological changes noted in some East German isolates.

Biotechnological engineering of potato to respond to INF1 is predicted to increase late blight resistance to INF1-producing strains of *P. infestans* (27,48). Based on our survey (Table 1), most isolates of *P. infestans* including all tested isolates from recent epidemics in Europe (various NL-genotypes [12,49]) and North America (US-6, US-7, and US-8 genotypes [17,18]) produce INF1 and should, therefore, be avirulent on a potato line responsive to INF1. However, it remains to be determined whether such INF1-responsive potato lines will have durable late blight resistance. The identification of

isolates deficient in INF1 suggests that *P. infestans* strains of the US-1 genotype can evolve loss of INF1 production and potentially gain virulence on elicitin-sensitive potato lines. Whether similar INF1-deficient races can readily evolve and reach high frequencies in sexual populations of *P. infestans* of various genotypes remains unclear. Experiments investigating potential fitness changes in INF1 mutants throughout the life cycle of *P. infestans* will address this question.

Even though races of *P. infestans* virulent on a wider range of plant genotypes have been described for 50 years (5), the molecular mechanisms underlying the evolution of new virulence traits in *Phytophthora* spp. remain unknown (25). Genetic crosses between *P. infestans* isolates with different virulence patterns suggest that avirulence to the *S. demissum* genes *R*-2 and *R*-4 is dominant (1), as documented in several plant-pathogenic fungi and bacteria. Previously, it was suggested that elicitins could act as dominant avirulence factors. Lack of elicitin production in *P. parasitica* was shown to correlate with increased virulence on sensitive plants (29). A re-
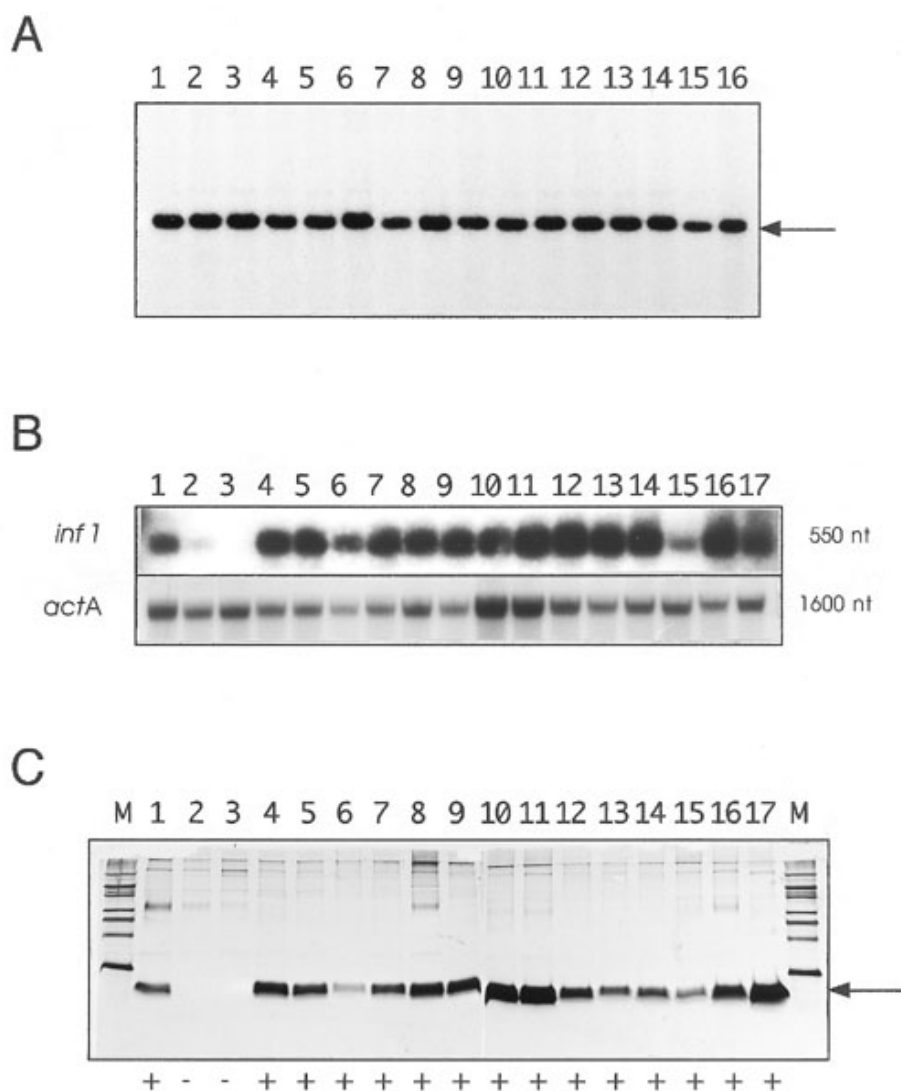

Fig. 4. Occurrence of the *inf1* gene, *inf1* mRNA, and INF1 protein in 17 isolates of *Phytophthora infestans*. **A,** Southern (DNA) blot containing *Bam*HI-digested total DNA from lane 1, DDR7601; lane 2, DDR7602; lane 3, DDR7702; lane 4, GER7401; lane 5, GER8451; lane 6, GER8501; lane 7, GER8601; lane 8, 46210; lane 9, 66006; lane 10, 68308; lane 11, 70001; lane 12, UK7225; lane 13, UK7818; lane 14, IT8001; lane 15, GE900083; and lane 16, GE900089 was hybridized with a probe from the *inf1* gene. The arrow indicates the 2.3-kilobase *Bam*HI fragment corresponding to the *inf1* gene. **B,** Northern (RNA) blot containing total RNA from the same isolates as in **A** (lanes 1 to 16) and lane 17, 88069 was hybridized with probes from the *inf1* and *actA* genes. The approximate sizes of the *inf1* and *actA* transcripts are shown on the right. **C,** Silver-stained polyacrylamide gels showing proteins present in culture filtrates from the same isolates as in **A and B**. Arrow indicates the position of the 10-kDa INF1 protein; + and – indicate the presence or absence of INF1, respectively.

cent finding in our laboratory strongly supports such an avirulence function for elicitins. We found that *P. infestans* strains engineered for INF1 deficiency have a broader host range, indicating that one of the molecular mechanisms underlying the evolution of a new race of *P. infestans* is based on mutations leading to loss of elicitin production (28). The results reported in this study, that INF1-nonproducing strains evolved independently in multiple lineages of *P. infestans* in a restricted geographic region (Table 1, Fig. 3), support this hypothesis. Future studies including the screening of potato germ plasm from the former East Germany for response to INF1 and comparative analyses of the virulence of INF1-producing and -nonproducing strains will help test this hypothesis.

ACKNOWLEDGMENTS

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