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Melanization and ageing are not drawbacks for successful agro-transformation of dark septate endophytes

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ABSTRACT

Dark septate endophytes (DSEs) are melanin-enriched ascomycetous fungi that are abundant in stressed environments. However, little is known about their physiology and metabolism, and DSE genes have not been functionally characterized yet. Therefore developing molecular genetic tools to investigate the biological function of genes of interest in DSEs is of major significance. We investigated *Agrobacterium tumefaciens*-mediated transformation (ATMT) efficiency in eight DSE strains belonging to *Cadophora* sp., *Cadophora malorum*, *Leptodontidium* sp., *Phialophora mustea*, and *Cladosporium cladosporioides*. ATMT efficiency was DSE-dependent and ranged from 0.6 to 125 %. We further focused on the effect of mycelium ageing on ATMT efficiency. *Leptodontidium* sp. Me07, *Leptodontidium* sp. Pr30, and *C. cladosporioides* CBS 101367 were significantly more transformed using 15-d-old mycelium (44.5, 6.9, and 1.1 %, respectively) as compared to 2-d-old mycelium (121, 28.7, and 25.1, respectively), whereas *P. mustea* Pr29 was more transformed using young mycelium (21.5 % compared to 5.3 % for the old mycelium). Finally, we focused on the effect of melanin content on ATMT efficiency. Melanin content in mycelium ranged from 0.9 to 3.2 mg g^{-1} DW. Tricyclazole negatively modulated melanin content, while copper positively modulated it. However there was no correlation between hyphal melanin content and ATMT efficiency.

Introduction

Dark septate endophytes (DSEs) are miscellaneous, ascomycetous fungi hosted by plant roots. They are distributed worldwide, under various climates ranging from polar to tropical ecosystems (Mandyam & Jumpponen 2005). They are common under harsh conditions such as arid, dry, saline, and polluted environments (Vrålstad, 2002; Knapp *et al.* 2012;

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Zhang *et al.* 2013). Their tolerance to these unfavourable conditions may be partially explained by the structure of their hyphae which possess melanin-enriched cell walls (Ban *et al.* 2012). The relatively high abundance of DSEs in stressed habitats suggests that they might have an important function for host survival in these ecosystems (Likar & Regvar 2013). However, their effects on plant growth are still poorly elucidated. Several studies and meta-analyses support the view that DSEs could have a beneficial role on plant growth and fitness (Addy *et al.* 2005; Alberton *et al.* 2009; Newsham 2011; Mayerhofer *et al.* 2013; Mandyam & Jumpponen 2015). Several studies report plant-growth-promoting abilities of DSEs through increased biomass production and protection against biotic (pathogen) (Tellenbach *et al.* 2013; Khastini *et al.* 2014) or abiotic stress (drought, salinity, pollution) (Barrow 2003; Sonjak *et al.* 2009; Deram *et al.* 2011; Likar & Regvar 2013). Although DSEs might be an important component of plant tolerance, little is known about the molecular mechanisms that underpin their interactions with host plants.

The sequencing of the genomes of *Cadophora* sp., *Leptodontidium* sp., *Microdochium bolleyi*, *Periconia macrospinosa*, and *Harpophora oryzae* (Xu *et al.* 2014; David *et al.* 2016) was completed recently. However, DSE genes/proteins have not been functionally characterized yet. Therefore developing molecular genetic tools to investigate the biological function of genes of interest in DSEs is of major significance. The generation and characterization of knock-out mutants represent a powerful strategy for deciphering the roles of these genes/proteins (Herrera-estrella *et al.* 2004; Jiang *et al.* 2013). Among the techniques used for fungal transformation (particle bombardment, electroporation, PEG transformation …), *Agrobacterium tumefaciens*-mediated transformation (ATMT) is considered as the most promising one (Mullins & Kang 2001). A number of fungi such as yeasts, or pathogenic, mycorrhizal, endophytic, and biocontrol fungi are indeed amenable to this transformation system (Michielse *et al.* 2005). Additionally, ATMT has several advantages. First, it does not require protoplasts or biolistic methods to transform cells and can be performed directly from mycelium. Second, it can generate a high percentage of transformants with a single-copy and randomly integrated DNA to facilitate the isolation of tagged genes. Finally, its application has been widely used for studies involving gene knock-out, over-expression, complementation, and generating random integrations (Michielse *et al.* 2005; Meyer 2008).

Up to now, ATMT of DSE has only been performed on the four species *Cladosporium cladosporioides*, *H. oryzae*, *Cadophora finlandica*, and *Phialocephala fortinii* (Gorfer *et al.* 2007; Zhang *et al.* 2011; Su *et al.* 2013), and more investigations are required to better understand the parameters that influence their transformation. ATMT protocols for various fungi indicate that a number of parameters affect transformation efficiency. These parameters include (i) fungal parameters such as the choice of the species and the initial material (*e.g.* spores, protoplasts, mycelium) (Michielse *et al.* 2005), (ii) bacterial parameters such as the *A. tumefaciens* strain and its cell density (Flowers & Vaillancourt 2005; Tzima *et al.* 2014), and (iii) cocultivation conditions such as the use of acetosyringone, incubation time, and temperature (Combier *et al.* 2003; Tzima *et al.* 2014). Among these factors, the addition of acetosyringone in the culture medium and the incubation time of the fungal-bacterial cocultivation are usually reported as important drivers of a successful ATMT. By contrast, the effect of mycelium ageing has been poorly investigated. Moreover, the effect of hyphal melanin content on ATMT efficiency has not been studied yet. Melanin is a dark pigment that provides structural rigidity to cell walls and protects cells against abiotic (UV, drought, metal) and biotic stress (lytic enzymes, pathogen attacks, antibiotics, drugs) (Butler & Day 1998; Van Duin *et al.* 2002; Gessler *et al.* 2014). According to Zhan *et al.* (2011) and Wheeler (1983), DHN-melanin, synthesized from 1,3,6,8-tetrahydroxynaphtalene, is admittedly the main form of melanin produced by ascomycetes, and tricyclazole was reported as an inhibitor of DHN-melanin synthesis. Conversely, copper treatment promoted melanin synthesis in cell walls of several fungi such as *Amorphotheca resinae*, *Aureobasidium pullulans*, and *Gaeumannomyces graminis* (Gadd & Griffiths 1980; Fogarty & Tobin 1996). DSEs are therefore suitable melanin-enriched fungal models to test whether a high melanin content in cell walls can affect ATMT. Finally, the majority of studies have investigated the transformation of a single strain or species using different protocols, so that generalizing the data is rather tricky.

We compared ATMT efficiency among eight strains belonging to four different DSE genera with an emphasis on the effect of (i) mycelium ageing, and (ii) the mycelium melanin content on ATMT efficiency.

Materials and methods

Strains, plasmid, and growth conditions

Eight DSEs were used. The species, their origin and accession numbers are detailed in Table 1. DSEs were cultured on malt extract agar (MEA) medium at 24 °C in the dark. The *Agrobacterium tumefaciens* strain EHA101 was used for fungal transformation. It harbours the pFAT-gfp binary vector that carries (i) the bacterial hygromycin B phosphotransferase (*hph*) ORF under the transcriptional control of the *Aspergillus nidulans trpC* promoter, and (ii) the green fluorescent protein (*gfp*) ORF under the control of the *A. nidulans* glyceraldehyde-3-phosphate promoter (Fitzgerald *et al.* 2003). The bacteria were grown at 28 °C on Luria–Bertani (LB) agar medium supplemented with 100 μg ml^{-1} spectinomycin.

Molecular identification of the fungal species

Fungal DNA was extracted using a REDExtract-N-Amp™ Plant PCR kit (Sigma–Aldrich, St-Quentin-Fallavier, France) according to the manufacturer's protocol. The internal transcribed spacer (ITS) region was amplified using the primers ITS1 and ITS4 (White *et al.* 1990). Twenty microlitres of a mixture containing 0.2 μM of each primer, 4 μl of genomic DNA, 5.2 μl of water, and 10 μl of REDExtract-N-Amp PCR ready mix were used for PCR amplification. The following PCR program was used: an initial denaturation step of 3 min at 94 °C, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR