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A novel and highly efficient method for genetic transformation of fungi employing shock waves

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ABSTRACT

Genetic transformation of filamentous fungi is an essential tool in many areas such as biotechnology, medicine, phytopathology and genetics. However, available protocols to transform fungi are inefficient, laborious and have low reproducibility. We report the use of underwater shock waves as a novel method to transform filamentous fungi. An experimental piezoelectric shock wave generator was designed to expose fungal conidia to heterologous DNA. The device was successfully tested in *Aspergillus niger, Fusarium oxysporum, Trichoderma reesei* and *Phanerochaete chrysosporium*. The transformation frequency per number of conidia was between two and four orders of magnitude higher in comparison to previously published methods. For example, the frequency of transformation in *A. niger* was improved up to 5400-fold as compared with *Agrobacterium* protocols. Transformation was verified by expression of the green fluorescent protein, PCR and Southern blot. Our method offers new possibilities for fast, easy and efficient genetic manipulation of diverse fungal species.

1. Introduction

Many fungi have had a great impact in several fields of biotechnology (Meyer, 2008). Industries such as paper, textile, food, pharmaceutical and others have benefited from many products and enzymes derived from fungi. Furthermore, the versatility of fungal metabolism has been continuously exploited to generate bioactive compounds and antibiotics. For decades, filamentous fungi, which make up the majority of known fungal species, have been considered the hosts of choice for heterologous protein production (Su et al., 2012; Ward, 2012). The high levels of secreted protein of these organisms may reduce the costs of production and the purification steps (Gouka et al., 1997). Over the last few years, introduction of novel genes and manipulation of specific metabolic routes of these organisms have had an increasing demand in diverse disciplines. Even though several species of fungi have been transformed successfully (Ward et al., 2012), genetic transformation of fungi in general still suffers from several drawbacks (Su et al., 2012). Current methodologies such as PEG-mediated protoplast fusion, electroporation, biolistic transformation and *Agrobacterium*-mediated transformation (AMT) usually have low frequency of transformation and problems of reproducibility (Lorito et al., 1993; Ozeki et al., 1994; Ruiz-Diez, 2002; Michielse et al., 2005). Also, there are many species of fungi that have proved recalcitrant to transformation by these methods (Meyer, 2008).

High frequency of transformation is the main requirement to produce valuable phenotypes of filamentous fungi. Frequency of transformation can been defined as the number of transformants per microgram of DNA or the number of transformants per number of cells as employed in AMT (de Groot et al., 1998; Mullins et al., 2001). Interestingly, increments of recombinant DNA are not correlated with an increased frequency of transformation (Koukaki et al., 2003; Ozeki et al., 1994).

The aim of our study was to use underwater shock waves as a novel method for efficient and fast transformation of filamentous fungi. As far as we know, this is the first report on shock wave-mediated transformation of fungi. Shock waves are mechanical waves that result from the sudden release of energy in a limited space and are routinely used in orthopedics to treat musculoskeletal diseases and in urology to disintegrate renal calculi without surgery (Haupt, 1997; Loske, 2007). For biomedical applications, shock waves are produced using electrohydraulic, piezoelectric or electromagnetic generators (Loske, 2007; Lingeman, 2007). Each shock wave consists of a single high-pressure peak with a steep onset and a gradual decline into a pressure trough. The positive pressure peak may be as high as 150 MPa, with phase duration of

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0.5–3 μs, followed by a decompression pulse of up to −20 MPa and phase duration of 2–20 μs. The piezoelectric shock wave generator designed for this study, produces underwater shock waves by exciting an array of piezoelectric crystals mounted on a hemispherical bowl-shaped aluminum backing (Fig. 1A). Water was used as a coupling medium because its acoustic impedance is similar to that of the conidial suspension. It is known that shock waves may cause a transient increase of cell membrane permeability (Gambihler et al., 1994; Lauer et al., 1997). The phenomenon responsible for this, referred to as acoustic cavitation, is the formation and violent collapse of microbubbles inside the fluid surrounding the cells. The microbubbles of air contained in the cell suspension get compressed by the positive pressure peak of each shock wave. After passage of a shock wave, the bubbles grow for a few hundred microseconds and finally collapse, emitting a high-speed jet of fluid. The impact of these microjets produces transient pores in the cell membranes allowing uptake of macromolecules contained in the fluid surrounding the cells (Arora et al., 2005; Ohl and Ikink, 2003). Insertion of heterologous DNA by shock waves has been successfully tested in *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* by other research groups (Divya Prakash et al., 2011; Loske et al., 2011). Either experimental device can be fabricated or clinical systems can be used, and the results demonstrate numerous advantages in comparison with the available methods (Divya Prakash et al., 2011).

Four important species of filamentous fungi were selected for this work: *Aspergillus niger* is commercially used worldwide as the producer of all the citric acid and is also an industrial workhorse for numerous enzymes (Lubertozzi and Keasling, 2009). *Trichoderma reesei* is employed in the production of cellulases and heterologous proteins for diverse industries (Meyer, 2008). The Federal Drug Administration (FDA) considers both species as GRAS (Generally Recognized as Safe). *Phanerochaete chrysosporium* is a basidiomycete that degrades lignin and other recalcitrant compounds (Martinez, et al., 2004). Finally, *Fusarium oxysporum* is a phytopathogen that causes devastating diseases in several crops like tomato, banana and bean (Michielse and Rep, 2009). *F. oxysporum* is also an efficient degrader of lignin and cellulose. The ability of this fungus to transform plant biomass to ethanol has increasing interest in biofuel production (Corrales Escobosa et al., 2011).

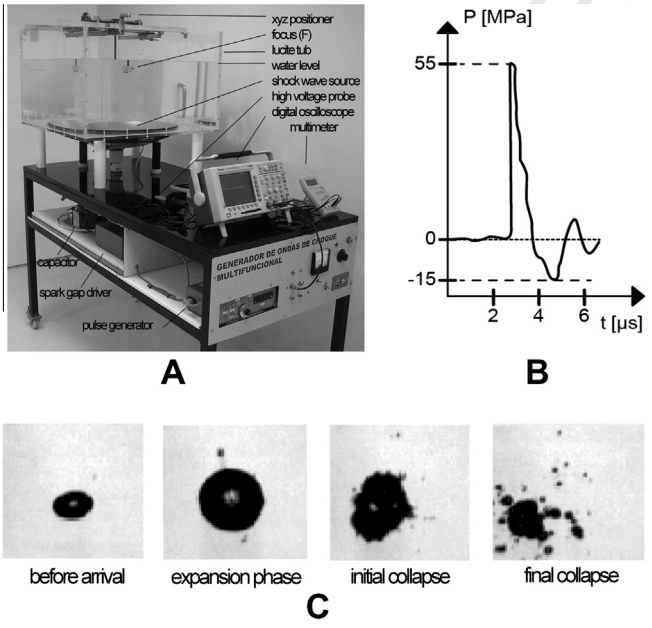

Fig. 1. Experimental arrangement and acoustic cavitation. (A) Photograph of the experimental shock wave generator used to transform filamentous fungi. (B) Typical pressure waveform generated by the shock wave generator at the focus F. (C) High speed photograph of an air bubble in water at the focus of the shock wave generator. Scale bar in (A) 50 cm and (C) 4 mm.

2. Methods

2.1. Research shock wave generator

A Piezolith 2300 (Richard Wolf GmbH, Germany) shock wave generator, having about 3000 piezoceramic crystals arranged on the concave surface of a spherical aluminum backing (radius about 0.35 m) was used. All crystals were connected in parallel and stimulated by high voltage pulses, using a pulse generator. Each electric discharge caused expansion of the crystals, producing a pressure wave that converged towards the center of the arrangement resulting in a shock front in the vicinity of F. A Lucite water tank (base 675 mm × 675 mm, height 450 mm) with an XYZ positioner was placed on top of the shock wave generator (Fig. 1A). The piezoelectric crystals were insulated from the water by a flexible polymer. The electric circuit consisted of a capacitor charging unit and a spark gap trigger. A high voltage transformer (input: 120 VRMS, 60 Hz; output: 5, 32 kVRMS) charged a 0.5 μF capacitor up to 7.6 ± 0.15 kV, through a 100 kΩ resistor. The capacitor maintained the voltage until the spark gap was fired. At this instant the energy was discharged through a 3 Ω resistor and excited the piezoelectric crystals. A diode prevented undesirable return of electric pulses, due to ringing of the piezoelectric crystal array. The spark gap consisted of a gas-filled cavity containing two main electrodes (input and output) separated a distance large enough to withstand more than 7.6 kV, and a trigger electrode, located next to the output electrode. A 12 kV, 1 mA, 5 μs pulse between the trigger electrode and the output electrode ionized the gas inducing the discharge of the capacitor. A special pulse generator was designed to trigger the system either in a manual or a repetitive mode at an adjustable rate between 0.1 and 1.0 Hz. In this experiment, shock waves were produced at a rate of 0.5 Hz. Water level and water temperature were set to 80 mm above F, and 23 °C.

2.2. Pressure measurements and high speed photographs

A polyvinylidene fluoride needle pressure gauge (Imotec GmbH, Würselen, Germany), having a 20 ns rise time was calibrated with a FOPH 2000 fiber optic hydrophone (RP Acoustics, Leutenbach, Germany), in accordance with the requirements of the International Electrotechnical Comission (Anonymous, 1998) and used to record 10 pressure profiles at F (focus). Signals coming from the hydrophone were fed into a 300 MHz digital oscilloscope (Tektronix Inc., Beaverton, OR, USA, model TDS3032). Ten waveforms provided sufficient data to compensate for shot-to-shot variations. The error in positioning the needle hydrophone was estimated to be ±0.5 mm. Water level was set 25 mm above F during pressure measurements. An APCI 8000S high speed camera (Redlake, Pasadena CA, USA) was used to register the dynamics of a single bubble immersed in water in the vicinity of F. To achieve this, the trigger of the high-speed camera was synchronized with the discharge circuit of the shock wave generator.

2.3. Plasmid constructions and vectors used in genetic transformation

For vector pANGFPHPH, the *gpdA* promoter (2.1 kb) of *Aspergillus nidulans* flanked by ApaI–NotI restriction sites was fused to the hygromycin resistance gene (*hph*) and the *trpC* terminator flanked by NotI–BstXI restriction sites. Also, a fragment of the *gpdA* promoter (405 bp), the sequence of the GFP and the NOS terminator (254 bp) flanked by BstXI–SpeI restriction sites were joined to