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Identification and characterization of DNA markers associated with a locus conferring virulence on barley in the plant pathogenic fungus *Cochliobolus sativus*

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Abstract *Cochliobolus sativus* is a plant pathogenic fungus that causes spot blotch on barley and wheat. Virulence of a pathotype-2 isolate (ND90Pr) on barley cultivar Bowman was previously determined to be controlled by a single locus. To identify DNA markers associated with this virulence locus, amplified fragment length polymorphism (AFLP) analysis was conducted on 104 progeny isolates derived from a cross between isolates ND90Pr (exhibiting high virulence on Bowman) and ND93-1 (exhibiting low virulence on Bowman). Among 115 AFLP markers identified, 14 were linked to the virulence locus *VHv1* in isolate ND90Pr, six of which co-segregated with *VHv1*. Two (E-AG/M-CA-207 and E-AG/M-CG-121) of the six co-segregating AFLP markers were cloned and used to probe genomic DNAs from the fungal parents and progeny. Both markers hybridized only with DNAs from ND90Pr and the virulent progeny. These two cloned markers were also used as probes to survey field isolates of *C. sativus* collected from different regions of the world and again only hybridized to DNAs from isolates that had the same virulence phenotype as ND90Pr. The results of this study indicate that E-AG/M-CA-207 and E-AG/M-CG-121 are closely linked to *VHv1* and are unique to isolates carrying the virulence locus. Development of a linkage group, coupled with the identification of closely linked molecular markers, will facilitate the cloning of the virulence gene *VHv1* in *C. sativus* by map-based cloning.

Keywords AFLP markers · Barley · *Cochliobolus sativus* · Spot blotch · Virulence gene

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Introduction

Cochliobolus sativus (Ito & Kurib.) Drechs. ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.)] is a plant pathogenic fungus with a wide host range in the Poaceae (Tinline 1988). It also is well known as the causal agent of spot blotch of barley and wheat (Wiese 1977; Mathre 1997). The development of methods for producing sexual crosses of the fungus in culture (Tinline 1951) facilitated conventional genetic studies on the inheritance of pathogenicity and virulence. Kline and Nelson (1971) studied the inheritance of pathogenicity in a cross between non-pathogenic and pathogenic isolates of *C. sativus* on six gramineous species. Their results suggested that pathogenicity for each of five species (*Cynodon dactylon*, *Dactylis glomerata*, *Eleusine indica*, *Panicum virgatum* and *Poa pratensis*) was controlled by one gene and for a sixth species (*Phalaris stenoptera*) by two genes. The seven genes identified for pathogenicity in this study segregated independently (Kline and Nelson 1971). From the evaluation of randomly isolated ascospores from a cross of two *C. sativus* isolates virulent on barley genotypes, NDB112 and Larker, Hosford et al. (1975) found that virulence was controlled by three and four genes, respectively.

Valjavec-Gratian and Steffenson (1997a) investigated cultivar-specific virulence in *C. sativus* on barley. They identified three pathotypes (designated 0, 1, 2) of the fungus among 36 isolates from North Dakota and other regions of the world on a differential host set consisting of barley lines NDB112, Bowman, and ND5883. Pathotype-1 isolates exhibit high virulence on ND5883, but low virulence on NDB112 and Bowman. Pathotype-2 isolates exhibit high virulence on Bowman, but low virulence on NDB112 and ND5883. Pathotype-0 isolates exhibit low virulence on all three differentials (Valjavec-Gratian and Steffenson 1997a). To determine the genetics of virulence of pathotype-2 isolates on barley cultivar Bowman, a cross was made between the *C. sativus* isolates ND90Pr (representing pathotype-2) and ND93-1 (representing pathotype-0). Genetic analysis showed that

a single locus (now designated *VHv1*) in isolate ND90Pr controls high virulence on Bowman (Valjavec-Gratian and Steffenson 1997b).

Little is known about the genetic and molecular interactions of *C. sativus* with its cereal hosts. To fully elucidate these interactions, it is important to catalog, isolate, and characterize genes for virulence or pathogenicity in the pathogen and genes for resistance in the host. Map-based cloning is one possible strategy for isolating virulence/avirulence loci from fungi (Sweigard et al. 1995; Valent 1997; Farman and Leong 1998; Orbach et al. 2000) and relies upon the identification of closely linked molecular markers. This paper describes the identification and characterization of AFLP markers associated with the cultivar-specific virulence locus (*VHv1*) in *C. sativus.*

Materials and methods

Fungal isolates and virulence evaluations

Parental *C. sativus* isolates, ND90Pr and ND93-1, and 104 progeny isolates were used in this study. The progeny isolates were derived from ascospores that were individually isolated from asci produced from the sexual cross between isolates ND90Pr and ND93-1 of this heterothallic haploid fungus (Valjavec-Gratian and Steffenson 1997b). The virulence phenotypes of 103 progeny were previously evaluated on the three differential barley genotypes Bowman, ND5883 and NDB112 (Valjavec-Gratian and Steffenson 1997b). These progeny isolates were re-tested in this study to confirm their virulence phenotype on the three differentials. Moreover, one additional isolate not tested by Valjavec-Gratian and Steffenson (1997b) was included in the virulence evaluation. The sowing and growing of the host lines, inoculum preparation, and inoculation protocols were as previously described (Valjavec-Gratian and Steffenson 1997a). The 0–9 rating scale of Fetch and Steffenson (1999) was used to score the infection responses of each isolate on the differential barley genotypes. This scale is comprehensive and considers both the size and type (degree of chlorosis/necrosis) of lesions. It has been useful for classifying infection responses in many types of studies in the barley spot blotch pathosystem (Fetch and Steffenson 1999).

Additionally, 58 field isolates of *C. sativus* collected around the world were used. This collection is not exhaustive, but does include isolates from the major barley growing regions where spot blotch is a disease problem. The virulence phenotypes of these field isolates were previously evaluated on the differential host set by Valjavec-Gratian and Steffenson (1997a) and Zhong and Steffenson (2001). Twenty-one geographically diverse isolates from this collection were selected for Southern hybridization with pathotype-2 specific AFLP markers and are listed in Table 1.

AFLP analysis

Genomic DNA was isolated from *C. sativus* using a procedure modified from Yoder (1988) and described by Zhong and Steffenson (2001). AFLP analysis was conducted using a modification of the procedure described by Vos et al. (1995). Template DNA for PCR was prepared by digesting genomic DNA (0.25 µg) with the restriction enzymes *Eco*RI and *Mse*I. The *Eco*RI and *Mse*I cohesive ends of the restriction fragments were ligated to adapters using the AFLP core reagent kit (Gibco BRL, Grand Island, N.Y.). Primers with one selective base (E-A, E-G, M-C and M-A) were used in pre-selective amplification. Eight *Eco*RI primers (E-AA, E-AG, E-AC, E-AT, E-GA, E-GG, E-GC and E-GT) and eight *Mse*I primers (M-CA, M-CG, M-CC, M-CT, M-AA, M-AG, M-AC and M-AT) were used for selective amplification. The DNA silver-staining system (Promega, Madison, Wis.) was used to detect the DNA fragments (Zhong and Steffenson 2000). Each AFLP marker was named by a combination of the extension bases used in the primer pair, and its approximate size in base pairs. For example, E-AG/M-CA-207 denotes an AFLP marker produced by the primer pair E-AG and M-CA with a molecular size of 207 base pairs. AFLP analysis was repeated using different batches of DNA from the same sample with the described AFLP detection system. The results were repeatable.

Cloning and sequencing of AFLP markers

AFLP bands of interest were excised from silver-stained gels and re-amplified by PCR using the same primer pair that was used for

Table 1. Field isolates of *C. sativus* used for characterization of pathotype-2 specific AFLP clones

Isolate	Geographical origin	Source	Pathotype[a]	Hybridization[b]	
				E-AG/ M-CA-207	E-AG/ M-CG-121
ND89-1	North Dakota	B. J. Steffenson	0	–	–
ND89-8	North Dakota	B. J. Steffenson	0	–	–
ND89-25	North Dakota	B. J. Steffenson	0	–	–
ND89-33	North Dakota	B. J. Steffenson	0	–	–
ND89-38	North Dakota	B. J. Steffenson	0	–	–
ND89-40	North Dakota	B. J. Steffenson	0	–	–
A20	Canada	R. D. Tinline	0	–	–
A18	Canada	R. D. Tinline	0	–	–
ND85F	North Dakota	B. J. Steffenson	1	–	–
ND95-17	North Dakota	B. J. Steffenson	1	–	–
KB002	North Dakota	B. J. Steffenson	1	–	–
KB364	Colorado	B. J. Steffenson	1	–	–
JPN95-1	Japan	B. J. Steffenson	1	–	–
CHN95-2	China	B. J. Steffenson	1	–	–
POL97-KD31	Poland	J. Chelkowski	1	–	–
ND92-8	North Dakota	B. J. Steffenson	2	+	+
ND92-10	North Dakota	B. J. Steffenson	2	+	+
ND92-11	North Dakota	B. J. Steffenson	2	+	+
ND93-12	North Dakota	B. J. Steffenson	2	+	+
ND95-31	North Dakota	B. J. Steffenson	2	+	+
ND91-Bowman	North Dakota	B. J. Steffenson	2	+	+

[a] Pathotypes characterized by Valjavec-Gratian and Steffenson (1997a) and Zhong and Steffenson (2001)

[b] DNA prepared from the isolates were hybridized with the two AFLP clones (E-AG/M-CA-207 and E-AG/M-CG-121). '+' indicates hybridization bands detected and '–' indicates no hybridization bands observed

generating the AFLP bands. PCR reactions were performed in a 50µl solution, which contained 1× buffer (10 mM Tris-HCl at pH 8.4 and 50 mM KCl), 1.5 mM of $MgCl_2$, 200 µM of dNTP, 1.0 unit of *Taq* DNA polymerase (Promega, Madison, Wis.), 36 ng of each AFLP primer, and one excised AFLP band. The PCR profile was denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. Re-amplified PCR products were verified by comparing with the respective AFLP bands in the denaturing polyacrylamide gels and then cloned into the plasmid vector pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. The boiling method (Sambrook et al. 1989) was used to isolate plasmid DNA from the bacterial host. Inserts were sequenced using the SequiTherm EXCEL II DNA Sequencing Kit with fluorescently labeled M13 primers (Epicentre, Madison, Wis.) in a LI-COR Sequencer (LI-COR Inc., Lincoln, Neb.). Double-strand sequencing was performed for each clone.

To convert AFLP markers into sequence-characterized amplified region (SCAR) markers, oligonucleotide primers were designed based on the sequences of the cloned AFLP fragments and used for PCR amplification with genomic DNA from the fungal isolates. Primers were synthesized by Life Technology Inc. of Grand Island, N.Y. PCR reactions were performed as described above for re-amplification of the excised AFLP bands. PCR products were separated in a 1.4% agarose gel and stained with ethidium bromide.

DNA hybridization

For Southern hybridization, total genomic DNA of *C. sativus* was digested with restriction enzymes according to the conditions recommended by the manufacturer (New England Biolabs, Beverly, Mass.). Digested DNA fragments (2 µg per lane) were separated on 0.9% agarose gels, treated with 0.25 N HCl for 10 min, and blotted to Hybond+filters (Amersham Pharmacia Biotech, Piscataway, N.J.) in 0.4 N NaOH for 12–18 h. Filters were pre-hybridized at 65°C in a pre-hybridization buffer (0.5 M $NaPO_4$, 7% SDS, and 1% BSA) for 2–3 h before they were probed with DNA clones that were labeled with α-[^{32} P]dCTP by the random hexamer labeling method (Feinberg and Vogelstein 1983). DNA–DNA hybridization was carried out at 65°C for at least 16 h. Filters were sequentially washed in 2× SSC/1% SDS at 65°C for 10 min, 1× SSC/0.5% SDS at 65°C for 10 min, and 0.5× SSC/0.25% SDS at 65°C for 1 h. Filters were then exposed to Kodak XAR-5 film for 16 to 96 h depending on the strength of the radioisotope signal. Labeled filters were stripped before re-use by washing in 0.5% SDS at 95°C for 1–2 min.

Data analysis and linkage construction

All AFLP markers detected between parental isolates ND90Pr and ND93-1 were evaluated on the 104 progeny isolates and tested for deviations from an expected 1:1 segregation ratio using the χ^2 test (α=0.05). Linkage analysis was performed using the computer program MAPMAKER version 2.0 (Lander et al. 1987). The 'Group' command was chosen to establish linkage groups with a logarithm of the odds (LOD) ratio score of 4.0 or greater and a maximum recombination frequency of 0.3. We chose to use a LOD value of 4.0 or larger in order to reduce the chance of obtaining a spurious linkage. Three-point analyses were used to establish the order of markers in a linkage group. The 'Try' command was used to determine the putative position of an additional marker in a linkage group, and then the 'Ripple' command was used to verify the order of the markers in that linkage group. The Kosambi mapping function (Kosambi 1944) was employed to convert recombination frequency to map distance in centimorgans (cM).

Results

Segregation of virulence in *C. sativus*

Every isolate evaluated in this study gave clear infection responses, allowing for the easy classification of progeny into low-virulence and high-virulence classes. Among the 104 progeny, 49 exhibited low virulence (infection responses ranging from 1 to 3) and 55 exhibited high virulence (infection responses ranging from 6 to 8) on Bowman. The segregation of high virulence versus low virulence was not significantly different from 1:1 (χ^2 = 0.35, P = 0.56), indicating that a single locus in isolate ND90Pr controls high virulence on Bowman. These results were identical to those reported by Valjavec-Gratian and Steffenson (1997b). The virulence locus in isolate ND90Pr was designated *VHv1* denoting virulence for *Hordeum vulgare*.

Identification of AFLP markers linked to *VHv1*

Sixty-four primer combinations (eight *Eco*RI primers and eight *Mse*I primers) were used to survey AFLPs between the *C. sativus* parental isolates ND90Pr and ND93-1. For each primer combination, 35–60 DNA fragments were detected, with an average of 42 scorable bands per primer combination. The number of polymorphic bands generated by a primer pair between isolates ND90Pr and ND93-1 ranged from 1 to 10, with an average of six per primer pair. Twenty primer pairs were ultimately used on the progeny population, and a total of

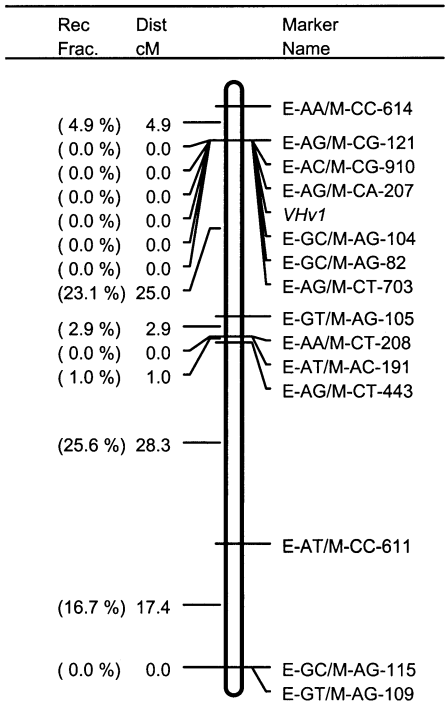

Fig. 1 Linkage group with AFLP markers associated with the barley cultivar-specific virulence locus *VHv1* in *C. sativus*

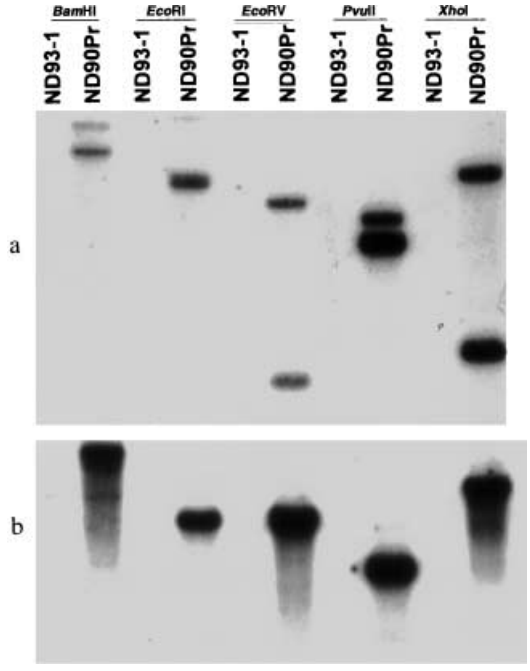

Fig. 2 Southern hybridization of the cloned AFLP markers E-AG/M-CA-207 (**a**) and E-AG/M-CG-121 (**b**) to genomic DNA from *C. sativus* parental isolates ND93-1 and ND90Pr. Genomic DNA was digested with five restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Pvu*II, *Xho*I), separated in a 0.9% agarose gel, and blotted to a Hybond N+ filter. The blot was probed with ^{32}P-radiolabeled clones E-AG/M-CA-207 (**a**). After stripping, the same blot was probed with E-AG/M-CG-121 (**b**)

115 segregating polymorphic AFLP markers was identified. Most (83%) of the AFLP markers showed a 1:1 Mendelian segregation ratio in the progeny population. Fourteen markers exhibited a distorted segregation ratio, 11 at the 5% significance level and three at the 1% significance level.

The virulence locus *VHv1* mapped on a linkage group with 14 AFLP markers when a minimum LOD value of 4.0 and a maximum theta of 0.3 were used (Fig. 1). Six AFLP markers co-segregated with *VHv1* in the progeny population. Two AFLP markers, E-AA/M-CC-614 and E-GT/M-AG-105, flanked *VHv1* at a distance of 4.9 cM and 25.0 cM, respectively (Fig. 1).

Characterization of AFLP markers associated with *VHv1*

Two AFLP markers (E-AG/M-CA-207 and E-AG/M-CG-121) that co-segregated with *VHv1* were cloned and used as probes to hybridize with the digested DNA of isolates ND90Pr and ND93-1. Both E-AG/M-CA-207 and E-AG/M-CG-121 hybridized only with genomic DNA of isolate ND90Pr, the parent exhibiting high virulence on Bowman. E-AG/M-CA-207 was present as two copies (Fig. 2a) and E-AG/M-CG-121 as a single copy (Fig. 2b) in the genome. When these cones were used to screen the 104 *C. sativus* progeny, they only hybridized with DNA of the 55 progeny that exhibited the same virulence pattern as parent ND90Pr (Fig. 3a and b), i.e., they were virulent on Bowman. These clones were also used to screen 21 geographically diverse field isolates of *C. sativus* from around the world (Table 1). Again, only DNA from isolates exhibiting high virulence on Bowman hybridized with these cloned AFLP markers (Fig. 4a and b).

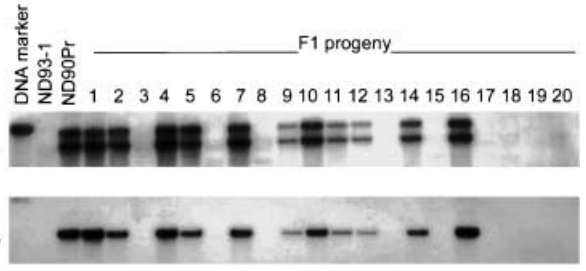

Fig. 3 Southern hybridization of the cloned AFLP markers E-AG/M-CA-207 (**a**) and E-AG/M-CG-121 (**b**) to genomic DNA from *C. sativus* parental isolates ND93-1 and ND90Pr and their progeny. Genomic DNA was digested with *Bam*HI (**a**) and *Eco*RV (**b**), respectively, separated in a 0.9% agarose gel, and blotted to Hybond N+ filters. The blots were probed with ^{32}P-radiolabeled E-AG/M-CA-207 (**a**) and E-AG/M-CG-121 (**b**), respectively. A subset sample of the progeny is shown

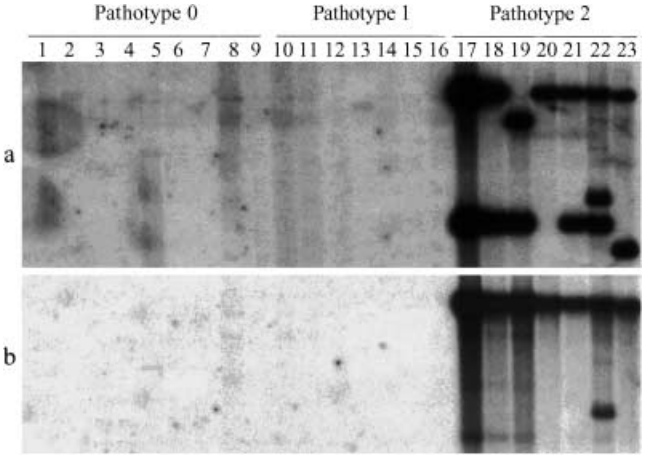

Fig. 4 Southern hybridization of the cloned AFLP markers E-AG/M-CA-207 (**a**) and E-AG/M-CG-121 (**b**) to genomic DNA from field isolates of *C. sativus*. Genomic DNA was digested with *Eco*RV, separated in a 0.9% agarose gel, and blotted to a Hybond N+ filter. The blot was probed with ^{32}P-radiolabeled E-AG/M-CA-207 (**a**). After stripping, the same blot was probed with E-AG/M-CG-121 (**b**)

Sequencing analysis indicated that E-AG/M-CA-207 and E-AG/M-CG-121 were derived from the *Eco*RI-*Mse*I fragments as expected for AFLP markers, and were 191-bp and 105-bp long, respectively. Primers were designed based on the sequence information of these two markers and used in PCR amplification with isolates ND90Pr and ND93-1 as well as their progeny. One primer pair from E-AG/M-CA-207 produced a band (about 160 bp) only in ND90Pr and the virulent progeny (Fig. 5a). Multiple bands were obtained with a primer pair from E-AG/M-CG-121, but a band (of about

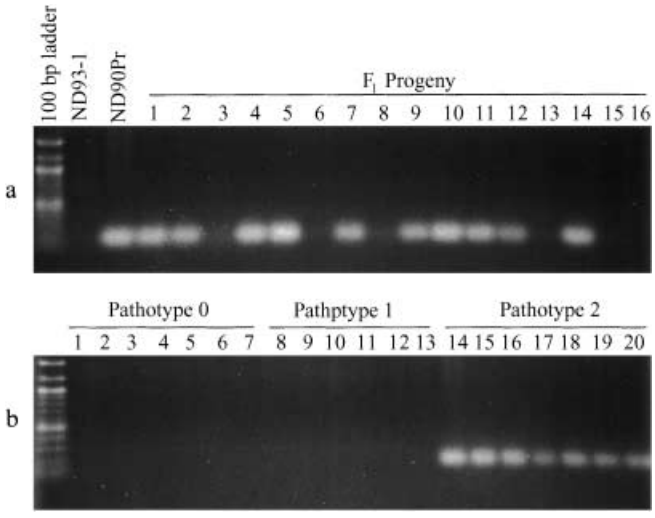

Fig. 5 PCR with E-AG/M-CA-207 specific primers, 5′ -GAATTCAGAGGGGCCTTC-3′ and 5′ -GACAAGCTGCTAGAGGAC-3′. (**a**) Amplification with DNA from *C. sativus* isolates ND93-1, ND90Pr and their progeny (1–16). (**b**) Amplification with DNA from a subset sample of field isolates of *C. sativus*

100 bp) unique to ND90Pr and the virulent progeny was identified (data not shown). We used these primers to screen field isolates collected from different regions of the world and found that the ND90Pr-specific bands were only amplified from pathotype-2 isolates. PCR amplification with primers from E-AG/M-CA-207 for a subset of field isolates is shown in Fig. 5b.

Discussion

The two cloned AFLP markers (E-AG/M-CA-207 and E-AG/M-CG-121) co-segregating with *VHv1* only hybridized with DNA of isolate ND90Pr, virulent progeny derived from ND90Pr, and pathotype-2 isolates collected from the field. This suggests that these DNA fragments around the *VHv1* locus are unique to isolates that carry virulence for Bowman. Hybridization of E-AG/M-CA-207 and E-AG/M-CG-121 with chromosomes separated by contour clamped homogeneous field (CHEF) electrophoresis localized the *VHv1* locus on a 2.80-Mbp chromosome in isolate ND90Pr, which is homologous with a 2.2-Mbp chromosome in isolate ND93-1 (Zhong 2000). The chromosome size difference may be due to unique DNA associated with virulence in isolate ND90Pr. Interestingly, in both *Cochliobolus heterostrophus* and *Cochliobolus carbonum*, whose pathogenicity depends on the production of a host selective toxin (HST), Tox1 and Tox2, respectively, the complex gene locus for toxin synthesis is located in a chromosome region that is unique to toxin-producing isolates (Ahn and Walton 1996; Kodama et al. 1999). In *C. heterostrophus*, about 1.2 Mbp of 'extra' DNA was found around the *Tox1* locus in race-T isolates that was absent in race-O isolates (non-toxin producer) (Kodama et al. 1999). In *C. carbonum*, more than 540 kb of DNA carrying the *Tox2* locus is missing in non-toxin-producing isolates (Ahn and Walton 1996). Although HSTs have not been isolated and characterized in *C. sativus*, they are presumed to be involved in the ability of the fungus to cause disease in various hosts based on the typical spot blotch symptoms of necrosis and chlorosis (Pringle 1979). Recent preliminary experiments with intracellular washing fluid from pathotype-2 infected barley leaves revealed a putative HST produced by the fungus (L. M. Ciuffetti and J.P. Martinez, personal communication). However, it is not known whether the toxin is synthesized by a gene complex similar to that found in *C. heterostrophus* (Kodama et al. 1999) and *C. carbonum* (Ahn and Walton 1996). Cloning of the *VHv1* locus and characterization of its gene product(s) will help address this question.

Pathogen virulence evaluations can be labor-intensive, time consuming, and prone to variation by various environmental factors. Development of pathotype-specific molecular markers would provide a convenient tool for pathotype identification. In the present study, the two markers co-segregating with the virulence locus *VHv1* were only present in the randomly sampled field isolates that carry the virulence locus based on the RFLP and PCR analysis. Although the collection of *C. sativus* isolates we tested in this study was not exhaustive, it was fairly diverse and included isolates from the major barley growing regions where spot blotch is a disease problem. Pathotype-2 isolates have only been identified in North Dakota and are thought to have arisen de novo relatively recently (Zhong and Steffenson 2001). Moreover, all pathotype-0 and pathotype-1 isolates evaluated were negative for these markers. Thus, the use of these two AFLP markers and their derived SCAR markers could provide a quick and convenient tool for identification of pathotype-2 isolates collected from the field.

Several genes from the rice blast fungus (*Magnaporthe grisea*) conferring specificity to different grass species and rice cultivars have been cloned using a map-based cloning strategy (Sweigard et al. 1995; Valent 1997; Farman and Leong 1998; Orbach et al. 2000). The construction of a linkage group and identification of closely linked DNA markers provide a solid starting point for the positional cloning of *VHv1* in *C. sativus.*

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