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Green Fluorescent Protein Expression in *Pseudogymnoascus destructans* to Study Its Abiotic and Biotic Lifestyles

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Abstract *Pseudogymnoascus destructans* (*Pd*) is the etiologic agent of bat White-nose syndrome, a disease that has caused the unprecedented reduction in the hibernating bat populations across eastern North America. The *Pd* pathogenesis appears to be a complex adaptation of fungus in its abiotic (caves and mines) and biotic (bats) environments. There is a general lack of experimental tools for the study of *Pd* biology. We described the successful expression of codon-optimized synthetic green fluorescent protein sGFP in *Pd*. The *sGFP*(*S65T*) gene was first fused in frame with the *Aspergillus nidulans* promoter in the tumor-inducing plasmid pRF-HUE, and the resulting plasmid pHUE-*sGFP*(*S65T*) was transformed into *Pd* by *Agrobacterium tumefaciens*-mediated transformation system. The integration of *sGFP*(*S65T*) in *Pd* genome was analyzed by PCR, and single integration frequency of approximately 66% was confirmed by Southern hybridization. Fluorescent microscopy and flow cytometric analyses of two randomly selected transformants with single integration revealed high expression of sGFP in both spores and hyphal structures. The biology of mutants as judged by sporulation, growth rate, and urease production was not altered indicating sGFP is not toxic to *Pd*. Thus, we have generated a valuable tool that will facilitate the elucidation of *Pd* biology, ecology, and pathogenicity in real time.

Keywords *Pseudogymnoascus destructans* · Green fluorescent protein (GFP) · White-nose syndrome (WNS) · ATMT · Confocal microscopy · Flow cytometry

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Introduction

White-nose syndrome (WNS) is an emerging cutaneous infectious disease of the hibernating insectivorous bats. The disease has caused unprecedented death in bat populations across eastern North America for almost a decade [1–4]. The etiologic agent of WNS is a slow growing psychrophilic fungus, *Pseudogymnoascus destructans* (*Pd*), which infects the more sparsely haired area on the skin of the hibernating bats and is capable of invading living bat tissues [1]. The fungus appears to have a clonal population in the USA [5, 6]. Environmental studies of *Pd* are rare, but an extensive survey of affected sites revealed the wide distribution of *Pd* DNA [7]. Our mycobiome studies revealed frequent recovery of *Pd* in the surveyed sites confirming other reports that the pathogen was distributed in WNS-afflicted bats habitats dominated by a few cosmopolitan fungal species [8]. The precise mechanisms by which *Pd* survives in the environment and invades living tissues of hibernating bats are far from clear. The lack of experimental tools further complicates our understanding of *Pd* biology and pathogenicity.

The green fluorescent protein (GFP) has been widely used as a reporter gene in many prokaryotes [9, 10] and eukaryotes [11, 12]. The GFP is a protein composed of 238 amino acid residues that exhibits bright green fluorescence when exposed to light in the blue to the ultraviolet range [13]. The successful expression of GFP was also extended to molecular genetic studies of yeast and filamentous fungi, including *Candida* [14, 15], *Ustilago* [16], *Colletotrichum* [17–19], *Trichoderma* [20], *Aspergillus* [21], *Magnaporthe* [22, 23], *Phytophthora* [24], and *Neurospora* [25, 26]. Several mutations in wild-type GFP have been created to improve detection and expression of the fluorescent protein, and one such mutation is by the replacement of the serine at position 65 with a threonine (S65T) has been shown in *E. coli* to result in enhanced brightness, faster chromophore formation and slower photobleaching [27]. This change was also introduced into synthetic GFP (sGFP) by site-directed mutagenesis, to create *sGFP*(*S65T*). The codon-optimized sGFP has been successfully used as a vital reporter in several plant pathogenic fungi [28] and in filamentous fungi *Aspergillus niger* [29].

In the present study, we describe the successful expression of sGFP in *Pd* by *Agrobacterium*-mediated transformation system (ATMT). Thus, we have generated a valuable tool that will facilitate the elucidation of *Pd* biology, ecology, and pathogenicity in real time.

Materials and Methods

Strains, Media, Plasmids, and Oligonucleotides

The information on strains, plasmids, and oligonucleotides used in this study is listed in Table 1. *Pseudogymnoascus destructans* wild-type (WT) and *sGFP*(*S65T*) expressing mutant strains were maintained on Sabouraud dextrose agar (SDA) and stored in 15% glycerol at − 80 °C. The induction medium (IM) for co-cultivation of *Pd* and *Agrobacterium tumefaciens* AGL-1 cells were prepared as described in our earlier publication [30]. Potato dextrose agar (PDA) supplemented with cefotaxime (200 µg/ml), and hygromycin (100 µg/ml) was used for the selection of hygromycin positive (hyg^{+}) mutants. The PDA medium alone was used for assessing sporulation, and Christensen's urea agar was used for urease enzyme production. All plasmids were maintained in *Escherichia coli* grown in Luria–Bertani (LB) broth containing kanamycin (50 µg/ml).

Construction of GFP Expression Plasmid

Previous attempt to express GFP in *Pd* using the pCT74 plasmid in which the codon-optimized *sGFP*(*S65T*) gene was under the control of promoter *ToxA* from *Pyrenophora tritici-repentis* was unsuccessful (personal communication). Therefore, a new GFP expression vector was constructed in the backbone of pRF-HUE plasmid. In brief, *sGFP*(*S65T*) gene was PCR-amplified from pCT74 plasmid using primer pair V2301/V2302 and PfuTurbo®Cx Hotstart DNA polymerase enzyme (Stratagene, Cedar Creek, TX). The 990-bp amplicon containing *sGFP*(*S65T*) gene and NOS terminator was cloned in frame into pRF-HUE plasmid at the downstream of strong constitutively expressed glyceraldehyde phosphate dehydrogenase promoter of *Aspergillus nidulans* (PgpdA) (Fig. 1).

Table 1 Strains, plasmids and oligonucleotides used in this study

Strains	Description	Source
E. coli	*lacx74 recA1 deoR F—mcrA*Δ (*mrr-hsdRMS-mcrBC*) ϕ*80 lacZ*Δ*M15*Δ*araD139*Δ*(ara-leu)7697 galU galK*	Invitrogen
A. tumefaciens AGL-1	Carrying pCAMBIA3301 plasmid, Kan^R	Dr. Seagchan Kang, University Park, PA, U.S.A.
P. destructans (Pd) M1379	Wild-type strain	This study
Pd-sGFP(S65T)-*M1*	*GFP* mutant of *Pd*	This study
Pd-sGFP(S65T)-*M2*	*GFP* mutant of *Pd*	This study

Plasmids	Description	Source
pCT74	GFP expression vector for fungi, hyg^R	[28]
pRF-HUE	Vector for ectopic overexpression of genes, hyg^R	[49]
pHUE-sGFP(S65T)	*sGFP* ectopic overexpression vector	This study

Oligonucleotides	Sequence (5′-3′)	Purpose
V2171	AGCTGCGCCGATGGTTTCTACAA	*Hyg* gene screening
V2172	GCGCGTCTGCTGCTCCATACAA	*Hyg* gene screening
V2301	GGACTTAAUGGTGAGCAAGGGCGAGGAG	*sGFP*(*S65T*) amplification from pCT74
V2302	GGGTTTAAUGATCTAGTAACATAGATGAC	*sGFP*(*S65T*) amplification from pCT74
V2327	CACAAGTTCAGCGTGTCCG	*sGFP* gene screening
V2328	GTTCACCTTGATGCCGTTC	*sGFP* gene screening

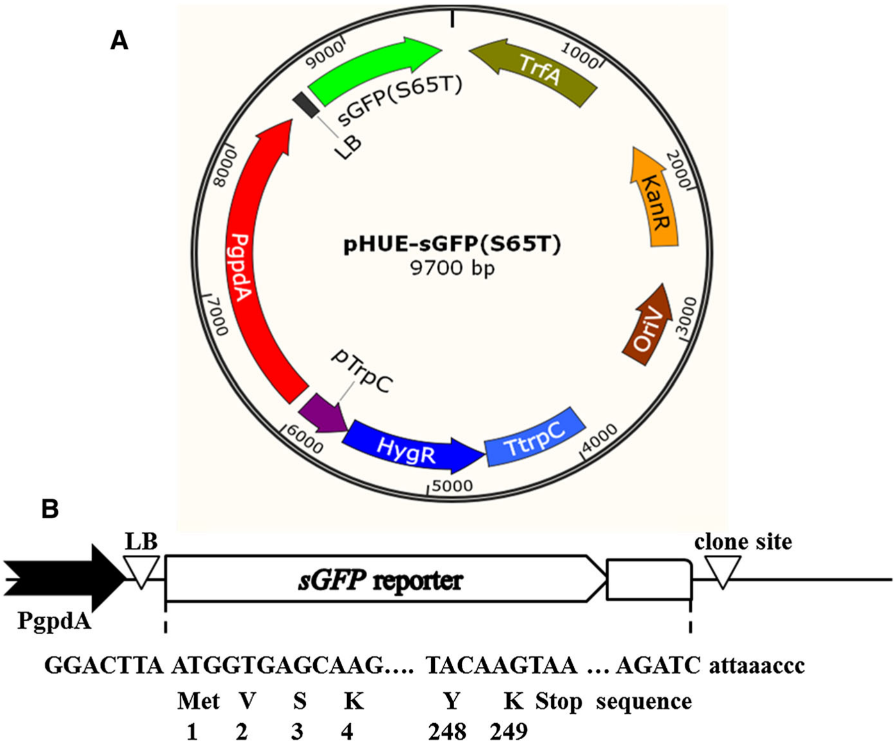
A. tumefaciens-Mediated Transformation and sGFP Expression in *Pd*

The *sGFP*(*S65T*) was transformed into *Pd* by ATMT system as described in a publication from our laboratory [30]. In brief, AGL-1 cells containing pHUE-*sGFP*(*S65T*) was grown in 5 ml of yeast extract broth (YEB) supplemented with 50 µg/ml kanamycin with shaking at 250 rpm for two days at 28 °C. Five hundred microliters of the culture were transferred to 5 ml of induction medium (IM) containing 200 µM acetosyringone (AS; Sigma-Aldrich, St. Louis, MO) and incubated for additional 6–7 h with shaking at 250 rpm at 28 °C until OD_{600} reached to approximately 0.8–1.0. For ATMT, *Pd* conidial suspension (10^7 conidia/ml) was prepared as described previously [30], and aliquots of 100 µl (10^6 conidia) were mixed with an equal volume of AGL-1 cells (10^7 cells) and this mixture was evenly spread onto sterilized nitrocellulose membranes (Cat. No. D9527, Sigma-Aldrich) placed onto PDA agar medium. Following co-cultivation at 15 °C for three days, the nitrocellulose membranes were lifted from PDA agar and transferred to PDA selection medium (200 µg/ml cefotaxime, and 100 µg/ml hygromycin B). Individual putative Hyg$^+$ transformants recovered were transferred to fresh PDA supplemented with 100 µg/ml hygromycin B, and plates were incubated at 15 °C for at least two weeks.

PCR and Southern Hybridization

The genomic DNA from the Hyg$^+$ transformants was extracted using MasterPure™ Complete DNA & RNA Purification Kit (Epicenter, Madison, WI). These transformants were first screened for the presence of *hyg* and *sGFP*(*S65T*) genes by PCR. The PCR conditions were: initial denaturation at 95 °C for 3 min followed by 30 cycles of 94 °C for 30 s, annealing at 58 °C for *hyg* or 56 °C for *sGFP*(*S65T*) and extension at 72 °C for 1 min. Twelve of the Hyg$^+$ transformants were assessed for t-DNA integration by

Fig. 1 Construction of pRF-HUE-sGFP(S65T) plasmid. The plasmid pRF-HUE was digested with restriction enzyme *PacI* and the nicking enzyme *Nt. BbvCI* followed by cloning of 990-bp *sGFP*(*S65T*) PCR amplicon. The resulting plasmid contained in-frame *sGFP*(*S65T*) downstream of strong constitutive *PgpdA* promoter (**a**). The diagrammatic representation of fusion construct of *PgpdA* and *sGFP*(S65T) reporter gene is shown (**b**)



Southern analysis. In brief, gDNA (~ 5 µg) from transformants and parent strains were digested with *Nde*I, electrophoresed on a 1.0% agarose gel, transferred onto nitrocellulose and analyzed using high-efficiency hybridization system (Molecular Research Center, Cincinnati, OH). The PCR-amplified fragment of *hyg* labeled with [^{32}P]-dCTP (PerkinElmer) was used as a probe. Hybridization was performed at 55 °C. DNA gel blots were visualized according to the procedure of Molecular Dynamics apparatus.

Flow Cytometry

Fungal conidial suspensions of *Pd* WT and *Pd* expressing sGFP(S65T) strains were prepared as described previously [30]. For FACS analysis, the conidial suspensions were suspended in phosphate buffered saline (PBS) at the concentration of 10^6 conidia/ml. *Pd* conidial suspensions were passed through a 488-nm laser for excitation, and fluorescence emissions were collected at 512 nm for GFP. The dead cells and debris were excluded from the analysis based on forward angle and side scatter light gating. Approximately 20,000 gated events were collected for analysis. Excitation of sGFP(S65T) was achieved using an argon ion laser emitting 488 nm light. All analyses were performed using BD FACS can benchtop flow cytometer with BD CellQuest software (BD Biosciences, San Jose, CA).

Fluorescence and Confocal Microscopy

The putative hyg$^+$ transformants were screened for sGFP(S65T) expression by an Olympus Provis AX70 fluorescence microscope with the excitation filter at 470–490 nm and barrier filter at 525–550 nm. Confocal imaging was performed on a Leica TCS SP5 confocal system. For hyphae and spores staining, 15 µl aqueous mounting medium (ProLong® Gold antifade reagent with DAPI, Molecular Probes®, Inc.) was used for each slide.

Genetic Stability of *Pd*-sGFP(S65T) Strains

The genetic stability of the mutant strains was assessed by monitoring *sGFP*(S65T) expression and conferring resistance to hygromycin B. The *Pd*-sGFP(S65T) strains were first passed through a non-selection medium (PDA without hygromycin B). After four successive passages, the fungal conidial suspensions were prepared from both the parent and the mutant strains, normalized to OD_{600} of 1.0, and 10 µl of suspensions was spotted onto PDA containing hygromycin B, SDA and Christensen's urea agar (Sigma-Aldrich, St. Louis, MO) media. All agar plates were incubated at 15 °C for seven days.

Results

Characterization Studies of the *sGFP* Transformants

A total of 87 hyg^+ transformants were recovered from plating of 2×10^7 *Pd* conidia on PDA selection medium, with the resulting transformation efficiency of 0.000435%. Of 87 hyg^+ transformants, 12 were randomly selected, sub-cultured, and PCR screened for T-DNA insertion using *hyg* and *sGFP*(*S65T*) genes. All the hyg^+ transformants displayed 600-bp amplicon against *hyg* gene and 400-bp amplicon against *sGFP*(*S65T*) gene, confirming the integration of T-DNA into the *Pd* genome (Supplementary Figure 1). Next, the T-DNA integration frequency was determined. The Southern analysis with *hyg* probe revealed that of 12 hyg^+ transformants, eight transformants displayed a single copy of T-DNA integration (67%), three transformants showed two copies of T-DNA integrations (25%), and one transformant exhibited three copies of T-DNA integrations (8%). A representative blot with five transformants with single, double and triple integrations of T-DNA is shown (Supplementary Figure 2). All the transformants had the typical emerald green fluorescence when observed under the microscope using the FITC filter (personal communication). Based on these results, two of the transformants with single integration event were chosen, and they were designated as *Pd*-sGFP(S65T)-M1 and *Pd*-sGFP(S65T)-M2. Both the mutant strains were confirmed to contain *hyg* and *sGFP*(*S65T*) amplicons (Fig. 2a) and they displayed single integration of *hyg* gene at different locations in the genome of *Pd* (Fig. 2b).

Microscopic Analysis of the *sGFP* Transformants and Flow Cytometry

The spores of *Pd*-sGFP(S65T)-M1 and *Pd*-sGF*P*(S65T)-M2 strains displayed high expression of green fluorescence as compared to the untransformed parent strain. The geometric mean fluorescence calculated from 20,000 events was 48.98 for M1 and 50.88 for M2 strain while it was 5.77 for the parent strain (Fig. 3). These results also confirmed that the codon-optimized sGFP(S65T) under the control of *A. nidulans* promoter *PgpdA* is highly expressed in *Pd*. Additionally, results also suggested that a single copy of sGFP(S65T) randomly integrated into *Pd* genome is adequate, as both the mutants showed comparable fluorescence. Since flow cytometry was applied to only dissociated cells (spores), we used confocal microscopy to determine GFP expression in the whole organism. The sGFP(S65T) expression in the hyphae appeared to be punctate and brightest around the nucleus while it was uniformly distributed in the spores. A representative mutant strain, i.e., *Pd-sGFP*(*S65T*)-M1, expressing GFP in hyphae and spores is shown (Figs. 4, 5).

Stable Expression of sGFP(S65T) in *Pd* Strains

Pd-sGFP(*S65T*)-M1 and *Pd-sGFP*(*S65T*)-M2 mutant strains grew efficiently on PDA containing hygromycin B after repeated sub-culture on non-selection medium (PDA alone). These results indicated that both the sGFP(S65T) expressing *Pd* strains were mitotically stable (Fig. 6a). The mutant strains did not show any defect of growth when compared to the WT strain on rich or poor medium (Fig. 6b). No morphological changes in size or shape of vegetative structures were observed (Fig. 6a, b). Similarly, other metabolic activities did not alter, as urease enzyme of the mutant strains converted urea as efficiently as the WT strain on Christensen's urea agar (Fig. 6c). These results indicated that single integration of *sGFP*(*S65T*) is not toxic to *Pd*.

Discussion

In this study, we describe the successful expression of codon-optimized *sGFP*(*S65T*) in *P. destructans,* an etiologic agent of WNS. The expression of *sGFP*(*S65T*) was found to be at all the stages of *Pd* growth including hyphae and spores. The *sGFP*(*S65T*) expressing *Pd* would likely to facilitate the understanding of the interactions of *Pd* with its biotic (bat) and abiotic (cave and mine) environments (Fig. 6).

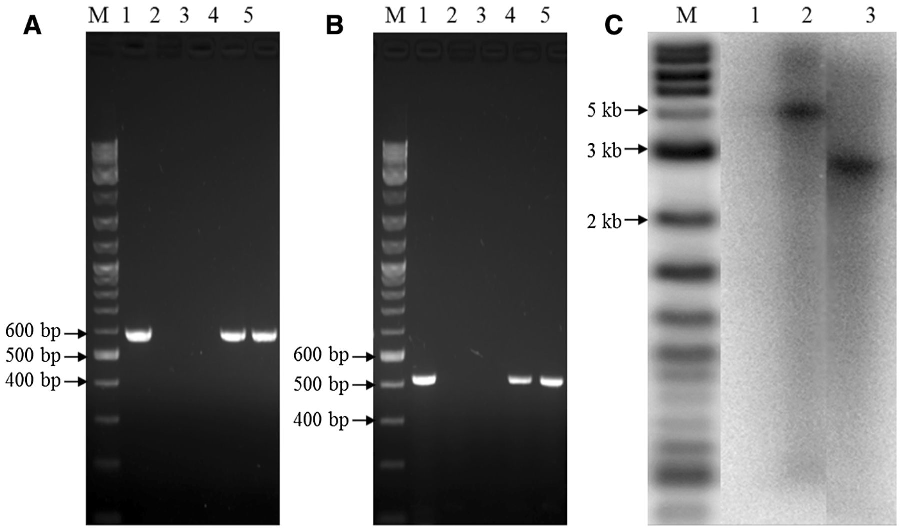


Fig. 2 Characterization of *Pd-sGFP*(*S65T*) expressing strains. **a** *hyg* diagnostic PCR analyses. DNA from *Pd-sGFP*(*S65T*)-M1 and *Pd-sGFP*(*S65T*)-M2 mutants yielded approximately 600-bp amplicons against *hyg* (V2171/V2172). DNA from *Pd* WT did not yield any PCR amplicon. The pHUE-sGFP(S65T) plasmid served as a positive control, and no DNA template control (NTC) served as a negative control. The marker was 2-Log DNA ladder (New England Labs). Lane 1: pHUE-*sGFP*(*S65T*); lane 2: *Pd* wild type; lane 3: NTC; lane 4: *Pd-sGFP*(*S65T*)-M1; lane 5: *Pd-sGFP*(*S65T*)-M2. **b** *sGFP*(*S65T*) gene diagnostic PCR analyses. DNA from *Pd-sGFP*(*S65T*)-M1 and *Pd-sGFP*(*S65T*)-M2 mutants yielded approximately 400-bp amplicons against *sGFP*(*S65T*) gene, respectively. DNA from *Pd* WT did not yield any PCR amplicon. The pHUE-sGFP(S65T) plasmid served as a positive control, and no DNA template control (NTC) served as a negative control. The marker was 2-Log DNA ladder (New England Labs). Lane 1: pHUE-*sGFP*(*S65T*); lane 2: *Pd* wild type; lane 3: NTC; lane 4: *Pd-sGFP*(*S65T*)-M1; lane 5: *Pd-sGFP*(*S65T*)-M2. **c** Southern blot analysis. Genomic DNA cut with *Nde*I and hybridized with 585-bp *hyg* probe yielding 5-kb fragment for *Pd-sGFP*(*S65T*)-M1- and 2.5-kb fragment for *Pd-sGFP*(*S65T*)-M2. These results confirmed a single T-DNA integration at two different locations in the *Pd* genome. The *Pd* WT strain was negative for *hyg* gene as no signal was detected against *hyg* probe. Lane 1, *Pd* wild type; lane 2, *Pd-sGFP*(*S65T*)-M1; lane 3, *Pd-sGFP*(*S65T*)-M2

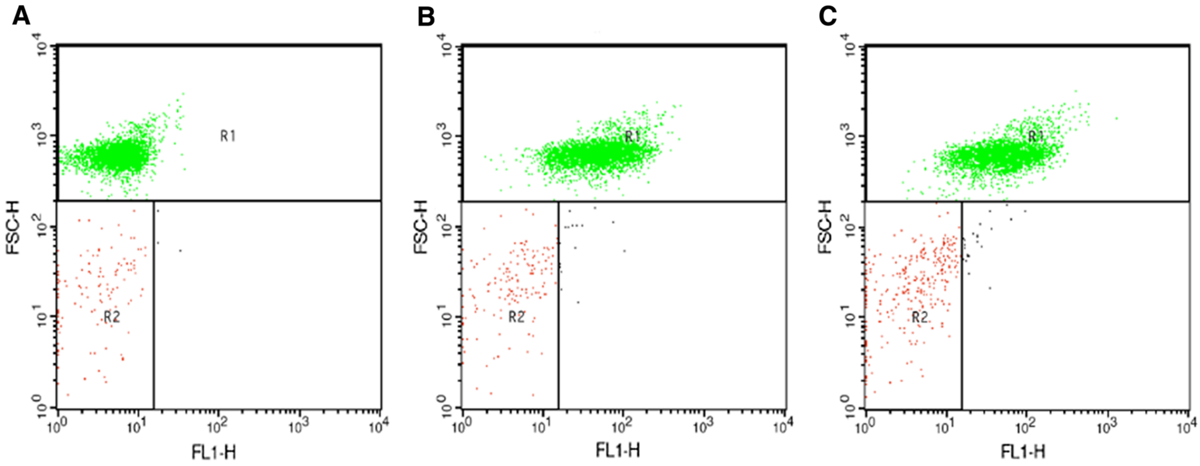


Fig. 3 Flow cytomteric analysis of *Pd*-sGFP(S65T) strains. The spores of *Pd* WT and mutant strains were harvested and analyzed by flow cytometry. FSC (*Y*-axis) is the forward scatter channel, which denotes the size of the cells; FL-1 (*X*-axis) is the fluorescence 1 channel, which is used for the detection of GFP. The spore populations were gated for live (R1) and dead or debris of dead (R2) populations. Both the mutant strains (*Pd-sGFP*(*S65T*)-M1 and *Pd-sGFP*(*S65T*)-M2) expressed significantly (tenfold) increased fluorescence (**b** and **c**) compared to the WT strain (**a**)

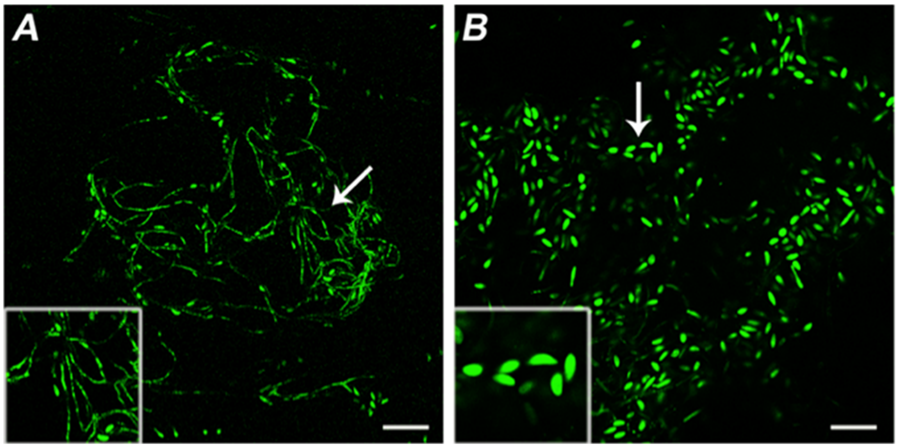


Fig. 4 Scanning Laser Confocal Microscopy of sGFP-expressing *Pd*. GFP expression was observed in *Pd* in both (**a**) mycelia (**b**) spores. Arrows indicate the magnified areas in the inset. Images of *Pd* GFP transformants were taken with a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). To prevent photobleaching, *Pd* was mounted on ProLong® Gold antifade reagent (Life Technologies, Green Island NY). Images were taken with a 40X oil objective with no zoom for (**a**) and a zoom of 2.1 for (**b**). Scale bars are 10 μm

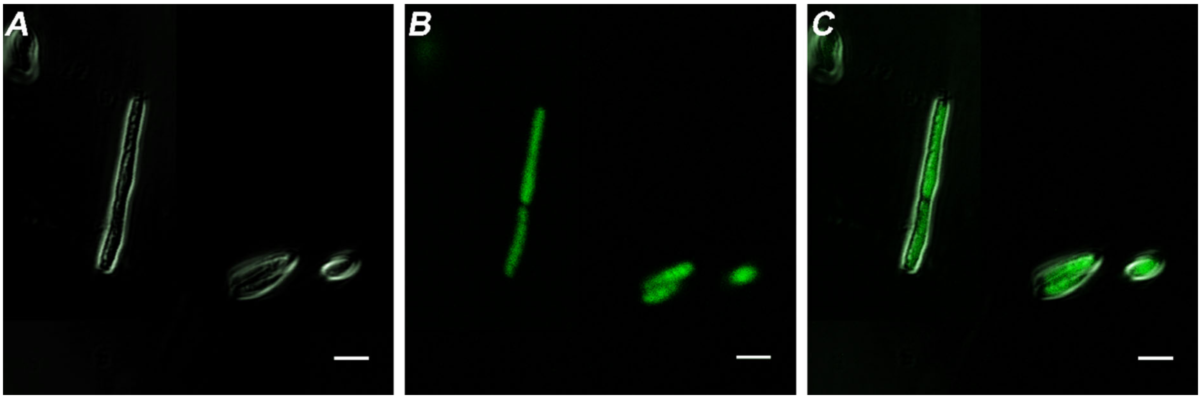


Fig. 5 Scanning Laser Confocal Microscopy using bright field and fluorescence shows sGFP-expressing *Pd*. *Pd* expresses sGFP (**a**) Bright field image of *Pd* (**b**) GFP expression of *Pd* and (**c**) Merge image of **a** and **b**. Images of *Pd* GFP transformants were taken with a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). To prevent photobleaching, *Pd* was mounted on ProLong® Gold antifade reagent (Life Technologies, Green Island NY). Images were taken with a 40X oil objective with no zoom. Scale bars are 5 μm

The wild-type jellyfish *Aequorea victoria* GFP does not confer discernible fluorescence to many fungi, primarily because it is not efficiently translated [21, 31]. Therefore, the modified form of GFP with optimized codon usage has been developed for yeast [32], plants [33], and mammals [34]. The optimized codon *sGFP*(*S65T*) under the control of promoter *ToxA* was successfully expressed in *Aspergillus niger* and several plant pathogenic fungi [29, 35].The successful expression of sGFP(S65T) under the control of constitutively expressed glyceraldehyde phosphate dehydrogenase promoter (*PgpdA*) from *A. nidulans* in *Pd* further complemented previous studies. We noticed the more intense expression of sGFP(S65T) in the spores than in the hyphal structures. The differential pattern observed could be due to spores being a final entity while hyphae are continuously growing and dividing structures.

The present study is the second report of the successful use of ATMT system for the expression of heterologous marker *sGFP*(*S65T*) in *Pd*. We have previously described the disruption of *URE1* by the homologous integration of *ure1::HYG* allele by ATMT system [30].These results indicated that the

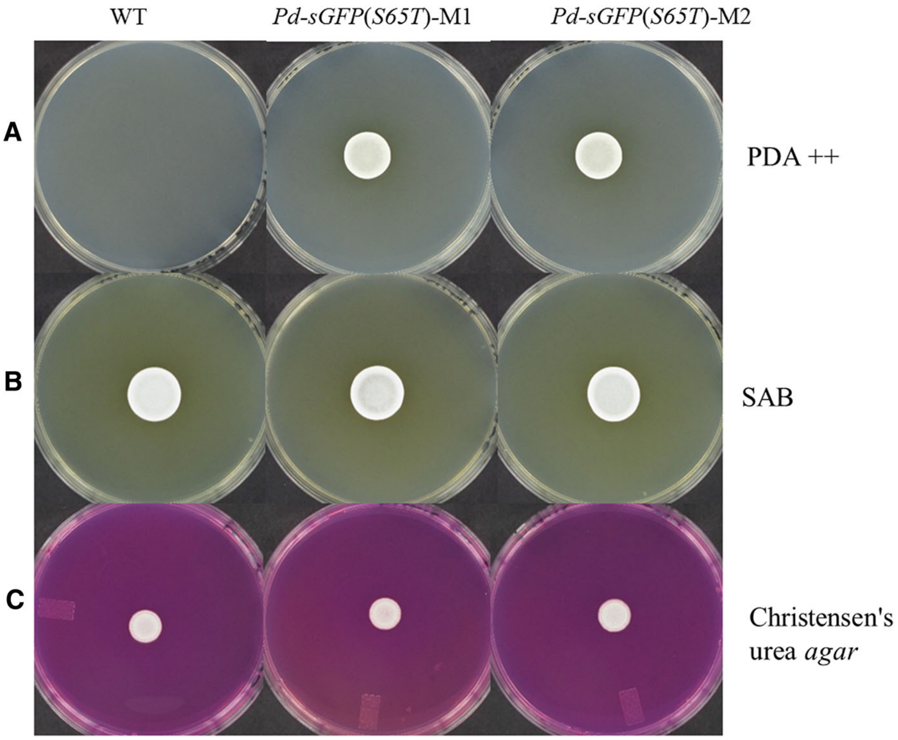


Fig. 6 Growth characteristics of *Pd-sGFP*(*S65T*) strains. Approximately 10 µl aliquots of the conidial suspension (10^7 spores/ml) of the WT and the sGFP-expressing mutants were spotted on selection (**a**), non-selection (**b**), and on Christensen's urea (**c**) agar. Plates were incubated at 15 °C for 14 days

ATMT system could be utilized for both homologous and heterologous integration of genes of interest in *Pd*. The Southern analysis showed that the frequency of a single copy of T-DNA integration was 66%. These results are in agreement with other filamentous fungi where the similar frequency of T-DNA integration was observed [36, 37]. Interestingly, both *sGFP*(*S65T*) expressing *Pd* mutants were genotypically and phenotypically stable, as they yielded amplicons against *hyg* and *sGFP*(*S65T*) genes as well expressed *sGFP*(*S65T*), despite repeated sub-culturing without selection pressure. The precise mechanisms of stable non-homologous integration of T-DNA in *Pd* are not well-understood. There are several host proteins involved in T-DNA integration into the yeast genome by non-homologous recombination [38]. We can only speculate that the non-homologous recombination/repair mechanisms are also operating at a low temperature in *Pd*. It is conceivable that the GFP intensity or expression in different mutants with single integration event is highly probable due to random integration of *sGFP*(*S65T*) at different chromosomal locations as suggested in previous studies [28, 39]. We did not find any difference in sGFP(S65T) expression at least in two of the *Pd* mutants investigated in present study.

It has been suggested that the high levels of GFP expression could be toxic to cell growth and development [40]. However, our results indicated that the high expression of sGFP(S65T) is not detrimental to *Pd* biology, as we did not observe any growth, sporulation, enzyme (urease) defects in mutants as compared to the parent strain. One possible reason could be that we chose mutants with single-copy integration. These results are encouraging, and sGFP(S65T) would allow tracking of *Pd* colonization and dispersion within bat tissues. This is achievable, as there is already a number of reports on the use of GFP as a tool for studying host-fungal pathogen interactions in vivo [41–44]. Although, current data indicate that *Pd* survives in caves and mines in the absence of bats as *Pd* was recovered or detected from these sites [8, 45]. Tracking sGFP(S65T) tagged *Pd* from cave soil would provide a more correct prediction of its association and interactions with cave substrates. Furthermore, *GFP*-based reporter systems have been developed successfully in bacteria and used in the environmental sampling studies including soil, water, rhizospheres, activated sludge, and biofilms [46]. This methodology has also been accomplished for studies on spatial dynamics of biocontrol strain *Trichoderma harzianum* ThzID1-M3 in soil and rhizoplane [47, 48].

Although, in the present study, the successful expression of sGFP(S65T) is based on the random integration of the marker in the *Pd* genome, the study provides a road map for future mechanistic studies where sGFP(S65T) can be tagged to genes involved in cold adaptation and pathogenicity. Additionally, *Pd* can serve as a model organism to understand cold adaptation and pathogenic mechanisms as necessary tools from whole genome sequencing to homologous, and heterologous gene expressions are now available.

In conclusion, this is the first report of successful expression of the sGFP(S65T) in psychrophilic fungus *Pd* by the ATMT system. The sGFP(S65T) could be used as the vital reporter marker for in-depth investigation of *Pd* biology, ecology, and pathogenicity in real time.

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