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# Development of an *Agrobacterium*-mediated transformation system for the cold-adapted fungi *Pseudogymnoascus destructans* and *P. pannorum*

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ABSTRACT

The mechanisms of cold adaptation by fungi remain unknown. This topic is of high interest due to the emergence of white-nose syndrome (WNS), a skin infection of hibernating bats caused by *Pseudogymnoascus destructans* (*Pd*). Recent studies indicated that apart from *Pd*, there is an abundance of other *Pseudogymnoascus* species in the hibernacula soil. We developed an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system for *Pd* and a related fungus *Pseudogymnoascus pannorum* (*Pp*) to advance experimental studies. *URE1* gene encoding the enzyme urease was used as an easy to screen marker to facilitate molecular genetic analyses. A Uracil-Specific Excision Reagent (USER) Friendly pRF-HU2 vector containing *Pd* or *Pp ure1::*hygromycin (*HYG*) disruption cassette was introduced into *A. tumefaciens* AGL-1 cells by electroporation and the resulting strains were co-cultivated with conidia of *Pd* or *Pp* for various durations and temperatures to optimize the ATMT system. Overall, 680 *Pd* (0.006%) and 1800 *Pp* (0.018%) transformants were obtained from plating of $10^7$ conidia; their recoveries were strongly correlated with the length of the incubation period (96 h for *Pd*; 72 h for *Pp*) and with temperature (15–18 °C for *Pd*; 25 °C for *Pp*). The homologous recombination in transformants was 3.1% for *Pd* and 16.7% for *Pp*. The availability of a standardized ATMT system would allow future molecular genetic analyses of *Pd* and related cold-adapted fungi.



## 1. Introduction

*Pseudogymnoascus destructans* (*Pd*) is the etiologic agent of white-nose syndrome (WNS), a skin infection of hibernating bats. This pathogen has caused unprecedented reductions in the abundance of the bat populations in the northeastern United States since 2006, with up to 95% mortality in some hibernacula (Blehert et al., 2009; Chaturvedi et al., 2010; Frick et al., 2010; Blehert, 2012). *Pd* is a newly recognized fungus that is well adapted to growing under cold conditions found in caves and mines. It secrets proteolytic enzymes documented in other pathogenic fungi to cause vertebrate skin infections. The pathogen appears to have a clonal population in the US (Rajkumar et al., 2011; Ren et al., 2012). However, the precise mechanisms by which *Pd* survives and invades the skin of the hibernating bats are not clear. Phylogenetic analyses of hibernacula soil revealed large number of *Pseudogymnoascus* spp., which have not been implicated in WNS (Minnis and Lindner, 2013). These results indicated that *Pd* possess certain unique virulence repertoire to cause disease in bats, which seems to be missing in other *Pseudogymnoascus* spp. It is also conceivable that mammalian or non-mammalian hosts are yet to be identified for other *Pseudogymnoascus* spp.

Recently, integrated genome profile analyses of *Pd* and *Geomyces pannorum* (re-named as *Pseudogymnoascus pannorum;* accession no. AYKR00000000) yielded preliminary information on the fundamental biological capabilities including pathogenicity, cold adaptation, and regulation mechanisms (Chibucos et al., 2013). However, follow-up experimental studies are not yet possible due to unavailability of an efficient transformation system. The lack of molecular tools also extends to other fungi inhabiting the cold environment.

The *Agrobacterium tumefaciens*-mediated transformation system (ATMT) has been successfully used for molecular genetic studies of yeasts and filamentous fungi, including *Saccharomyces* (Piers et al., 1996), *Cryptococcus* (McClelland et al., 2005), *Coccidioides* (Abuodeh et al., 2000), *Aspergillus* (Gouka et al., 1999; Michielse et al., 2005), *Trichophyton* (Yamada et al., 2009), *Fusarium* (Mullins et al., 2001), *Glomus* (Helber and Requena, 2008), *Oculimacula* (Eckert et al., 2005) and *Verticillium* (Dobinson et al., 2004). ATMT also has been shown to be an important alternative to other fungal transformation systems, as it yields a high number of transformants, is easier to perform, does not require special

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equipment, and is characterized by a high frequency of homologous recombination (Frandsen, 2011).

The enzyme urease encoded by *URE1* gene is an important virulence factor for the fungal pathogens *Cryptococcus neoformans* and *Coccidioides posadasii* (Olszewski et al., 2004; Mirbod-Donovan et al., 2006) and the bacterial pathogens *Helicobacter pylori* and *Proteus mirabilis* (Jones et al., 1990; Eaton et al., 1991; Tsuda et al., 1994). It has been reported that *Pd* also secretes significant amounts of urease, but its precise function in either the saprophytic or parasitic life of this fungus is not clear (Chaturvedi et al., 2010; Reynolds and Barton, 2014). In this report, we have used the urease-encoding gene *URE1* as an easily screenable marker to develop an ATMT system for cold-adapted fungi, which would prove valuable for future molecular genetics and pathogenic studies of *Pd* and other related species in this genus.

## 2. Materials and methods

### 2.1. Strains, plasmids and media

All strains and plasmids used in this study are listed in Table 1. *P. destructans* (*Pd*) and *P. pannorum (Pp)* strains were routinely maintained on yeast extract peptone dextrose (YPD) agar and stored in 15% glycerol at −70 °C. Potato dextrose agar (PDA; Difco), Christensen's urea agar (Sigma–Aldrich), YNB medium [2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Cat. no. 233520) and 2% agar], YNB-U (0.227% urea as sole source of nitrogen) were used to assess phenotypes of mutants and wild type (WT) strains of *Pd* and *Pp*. The induction medium (IM) used for co-cultivation experiments was composed of $K_2HPO_4{\cdot}3H_2O$ 1.9 g, $K_2HPO_4$ 2.05 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 0.5 g, glucose 1.8 g, 4% (w/v) glycerol and a final concentration of 40 mM MES hydrate (Sigma–Aldrich) per liter (Zhang et al., 2013). Yeast extract broth (YEB) used for culture of *A. tumefaciens* (AGL-1) cells contained 10 g peptone, 1 g yeast extract, 5 g sucrose and 0.5 g $MgSO_4{\cdot}7H_2O$ per liter and adjusted to pH 7.0.

### 2.2. URE1 gene characterization from Pd and Pp

DNA sequences of *PdURE1* and *PpURE1* were first obtained from *Pd* (http://broadinstitute.org/annotation/genome/Geomyces_destructans/MultiHome.html) and *Pp* (http://fungalgenomics.concordia.ca/fungi/Gpan.php) genome databases and then blasted

**Table 1**
Strains, plasmids and oligonucleotides used in this study.

| Strains | Description | Source |
|---|---|---|
| *E. coli* Top10 | lacϒ74 recA1 deoR F – mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80 lacZΔM15Δ araD139Δ (ara-leu)7697 galU galK | Invitrogen |
| *Agrobacetrium tumefaciens* (AGL-1) | Genotype-AGL0 recA::bla pTiBo542deltaT Mop+ CbR | Dr. Seogchan Kang, University Park, PA, USA |
| *Pseudogymnoascus destructans* (*Pd*) M1379 | Wild type strain | Bat skin (Chibucos et al., 2013) |
| *Pseudogymnoascus pannorum* (*Pp*) M1372 | Wild type strain | Soil (UAMH1062) (Chibucos et al., 2013) |
| *Pdure1*–10 | ure1 mutant of *Pd* | This study |
| *Pdure1*–25 | ure1 mutant of *Pd* | This study |
| *Ppure1*–4 | ure1 mutant of *Pp* | This study |
| *Ppure1*–11 | ure1 mutant of *Pp* | This study |
| Plasmid | Description | Source |
| pRF-HU2 | Vector for targeted gene replacement containing *HYG* gene as selection marker | (Frandsen et al., 2008) |
| *pPdure1::HYG* | 5′ and 3′ prime arms of *PdURE1* gene cloned into pRF-HU2 | This study |
| *pPpure1::HYG* | 5′ and 3′ prime arms of *PpURE1* gene cloned into pRF-HU2 | This study |
| Oligonucleotides | Sequence (5′–3′) | Purpose |
| V2149a | GGTCTTAAUTTTAGGCGGAGCTATGAC | *pPdure1::HYG* vector construction |
| V2150a | GGCATTAAUAAGGGCGTTGATGATGAC | *pPdure1::HYG vector construction* |
| V2151a | GGACTTAAUTATGTTGCAGGCTTTGGA | *pPdure1::HYG* vector construction |
| V2152a | GGGTTTAAUGATCTGGCATCATATCGTC | *pPdure1::HYG* vector construction |
| V2171 | AGCTGCGCCGATGGTTTCTACAA | $Hyg^+$ transformant screening and *HYG* probe for Southern analysis of *Ppure1* mutants |
| V2172 | GCGCGTCTGCTGCTCCATACAA | $Hyg^+$ transformant screening and *HYG* probe for Southern analysis of *Ppure1* mutants |
| V2173 | GGGTTTAAUTGCCACCGCCAATGTATC | *pPpure1::HYG* vector construction |
| V2174 | GGACTTAAUTCATCATCAACGCCCTTA | *pPpure1::HYG* vector construction |
| V2175 | GGCATTAAUACGGTGACGAGGTAGGTT | *pPpure1::HYG* vector construction |
| V2176 | GGTCTTAAUGACGAGGCGGTTGAGGAA | *pPpure1::HYG* vector construction |
| V2199 | CCATACCGTCACGCAGAG | *Ppure1* mutant screening |
| V2200 | AATAAAGGGCGGAAGAGG | *Ppure1* mutant screening |
| V2201 | TAAATCCGTTCTTGATGCCGATATC | *Ppure1* mutant screening |
| V2202 | AATGTTGCGGATCTCATG | *Ppure1* mutant screening |
| V2203 | ATTGTTGATTGGAGTGGTATT | *Pdure1* mutant screening |
| V2204 | CCGCATGTTGTTCTTGCC | *Pdure1* mutant screening |
| V2205 | CATCATCAACGCCCTTAT | *Pdure1* mutant screening |
| V2206 | AGCTCCCATGTCGTGTAA | *Pdure1* mutant screening |
| V2281 | GACCAGTTGCCTAAATGAA | *Pdure1* mutant screening |
| V2282 | GTAAGTATGGGAAGCAGAAA | *Pdure1* mutant screening |
| V2285 | TTTCGGCGTGGGTATGGT | *Ppure1* mutant screening |
| V2286 | TCGGATTCAAGCGACTGG | *Ppure1* mutant screening |
| V2370 | TTCAGTAACGTTAAGTGGATCC | *HYG* probe for Southern analysis of *Pdure1* mutants |
| V2372 | CGCAAGGAATCGGTCAATAC | *HYG* probe for Southern analysis of *Pdure1* mutants |

The underlined sequences denote 9-bp long 2-deoxyuridine containing overhangs for directional cloning into pRF-HU2 vector (Frandsen et al., 2008).