Molecular Characterization of Vegetative Compatibility Group 4A and 4B Isolates of *Verticillium dahliae* Associated with Potato Early Dying

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ABSTRACT

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Forty isolates of *Verticillium dahliae*, collected from potato seed tubers and potato plants from various regions in North America and previously assigned to vegetative compatibility groups (VCGs) 4A or 4B, were characterized using molecular markers. The VCG 4A isolates were previously shown to be a highly virulent pathotype of potato and to interact synergistically with the root-lesion nematode *Pratylenchus penetrans* to cause potato early dying. All but one of the VCG 4A isolates characterized in this study lacked the subspecies-specific repetitive DNA sequence E18 and could be differentiated from the remaining isolates by restriction fragment length polymorphisms (RFLPs) in the nuclear rDNA and *Trp1* loci. The E18 RFLP patterns of several VCG 4B isolates from Maine and New York were highly similar to those of VCG 4B isolates previously collected from potato and tomato fields in Ontario. The data presented here suggest that the molecular markers will be useful for the detection and classification of isolates of *V. dahliae* associated with potato early dying.

Additional keywords: DNA fingerprints, PED

The soilborne fungus *Verticillium dahliae* Kleb. causes vascular wilt disease in a variety of economically important agronomic and horticultural crops and is the principal pathogen responsible for potato early dying (PED) in North America (23). Strains of *V. dahliae* that are associated with this disease can be classified into a few subspecific groups (9,10,12,16,20,21,25). These groups are differentiated on the basis of vegetative compatibility, a multigene trait that determines the capacity of distinct fungal isolates within a species to anastomose and form heterokaryons, and to potentially exchange genetic information (13,14). Because *V. dahliae* has no known sexual reproductive cycle, isolates in a single vegetative compatibility group (VCG) are thought to comprise a biologically distinct population, members of which often share specific traits. Four ma-

jor VCGs have been identified within *V. dahliae*; VCGs 2 and 4 have been further divided into subgroups based on differential interactions among tester strains within each group (9,10). VCG 2 includes subgroups 2A and 2B, while VCG 4 includes subgroups 4A, 4B, and 4A/B. The majority of isolates associated with PED are in VCGs 4A and 4B (1,10,25). Although there is considerable variation in the aggressiveness of individual isolates of *V. dahliae* on potato, the VCG 4A isolates are of the most concern to potato production in North America, as they are more aggressive than VCG 4B isolates (10,20, 21,25) and interact synergistically with the root-lesion nematode *Pratylenchus penetrans*, resulting in premature plant senescence and low tuber yields (1).

Vegetative compatibility analysis, the only method currently available to differentiate isolates of *V. dahliae* associated with PED, is a laborious procedure that requires generation of nitrate nonutilizing (*nit*) mutants, mutant phenotype characterization, and multiple complementation tests (9,10,25). The assignment of an isolate to a specific VCG may also be complicated by the inability to obtain suitable *nit* mutants, or by differences in the ability of a particular *nit* mutant to complement different tester strains within a VCG (9,25). Studies of potato early dying would therefore be facilitated if additional methods could be developed to rapidly differentiate

the VCG 4A isolates from isolates in other subgroups of *V. dahliae*.

Omer et al. reported that both VCG 4A and 4B isolates of *V. dahliae* frequently can be recovered from commercial potato seed tubers produced in North America (20,21). Data from recent molecular studies of *V. dahliae* have suggested that potato may act as a reservoir for the VCG 4A and 4B isolates that are prevalent in some potato and tomato fields in Ontario, Canada, and that specific molecular markers may be diagnostic for the VCG 4A isolates (5,7,8,16). The objectives of the current study were therefore to evaluate the strength of the association of VCG 4A with selected molecular markers and to characterize, at the molecular level, a collection of *V. dahliae* isolates from potato seed tubers from across North America.

MATERIALS AND METHODS

Verticillium isolates. *V. dahliae* tester strains Dvd-2 (VCG 4B, from tomato), Dvd-T5 (VCG 2A, from tomato; ATCC 201177), Dvd-3 (VCG 4B, from potato), and Dvd-E6 (VCG 4A, from eggplant) are from Ontario, Canada (5,6). Tester strains MT (VCG 4B, from maple), BB (VCG 4A, from potato), and CW (VCG 4A/B, from cherry) are from Canada, Idaho, and Washington State, respectively (9). All other isolates of *V. dahliae* are from commercial potato seed tubers grown in 10 U.S. states and two Canadian provinces, or from potato plants from Ohio, and are listed in Table 1.

Vegetative compatibility analysis. Isolates listed in Table 1 were assigned to VCGs 4A or 4B in previous studies (21; M. Omer and R. C. Rowe, unpublished). Vegetative compatibility groups of potato isolates 99-1 and 66-12 were retested using the *nit* mutants generated by Omer et al. (21). The *nit* mutants were selected by growth on cornmeal agar medium containing 20 to 30% potassium chlorate, and chlorate-resistant sectors were subcultured onto minimal medium. Colonies with thin, nonaerial mycelial growth were considered to be *nit* mutants; mutant phenotypes were determined by growth on minimal media amended with various nitrogen sources, as previously described (9,10). For VCG tests, the mutants were paired on minimal medium containing 0.2% sodium nitrate (5,9,10) with complementary mutants of

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tester strains BB, MT, CW, Dvd-3, or Dvd-E6. Compatibility was detected as a region of dense prototrophic growth, often associated with abundant microsclerotia production, at the contact site between mycelia of the paired isolates, as described previously (9).

Restriction fragment length polymorphism (RFLP) analysis. Isolates of *V. dahliae* shown in Table 1 were characterized by RFLP analysis, using molecular markers, in a blind study in which VCG designations of the isolates were not disclosed prior to the analysis. Genomic DNA from each isolate was purified by a glass bead breakage method, from spores grown in liquid complete medium (4). The DNA was digested with the restriction

enzymes *EcoRI* or *XhoI*, fractionated by electrophoresis through 0.8% agarose gels, and DNA blots prepared for hybridization as previously described (5). High-stringency hybridizations and washes, and detection of hybridized probe, were carried out as previously described (5). Hybridization probes were: (i) plasmid pTZ19/VtITS, which contains the nuclear ribosomal DNA intervening transcribed spacer and 5.8S rDNA sequences from *Verticillium tricornutum* (15), (ii) E18, a nuclear repetitive DNA sequence from the Dvd-2 race 2 tomato isolate of *V. dahliae* (22), and (iii) a 364-bp restriction fragment of the putative trypsin gene *Trp1* (K. F. Dobinson, unpublished) from the Dvd-T5 race 1 isolate of *V. dahliae*, cloned into

pBluescript II KS (+). DIG-labeled pTZ19/VtITS probe was prepared as previously described (5). The E18 and *Trp1* probes were prepared by polymerase chain reaction (PCR) amplification of the cloned sequences, using the PCR-DIG Labeling Kit (Roche Diagnostics, Laval QC), with 24-base primers complementary to the T7 and T3 promoter sequences of the plasmid vector and an annealing temperature of 65°C.

RESULTS

Three *V. dahliae* strains of known VCG (5) were used as standards for the RFLP analyses. The ribosomal DNA sequence from *V. tricornutum* hybridized to two *EcoRI* fragments of genomic DNA from each of these strains, including one fragment of approximately 3.2 kb and one of either 2.2 or 0.9 kb (Fig. 1A). As shown in Fig. 1B, the *Trp1* probe hybridized to two fragments of *XhoI*-digested genomic DNA from the same strains of *V. dahliae*, including one fragment of approximately 4.0 kb and one of either 1.5 or 0.9 kb. The VCG 4A isolate Dvd-E6 contained the 0.9-kb rDNA and 0.9-kb *Trp1* fragments. Both the VCG 2A isolate Dvd-T5 and the VCG 4B isolate Dvd-2 contained the 2.2-kb rDNA fragment and the 1.5-kb *Trp1* fragment. As shown in Table 1, 18 of the potato isolates contained both the 0.9-kb *EcoRI* rDNA and the 0.9-kb *XhoI* *Trp1* fragments; 22 of the isolates contained the 2.2-kb *EcoRI* rDNA and the 1.5-kb *XhoI* *Trp1* fragments.

The repetitive DNA sequence E18 was present at low to moderate copy number in all but three isolates (BC isolates 149, 165, and 166) having the 2.2-kb *EcoRI* rDNA/1.5-kb *XhoI* *Trp1* RFLP profile (Table 1; Fig. 2A, lanes 1 to 18; Fig. 2B, lanes 3 and 4) and absent from all isolates containing the 0.9-kb *EcoRI* rDNA and 0.9-kb *XhoI* *Trp1* fragments (Table 1; Fig. 2B, lane 5). Four isolates from New York and Maine had E18 profiles that were highly similar (>80% band identity) to those of isolates that are prevalent in some tomato and potato fields in southwestern Ontario (Fig. 2A, compare lanes 11 to 13, 18, and 19; see also references 5,8,16).

In recent studies, VCG 4A isolates of *V. dahliae* have been found to lack E18 sequences and to contain the 0.9-kb *EcoRI* rDNA fragment (5,16). Based on those data, the 18 isolates listed in Table 1 that contained the 0.9-kb rDNA and did not contain E18 sequences were predicted to be members of VCG 4A. Similarly, based on previous experiments showing that VCG 4B isolates are characterized by the 2.2-kb *EcoRI* rDNA fragment (5,7,16), the 22 isolates with this RFLP (Table 1) were predicted to be members of VCG 4B.

The VCG predictions were correct for 38 of the 40 isolates, including all of those predicted to be in VCG 4A. However, while the rDNA RFLP profiles of isolates

Table 1. Origin, molecular variation, and vegetative compatibility groups (VCGs) among isolates of *Verticillium dahliae* collected from potato seed tubers and plants in North America

Isolate ^a	Geographic origin ^b	rDNA/ <i>EcoRI</i> ^c RFLP	<i>Trp1/XhoI</i> ^c RFLP	E18 sequences ^d	VCG ^e
149	BC	2.2	1.5	—	4B
165	BC	2.2	1.5	—	4B
166	BC	2.2	1.5	—	4B
152	OR	2.2	1.5	+	4B
193	OR	2.2	1.5	+	4B
155	OR	2.2	1.5	+	4B
157	OR	2.2	1.5	+	4B
197	ID	2.2	1.5	+	4B
201	MT	2.2	1.5	+	4B
P3	OH	2.2	1.5	+	4B
P273	OH	2.2	1.5	+	4B
62-8	NB	2.2	1.5	+	4B
101-1	NY	2.2	1.5	+	4B
102-1	NY	2.2	1.5	+	4B
105-1	NY	2.2	1.5	+	4B
11-11	ME	2.2	1.5	+	4B
16-1	ME	2.2	1.5	+	4B
42-1	ME	2.2	1.5	+	4B
42-7	ME	2.2	1.5	+	4B
45-11	ME	2.2	1.5	+	4B
66-12	ME	2.2	1.5	+	4A
99-1	NY	2.2	1.5	+	4A/B
170	BC	0.9	0.9	—	4A
86	WA	0.9	0.9	—	4A
87	WA	0.9	0.9	—	4A
129	WA	0.9	0.9	—	4A
131	WA	0.9	0.9	—	4A
111	MT	0.9	0.9	—	4A
108	WY	0.9	0.9	—	4A
110	WY	0.9	0.9	—	4A
90	SD	0.9	0.9	—	4A
92	SD	0.9	0.9	—	4A
97	SD	0.9	0.9	—	4A
83-1	WI	0.9	0.9	—	4A
88	WI	0.9	0.9	—	4A
21-18	MI	0.9	0.9	—	4A
P103	OH	0.9	0.9	—	4A
P107	OH	0.9	0.9	—	4A
30-6	NB	0.9	0.9	—	4A
66-13	ME	0.9	0.9	—	4A

^a Isolates with designations prefixed by P are from potato plants.

^b OR, Oregon; ID, Idaho; MT, Montana; MI, Michigan; OH, Ohio; ME, Maine; BC, British Columbia; NY, New York; WA, Washington; WY, Wyoming; SD, South Dakota; WI, Wisconsin; NB, New Brunswick.

^c Hybridization probe/restriction enzyme combination used for the restriction fragment length polymorphism (RFLP) analysis. Sizes of polymorphic fragments (kb) are indicated. Fragment sizes were estimated by agarose gel electrophoresis.

^d Presence (+) or absence (—) of E18 sequences determined by DNA blot hybridization of the E18 probe to *EcoRI*-digested genomic DNA.

^e Data from reference 21. Isolate 99-1, previously classified as VCG 4A (21), was reclassified as VCG 4A/B (see Table 2).

99-1 and 66-12 predicted their inclusion in VCG 4B (Table 1), both isolates had been previously assigned to VCG 4A (21). The vegetative compatibility of these isolates was therefore re-evaluated. The VCG 4A classification of isolate 66-12 was verified (Table 2). The vegetative compatibility classification of isolate 99-1 was less straightforward; *nit* mutants complemented

some VCG 4B tester strains, but also complemented some VCG 4A testers. This isolate has therefore been tentatively reclassified VCG 4A/B (Table 2).

DISCUSSION

Molecular techniques, including RFLP and randomly amplified polymorphic DNA (RAPD) analysis, have been widely used to

differentiate *V. dahliae* from other *Verticillium* species (15,17) and to assess genetic diversity within *V. dahliae* (2,5,8,11,16, 18,19,24). With few exceptions, variation at the molecular level has not been correlated with aggressiveness, host, or geographic origin (2,5,8,11,16,18,19,24).

In the case of vegetative compatibility, few data are available to adequately assess the relationship(s) between VCG and molecular variation. VCGs 2A, 2B, and 4B have been previously shown to be genetically heterogeneous groups comprised of isolates that may be further differentiated by their host range, aggressiveness on cotton, pathogenicity to tomato, or arrays of repetitive DNA sequences (3,5,8,12,16). The data presented in the current study suggest that VCG 4A includes at least two genetically distinct types of isolates, one of which is characterized by the 0.9-kb *EcoRI*/rDNA and 0.9-kb *XhoI*/*Trp1* markers and is prevalent across North America (16,21). The results of this study also indicate that rDNA or *Trp1* marker analysis can be used to rapidly identify this VCG 4A genotype within collections of *V. dahliae* obtained from soil or various potato tissues. It may also be possible in the future to exploit the polymorphisms at the rDNA or *Trp1* loci, to develop a PCR-RFLP assay(s) for diagnostic purposes.

The results of the VCG retests for isolate 99-1 (Table 2) highlight one of the potential difficulties in using vegetative compatibility to classify isolates. The RFLP analysis has several advantages over vegetative compatibility analysis; it is faster, less labor intensive, and less subject to variables inherent in the latter, such as the inability to obtain *nit* mutants or variation in the strength of complementation reactions with different tester strains. However, the utility of the rDNA and *Trp1* markers also has its limitations. Since no polymorphisms at the rDNA and *Trp1* loci have been identified between VCG 4B isolates and isolates of vegetative compatibility groups 1, 2A, 2B, 3, and 4A/B (Fig. 1, data not shown, and references 5,7), these markers cannot be used to definitively identify isolates within VCG 4B. Similarly, because E18 sequences are absent from some VCG 4B isolates (Table 1) and present in some isolates of VCGs 2A and 2B (Fig. 2A, lane 21, and data not

Fig. 1. DNA blots showing hybridization of the *Trp1* and rDNA probes to genomic DNA from isolates of *Verticillium dahliae* of vegetative compatibility groups 2A, 4A, and 4B. (A) DNA was digested with *EcoRI*, and the blot was hybridized with the rDNA probe. Sizes of the hybridizing fragments (kb) are indicated to the right of the blot. Lanes: 1, Dvd-2 (VCG 4B); 2, Dvd-T5 (VCG 2A); 3, Dvd-E6 (VCG 4A). Sizes of the fragments (kb) are indicated to the right of the blot. (B) DNA was digested with *XhoI*, and the blot was hybridized with the *Trp1* probe. Lanes: 1, Dvd-T5 (VCG 2A); 2, Dvd-2 (VCG 4B); 3, Dvd-E6 (VCG 4A); 4, no DNA; 5, DIG-labeled Lambda/*HindIII* molecular markers. Sizes of markers (kb) are indicated to the right of the blot.

Table 2. Reassessment of vegetative compatibility groups of 99-1 and 66-12 isolates of *Verticillium dahliae*^a

Isolate	Tester strains					VCG
	VCG 4B Dvd-3	VCG 4B MT	VCG 4A Dvd-E6	VCG 4A BB	VCG 4A/B ^b CW	
99-1	+	+/- ^c	-	+, +/- ^c	+	4A/B
66-12	-	-	+	+	+	4A

^a Strength of the complementation reaction is indicated as strong (+), weak (+/-), or not detectable (-) according to references 9 and 10. Data are from at least two tests between complementary mutants. In pairings where the amount of prototrophic growth varied between experiments, all types of interactions have been indicated.

^b 4A/B designation indicates ability to complement both 4A and 4B isolates.

^c Prototrophic growth developed over 2- to 3-week period.

shown), their presence is not a diagnostic marker for VCG 4B. In most cases it would therefore be necessary to use VCG and/or other molecular markers to further classify isolates having the 2.2-kb rDNA/1.5-kb *Trp1* polymorphisms.

Five E18 RFLP profiles were detected among the VCG 4B and 4A/B isolates (isolates having >80% identical bands were considered to have the same profile). Of particular interest was the observation that the E18 profiles of three New York isolates (101-1, 102-1, and 105-1) and one Maine isolate (11-11) were highly similar to that of the Ontario isolate Dvd-2 (Fig. 2) and to those of other isolates prevalent in Ontario potato and tomato fields (8,16). It has recently been proposed that isolates of this type have been distributed throughout Ontario with seed potato (7,8). Although only a small number of isolates from potato seed tubers were characterized in the present study, the data presented here are consistent with that hypothesis. More extensive studies will be required to determine the extent to which this genotype has been distributed across North America.

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Fig. 2. DNA blots showing hybridization of probe E18 to genomic DNA from potato isolates of *Verticillium dahliae*. Genomic DNA from all E18-containing isolates listed in Table 1 and from VCG 4A isolate 66-13, which does not contain E18 sequences, was digested with *EcoRI*, and the blots were hybridized with DIG-labeled probe. (A) Lanes: 1, 2, 4, and 5, Oregon isolates 157, 193, 155, and 152, respectively; 3, Idaho isolate 197; 6, Montana isolate 201; 7, New Brunswick isolate 62-8; 8 and 9, Ohio isolates P3 and P273; 10 to 13, New York isolates 99-1, 101-1, 102-1, and 105-1; 14 to 18, Maine isolates 16-1, 42-1, 42-7, 45-11, and 11-11; 19 to 21, Ontario isolates Dvd-2 (VCG 4B), Dvd-3 (VCG 4B), and Dvd-T5 (VCG 2A). (B) Lanes: 1 and 2, Ontario isolates Dvd-2 and Dvd-3; 3, New Brunswick isolate 62-8; 4 and 5, Maine isolates 66-12 and 66-13. Ethidium bromide staining of the gels indicated that equivalent amounts of DNA were in each lane (not shown). Sizes of molecular weight standards (kb) are indicated to the right of the blot.

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