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Short communication

Development of a pathotype specific SCAR marker in *Plasmodiophora brassicae*

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Abstract

Plasmodiophora brassicae is an obligate biotroph that causes clubroot, one of the most damaging diseases of crucifers. Breeding of clubroot-resistant plants has been hampered by the large variation of pathogenicity in *P. brassicae* and by the lack of an efficient means for detecting specific isolates. To improve the practicality of *P. brassicae* pathotype-identification, a molecular approach was developed. RAPD profiles of 37 single-spore-derived isolates belonging to seven different pathotypes were compared. A RAPD marker, $OPL14_{1200}$, was found in the molecular pattern of all the isolates belonging to one particular pathotype (P1), pathogenic on all differential hosts tested. The DNA band corresponding to this marker was cloned and sequenced. No significant homology to previously characterised nucleotide sequences was found. Primers were designed to specifically amplify the $OPL14_{1200}$ band. The SCAR marker was observed in all isolates belonging to pathotype P1 and was absent in isolates belonging to other pathotypes and in the different plant hosts analysed. The SCAR marker was also generated from direct amplification of DNA from clubs (mixture of host and pathogen DNA) developed after infection by P1 isolates. This molecular marker may be a valuable tool for rapid and reliable identification of *P. brassicae* P1 isolates in areas where resistant varieties are cultivated.

Plasmodiophora brassicae Woron. is a soilborne obligate biotroph, that causes clubroot, one of the most damaging diseases of crucifers in the world. The pathogen causes swelling of the roots and the resultant clubs inhibit nutrient and water transport, stunt the growth of the plant and increase the susceptibility to wilting. Cultural practices or chemical treatments are not efficient or too expensive to keep the crops healthy. Therefore, the development of resistant cultivars is now considered essential to control this disease for all *Brassica* species. The knowledge of the virulence pattern of the pathogen populations in areas where resistant varieties are cultivated is one of the requisites for effective deployment of durable plant resistance. Thus, the large variation of pathogenicity in *P. brassicae* (Crute et al., 1980; Somé et al., 1996; Kuginuki et al., 1999) and the lack of a satisfactory means for detecting specific virulent isolates (specific pathotypes) would not be a limitation for the control of clubroot. Pathogenicity studies using sets of differential hosts are time-consuming, labour intensive and subject to varying environmental conditions. Notably, the interpretation of results is often limited by non-homogeneous reactions owing to the heterogeneity of *P. brassicae* field isolates (Jones et al., 1982a; Crute and Pink, 1989). In fact, field isolates (i.e., *P. brassicae* isolated from a single root gall) are heterogeneous resting spore populations, resulting in mixtures of pathotypes as demonstrated by using single-spore isolates (SSIs) (Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Somé et al., 1996). This implies that the characterisation of a field isolate using a differential

test series is only valid for the inoculum used in the test (Voorrips, 1995). Moreover, pathogenic genotypes might be masked by non-pathogenic ones in a club, and a plant could be noted as resistant only for the major component of the heterogeneous pathogen isolate (Jones et al. 1982b; Somé et al., 1996; Voorrips, 1996). Lastly, isolation and biological characterisation of genetically uniform SSIs is not feasible in the routine identification of the virulence pattern of a large number of collections because of the time required and the variable success rate of the SSI method (Haji Tinggal and Webster, 1981; Jones et al., 1982b; Manzanares et al., 1994; Narisawa et al., 1996; Voorrips, 1996).

Molecular markers specific to isolates or group of isolates (pathotypes) would be a valuable tool to identify and monitor *P. brassicae* populations. Recently, specific PCR primers for *P. brassicae* have been developed as potential markers of genetic variation (Buhariwalla et al., 1995) or for diagnostic and detection of resting spores in soil, water and plant material (Ito et al., 1997; 1999; Faggian et al., 1999), but no pathotype specific sequence was available.

In this study, a putative RAPD marker was found specific to isolates belonging to pathotype P1, pathogenic on all differential hosts tested, and was converted into a sequence characterised amplified region (SCAR). The identification of a reliable PCR band marker allowed direct detection of pathotype P1 isolates from clubs consisting of a mixture of host and pathogen DNA.

Molecular characterisation of *P. brassicae* by RAPD analysis was performed on 37 SSIs from different hosts and geographical origins (Table 1). Pathotype classification of the isolates was performed according to the disease reaction of three differential *B. napus* cultivars as described previously (Table 1) (Somé et al., 1996). The SSIs belonging to 7 different pathotypes were propagated on Chinese cabbage (*Brassica rapa* spp. *campestris* cv. Granaat). Clubs were washed, superficially disinfected and stored at $-20\,^{\circ}$C until required. Genomic DNA of *P. brassicae* was isolated from resting spores. The spores were washed five times in sterile distilled water, dispersed in 100 mM $MgCl_2$, 200 mM Tris pH 7.4 buffer and treated with DNase I (30 μg ml^{-1}) for 3 h at 37 $^{\circ}$C to eliminate contaminating host DNA. The solution was centrifuged at 2500 *g* for 5 min, and the pellet was recovered in 5 mM EDTA, 0.5% SDS, 10 mM Tris pH 7.8 buffer containing 20 μg ml^{-1} of proteinase K for 30 min at 37 $^{\circ}$C. After repeated centrifugation, the final pellet was frozen in liquid nitrogen and lyophilised. DNA was extracted from lyophilised resting spores using a CTAB protocol (Doyle and Doyle, 1990). DNA was also extracted using the same CTAB protocol from young leaves and from roots of uninfected or infected hosts: Chinese cabbage, cauliflower (*B. oleracea*) and rapeseed (*B. napus*). Roots were superficially disinfected, cut into small fragments using a razor blade and ground in liquid nitrogen.

Pathogen and host DNA amplifications were performed with 19 single decamer primers obtained from Operon Technologies Inc. (Alameda, CA). The reaction mix (12.5 μl) contained 0.4 units of Taq DNA polymerase (Eurobio), 1 $\times$ polymerase buffer (10$\times$), 1.9 mM $MgCl_2$, 100 μM dNTPs, 0.2 μM of primer, and approximately 12.5 ng of genomic DNA. Amplifications were carried out in a DNA thermal cycler (Perkin Elmer Cetus) programmed for an initial denaturation cycle (94 $^{\circ}$C for 30 s) followed by 45 cycles of 30 s at 92 $^{\circ}$C, 1 min at 35 $^{\circ}$C, and 2 min 30 s at 72 $^{\circ}$C, with a final extension at 72 $^{\circ}$C for 5 min. RAPD products were separated on a 1.8% agarose gel. Gels were stained with ethidium bromide and viewed with ultraviolet light. All RAPD profiles were fully reproducible in at least two independent assays using two independent DNA extractions. In all cases, host and pathogen fingerprints were clearly distinct.

One out of the 19 arbitrary primers tested, OPL14, generated a polymorphic pattern displaying a 1200-bp DNA fragment specific to isolates belonging to pathotype P1 regardless of host or geographical origin. P1 isolates were virulent and highly aggressive on all three differential hosts tested (Table 1). The marker $OPL14_{1200}$ was absent in all other isolates and in the amplifications with the different host plant DNA (Figure 1).

As RAPD markers have been described to be Taq polymerase and DNA extraction protocol dependent (Pandey et al., 1996), problems might occur in the recovery of these markers in other laboratories. Moreover, another possible disadvantage of the RAPD technique is the extreme sensitivity to contamination by other non-target organisms. This is particularly important when obligate parasites are studied and host DNA may also be present. For this reason, the RAPD fragment was cloned and converted into a SCAR marker to improve the detection method. After electrophoresis of RAPD fragments, the gel was de-stained in distilled water for 1 h to eliminate TAE buffer. The $OPL14_{1200}$ band originating from the eight different isolates belonging to pathotype P1 (Table 1) was

Table 1. Pathotype classification, host and geographical origins of the 37 SSIs of *P. brassicae* used in this study

Isolate No.	Differential host*				Host	Geographical origin (French district)
	Nevin	Wilhelmsburger	Brutor	Pathotype		
Ms6	+	+	+	P1	Rapeseed	Côtes d'Armor
M651	+	+	+	P1		
M727	+	+	+	P1		
M746	+	+	+	P1		
M774	+	+	+	P1		
K92-42	+	+	+	P1	Cauliflower	Finistère
K92-33	−	−	+	P3		
K92-18	−	−	+	P3		
K92-16	−	−	−	P4		
K92-19	−	−	−	P4		
K92-118	−	−	−	P4		
K92-8	+	−	−	P6		
K92-24	−	+	−	P7		
K92-32	−	+	−	P7		
Pb137-436	−	−	−	P4	Cauliflower	Finistère
Pb137-437	−	−	−	P4		
Pb137-514	−	−	−	P4		
Pb137-538	−	−	−	P4		
Pb137-560	−	−	−	P4		
Pb137-524	−	+	+	P5		
Pb137-418	−	+	−	P7		
Pb137-434	−	+	−	P7		
Pb137-439	−	+	−	P7		
Pb137-499	−	+	−	P7		
Pb137-522	−	+	−	P7		
Pb137-532	−	+	−	P7		
Pb137-543	−	+	−	P7		
Pb137-555	−	+	−	P7		
Pb137-566	−	+	−	P7		
Pb137-574	−	+	−	P7		
Pb137-576	−	+	−	P7		
Pb137-483	+	+	−	P8		
SJ92-225	+	+	+	P1	Cauliflower	Ille-et-Vilaine
SJ92-336	+	+	+	P1		
SJ92-256	−	−	−	P4		
SJ92- 233	−	+	+	P5		
SJ92-370	−	+	−	P7		

*Pathotype classification according to the disease reaction of three differential *B. napus* cultivars to the *P. brassicae* isolates (Somé et al., 1996).
+: Indicates a susceptible host reaction.
−: Indicates a resistant host reaction.

excised with a sterile cutter. DNA was recovered from agarose by Sephaglass band preparation (Pharmacia). Approximately 0.1 ng of DNA was subjected to re-amplification with the same protocol as for the RAPD analysis with the exception that 30 cycles were employed instead of 45. A final extension time of 90 min was applied to maximise the formation of poly-A ends (Li and Guy, 1996). The PCR product was cloned using the TA cloning kit (Invitrogen) (vector pCR 2.1). Recombinant white colonies obtained were grown at 37 °C/300 rpm in 300 µl of Terrific Broth buffer (Sambrook et al., 1989) for 8 h and subjected to PCR amplification as described by Barret et al. (1998). After agarose electrophoresis,

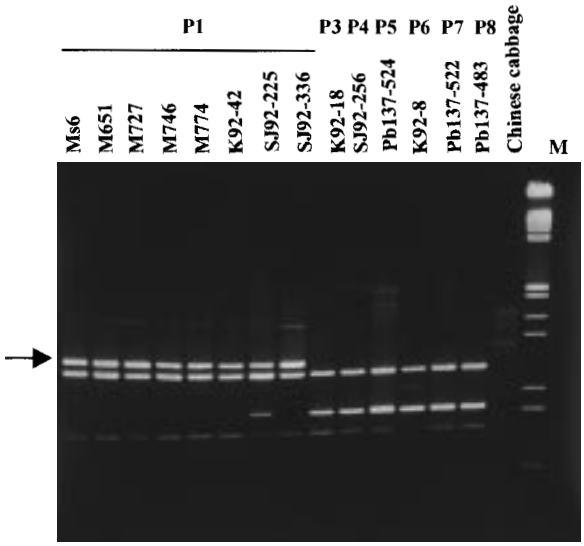

Figure 1. Amplification patterns of different isolates of *P. brassicae* with the random primer OPL14. P: pathotype; M: molecular weight marker (lambda DNA digested by *Eco*RI and *Hin*dIII). The polymorphic fragment specific to isolates belonging to pathotype P1 is indicated by an arrow.

the PCR products were transferred to Hybond N+ membrane (Amersham) and hybridised with the $OPL14_{1200}$ band recovered from the RAPD gel using the direct ECL kit (Amersham). Seven positive clones of the approximately expected size (1200 bp) which cross-hybridised with the purified $OPL14_{1200}$ band were recovered among 30 white colonies tested. The relatively low percentage of colonies containing the fragment of interest could be explained by the use of crude PCR amplification DNA for cloning.

Sequencing of three of these clones was performed by Genome Express (Grenoble, France). Sequence analysis of the $OPL14_{1200}$ fragment revealed that it contained 1214 bp. The presence of the OPL14 RAPD primer at the two extremities was checked (Figure 2). Sequence homologies were analysed with the GCG package (Genetics Computer Group, University of Wisconsin, Madison) using the FASTA subroutine (Pearson and Lipman, 1988). No significant homology was found with previously characterised nucleotide sequences in the EMBL, DDBJ and Genebank databases.

From this sequence, PCR-specific primers were designed using Oligo 4 software. The sequences of the two oligonucleotides selected were: SCL14UP: 5′-GTG ACA GGC TGA CCC GAA GGA TTA-3′; and SCL14LP 5′-GTG ACA GGC TGT GAT CAG TCC-3′. All isolates and hosts were amplified using these primers. The PCR reaction mixture (50 µl) contained 1× Taq polymerase buffer (Gold, Perkin Elmer), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 µM of each primer, 1.25 U of Gold Taq polymerase (Perkin Elmer) and 20 ng of *P. brassicae* or host plant DNA. PCR was performed in Perkin Elmer apparatus programmed for an initial denaturation cycle (94 °C for 10 min) followed by 30 cycles of 94 °C/1 min, 60 °C/1 min, 72 °C/3 min and a final extension step of 72 °C/5 min. PCR products were resolved on 1.8% agarose gel.

The 1214 bp fragment corresponding to the expected size was monomorphic in all isolates belonging to pathotype P1 (Figure 3). Using DNA dilution series, we obtained visible products from 100 pg of template. The DNA from plant hosts and isolates belonging to other pathotypes gave no amplification products with SCL14UP+SCL14LP primers. We have also tested SCL14UP+SCL14LP amplification with Eurobio Taq polymerase and from different batches of DNA extractions and we observed no difference on amplification products.

In order to test whether this SCAR marker could be used to detect the isolates belonging to pathotype P1 directly from clubs, several infected plants of different species were tested (Chinese cabbage, cauliflower and rapeseed). Amplifications with DNA directly extracted from clubs developed after infection with isolates of pathotype P1 resulted in a single band of the expected size. No amplification was observed either from DNA from clubs of plants infected with isolates belonging to other pathotypes or from uninfected roots (Figure 3). The SCAR marker was also detected after successive propagation of the *P. brassicae* P1 isolates through the susceptible host Chinese cabbage (results not shown).

In France, P1 isolates are relatively frequent in the areas with intensive *Brassica oleracea* growing crops (Somé et al., 1996). In previous work, we have showed that P1 isolates were highly aggressive on cole crops (notably cauliflower) and that several *B. oleracea* resistance sources described as possessing a high level of resistance were completely ineffective against P1 isolates (Manzanares-Dauleux et al., 2000). In order to avoid the time-consuming task of testing the pathogen on differential hosts, the SCAR marker obtained here could be useful for following P1 isolates and thus

Figure 2. The OPL14-1214 fragment sequence, with OPL14 RAPD primer position (in bold type) and the positions of the primers used for specific PCR amplification (italics).

for monitoring its dispersal in regions where resistant cultivars will be developed. The greatest important advantage is that amplification could be performed rapidly and directly from clubs without previous purification of resting spores. Future studies should include a larger number of isolates (from different hosts and geographic origins) and markers to validate this SCAR marker and to find other specific markers for different pathotypes. Molecular markers may also be used to gain a better understanding of the genetic variability and the structuring factors within populations of *P. brassicae*.

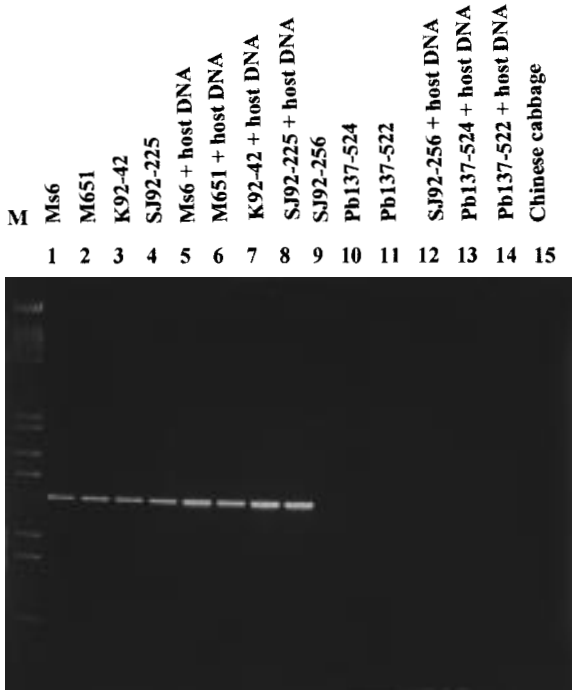

Figure 3. Amplification patterns with PCR-specific primers SCL14UP and SCL14LP of DNA extracted from (i) isolated resting spores of *P. brassicae* (lanes 1–4 pathotype P1, 9–11 pathotypes P4, P5, P7), (ii) infected roots of Chinese cabbage (lanes 5–8 pathotype P1, 12–14 pathotypes P4, P5, P7) and (iii) uninfected roots of Chinese cabbage (lane 15). M: molecular weight marker (lambda DNA digested by *Eco*RI and *Hin*dIII).

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