

Development of Microsatellite Markers for the Characterisation of *Phaeoisariopsis griseola* (Bean Angular Leaf Spot Agent) Populations in Central America

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Abstract

Although several researches revealed an important diversity within *Phaeoisariopsis griseola*, the bean angular leaf spot (ALS) agent, no sexual recombination was already detected for this fungus. That apparent contradiction gave rise to the interest to develop codominant markers in order of a more precise analysis of the pathogen populations. Microsatellites were expected to allow characterising *P. griseola* populations in terms of allele frequencies. A genomic library was constructed by ligating DNA fragments, previously prepared by enzymatic restriction of total DNA of two pathogen strains, into a pZERO plasmid. After transformation of TOP 10F' strains of *E. coli* with the recombinant plasmid, a total of 448 colonies were selected for zeocin resistance. The probe mixture [(GT)₁₅, (GA)₁₅, (GATA)₈, (GTG)₁₀], previously labelled with ³²P, was used to screen the genomic library for the presence of microsatellite sequences. The vector DNA was then extracted from the positive colonies and sequenced. Based on the sequences, a first group of 10 microsatellite loci was identified and the corresponding primers designed. A size analysis using an ALF express system exhibited 3 polymorphic microsatellites among a total of 4 loci already considered. The identification of other polymorphic microsatellites is continuing before a large scale analysis of our pathogen collection by using this new molecular tool developed for *P. griseola*.

Keywords: angular leaf spot; *Phaeoisariopsis griseola*; *Phaseolus vulgaris*; microsatellite

INTRODUCTION

Angular leaf spot (ALS) disease, caused by the fungal pathogen *Phaeoisariopsis griseola* (Sacc.) Ferr. (*Isariopsis griseola* Sacc.) can induce yield losses reaching up to 80% (SCHWARTZ *et al.* 1981). Among the different potential strategies of ALS management, genetic resistance remains the most appropriate one for developing countries. A sustainable use of resistant varieties requires to take into account the diversity within the pathogen populations. Different works revealed that *P. griseola* is highly variable in terms of virulence (ALVAREZ-AYALA & SCHWARTZ 1979), isoenzymatic (BOSHOF *et al.* 1996) and molecular patterns (MAHUKU *et al.* 2002).

The origin of a such high diversity within a species without a known sexual stage remains unclear. In this context, the present work aims at developing microsatellite or simple sequence repeat (SSR) markers (MILBOURNE *et al.* 1998) to generate data which can allow characterising the genetic structure in terms of allele frequencies.

MATERIALS AND METHODS

Two *P. griseola* isolates (BR2 and BR5), collected during 1995 in Brazil, were conserved in a 25% glycerol solution at -80°C. They were firstly grown on a V8 agar medium before a multiplication of mycelium in liquid medium followed by DNA isolation according

to the protocol previously optimised for this pathogen (BUSOGORO *et al.* 1999). *P. griseola* inserts were then prepared by digesting the isolated DNA with the restriction enzyme MboI (Roche). A size-fractionation on a 1% agarose gel was performed to separate the DNA fragments. Restriction fragments ranging from 100 to 800 bp were recovered by a gene clean protocol for each of the two isolates. To constitute the insert preparation, restriction fragments of the two *P. griseola* isolates were mixed.

This preparation was then overnight ligated (14°C) into a pZERO plasmid previously digested with BamHI (Boehringer Mannheim). The ligation mixture was transformed into *Escherichia coli* competent cells TOP10F' (Invitrogen). The transformation mixture was then transferred onto LB-Zeocin plates and incubated overnight at 37°C. Screening of the zeocin resistant colonies was performed according to the general protocol (DOW *et al.* 1995) by using a 4 oligonucleotides mixture probe labelled with γ ^{32}P . The vector DNA of the putative positive clones was purified by using the QIAprep Miniprep System protocol (Westburg QIAGEN). The so purified DNA was sequenced (Eurogentec) to determine the position of microsatellite sequences as well as their surrounding nucleotide sequences. The sequence data was finally analysed in order to design the primers which amplify microsatellite loci. Optimisation of the PCR conditions was then followed by a preliminary size analysis of the amplified fragments with an ALF express system (Pharmacia Biotech).

RESULTS

Plating the transformation mixture gave rise to a series of 448 *E. coli* colonies. This bacterial growth in presence of the zeocin antibiotic confirmed the integration of the recombinant pZERO plasmid into the *E. coli* competent cells. In fact, insertion a DNA fragment within the pZERO multiple cloning site allows growth of only positive recombinants (Invitrogen). The screening procedure of the so obtained library revealed 8 clones showing very strong hybridisation signals while only a low background was observed for all the other clones.

After purification of the vector DNA from the putative positive clones, the presence of inserts within the plasmid DNA was confirmed by using a PCR reaction control as well as by an enzymatic restriction one. Sequence data obtained through the sequencing process allowed identifying different microsatellite regions within the analysed *P. griseola* inserts. In the Table 1,

Table 1. Localisation of a microsatellite sequence within a *P. griseola* insert. The sequenced DNA was purified from one positive clone identified by the hybridisation screening process of the genomic library

Sequence of a <i>P. griseola</i> insert
GATCTTGTGTGTGTGTGTGTGTGTGCTCGGGTGGTC TATGCTAGGCCAGGAGGAGTGATGAATCAAATT GCCCTTCTGCCTCTCCGCATCCGATATCAGTG TTTCTCTATCCTCTAATA

an example of insert sequence containing a microsatellite is shown. In this case, the dinucleotide motif TG, which is the most frequently represented microsatellite motif (BRENIG & BREM 1991) is tandemly repeated 9 times. Based on the conservation of the nucleotide sequences surrounding microsatellites (RONGWEN *et al.* 1995), it was possible to design oligonucleotide primers specific for each microsatellite identified since the sequence data was available. A group of 10 primer pairs was designed and the PCR amplification conditions were optimised to amplify the corresponding microsatellite loci by using the *P. griseola* DNA as target. According to a size analysis already performed on 4 microsatellite loci, 3 microsatellites revealed a polymorphism within the ALS agent.

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