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Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus *Piriformospora indica*

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ABSTRACT

Piriformospora indica (Basidiomycota, Sebacinales) is a root colonizing fungus which is able to increase biomass and yield of crop plants and to induce local and systemic resistance to fungal diseases and tolerance to abiotic stress. A prerequisite for the elucidation of the mode of action of this novel kind of symbiosis is knowledge of the genome organization as well as the development of tools to study and modify gene functions. Here we provide data on the karyotype and genetic transformation strategies. The fungus was shown to possess at least six chromosomes and a genome size of about 15.4–24 Mb. Sequences of the genes encoding the elongation factor 1-α (TEF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for genome size estimation through real-time PCR analysis. Chromosomal location investigated by Southern blot and expression analysis suggested that *TEF* and *GAPDH* are single-copy genes with strong and constitutive promoters. A genetic transformation system was established using a fragment of the TEF promoter region for construction of vectors carrying the selectable marker hygromycin B phosphotransferase. Results demonstrate that *P. indica* can be stably transformed by random genomic integration of foreign DNA and that it posses a relative small genome as compared to other members of the Basidiomycota.

1. Introduction

Piriformospora indica is a filamentous fungus that belongs to the order Sebacinales (fam. Hymenomycetes). This order represents the earliest Basidiomycota lineage with mycorrhizal members (Weiss et al., 2004). Taxa included in this basal order occur in many different habitats and are frequently associated with plants via an extraordinary diversity of mycorrhization types, including ecto-, orchid-, ericoid-, cavendishioid-, and jungermannioid mycorrhizae (Kottke et al., 2003; McKendrick et al., 2002; Selosse et al., 2007; Setaro et al., 2006; Urban et al., 2003). Many of these fungi are not obligate but rather facultatively interacting with the plant, and some of them, including *P. indica*, can grow in axenic culture by exploiting their saprotrophic capabilities. *P. indica* resembles arbuscular mycorrhiza (AM) fungi in several but not all functional and physiological characteristics (Schäfer et al., 2007; Schäfer and Kogel, 2009). The fungus has attracted interest since its discovery, a decade ago, because of the beneficial effects it conveys on a broad variety of mono- and dicotyledonous hosts (Sahay and Varma, 1999; Serfling et al., 2007; Varma et al., 1999; Waller et al., 2005). Along with promotion of plant biomass and increase in abiotic stress tolerance (Baltruschat et al., 2008), *P. indica* mediates disease resistance in plants to root pathogens such as *Fusarium graminearum* (Deshmukh and Kogel, 2007). Moreover, and in contrast to AM fungi, *P. indica* confers systemic resistance to leaf pathogens such as powdery mildew fungi (Waller et al., 2005) and is able to colonize the roots of the crucifer *Arabidopsis thaliana*, enabling a straight forward genetic dissection of the molecular basis of the symbiosis. Accordingly, we could shown that the systemic resistance conferred in *Arabidopsis* by *P. indica* is mediated by the Induced Systemic Resistance (ISR) pathway, since it required jasmonic acid signaling and the cytoplasmic function of NPR1 (Stein et al., 2008). A further distinction between AM fungi and *P. indica* concerns their cellular interaction with the plant root. *P. indica* does not form arbuscles and vesicles but instead intracellular coiled-like structures that might facilitate nutrient exchange (Blechert et al., 1999; Schäfer and Kogel, 2009). Cytological and molecular evidence indicate that root colonization and chlamydospore formation by *P. indica* is functionally associated with cell death in cortex and rhizodermal host cells (Deshmukh et al., 2006). Taken all together these data suggest that *P. indica* forms a novel type of mutualistic symbiosis with plants.

Understanding the biochemical and molecular mechanisms that provide the mutual benefits for the fungus and the plant requires elucidation of the biochemistry, molecular biology and genetics

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of the symbiosis. The present work aimed at identifying the chromosomes and genome size of *P. indica*. To this end, we cloned and sequenced the promoter regions and the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and translation elongation factor 1-α (TEF) to serve as genetic markers. Further, we used the TEF promoter region for establishing an efficient genetic transformation system protocol which is a prerequisite for functional studies of symbiosis-related genes from the fungus and the plant.

2. Materials and methods

2.1. Fungal strains, culture techniques and DNA isolation

Fungal isolates were propagated in liquid complete medium (Pham et al., 2004) at 28 °C with shaking. Genomic DNA from *P. indica* (DSM11827; from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), the haploid *Saccharomyces cerevisiae* genotype BY4741, MATa (ACC. No. Y02321, Euroscarf, Frankfurt), and the diploid *S. cerevisiae* genotype FY1679, MATa/MATα (ACC. No. 10000D, Euroscarf, Frankfurt) was isolated using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany) according to the instruction manual, or from 10 g material using a modified method from Doyle and Doyle (1987) followed by a CsCl centrifugation in 5 ml quick-seal centrifuge tubes (Beckman) at 56,000 rpm for 24 h at 20 °C in a Beckman XL70 centrifuge with rotor VTI 90. The quality and concentration of DNA was analyzed by electrophoresis and spectrophotometry (ND 1000, NanoDrop Technologies, Wilmington, USA).

2.2. Estimation of P. indica genome size by real-time PCR analysis

For the preparation of standard DNA, specific PCR products were generated for the genes *RPS3* of the haploid *S. cerevisiae* (BY4741), and the diploid *S. cerevisiae* (FY1679), as well as for *TEF* and *GAPDH* of *P. indica* (DSM11827), using the respective outer primer pairs RPS3-F1/R1 (Wilhelm et al., 2003), tef420f/tef420r, and gpd383f/gpd383r (see Table 1 from Supplemental material). These PCR products contain the binding sites for the nested primers used in real-time PCR analysis. The reaction mixtures included 0.3 μM each forward and reverse primer, 200 μM each deoxynucleotide (dATP, dCTP, dGTP, and dTTP), 2.5 mM $MgCl_2$ (DNA Cloning Service, Hamburg, Germany), 0.5 U Taq DNA polymerase (DNA Cloning Service), and 50 ng genomic template DNA in a total volume of 25 μl 1× reaction buffer (DNA Cloning Service). The PCR protocol consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 amplification cycles with 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C for the GAPDH and RPS3 primers; and an initial denaturation step of 95 °C for 5 min, followed by 35 amplification cycles with 30 s at 95 °C, 30 s at 57 °C and 1 min at 72 °C for the TEF primers. The PCR products were gel purified using the NucleoSpin Extract II (Macherey-Nagel GmbH, Düren, Germany) and eluted in water. Quality and concentration of all purified standard DNA samples were determined by NanoDrop.

Quantitative PCR amplifications with the primer pairs PRS3-F2/R2; tef-f/tef-r and gpd-f/gpd-r were performed in 20 μl SYBR green JumpStart Taq ReadyMix (Sigma–Aldrich, München, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler (Stratagene, La Jolla, USA). Each run contained a fresh made series of five standards (10-fold serial dilutions) and 1 μl of three different dilutions of the genomic DNA samples in 2–3 technical repetitions. PCR condition for the *TEF* and *RPS3* genes were: initial denaturation for 10 min at 95 °C, followed by 35 cycles with 30 s at 95 °C, 1 min at 57 °C, 30 s at 72 °C, and for the *GAPDH* gene 10 min at 95 °C, followed by 35 cycles with 30 s at 95 °C, 30 s at 58 °C, 1 min at 72 °C. The melting curve was analyzed at the end of cycling to ensure amplification of only a single PCR product. *Ct* values were determined with the Mx3000P V2 software (Stratagene, Heidelberg) supplied with the instrument. The estimation of the genome size based on the *C* values was calculated as described before by Wilhelm et al. (2003). Briefly, the amount of DNA which corresponds to the size of one haploid genome (*C* value) was derived from the ratio of the mass of template DNA (*m*, determined by UV absorbance) and the copy number of the target sequence (*N*, determined by real time PCR), $C = m \times N^{-1}$. The genome size was calculated by $\Gamma = C \times N_A \times M_{Bp}^{-1}$, where N_A is Avogadro's number ($6.022 \times 10^{23}\ mol^{-1}$) and M_{Bp} is the mean molar mass of a base pair ($660\ g\ mol^{-1}$).

2.3. Pulsed field gel electrophoresis (PFGE) separation of P. indica chromosome-sized DNA molecules

Four-week-old mycelium from *P. indica* was crashed using a sterile blender, transferred to complete medium (CM) and incubated at 24 °C with shaking. After 48 h the mycelium was collected by filtration, washed using 0.9% NaCl and incubated 1 h at 37 °C in a protoplasting solution containing 2% lysing enzymes from *Trichoderma harzianum* (L1412 Sigma) dissolved in SMC buffer (1.33 M sorbitol, 20 mM MES, 50 mM $CaCl_2$, pH = 5.8). Protoplasts were filtered through a miracloth filter (Merck, Eurolab, Darmstadt, Germany) and washed three times with STC buffer (1.33 M Sorbitol, 10 mM Tris–HCl, 50 mM $CaCl_2$, pH = 7.5). For the preparation of chromosomal DNA the protoplast suspension was mixed with equal volume of 1.8% BioRad pulsed field (PF) certified agarose gel at 55 °C. The solidified plugs were incubated in proteinase K buffer for 12 h (50 mM Tris–HCl, 50 mM EDTA, 1% SDS, proteinase K 10 mg/ml, pH = 8) and washed three times with washing buffer (0.1 mM Tris–HCl, 0.1 M EDTA, 0.15 M NaCl). This step was repeated two times. Plugs were stored in washing buffer at 4 °C. Experiments were performed on a Bio-Rad CHEF DR III apparatus. The plugs were loaded into a 1.2% BioRad PF certified agarose gel in 1× TAE. The running buffer was 1× TAE, with continuous recirculation at 14 °C. The best separation was achieved after 96 h at 2 V: phase 1 – pulse time 1–1800 s, angle 100°, 48 h; phase 2 – pulse time 1–2000 s, angle 106°, 48 h. After electrophoresis, gels were stained with 0.5 μg/ml of ethidium bromide and photographed. Chromosomal DNA from *S. cerevisiae* (Bio-Rad) and *Schizosaccharomyces pombe* (Bio-Rad) were used as size standards.

2.4. Southern blot

The ethidium-bromide-stained whole chromosomes PF gels or agarose gels containing overnight digested *P. indica* DNA (with the restriction enzymes: *Hind*III, *BamH*I or *Sac*I) were scanned, photographed and then processed for Southern transfer onto a nylon membrane (Amersham Biosciences Hybond-N+) according to the manufacturer's instructions. Membrane was cross-linked using BioRad GS Gene Linker (two times, 50 s, 125 mJ). The DNA probes were labeled with [−32P]dCTP by the random oligonucleotide priming labeling procedure (Amersham Ready-to-Go DNA Labeling Beads) and cleaned using Amersham Illustra Microspin G-25 Columns. The pre- and hybridization solution contained 10% SDS, 5× HSB (PIPES, 5 M NaCl, 0.5 M EDTA, pH 6.8) and Denhardts III (2% BSA, 10% SDS, 2% Ficall-400, 2% PVP-360, 10% $Na_2P_2O_7 \times 10H_2O$ in water) and was mixed together with freshly boiled Carrier DNA (Salmon testis DNA, Sigma). Prehybridisation and hybridisation was carried out at 65 °C for 16–20 h. The filters were washed twice for 30 min in preheated 2× SSC/1% SDS at 65 °C and once in 1× SSC/0.5% SDS for 30 min at 65 °C. The filters were then exposed to an X-ray film (X0MAT, KODAK) for 3–4 h at room temperature in darkness for autoradiography.