Identification of rye- and wheat-types of *Pseudocercospora herpotrichoides* with DNA probes

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A partial genomic library of the plant-pathogenic fungus *Pseudocercospora herpotrichoides* was prepared in a bacterial plasmid vector. Plasmids from 20 random recombinant clones were radioactively labelled and used as probes in Southern blot experiments with genomic DNA isolated from seven isolates belonging to the rye-type and four isolates of the wheat-type of *P. herpotrichoides*. Most of the probes tested revealed polymorphisms between the two types. In addition, some of them distinguished different isolates within each type. One probe that gave strong hybridization signals may correspond with repetitive DNA sequences. It was shown by dot-blot hybridization to be specific to all the wheat-type isolates tested.

Introduction

Pseudocercospora herpotrichoides (Fron) Deighton causes eyespot, a widespread disease of wheat, barley and rye in temperate areas (reviewed by Fitt *et al.*, 1988). It is an important component of the parasitic complex of cereal stem-base rot in association with *Rhizoctonia* spp. and *Fusarium* spp. (Goulds & Polley, 1990). After germination, the mycelia spreads through successive leaf sheets to reach the inside of the stem. Economic damage does not occur until the fungus penetrates the stem and interferes with sap circulation. Yield losses in untreated fields can reach 4–30% of total production and vary greatly according to field location and season (Lagneau *et al.*, 1986).

Pathogenicity, growth and morphological variations within the species have given rise to several attempts at subspecific classification (Lange-De La Camp, 1966; Scott *et al.*, 1975). Possibly the most accepted system divides the species into wheat type (W-type) and rye type (R-type). The W-type is more pathogenic to wheat and barley than to rye seedlings and produces green-to-grey colonies with smooth margins and a high growth rate when grown on agar media. The R-type is equally pathogenic to wheat as to rye seedlings, its colonies are beige-to-brown with feathery margins, and it grows on agar media at approximately half the rate of W-type

isolates. Nirenberg (1981) distinguished two varieties based on spore size and morphology: *P. herpotrichoides* var. *herpotrichoides*, characterized by small and generally incurved conidia, and *P. herpotrichoides* var. *acuformis*, characterized by larger, straight conidia. Leroux & Gredt (1988) proposed an identification of isolates according to their resistance to fungicides and their growth rate. King & Griffin (1985) and Sanders *et al.* (1986) considered that slow-growing colonies could be classified as R-type or *P. herpotrichoides* var. *acuformis*, and fast-growing colonies as W-type or *P. herpotrichoides* var. *herpotrichoides*, but this was invalidated by Mauler & Fehrmann (1987) as the growth-rate ranges of the two types appear to overlap.

Typing of *P. herpotrichoides* strains occurring in the field is important for decision-making in eyespot control. The sensitivity of R-type strains to triazole fungicides is low (Leroux & Gredt, 1988). Resistance to benzimidazole fungicides is generally more frequent among R-type than W-type strains (King & Griffin, 1985; Maraite *et al.*, 1985; Fitt *et al.*, 1988). This can, to some extent, explain the population shift towards the R-type observed since the early eighties (Hollins & Scott, 1987).

Better understanding of variations in field populations and fungicide resistance requires identification of a large number of isolates. However, pathogenicity tests are time-consuming and the results tend to vary with experimental conditions (Fitt *et al.*, 1988; Creighton, 1989; Creighton & Bateman, 1991); in addition, morphological criteria vary widely within subspecific taxa of

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Abbreviation: RFLP, restriction fragment length polymorphism.

P. herpotrichoides (Maraite *et al.*, 1985; Hollins *et al.*, 1985; Creighton, 1989; Creighton & Bateman, 1991). New and reliable identification methods are thus required. Electrophoresis of total proteins has been used, but the resulting band patterns are complex, and only a few bands are truly characteristic of each type of *P. herpotrichoides*. So far, isoenzyme patterns have given variable results according to the enzymes analysed (Wegge *et al.*, 1991). Serological identification methods have been studied (Lind, 1990), but their discriminatory power at the subspecific level is low. For these reasons, a molecular approach to the problem was adopted, to seek varietal differences at the genomic level.

This paper describes the preliminary results of a molecular approach which allows the varietal differentiation of *P. herpotrichoides* by restriction fragment length polymorphism (RFLP) and dot-blot hybridization, avoiding misidentification with fungi such as *Microdochium nivale* and *Rhizoctonia cerealis* which cause similar symptoms on the cereal foot (Goulds & Polley, 1990).

Methods

Fungal strains. Eleven isolates of *Pseudocercospora herpotrichoides* were chosen from our collection according to their morphology, growth rate and fungicide sensitivity. One strain each of *Rhizoctonia cerealis* and *Microdochium nivale* were also included (Table 1). Cultures were stored at room temperature on 3.9% (w/v) potato dextrose agar (Difco) and covered with sterile water and paraffin oil. After regeneration of the mycelia on potato dextrose agar, sporulation was obtained on a poor medium [Sach's nutrient agar: 0.1% KH_2PO_4 , 0.1% KNO_3 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 1.7% w/v, agar (Oxoid)], after incubation for 3 weeks at 8 °C by exposure to near-ultraviolet radiation. Spore suspensions were used to inoculate 100 ml of MM medium (2% w/v, glucose, 0.15% asparagine, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% FeCl_3 , 0.1% yeast extract). After culture in the dark for 17 d at 16 °C, mycelium was harvested by filtering through Whatman paper no. 4, rinsed with sterile distilled water, frozen in liquid nitrogen, and stored at -70 °C.

Preparation of DNA. DNA extraction from *P. herpotrichoides* and *Penicillium* sp. was carried out according to the procedures of Rogers & Bendich (1988), modified as follows. Frozen mycelium (10 g) was ground in a mortar and pestle with 2.5 g Al_2O_3 in the presence of liquid nitrogen and mixed with 18 ml of 2 × CTAB solution [2% w/v, cetyltrimethylammonium bromide (CTAB), 100 mM-Tris/HCl, 1.4 M-NaCl, 20 mM-EDTA, 1% w/v, polyvinylpyrrolidone (PVP), pH 8.0] prewarmed to 65 °C. After incubation for 5 min at 65 °C, the suspension was divided and placed into two glass tubes. The aqueous phase was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) and centrifuged at 7000 r.p.m. for 10 min at room temperature (Beckman JS 13.1 rotor). The aqueous phase was transferred to another tube and mixed with one-tenth its volume of 10% CTAB solution (10% w/v, CTAB, 0.7 M-NaCl) prewarmed to 65 °C. After incubation for 5 min at 65 °C, the aqueous phase was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) and centrifuged at 7000 r.p.m. for 10 min at room temperature. The aqueous phase was transferred to another tube, mixed with one-tenth its volume of 10% CTAB solution prewarmed to 65 °C, and the extraction repeated. The aqueous phase was transferred to another tube

Table 1. *Origin and characteristics of the fungal strains*

Radial growth rate was determined on PDA after 10 d at 17 °C. Sensitivity to the fungicides benomyl (B), diethofencarb (D) and tebuconazole (T) was determined from the reduction in growth rate in the presence of 1 µg ml⁻¹ of the fungicide: <19%, strain is highly resistant (R); 20–59%, weakly resistant (r); 60–79%, weakly sensitive (s); 80–100%, highly sensitive (S). NT, Not tested.

UPM no.*	Isolation			Radial growth rate (mm d ⁻¹)	Fungicide sensitivity
	Date	Host	Location (Belgium)		
<i>Pseudocercospora herpotrichoides</i> : rye-type†					
662	7/89	Wheat	Orbais	0.55	B ^R D ^S T ^r
663	7/89	Wheat	Orbais	0.42	B ^R D ^S T ^s
664	7/89	Wheat	Villers	0.37	B ^S D ^R T ^s
666	7/89	Wheat	Seraing	NT	NT
667	6/89	Wheat	Seraing	0.40	B ^S D ^R T ^R
668	6/89	Wheat	Bierwart	0.52	B ^R D ^R T ^R
670	4/89	Wheat	Villers	0.58	B ^R D ^S T ^R
<i>Pseudocercospora herpotrichoides</i> : wheat-type†					
672	7/89	Wheat	Villers	0.55	B ^R D ^S T ^S
673	7/89	Wheat	Villers	0.58	B ^S D ^R T ^S
674	7/89	Wheat	Villers	0.70	B ^S D ^R T ^s
678	4/89	Wheat	Thorembais	1.18	B ^S D ^R T ^r
<i>Microdochium nivale</i>					
645	11/90	Turf grass	Brusen	NT	NT
<i>Rhizoctonia cerealis</i>					
607	5/90	Wheat	Thorembais	NT	NT

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† *P. herpotrichoides* strains are classified as rye-type or wheat-type depending on the feathery or smooth colony margins, respectively.

containing an equal volume of CTAB precipitation solution (1% w/v, CTAB, 50 mM-Tris/HCl, 10 mM-EDTA, pH 8.0) prewarmed to 65 °C. The suspension was then gently mixed to allow precipitation and centrifuged at 7000 r.p.m. for 20 min at room temperature. The supernatant was then discarded and the pellet resuspended in 3 ml of TE high-salt solution (10 mM Tris/HCl, 0.1 mM-EDTA, pH 8.0), prewarmed to 65 °C, and incubated for 10 min at 65 °C. The DNA was precipitated by adding a one-tenth volume of 3 M-sodium acetate and 2.5 vols cold ethanol, followed by gentle mixing and incubation for 12 h at -20 °C. After centrifugation at 7000 r.p.m. for 20 min at 4 °C, the supernatant was discarded and the pellet washed with 6 ml cold 70% (v/v) ethanol. Centrifugation was repeated for 10 min and the supernatant discarded. The pellet was dried for 3–4 min in a speed vacuum and the DNA resuspended in 80 µl of 1 × TE solution (10 mM Tris/HCl, 1 mM-EDTA, pH 8.0) and stored at -20 °C.

Vicia faba DNA was solubilized from isolated nuclei and purified by centrifugation on a CsCl gradient as described by Boutry & Chua (1985). *Saccharomyces cerevisiae* DNA was prepared from lysed protoplasts as described by Ausubel *et al.* (1989).

DNA required for the construction of the genomic library was purified and fractionated by isopycnic centrifugation in a CsCl density gradient containing the fluorescent dye bisbenzimidazole (Hoechst 33258, Sigma) according to Garber & Yoder (1983), but with an initial CsCl density of 1.6 g ml⁻¹. The nucleic acid concentration was estimated by a fluorimetric method involving specific binding of bisbenzimidazole to DNA.

Construction of a DNA library and probe preparation. Unless otherwise indicated, standard DNA techniques were performed according to

Sambrook *et al.* (1989). DNA (20 µg) was digested with *Bam*HI (BRL) as recommended by the manufacturer and fragments separated on a 2% (w/v) agarose gel in TBE [0.13 M-Tris, 0.15 M-boric acid, 3 mM-EDTA, ethidium bromide (5 µg ml⁻¹)]. DNA fragments 0.5–1 kb long were recovered from gel slices by centrifugation through siliconized sterile glass wool according to Heery *et al.* (1990), ligated into the *Bam*HI site of pBluescript SK⁺ (Stratagene), and used to transform competent cells of *Escherichia coli* 71-18. Recombinant plasmids were isolated by a LiCl miniprep procedure according to Brent (1990) and analysed by agarose gel electrophoresis. Clones were arbitrarily chosen as probes and labelled with [α -³²P]dCTP [3000 mCi mmol⁻¹ (111 GBq mmol⁻¹); 10 µCi µl⁻¹ (370 kBq ml⁻¹)] by the nick-translation method as recommended by the manufacturer (BRL).

Southern blot and dot-blot hybridization. For Southern blot experiments, DNA was digested with *Eco*RI or *Hind*III, separated by electrophoresis through a 1.5% (w/v) agarose gel in TBE, and transferred to nylon membranes (Hybond N⁺, Amersham) according to the manufacturer's instructions. For dot-blot experiments, DNA was directly spotted onto nylon membranes as recommended by the manufacturer. Hybridizations were performed at 42 °C (Sambrook *et al.*, 1989), the probe activity being 4×10^6 c.p.m. ml⁻¹. Blots were washed in 0.5 × SSC and 1% (w/v) SDS for 3 h at 65 °C and exposed to X-ray films (X-OMAT AR, Kodak) at -80 °C with an intensifying screen. Re-use of blots was possible after removal of the probe by immersion of the membrane in boiling 0.5% SDS and cooling to room temperature.

Results

To obtain DNA probes capable of revealing polymorphisms among *P. herpotrichoides* isolates, we constructed a partial genomic library of the W-type isolate UPM672. Purified nuclear DNA was cut with the restriction enzyme *Bam*HI. DNA fragments 0.5–1 kb in length were isolated from preparative gel electrophoresis and cloned in a bacterial plasmid.

Twenty recombinant clones were randomly chosen, ³²P-labelled, and hybridized with *Hind*III- or *Eco*RI-cleaved genomic DNA from R-type (UPM662) and W-type (UPM672) isolates. One of the probes (Psh19.42, Fig. 1 probe 1) gave a strong signal, and thus might contain a DNA repeat sequence. All the other probes but one gave one or two hybridizing bands, differing in size between the R-type and W-type of *P. herpotrichoides*, regardless of the restriction enzyme used. Fig. 1 illustrates the hybridization patterns obtained with five of the probes tested. Two probes (Psh20.37 and Psh19.42) gave a signal with the W-type only. We conclude that these sequences are specific to the W-type or that the corresponding sequences in the R-type are highly heterologous.

The general ability of our probes to reveal polymorphisms between closely related individuals was tested with a combination of five clones producing signals of different sizes in Southern hybridization experiments performed with *Eco*RI-digested total DNA (Fig. 2a). Such a combination allowed easy determination of the

Fig. 1. Southern blot hybridization of R- and W-type genomic DNA with probes derived from a W-type isolate of *P. herpotrichoides* UPM672. Genomic DNA (1 µg) extracted from the UPM662 R-type (R) and UPM672 W-type (W) isolates was digested with the restriction enzymes *Hind*III (H) or *Eco*RI (E), electrophoresed and transferred to nylon membranes. Hybridizations were performed with ³²P-labelled DNA of the following clones: Psh19.42 (1), Psh20.37 (2), Psh20.39 (3), Psh20.40 (4) or Psh20.38 (5).

two types of *P. herpotrichoides* within a group of 11 field isolates, while no signal was obtained with DNA from two other pathogenic fungi of the cereal stem base, *Rhizoctonia cerealis* and *Microdochium nivale*. Furthermore, several isolates within each morphological type could be singled out by their specific restriction fragment pattern (Fig. 2a). Three classes were observed for the seven R-type isolates while each of the four isolates of W-type gave a different pattern. Similar differences were detected among four monocaryotic ascospores of each type (results not shown). As shown in Fig. 2(b), signals were obtained with W-type isolates only, when a similar blot was hybridized with the W-type-specific probe Psh19.42 (Fig. 1, lane 1). With this probe, it was possible to distinguish between the four isolates, as the hybridization patterns varied considerably.

The high signal intensity and the W-type specificity observed with the Psh19.42 probe, whose restriction map is shown in Fig. 3, led us to test this DNA fragment in dot-blot hybridizations with DNA extracts derived from the two morphological types of *P. herpotrichoides* and to evaluate its possible use in diagnosis. Thus, serial dilutions of DNA were hybridized from the R- and W-types analysed in Fig. 2, alongside *M. nivale* and *R. cerealis* and three additional controls: a *Penicillium* sp.,

Fig. 2. Southern blot analysis of several R- and W-type isolates of *P. herpotrichoides*. Total DNA from the seven R-type and the four W-type isolates of *P. herpotrichoides* listed in Table 1 and from one isolate each of *R. cerealis* (*R.c.*) and *M. nivale* (*M.n.*) was hybridized with a combination of the Psh19.13, Psh19.48, Psh19.17, Psh19.22 and Psh19.29 clones (a) or with the Psh19.42 clone alone (b). From left to right, DNAs were from isolate UPM607 of *R. cerealis* (1); from isolates UPM670 (2), UPM668 (3), UPM667 (4), UPM664 (5), UPM666 (6), UPM663 (7) and UPM662 (8) of the R-type of *P. herpotrichoides*; from isolates UPM672 (9), UPM673 (10), UPM674 (11) and UPM678 (12) of the W-type of *P. herpotrichoides*; and from isolate UPM645 of *M. nivale* (13).

Fig. 3. Restriction map of the *P. herpotrichoides* BamHI DNA insert of the Psh19.42 clone. Total length of the insert is 0.95 kb.

Saccharomyces cerevisiae and *Vicia faba* (broad bean). Signals were obtained with DNA from the W-type only (Fig. 4a). For all four W-types, 0.5 µg of DNA was sufficient to produce a signal after a 1 d exposure. As a control, we hybridized the same blot with another probe (Psh20.38, lane 5 of Fig. 1) which gave a signal with all isolates of *P. herpotrichoides* analysed (Fig. 4b).

Discussion

We have hybridized several DNA probes derived from a W-type *P. herpotrichoides* strain with DNA prepared from strains, either of the same type or the R-type. Most of the probes gave one- or two-band signals of similar intensity, showing that they probably came from a single-copy sequence. In no case was the size of hybridizing fragments the same for both *P. herpotrichoides* types, which reflects a high degree of polymor-

Fig. 4. Dot-blot hybridization of total DNA of the R- and W-types of *P. herpotrichoides* with a R-type specific probe. Total DNA from the following was spotted on a nylon membrane: isolate UPM607 of *R. cerealis* (1, *R.c.*); isolates UPM670 (2), UPM668 (3), UPM667 (4), UPM664 (5), UPM666 (6), UPM663 (7) and UPM662 (8) of the R-type of *P. herpotrichoides*; isolates UPM672 (9), UPM673 (10), UPM674 (11) and UPM678 (12) isolates of the W-type of *P. herpotrichoides*; isolate UPM645 of *M. nivale* (13, *M.n.*); *Saccharomyces cerevisiae* (14, *S.c.*); *Vicia faba* (15, *V.f.*); and a representative of the genus *Penicillium* (16, *P.sp.*). The same blot was hybridized with the Psh19.42 clone (a) and with the Psh20.38 clone (b). The amount (µg) of DNA spotted is indicated on the left.

phism between their respective genomes. This molecular-level observation is in keeping with the reported vegetative incompatibility of these two types (Hocart *et al.*, 1989). It raises the possibility of distinguishing

between the two types. Furthermore, two probes were found to hybridize with the W-type only. One, (Psh19.42), which probably contains a multicopy sequence, was able to distinguish W-types from R-types in a dot-blot hybridization experiment. In Southern blot experiments, a mixture of five probes could distinguish the four representatives of the W-type and three different patterns among the seven representatives of the R-type. Further testing is required to ensure that these probes can distinguish between a wide variety of W- and R-type isolates. In addition, such tests should be carried out in concert with rigorous pathogenicity testing to ensure complete compatibility between the two characterization methods. It is interesting to note that the 11 strains used in this study could not clearly be classified according to phenotypic traits such as growth rate and fungicide sensitivity (Table 1).

Recently, DNA polymorphism in *P. herpotrichoides* has been investigated with a ribosomal RNA probe derived from the wheat genome (Nicholson *et al.*, 1991). In agreement with the results presented here, a higher degree of polymorphism was also observed in the W-type. In comparison to other classification methods, the advantages of RFLP technology are numerous. Paramount is the fact that DNA hybridization signals are fully reproducible and do not vary with culture age or growth conditions. The high sensitivity of the method is another great advantage. With powerful techniques such as the polymerase chain reaction (PCR), it is conceivable that molecular approaches at DNA level will enable us to detect the presence of specific types in the field without requiring any *in vitro* culture.

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