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Screen for soil fungi highly resistant to dichloroaniline uncovers mostly *Fusarium* species

Laetitia Chan Ho Tong [a], Julien Dairou [b], Linh-Chi Bui [b], Julien Bouillon [a], Fernando Rodrigues-Lima [b], Jean-Marie Dupret [b], Philippe Silar [a,*]

[a] Univ. Paris Diderot, Sorbonne Paris Cité, Institut des Energies de Demain (IED), F-75205 Paris, France
[b] Univ. Paris Diderot, Sorbonne Paris Cité, Unit of Functional and Adaptive Biology (BFA), CNRS UMR 8251, F-75205 Paris, France

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ABSTRACT

Arylamines are frequent pollutants in soils. Fungi have proven to be efficient in detoxifying these chemicals by acetylating them using arylamine N-acetyl transferase enzymes. Here, we selected from natural soils fungi highly resistant to 3,4-dichloroaniline (DCA). *Fusarium* species were the most frequently isolated species, especially *Fusarium solani*. The sequenced strain of *F. solani* contains five NAT genes, as did all the DCA-resistant isolates. RT-PCR analysis showed that the five genes were expressed in *F. solani*. Expression of the *F. solani* genes in *Podospora anserina* and analysis of acetylation directly in *F. solani* showed that only the *NhNAT2B* gene conferred significant resistance to DCA and that *F. solani* likely uses pathways different from acetylation to resist high doses of DCA, as observed previously for *Trichoderma*.

1. Introduction

Arylamines are chemicals that are formed naturally, during woodland fires or by plants to defend themselves, or are produced by man. While some have important roles in industry and agronomy as antibiotics, herbicides or dyes, many are toxic and accumulate in the environment where they have harmful effect (Dupret et al., 2011). 3,4-dichloroaniline (DCA) is formed by the transformation of diuron and linuron, two widely used herbicides. DCA is hundred times more toxic than diuron, persists in soil and water reservoirs and may be difficult to remove. Fungi may prove efficient for this task, since they represent the major biomass of soil microorganisms and produce a plethora of enzymes able to break down many xenobiotics (Harms et al., 2011; Silar et al., 2011). For arylamine, removal may be initiated by the transformation of the toxic arylamine into its less toxic acetylated form. Arylamine N-acetyl transferases (NAT) are intracellular enzymes present in many living forms ranging from bacteria to humans and catalyze this reaction using acetyl-CoA as acetyl donor. They are especially efficient in fungi, where they are present in most species, often encoded by small gene families (Martins et al., 2010).

In the ascomycetes, these enzymes have particularly diversified and can be classified into at least seven families (Martins et al., 2010). In the maize pathogen *Fusarium verticillioides*, the *FDB2* gene encoding a NAT of family III is essential to remove two benzoxazinones synthesized by the plant against microbes and insects, thus enabling the fungus to infect the plant (Glenn and Bacon, 2009). In the dung fungus *Podospora anserina*, two NAT genes, *PaNAT1* and *PaNAT2* from family I and II, respectively, are present, one of which (*PaNAT2*) is sufficiently active toward DCA to envision remediation strategies (Martins et al., 2009). In this species, it was shown that acetylation is likely the only way by which DCA is rendered less toxic (Martins et al., 2009). On the contrary, in *Trichoderma virens*, a mycoparasite used in agronomy, and *Trichoderma reesei*, a saprobe used in industry to produce enzymatic cocktails, NAT play a less prominent role and other as yet unknown pathways removing altogether DCA and acetyl DCA are likely used, although the NAT pathway still plays a significant role in *T. reesei* (Cocaign et al., 2013). Because these two fungi are highly resistant to DCA, they are also promising candidates for remediation strategies. However, they may transform DCA into other chemical(s) with unidentified toxicity.

At the present time, the fungi that are best suited for remediation are unknown and those that have been tested were so because of educated guesses. *P. anserina* was chosen because of the facility to inactivate genes in this species, enabling rapid answers regarding the role of NAT in fungi, while the two *Trichoderma* were chosen because these organisms are already used in industry and

* Corresponding author at: Univ Paris Diderot, Sorbonne Paris Cité, Laboratoire Interdisciplinaire des Energies de Demain, case courrier 7040 Lamarck, 75205 Paris CEDEX 13, France. Tel.: +33 1 57 27 84 72.
E-mail address: philippe.silar@univ-paris-diderot.fr (P. Silar).

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agronomy. Here, we selected directly from soil fungi that are naturally highly resistant to DCA with the rational that these fungi may prove even better candidates than those already available.

2. Materials and methods

2.1. Fungal strains and growth conditions

The wild-type *P. anserina* strain and *ΔPaNAT1/2*, its derivative inactivated for the *PaNAT1* and *PaNAT2* genes, used in this study derived from the "S" (uppercase S) wild-type strain (Martins et al., 2009; Rizet and Delannoy, 1950) used for sequencing (Espagne et al., 2008; Grognet et al., 2014). Standard culture conditions, media and genetic methods for *P. anserina* have been described (Rizet and Engelmann, 1949; Silar, 2013) and the most recent protocols can be accessed at http://podospora.igmors.u-psud.fr/methods.php. The M2 medium has the following composition KH2PO4 0.25 g/l, K2HPO4 0.3 g/l, MgSO4/7H2O 0.25 g/l, Urea 0.5 g/l, Thiamine 0.05 mg/l, Biotin 0.25 μg/l, Citric Acid 2.5 mg/l, ZnSO4 2.5 mg/l, CuSO4 0.5 mg/l, MnSO4 125 μg/l, Boric Acid 25 μg/l, Sodium Molybdate 25 μg/l, Iron Alum 25 μg/l, Dextrine 5 g/l, Agar 12.5 g/l. The same media and protocols (Rizet and Engelmann, 1949; Silar, 2013) were used for the culture and analysis of the other fungi, i.e., all the strains isolated in this study, as well as the *Fusarium solani* reference strain 77-13-4. Strain 77-13-4 was a kind gift from Dr. Hans D. VanEtten.

For isolation of soil fungi resistant to DCA, minimal M2 medium plates were supplemented with 500 μM of DCA (Sigma Aldrich cat n° D55407) and 50 μg/ml of tetracycline and 25 μg/ml of chloramphenicol, inoculated with soil particles at one point near the edge and incubated on the bench for three weeks. On some plates, a fungal culture originating from the soil particles were observed. For each positive culture, an explant was excised from the growing edge and used for further analysis. The explants were inoculated on M2 medium containing 500 μM DCA, 50 μg/ml of tetracycline, 25 μg/ml chloramphenicol and 50 μg/ml of kanamycin. Bacteria were still present in the resulting cultures. Explants of the cultures were thus inoculated on fresh M2 medium devoid of DCA and containing 50 μg/ml of tetracycline, 25 μg/ml of chloramphenicol and 50 μg/ml of kanamycin. This resulted in bacteria-free fungal cultures. Bacteria were isolated from three positive cultures by streaking on LB agar medium.

2.2. Strain identification and analysis

DNA was extracted from fungi as described (Lecellier and Silar, 1994) and the ITS region was amplified using primers ITS1 and ITS4 (White et al., 1990). Sequences were then compared to GenBank with BLAST (Altschul et al., 1990). To check the presence of the NAT genes in the isolated strains, the verification primers of Table S1 were used to amplify the genes by PCR. RNA was extracted using the RNeasy Plant Mini Kit from Qiagen (Venlo, Netherlands) and treated with the RNAse-free DNAse Set also from Qiagen (Venlo, Netherlands). It was reverse transcribed using the RevertAid Reverse Transcriptase from Thermo Scientific (NYSE: TMO) and amplified using the GoTaq® G2 Flexi DNA Polymerase from Promega (Madison, WI, U.S.A.). Sequencing of the PCR products confirmed that transcripts of the relevant genes were actually amplified.

2.3. Transgenic expression in P. anserina

The five genes were amplified by PCR using strain 77-13-4 as template and the amplification primers from Table S1 using a high-fidelity DNA polymerase. Each coding sequence was amplified with ~500 bp of 5′-upstream and ~500 bp of 3′-downstream of the start and stop codons, respectively, including thus the potential promoters and terminators. The PCR fragments were then introduced independently in the *P. anserina ΔPaNAT1/2* strain using the same protocol as the one set up for *Trichoderma* (Cocaign et al., 2013). Briefly, the fragments were cloned into the pCIB4 plasmid digested with *Swa*I. This plasmid enabled integration in single copy into an expressed region of the *P. anserina* genome, as shown previously (Déquard-Chablat et al., 2012). The five resulting inserts, each carrying a different *F. solani* NAT gene, were sequenced to confirm that no mutation was generated during amplification and subsequent cloning. *Asc*I-linearized recombinant plasmids were then transformed into a *ΔPaNAT1/2 Δmus52::GenR* mutant strain, which lacked the mus52 subunit of the KU complex involved in non-homologous recombination. In such strain, DNA integration proceeded almost exclusively by homologous recombination. Nourseothricin-resistant transformants were obtained and two randomly-chosen transformants for each construct were crossed with the *ΔPaNAT1/2* mutant. In the F1 progeny, we obtained strains devoid of the *P. anserina* NAT genes and lacking the *Δmus52::GenR* mutation, but containing in a single copy either one of the *F. solani* NAT genes. These strains were sensitive to geneticin and resistant to nourseothricin, but also to hygromycin B and phleomycin, as hygromycin B and phleomycin resistance markers were used to inactivate *PaNat1* and *PaNat2*, respectively (Martins et al., 2009). Correct integration of each of the constructs was checked by PCR amplification in the F1 progeny with the primers used for amplifying the genes. In each of the tested transgenic strains (2 per constructs), a complete copy of the relevant *F. solani* gene was present.

To check for expression of the *F. solani* NAT genes in *P. anserina*, one strain for each gene was randomly selected. Total RNA was extracted and amplified as for *F. solani* (see above). The control used was the *Pa_6_9020* gene, which was amplified using primers 9020RTF (5′-ggccgagaaagctgccaaag-3′) and 9020RTR (5′-agagggcat tgtggctagg-3′). This gene encodes a component of the inner-mitochondrial membrane translocon (TIM complex) and is well expressed.

2.4. Detection of NAT activity

Fungi were grown for 2 days at 27 °C on M2 medium covered with a sterile cellophane sheet and harvested by scraping. The fungal biomass was mixed with glass beads in 1 vol. of lysis buffer (Tris HCl 20 mM, pH = 7.5; NaCl 100 mM; EDTA 1 mM; 0.05% Triton X-100) and broken using the "TeSeE™ PRECESS 24™" (Bio-Rad, Marnes-la-Coquette, France) for 45 s at 5000 rpm. Samples were centrifuged at 13,200 rpm for 15 min at 4 °C. Protein concentrations were determined in the supernatants with the Bradford assay from Bio-Rad (Marnes-la-Coquette, France). Enzymatic activity was measured with the 2-aminofluorene (2-AF) assay (Cocaign et al., 2013). Crude extracts (500 μg) and 2-AF (1 mM final concentration) in 150 μL assay buffer (Tris HCl 20 mM, pH = 7.5) were incubated for 5 min at 37 °C in a 96-well plate. Acetyl-CoA (1 mM final concentration) was added and the plate was further incubated at 37 °C for 15 min. 50 μL of the reaction were withdrawn and the reaction stopped by adding 50 μL of 15% perchloric acid. The acetylation rate of 2-AF by the fungal extracts was measured by reversed-phase HPLC (Shimadzu HPLC system interfaced with the LabSolution software). Samples were injected onto a C18 column (length = 100 mm; internal diameter = 4.6 mm; particle size = 2.5 μm) at 40 °C. The mobile phase used for separation consisted of 60% methanol in 20 mM sodium perchlorate buffer (v/v), pH = 3. The flow rate was kept constant at 0.5 ml/min. The products were monitored spectrophotometrically at 280 nm and quantified by integration of the peak