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Characterization of NpgA, a 4′-phosphopantetheinyl transferase of *Aspergillus nidulans*, and evidence of its involvement in fungal growth and formation of conidia and cleistothecia for development[§]

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The null pigmentation mutant (*npgA1*) in *Aspergillus nidulans* results in a phenotype with colorless organs, decreased branching growth, delayed of asexual spore development, and aberrant cell wall structure. The *npgA* gene was isolated from *A. nidulans* to investigate these pleiomorphic phenomena of *npgA1* mutant. Sequencing analysis of the complementing gene indicated that it contained a 4′-phosphopantetheinyl transferase (PPTase) superfamily domain. Enzymatic assay of the PPTase, encoded by the *npgA* gene, was implemented *in vivo* and *in vitro*. Loss-of-function of *LYS5*, which encoded a PPTase in *Saccharomyces cerevisiae*, was functionally complemented by NpgA, and *Escherichia coli*-derived NpgA revealed phosphopantetheinylation activity with the elaboration of 3′5′-ADP. Deletion of the *npgA* gene caused perfectly a lethal phenotype and the absence of asexual/sexual sporulation and secondary metabolites such as pigments in *A. nidulans*. However, a cross feeding effect with *A. nidulans* wild type allowed recovery from deletion defects, and phased-culture filtrate from the wild type were used to verify that the *npgA* gene was essential for formation of metabolites needed for development as well as growth. In addition, forced expression of *npgA* promoted the formation of conidia and cleistothecia as well as growth. These results indicate that the *npgA* gene is involved in the phosphopantetheinylation required for primary biological processes such as growth, asexual/sexual development, and the synthesis of secondary metabolites in *A. nidulans*.

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Introduction

Fatty acid synthases (FASs), polyketide synthases (PKSs), and nonribosomal peptide synthetases (NRPSs) are central components of primary and secondary metabolisms, and 4′-phosphopantetheinyl transferases (PPTase) are required for the action of the FASs, PKSs, and NRPSs that have been characterized in eukaryotic cells (Lambalot *et al.*, 1996; Walsh *et al.*, 1997). PPTases catalyze the post-translational modification of proteins by the covalent attachment of a 4′-phosphopantetheine (P-pant) moiety of coenzyme A to a conserved serine residue of an inactive form of acyl carrier protein (ACP), peptidyl carrier protein (PCP), or aryl carrier protein (ArCP), resulting in conversion to the active form during the biosynthesis of various metabolites (Lambalot *et al.*, 1996). Phosphopantetheinylation catalyzed by PPTase is required for the biosynthesis of some primary metabolites such as fatty acid and lysine, and some secondary metabolites such as polyketides and nonribosomally synthesized peptides (Walsh *et al.*, 1997).

Almost all organisms that utilize more than one p-Pant-dependent pathway also have more than one PPTase (Gehring *et al.*, 1998; Quadri *et al.*, 1998). In general, one of these PPTases is optimized for the pantetheinylation of carrier proteins of primary metabolism and excludes those of secondary metabolism (AcpS-type), while the other, Sfp-type PPTase, effectively modifies carrier proteins that participate in secondary metabolism (Lambalot *et al.*, 1996; Reuter *et al.*, 1999). However, a few of the sequenced fungi appear to have sorted out their PPTase of primary metabolism, such as characterization of *npgA* in *A. nidulans* and PcpS in *P. aeruginosa*, the first examples of PPTases that can perform primary as well as secondary metabolism (Kim *et al.*, 2001; Finking *et al.*, 2002). In filamentous fungi, it has been reported that *Neurospora crassa*, *A. fumigates*, *A. nidulans*, and *F. fujikuroi* harbor only a single PPTase (Keszenman-Pereyra *et al.*, 2003; Wiemann *et al.*, 2012). The broad specificity of fungal PPTases is necessary to participate in the biosynthesis of primary metabolites such as fatty acid and lysine, and secondary metabolites such as pigments, antibiotics, siderophores, and mycotoxins. However, many primary and secondary metabolites as well as the enzymes involved in their production are yet to be revealed.

Following the vegetative growth, *A. nidulans* deposits typi-

cal pigments in the each cell type during the developmental process as the conidia, conidiopore, cleistothecia, and ascospore are colored dark green, dark brown, black, and purple, respectively. The *npgA1* mutant of *A. nidulans* resulted in including no pigment in any cell type, the retardation of hyphal branching, hypersensitivity to Novozyme 234^{TM}, and a remarkable reduction in the viscosity of the culture filtrate (Han and Han, 1993, 1994; Chung *et al*., 1996). Ultrastructural observation has shown that these pleomorphic phenotypes are caused by structural defects in cell walls (Chung *et al*., 2003). In addition, mutation of *cfwA2*, which is an allele of *npgA* impaired penicillin biosynthesis at the nonpermissive temperature, showing that the *npgA* gene is required for synthesis of metabolites such as lysine, penicillin, and siderophore, which involves an NRPS step (Keszenman-Pereyra *et al*., 2003; Oberegger *et al*., 2003). NpgA, a PPTase, is a single enzyme and a broad- range enzyme with acyl or peptidyl carrier protein domains (Keszenman-Pereyra *et al*., 2003). It has been proposed that undiscovered CfwA-dependent PKSs and NRPSs are associated with biosynthesis of unconfirmed metabolites required for asexual sporulation (Márquez-Fernández *et al*., 2007). However, correlations between PPTases and asexual/sexual development, especially sexual sporulation in *A. nidulans*, have not been fully determined. FfPpt1, a PPTase of *F. fujikuroi*, was reported to be involved in asexual sporulation and sexual mate recognition (Wiemann *et al*., 2012). Further, biosynthesis of metabolites has been implicated in fungal differentiation or development; more especially, secondary metabolism is associated with sporulation processes in microorganisms (Hopwood, 1998; Hicks *et al*., 2001). However, few studies of filamentous fungi have reported a similar role for metabolites in fungal asexual/sexual sporulation.

In this study, we isolated and characterized the *npgA* gene to investigate a correlation between PPTase and development. A point mutation (e.g., *npgA1*) results in a colorless phenotype throughout the entire life cycle (null-pigment mutant: NPG) in *A. nidulans.* The work focused on the measurement of enzyme activity as PPTase and its correlations with growth, such as dependency of deletion mutant on culture filtrate and developmental features, as well as the characterization of an over-expression mutant. We suggest that NpgA is essential for the post-translational modification of diverse enzymes that are required for biological processes such as growth, and asexual and sexual development in *A. nidulans.*

Materials and Methods

Fungal strain and growth

Aspergillus nidulans VER7 strain (*pabaA1, yA2; ΔargB::trpC; trpC801*) was used to construct the *npgA* deletion and overexpression mutants in this study (Han *et al*., 2001). The SNT611 strain, which was progeny from a cross between *A. nidulans* wild type and SN602, was used to isolate the *npgA* gene from *A. nidulans.* The SN602 (parent) strain was a temperature-sensitive suppressor mutant of *npgA*1 (Han *et al*., 2005). *A. nidulans* strains were maintained on complex medium (CM) and minimal medium (MM) at 37°C, except for SNT611, which was maintained at 30°C. Media were prepared as described previously (Pontecorvo *et al*., 1953; Kafer, 1977) with slight modifications. Mutants were isolated on MM or CM with 0.01% sodium deoxycholic acid added to reduce colony size (Mackintosh and Pritchard, 1963).

Saccharomyces cerevisiae BY4741 (*mat a*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and *lys5* knockout strains (*mat a*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *lys5::His3*) were grown under standard conditions and transformed according to the standard protocol (Geraghty *et al*., 1999; Praphanphoj *et al*., 2001).

Nucleic acid manipulation and PCR primers

Small-scale genomic DNA isolation from *A. nidulans* mycelium for Southern blot hybridization and PCR were carried out as described previously (Lee and Taylor, 1990). Twenty to sixty milligram of ground lyophilized mycelia or 0.1 to 1.0 g of fresh mycelium ground in liquid N_2 was transferred to a microcentrifuge tube. Five hundred microliters of lysis buffer was added, and mixed well. The tube was incubated at 65°C for 1 h, and then genomic DNA was extracted twice with phenol/chloroform, and isolated with ethanol (Lee and Taylor, 1990). DNA (10 mg) was digested with *Sac*I, *Sal*I, *Nru*I, and *Pst*I blotted onto a nylon membrane, and hybridized with radioactive probes (Sambrook *et al*., 1989).

Total RNA was prepared by the CsCl density gradient ultracentrifugation method. The expression pattern of *npgA* was examined by Northern blot analysis using total RNA extracted from the *npgA* deletion and over-expression mutant strains, as described previously (Jun *et al.,* 2011). The primers (Supplementary data Table S1) used in this study were synthesized by a commercial oligonucleotide synthesis facility (Bioneer, Korea).

Cloning of the *npgA*1-complementing gene, *npgA*

The *A. nidulans* genomic library, which constructed in pKBY2, was introduced into the SNT611 strain according to the procedure for protoplast preparation and transformation of *A. nidulans* (Yelton *et al*., 1984; Timberlake, 1990). The *npgA1*-complementing gene was isolated from the transformant by *in vitro* packaging of genomic DNA of the transformant. The size of the DNA fragment showing the *npgA1*-complementing activity was minimized to a 3.0-kb *Hin*cII-digested fragment. The fragment was cloned into the *Hin*cII site of the pBluescript SK (-) vector (Stratagene), generating pNPH, and sequenced using the dideoxynucleotide method with universal and synthetic oligonucleotide primers.

To obtain the cDNA clone of *npg*A, PCR using reverse transcriptase PCR (RT-PCR) was performed with primers Npg-F1 and Npg-R1. The resulting 1.6-kb cDNA amplicon was cloned and sequenced.

Functional characterization of PPTase activity in yeast

The *S. cerevisiae* BY4741 was used for disruption of the *LYS5* gene by the insertion of the *HIS3* gene. The *lys5*-knockout mutant was generated by one-step PCR-mediated gene disruption (Praphanphoj *et al*., 2001). To generate a yeast expression construct of the *npgA* gene, the open reading frame (ORF) was amplified by PCR using the *npgA* cDNA clone as template and oligonucleotides Npg-eF1 and Npg-eR1. The

resulting 1.0-kb PCR amplicon was digested with *Sal*I/*Not*I, and then cloned into the yeast expression vector pBG130 (Praphanphoj *et al*., 2001), generating pBGNPG. pBGNPG was transformed into *S. cerevisiae* the *lys5*-knockout mutant and transformants were grown for complementation analysis on synthetic dextrose (SD) medium, supplemented with leucine, methionine, uracil, and lysine, when necessary.

In vitro phosphopantetheinylation of coenzyme A by PPTase

For over-expression of NpgA protein in *E. coli*, the complete *npgA* coding sequence was amplified using the pNPSH as a template and the specific oligonucleotide pair Npg-eF2 and Npg-eR2 as primers. A 1.0-kb *Sal*I-digested PCR amplicon was cloned into the expression vector pQE30 containing 6xHis affinity tag (Qiagen), generating pQENP (Supplementary data Fig. S2A). Over-expression from pQENP was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactoside for 4 h. The NpgA protein was purified by using the Ni-NTA spin kit (Qiagen), following the manufacture's protocol. Purified NpgA protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel (Supplementary data Fig. S2B).

In vitro phosphopantetheinylation was carried out at 37°C in a total volume of 40 μl containing 10 μl of 50 μm coenzyme A, 5 μl of 2 μg NpgA dissolved in elution buffer, and 25 μl Tris-HCl (pH 6.8) containing 40 mM $MgCl_2$. This suspension was incubated for 10 min after mixing. The sample was then injected onto an analytical reversed phase high-performance liquid chromatography (HPLC) column (Pinnacle II C18, 150 mm, 5 μm, RESTEK) that had been equilibrated with methanol. Absorbance at 254 nm was monitored.

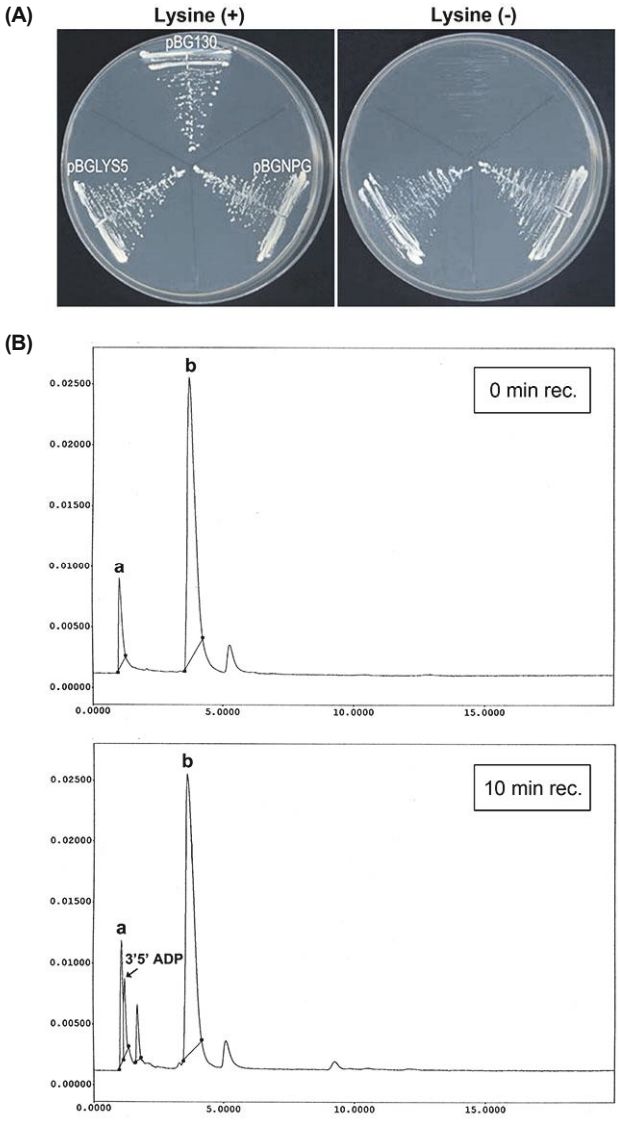

Fig. 1. Determination of 4'-PPTase activity of *A. nidulans* NpgA *in vivo* and *in vitro*. (A) *S. cerevisiae* lys5 knockout strain harbors the wild type *LYS5* gene of yeast (pBGLYS5) or the *npgA* cDNA of *A. nidulans* (pBGNPG) that are controlled under the GAL promoter. A strain, the yeast expression vector the pBD130 was transformed into the *lys5* knockout strain was used as a negative control (Praphanphoj *et al*., 2001). Minimal medium containing galactose (SGal) is supplemented with or without lysine (B) NpgA protein activity was measured by the HPLC analysis with coenzyme A. The reaction mixture included 50 μm coenzyme A, 10 mM $MgCl_2$, and 2 μg NpgA protein, with 'a' and 'b' peaks contained an imidazole and coenzyme A, respectively.

Construction of the *npgA* deletion mutant

The replacement vector pNPdel, which was designed to favor double-crossover integration events, was constructed as follows: A 2.0-kb *Hinc*II/*Sac*I fragment containing the near-full-length *npg*A was ligated into *Hinc*II/*Sac*I-digested pBS SK, and the pNPSH plasmid was used as a template for inverted PCR using the primers Npg-dF1 and Npg-dR1. The PCR amplicon was digested with *Xba*I/*Sma*I and fused with a 3.0-kb *Xba*I/*Pvu*II fragment containing the *argB* gene (Jun *et al*., 2011). In the replacement vector, pNPdel, the *argB* gene was inserted between sites 18 bp and 1,035 bp of the *npgA* gene, relative to the start codon, and was flanked by approximately 338 bp and 562 bp of 5′- and 3′-sequences, respectively. A 3.9-kb DNA fragment containing the 5′ untranslated region (5′ UTR) of the *npgA* gene, the *argB* gene, and the 3′ UTR of the *npgA* gene was amplified using the pNPdel as a template and the specific oligonucleotide pair Npg-dF2 and Npg-dR2 (Fig. 2A). Amplified PCR fragments were introduced into the *A. nidulans* VER7 strain. PCR and Southern blot analysis were conducted with genomic DNA from the transformants to confirm the replacement of the *npg*A gene. For the final confirmation, meiotic segregants between the *npgA* deletion mutant (DNPG) and the wild type strains were isolated and analyzed by Southern and Northern hybridization.

Functional complementation of DNPG was performed by the introduction of the wild-type allele. PCR amplification of the 2.4-kb *npg*A gene was performed with primers Npg-cF1 and Npg-cR1 to amplify a fragment between sites -338 bp and 996 bp of the *npgA* gene, relative to the start codon, and which incorporate *Hin*dIII and *Sma*I sites, respectively. The resulting 2.4-kb PCR amplicon was cloned into the *Hin*dIII/*Sma*I-digested pPTRI vector that had a pyrithiamine resistance gene, *ptrA* as a selective marker (TaKaRa). The complementing vector, pTRNP, was then used to transform into the DNPG strain. Transformants were selected from agar plates that were supplemented with 0.01% pyrithiamine (TaKaRa) as appropriate, passaged three or four times on

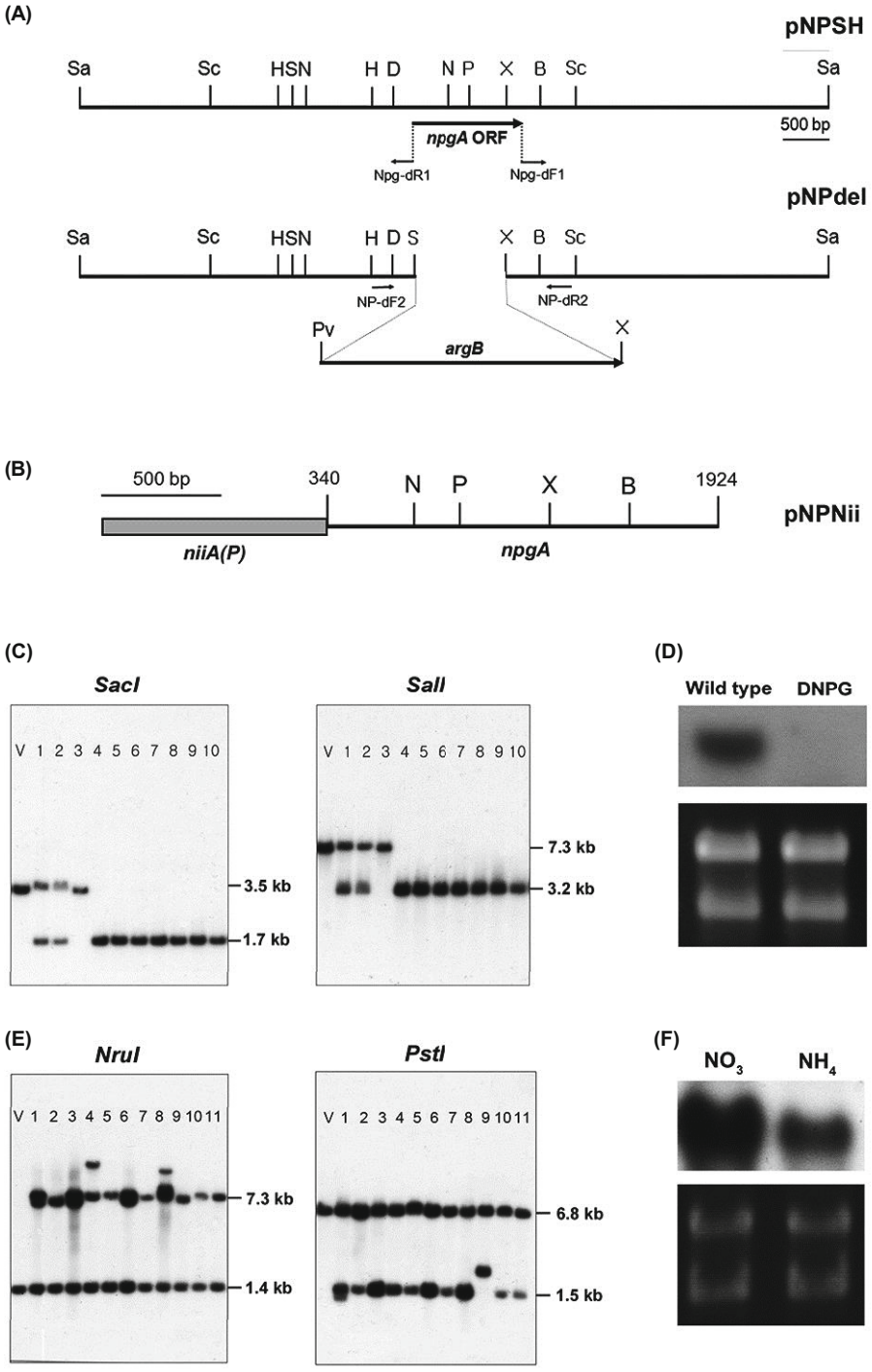

Fig. 2. Deletion and over-expression constructs of the *npgA* gene in *A. nidulans*. (A) Restriction map of the *npgA* genomic region and the gene replacement vector pNPdel. (B) A plasmid, pNPnii, harbors the entire *npgA* genomic DNA under the *niiA* promoter of the *A. nidulans*. Arrows indicate the direction of transcription (B, *Bgl*II; D, *Dra*I; H, *Hin*cII; N, *Nru*I; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sa, *Sal*I; S, *Sma*I; X, *Xba*I). (C) Southern blot analysis of the *npgA*-null mutant strains. The genomic DNA from wild-type strains VER7/ pILJ16 and meiotic segregants (lanes 1–10) between DNPG and the wild-type strain were isolated, restricted with *Sac*I or *Sal*I, and probed with a 410-bp *Bgl*II/*Sac*I fragment that locates just downstream of the *npgA* gene. Lane V represents as VER7/pILJ16 control strain. Lanes 4–10 represent as segregants having the desired gene replacement events. (D) Expression of the *npgA* gene in DNPG. (E) Southern blot analysis of the *npgA* over-expression mutant strains. The genomic DNA from wild type and transformed strains (lanes 1–11) were isolated, restricted with *Nru*I or *Pst*I, and hybridized with the *npgA*-specific probe which is a 530-bp fragment obtained by digestion of pNPSH2 with *Dra*I and *Nru*I. Lane V represents as VER7/pILJ16 control strain. Lanes 5, 7, and 11–12 represent as transformants having the desired gene integration event for over-expression of the *npgA* gene. (F) Total RNA was isolated from the *npgA* ONPG and analyzed by the same method.

selective media, and single-spore-isolated. PCR and Southern blot analyses were conducted with genomic DNA from the transformants to confirm the replacement and *in trans* complementation of the *npg*A gene. The phenotypic characteristics of DNPG were compared with the wild type VER7/ pILJ16 strain.

Characteristics of the *npgA* deletion mutant using a cross-feeding effect

The *npgA* deletion mutant strain was observed with supplements consisting of three kinds of culture filtrates (CF, ASEXCF, and SEXCF) from *A. nidulans* wild type, in order to characterize the biological function of the *npgA* gene.

CF was obtained after *A. nidulans* wild type was grown in CM liquid shake cultures at 37°C for 2 days. ASEXCF was an asexual stage-specific culture filtrate. Harvested mycelial balls, which were cultured in CM liquid shake cultures at 37°C for 14 h, were spread onto 3M paper disks in Petri dishes, and then CM liquid medium was added to the bottom of the disk. ASEXCF was taken every 12 h after the my-

celial balls were grown in standing culture at 37°C for 2 days. SEXCF was a sexual stage-specific culture filtrate. After 24 h, the same plates as those used for ASEXCF were sealed with parafilm and aluminum foil to induce sexual development, and sexual-induced culture filtrate was taken every 24 h for 5 days. Asexual and sexual development was induced as previously described (Jeong *et al*., 2000; Han *et al*., 2001).

Conidial inocula for all observations of DNPG were obtained from the cultured plate with DNPG in CM containing ASXCF at 37°C for 30 h.

Construction of an *npgA* over-expression mutant strain

pNPnii was constructed to over-express the *npgA* gene. The *npgA* gene was amplified by PCR using the primer pair Npg-F1 and Npg-R1, digested with *Xho*I, and inserted into the *Xho*I site of pRB2-1.The pRB2-1 constructed in this study was a derivative of pRB2 (Punt *et al*., 1995). From pRB2, a fragment containing the *niiA* promoter and the *Nco*I site (5′-CCATGG-3′) was removed by digestion with *Bam*HI and *Xho*I, generating a 4.4-kb fragment. A PCR amplicon containing the *niiA* promoter was inserted into the 4.4-kb fragment to generate pRB2-1 (Fig. 2B). The sequences of the PCR oligonucleotide pair were NII-F and NII-R. The pNPnii construct was introduced into the VER7 strain, and transformants were selected and analyzed by Southern and Northern hybridization. Expression of the *npgA* gene by the *niiA* promoter was induced by addition of 0.6% sodium nitrate. As a control for this induction, the same strain was cultured on the identical medium, except with 0.2% ammonium tartrate replacing sodium nitrate (Johnstone *et al*., 1990).

Measurement of conidia and cleistothecia yield in the over-expression mutant

Phenotypic changes in growth, and asexual and sexual developmental stages were measured as described previously (Han *et al*., 2001; Jun *et al*., 2011). The conidia yield of ONPG was examined by removing the agar block with a corker border to Tween 80-containing vials, and counting them with a hemacytometer after vigorous vortexing. Next, the total number of conidiophores was determined per fixed area under a dissection microscope. In addition, wild-type and ONPG strains were incubated in sexual-induced condition, and the total number of cleistothecia per fixed area was counted under a stereoscopic microscope to determine the rate of development of the sexual organ.

Results

Isolation and characterization of *npgA* in *A. nidulans*

The null pigmentation (NPG) mutant of the *A. nidulans*, which failed to synthesize pigment at any stage of its life cycle, was isolated and characterized (Han and Han, 1993). A temperature-sensitive suppressor of *npgA*1, the SNT611 strain, was used to obtain the *npgA* gene which was able to complement phenotypes derived from the *npgA*1 mutation. The minimum *npgA*1-complementing 3.0-kb *Hin*cII fragment was cloned to investigate unknown biological functions of the *npgA* gene. Based on genomic sequence analysis, a near full-length cDNA clone was obtained using RT-PCR with the primer pair Npg-F1 and Npg-RI, at nucleotide (nt) positions 1 to 21 and 1,565 to 1,585 (relative to the start codon), respectively, and the resulting 1,585-bp amplicon was cloned into the PBS SK(-) vector. Comparison with the corresponding genomic sequence revealed that the cloned gene contained one exon with no introns.

Sequence analysis of the cloned cDNA showed an ORF of 344 amino acids (aa), with an estimated molecular mass of 37.7 kDa and an isoelectric point of 5.65, named "*npgA*". The GenBank accession number for *npgA* is AF198117. Analysis of the deduced *npgA* gene product (NpgAp) revealed the presence of a PPTase superfamily domain, which contained a conserved sequence of (V/I)G(V/I)DX(78)(F/W)(S/C/T)XKE(A/S)hhK between aa 187–277, similar to PPTases of other organisms (Supplementary data Fig. S1). The *npgA* gene was an allele of *cfwA*2 which encodes a PPTase (Keszenman-Pereyra *et al*., 2003).

Function of NpgA protein as a PPTase in *A. nidulans*

The PPTase of *S. cerevisiae* (Lys5p) has been known to catalyze the transfer of a 4′-phosphopantetheinyl moiety from coenzyme A to α-aminoadipate reductase (Lys2p) (Ehmann *et al*., 1999). The ability of NpgA to complement, *in vivo*, the *S. cerevisiae* PPTase encoded LYS5, was tested to determine whether the *npgA* ORF codes for a PPTase. The *S. cerevisiae*

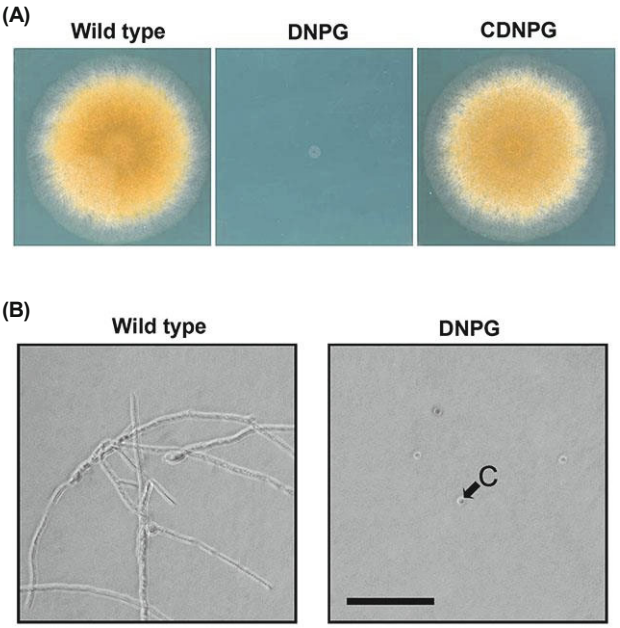

Fig. 3. Phenotypic characteristics of *npgA*-null mutant strain. (A) Colony morphology of the wild type strain VER7/pILJ16, *npgA*-null mutant DNPG, and a complemented strain of *npgA*-null mutant, CDNPG. The strains were cultured on a MM supplemented with para-aminobenzoic acid at 37°C for 4 days. (B) Microscopic observations of germination of DNPG compared with the wild-type strain. Conidia (1×10^6/ml) germination of *A. nidulans* strains VER7/pILJ16 and DNPG (*ΔnpgA*) on coverslips incubated in liquid MM. Micrographs were taken after 18 h of growth at 37°C. Representative micrographs obtained by light microscopy are presented. A conidium is represented in (C) and indicated by the arrow. The scale bar shown in a right panel is 40 μm.

Table 1. Relative growth rates of *npgA* mutant strains on culture filtrates

Strain	Relevant genotype	Growth rate (%)					
		Primary branch (N/colony)[a]		Radial extension (mm/h)[b]		Cell mass (% of the wild type)[c]	
		CM	CM+CF[d]	CM	CM+CF	CM	CM+CF
Wild type	*npgA*$^+$	100.0 ± 0.0	111.6 ± 3.0	100.0 ± 0.0	102.7 ± 1.4	100.0 ± 0.0	102.3 ± 3.1
ONPG	*niiA*(p)::*npgA*	127.7 ± 3.3	123.1 ± 4.1	102.9 ± 0.8	105.4 ± 0.4	105.0 ± 4.4	106.9 ± 3.8
DNPG	Δ*npgA*	0.0 ± 0.0	70.4 ± 3.4	0.0 ± 0.0	103.7 ± 0.0	0.0 ± 0.0	79.5 ± 3.4

[a] The numbers of primary branch (hyphal element) determined per a colony under a light microscope.
[b] Apical extension rates were determined for each strain by measuring colony diameter. Wild type growth rate (mm/h) was 0.63. Data from 3 different colonies were averaged.
[c] Amount of mycelial dry weights were determined after each strain cultured for 3 days.
[d] Culture filtrate was obtained without mycelial balls.
All data from 3 different colonies were averaged.

lys5-null mutant was unable to grow unless lysine was provided. Therefore, pBGNPG, which contained the *npgA* gene under the control of the *GAL* promoter, and the *URA3* wild type gene was introduced into the *lys5*-null mutant, and uracil prototrophy transformants were able to grow on medium not supplemented with lysine (Fig. 1A). Therefore, the *npgA* gene was able to complement the *LYS5* deletion mutation of *S. cerevisiae* as a functional PPTase.

To determine the P-pant transferase activity of this enzyme, NpgA protein (37.7 kDa) was overproduced and purified using hexa histidine-tagged proteins. PPTase cleaved coenzyme A, transferred the P-pant moiety to a conserved residue of inactive substrates, and produced 3′5′-ADP. In order to determine whether one of new products in the mixture reaction was obtained from 3′5′-ADP, each peak of the products was compared with the peak of first-hand CoA in reversed-phase HPLC analysis. There was a new product generated by NpgA activity, which was identified as having the same peak as that of the 3′5′-ADP in HPLC (Fig. 1B).

Construction of the *npgA* deletion and over-expression mutants

To examine the effects of deleting the *npgA* gene, an *npgA*-null mutant was constructed by site-directed recombination during integrative transformation. Linear DNA that contained a disrupted *npgA* gene with 338-bp 5′- and 526-bp 3′-flanking regions was used to transform the *A. nidulans* VER7 strain using an *argB* marker. Several transformants, when approximated around the wild type strain, showed *npgA*1 phenotype. However, deletion of *npgA* resulted in a

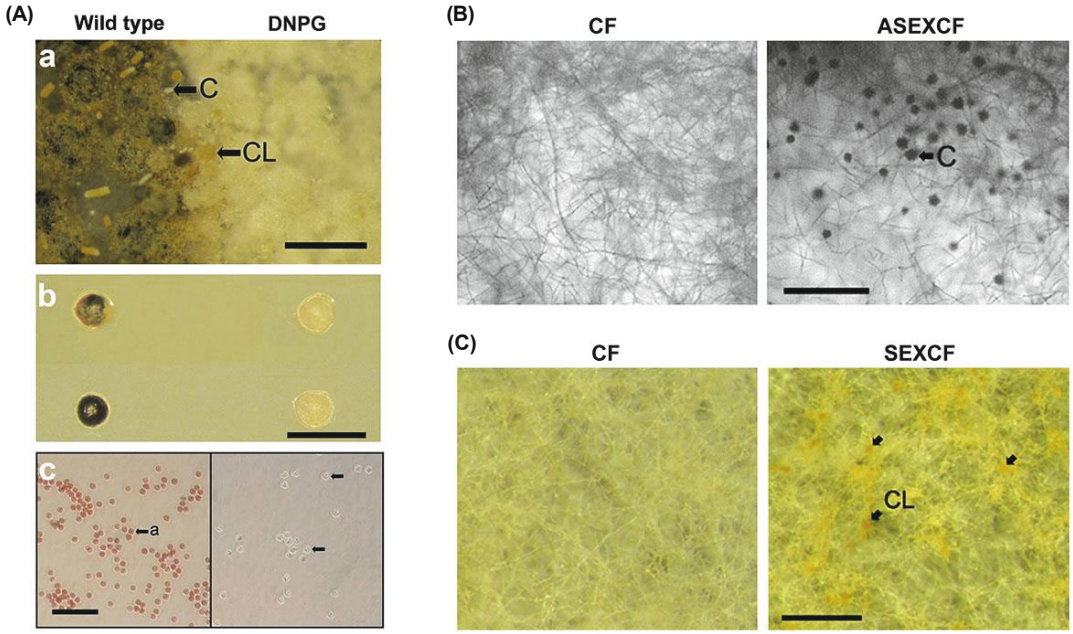

Fig. 4. Cross-feeding effects between *A. nidulans* wild type strain and lethal phenotype *npgA*-null mutant. (A) To examine cross-feeding effects, inoculated wild-type strain VER7/pILJ16 and nearby the *npgA* deletion mutant DNPG on CM plate that was supplemented with culture filtrates from the wild-type strain. Conidiophore and cleistothecia are represented as C and CL, and indicated by the arrows. The scale bar shown is 400 μm (A-a). Cleistothecium of the wild type strain and DNPG were taken from a plate using a needle. The scale bar shown is 250 μm (A-b). Cleistothecium from each strain was broken to reveal contents. Ascospores are represented as 'a', and indicated by the arrow. The scale bar shown is 30 μm (A-c). (B) Effects of culture filtrates, which were obtained from different developmental stages, for characteristics of DNPG. To observe an asexual organ, DNPG strain was point-inoculated on CM plates that were supplemented with CF or the asexual stage-specific culture filtrates from *A. nidulans* wild type strain (ASEXCF), and incubated at 37°C for 30 h. Conidiophore is represented as C and indicated by the arrow. The scale bar shown is 150 μm. (C) To observe a sexual organ, tested strains were point-inoculated on CM plates that were supplemented with CF or the sexual stage-specific culture filtrates of FGSC4 (SEXCF), and incubated at 37°C for 3 weeks. Cleistothecium is represented as CL and indicated by the arrow. The scale bar shown in is 300 μm.

strain that was entirely unable to grow on any medium unless supplemented with cultured filtrate (described in the next section) or brought into close contact with the wild type strain. Therefore, all *npgA*-null mutant candidates were cultured on CM and MM including cultured filtrates to obtain the mycelia and conidia for follow-up experiments.

To confirm the pure replacement of the wild-type allele with the disrupted allele, 7 transformants were further examined by genetic crossing with the wild type. Meiotic segregants of the *npgA* deletion type were then verified by Southern and Northern blot analyses (Fig. 2C and D). As shown in Fig. 2C, the hybridization pattern of *Sac*I-digested genomic DNA or *Sal*I-digested genomic DNA of 7 transformants of DNPG with a probe prepared using the 530-bp *Dra*I/*Nru*I-digested *npgA* fragment differed from that of the wild type. These results suggested that the transforming vector had integrated at the *npgA* locus by site-directed homologous recombination. Moreover, the *Sac*I- and *Sal*I-digested genomic DNA of the 7 transformants of the *npgA*-null mutant had hybridizing bands at 1.7 kb and 3.2 kb, respectively, corresponding to the expected sizes of the replaced alleles. In addition, the *npgA*-null mutant showed no transcripts at all (Fig. 2D).

In addition, we examined the effect of forced expression of the *npgA* gene in *A. nidulans*. This was accomplished by fusing the *npgA* gene to the *niiA* promoter followed by transformation into a wild-type strain. Over-expression of the *npgA* gene was verified by genomic Southern blot analysis, and Northern blot analysis using the specific region of *npgA* as a probe (Fig. 2E and F). As shown in Fig. 2F, the *npgA* transcript was greatly increased in ONPG when cultured on sodium nitrate as a sole nitrogen source.

Essential role of *npgA* for growth in *A. nidulans*

A typical *npgA*-null mutant, DNPG, was unable to grow on either MM or CM. Therefore, deletion of the *npgA* gene resulted in a lethal phenotype in *A. niduals* (Fig. 3A). Compared to conidial germination of the wild type VER7/pILJ16 strain, DNPG neither produced a germ tube nor did it undergo conidial swelling (Fig. 3B). Complementation of DNPG was conducted using a wild-type *npgA* gene. The complemented strains (CDNPG) restored the characteristics of growth, development, and pigmentation (Fig. 3A). Southern blot analysis revealed that all complemented transformants contained an additional wild-type allele of *npgA* (data not shown). Thus, the functional complementation using a wild-type allele of the *npgA* gene unequivocally confirmed that the phenotypic differences of DNPG were due to knockout of *npgA*.

However, most interestingly, mycelial growth was recovered in DNPG by a cross-feeding effect, although recovery was imperfect (Fig. 4A). Consequently, the culture filtrate from the *A. nidulans* growth stage (CF) restored to mycelial growth without development (CF in Fig. 4B and C). Growth of the DNPG strain, rescued by the CF effect, revealed an increase of about 46% in the branching rate, 97% in the radial extension rate, and 20% in cell mass (Table 1). Secreted-extracellular materials, which were not produced by the *npgA*-null mutant, were provided by culture filtrate of wild-type *A. nidulans* to DNPG, and the *npgA*-null mutant was able to grow. These results indicated that the *npgA* gene is essential for growth in *A. nidulans*, and verified that NpgA protein was needed to produce secreted metabolites such as lysine.

In addition, to reconfirm the role of *npgA* was in a growth, we examined the effect of forced expression of the *npgA* gene in *A. nidulans*. The ONPG strain expressed elevated mycelial growth on MM (Fig. 5A), we determined the primary branching rate, radial extension rate, and cell mass of ONPG were compared to those of wild-type strains, confirming that over-expression of the *npgA* gene enhanced the growth of *A. nidulans*. As shown in Table 1, the primary branching rate of the ONPG strain increased by approximately 25% compared with the wild type, and the radial extension rate and cell mass of the ONPG strain increased minimally. Taken together, these results indicated that over-expression of *npg*A led to an increase in NpgA protein yield, and that this elevation increased the yield of secreted metabolites, including lysine. Therefore, increasing expression of this growth factor enhanced cell growth.

Essential role of *npgA* for formation of conidia and cleistothecium in development

While secreted metabolites containing lysine rescued growth from NpgA loss-of-function, asexual and sexual development stages were not entirely recovered on culture media containing growth-stage CF even when the *npgA* deletion mutant DNPG was incubated in these media for more than three weeks (Fig. 4B and C). These results indicated that deletion of *npgA* impaired the asexual/sexual development as well as growth in *A. nidulans*. However, interestingly, development of conidia and cleistothecia in DNPG were rescued the formation of by a cross-feeding effect, although recovery was imperfect (Fig. 4A). Conidia and cleistothecia of DNPG were observed in a region contiguous with wild-type strain because metabolites, which were produced and secreted from developmental stages, were supplied directly to DNPG strains.

In order to verify that the *npgA* gene was associated with formation of metabolites that were essential for asexual/

Table 2. **Influence of over-expression of *npgA* in asexual and sexual development**

Strain	Relevant genotype	Asexual and sexual organs per field (% of the wild type)		
		Conidia[a]	Conidiophores[a]	Cleistothecia[b]
Wild type	*npgA*$^{+}$	100 ± 0	100 ± 0	100 ± 0
ONPG	*niiA*(p)::*npgA*	290 ± 26	269 ± 12	201 ± 11

The numbers of asexual and sexual organs per field are expressed as the percentage. All data from 5 different fields of a plate were averaged. Values are mean ± standard error from three independent plates.
[a] Quantitation of conidial yield in the wild type strain and ONPG. The total numbers of conidiophores were determined per 1 mm^2 area under a dissection microscope.
[b] Quantitation of produced cleistothecia yield in the wild type strain and ONPG. The numbers of formed cleistothecia were determined per 1 mm^2 area.

sexual development as well as growth, we artificially induced conidia of the DNPG strain, and conidia were inoculated on CM containing stage-specific CF and observed for 3 weeks. As expected, formation of asexual (conidia) and sexual organs (cleistothecia) was restored by ASEXCF and SEXCF, respectively (Fig. 4B and C). These results indicated that the *npgA* gene was associated with the presence of specific metabolites required for development as well as growth. Development was restored imperfectly through exposure to ASEXCF and SEXCF. Cell walls of the rescued sexual organ, the cleistothecia, were easily destroyed by contact (data not shown), and showed a null pigment phenotype. Furthermore, the number of ascospores per cleistothecia was less than in the wild type, and the ascospore had an irregularly external shape (Fig. 4A). These results suggested that metabolites in CF partially recovered development of organs from defects caused by deletion of *npgA*, and other factor(s) should be required to interior to success perfect development and *npgA* gene was intimately associated with production of these metabolites.

To study the effects of forced expression of *npgA* in asexual and sexual developments, we examined formation of conidia and cleistothecia in the ONPG strain. Over-expression of *npgA* resulted in a level of conidiation more than two times that of the wild type (Fig. 5B and Table 2). In addition, cleistothecia of the ONPG strains were produced more abundantly than in the wild type, and over-expression of *npgA* resulted in a two-fold increase in formation of cleistothecium (Fig. 5C, D, and Table 2), indicating that over-expression of *npgA* accelerated asexual and sexual development. These results verified that over-expression of the *npgA* gene caused a substantial increase in formation of conidia and cleistothecia, supporting the idea that increase in a developmental factor yielded enhanced development of asexual and sexual organ. Therefore, the *npgA* was closely associated with both asexual and sexual developments in *A. nidulans*.

Discussion

The *npgA*1 mutation was previously isolated and its diverse phenotypes were characterized as having no pigmentation in any cell types, retardation of hyphal branching and differentiation, hypersensitivity to degrading enzyme, and the absence of a specific layer in the hyphal and conidial walls (Han and Han, 1993, 1994; Chung *et al.*, 2003). These pleiotrophic phenotypes support the concept that the *npgA* gene product functions as a central regulator (Chung *et al.*, 2003). In order to understand the biological functions of a central regulator involved in these pleiotrophic factors in *A. nidulans*, the *npgA* gene was investigated. Structural analysis of the *npgA* gene, completely rescued from the *npgA1* mutant, encoded a putative PPTase. The *npgA* gene, which is an allele gene of *cfwA*, has been reported to regulate the activity of various target enzymes involved in the synthesis of metabolites such as fatty acids, polyketides, and nonribosomal peptides; lysine, sideropore, penicillin, sterigmatocystin, and pigments in *A. nidulans* (Mootz *et al.*, 2002; Keszenman-Pereyra *et al.*, 2003; Oberegger *et al.*, 2003; Márquez-Fernández *et al.*, 2007). Thus, the loss of *npgA* would result se-

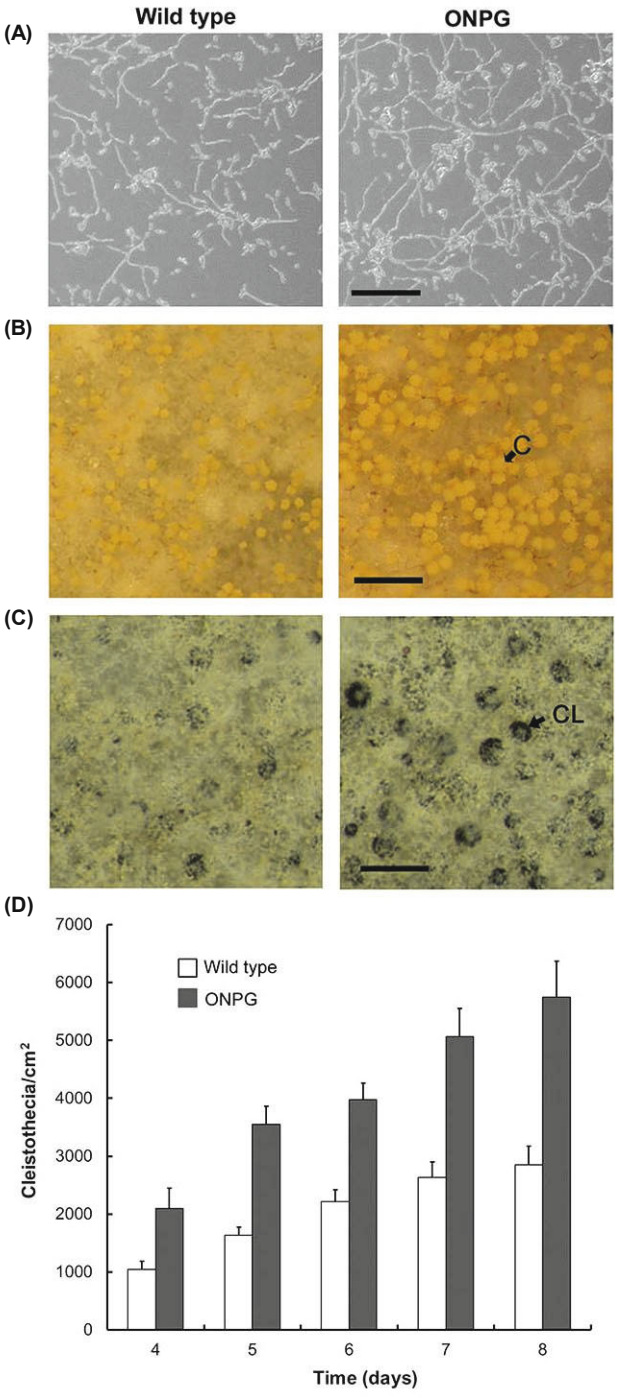

Fig. 5. Microscopic observations of *npgA* over-expression mutant strain. Conidia (1×10^6) of the wild-type strains VER7/pILJ16 and *npgA* over-expression mutant ONPG were spread on MM (A) and CM (B–D) containing 0.6% sodium nitrate as the sole nitrogen source and incubated at 37°C. Micrographs were taken at 12 h for hyphal growth (A), 48 h for production of conidiophores (B), or 5 days for production of cleistothecia (C). (D) After 24 h, plates were sealed with Parafilm and aluminum foil to induce the sexual development and incubated at 37°C for 7 days. (E) Total number of cleistothecia per fixed area was counted under a stereoscopic microscopy and used to calculate cleistothecia/cm^2. Conidiophore and cleistothecium are indicated by the arrow and represented as C and CL, respectively. The scale bar shown in the right panel is 250 μm.

vere defects in *A. nidulans* because if its function as a PPTase in producing various metabolites. However, enzymatic activity of a PPTase in filamentous fungi, and specifically *A. nidulans*, has never been shown. There is also insufficient information about the correlation between phosphopantetheinylation according to NpgA, PPTases and asexual and sexual sporulation.

In the present study, the *in vitro* enzymatic function of NpgA as a PPTase in *A. nidulans* was verified. First, NpgA was expressed in a *LYS5* knockout mutant strain of *S. cerevisiae*, and its ability to complement Lys5p was demonstrated *in vivo*. NpgA modified the α-aminoadipate reductase (Lys2p) to its active holo form thereby conferring the ability to synthesize lysine on the *LYS5* deletion strain. NpgA was homologous to Lys5p, a PPTase that is essential for post-translational modification of the Lys2p with the P-pant cofactor in *S. cerevisiae* in synthesizing lysine as a primary metabolite as in complementation test of *S. cerevisiae LYS5*-null mutant with different genetic background (Mootz *et al*., 2002). Second, NpgA transferred the 4′-phosphopantetheinyl moiety of coenzyme A to the side chain hydroxyl of the invariant serine residue in PCPs and ACPs that generated 3′5′-ADP. In general, PPTases catalyze the nucleophilc attack of the hydroxyl side chain of the conserved carrier protein serine residue onto the 5′-β-pyrophosphate linkage of coenzyme A (CoASH) in a magnesium ion-dependent reaction, and then during polyketide, or nonribosomal peptide synthesis, the activated substrates and elongation intermediates are covalently linked as thioesters to the thiol moiety of the 4′-P-pant.

PPTases are classified, according to structure and characteristics of the carrier protein, into three main groups: AcpS type, Sfp type, and fatty acid synthase type (Sanchez *et al*., 2001). Specific PPTase are optimized for the pantetheinylation of carrier proteins of primary metabolism and exclude those of secondary metabolism, while the other PPTases efficiently modify carrier proteins involved in secondary metabolism (Reuter *et al*., 1999). Most organisms use more than one phosphopantetheinyl-dependent pathway, and they possess more than one PPTase (Gehring *et al*., 1998; Quadri *et al*., 1998). However, some more the recently investigated PPTases, such as PcpS in *P. aeruginosa*, have been found to contribute to primary as well as secondary metabolism (Finking *et al*., 2002). NpgA is a new type of PPTase in *A. nidulans*, an organism that harbors only one PPTase as an independent gene with broad-ranging function (Keszenman-Pereyra *et al*., 2003). It has been suggested that NpgA might be responsible for the modification of ACPs of fatty acid or polyketide synthases, which are involved in the synthesis of primary metabolites, as well as PCPs of nonribosomal peptide synthetases, which are involved in the synthesis of secondary metabolites.

Loss-of-function mutation of PPTase-homologous genes in *A. fumigatus*, *A. niger*, *C. graminicola*, *C. sativus*, *F. fujikuroi*, *M. oryzae*, *P. chrysogenum*, and *P. aeruginosa* reduce the production of primary and secondary metabolites such as lysine, fatty acids, and sideropore (Finking *et al*., 2002; Wiemann *et al*., 2012). In particular, diverse primary and secondary metabolites that are essential for growth are impaired by deletion of the *pcpS*-null mutant. In *A. nidulans*, deletion of the *cfwA* gene also results in an absolute requirement for lysine and sideropore, however, even supplementation with these two metabolites is unable to fully recover the growth defect of *cfwA* mutant strains. Restored strain shows a cotton-like "fluffy" morphology and little conidiation (Oberegger *et al*., 2003; Márquez-Fernández *et al*., 2007). Deletion of the *npgA* gene showed a lethal phenotype and an absence of growth that was impossible to restore by supplementation of lysine or siderophore only. Interestingly, however, *A. nidulans* was rescued from this lethal phenotype by supplementation of CF from *A. nidulans* in a vegetative growth stage. These results indicate that *npgA* is essential for the biosynthetic pathway of other primary metabolite and secondary metabolites such as pigments and fatty acid. As an example, a reduction in lipid content associated with the *npgA1* mutant has been shown previously (Chung *et al*., 1996). The results of this study suggest, first, that the *npgA1* mutantion negatively affects lipid biosynthesis caused by the impairment of fatty acids, and that *npgA* is involved in the modification of fatty acid synthetase in fatty acid biosynthesis. Second, melanin was completely absent in the cell wall of the *npgA1* mutants, indicating that PPTases play an essential role in priming PKSs, which catalyze of production of polyketides such as pigments that contain melanin (Chung *et al*., 1996). Recover of the null pigmentation phenotype by a full *npgA* gene indicates that a complete PPTase is essential for pigmentation in the developmental stages of *A. nidulans*. In addition, the *npgA*-null mutant showed the absence of the developmental organs conidia and cleistothecia, even when CF from vegetative growth stage was added. The defective development caused by deletion of *npgA* was recovered only by an asexual-stage CF or sexual-stage CF. Deletion of *npgA* impairs asexual and sexual developments as well as growth in *A. nidulans*, indicating that the various produced by NpgA are essential for both the growth and development stages. The possibility of unknown CfwA-dependent PKSs and/or NRPSs involved in biosynthesis of unknown metabolites required for asexual sporulation in *A. nidulans* (Márquez-Fernández *et al*., 2007), and FfPpt1 of *F. fujikuroi* is found be involved in asexual sporulation and sexual mating recognition (Wiemann *et al*., 2012). In general, biosynthesis of metabolites is implicated in fungal differentiation or development, and more specifically, secondary metabolism is associated with sporulation processes in microorganisms (Hopwood, 1998; Hicks *et al*., 2001). Several studies of filamentous fungi have reported a role for polyunsaturated fatty acids (i.e. linoleic acid) in fungal sporulation (Roeder *et al*., 1982). In *A. nidulans*, linoleic acid-derived signal molecules control the development of conidiophores and cleistothecia (Champe and El-Zayat, 1989; Mazur *et al*., 1991). However, not much else is known about the correlation between PPTases and asexual/sexual development, especially sexual sporulation in filamentous fungi.

In the current study, *npgA* gene over-expression was shown to causes an elevated yield of conidiophores and cleistothecia. Due to action of the FASs, PKSs, and NRPSs caused by over-expression of *npgA*, biosynthesis of natural products, metabolites associated with asexual/sexual development, are increased in *A. nidulans*, and an increase in specific metabolites may promote asexual/sexual sporulation. However, be-

yond fatty acids, the particular natural products that are dependent on phosphopantetheinylation and are associated with development in *A. nidulans* have not been investigated.

Finally, the filamentous ascomycete *A. nidulans* is a model organism for the control of metabolic pathways and development in eukaryotes, because this fungus establishes highly specialized cell types and produces various secondary metabolites. The *npgA* gene is involved in production of various natural factors associated with metabolic pathways and development in *A. nidulans*. The sole PPTase of *A. nidulans*, NpgA, has catalytic properties significantly different from other enzymes of the PPTase superfamily. We conclude that NpgA, with broad-specificity in PPTase activity, is essential for the biosynthesis of some primary metabolites such as fatty acid and lysine, and for some secondary metabolites such as any pigment materials, siderophore, and penicillin. Phosphopantetheinyl transferases may be used *in vitro* or *in vivo* by coexpression with synthase genes to produce large, usable quantities of properly modified, active synthases for further studies on PPTase activity. An understanding of the *A. nidulans* PPTase examined in this study could lead to elimination of pathogenicity is in fungal pathogens, or to the biosynthesis of natural products, including antibiotics in *Aspergillus* species or in other microorganisms.

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