Characterisation of Sunflower Downy Mildew (*Plasmopara halstedii*) Races by RAPD

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Abstract

The molecular variability of *Plasmopara halstedii* isolates from 4 countries was studied by RAPD (Random Amplified Polymorphic DNA). Fifteen primers revealed 107 different bands, and the patterns of these according to isolate were used to construct a dendrogramme. The isolates appeared divided into two groups. The first included the Moroccan isolates and the French isolate of the pathotype "race 1". The second group included the other isolates, from the U.S.A., Spain and French races A and B. The possible origins and interrelations between the races are discussed. Some amplified DNA fragments were specific to particular races, so that after cloning and sequencing, it may be be possible to use them as specific markers of downy mildew races.

Introduction

Plasmopara halstedii, the pathogen causing sunflower downy mildew is endemic in North America (Delanoe, 1971). Resistance to the disease appears to be race specific, corresponding approximately to the gene-for gene system first described by Flor in 1955. Until 1981, only two races were known, race 1 in Europe and race 2 in North America. Vranceanu *et al.* (1981) described a race with different characteristics at the Plant Breeding station at Fundulea in Romania. The same year, Fick and Auwater (1981) isolated a new race in North Dakota, USA, (race 3). Gulya and Urs (1985) identified race 4 in South Dakota, USA, and then Gulya *et al.* (1991) described three new races. The last authors cited races 4 and 6 in France in addition to race 1 whereas Tourvieille *et al.* (1993) isolated two races, A and B whose reactions with differential sunflower lines are not those published for 4 and 6. These apparently contradictory results may be due to different resistance test procedures, in particular definitions of symptoms of resistance and susceptibility (Mouzeyar *et al.*, 1994).

This paper reports studies of the molecular characteristics of *P.halstedii* isolates from 4 different countries to analysis their relationships and to determine whether it is possible to use molecular analyses to identify races.

Material and methods

Fungal isolates : The origins and pathotypes of the downy mildew isolates studied are presented in Table 1. For security reasons, no race was imported live into France. Samples of zoosporangia and

zoospores were provided in 80% ethanol. Pathotype determination was made in the country of origin.

Table 1 : Origins and pathotypes of *P. halstedii* isolates analysed by RAPD.

Name	INRA code	Origin	Correspondant	Pathotype
Fr1	Cl 99	France	Mis Philippon	race 1
Fr2	Cl 35	France	Mr Walser	race A
Fr3	Cl 21	France	Mr Walser	race B
Am1	R2	U.S.A.	Dr Gulya	race 2
Am2	R3	U.S.A.	Dr Gulya	race 3
Am3	R4	U.S.A.	Dr Gulya	race 4
Es1	EsB	Spain	Dr Melero	?
Es2	EsC	Spain	Dr Melero	?
Es3	EsD	Spain	Dr Melero	?
Ma1	Cl 176	Morocco	Dr Achbani	race 1
Ma2	Cl 177	Morocco	Dr Achbani	race 1
Ma3	Cl 178	Morocco	Dr Achbani	race 1

DNA extraction : The method of Rogers and Bendich (1985) was used, modified as follows:

650µl of extraction buffer containing 100mM of HCl-Tris (pH 9), 20mM EDTA (pH 8), 4mM NaCl, 2% CTAB and 0.2% ß-mercaptoethanol were added to 10 mg downy mildew zoosporangia. After grinding, 10µl of proteinase K (20mg/ml) were added to the mixture which was incubated for 30 min. at 55°C. The solid debris were eliminated by centrifugation (20 min. at 13 000g). 600µl phenol/chloroform/isoamylic acid (25/24/1) were added to the supernatant and centrifiged (10 min. at 13 000g). The upper phase containing DNA was treated with neutral chloroform to eliminate possible traces of phenol and then centrifuged again (10 min at 13 000g). 300µl of isopropanol were added to the upper phase to precipitate the DNA. The mixture was incubated 1h at -20°C, then centrifuged 30 min. at 13 000g. The solid phase was washed with 500µl of 70% ethanol followed by centrifugation for 10 min. at 13 000g, drying under vacuum for 5 min. and solubilisation in 50µl buffer (Tris : 10mM+EDTA pH8 : 1mM).

Primers : Fifteen Operon technologies primers were used.

RAPD amplification : The amplification reaction was carried out in 25µl of the following mixture :

Tris HCL : 1mM
MgCl2 : 0.15mM
Gelatine : 0.02%
Primer (Operon) : 0.2µM
DNA : 50ng
KCl : 5mM
Triton X 100 : 0.01%
d NTPS (Pharmacia) : 100µM
Taq polymerase(Appligene) : 0.5UI
UP Sterile H20 to give : 25µl

Analyses of amplification products : Analyses were made on 1.4% agarose plates in TAE buffer (0.04M Tris-acetone : 1mM EDTA, pH8). Migration occurs under an 80volt current for 4h. The DNA fragments were stained by addition of 0.5µg/µl Ethidium bromide. The DNA bands were observed by trans-illumination under Ultra-Violet light.

Analysis of RAPD profiles : Analysis was made of the presence/absence of each of the bands revealed by the 15 primers. *P.halstedii* isolates were grouped according to an ascendant hierarchical classification (soft-ware : NTSYS).

Results

Amplification : The number of DNA fragments amplified by any one primer varied from 3 to 13. Three primers showed no polymorphism. A total of 107 bands were revealed.

Classification of isolates : The dendrogramme is given in Fig. 1. It shows that the isolates form two groups. The first includes the three Moroccan isolates and the one belonging to race 1 in France. These four isolates show a high coefficient of similarity (>95%). The second group is more complex, with the isolates Fr2 (race A) and Am3 (race 4) separated from the others. The isolates Am1 and Am2 (races 2 and 3 respectively) are close together, the three Spanish isolates are all within this group but they can be separated, one isolate being very close to Fr3 (race B).

Discussion

The RAPD technique only requires a few nanogrammes of DNA (Rafalski *et al.*, 1993), so it is quite appropriate in the study of obligate parasites which cannot be cultivated on artificial medium. DNA is extracted from spores which are produced by the fungus outside the plant host.

DNA amplification of 12 isolates from 4 different countries shows considerable similarities in RAPD profiles obtained. These results are rather different from those of Borovkova *et al.* (1992) who found frequent polymorphism within and between downy mildew races, on 22 isolates mainly collected in the U.S.A. and belonging to 6 different races. In the present study, there was at least 88% similarity between the 3 American isolates, each belonging to a different race. This is probably an under-estimation of similarity since only primers likely to show polymorphism were used (Coelho, 1994). It would be interesting to complete the present studies using the same isolates as Bokorova *et al.*

The hierarchical classification indicates close genetical relations between the Moroccan isolates and the French isolate Fr1, which all belong to race 1. These four isolates probably have a common European origin (race 1 is the "European "race). The other isolates appear genetically quite close to each other, with the exception of the American isolate Am3 (race 4). This suggests that the appearance in Europe of the French isolates Fr2 and Fr3 (races A and B) and of all the Spanish isolates, may be due to importation of contaminated seed, a means of transmission described by Anselme and Planque (1972) rather than to mutation of the European race. Evidence for this hypothesis comes from the fact that races A and B, which show virulence patterns similar to those of the American races 3, and 4 appeared in France after races 3 and 4 had been observed in the U.S.A..

Further, when races A and B appeared in France, isolates of race 2, or similar, had not been described.

Some race specific amplified DNA fragments have been cloned and sequenced (SCAR technique described by Paran and Michelmore, 1993). Studies of their specificity for each race are in progress. They may provide a new method of pathotype identificaton which would limit the risk of errors due to difficult observations of reactions of some differential sunflower lines to the downy mildew resistance test.

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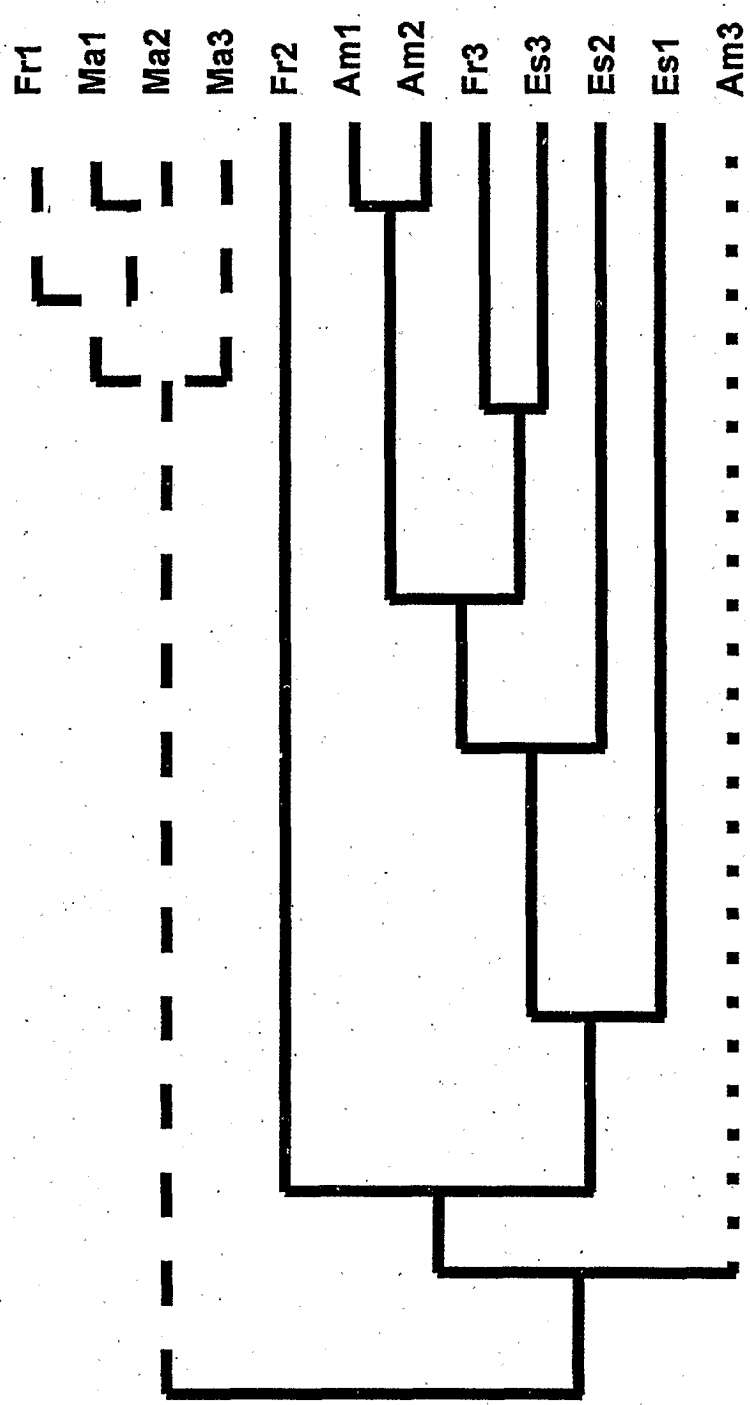

Fig 1 : Dendrogramme of 12 isolates of *Plasmopara halstedii* (107 bands revealed by 15 primers in RAPD)