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A Simple and Rapid Method to Extract Genomic DNA from Urediniospores of Rust Diseases for Molecular Analysis

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Abstract: A simple and rapid procedure for efficiently isolating DNA from urediniospores of rust fungi suitable for use as a template for PCR amplification and other molecular assays specifically for sequencing rDNA IGS1 is presented. Urediniospores of 11 samples of brown rust collected from infected leaves of commercial wheat cultivars from different parts of Iran purified and propagated on wheat seedlings of the susceptible wheat cultivar Bolani. To DNA extraction 200 mg of desiccated urediniospores and 20 mg autoclaved carborundum powder (for crushing the urediniospore cell walls) were added to 1.5 ml microcentrifuge tubes. The tubes were then placed into a medium-sized mortar (used for plant virus experiments) and liquid nitrogen was added. Frozen urediniospores were ground using plastic mini-pestles mounted in an electric drill under a low speed for 20 sec. After grinding, 500 µl of extraction buffer was added to the cracked spores. DNAs were extracted by using phenol, chloroform, isopropanol and ethanol. PCR amplification of rDNA IGS1 was performed by using L318 and 5SK primers. The amplification products were electrophoresed on 1% TBE agarose gel, stained with ethidium bromide and visualized under UV light. The amount and quality of the DNA obtained by this procedure were suitable for the PCR amplification and sequencing of IGSI and could be relevant for other molecular assays. Significance and Impact of the Study: Use of this procedure will enable researchers to obtain DNA from urediniospores of rust fungi quickly and inexpensively for use in molecular assays and replaces current expensive and time-consuming procedures.

Zusammenfassung: Eine einfache und schnelle Methode zur DNA-Isolierung aus Uredinoiosporen von Rostpilzen, die in molekularen Techniken wie PCR-Amplifikation und rDNA-Sequenzierung angewandt werden kann wird vorgestellt. Urediniosporen von 11 Braunrostproben wurden von infizierten Blättern von kommerziellen Weizensorten in verschiedenen Regionen Irans gesammelt und auf der empfänglichen Varietät Bolani vermehrt. Zur DNA-Extraktion wurden 200 mg getrocknete Urediniosporen mit 20 mg autoklaviertem Carborund-Pulver versetzt und in 1.5 ml Eppendorfgefäßen unter flüssigem Stickstoff mit Plastikstösseln aufgebrochen. Nach dem Aufschluss wurden 500 µl Extraktionspuffer zugesetzt und die gelöste DNA mit Phenol, Chloroform, Isopropanol und Äthanol gereinigt. PCR-Amplifikation der rDNA wurde mit den Primern L318 und 5SK durchgeführt und die Amplifikationsprodukte auf einem 1% TBE-Agarose-Gel mit Ethidiumbromid sichtbar gemacht. Die beschrieben Methode stellt eine einfache und kostengünstige Alternative zu bestehenden Extraktionsmethoden dar.

Key words: rDNA, IGS1, *Puccinia triticina*, sequencing.

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Introduction

The rust fungus *Puccinia triticina* Eriksson is the cause of brown rust disease, which affects wheat (*Triticum sativum*) and has drastically decreased wheat production in most parts of the world (Kolmer 2005). *Puccinia triticina* is an endemic disease of wheat in many regions of Iran (Afshari 2008). Although great effort has been made to understand the biological and molecular basis of brown rust infection, there is a lack of standardized and specific protocols for routine molecular biology research of this organism, although these are commonly available for other fungal research. Current methods of DNA extraction from *P. triticina* and other fungal pathogens are either time-consuming and or are based on expensive technologies (Muller et al. 1998; Faggi et al. 2005; Borman et al. 2006; Cheng and Jiang 2006). They include the use of SDS/CTAB/proteinase K (Wilson 1990), SDS lysis (Syn and Swarup 2000), lysozyme/SDS (Flamm et al. 1984), high-speed cell disruption (Muller et al. 1998) and bead-vortexing/SDS lysis (Sambrook and Russel 2001). Additionally,

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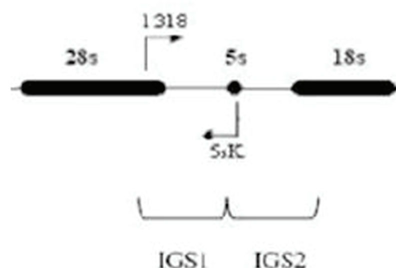

Fig. 1: Schematic representation of primer positions on rDNA.

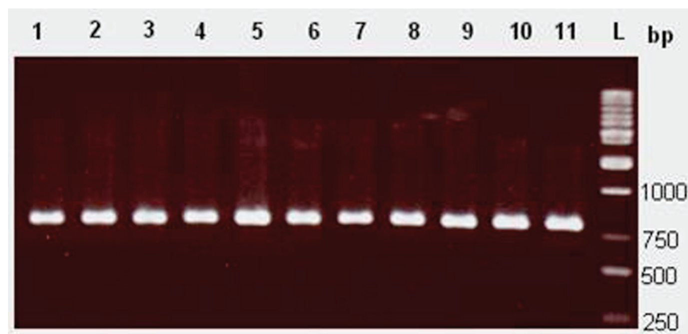

Fig. 2: Electrophoresis of PCR products from the IGS1 region of ribosomal DNA of 11 isolates of *Puccinia triticina* on 1% agarose gel.

some result in poor DNA yields, as the cell walls are difficult to lyse (Muller et al*.* 1998).

The major challenges to isolating DNA of good quality and quantity from rust fungi (an obligate parasite) are the lack of mycelium produced in cultural mediums and the need to break the rigid cell walls of urediniospores, as they are often resistant to traditional DNA extraction procedures. There are currently numerous DNA isolation kits available commercially, but they often have a high cost per sample (Ahmed et al. 2009; Kang et al. 2004). According to Fredricks et al. (2005) no single extraction method among those currently available is optimal for all fungi analyzed so far.

The polymerase chain reaction (PCR) procedure used for fungi analyses, especially during genetically modified (GM) screening, requires high-quality DNA to ensure successful amplification with reproducible results. Furthermore, high purity DNA is required for PCR and other PCR-based techniques, such as random amplified polymorphic DNA (RAPD), micro- and macrosatellite analyses, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) used for genome mapping, DNA fingerprinting (Khanuja et al., 1999) and DNA sequencing used for genomic analysis.

Here, we report the development of an alternative and rapid DNA isolation method adapted from the protocol of Reader and Broda, and its successful application to urediniospores of *P. triticina*. We also report the amplification of extracted DNA of 11 isolates of *P. triticina* collected from different parts of Iran, which were then used to amplify the intergenic spacer 1 (IGS1) region of rDNA and for sequencing analysis.

Material and Methods

Samples collection and multiplication of urediniospores During Spring 2009, samples of brown rust-infected leaves were collected from commercial wheat cultivars from different parts of Iran. Under controlled conditions in a green house, 11 samples were purified and propagated on wheat seedlings of the susceptible wheat cultivar Bolani. Urediniospores of single pustules were isolated and propagated on Bolani for each sample. Propagated urediniospores were stored in aluminum foil packets. The collected spores were desiccated in desiccators contained silica gel and were then placed in the freezer (-20°C) until needed.

DNA extraction

The DNA extraction procedure was adopted, with some modifications, from the method of Reader and Broda (Reader and Broda, 1985): 200 mg of stored urediniospores and 20 mg autoclaved carborundum powder (for crushing the urediniospore cell walls) were added to 1.5 ml microcentrifuge tubes. The tubes were then placed into a medium-sized mortar (used for plant virus experiments) and liquid nitrogen was added. Frozen urediniospores were ground using plastic mini-pestles mounted in an electric drill under a low speed for 20 sec. After grinding, 500 µl of extraction buffer [200 m mol l^{-1} Tris-HCl (pH 7.5), 250 m mol l^{-1} NaCl, 25 m mol l^{-1} EDTA, 0.5% SDS; Edwards et al., 1991] was added to the cracked spores, which were then homogenized for 5 min at maximum speed using Vortex Genie 2. Then, 350 µl phenol was added to each tube, which was inverted gently four times. Subsequently, 250 ul chloroform was added and the tubes inverted gently 40 times. The tubes were then centrifuged at 4°C for 30 min at 2834 g. After centrifugation, the aqueous supernatants were decanted into new tubes. The samples were treated with 2 µl RNase (20 ug ml^{-1} TE) and incubated at 37°C for 10 min. An equal volume of chloroform was added and mixed gently. The tubes were centrifuged at 4°C for 10 min at 2834 g. The aqueous layer was transferred to new Eppendorf tubes, to which a 0.54 volume of cold isopropanol was added for precipitation of the DNA. The tubes were centrifuged at 4°C for 5 min at 2834 g. The supernatants were poured into a sink gently and 100 µl cold 70% ethanol was added to pellets, which were then centrifuged at 4°C for 5 min at 2834 g; the ethanol was then removed from tubes. Each pellet was dried in an incubator at 37°C for 30 min and dissolved in (50 µl) sterile double-deionized water. The subsequent DNA yields and quality were assessed by standard electrophoresis through a 1% (w/v) ethidium bromide-stained agarose gel.

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PCR amplification of rDNA

PCR amplification was performed in a Mastercycler® gradient machine (Eppendorf). The primers for the reactions were as follows: forward primer: L318 (GCTACGATCCACTGAGGTTC) and reverse primer: 5SK (CTTCGCAGATCGGACGGGAT). Localization of the primers to fungal rDNA is presented in Fig. 1.

Each PCR reaction mixture had a total volume of 50 µl and contained genomic DNA (50 ng of DNA solution); 1.5 mM $MgCl_2$; 5 µl of 10x PCR Buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl); 50 pmol of each primer (L318 and 5SK Fig.1); and 2 units of Taq polymerase. The samples were subjected to 30 cycles of 95°C for 1 min, 55°C for 3 min and 71°C for 2 min for the denaturation, annealing and elongation steps, respectively. The PCR products were then stored at 4°C. The amplification products were electrophoresed on 1% TBE agarose gel, stained with ethidium bromide and visualized under UV light (Fig. 2).

DNA sequence

PCR products were transferred to MWG Biotech Pvt. Ltd. for sequencing. Multiple sequence alignments were carried out using the MPEG4 software.

Results

DNA extraction

By using this simple and rapid protocol, it was possible to extract DNA from *P. triticina* urediniospores and to perform PCR for amplification of the IGSI of a large number of samples in a single working day. The efficiency and the speed of this method, together with the use of inexpensive facilities, make it an attractive alternative for the extraction of urediniospore DNA. The results show that the DNA produced by this simple, low-cost, fast and safe protocol can be used in PCR-based techniques examining a wide range of rust diseases, and in laboratories with inexpensive equipment and technology.

IGS1 PCR

Using the L318 and 5Sk primer pair yielded an 870-bp PCR product fragment for all isolates (Fig. 2). The presence of these similar products suggests that homogeneity in IGS1 length exists within individual samples of *P. triticina*.

Sequence analysis

The IGS1 sequences were submitted to the NCBI database using the Blast search program (BLASTN 2.0.14) (ALTSCHUL et al. 1997). Significant alignment was only found for the first 62 and the last 148 bases, corresponding to the 28S, end of IGS1 and 5S ribosomal RNA, respectively. The most similar sequence was the 28S and 5S ribosomal RNA of *Puccinia striiformis* f.sp. *tritici* but it was not sufficient to identify the present fungi. The sequences were submitted to GenBank using Bankit and the accession numbers of isolates were registered as: HM590475, HM590476, HM590477, HM590485, HM590482, HM590483, HM590484, HM590480, HM590479, HM590481 and HM590478.

Discussion

The method presented in this paper eliminates much of the laborious and time-consuming steps of most other DNA extraction protocols (Van Burik et al. 1998; Haugland et al. 1999; Al-Samarrai and Schmid 2000). DNA was isolated immediately from urediniospores. In this procedure, cell walls are broken by plastic mini-pestles mounted in an electric drill with the addition of carborundum powder. The amount and quality of the DNA obtained by this procedure were suitable for the PCR amplification and sequencing of IGSI and could be relevant for other molecular assays. One of the advantages of this procedure is that many samples can be simultaneously processed. Of particular interest is the recovery of IGS1 RNA as visualized by electrophoresis in ethidium bromide-stained gel. The DNA extraction procedure can be completed within 4 hours. Therefore, many samples can be simultaneously processed in a short period of time. This method is applicable to various species of rust fungi isolated from diverse hosts.

The DNA yields were high and pure enough to be readily amplified by PCR and the PCR products were suitable for sequencing. It is likely that this procedure could be applied to many other rust fungal urediniospores. It provides a rapid, reliable and low-cost alternative to the existing DNA purification protocols used in phytopathological laboratories (VAN BURIK et al. 1998; HAUGLAND et al. 1999; MANIAN et al. 2001; CASSAGO et al. 2002). Use of this procedure will enable researchers to obtain DNA from urediniospores of rust fungi quickly and inexpensively for use in molecular assays and replaces current expensive and time-consuming procedures (HAUGLAND et al. 1999; LIU et al. 2000; GRIFFIN et al. 2002). The results obtained also represent the first evidence of amplification of the IGS1 from *P. triticina*.

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