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RESEARCH ARTICLE

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A Novel Rapid Fungal Promoter Analysis System Using the Phosphopantetheinyl Transferase Gene, *npgA*, in *Aspergillus nidulans*

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ABSTRACT

To develop a convenient promoter analysis system for fungi, a null-pigment mutant (NPG) of *Aspergillus nidulans* was used with the 4′-phosphopantetheinyl transferase (PPTase) gene, *npgA*, which restores the normal pigmentation in *A. nidulans*, as a new reporter gene. The functional organization of serially deleted promoter regions of the *A. nidulans trpC* gene and the *Cryphonectria parasitica crp* gene in filamentous fungi was representatively investigated to establish a novel fungal promoter assay system that depends on color complementation of the NPG mutant with the PPTase *npgA* gene. Several promoter regions of the *trpC* and *crp* genes were fused to the *npgA* gene containing the 1,034-bp open reading frame and the 966-bp 3′ downstream region from the TAA, and the constructed fusions were introduced into the NPG mutant in *A. nidulans* to evaluate color recovery due to the transcriptional activity of the sequence elements. Serial deletion of the *trpC* and *crp* promoter regions in this PPTase reporter assay system reaffirmed results in previous reports by using the fungal transformation step without a laborious verification process. This approach suggests a more rapid and convenient system than conventional analyses for fungal gene expression studies.

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1. Introduction

Reporter genes are extensively employed in reporter assay systems to investigate transcriptional regulatory elements for gene expression from bacteria to higher eukaryotes [1]. Promoters and other regulatory elements are widely fused to reporter genes and introduced into target cells. Application of reporter genes can analyze promoter activities both qualitatively and quantitatively within the cell based on specific physiological conditions [1,2]. The most representative reporter genes, green fluorescent protein (*gfp*), luciferase (*luc*), β-galactosidase (*lacZ*), and β-glucuronidase (*gusA*), are used in reporter systems that measure the transcriptional activity of promoter fragments in genes of interest based on the amount of reporter protein expressed [3–8].

The *gfp*, *lacZ*, *luc*, and *gusA* gene are also commonly used to study the structure and function of an individual gene in a fungal reporter system [9]. Reporter genes are continuously improved by directed molecular evolution and dual reporter methods for easy detection and a wider range of applications [2,10,11]. Several advanced genes have been selected and applied for sensitive and effective fungal promoter analysis [12,13]. In addition, other reporter systems, such as glucose oxidase, laccase, and β-lactamase enzyme assays, are frequently used for better monitoring in fungi [14–16]; however, reporter genes in fungi are not always successful and it is necessary to verify genetically for further promoter analysis. The disadvantages of using the *lacZ, luc,* and *gus* reporters are their cost and potential interference with reporter activity assays. Unlike those reporters, GFP and its derivatives do not require a substrate. However, because fluorescent protein reporters have a long half-life and background expression, low-intensity signals can be difficult to measure [1,17,18]. In addition, in fungi, the *lacZ* or *uidA* reporter generally produces considerable background because of endogenous fungal enzymes [16]. Thus, glucose oxidase is occasionally used as a reporter system. However, even the system requires a peroxidase as a helper enzyme as it involves two enzymes [16,19]. The use of these systems in fungi is hampered by the need for

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Supplemental data for this article can be accessed here.

genetic validation prior to determination of promoter activity. Therefore, a novel and simple fungal reporter system is needed to enable study of a wider range of promoters.

4′-Phosphopantetheinyl transferases (PPTases) catalyze the post-translational modification of fatty acid synthases (FASs), polyketide synthases (PKSs), and non-ribosomal peptide synthetases (NRPSs), which are important for the biosynthesis of various metabolites. PPTases are essential enzymes in all three domains of life as they functionalize the CPs of FASs, PKSs, and NRPSs [20]. In filamentous fungi, PPTases are required for the biosynthesis of natural products, such as primary and secondary metabolites [21]. Thus, fungal PPTases have been used to optimize the product yields of fungal PKSs and NRPSs [22]. In addition, *Δ*PPTase heterologous reporter strains have been developed for use in NRPS/PKS gene screening by PPTase complementation in *Streptomyces* [23]. PPTases are used in various ways. The filamentous fungus *Aspergillus nidulans* also harbors a broad-range single PPTase, NpgA, which is involved in phosphopantetheinylation in the biosynthesis of secondary metabolites such as pigment, penicillin, and siderophore, as well as primary metabolites such as lysine and fatty acids [21,24]. Thus, the *npgA* gene is essential for fungal growth and the formation of conidia and cleistothecia, which are necessary for development [24]. Our previous studies of a nonsense mutant of *npgA* showed a colorless phenotype throughout the whole life cycle (null-pigment mutant: NPG) in *A. nidulans* due to the lack of formation of pigment and cell wall components [25,26]. Of note, the NPG phenotype entirely restored color by introducing the wild-type copy of the *npgA* gene in *A. nidulans* [24].

We herein report the establishment of a novel reporter system using the PPTase gene, *npgA*, with a colorless mutant in *A. nidulans* to construct a rapid fungal promoter assay system based on color complementation. The PPTase reporter assay in fungi presents results are consistent with conventional assays (i.e., β-galactosidase and GFP). The PPTase reporter system is less time consuming than conventional reporter systems. We aimed to develop a simple reporter system for use in studies of the regulation of gene expression in fungi.

2. Materials and methods

2.1. Fungal strains and culture condition

Aspergillus nidulans FGSC A4 strain (fungal genetics stock center, KS, USA) was used as the wild type strain. A null pigmentation mutant strain, WX17 (*biA, npgA1; sB3; chaA1; veA1*) [26,27] was used as the recipient strain in fungal transformation to analyze various promoter deletion constructs. *A. nidulans* strains were maintained on complete medium (CM) and minimal medium (MM) supplemented with biotin 0.08 μM, 1 mM methionine, and 4 mM tryptophan at 37 °C. Media were prepared as described previously [28,29] with slight modifications. Mutants were isolated on CM or MM with 0.01% sodium deoxycholic acid (SD) added to reduce colony size for single colony isolation [30]. For mycelial cultures in this study, an equal numbers of conidia (10^8 conidia per 100 ml) from each strain were cultured on complete liquid medium for 14 h.

2.2. Constructs for npgA complementation test

A series of plasmid clones was constructed through specific digestions of a previous pNPH3.0 plasmid using various restriction enzymes (Takara, Tokyo, Japan) to investigate the functional complementing region of *npgA*. The pNPH3.0 plasmid contains a 3.0-kb *Hinc*II-digested fragment harboring *npgA1* complementing activity [24]. The pNPHS2.0, pNPHB1.6, pNPHX1.2, pNPDS1.8, pNPNS1.2, pNPPS1.1, pNPXH1.8, pNPRH1.9, and pNPDH2.8 plasmids used for fungal transformation were constructed by digestion of pNPH3.0 (*Hinc*II/*Sac*I (2.0 kb), *Hinc*II/*Bgl*II (1.6 kb), *Hinc*II/*Xba*I (1.2 kb), *Dra*I/*Sac*I (1.8 kb), *Nru*I/*Sac*I (1.2 kb), *Pst*I/*Sac*I (1.1 kb), *Xba*I/*Hinc*II (1.8 kb), *Eco*RI/*Hinc*II (1.9 kb), and *Dra*I/*Hinc*II (2.8 kb)), and then ligated into the 3.0-kb *Hinc*II/*Sac*I-, *Hinc*II-, *Hinc*II/*Xba*I-, *Hinc*II/*Sac*I-, *Eco*RV/*Sac*I-, *Pst*I/*Sac*I-, *Hinc*II/*Xba*I-, *Hinc*II/*Eco*RI-, and *Hinc*II-digested pBluescript II SK (–) vector (Stratagene, La Jolla, CA), respectively. The 1.6-kb *Hinc*II/*Bgl*II fragment was used after end blunting. The resulting plasmids were verified by enzyme mapping and sequencing methods to transform into colorless mutant strains (NPG) in *A. nidulans*.

2.3. Serial 5′-deletion constructs for promoters analysis

For the series of progressive 5'-deletion constructs of *trpC* in *A. nidulans*, the 1,287-bp promoter region upstream of the translation start codon (ATG) to nt −1,287 of the *trpC* gene was first amplified and cloned into the pGEM-T easy vector (Promega, Madison, WI), generating the pGTP vector. Next, the 1,287-bp PCR amplicon of the *trpC* promoter from pGTP was fused with the PPTase gene *npgA* (GenBank accession no. AF198117), which contains a 1,034-bp open reading frame and a 966-bp 3' downstream region from the TAA from pNPHS2.0 [24], using an overlap extension PCR

method [31]. The 3,287-bp fused PCR amplicon fragment including the 1,287-bp *trpC* promoter *npgA* coding region and *npgA* terminator (in order) was cloned into the pGEM-T easy vector, generating the pTNP1287 vector. To construct six other vectors, promoter sequences of 824-bp, 590-bp, 405-bp, 287-bp, 109-bp, and 82-bp PCR amplicons of the *trpC* promoter were amplified and fused to the same fragment of *npgA*, generating pTNP824, pTNP590, pTNP405, pTNP287, pTNP109, and pTNP82, respectively.

In addition, for the series of progressive 5'-deletion constructs of *crp* in *C. parasitica*, the 1,282-bp promoter sequence of the *crp* gene was first amplified and cloned into the pGEM-T easy vector, generating the pGCP vector. Next, the 1,282-bp PCR amplicon of the *crp* promoter from pGCP was fused on the same region of *npgA* as the *trpC* system. The 3,282-bp fused PCR amplicon fragment including the 1,282-bp *crp* promoter was cloned into the pGEM-T easy vector, generating the pTCP1282 vector. To construct five other vectors, promoter sequences of 902-bp, 689-bp, 641-bp, 425-bp, and 188-bp PCR amplicons of the *crp* promoter were amplified and fused to the same fragment of *npgA*, generating pCNP902, pCNP689, pCNP641, pCNP425, and pCNP188, respectively. Gene-specific primers shown in Supplementary Table S1 were used to amplify each fragment to construct all vectors analyzed in this study. All constructs were confirmed by restriction enzyme mapping and sequencing. The resulting vectors were then used to transform the recipient strain to investigate its use as a novel system for fungal promoter analysis.

2.4. Fungal transformation

Transformation of *A. nidulans* was conducted according to a previously described method for the preparation of protoplasts and transformation of *A. nidulans* [32], with slight modifications. Each transforming vector was introduced into the NPG mutant strain (WX17) and cultured under non-selective culture conditions for 3 d at 37 °C. To minimize the differences between the constructs, equal numbers of protoplasts (10^8 per reaction) and amounts of plasmid DNA (1 μg per reaction) were quantified using a hemacytometer, the light microscope (Leica, Berlin, Germany), and UV microplate reader (SPECTROstar, BMG Labtech, Ortenberg, Germany), before use for transformation. The transformed isolates were single-spored to facilitate further analyses. Fungal transformations for complementation tests were performed in three biological and three technical replicates. Statistical analyses were conducted by one-way ANOVA using SPSS version 18 (SPSS, Chicago, IL), and the significance of differences between constructs was assessed by determining the magnitude of the F value at $p < 0.05$. The significance of all effects was determined using a Duncan's multiple-range test at $p < 0.05$.

2.5. Real-time quantitative PCR analysis

To prepare the genomic DNA and total RNA, an equal numbers of conidia from each single-spore green-color and/or non-color isolate were cultured on complete liquid medium and the mycelia were harvested. Genomic DNA and total RNA from *A. nidulans* isolates were extracted as previously described [24]. Real-time qPCR was performed using FastStart Essential DNA Green Master Mix (Roche, Basel, Switzerland) on a LightCycler 96 system (Roche). Relative DNA abundances and gene expression levels were calculated by the comparative threshold cycle method using the LightCycler 96 qualitative detection module (Roche) as described previously [33]. qRT-PCR experiments were performed three biological and technical replicates. The housekeeping gene β-actin (GenBank accession no. M22869.1) was used as the reference gene [34]. Significant differences at $p < 0.01$ were statistically analyzed using Student's t-test.

3. Results and discussion

3.1. Functional complementation assay of the npgA gene

The functional complementing domain of *npgA* in *A. nidulans* was investigated before developing a novel fungal promoter system that utilizes *npgA* and its mutant strain as a reporter and a host for transformation. The *npgA* gene, which encodes a novel PPTase, is broadly required for post-translational modification in the biosynthetic processing of primary and secondary metabolites [21,24]. Accordingly, the complete loss-of-function mutation of *npgA* reveals causes a lethal phenotype because NpgA protein, the PPTase of *A. nidulans*, synthesizes essential primary metabolites for fungal growth (e.g., lysine). Even a point mutation of *npgA* leads to the colorless mutant (NPG), which produces no pigment throughout the whole life cycle in *A. nidulans*, and the wild-type full-length gene of *npgA* completely restores the NPG phenotype normal pigmentation [24]. However, no functional complementing region of *npgA* has been reported.

We accurately determined an essential region with in *npgA,* which was required for complementation of *A. nidulans* NPG strain, before development of a new reporter system. Analysis of the deduced *npgA* gene product revealed the presence of

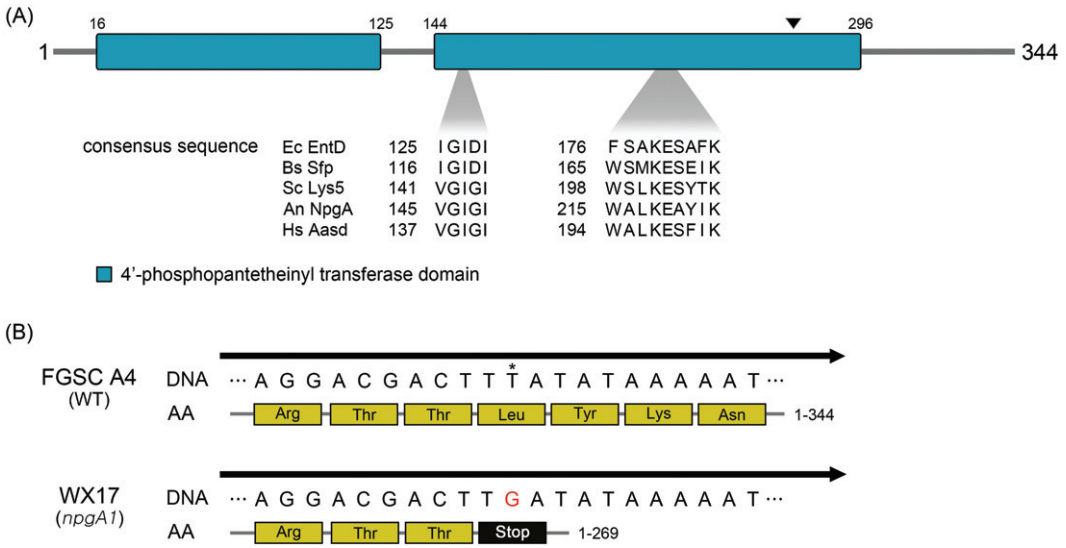

Figure 1. Domain diagram of *A. nidulans* PPTase, NpgA (A). The 4′-phosphopantetheinyl transferase (PPTase) domains are colored green. The domain was retrieved from the NCBI databases and InterPro (http://www.ebi.ac.kr). Consensus sequence of the PPTase, NpgA in *A. nidulans* (An NpgA, AAF12812) was displayed inside the PPTase domain. Other PPTase proteins: Ec EntD, *E. coli* (BAA35224); Bs Sfp, *B. subtilis* (NP_388239); Sc Lys5, *S. cerevisiae* (AAC49449); Hs Aasd, *H. sapiens* (NP_056238). The arrowhead represents the *npgA*1 point mutation site (point-nonsense mutation) that results in a colorless phenotype in *A. nidulans*. Sequencing analysis of the *npgA* open reading frame in the wild-type (WT) and WX17 (*npgA1*) mutant *A. nidulans* (B). The star represents the transition site in the *npgA1* mutation.

4′-phosphopantetheinyl transferase (PPTase) domain, related to a phosphopantetheinylation between aa 16 and 296 (Figure 1(A)) including a conserved sequence of (V/I)G(V/I)DX (40-45) (F/W)(S/C/T)XKE(A/S)hhK as other PPTases of other fungi [24]. A point-nonsense mutation of the NPG mutant revealed T to G transition in the coding nucleotide 1,145 in the mutant strain that resulted in a premature stop codon (position 269) (Figure 1(B)). Various phenotypes of the *npgA*1 mutant strain were showed because the point-nonsense mutation site of the *npgA1* mutant was included in the PPTase domain. It suggests that the whole PPTase domain is necessary to function as Npgp, PPTase enzyme.

In addition, to investigate the functional complementing region of *npgA*, we constructed a series of plasmid clones (Figure 2). The resulting plasmids were introduced into colorless mutant strains in *A. nidulans*. Each plasmid was visually analyzed for its capacity for color restoration from the colorless phenotype to the wild-type green phenotype (FGSC A4). Fungal cells of the NPG mutant showing the colorless phenotype covered the entire non-selective medium with sparsely green-colored transformants if *npgA* transcriptional activity was successful (Figure 2(B)). The qualitative results showed that the 1.4-kb *Dra*I/*Bgl*II fragment was minimally required for complementation of the null pigmentation mutation in both NPG mutant strains and partial deletion of the *npgA* coding region. *Nru*I/*Sac*I (1.2 kb), *Pst*I/*Sac*I (1.1 kb), *Xba*I/*Hinc*II (1.8 kb), and *Eco*RI/*Hinc*II (1.9 kb) caused a failure of complementation. The minimum fragment harboring complementing activity included the full-length *npgA* coding sequence with a functional DNA terminator sequence (Figure 2). PPTase complementing activity through color restoration was conveniently visible with the unaided eye visually confirmed at the transformation step without any additional experiment (in contrast to conventional fungal reporter systems such as GFP, luciferase, β-galactosidase, β-glucuronidase, glucose oxidase, laccase, and β-lactamase) [9,16,17]. As mentioned above, a non-selective scheme using the NPG mutant is practical. In addition, PPTase deletion mutants of *Streptomyces* spp. as model heterologous expression reporter strains have been developed to screen for biosynthetic gene clusters (BGCs) that include NRPS/PKS genes [23]. The approach uses PPTase-dependent pigment production by the PPTase gene to screen for BGCs containing clones. The PPTase mutant strains carry a PPTase-dependent blue pigment (indigoidine) synthase A gene in a PPTase deletion background [23]. Indigoidine is a suitable reporter for NRPS and PPTase function because its production in *Streptomyces* cells is visible to the naked eye [35]. Therefore, our scheme using PPTase-dependent pigment production may enable the development of a novel fungal promoter assay system.

3.2. Application of the PPTase gene, npgA, as a reporter

Serially deleted promoter modules can be functionally verified *in vivo* (promoter analysis) not only to investigate the potential regulatory element of the

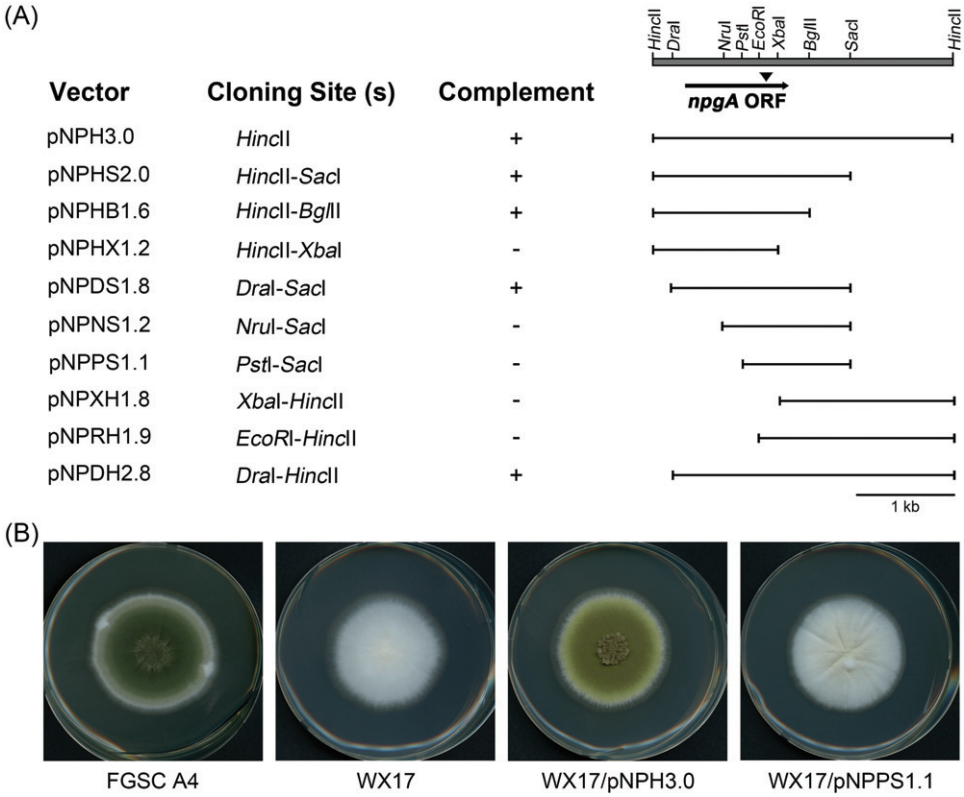

Figure 2. Functional complementation assay of the 4′-phosphopantetheinyl transferase (PPTase) gene, *npgA*, in the *Aspergillus nidulans* NPG mutant WX17 and colony morphology. (A) The restriction fragments shown were subcloned into plasmid vectors (pNPH3.0, pNPHS2.0, pNPHB1.6, pNPHX1.2, pNPDS1.8, pNPNS1.2, pNPPS1.1, pNPXH1.8, pNPRH1.9, and pNPDH2.8) and their capability of color restoration was examined. All plasmids were constructed by restriction enzyme digestion and ligation. "+" and a "−" represent successive and non-successive color complementation of the NPG strain, respectively. The location and direction of the *npgA* coding region and open reading frame (ORF) are indicated by a bold arrow below the restriction map. The arrowhead represents the *npgA*1 point mutation site in *A. nidulans*. (B) Strains, as indicated on the panels, are wild-type FGSC4, a null-pigment mutant (NPG) WX17, a restored green transformant of WX17 (WX17/pNPH3.0), a no restore transformant of WX17 (WX17/pNPPS1.1). Colonies are shown after 4 d of cultivation at 37 °C.

fungal target genes but also to develop a fungal promoter analysis system using a novel reporter [9,16,18]. The potential biotechnological application of *A. nidulans* PPTase as a fungal specialized reporter gene requires the development of a novel rapid system for fungal promoter analysis. Thus, we tested the applicability of PPTase for system development using two different promoters of the *trpC* gene and the cryparin (*crp*) gene. The *trpC* gene encodes a multifunctional enzyme involved in tryptophan biosynthesis, glutamine amidotransferase, phosphoribosylanthranilate isomerase (PRAI), and indole glycerol phosphate synthase (IGPS) in *A. nidulans* [36]. The *crp* gene encodes the cell surface-abundant hydrophobin protein cryparin in *Cryphonectria parasitica* [37]. Each promoter analysis of the *trpC* gene and the *crp* gene showed serial deletions of the *trpC* and *crp* promoters by the β-galactosidase and GFP reporter systems, respectively [38,39]. For the purpose of this study, each chimeric promoter from the two different genes (*trpC* and *crp*) connected with the *npgA* coding region was constructed identical to serial deletions of the two promoters based on previous reports [38,39] (Figure 3). The resulting plasmid constructs were then introduced into the recipient strain, NPG mutant strains, WX17 to investigate its use as a novel system for fungal promoter analysis.

Complementation tests were conducted to examine whether the deleted promoter region contained an essential activating element for expression of the *npgA* reporter gene. First, non-selective transformation of the *A. nidulans* WX17 strain (NPG) was performed for promoter analysis of the *trpC* gene. Equal DNA amounts of seven transforming vectors, including a serially deleted *trpC* promoter, were separately introduced into the same number of protoplasts of the NPG mutant strain and then cultured on non-selective medium for 4 d. If the null pigmentation phenotype of NPG was successfully restored by complementation of the wild-type *npgA*, restored green-color transformants were observed. Transcriptional activity by functional complementation in which the wild-type *npgA* gene was artificially introduced into the mutant resulted in restored green colonies (Figure 2). Non-selective transformants were sparsely found; wild-type colonies of green color in *A. nidulans* were accompanied by colorless cells of NPG

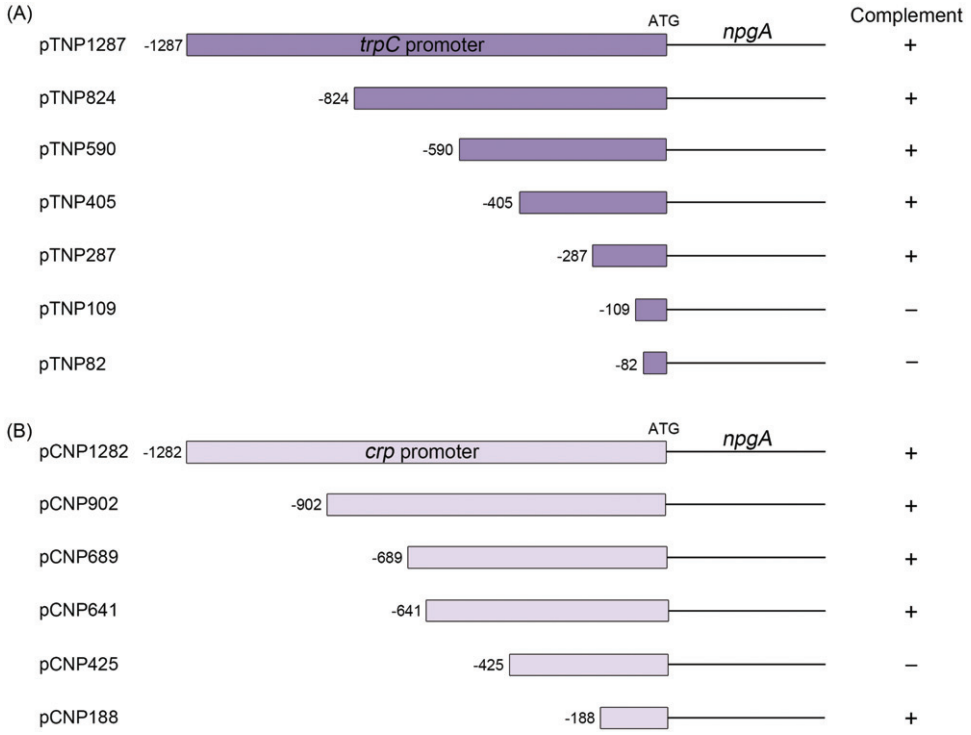

Figure 3. Schematic diagrams of each series of progressive 5′-deletion constructs of the *trpC* (A) and *cryparin (crp)* (B) promoter regions and promoter locations for the complementation activity of the 4′-phosphopantetheinyl transferase (PPTase) gene, *npgA*. Various deletion derivatives of the *trpC* and *crp* promoters were fused to the coding region of the *npgA* gene with the terminator region using fusion PCR, and all fused fragments were cloned into a general T vector. Each clone was evaluated to examine color complementation activity of the null-pigmentation mutant (NPG) strain by fungal transformation. Purple and light purple bars indicate the *trpC* and *crp* promoters, respectively. Numbers at the end of each bar indicate the distance (in nucleotides) from the translation start codon (ATG). "+" and a "−" represent successive and non-successive color complementation of the NPG strain, respectively.

throughout the entire culture plate. As shown in Figure 3(A), we initially constructed a region of 1,287 bp upstream from the start codon (pTNP1287). Such a functional element of the *trpC* gene has already been reported [36,38]; therefore, the region appeared to be long enough for promoter analysis. The non-pigment phenotype of the WX17 mutant was restored to the wild-type green color (Figure 4(A); top panel). These results suggest that the *trpC* promoter region of 1,287 bp is sufficient for *npgA* transcription activity. Furthermore, the deletion of nt −1,287 to −825 (pTNP824), nt −1,287 to −591 (pTNP590), nt −1,287 to −406 (pTNP405), and nt −1,287 to −288 (pTNP287) resulted in successive color complementation, confirming the presence of a sufficient region for *npgA* expression located between nt −1,287 and −288, consistent with previous reports [38]. However, the deletion of nt −1,287 to −110 (pTNP109) and nt −1,287 to −83 (pTNP 82) failed to restore the mutant color, confirming the presence of essential acting elements related to transcription activity located between nt −287 and the translation start codon, also consistent with previous reports (Figure 3(A)) [36]. These results indicate that this novel system is practical for use as a fungal promoter assay system.

For additional verification, six transforming vectors, including a serially deleted *crp* promoter of other fungal species of *C. parasitica*, were constructed using the identical scheme, and the possibility of heterologous complementation was examined. We initially constructed a region of 1,282 bp upstream from the start codon (pCNP1282) (Figure 3(B)) and the pCNP clone, which restored color (Figure 4(B), top panel). Because the functional element of the *crp* gene has already been reported [39], we hypothesized that the region would be sufficient to perform additional promoter analyses. Our results suggest that the *crp* promoter region of 1,282 bp in other fungal species is sufficient for *npgA* transcription activity. In addition, the deletion of nt −1,282 to −903 (pCNP902), nt −1,282 to −690 (pCNP689), and nt −1,282 to −642 (pCNP641) resulted in successive restored green colonies, confirming the presence of elements related to transcription activity located between nt −1,282 and −641, consistent with previous reports [39]. However, the deletion of nt −1,282 to −426 (pCNP425) failed to restore the mutant color, confirming the presence of essential acting elements related to transcription activity located between nt −641 and −426, also consistent with previous reports. Surprisingly, the fragment between nt −188

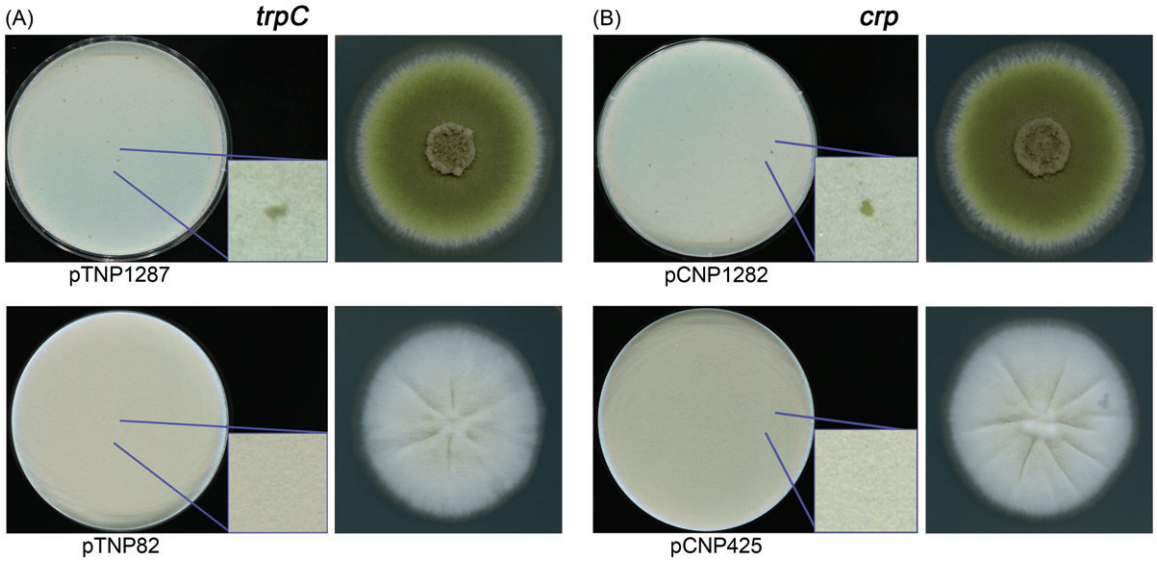

Figure 4. Isolation and colony morphology of transformants that restored the wild-type green color on NPG complementation using the PPTase promoter assay system. Protoplasts of *A. nidulans* strain WX17 (*npgA1* mutant) carrying the *A. nidulans trpC* promoter (A) or the *C. parasitica crp* promoter (B) deleted constructs were plated on non-selective culture medium. Representative transformation plates are shown after 4 d of cultivation at 37 °C (left panels), and single-spore isolates of those are shown under the same cultivation conditions (right panels). Successive and non-successive complementation of the NPG strain depending on *npgA* transcriptional activity is shown: green (top panels) and no-color phenotype (bottom panels), respectively.

and the translation start codon appeared to be sufficient for complementation (Figure 3(B)). The presence of a strong negative genetic element and the core element of the cryparin promoter in the previous report was verified once again through this novel system.

As previously reported, various promoters from different species, such as the bacteriophage T5 promoter of *Escherichia coli* RNA polymerase, the *GAL1* promoter of *Saccharomyces cerevisiae*, the *AOX1* promoter of *Pichia pastoris*, and the *niiA* promoter of *A. nidulans*, completely express the *A. nidulans* PPTase gene, *npgA* [24,40,41]. Moreover, *A. nidulans*, as a host strain, has been developed for use with an efficient system for heterologous expression of any fungal secondary metabolite gene. Gene clusters related to biosynthesis of secondary metabolites in other fungi are particularly suitable for high-yield heterologous expression in *Aspergillus* [22,42]. Therefore, use of *npgA* as a reporter gene would enable investigation of a wide range of promoters. There was also no background activity; the cells either did or did not be complemented. Production of green pigment as a visual indicator was used to detect PPTase activity due to its sensitivity. This novel system eliminates any laborious step of validation after fungal transformation required to confirm the presence of essential elements at the promoter. Thus, these results indicate that this system is a sufficient and practical fungal promoter assay system. Fungal transformation suggests the potential use of PPTase as a reporter of a novel system for fungal promoter analysis, which can be verified by a very fast and simple experimental process.

3.3. Evaluation of the fungal promoter assay using the PPTase gene and NPG strain

Successive expression of the PPTase gene, *npgA*, with *trpC* or *crp* promoter activity was observed depending on the presence of green wild-type color transformants in which the non-pigmentation phenotype of the NPG mutant was restored in the previous step. To verify these results further *in vivo*, we counted the number of restored green-color transformants on each transformation plate (Figure 4, top panels). Identification of regulatory elements in the *trpC* and *crp* promoters was performed by validating the PPTase activities driven by the *trpC* and *crp* promoter-deleted constructs (Figure 5). Based on the stepwise decrease of PPTase activity driven by serial deletions of the *trpC* and *crp* promoters, the promoters were functionally verified, and the results were consistent with previous reports [38,39]. In the *trpC* promoter analysis, the effects of the seven deleted constructs, pTNP1287, pTNP824, pTNP590, pTNP405, pTNP287, pTNP109, and pTNP82 (Figure 5(A)), on *trpC* promoter activity were non-selectively determined by counting the number of restored green-color colonies in the NPG mutant. Serially deleted constructs (pTNP1287, pTNP824, pTNP590, and pTNP405) among the promoter region of nt −1,287 to −406 resulted in almost identical numbers of color-restored transformants (no. of green colonies:

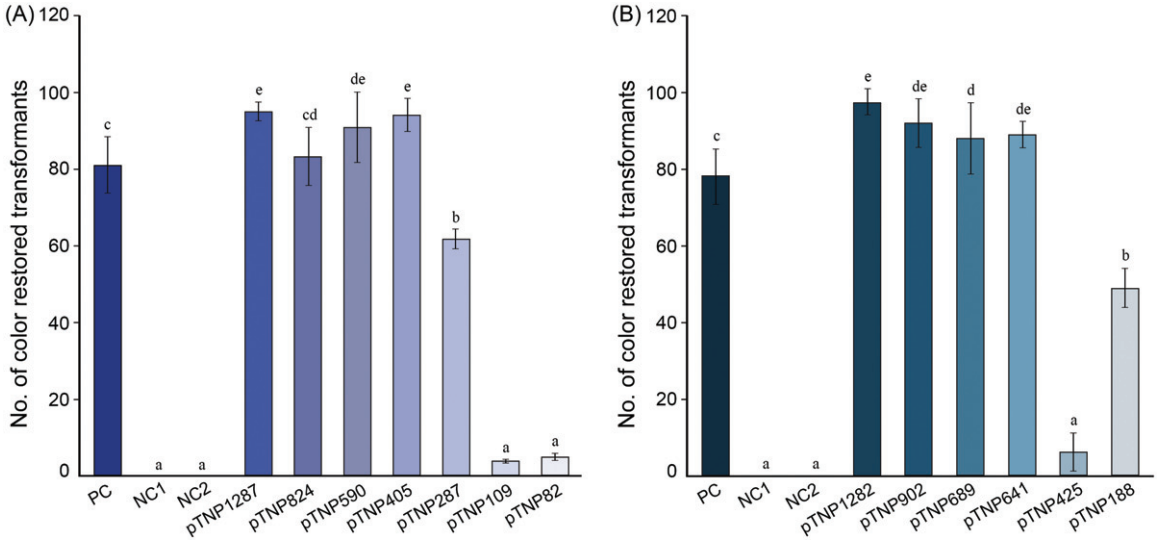

Figure 5. Promoter activity assay for PPTase activities driven by various *trpC* (A) and *crp* (B) promoter deleted constructs. Seven constructs (pTNP1287, pTNP824, pTNP590, pTNP405, pTNP287, pTNP109, and pTNP82) for the *trpC* promoter assay and six constructs (pCNP1282, pCNP902, pCNP689, pCNP641, pCNP425, and pCNP188) for the *crp* promoter assay were transformed into the fungal host strain and the NPG mutant, respectively. PPTase activities were analyzed by counting the numbers of green wild-type color transformants in which the non-pigmentation phenotype of the NPG mutant was restored to green color. PC and NC represent the positive (pNPH3.0 plasmid) and negative (pGEM-T easy vector and the *trpC/crp* full gene vector) control samples for the complementation tests, respectively. Bars indicate the number of color-restored transformants per plate. Data are the means ± standard deviations of from three replicates in three independent experiments. The significance of all effects was determined using a Duncan's multiple-range test at $p < 0.05$.

83.3 ± 7.6–94.7 ± 2.5). Maximum number of the green-color restored transformants was observed using fragments between 405 bp and 1,287 bp. The deletion of nt −1,287 to −288 (pTNP287) resulted in an approximate 0.6-fold decrease in generation of transformants (no. of green colonies: 62.0 ± 2.7) compared with those produced by pTNP1287, confirming the presence of regulatory elements located at or near nt −300, as previously reported [36]. Two deletion constructs, pTNP109 and pTNP82, which contain the fragment between nt −286 and the translation start codon, showed an almost total loss of promoter activity for normal expression of the *npgA* gene (no. of green colonies: 3.7 ± 0.6–4.7 ± 0.6) (Figure 5(A)). A few green transformants may be produced by homologous gene targeting. Thus, the results were statistically in agreement with the absence of PPTase activity in the negative controls. The absence of the essential acting elements causes failure of transcriptional activity for PPTase complementation. However, the yield numbers did not provide clarity on the relationship between promoter strength and the increased the number of restored green transformants. This novel reporter system cannot quantify transcriptional or promoter activities. Nevertheless, these results suggest that PPTase has potential as a system for visual analyses of fungal promoters and involves a rapid and simple experimental process.

In addition, for the *crp* promoter analysis, the six deletion constructs, pCNP1282, pCNP902, pCNP689, pCNP641, pCNP425, and pCNP188 (Figure 5(B)), were examined, and the number of restored green colonies on non-selective medium was counted. Serially deleted constructs (pCNP1282, pCNP902, pCNP689, and pCNP641) containing the promoter region from nt −1,282 to −641 showed similar results, and maximum number of the green-color restored transformants was observed using these fragments (no. of green colonies: 87.7 ± 3.5–96.7 ± 3.5). The deletion of nt −1,282 to −424 (pCNP425) resulted in a total loss of promoter activity for normal expression of the *npgA* gene (no. of green colonies: 6.0 ± 3.6). The deleted construct pCNP188, which contains the fragment between nt −188 and the translation start codon, resulted in an approximate 8.1-fold increase in generation of transformants (no. of green colonies: 48.3 ± 5) compared with those produced by pCNP425, confirming the presence of a strong negative genetic element located from nt −427 to −189, as previously reported [36]. Although the level of promoter strength can be generally inconsistent or different depending on the culture media [35], the presence of essential elements of the *trpC* and *crp* promoters in the previous report were reconfirmed through this novel system. However, these results do not demonstrate as ability to quantify transcriptional or promoter activities as the results of the *trpC* and *crp* promoter analyses. Analyses of promoter strength would support its use in practice.

Of note, Lubertozzi and Keasling reported that the copy numbers of fungal transformants in

A. nidulans could be measured, and the connection between gene copy number and gene expression level was investigated using real-time quantitative reverse transcription PCR (qRT-PCR). There is occasionally no association between increased gene copy number and increased gene expression [42]. Therefore, to prove that the comparison of promoter activity in the current study was not attributable to the multi-copy integration of *npgA* into the host genome, we investigated the effect of gene dosage on transgene expression levels by qRT-PCR (Supplementary Figures S1 and S2). Genomic DNA and total RNA of single-spore isolates from each non-selective transformation plate (Figure 4) were used to evaluate transgene dosage and transgene expression level, respectively. The individual fungal colonies, which appeared as green-color or non-color cells, were obtained from the promoter analysis of *trpC* and *crp*.

As shown in Supplementary Figure S1, randomly selected non-color colonies from each deleted construct for promoter analyses of *trpC* and *crp* showed the same dosage of the *npgA* gene relative to the host strain, WX17. We assumed these were not transformants, which were only contained in the original mutant gene copy of *npgA* in the genome. In contrast, restored green-color colonies generally involved the integration of at least one additional gene copy of *npgA* DNA into WX17 (Supplementary Figure S1). In addition, all isolated green colonies were of the expected color despite their different levels of *npgA* (Supplementary Figure S2). These findings confirm that there was no association between increased gene copy number of *npgA* and increased number of restored green transformants, as previously reported [43]. These results also indicate that any prior verification of a single-copy integrant is not essential for the promoter analysis using this promoter assay system because of the absence of relation between the numbers of *npgA* gene copies and the restoring effects of the NPG mutant.

The green color of wild-type *A. nidulans* spores is due to the production of a spore-specific green pigment, which results in the generation of green colonies. *wA* and *yA*, which are required for spore pigmentation, encode polyketide synthase and laccase, respectively. Mutation of *npgA* is epistatic to mutations of *wA*, *yA*, and *chaA*. Although the expression levels of these genes and the activities of their products are normal, the *npgA1* mutant never produce no pigment [25–27]. PPTase, an NpgA enzyme, post-translationally modifies NRPS/PKS synthases, which are required for pigment production by *A. nidulans* [24]. This is why all isolated colonies were of an identical color despite their different gene copy numbers and expression levels. However, we did not determine the color distribution of colonies according to the promoter region. Further studies of our novel reporter system are needed to develop a quantitative assay based on the amount of pigment.

Based on these results, it is not necessary to introduce a selective marker gene and conduct genetic verification to confirm transformants before performing promoter analyses of the gene of interest, as additional isolation and culture steps are unnecessary. This system will reduce the number of procedures that comprise multiple laborious steps. In addition, because the NPG mutation results in hypersensitivity to Novozyme234 as a result of abnormal cell wall composition, the mutant produces a larger number of protoplasts than the wild-type [25,44]. Thus, the *npgA*1 null-pigmentation mutant would be a useful host for fungal transformation in this system. Furthermore, because *Aspergillus* spp. possess NRPSs/PKSs, including a functional endogenous PPTase [22], our PPTase mutant strain could be developed into an NRPS/PKS gene screening system based on PPTase complementation in *A. nidulans* as bacterial reporter system.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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