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Cadophora finlandia and *Phialocephala fortinii*: *Agrobacterium*-mediated transformation and functional GFP expression

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ABSTRACT

Hygromycin B resistance was transferred to the sterile mycelia of *Cadophora finlandia* and *Phialocephala fortinii* by co-cultivation with *Agrobacterium tumefaciens*. Constitutively expressed green fluorescent protein (GFP) was also introduced using the same vector. Confocal laser scanning microscopy (CLSM) revealed strong fluorescence of transformants. Both traits were mitotically stable during one year of subculturing on non-selective growth medium. Southern blot analysis showed that the majority of the transformants contained single-copy integrations at random sites in the genome.

Introduction

Cadophora finlandia and *Phialocephala fortinii* both belong to the order *Helotiales* in the phylum *Ascomycota*. Both fungi have a darkly pigmented mycelium and show rather slow growth rates. Neither sexual nor asexual reproduction is readily detected under standard laboratory conditions.

C. finlandia (previously named *Phialophora finlandia*; Harrington & McNew 2003) can form both ericoid mycorrhiza (ERM) with ericoid hosts and ectomycorrhiza (ECM) with ectomycorrhizal hosts (Vrålstad *et al.* 2002a). It is frequently found in heavy-metal polluted habitats, and a possible functional role in heavy metal resistance has been suggested (Vrålstad *et al.* 2002b). The isolate used in this study (*C. finlandia* PRF15) was obtained from an ectomycorrhizal root tip from *Salix caprea* growing on soil heavily contaminated with cadmium, lead, and zinc (Dos Santos Utmazian *et al.* 2007).

P. fortinii belongs to the group of so-called dark septate endophytes (DSE), a group of fungi with a worldwide distribution but uncertain ecological role (Mandyam & Jumpponen 2005). In temperate forest systems, *P. fortinii* is a common endophyte of tree roots (Queloz *et al.* 2005). The isolate used in this study was obtained from an ectomycorrhizal *Quercus petrea* root tip growing on a serpentine soil. At this site, *P. fortinii* could be detected as an 'ectomycorrhiza-associated fungus' in 13 out of 29 ectomycorrhizal root samples from *Pinus sylvestris* and

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Q. petrea (Markus Gorfer, Alexander Urban, & Joseph Strauss, unpubl. results).

To allow further research on this important group of fungi, a genetic transformation system based on *Agrobacterium tumefaciens* was developed. Since the first demonstration that *A. tumefaciens* can transfer its T-DNA not only to plants, but also to yeast (Bundock *et al.* 1995) and filamentous fungi (de Groot *et al.* 1998), there have been several reports of successful transformation of different filamentous fungi (e.g. Chen *et al.* 2000; Weld *et al.* 2006), including ectomycorrhizal basidiomycetes (Hanif *et al.* 2002; Pardo *et al.* 2002; Kemppainen *et al.* 2005), but ectomycorrhizal ascomycetes have not, thus far, been stably transformed by *Agrobacterium*. Only transient transformation leading to transient expression of enhanced green fluorescent protein (EGFP) has been reported for *Tuber borchii* (Grimaldi *et al.* 2005). Recently, the successful expression of autofluorescent proteins has been reported for ectomycorrhizal basidiomycetes. EGFP was expressed in *Pisolithus tinctorius* (Rodríguez-Tovar *et al.* 2005) and *Hebeloma cylindrosporum* (Müller *et al.* 2006), and *Discosoma striata* red fluorescent protein (DsRed) expression was reported in *Suillus grevillei* (Murata *et al.* 2006).

To our knowledge, this is the first report on the successful genetic transformation of fungi belonging to ectomycorrhizal and ericoid ascomycetes and DSE (cf. clade 1 in the *Helotiales* according to Vrålstad *et al.* 2002b). Using this technique the functional roles of genes associated with mycorrhiza formation, heavy-metal tolerance, and others, can be studied. A genomic array from *C. finlandia* PRF15 has been developed in the authors' laboratory and is currently used for the identification of heavy metal-regulated genes (unpubl.).

Strong green fluorescent protein (GFP)-expression will contribute to the elucidation of colonization patterns of DSE fungi. Preliminary evidence from our laboratory suggests *P. fortinii* frequently colonizes roots that form ECM with other fungi (Markus Gorfer, Alexander Urban, & Joseph Strauss, unpubl. results). Specific visualization will facilitate detection of the distinct structures *P. fortinii* forms in this complex habitat composed of tree roots, ECM-forming fungi, ECM-associated fungi, and bacteria (Nurmiaho-Lassila *et al.* 1997).

Materials and methods

Strains and culture conditions

Cadophora finlandia PRF15 was isolated from an ECM root tip from *Salix caprea* growing in Arnoldstein (Carinthia, Austria), a site heavily contaminated with cadmium, lead, and zinc (Dos Santos Utmazian *et al.* 2007). *Phialocephala fortinii* RSF-Q104 was isolated from an ECM root tip from *Quercus petrea* growing on a serpentine site in Redlschlag (Burgenland, Austria; for a field site description see Wenzel *et al.* 2003). Both strains were grown on malt extract agar (MEA) or modified Moser medium (MMM, 1 % glucose; 2 g l^{-1} peptone, 0.2 g l^{-1} yeast extract, 0.5 g l^{-1} KH_2PO_4, 0.05 g l^{-1} myo-inositol, 75 mg l^{-1} $CaCl_2 \cdot 2\ H_2O$, 10 mg l^{-1} $FeCl_3 \cdot 6\ H_2O$, 150 mg l^{-1} $MgSO_4 \cdot 7\ H_2O$, 10 mg l^{-1} $MnSO_4 \cdot H_2O$, 1 mg l^{-1} $ZnSO_4 \cdot 7\ H_2O$, 2 % agar, pH~6) at room temperature. The following supplements were added to growth media when necessary (final concentrations in parentheses): ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), hygromycin B (HygB, 50 μg ml^{-1}), cefotaxime (Cef, 100 μg ml^{-1}) and acetosyringone (AS, 200 μM).

To prepare fungal cells for long-term storage, colonies were grown on MEA + 5 % (v/v) glycerol at room temperature for two to four weeks and then transferred to 8 °C for 4 d. Agar blocks containing fungal mycelium were cut from these plates, immersed in 20 % glycerol, and slowly frozen to −80 °C. The glycerol-cooling pre-treatment increases viability of mycelium, especially from *P. fortinii*, dramatically.

Agrobacterium tumefaciens AGL-1 was grown on tryptic soy agar (TSA) or *Agrobacterium*-induction medium (AtIND; 10.5 g l^{-1} K_2HPO_4, 4.5 g l^{-1} KH_2PO_4, 1 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} sodium citrate, 0.2 % glucose, 8 mM $MgSO_4$, 1 mg l^{-1} thiamine, 200 μM AS, 40 mM MES; pH 5.3) at 28 °C. *Escherichia coli* JM109 was used for cloning procedures.

For interaction experiments, *Pinus sylvestris* W.Ki P5(III/4–7) seeds were surface sterilized for 15 min in 15 % hydrogen peroxide, washed in several changes of sterile water and put on water-soaked filter paper in Petri dishes for germination. Germlings were transferred axenically to glass tubes (25 × 200 mm) filled with sterile perlite, and soaked with modified Melin Norkrans medium (MNM) (Guttenberger & Hampp 1992). Fungal inoculum (three to five small agar blocks) was added at the same time. Plants with strong root development and extensive fungal growth were harvested after approximately two months for microscopic inspection of the root system.

All biological materials described in this publication are held in the Fungal Genomics Unit, Department of Applied Plant Sciences and Plant Biotechnology, University of Natural Resources and Applied Life Sciences in Vienna and will be made available upon request subject to material transfer agreements where appropriate.

Molecular techniques

Standard procedures were used in the transformation and electroporation of bacteria, PCR, gel electrophoresis, and cloning steps (Sambrook & Russell 2001). Plasmid pCBCT (Fig 1) was constructed by inserting the *Eco*RI/*Xho*I fragment containing the hygromycin resistance gene *hph* under the control of the *Aspergillus nidulans trpC* promoter (Carroll *et al.* 1994) and the SGFP-gene (plant-optimized version of GFP containing the S65T mutation) under the control of the *tox*A-promoter from Pyrenophora tritici-repentis (Lorang *et al.* 2001) from plasmid pCT74 into the EcoRI/SalI sites in the multiple cloning site from the mini binary vector pCB301 (Xiang *et al.* 1999). Genomic DNA from fungal mycelia was isolated by a standard phenol–chloroform extraction after grinding in liquid nitrogen. For Southern blotting, DNA was digested with *Eco*32I (isoschizomer of *Eco*RV, Fermentas St. Leon-Rot, Germany), size-fractionated on a 0.8 % agarose gel and transferred to Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA) and hybridized according to manufacturer's instructions. Probe DNA was generated by PCR amplification of the whole T-DNA from plasmid pCBCT with the primer pair CB01 (5′-GTGGTTGGCATGCACATACA-3′) and CB02 (5′-GCCTGTATCGAGTGGTGATT-3′) and radiolabelled with α^{32}P-dCTP and Ready-To-Go™ DNA Labelling Beads (GE Healthcare, Amersham, UK).