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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.

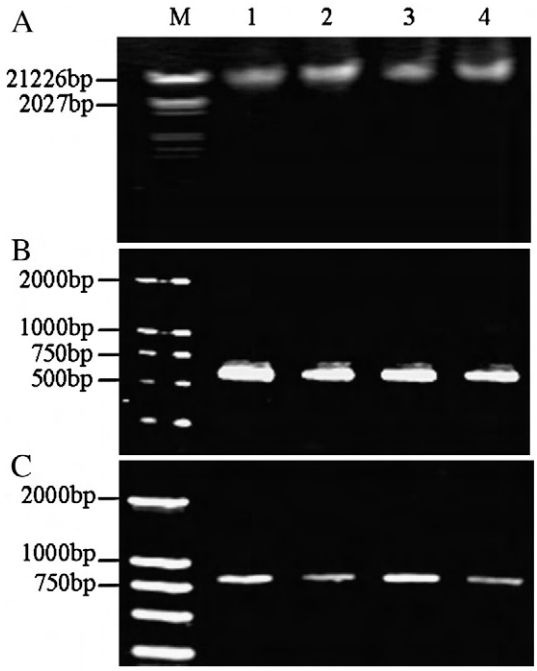

Fig. 1. Whole genome amplification (WGA) of single spores of *Puccinia striiformis* by multiple displacement amplification (MDA) and PCR amplification of genetic markers from single-spore WGA reaction templates. A. Total DNA extractions (2 μl) and WGA reaction products (2 μl) fractionated on a 1% agarose 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.

2.5. Sequence analysis

The PCR products were purified using the HQ&Q Gel Extraction Kit (U-gene Biotechnology, Anhui, China), cloned into the pMD19-T vector (TaKaRa Biotechnology Co. Ltd., Dalian, China) as described by the manufacturer's protocols, and sequenced by dideoxy sequencing method (Sangon, Shanghai, China) in both forward and reverse directions. The sequence data were viewed using Chromas software

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Baihua(T-DNA)       CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG 60
Baihua(S-DNA)       CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG 60
Shui5(S-DNA)        CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG 60
Shui5(T-DNA)        CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG 60
                    ************************************************************

Baihua(T-DNA)       GATCATTATTAAAAGAACTAGAGTGCACTTTATTGTGGCTCGACCCCTTTTAAAAATCTC 120
Baihua(S-DNA)       GATCATTATTAAAAGAACTAGAGTGCACTTTATTGTGGCTCGACCCCTTTTAAAAATCTC 120
Shui5(S-DNA)        GATCATTATTAAAAGAACTAGAGTGCACTTTATTGTGGCTCGACCCCTTTTAAAAATCTC 120
Shui5(T-DNA)        GATCATTATTAAAAGAACTAGAGTGCACTTTATTGTGGCTCGACCCCTTTTAAAAATCTC 120
                    ************************************************************

Baihua(T-DNA)       ACCCAAACTTTTAAGACTTGGTTGCATGATTTGAAAGAATCATTGCAATTGAGTAGACGT 180
Baihua(S-DNA)       ACCCAAACTTTTAAGACTTGGTTGCATGATTTGAAAGAATCATTGCAATTGAGTAGACGT 180
Shui5(S-DNA)        ACCCAAACTTTTAAGACTTGGTTGCATGATTTGAAAGAATCATTGCAATTGAGTAGACGT 180
Shui5(T-DNA)        ACCCAAACTTTTAAGACTTGGTTGCATGATTTGAAAGAATCATTGCAATTGAGTAGACGT 180
                    ************************************************************

Baihua(T-DNA)       AACTTCTTTATTGAATGTTGCATTACCCTCCCTTTTTTTTTTT---ATTAAAAATTACAC 237
Baihua(S-DNA)       AACTTCTTTATTGAATGTTGCATTACCCTCCCTTTTTTTTTTTT--ATTAAAAATTACAC 238
Shui5(S-DNA)        AACTTCTTTATTGAATGTTGCATTACCCTCCCTTTTTTTTTTTT--ATTAAAAATTACAC 238
Shui5(T-DNA)        AACTTCTTTATTGAATGTTGCATTACCCTCCCTTTTTTTTTTTTTTATTAAAAATTACAC 240
                    ****************************************   **************

Baihua(T-DNA)       AAAACACAAGTTTAAATGAATGTAACCAAACCTTTAATTATAAATAACTTTTAACAATGG 297
Baihua(S-DNA)       AAAACACAAGTTTAAATGAATGTAACCAAACCTTTAATTATAAATAACTTTTAACAATGG 298
Shui5(S-DNA)        AAAACACAAGTTTAAATGAATGTAACCAAACCTTTAATTATAAATAACTTTTAACAATGG 298
Shui5(T-DNA)        AAAACACAAGTTTAAATGAATGTAACCAAACCTTTAATTATAAATAACTTTTAACAATGG 300
                    ************************************************************

Baihua(T-DNA)       ATCTCTAGGCTCTCACATCGATGAAGAACACAGTGAAATGTGATAAGTAATGTGAATTGC 357
Baihua(S-DNA)       ATCTCTAGGCTCTCACATCGATGAAGAACACAGTGAAATGTGATAAGTAATGTGAATTGC 358
Shui5(S-DNA)        ATCTCTAGGCTCTCACATCGATGAAGAACACAGTGAAATGTGATAAGTAATGTGAATTGC 358
Shui5(T-DNA)        ATCTCTAGGCTCTCACATCGATGAAGAACACAGTGAAATGTGATAAGTAATGTGAATTGC 360
                    ************************************************************

Baihua(T-DNA)       AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCAAAAGG 417
Baihua(S-DNA)       AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCAAAAGG 418
Shui5(S-DNA)        AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCAAAAGG 418
Shui5(T-DNA)        AGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCAAAAGG 420
                    ************************************************************

Baihua(T-DNA)       CACACCTGTTTGAGTGTCATGAAACCCTCTCATTAAATAATTTTGATTAATTATTTTCAA 477
Baihua(S-DNA)       CACACCTGTTTGAGTGTCATGAAACCCTCTCATTAAATAATTTTGATTAATTATTTTCAA 478
Shui5(S-DNA)        CACACCTGTTTGAGTGTCATGAAACCCTCTCATTAAATAATTTTGATTAATTATTTTCAA 478
Shui5(T-DNA)        CACACCTGTTTGAGTGTCATGAAACCCTCTCATTAAATAATTTTGATTAATTATTTTCAA 480
                    ************************************************************

Baihua(T-DNA)       TGGATGTTGAGTGCTGCTGTAATTAGCTCACTTTAAATATATAAGTCACTTTTCTATAAG 537
Baihua(S-DNA)       TGGATGTTGAGTGCTGCTGTAATTAGCTCACTTTAAATATATAAGTCACTTTTCTATAAG 538
Shui5(S-DNA)        TGGATGTTGAGTGCTGCTGTAATTAGCTCACTTTAAATATATAAGTCACTTTTCTATAAG 538
Shui5(T-DNA)        TGGATGTTGAGTGCTGCTGTAATTAGCTCACTTTAAATATATAAGTCACTTTTCTATAAG 540
                    ************************************************************

Baihua(T-DNA)       TTGGATTGACTTGGTGTAATAATTTTATCATCACATCAAGGATTGTAGCAATACTGCCAT 597
Baihua(S-DNA)       TTGGATTGACTTGGTGTAATAATTTTATCATCACATCAAGGATTGTAGCAATACTGCCAT 598
Shui5(S-DNA)        TTGGATTGACTTGGTGTAATAATTTTATCATCACATCAAGGATTGTAGCAATACTGCCAT 598
Shui5(T-DNA)        TTGGATTGACTTGGTGTAATAATTTTATCATCACATCAAGGATTGTAGCAATACTGCCAT 600
                    ************************************************************

Baihua(T-DNA)       CTTATTTAAGGGAGACTCCTAAAAACCCAATTTTAACCTTAAGACCTCAAATCAGGTGGG 657
Baihua(S-DNA)       CTTATTTAAGGGAGACTCCTAAAAACCCAATTTTAACCTTAAGACCTCAAATCAGGTGGG 658
Shui5(S-DNA)        CTTATTTAAGGGAGACTCCTAAAAACCCAATTTTAACCTTAAGACCTCAAATCAGGTGGG 658
Shui5(T-DNA)        CTTATTTAAGGGAGACTCCTAAAAACCCAATTTTAACCTTAAGACCTCAAATCAGGTGGG 660
                    ************************************************************

Baihua(T-DNA)       ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 695
Baihua(S-DNA)       ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 696
Shui5(S-DNA)        ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 696
Shui5(T-DNA)        ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA 698
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Fig. 2. Alignment of the ITS regions from *Puccinia striiformis* f. sp. *tritici* isolates Baihua and Shui5 amplified by PCR using total DNA (T-DNA) and whole genome amplified DNA from single spores (S-DNA) as templates. The sequences have been deposited in GenBank (EU924747, FJ224375, FJ224376, and FJ224378).

```
Baihua(T-DNA)       CGAATTAGTTGACTCCGTACTGGATGTCGTCCGCAAAGAAGCAGAAGGATGTGACTGTCT 60
Baihua(S-DNA)       CGAATTAGTTGACTCCGTACTGGATGTCGTCCGCAAAGAAGCAGAAGGATGTGACTGTCT 60
Shui5(S-DNA)        CGAATTAGTTGACTCCGTACTGGATGTCGTCCGCAAAGAAGCAGAAGGATGTGACTGTCT 60
Shui5(T-DNA)        CGAATTAGTTGACTCCGTACTGGATGTCGTCCGCAAAGAAGCAGAAGGATGTGACTGTCT 60
                    ************************************************************

Baihua(T-DNA)       TCAAGGATTTCAGATCACCCACTCCCTCGGTGGTGGAACTGGTGCCGGAATGGGTACCTT 120
Baihua(S-DNA)       TCAAGGATTTCAGATCACCCACTCCCTCGGTGGTGGAACTGGTGCCGGAATGGGTACCTT 120
Shui5(S-DNA)        TCAAGGATTTCAGATCACCCACTCCCTCGGTGGTGGAACTGGTGCCGGAATGGGTACCTT 120
Shui5(T-DNA)        TCAAGGATTTCAGATCACCCACTCCCTCGGTGGTGGAACTGGTGCCGGAATGGGTACCTT 120
                    ************************************************************

Baihua(T-DNA)       GTTGATTTCCAAGATCCGAGAAGAATTTCCTGATCGTATGATGGCCACCTTCTCCGTTGT 180
Baihua(S-DNA)       GTTGATTTCCAAGATCCGAGAAGAATTTCCTGATCGTATGATGGCCACCTTCTCCGTTGT 180
Shui5(S-DNA)        GTTGATTTCCAAGATCCGAGAAGAATTTCCTGATCGTATGATGGCCACCTTCTCCGTTGT 180
Shui5(T-DNA)        GTTGATTTCCAAGATCCGAGAAGAATTTCCTGATCGTATGATGGCCACCTTCTCCGTTGT 180
                    ************************************************************

Baihua(T-DNA)       CCCCTCGCCTAAGGTGTCTGATACCGTTGTCGAGCCTTACAACGCCACCTTGTCGGTTCA 240
Baihua(S-DNA)       CCCCTCGCCTAAGGTGTCTGATACCGTTGTCGAGCCTTACAACGCCACCTTGTCGGTTCA 240
Shui5(S-DNA)        CCCCTCGCCTAAGGTGTCTGATACCGTTGTCGAGCCTTACAACGCCACCTTGTCGGTTCA 240
Shui5(T-DNA)        CCCCTCGCCTAAGGTGTCTGATACCGTTGTCGAGCCTTACAACGCCACCTTGTCGGTTCA 240
                    ************************************************************

Baihua(T-DNA)       TCAACTGGTCGAAAACTCGGACGAAACCTTTTGTATCGATAACGAGGCGCTTTACGATAT 300
Baihua(S-DNA)       TCAACTGGTCGAAAACTCGGACGAAACCTTTTGTATCGATAACGAGGCGCTTTACGATAT 300
Shui5(S-DNA)        TCAACTGGTCGAAAACTCGGACGAAACCTTTTGTATCGATAACGAGGCGCTTTACGATAT 300
Shui5(T-DNA)        TCAACTGGTCGAAAACTCGGACGAAACCTTTTGTATCGATAACGAGGCGCTTTACGATAT 300
                    ************************************************************

Baihua(T-DNA)       CTGCTTCCGCACCCTGAAATTGGCTACACCTACCTACGGTGACCTCAATCACTTGGTCTC 360
Baihua(S-DNA)       CTGCTTCCGCACCCTGAAATTGGCTACACCTACCTACGGTGACCTCAATCACTTGGTCTC 360
Shui5(S-DNA)        CTGCTTCCGCACCCTGAAATTGGCTACACCTACCTACGGTGACCTCAATCACTTGGTCTC 360
Shui5(T-DNA)        CTGCTTCCGCACCCTGAAATTGGCTACACCTACCTACGGTGACCTCAATCACTTGGTCTC 360
                    ************************************************************

Baihua(T-DNA)       GATCGTCATGAGTGGGATCACCACCTGTCTTCGATTCCCCGGTCAGCTTAACTCTGATCT 420
Baihua(S-DNA)       GATCGTCATGAGTGGGATCACCACCTGTCTTCGATTCCCCGGTCAGCTTAACTCTGATCT 420
Shui5(S-DNA)        GATCGTCATGAGTGGGATCACCACCTGTCTTCGATTCCCCGGTCAGCTTAACTCTGATCT 420
Shui5(T-DNA)        GATCGTCATGAGTGGGATCACCACCTGTCTTCGATTCCCCGGTCAGCTTAACTCTGATCT 420
                    ************************************************************

Baihua(T-DNA)       CCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATT 400
Baihua(S-DNA)       CCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATT 480
Shui5(S-DNA)        CCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATT 480
Shui5(T-DNA)        CCGTAAACTAGCTGTCAACATGGTTCCTTTCCCTCGATTGCACTTCTTCATGGTCGGATT 480
                    ************************************************************

Baihua(T-DNA)       CGCTCCGCTTACCGCTCGTGGAAGCCAACAATACCGGGCAATCACCGTCCCAGAGTTGAC 540
Baihua(S-DNA)       CGCTCCGCTTACCGCTCGTGGAAGCCAACAATACCGGGCAATCACCGTCCCAGAGTTGAC 540
Shui5(S-DNA)        CGCTCCGCTTACCGCTCGTGGAAGCCAACAATACCGGGCAATCACCGTCCCAGAGTTGAC 540
Shui5(T-DNA)        CGCTCCGCTTACCGCTCGTGGAAGCCAACAATACCGGGCAATCACCGTCCCAGAGTTGAC 540
                    ************************************************************

Baihua(T-DNA)       ATCGCAAATGTTTGATGCCAAGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATA 600
Baihua(S-DNA)       ATCGCAAATGTTTGATGCCAAGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATA 600
Shui5(S-DNA)        ATCGCAAATGTTTGATGCCAAGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATA 600
Shui5(T DNA)        ATCGCAAATGTTTGATGCCAAGAACATGATGGCCGCTTCTGACCCGAGACACGGCCGATA 600
                    ************************************************************

Baihua(T-DNA)       CTTGACCGTTGCCGCTTACTTCCGTGGAAAGGTTTCCATGAAAGAAGTCGAAGAGAACAT 660
Baihua(S-DNA)       CTTGACCGTTGCCGCTTACTTCCGTGGAAAGGTTTCCATGAAAGAAGTCGAAGAGAACAT 660
Shui5(S-DNA)        CTTGACCGTTGCCGCTTACTTCCGTGGAAAGGTTTCCATGAAAGAAGTCGAAGAGAACAT 660
Shui5(T-DNA)        CTTGACCGTTGCCGCTTACTTCCGTGGAAAGGTTTCCATGAAAGAAGTCGAAGAGAACAT 660
                    ************************************************************

Baihua(T-DNA)       GCTGTCCGTTCAAAGCAAGAACTCCAACTACTTTGTTGAGTGGAGTAAGTCCGGCTTTCG 720
Baihua(S-DNA)       GCTGTCCGTTCAAAGCAAGAACTCCAACTACTTTGTTGAGTGGAGTAAGTCCGGCTTTCG 720
Shui5(S-DNA)        GCTGTCCGTTCAAAGCAAGAACTCCAACTACTTTGTTGAGTGGAGTAAGTCCGGCTTTCG 720
Shui5(T-DNA)        GCTGTCCGTTCAAAGCAAGAACTCCAACTACTTTGTTGAGTGGAGTAAGTCCGGCTTTCG 720
                    ************************************************************

Baihua(T-DNA)       TCTGTTTCTCTCATGTCGGCTTATGATTATGATACTCATATCTGTGAACACTTGGTATAA 780
Baihua(S-DNA)       TCTGTTTCTCTCATGTCGGCTTATGATTATGATACTCATATCTGTGAACACTTGGTATAA 700
Shui5(S-DNA)        TCTGTTTCTCTCATGTCGGCTTATGATTATGATACTCATATCTGTGAACACTTGGTATAA 780
Shui5(T-DNA)        TCTGTTTCTCTCATGTCGGCTTATGATTATGATACTCATATCTGTGAACACTTGGTATAA 780
                    ************************************************************

Baihua(T-DNA)       CCTCAGTTCCGAACAACGTC 800
Baihua(S-DNA)       CCTCAGTTCCGAACAACGTC 800
Shui5(S-DNA)        CCTCAGTTCCGAACAACGTC 800
Shui5(T-DNA)        CCTCAGTTCCGAACAACGTC 800
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Fig. 3. Alignment of the β-tubulin gene from *Puccinia striiformis* f. sp. *tritici* isolates Baihua and Shui5 amplified by PCR using total DNA (T-DNA) or whole genome amplified DNA from single spores (S-DNA) as templates. The sequences have been deposited in GenBank (FJ612008, FJ612006, FJ612007, and FJ612005).

version 2.3 (Technelysium, Queensland, Australia), and manually adjusted in a text editor, and subjected to BLASTN (Altschul et al., 1990) analysis against the GenBank NR database.

3. Results

3.1. Whole genome amplification from single spores

The WGA reactions were set up using the REPLI-g mini kit and single-spore denatured solution. An aliquot (2 μl) of the reaction was run on an agarose gel and the amplification products were visualized. The single-spore WGA-DNA (S-DNA) was of similar size to that of extracted genomic DNA by conventional methods (over 20 kb) (Fig. 1A).

3.2. Primer design for β-tubulin and PCR analysis of specific genes

The β-tubulin gene primers were designed based on existing sequences for *P. striiformis* in GenBank, and the primer sequences were as follows: TURustF: 5′-GTCAYTAYACYGARGGWGC-3′, TURustR: 5′-TGTCACARTGDGCRRTTTG-3′. The primers were synthesized by the Generay Biotech Co., Ltd. (Shanghai, China).

The MDA generated samples were analyzed for fidelity to source by PCR amplification of two DNA markers, nuclear rDNA-ITS (~700 bp, Fig. 1B), and a part of the β-tubulin gene (~800 bp, Fig. 1C). As a positive control, these genes were also amplified from the genomic DNA extracts from multiple spores, allowing direct comparison of the efficiency of the single spore method. The size of bands and their intensity were similar for both the multi-spore and single-spore DNA extractions and amplifications. Over 100 separate DNA extractions from single spores using conventional methods were made to amplify these sequences, but no amplified DNA was observed.

3.3. Sequence analysis of ITS and β-tubulin gene fragments

To check the sequence fidelity of the method of amplification from whole genome DNA extractions of single spores of *P. striiformis* f. sp. *tritici*, the DNA fragments were sequenced in forward and reverse directions with the primers M13+ and M13− situated in pMD19-T. Fig. 2 shows the alignments of the 700-bp ITS region from isolates Baihua and Shui5 amplified from total DNA (T-DNA) and whole genome DNA from single spores (S-DNA). The sequences from Baihua (T-DNA), Shui5 (T-DNA), Baihua (S-DNA), and Shui5 (S-DNA) have been deposited in GenBank (EU924747, FJ224375, FJ224376, and FJ224378). The sequence of ITS fragments amplified from T-DNA showed only a single mismatch with the sequence from S-DNA of Baihua. For Shui5, there were two mismatches between T-DNA and S-DNA. The mismatches were all found in a poly T (>10) region.

A similar analysis was carried out for an 800-bp portion of the β-tubulin gene. The different sequence runs yielded the same results. Fig. 3 shows the alignments of the β-tubulin sequence from isolates Baihua and Shui5 amplified from T-DNA and S-DNA. The sequences of Baihua (T-DNA), Shui5 (T-DNA), Baihua (S-DNA), and Shui5 (S-DNA) have been deposited in GenBank (FJ612008, FJ612006, FJ612007, and FJ612005). The sequences of β-tubulin fragments amplified from T-DNA and S-DNA were identical.

4. Discussion

There are several reasons for using DNA extracted from single spores for genetic analysis. First, it is difficult to produce a large number of spores because of time and expense. Secondly, it is difficult to ensure that spores are all genetically uniform even if attempts were made to collect from a single uredinium. Thirdly, using a single spore for DNA extraction allows for a reduced possibility of contamination by other organisms, such as cross-contamination in open pot cultures by other stripe rust isolates and potential somatic hybridization in the asexual reproduction process. Application of an efficient whole genome amplification method can overcome these inherent shortcomings when dealing with a non-culturable obligately parasitic organism.

The extraction of DNA from single spores of *P. striiformis* by the multiple displacement amplification (MDA) reaction is a suitable and reliable method to obtain DNA for genetic analysis. MDA relies upon the high fidelity and strand displacement ability of DNA polymerase of the bacteriophage phi29 (Esteban et al., 1993) and the exonuclease resistant random primers (Dean et al., 2001) to amplify DNA fragments of high molecular weight (>10 kb) with low error rates ($<10^{-6}$). After only a few hours, it can provide consistent DNA amplification even with very low amounts of starting template (Peng et al., 2007). The sensitivity is so high, that great care must be taken in picking up the desired single spore and throughout the extraction process, since any minute amounts of contaminating DNA would also be amplified.

The nuclear rDNA-ITS region is commonly used for the identification of many fungal species (Roose-Amsaleg et al., 2002), and is the most frequently sequenced region among fungi. This region is situated between the 18S and 28S ribosomal genes, which are found in multiple copy number in eukaryotes (Weider et al., 2005). In addition to a multi-copy gene, we selected the β-tubulin gene because it also has been used frequently in fungal systematics (van der Merwe et al., 2007), and has single or low-copy number (Ayliffe et al., 2001). Only a single β-tubulin gene has been found among most fungi studied, but two β-tubulin genes have been found in the genomes of several ascomycota and the rust, *Melampsora lini* (Ayliffe et al., 2001). However, Wirsel et al. (2004) did not find evidence of a second copy in the rust, *Uromyces fabae*. We assume that the β-tubulin is a single-copy gene in *P. striiformis*, but more research is needed to confirm this.

For rDNA-ITS, up to two mismatches were found between T-DNA and S-DNA of the two isolates investigated. These differences may be real, since polymorphism has been observed in the multiple copies of rDNA sequences of rust fungi (Roose-Amsaleg et al., 2002). A single uredospore is a dikaryon composed of two haploid nuclei, and polymorphisms may exist between these two nuclei. Or these differences may have arisen during the PCR amplification and sequencing process. For the β-tubulin gene, the sequences of T-DNA and S-DNA were identical in multiple sequencing attempts, although the primer regions did show variability because degenerate primers were used.

Successful amplification of the multi-copy ITS region and the single copy β-tubulin gene in *P. striiformis* confirmed that the nuclear DNA was copied during the MDA reaction using single spores, while attempts at amplification of DNA from conventional single spore extractions never yielded any products. The band intensities of the ITS or β-tubulin amplicons using MDA of single spores were similar to those obtained using genomic DNA from multiple spores. Furthermore the gene sequences were almost identical confirming the genome coverage of the MDA technique. The MDA-synthesized template has been used for a diverse set of downstream applications such as allele amplification, single nucleotide polymorphism (SNP) analysis (Dean et al., 2002), restriction fragment length polymorphisms (RFLP) analysis (Gadkar and Rilling, 2005b), polymorphism discovery, single sperm typing, chromosome translocation, and gene mutation analysis (Jiang et al., 2005; Luthra and Medeiros, 2004; Murthy et al., 2005). Future research in these fields on *P. striiformis* f. sp. *tritici* will be facilitated through the use of the MDA method, since it reliably amplified genomic DNA from a single fungal spore. The use of MDA-DNA makes it possible to perform large scale genetic analyses of single spores of the wheat yellow rust pathogen, and possibly of other rust pathogens and other obligately parasitic fungi.

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