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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).

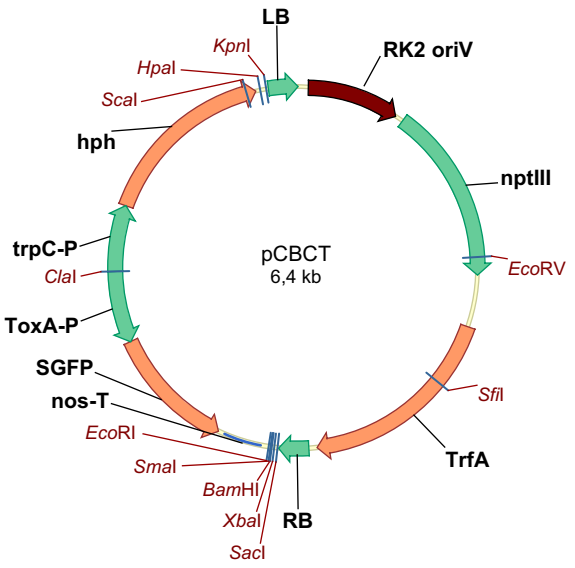

Fig 1 – Binary vector pCBCT. Plasmid used for *Agrobacterium*-mediated transformation of *Cadophora finlandia* and *Phialocephala fortinii*. It contains the HygB-resistance marker (*hph*) under the control of the *Aspergillus nidulans trpC*-promoter, and the green fluorescence protein (SGFP) under the control of the *Pyrenophora tritici-repentis toxA*-promoter between the left border (LB) and the right border (RB) of *A. tumefaciens* T-DNA. The vector backbone containing RK2 oriV, *nptIII* (neomycin phosphotransferase) and TrfA originates from the mini binary vector pCB301. Unique restriction sites are indicated.

Agrobacterium-mediated transformation

Agrobacterium-transformation was based on the protocol described by Hanif *et al.* (2002). Briefly, fungal cultures actively growing on a MMM plate covered by a cellophane sheet were transferred with the cellophane sheet to MoserIND (as MMM but only with 0.2% glucose instead of 2% and 10 mM MES to obtain a pH of 5.3; additionally, after autoclaving glycerol was added to a final concentration of 0.5% and AS to a final concentration of 200 μM). Fungal mycelium was then covered with 2 ml of an induced culture of *A. tumefaciens* AGL-1 [pCBCT] and allowed to completely wet the hydrophobic mycelium, excessive liquid removed, and co-cultivation plates incubated at room temperature for 2 d. Cellophane sheets bearing the fungal mycelium were transferred to selection plates (MEA + 50 μg ml^{-1} HygB +100 μg ml^{-1} Cef). After 5 and 10 d the cellophane sheets were transferred to fresh selection plates. After two weeks, emerging hygromycin-resistant mycelium was subcultured on MEA + HygB for three selective rounds.

Confocal microscopy

Imaging of GFP was performed on a Leica TCS SP2 Leica Microsystems, Wetzlar, Germany confocal laser scanning microscope. Serial optical sections, as described in legend to Fig 3, were obtained using an excitation wavelength of 488 nm, with emission signals being collected at the emission maxima of 500–555 nm. A combined extended focus image was produced using ImageJ 1.36b software (http://rsb.info.nih.gov/ij).

Results and discussion

Both fungal strains used in this study showed high sensitivity towards HygB: growth of *Cadophora finlandia* PRF15 was fully suppressed at a concentration of 25 μg ml^{-1} HygB and 10 μg ml^{-1} HygB was sufficient to suppress the growth of *Phialocephala fortinii* RSF-Q104. For this reason, the hygromycin resistance marker *hph* (hygromycin phosphotransferase) driven by the *Aspergillus nidulans trpC*-promoter was used to construct an appropriate transformation vector. In addition, SGFP driven by the strong and constitutive *Pyrenophora tritici-repentis toxA*-promoter (Lorang *et al.* 2001) was inserted into the plasmid. Both fungal promoters direct high-level constitutive gene expression and were already shown to be functional in a variety of hosts (Lorang *et al.* 2001). The resulting binary vector was called pCBCT (Fig 1).

Fungal mycelium growing on MoserIND-induction medium (20 colonies per petri dish) was co-cultivated with AS-induced *Agrobacterium tumefaciens* AGL-1 cells [pCBCT]. Controls included *A. tumefaciens* AGL-1 omitting the plasmid and non-induced *A. tumefaciens* [pCBCT]. Only *A. tumefaciens* AGL-1 [pCBCT] induced with AS gave rise to HygB-resistant colonies. Occasionally, on control plates, fresh mycelium started to grow from the margins of the colonies due to loosening of contact between the cellophane sheet and the selective growth medium, but stopped growing upon prolonged incubation. Growth did not resume after transfer of such colonies onto fresh selection plates. In contrast, all strongly HygB-resistant colonies continued growth after transfer to fresh selective growth medium. From 20 colonies, which were treated with *A. tumefaciens* AGL-1 [pCBCT], on average ten HygB-resistant sectors were obtained. Extending the incubation time (about two months) of the primary selection plates gave rise to additional HygB-resistant sectors. One year of subculturing transformants on non-selective medium (MEA) did not result in loss of the HygB resistance. For this reason, the transformants obtained by this method are considered as having high mitotic stability.

Southern-blot analysis was carried out to assess the fate of the T-DNA insertions in the transformants. Genomic DNA was digested with Eco32I (isoschizomer of EcoRV), which cuts once inside the plasmid pCBCT (in the kanamycin resistance marker nptIII) but not in the T-DNA. Integration of the complete T-DNA into the genome at random sites should therefore result in bands with differing sizes > 3 kb. All investigated HygB-resistant transformants from *C. finlandia* PRF15 and *P. fortinii* RSF-Q104 gave rise to hybridizing bands of >3 kb (see Fig 2). Different band sizes in the transformants are indicative of random integration events. The majority of the transformants had the T-DNA integrated at a single locus. Whether some transformants contain tandem copies of the T-DNA integrated at one locus has not been determined. Some transformants showed double bands (*C. finlandia* PT15 and

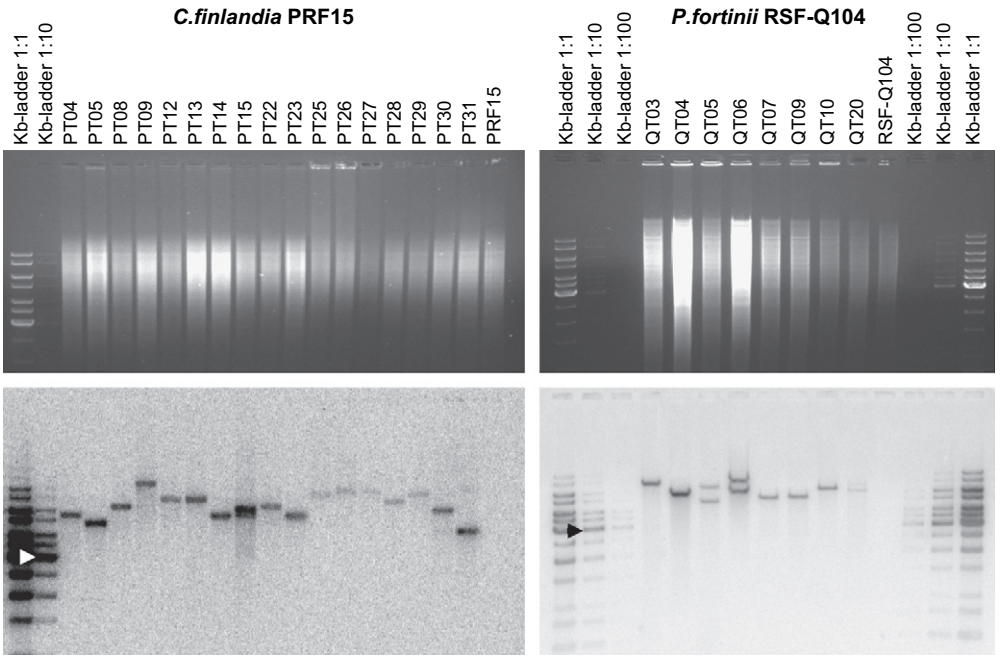

Fig 2 – Southern-blot analysis of transformants. Genomic DNA of HygB-resistant transformants from *Cadophora finlandia* PRF15 (left) and *Phialocephala fortinii* RSF-Q104 (right) was digested with Eco32I, size-fractionated on a 0.8 % agarose gel (upper panel), transferred to nylon membrane and hybridized to radiolabelled T-DNA from plasmid pCBCT (lower panel). DNA from the kb-ladder was also radiolabelled. PT and QT indicate different transformants, PRF15 and RSF-Q104 indicate the untransformed controls. The 3-kb-band of the kb-ladder (Fermentas, band sizes in kb: 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25) is marked by an arrowhead. All hybridizing bands have a size > 3 kb, the size of the T-DNA. The majority of the transformants appear to contain a single copy of the T-DNA integrated in their genomes. Only transformants PT15, QT05 and QT06 contain two copies of the T-DNA integrated at independent loci. QT20 is probably a mixture of two independently transformed mycelia. No hybridization signals are seen with the untransformed control strains *C. finlandia* PRF15 and *P. fortinii* RSF-Q104.

P. fortinii QT05, QT06 and QT20). It is likely that QT20 is a mixed mycelium having arisen from two independent T-DNA transfer events, as the intensities of the bands are different. In the other transformants with double bands (PT15, QT05, and QT06) hybridization intensities of the bands are similar, which would be in agreement with two T-DNAs integrated at independent sites in the genome of these transformants.

Randomly selected transformants of both strains were investigated for SGFP fluorescence by confocal laser scanning microscopy. In all cases, all hyphae showed a strong fluorescence (Fig 3A). Untransformed recipient strains did not show any fluorescence at the applied excitation and detection conditions. No hyphae without fluorescence could be found in transformed mycelium, although sometimes fluorescence was attenuated by the dark pigments of the fungi. The high mitotic stability and the strong hybridization signals in Southern analysis indicate that the mycelium of transformants only contains transformed nuclei and does not contain stowaways without T-DNA. This is of particular interest as single cell stages were not subcultured at any point during the transformation procedure,. The high sensitivity of *C. finlandia* and *P. fortinii* to HygB indicates a strong toxic effect of this compound and this might lead to insufficient nuclear cross-complementation thus abolishing the co-existence of untransformed nuclei under the selective pressure applied.

Growth of *C. finlandia* PRF15 transformants in the presence of pine roots resulted in the formation of a dense mesh around the roots (Fig 3B), but structures typical for an ECM-like true mantle and a Hartig net were not observed. No penetration of hyphae into roots was detected. It should be noted that the mycorrhizal status of *C. finlandia* PRF15 is still unclear and a true ECM was not formed with pine under our laboratory conditions (unpubl. observation). However, PRF15 was originally isolated from an ECM root tip of willow and experiments using willow as an interaction partner are underway.

Association with pine roots was less pronounced in the case of *P. fortinii* Q104 transformants. Similarly, no hyphae could be detected inside the pine roots, as would be expected from a DSE fungus. However, transformants frequently formed distinct structures resembling spores or appressoria on the surface of pine roots (Fig 3C).

The herein presented transformation system will certainly contribute to facilitate molecular analyses of an important group of ascomycetes, which contains saprophytic, endophytic, ectomycorrhizal, and ericoid mycorrhizal fungi.

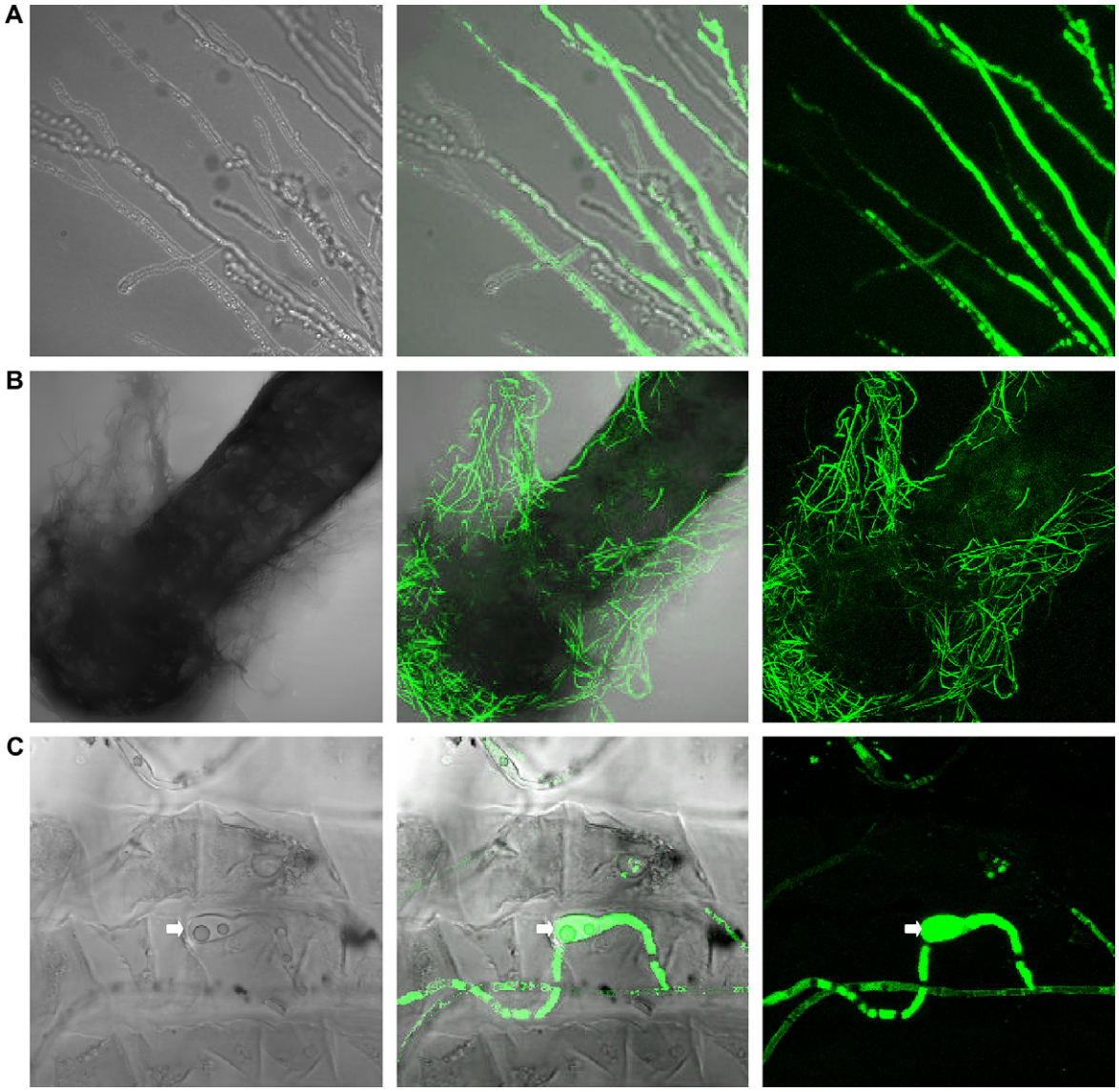

Fig 3 – CSLM images of transformants. The upper panel (A) shows free-living mycelium of *Cadophora finlandia* transformant PT09. Strong fluorescence due to SGFP expression is detected in the whole mycelium. The picture size is 150 × 150 μm, the depth is 10 μm; 8 separately scanned layers were stacked. The central panel (B) shows *C. finlandia* transformant PT09 grown in the presence of pine roots. Formation of a dense hyphal network around the root can be seen. The picture size is 750 × 750 μm, the depth is 120 μm; 30 separately scanned layers were stacked. The lower panel (C) shows *Phialocephala fortinii* transformant QT01 growing on the surface of a pine root. A structure resembling an appressorium or a spore is indicated by an arrow. For the light microscope image, only one layer with this special structure in focus was taken, whereas for the fluorescent image 20 layers over a depth of 20 μm were stacked. The picture size is 75 × 75 μm. The left row shows light microscopy images, the right row shows the corresponding fluorescent images, and the central row is a merge of both images.

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