RESTRICTION FRAGMENT LENGTH POLYMORPHISMS AND RAPD MARKERS IN DNA OF *PLASMOPARA HALSTEDII*, THE DOWNY MILDEW FUNGUS OF SUNFLOWER.

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SUMMARY

A molecular analysis of the genome of six races of *Plasmopara halstedii* was performed using cloned restriction fragments of genomic DNA from each race. Dot blot hybridization of clones with total genomic DNA from each race showed differences among several races. Forty-four clones were analyzed by Southern hybridization with total DNA of each race. Two clones were identified which produced diagnostic hybridization patterns with *Bam*HI digests of the DNA, and could be used to distinguish five of the six races by RFLP. Hybridization of these clones with DNA from different isolates of each race revealed identical patterns. Hybridization of clone 1-13B to *Eco*RI digests of total DNA from each race produced a ladder of fragments that differed by 0.75 kb, a pattern which suggested the presence of family of direct tandem repeats.

Analysis of 22 isolates of six races for randomly amplified polymorphic DNA (RAPD) revealed extensive polymorphism. Two random primers differentiated all isolates from each other. Two other primers identified specific RAPD markers for a group of race 3 isolates. An isolate of race 3 from Bulgaria was different from race 3 isolates which originated in the USA. In contrast, the isolates of race 6 from France and Canada did not show substantial differences. The RAPD markers showed consistent patterns between different generations of one isolate.

INTRODUCTION

The obligate parasitic fungus *Plasmopara halstedii* is the causal agent of downy mildew in sunflower, and is considered one of the most serious diseases of sunflower. Nine races are known, and the corresponding resistance genes have been identified (Gulya et al., 1991). Physiologically, races of *P. halstedii* have been characterized on the basis of the disease response with a set of differential sunflower lines. However, little is known about the degree to which races differ from one another at the molecular level, or whether differences exist between geographical isolates of a race.

In this paper we describe our initial investigation of different races and isolates of *Plasmopara halstedii* using restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) techniques. The studies form a basis for the development of a procedure for rapid race identification and for analysis of similarity and relatedness of isolates with different geographic origin.

MATERIALS AND METHODS

Fungal Material. The isolates of six *Plasmopara halstedii* races used in this study are listed in Table 1. Isolates of *P. halstedii* were maintained by the Oilseeds Research Unit, U. S. Department of Agriculture, Agricultural Research Service, Fargo, ND. Spores were propagated and isolated as described by Gulya et al. (1991).

DNA Procedures. DNA of *P. halstedii* for RFLP analysis was extracted by the procedures described by Shure et al. (1983). For polymerase chain reaction (PCR) analysis, DNA was isolated by the combined procedures of Hulbert and Michelmore (1988) and Garber and Yoder (1983). Partial genomic libraries in a pUC19 plasmid vector were made by standard procedures (Maniatis et al., 1982) from DNA of all six races by digestion of each race with *Bam*HI, *Eco*RI, *Hin*dIII, or *Sal*I. The first number for each clone designation represents the race of *P. halstedii* from which it was isolated; the second number is arbitrary.

Colony hybridization of a total of 334 recombinant clones was performed on nylon membranes (Biotrace RP, Gelman) with nick-translated total DNA of each race. The hybridization intensity of each clone with each race was characterized as strong, weak, or not detected. Several clones from each of the three classes of hybridization were selected for RFLP analysis of the six races. For dot blot hybridization, 1 μg of total DNA was applied to a nylon membrane with a Bio-Dot Microfiltration Apparatus (Bio-Rad). For genomic Southern hybridizations, 2 to 3 μg of total DNA was digested to completion by the appropriate enzyme, size-fractionated on a 1% agarose gel in TAE buffer, and transferred to a nylon membrane via the alkaline transfer technique (Reed and Mann, 1985). The hybridization was performed in 5X SSC at 65°C overnight with nick-translated recombinant plasmid or phage DNA. The final wash after hybridization was done in 0.2X SSC at 65°C.

Analysis of RAPD markers was done according to the procedures of Martin et al. (1991) with small modifications. Oligonucleotides from RAPD Kits (V,I,C,K sets) purchased from Operon Technologies Inc. were used as primers. The amplification reaction was performed in 10 μl volume in a Coy thermal cycler, and consisted of 45 cycles of 1 min at 94°C, 30 sec at 35°C, 2 min at 72°C, followed by one cycle for 7 min at 72°C. Products were separated by electrophoresis in 1.2% agarose in 1X TAE buffer. A total of 80 primers were tested.

RESULTS AND DISCUSSION

Dot blot analysis of *P. halstedii* DNA. Among 334 recombinant clones, ten clones that hybridized strongly to the DNA of the race from which they were isolated were selected. These clones were used as probes to determine if race-specific identification could be performed via dot blot analysis. This analysis revealed that the clones were homologous to all races but exhibited different hybridization intensities in different races.

RFLP analysis of *P. halstedii* DNA. Forty-one clones, including representatives from each class of hybridization intensity in the dot blot test, were evaluated for RFLP among six races. The ten which were chosen by dot blot hybridization were hybridized to *Bam*HI, *Eco*RI, or *Hin*dIII-digested DNA from the six races, but polymorphism was not detected. Thirteen of the remaining 31 clones revealed race-diagnostic RFLPs in *Bam*HI-digested DNA. In *Eco*RI or *Hin*dIII-digested DNA, eight clones were diagnostic only for race 6; the rest were not diagnostic.

Several clones could be used to distinguish more than one race. For example, hybridization with clone 6-36 allowed the discrimination of three of the six races. Another diagnostic clone, 1-13, exhibited strong hybridization to race 1 DNA. The initial screening of the *Bam*HI digests of the six races with clone 1-13 showed that the clone could distinguish among races 1, 2, 5, and 6. Hybridization of clone 1-13 with *Sal*I-digested DNA detected a 0.75 kb fragment in all races, and in addition, race 5 could be distinguished from the other races by the presence of a 3.75 kb fragment, race 2 by a 3.0 kb fragment, and race 6 by 3.0 and 4.5 kb fragments. To ensure that patterns were race-specific, but not isolate-specific, we made a comparison of three isolates of race 1, two isolates of race 5 (one greenhouse isolate and one field isolate, #2098), and four isolates of race 6. Each showed a characteristic pattern for its race. Clone 2-24 hybridized to a 0.6 kb *Bam*HI fragment common to all six races. But in addition, this clone also hybridized to a 1.5 kb *Bam*HI fragment in races 4 and 5, and to a 3.6 kb BamHI fragment in race 5. Thus, clone 2-24 could distinguish between races 4 and 5. Therefore, with clones 1-13 and 2-24 we were able to differentiate five of the six *P. halstedii* races.

Tandem repeats. The ladder-like patterns of the hybridization of some clones to downy mildew DNA suggested that downy mildew DNA contained fragments of tandemly repeated sequences. Examination of these clones by Southern hybridization with downy mildew DNA which had been partially digested with different enzymes showed four different tandem repeats: 1-13B, 33H, 90H and 103B. Each contained an elementary unit tandemly repeated in the genome more than ten times. Restriction analysis and sequencing showed that 1-13B and 90H had internal fine structure. The 90H repeat could be divided into two similar subunits, while the 1-13B repeat could be divided into four subunits. The 1-13B repeat has an open reading frame, which can encode for an 80 amino acid protein with a predicted pI of 11.6. There is a 200 bp hairpin upstream of this

reading frame. It has been proposed that obligate parasites have genomes that differ in organization from other filamentous fungi by relatively large proportions of repetitive DNA (Christiansen and Giese, 1990). Our data supports this hypothesis.

RAPD markers. RAPD markers were used for analysis of isolates of different geographic origin for each *P. halstedii* race to determine whether RAPD analysis could provide race-specific markers and/or demonstrate differences among isolates. A survey conducted with 11 primers revealed considerable polymorphism among the amplification products. The size of the fragments ranged from 0.2 to 4.5 kb, and up to 15 fragments were amplified.

Among the primers analyzed, four appeared to be the most interesting. For example, primers V03 and I08 both differentiated all isolates studied. With these primers each isolate of each race had a distinguishing pattern of amplification products. No race-specific fragments were observed.

Two other primers, C08 and C09, showed specific RAPD markers for a group of race 3 isolates. Six of the nine isolates of race 3 lacked some fragments characteristic of the other isolates, but had one extra band in the 1 kb zone. Isolates #1030 and #1053 of race 3 had the same pattern as isolates of races 1, 4, and 5, but, like the other race 3 isolates, they did not have a 1.4 kb fragment. One pair of race 3 isolates (#1459 and #1461) produced nearly identical amplification products using primer V03. We later learned that both pairs of isolates, #1459/#1461 and #1030/1053, were different generations of one isolate. Therefore, it appears that the patterns provided by RAPD markers do not change significantly from one generation to the next. With primer C09, all of the isolates of race 3 tested (except #1459) differed from all the isolates of all other races by the absence of a 2.8 kb fragment and the presence of additional fragments in the 1.6 to 1.8 kb zone. These primers (C08 and C09) are the only primers identified so far that can distinguish some isolates of one race from isolates of other races. It is important to note that with all the primers tested, the race 3 isolate from Bulgaria had patterns different from all the other isolates of race 3. Because of its dissimilarity to the race 3 isolates of North American origin, these differences may be associated with geographic origin. This is not always the case, however, because isolates of race 6 from France and Canada showed only minor differences with primers V03 and I08.

Large differences in the structure of pathogen populations have been shown by a number of morphological markers, such as specific virulence, and by genetic markers, such as RFLP. Therefore, the considerable variability revealed by RAPD markers among sunflower downy mildew races and isolates is not surprising. Although RAPD markers cannot be used in *P. halstedii* homothallic isolates as genetic markers, they can be used for determination of relatedness among isolates.

Table 1. Isolates of *Plasmopara halstedii* used for determining DNA restriction length polymorphism and RAPD markers.

Pathotype	Isolate Designation	Origin
Race 1	R1	unknown
Race 1	H1	Hungary, 1976-1980
Race 1	#1555	Yugoslavia, Novi Sad, 1988
Race 1	H49	Hungary, Kisszallas, 1988
Race 3	R3	unknown
Race 3	R3 Bul	Bulgaria
Race 3	#1030	USA, Fargo, ND, 1983
Race 3	#1053	USA, Fargo, ND, 1984
Race 3	#1236	USA, Fargo, ND, 1984
Race 3	#1244	USA, Fargo, ND, 1985
Race 3	#1461	North America, 1986
Race 3	#1459	North America, 1986
Race 3	#1574	North America
Race 4	R4	unknown
Race 4	H50	Hungary, Heki, 1989
Race 4	#1677	France, 1990
Race 5	R5	USA, Fargo, ND, 1990
Race 5	#2098	USA, Crookston, MN, 1991
Race 6	R6	France
Race 6	#1667	France
Race 6	#1678	Canada, Morden, Manitoba
Race 6	#1923	Canada, Morden, Manitoba

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