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Whole genome amplification of the rust *Puccinia striiformis* f. sp. *tritici* from single spores

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ABSTRACT

Rust fungi are obligate parasites and cannot be routinely cultured to obtain sufficient biomass for DNA extractions. Multiple displacement amplification (MDA) was demonstrated in this study for whole genome amplification from single spores of the rust fungus, *Puccinia striiformis*. The genomic DNA coverage and fidelity of this method was evaluated by PCR amplification and sequencing of two genetic markers: portions of the multi-copy nuclear ribosomal DNA internal transcribed spacer region (ITS) and the single copy β-tubulin gene from two geographical diverse isolates. Our results show that MDA is a valuable tool for whole genome amplification from single spores, and we propose that MDA-amplified DNA can be used for molecular genetic analysis of the wheat yellow rust fungus.

1. Introduction

Puccinia striiformis f. sp. *tritici*, is a highly specialized obligately biotrophic pathogen (Roose-Amsaleg et al., 2002) that causes yellow rust of wheat. It is one of the most devastating diseases of wheat throughout the world and is considered a major problem in wheat production, particularly in China (Shan et al., 1998). Because of their nature as obligate parasites, rusts cannot be readily cultured on nutrient media, and the strict asexual propagation by uredospores has significantly hampered research from a molecular genetics viewpoint (Roose-Amsaleg et al., 2002). A basic requirement of molecular tools is the availability of suitable quality and sufficient quantity of DNA (Fernández-Ortuno et al., 2007). Although many molecular genetic analyses of fungi have been reported, studies of large populations of rust fungi are limited (Shan et al., 1998). Genomic DNA can be extracted from single uredospores (de Vallavieille-Pope et al., 1990) and subjected to amplification, however there are many disadvantages, such as low reproducibility, possible contamination by other organisms, and somatic hybridization among others.

DNA extraction from single spores requires a mechanical step to break up the spore wall, and then the suspension containing the crushed spores can be used directly for PCR, which has been described in the studies of the large-spored, multinucleate arbuscular mycorrhizal fungi (AMF) (Vanderkoornhuyse and Leyval, 1998; Schwarzott and SchuBler, 2001). The limited DNA content in a single spore may restrict analyses to only a few PCR amplifications. Moreover, PCR amplification often fails because of the variable efficiency of single spore DNA extractions (Gadkar and Rilling, 2005a), and hence this method cannot meet the needs of many research projects (Zhang et al., 1992; Peng et al., 2007).

Another approach is to utilize whole genome amplification (WGA) strategies which can produce abundant quantities of DNA from a limited source, even single spores (Gadkar and Rilling, 2005a). Previously, several PCR-based WGA techniques have been developed, such as primer extension preamplification (PEP) (Zhang et al., 1992), and degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius et al., 1992). Although PCR-based WGA techniques have been successfully applied to whole genome amplification even from single cells, these methods have limitations, such as amplification bias, generation of relatively short DNA fragments and possible introduction of mutations into the amplified products (Peng et al., 2007).

Recently, multiple displacement amplification (MDA) has provided high yield, faithful representation of the original template, and complete coverage of the genome in a relatively simple procedure. MDA has been used for accurate WGA from single cells and spores (Handyside et al., 2004; Hellani et al., 2005; Paez et al., 2004; Raghunathan et al., 2005; Spits et al., 2006; Wang et al., 2004).

To develop methodology that can shed some light on the molecular genetics of wheat stripe rust fungi, we have used MDA to amplify the

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whole genome from single spores. We then tested the genome coverage and fidelity of the MDA process by sequencing of two genetic markers: portions of the multi-copy nuclear ribosomal DNA internal transcribed spacer (rDNA-ITS) region and the single copy β-tubulin gene. Our results show that amplification of genomic DNA from a single spore using MDA is feasible.

2. Materials and method

2.1. Fungal strains

Two isolates of *P. striiformis* f. sp. *tritici*, strain Baihua from the USA and strain Shui5 from Gansu, China, were used in this study. Each isolate was cloned from a single uredospore by a series of mono-uredospore transfers on wheat seedlings (de Vallavieille-Pope et al., 1990).

2.2. Isolation of total DNA

Total DNA (T-DNA) was extracted according to Roose-Amsaleg et al. (2002) with slight modifications. Approximately 1 mg non-germinated uredospores were crushed in a 1.5 ml microcentrifuge tube with 50 μl of extraction buffer [100 mM Tris–HCl, pH 9.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 2% cetyltrimethylammonium bromide (CTAB)] by using plastic pestles (Bio Basic Inc. Markham, Ontario, Canada). Then, 550 μl of extraction buffer was added to each tube, and the solution was incubated for 2 h at 65 °C. Proteins were denatured and removed by repeated extractions with 600 μl Tris saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Phases were separated by centrifugation, the aqueous phase removed and DNA precipitated with 700 μl of isopropanol. DNA was pelleted by alcohol precipitation, dried and resuspended in 40 μl of Tris–EDTA buffer (10 mM Tris–HCl, pH 7.6; 1 mM EDTA). An aliquot of the extracted DNA was separated by electrophoresis on 1% agarose gels for visualization and quantification.

2.3. Isolation of single spores and whole genome amplification from single spores

The single spore extraction method followed Hahn et al. (2000) with slight modifications. A 0.5 mm diameter nickel–chromium vaccination needle (Hexing A&F Science Equipment Factory, Jiangsu, China) was flamed with ethanol. A glass slide was immersed into ethanol, and then air dried. A few spores were picked up with the vaccination needle and applied onto the glass slide, and gently teased apart. A transfer needle was made from a 200 μl microcentrifuge tube (Axygen PCR-02-C, Shanghai BioScience, Shanghai, China), by first cutting the tube along its length. Repeated gentle shaving of one half with a sharp scalpel resulted in needles approximately 3 mm long and 100 μm wide at the tip. These minute shards were picked up at one end with tweezers, and the tip was used to pick up single spores from the glass slide while viewed at 60× under a dissecting microscope (Leica MZ9.5, All Microscopy, Beijing, China). The shard with the single spore was then placed into a PCR tube.

Whole genome amplification of single spores was carried out using the REPLI-g Mini Kit (Gene Company Ltd., Beijing, China) following manufacturer's instructions with slight modifications. A 3.5 μl aliquot of denaturation buffer was added into the microcentrifuge tube containing the target single spore and 3 μl phosphate buffered saline (PBS) included in the kit. This solution was vortex-mixed, centrifuged briefly, incubated for 10 min on ice, and then 3.5 μl of a stop solution was added. To the denatured sample, 40 μl of a blend of REPLI-g Mini DNA polymerase and REPLI-g Mini reaction buffer was added, mixed and incubated at 30 °C for 16 h. After incubation, the enzyme was heat-inactivated at 65 °C. The amplified product was run out on a 1% agarose gel to verify the reaction. The remaining amplified DNA was stored at 4 °C for short-term use or at −20 °C for longer-term periods.

2.4. Primer design and PCR amplification

The β-tubulin primers were designed with the Primer Premier 5.0 (Premier Biosoft International, Palo Alto, California, USA), using β-tubulin gene sequences from GenBank (*Aecidium brachycomes*, EF570791.1; *Puccinia aegopodii*, DQ983205.1; *P. bistortae*, EF570794.1; *P. calthae*, EF570798.1; *P. cnici*, EF570803.1; *P. dioica*, EF570804.1; *P. fergussonii*, DQ983210.1; *P. fergussonii*, EF570806.1; *P. hieracii*, EF570811.1; *P. lagenophorae*, EF635884.1; *P. ludwigii*, EF570816.1; *P. luzulae*, EF570819.1; *P. morrisoni*, EF570841.1; *P. obscura*, EF570818.1; *P. ribesii-caricis*, EF570802.1; *P. rupestris*, DQ983207.1; *P. saccardoi*, EF570835.1; *P. septentrionalis*, EF570838.1; *P. stylidii*, EF570844.1; *P. vaginatae*, EF570846.1; *P. uliginosa*, EF570801.1; *P. xanthii*, EF635885.1; and *Uromyces sommerfeltii*, EF570859.1). To amplify the ITS region, the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) were used. The PCR reactions were set up in a 25 μl volume containing 2 μl of DNA extract, 2 mM $MgCl_2$, 1 U of DNA polymerase, 1 μM of each of the primer pair, 200 μM of dNTP mixture and 2.5 μl of 10× reaction buffer. The cycling conditions were as follows: for rDNA-ITS, 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 7 min; for β-tubulin, 94 °C for 5 min, followed by 20 cycles of 94 °C for 1 min, 65 °C for 1 min (with a decrease of the annealing temperature every cycle by 1 °C) and 72 °C for 1 min, followed 20 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gels to visualize and quantify the reaction products.

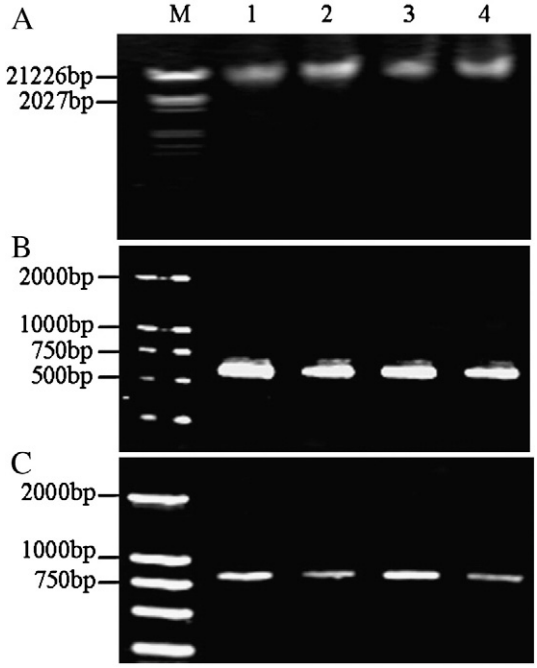

Fig. 1. Whole genome amplification (WGA) of single spores of *Puccinia striiformis* by multiple displacement amplification (MDA) and PCR amplification of genetic markers from single-spore WGA reaction templates. A. Total DNA extractions (2 μl) and WGA reaction products (2 μl) fractionated on a 1% agarose gel. B. Amplification of the ITS regions of nuclear rDNA (ITS) from total DNA and single-spore WGA-DNA. C. Amplification of a fragment of the β-tubulin gene from total DNA and single-spore WGA-DNA. Lane M: molecular size markers: A. λDNA/HindIII+EcoR I Marker; B and C. DL2000 marker. Lanes 1 and 3, total DNA of *P. striiformis* strain Baihua and Shui5 genomic DNA, respectively. Lanes 2 and 4, whole genome amplification products of single spores of *P. striiformis* strains Baihua and Shui5, respectively.