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

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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

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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

μL and the final concentration of the components was HotMaster Taq buffer (pH 8.5), 45 mM KCl, 2.5 mM $Mg(OAc)_2$, 200 μM dNTP, 1 U *Taq* DNA polymerase, appropriate concentration of PCR primers (1 μM for the Giβ-tubulin1 primers) and 2 μL of DNA appropriately diluted. The PCR cycling was carried out on a Mastercycler™ thermal cycler (Brinkmann Instruments, NY). The cycling condition for the β-tubulin1 gene was as follows: 95 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 1.0 min and 72 °C for 1.0 min and a final extension of 72 °C for 7 min.

The SDA reactions were diluted 1/50 times in TE buffer prior to PCR amplification. The genomic DNA extracted from each individual AM fungal spore, which was used for the SDA reaction served as positive controls. Dilution of the genomic DNA template to the same level as the SDA template (1/50), to compare the amplification efficiency, was not successful as the template became too dilute for PCR amplifications. Empirically, the maximum dilution for the genomic DNA template was found to be 1/20. The PCR amplicons were fractionated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

2.4.2. Sequence analysis of G. intraradices *ITS1 region*

The ITS spacer region from *G. intraradices* amplified as described above was cloned into the pGEM-T T/A plasmid vector (Promega, Madison, WI) and transformed in JM109 *Escherichia coli* bacterial cells as per the manufacturer's instructions. Ten white colonies were randomly isolated, and presence of insert checked using the ITS1/4 primers and PCR cycling conditions as described earlier [16]. The plasmids were purified using the Qiagen mini prep kit (Qiagen, Valencia, CA) and submitted for cycle sequencing using the ABI Prism Big Dye™ chemistry (Applied Biosystems, Foster City, CA) at the Murdock lab core sequencing facility (University of Montana, Missoula). The sequencing data were edited using the Chromas™ software version 1.45 (Griffith University, Queensland, Australia) and aligned with the four ITS1 sequence variants previously deposited in GenBank (Accession Nos. AY394030, AY394031, AY394032, and AY394033) using the Clustal W sequence alignment programme [18].

3. Results

3.1. Strand displacement amplification

The SDA reactions were setup using the Phi29 DNA polymerase and random hexamers on genomic DNA extracted from single spores of AM fungi, belonging to the genera *Glomus* and *Gigaspora*. The genomic DNA for the amplification was used directly for the SDA reaction without any additional purification step. An aliquot (1.5 μL) of the reaction was run on 1.5% agarose gel and the amplification products visualized. The amplified product was found to be ca. 10 kb in size (Fig. 1) for all the AM fungal templates used for the reaction. The four additional genomic DNA extractions, performed on single spores of the AM fungus *G. intraradices* also produced a single amplicon of ca. 10 kb (Fig 1). The amount of amplified SDA product ranged from 3.8 to 5.4 μg per reaction for the *Glomus* species and 5.8 μg for *G. rosea*.

3.2. PCR amplification of specific genes

The SDA generated templates were analyzed for representativeness by PCR amplification of the β-tubulin1 gene and the ITS and 18S rDNA fragment. As a positive control, these genes were also amplified from the same genomic DNA templates used for setting up the SDA reactions, allowing us to directly compare the efficiency of the SDA reactions. The *G. intraradices* specific β-tubulin1 primers amplified a 301 bp fragment both from the genomic DNA and SDA reaction templates (Fig. 2). The band intensity of the amplified products was consistently low when genomic DNA was used as a template, as compared to the amplicons from the SDA amplified templates (Fig. 2). Due to the highly conserved nature of the β-tubulin1 gene, a 301 bp fragment from the SDA reactions was generated from the other three *Glomus* species as well.

The multicopy ITS and 18S rDNA sequence were amplified from both the genomic and SDA DNA templates. The size of the ITS fragments was ca. 600 bp for the four *Glomus* species and ca. 550 bp for the one *G. rosea* isolate (Fig. 3(a)). Also, the Ef3/4 primer pair amplified a 1.65 kb fragment from all the AM fungal species used in our study (Fig. 3(b)). In both the cases, the PCR products amplified from the genomic DNA had a lower intensity, when compared to the amplicons amplified form the SDA generated template.

3.3. Sequence analysis of ITS1 fragment

The 10 ITS1 sequences (deposited in GenBank with the following Accession Nos. AY728199 to AY728208) amplified from the *G. intraradices* SDA reactions were aligned with the four ITS1 sequence variants previously deposited in the GenBank (Accession Nos. AY394030, AY394031, AY394032, and AY394033). Out of these four possible ITS1 variants, No. 1 (AY394030), 3 (AY394031) and 4 (AY394033) variants were detected (Fig. 4). The distribution of the 10 sequences, according to the clone designation number, into each variant type was as follows: variant 1: Gi_ITS11,9; variant 3: Gi_ITS6,7,8 and variant 4: Gi_ITS3,4,5,10,11,12.

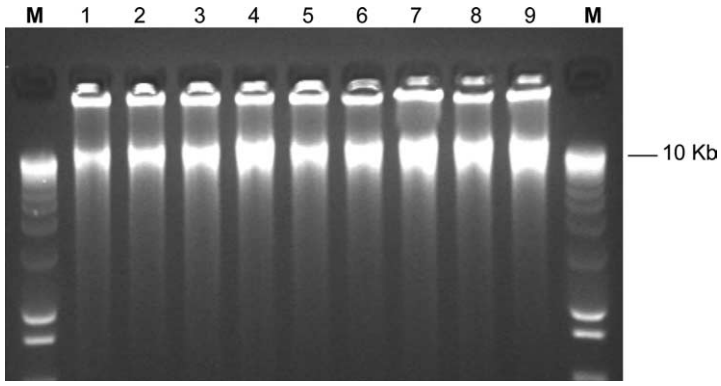

Fig. 1. SDA reaction products fractionated on 1.5% agarose gel. Lanes 1–5: *G. intraradices*, lane 6: *G. proliferum*, lane 7: *G. cerebriforme*, lane 8: *G. lamellosum*, lane 9: *G. rosea*. M: Hyperladder marker I (Bioline, Boston, MA).

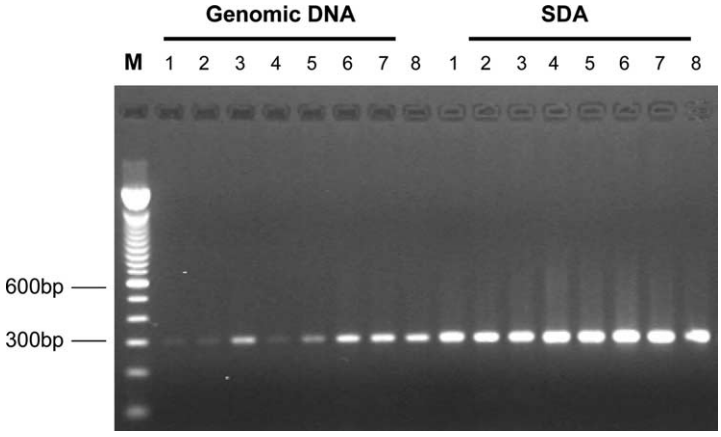

Fig. 2. Amplification of 301 bp β-tubulin1 gene fragment from the genomic DNA and SDA reaction templates from four *Glomus* species. The dilution factor for genomic DNA and SDA templates was 1/20 and 1/50, respectively. Lanes 1–5: *G. intraradices*, lane 6: *G. proliferum*, lane 7: *G. cerebriforme*, lane 8: *G. lamellosum*, lane 9: *G. rosea*. M: 100 bp ladder (Invitrogen, Carlsbad, CA).

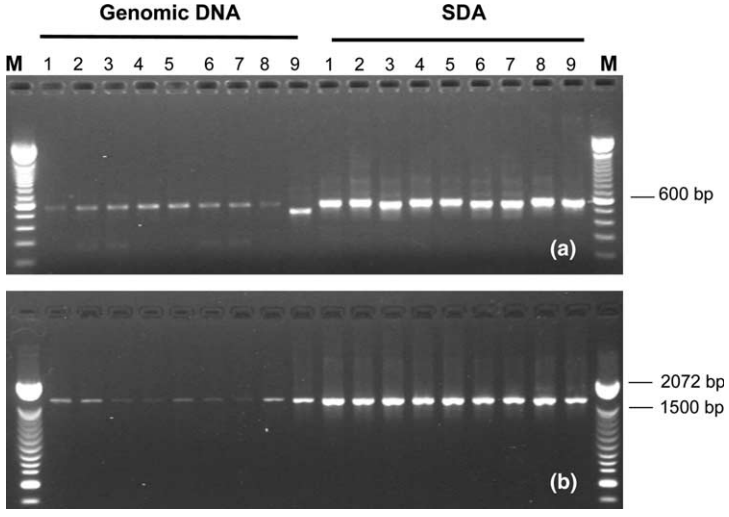

Fig. 3. Amplification of ITS spacer (a) and 18S rDNA (b) from the genomic DNA and SDA reaction templates. The dilution factor for genomic DNA and SDA templates was 1/20 and 1/50, respectively. Lanes 1–5: *G. intraradices*, lane 6: *G. proliferum*, lane 7: *G. cerebriforme*, lane 8: *G. lamellosum*, lane 9: *G. rosea*. M: 100 bp ladder (Invitrogen, Carlsbad, CA).

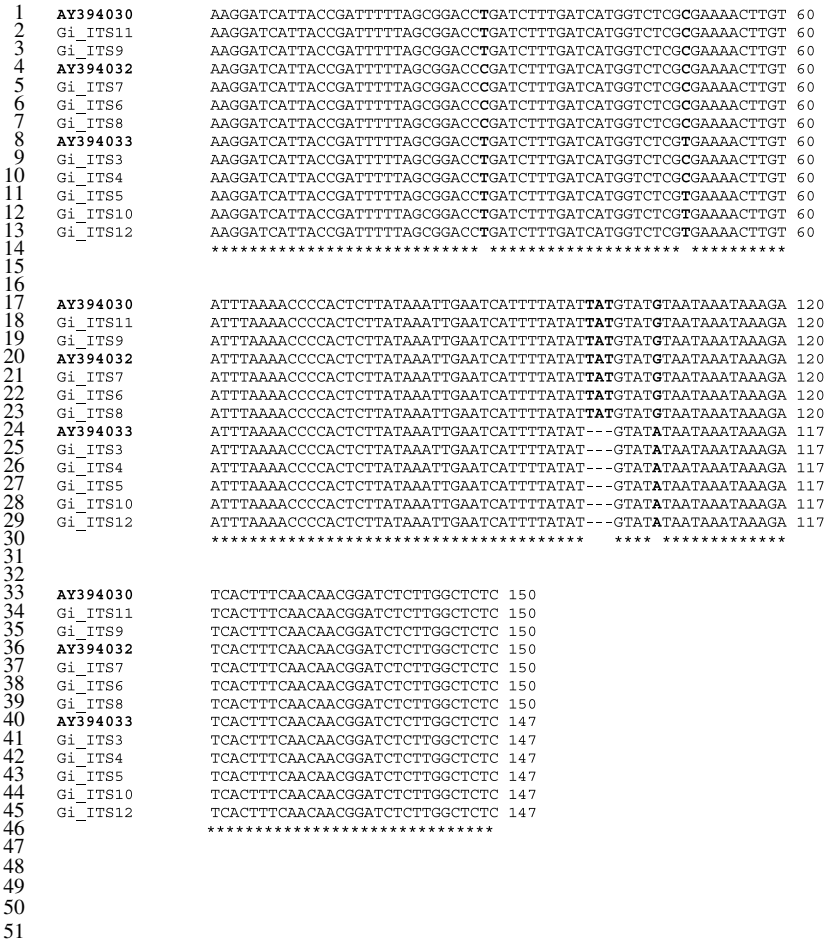

Fig. 4. Alignment of the 10 ITS1 sequences from *G. intraradices* amplified by SDA amplification. The exact location of the variable nucleotides in each of the ITS1 variants is depicted in bold. The accession numbers, AY394030, AY394032, and AY394033, represent the three ITS1 sequence variants previously deposited in the GenBank for this fungus.

4. Discussion

The molecular genetics of AM fungi involves the difficult procedure of extracting DNA from single individual spores. The low DNA content of an individual spore, inconsistent extraction efficiencies and low availability of axenically cultured fungal material imposes a severe limitation in our ability to perform large scale genetic analyses from a single sample. Application of an efficient whole genome amplification method is a possible alternative to address this intrinsic shortcoming.

To be practical, a WGA method should have the following features: whole coverage of the genome, generation of long fragments, successful amplification of small amounts input template DNA (pg to ng range), high amplification fidelity and copious yield. The two most widely used methods of WGA are based on PCR [19,20]. Both these methods though technically straightforward suffer from certain shortcomings, due to their reliance on thermocylcing and annealing kinetics of the degenerate primer during the PCR amplification procedure. This makes it highly susceptible to sequence bias, low fidelity in copying targets not in its entirety, smaller amplicon size, preferential amplification of certain loci and incorporation errors intrinsic to *Taq* polymerase functioning. To address these limitations, non-PCR based WGA techniques have been proposed [11,20].

The SDA technique is also called multiple displacement strand amplification (MDA) is a non-PCR based WGA. It uses the principle of the rolling circle type of replication, a process of amplification by which circular molecules such as plasmids and viruses replicate [21]. In lieu of *Taq* polymerase, the SDA use Phi29 DNA polymerase or a blend of *Bst* polymerase and T4 gene proteins [22,23]. In combination with random primers and a fixed temperature of incubation (30 °C), the polymerase amplifies the target DNA via a hyperbranching mechanism. This generates hundreds or thousands of copies of the input DNA template in only a few hours, resulting in micrograms quantities

of DNA from as little as nanogram of the starting material.

The Phi29 polymerase mediated SDA technique has recently been demonstrated [24] to successfully copy ca. 99.82% of the total genome from normal and cancerous human cells. This is in stark contrast to PCR-WGA methods, which do not provide such wide genome coverage. Apart form high sequence representation, the SDA method has other advantages some of which include, low polymerase error rate ($<10^{-7}$), negligible sequence bias during amplification, scalability of the reaction to any volume, tolerance to low quality of DNA template, bigger amplicon size and a very high DNA yield [12].

Successful PCR amplification of specific genes present in AM fungi confirmed that the genomic DNA was successfully copied during the SDA reaction. As a control, the same genomic DNA was subjected to PCR amplification to assess the efficiency of the SDA. The consistent low band intensity of the PCR amplicons when genomic DNA was used as a template, as compared to the amplicons from SDA derived templates, shows that copies of the target genes were substantially increased during the SDA reaction. In the post-SDA analysis using PCR, the β-tubulin1 gene was better or equally well amplified as compared to the ITS and 18S rDNA, even though the β-tubulin1 is present at a low-copy number in the AM fungal genome. This could be best explained by the fact that the primers used for the rDNA amplification were universal, fungal specific, as compared to β-tubulin1 primers which were designed on a specific sequence from *G. intraradices* in the present study. This differential specificity could have resulted in apparent amplification variability, considering the complex binding kinetics of a primer during a typical thermocylcing reaction. Since the amount of DNA present in a single AM fungal spore ranges from 0.05 to 0.61 ng, depending upon the species, the amount of DNA generated in micrograms quantities (3.8–5.8 μg) by the SDA technique in our study represents a substantial fold amplification of the input template. In our hands, the amount of template generated by a single SDA reaction was sufficient for at least 3000 PCRs, as compared to ca. 50 reactions from a single genomic DNA preparation maximally diluted. This empirically allows for a large scale genetic analysis from a single spore.

The size of the ITS fragment was in the range of 550–600 for *Glomus* species as observed earlier [25]. Sequence analysis of the SDA amplified ITS1 region revealed three out of the four possible ITS1 variants previously identified in *G. intraradices*, by sequencing a total of 78 ITS1 clones from a single spore [6]. We attribute the failure to detect the fourth ITS1 variant to the limited number of sequencing reactions (a total of 10) performed in our study.

5. Conclusion

The Phi29 based WGA is an attractive method for amplifying highly limiting quantities of DNA from single spores of AM fungi. With the low hands-on time required to set up SDA reactions (<30 min), excellent genomic coverage and generation of μg quantities of DNA (from pg levels of starting material), use of SDA would make it possible to perform large scale genetic analysis of single AM fungal spores, not possible previously.

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