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Genetic transformation of the white-rot fungus *Dichomitus squalens* using a new commercial protoplasting cocktail

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ABSTRACT

D. squalens, a white-rot fungus that efficiently degrades lignocellulose in nature, can be used in various biotechnological applications and has several strains with sequenced and annotated genomes. Here we present a method for the transformation of this basidiomycete fungus, using a recently introduced commercial ascomycete protoplasting enzyme cocktail, Protoplast F. In protoplasting of *D. squalens* mycelia, Protoplast F outperformed two other cocktails while releasing similar amounts of protoplasts to a third cocktail. The protoplasts released using Protoplast F had a regeneration rate of 12.5% (± 6 SE). Using Protoplast F, the *D. squalens* monokaryon CBS464.89 was conferred with resistance to the antibiotics hygromycin and G418 via polyethylene glycol mediated protoplast transformation with resistance cassettes expressing the hygromycin phosphotransferase (*hph*) and neomycin phosphotransferase (*nptII*) genes, respectively. The *hph* gene was expressed in *D. squalens* using heterologous promoters from genes encoding β-tubulin or glyceraldehyde 3-phosphate dehydrogenase. A Southern blot confirmed integration of a resistance cassette into the *D. squalens* genome. An average of six transformants (± 2 SE) were obtained when at least several million protoplasts were used (a transformation efficiency of 0.8 (± 0.3 SE) transformants per μg DNA). Transformation of *D. squalens* demonstrates the suitability of the Protoplast F cocktail for basidiomycete transformation and furthermore can facilitate understanding of basidiomycete gene function and development of improved strains for biotechnological applications.

1. Introduction

D. squalens is a white-rot fungus that efficiently degrades lignocellulose in nature and produces an extensive set of plant biomass modifying enzymes (Rytioja et al., 2015). This basidiomycete has potential for several biotechnological applications, such as biological pretreatment of various plant biomasses (Bak et al., 2010; Itoh et al., 2003), removal of pollutants from the environment (Čvančarová et al., 2013) and synthetic dye-decolourisation (Eichlerová et al., 2006; Novotny et al., 2012).

D. squalens has a growing body of genomic, molecular and biochemical research to which a genetic transformation system could facilitate testing of hypotheses. For example, the molecular response of *D. squalens* to various complex carbon sources has been studied (Rytioja et al., 2017) and several oxalate-metabolising genes have been identified as putatively involved in detoxification as well as other functions in *D. squalens* (Mäkelä et al., 2014). After the sequencing of one *D. squalens* strain as part of a large comparative study (Floudas et al., 2012), three additional strains have been sequenced more recently (http://genome.jgi.doe.gov/programs/fungi/index.jsf), providing the best coverage of a filamentous basidiomycete species to date. The strains produce a wide array of lignocellulose degrading enzymes with variability in growth and activities between the strains (Casado-López et al., 2017).

Transformation systems have previously been developed for other lignocellulose and/or toxic compound degrading fungi, such as *Schizophyllum commune* (Bartholomew and Marion, 1996), *Pleurotus ostreatus* (Peng et al., 1993), *Phanerochaete chrysosporium* (Alic et al., 1991) and *Trametes versicolor* (Bartholomew et al., 2001), where the latter is the most closely related species to *D. squalens*. There are several strategies for the transformation of filamentous fungi with the preparation of protoplasts being the most common method for transformation of cells (Ruiz-Díez, 2002). A critical component for the preparation of protoplasts is an appropriate protoplasting enzyme cocktail, which releases sufficient numbers of viable protoplasts that can regenerate and are competent for uptake of DNA. Finding appropriate protoplasting cocktails can be challenging such as when replacements

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were sought for the widely used but no longer available Novozyme 234 cocktail (de Bekker et al., 2009; Jung et al., 2000). We tested and compared a recently introduced protoplasting cocktail that is marketed for transformation of the ascomycete *Aspergillus niger* for the basidiomycete *D. squalens* and used it to develop a transformation system for this fungus. Our results indicate that Protoplast F may be a strong candidate for the development of transformation systems for other basidiomycete species.

2. Materials and methods

2.1. Strain and growth conditions

The monokaryotic *D. squalens* strain CBS464.89 (Casado-López et al., 2017; Pham et al., 1990) was used throughout. *D. squalens* was cultured on malt extract agar (MEA; Oxoid) plates at 28 °C. For liquid precultures, five 0.5 cm plugs from a fresh culture on a MEA Petri dish were added to 50 mL of 2% w/v malt extract broth (MEB; Oxoid) and blended at 8000 rpm for 5 s using a Waring blender. The mixture was then transferred into a 250 mL flask (without the addition of any extra medium) and incubated at 28 °C, 120 rpm, for 72 h. The precultures were re-blended as previously (50 mL at a time) and 30 mL of the re-blended preculture was used to inoculate 300 mL of MEB in a 2 L plastic flask, to which a small marble was added to prevent clumps of mycelia forming, and incubated at 28 °C, 120 rpm, for 16–20 h.

2.2. Comparison of protoplasting cocktails

Mycelia grown as described in the previous section were filtered through Miracloth, then divided into three parts so as to wash mycelia with only the buffer subsequently used to protoplast that mycelia as listed in Table 1. Two hundred milligram wet weight of mycelia (~30 mg dry weight), appropriate buffer and the appropriate dosage of enzymes as listed in Table 1 were added to bring the total volume to 1 mL in 2 mL tubes. Mycelia were protoplasted in triplicate for each enzyme cocktail. The tubes were shaken vigorously for 2–3 s and then incubated horizontally for 4 h at the temperature listed in Table 1, with shaking at 100 rpm. Aliquots were taken at 1 h, 2.5 h and 4 h and the protoplasts (> 1.5 μM in diameter) were counted using a haemocytometer (Neubauer improved; 0.1 mm depth). Mycelia without protoplasting enzymes were used as controls and no protoplasts were observed here. For statistical analysis, Student's *t*-test was used in Excel.

2.3. Comparison of promoter sequence identities and preparation of plasmid DNA for transformation

The promotor sequence of β-tubulin (1678 bp) from pTMS14 and *gpd* (744 bp) from pFungiway8 were compared to the same length of promotor sequence of the respective *D. squalens* genes (*Dicsqu464_1_PID_938462* and *Dicsqu464_1_PID_934352*) using the EMBOSS Needle global alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). The alignment was visualized using ESPript (Robert and Gouet, 2014). Plasmid DNA was purified using either Miniprep or Midiprep kits (Promega). *BglII* (Promega) and *NheI* (NEB) were used to linearise the pTMS14 and pFungiway8 plasmids, respectively.

2.4. Release and purification of protoplasts for transformation

From five 300 mL overnight cultures, the mycelia were filtered through Miracloth and washed with Megazyme Protoplast F lysis buffer. The mycelia were collected in a 50 mL tube (~7.5 g mycelial wet weight in total) and Protoplast F (0.27 $mL \cdot g^{-1}$ mycelial wet weight) enzyme mix with BSA (100 $mg \cdot g^{-1}$ mycelial wet weight) in Megazyme Protoplast F lysis buffer was added by filter sterilising through a 0.45 μM filter. The final volume was adjusted to 50 mL with Megazyme Protoplast F lysis buffer. The tube was shaken vigorously for 2–3 s, transferred to a 500 mL flask and incubated at 100 rpm, 30 °C, for 3–4 h. The release of protoplasts was monitored periodically during the protoplasting.

The protoplasts were purified by filtering through funnels loosely packed with fine glass wool, collected and adjusted to 50 mL with ice-chilled 1.33 M sorbitol, 50 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 (STC). The filtered protoplasts were centrifuged with a swing-out rotor for 10 min at 4 °C, 805 × *g*. The supernatant was decanted and the protoplast pellet was re-suspended in 50 mL ice-chilled STC and centrifuged again as in the previous step. After decanting the supernatant, the protoplast pellets were re-suspended in 200 μL of ice-chilled STC, pooled and counted using a haemocytometer.

2.5. Polyethylene glycol (PEG) mediated transformation

Between 2 and 20 million protoplasts in STC were added to each transformation reaction in a total volume of 620 μL that also included 20 μL 0.4 M aurintricarboxylic acid ammonium salt (ATA), 100 μL 20% w/v PEG and DNA (a mixture of 10 μg of circular and 3 μg of linearized plasmid). The 20% w/v PEG solution was prepared by diluting with STC from a 60% w/v PEG solution (PEG 4000 in 50 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5). A control without DNA was also prepared. The reactions were transferred to 15 mL tubes and incubated for 10 min at RT, followed by the addition of 3.75 mL of 60% w/v PEG with gentle mixing, and a further incubation for 20 min at RT. STC (not cold) was then added to a final volume of 15 mL, mixed by inversion and the reactions were centrifuged for 10 min at 3220 × *g*, RT. The supernatant was discarded and the pellet re-suspended in 500 μL STC.

For plating, the protoplasts were mixed to 9 mL of regeneration soft agar (2% w/v MEB, 0.5 M sucrose and 0.5% w/v Select agar (Invitrogen)), and poured onto regeneration base agar (2% w/v MEB with 1.5% w/v agar, and 0.5 M sucrose) in 9 cm Petri dishes. For selection of transformants, the same concentration of antibiotics was added to both the soft and base agars. Depending of the resistance cassette used, Hygromycin Gold (Invivogen) or G418 Sulfate (Gibco by

Table 1
Details of the experimental set-up for comparison of protoplasting cocktails.

Protoplasting cocktail	Details (product code if applicable) and manufacturer(s)	Dosage	Protoplasting (lysis) buffer	Incubation temperature	Reference(s) if applicable
Protoplast F	E-PROTOF, Megazyme	50 μL	25 mM potassium phosphate (pH 5.8) and 0.7 M potassium chloride	30 °C	N/A
Yatalase	T017, Takara	20 mg	50 mM maleate (pH 5.5) and 0.6 M magnesium sulfate	30 °C	N/A
VinoTaste® Pro	Novozymes	50 mg	200 mM sodium phosphate (pH 6.0) and 0.8 M sorbitol	30 °C	(Kowalczyk et al., 2017)
Supplemented *T. harzianum* mix	Lysing enzymes *Trichoderma harzianum* (L1412, Sigma)	5 mg	As for VinoTaste® Pro	37 °C	(de Bekker et al., 2009)
	β-Glucuronidase (G0751, Sigma)	460 units			
	Chitinase (C6137, Sigma)	0.15 units			