FEMS Microbiology Letters 242 (2005) 65–71

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Application of Phi29 DNA polymerase mediated whole genome amplification on single spores of arbuscular mycorrhizal (AM) fungi

Vijay Gadkar *, Matthias C. Rillig

Microbial Ecology Program, Division of Biological Sciences, 32 Campus Drive # 4824, University of Montana, Missoula, MT 59812, USA

Received 20 August 2004; received in revised form 11 October 2004; accepted 21 October 2004

First published online 4 November 2004

Edited by S. Rimsky

Abstract

Genetic analysis of arbuscular mycorrhizal (AM) fungi relies on analysis of single spores. The low DNA content makes it difficult to perform large scale molecular analysis. We present the application of Phi29 DNA polymerase mediated strand displacement amplification (SDA) to genomic DNA extracted from single spores of *Glomus* and *Gigaspora* species to address this problem. The genome coverage of the SDA process was evaluated by PCR amplification of the β-tubulin1 gene and part of the rDNA cluster present in AM fungi. The fidelity of SDA was evaluated further by sequencing the *Glomus intraradices* ITS1 variants to detect the four ITS1 variants previously identified for this fungus.

Keywords: AM fungi; Phi29 DNA polymerase; Strand displacement amplification; Whole genome amplification

1. Introduction

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are thought to be the oldest group of asexual multicellular organisms, and circumstantial evidence indicates that AM-like fungi played a pivotal role in the establishment of the terrestrial flora [1]. These fungi are widely distributed, and have many roles ranging from effects on host plant physiology influence on plant communities [2] and ecosystem processes [3].

AM fungi are coenocytic, where many nuclei coexist in a common cytoplasm. Studies have revealed that these nuclei differ genetically, with various alleles of a single gene being present in the nuclear population [4]. Not surprisingly, a remarkably high amount of genetic variability exists in an AMF species and within individual spores [5–7]. Studies have tried to elucidate the mechanism by which the various alleles of a particular gene are distributed into daughter progeny spores. If this transmission is faithful, all the alleles would be present in the new daughter spore (homokaryosis model); if not, the progeny spores would receive an unequal complement making them genetically variable from its progenitor spore (heterokaryosis model). These two models of clonal propagation are subject to much debate and studies supporting both of them have been published [4,6].

In order to address the basic functioning of genetic transmission in AM fungi per se, PCR based molecular techniques have been extensively used to detect genetic

* Corresponding author. Tel.: +1 406 243 2393; fax: +1 406 243 4184.

E-mail address: vijay.gadkar@mso.umt.edu (V. Gadkar).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.10.041

differences from single individual spores. The low DNA content in a single spore, e.g. 5–19 pg in a *Glomus* sp. [8], imposes severe limitations to perform multiple genetic analyses on genomic DNA extracted from a single sample. Powerful techniques capable of detecting genome wide polymorphism like amplified length fragment polymorphism (AFLP) [9,10] have to incorporate additional PCR amplification steps, resulting in the generation of a large number of false positives. Moreover, the delicate methodology of single spore DNA extractions is invariably inefficient, many times leading to failed PCR amplifications.

In this work, we have sought to address the fundamental bottleneck of the low amount of starting template by using strand displacement amplification (SDA) [11] in order to preamplify the DNA template prior to PCR analysis. SDA is a new technique of whole genome amplification (WGA) [12] which does not suffer from drawbacks of the traditional PCR based WGA. We have used the SDA method on genomic DNA extracted from single spores of four different species of *Glomus* and one species of *Gigaspora*. The amplified DNA was then used as template for PCR amplification of genes present in the AM fungal genome to assess the representativeness of the SDA amplification.

2. Materials and methods

2.1. Fungal material

The AM fungi *Glomus intraradices* Schenck & Smith (DAOM 181602), *G. proliferum* Dalpé & Declerck (MUCL 41827), *G. lamellosum* Dalpé, Koske & Tews (MUCL 43195), *G. cerebriforme* McGee (MUCL 43208) and *Gigaspora rosea* Nicolson and Schenck (DAOM 194757) were cultivated in association with Ri T-DNA derived hairy roots of carrot using a procedure as described earlier [13,14]. Spores were harvested from split plates by dissolving the gelling agent (Phytagel) using 10 mM citrate buffer, pH 6.0, as described earlier [15].

2.2. Extraction of genomic DNA

Individual single spores of four *Glomus* and one *Gigaspora* species were picked under a stereo zoom microscope and carefully dispensed into 0.5 mL Eppendorf tubes pre-dispensed with 2.5 μL of KOH (0.25N). Each individual spore was ruptured using a sterile micropipette tip under a stereo zoom microscope, the contents vortexed, spun briefly to settle the content and boiled at 90 °C for 10 min. To this solution, 2.5 μL of neutralizing solution (0.5 M Tris–HCl, pH 8.0 and 0.25 N HCl) was added, and the contents were boiled for another 10 min for 90 °C. The DNA extracted via this method was used immediately for the SDA reaction or stored at −20 °C prior to use. To test the efficiency of the SDA amplifications, four separate DNA extractions and SDA amplification in addition were set up for single spores of *G. intraradices*.

2.3. Strand displacement amplification

The SDA was carried out using the GenomiPhi™ DNA amplification kit (GE Healthcare Bioscience, Piscataway, NJ). The amplification reaction was set up as per the manufacturer's protocol using 1 μL of the genomic DNA supernatant extracted from a single spore as described above and 9 μL of sample buffer containing random hexamers. After an initial denaturation at 95 °C for 3 min, the sample was snap chilled on ice for a few minutes. To this chilled sample, a pre-optimized blend (10 μL) of Phi29 DNA polymerase enzyme, buffer and nucleotides was added, mixed carefully and incubated for 30 °C for 16 h. The Phi29 DNA polymerase enzyme was inactivated by heating the reaction mixture at 65 °C for 10 min. An aliquot (1.5 μL) of the amplified sample was run on 1.5% agarose gel to verify the amplification. The remaining sample was precipitated using sodium acetate/EDTA buffer protocol as recommended by the manufacturer. The DNA pellet was air dried, suspended in 20 μL of TE, and quantified spectrophotometrically at 260/280 nm and stored at −20 °C prior to further analysis.

2.4. Analysis of SDA generated amplicons

2.4.1. PCR amplification of β-tubulin 1 gene, the ITS and 18S rDNA cluster.

The SDA reaction products were subjected to specific amplification of genes using regular PCR. The ribosomal DNA cluster present in all AM fungi, namely internal transcribed spacer (ITS) and 18S rDNA were amplified. The ITS region including the 5.8S rDNA cluster was amplified using the ITS 1/4 primer pair with PCR cycling conditions as described earlier [16]. The 18S rDNA cluster was amplified using the EF 3/4 primer pair using the cycling conditions as described by Smit et al. [17]. In addition a primer pair amplifying a 301 bp fragment was designed for the *G. intraradices* β-tubulin1 gene using sequence previously deposited in GenBank (Accession No. BE603903). The sequences of the forward and reverse primers are as follows: Giβ-tub1-F2: TGTTACCGACAAAAGTGCCA and Giβ-tub1-R2: GCTCCATTAACTTCTCGCAG. All primers described above were custom synthesized from Sigma-Genosys (Sigma-Genosys, Woodlands, TX).

The PCR was set up using the 2.5× HotMasterMix™ (Eppendorf, Westbury, NY), which contains HotMaster Taq DNA polymerase, buffer, dNTP's and a proprietary chelator, which adjusts magnesium concentration during the reaction. The reaction volume was typically 20