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SHORT COMMUNICATION

Analysis and functional expression of NPP pathway-specific regulatory genes in *Pseudonocardia autotrophica*

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Abstract Using the genomics-guided polyene screening method, a rare actinomycetes called *Pseudonocardia autotrophica* was previously identified to contain functionally clustered nystatin-like biosynthetic genes and to produce a presumably novel polyene compound named nystatin-like Pseudonocardia polyene (NPP) (Kim et al., J Ind Microbiol Biotechnol 36:1425–1434, 2009). Since very low NPP productivity was observed in most *P. autotrophica* culture conditions, its biosynthetic pathway was proposed to be tightly regulated. Herein we report *in silico* analysis of six putative NPP pathway-specific regulatory genes present in its biosynthetic gene cluster, followed by functional overexpression of these regulatory genes in *P. autotrophica*. Three pathway-specific regulatory genes (*nppRI*, *RIII*, and *RV*) were predicted to belong to a typical LAL-type transcriptional family. Each regulatory gene was cloned under the strong constitutive *ermE** promoter in a *Streptomyces* integrative pSET152 plasmid, followed by direct intergeneric conjugation from a plasmid-containing *E. coli* donor cell to *P. autotrophica*. While all the *P. autotrophica* exconjugants exhibited improved NPP productivity, the one containing *nppRIII* showed the highest NPP productivity improvement. In addition, culture-time-dependent analysis revealed that the *nppRIII*-stimulated NPP biosynthesis was more significant in the late exponential growth stage than in the stationary stage. Moreover, the *P. autotrophica nppRIII*-disruption mutant failed to produce NPP, with significantly reduced transcription levels of most *npp* biosynthetic genes. The results described suggest that identification and overexpression of key pathway-specific regulatory gene, followed by optimum harvest timing, should be critical factors to maximize the productivity of an intrinsically low-level metabolite such as NPP produced by rare actinomycetes species.

Keywords *P. autotrophica* · Nystatin · Polyene · Pathway-specific regulator

Introduction

Streptomycetes and its physiologically related actinomycetes are Gram-positive filamentous soil bacteria with a complex lifecycle involving morphological differentiation and have been appreciated as rich natural sources of a variety of commercially valuable enzymes and secondary metabolites, including antibiotics, antitumor agents, immunosuppressants, and enzyme inhibitors [26]. Since regulation of secondary metabolite production usually involves tightly regulated multiple and parallel networks, most wild-type species produce their secondary metabolites at very low levels in typical laboratory culture conditions [3].

Within this regulatory network, a gene working at the proximal level usually residing within the respective biosynthetic gene cluster is called a pathway-specific regulatory gene, only affecting the expression of a single secondary metabolite biosynthetic pathway. A family of genes whose products contain OmpR-like DNA-binding domain was named SARP (*Streptomyces* antibiotic regulatory proteins),

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typically found in non-macrolide polyketide biosynthetic gene clusters [2, 16–18, 24]. Unlike the SARP family, a different transcriptional family of regulators was identified to contain a relatively large protein with an N-terminal adenosine triphosphate (ATP)-binding domain represented by discernible Walker A and B motifs, and a C-terminal LuxR-type DNA-binding domain [23]. Regulators belonging to this so-called LAL (large ATP-binding regulators of the LuxR) family, whose prototype member is the *Escherichia coli* MalT involved in uptake and catabolism of maltodextrins [6], have been identified and characterized in several macrolide antibiotic pathways [9, 10, 14, 25]. Moreover, another type of pathway-specific regulatory family containing an N-terminal PAS doamin and a C-terminal HTH domain was also identified in a polyene macrolide pathway cluster such as NysRIV for nystatin, PimM for pimaricin, was identified, respectively [1, 21].

Previously, using a polyene cytochrome P450 hydroxylase-specific genome screening strategy, *Pseudonocardia autotrophica* KCTC9441 was determined to contain genes potentially encoding polyene biosynthesis [15]. In addition, sequence information of an approximately 125.7-kb contiguous DNA region in five overlapping cosmids isolated from the *P. autotrophica* KCTC9441 genomic library revealed a total of 22 open reading frames, which are presumably involved in the nystatin-like Pseudonocardia polyene (NPP) biosynthesis of a nystatin-like compound tentatively named NPP (Fig. 1a) [12]. Herein we report *in silico* analysis of six putative NPP pathway-specific regulatory genes present in its biosynthetic gene cluster, followed by functional overexpression of these regulatory genes in *P. autotrophica*. The results described suggest that identification and overexpression of key pathway-specific regulatory gene, followed by optimum harvest timing, should be critical factors to maximize the productivity of an intrinsically low-level metabolite such as NPP produced by rare actinomycetes species.

Materials and methods

Bacterial strains and growth conditions

Pseudonocardia autotrophica KCTC9441, purchased from the Korean Collection, was used as an NPP-producing strain [12]. *P. autotrophica* strains were grown at 28°C on GSMY agar medium or ISP2 agar medium for sporulation [13, 21], and grown in GSMY liquid medium for DNA isolation. For NPP production, the strain was grown in YEME liquid medium [12, 13]. *Escherichia coli* DH5α strain was primarily used for DNA cloning and plasmid propagation. The methylation-deficient *E. coli* ET12567/pUZ8002 was used as donor in intergeneric conjugations [7]. Standard genetic techniques with *E. coli* and in vitro DNA manipulations were as described by Sambrook et al. [20]. Most of the recombinant DNA techniques in *P. autotrophica* were performed as described in the *Streptomyces* manual by Kieser et al. [12].

Analysis and expression of NPP pathway-specific regulatory genes

In silico analysis of the amino acid sequences was performed by using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov), Frameplot 2.3.2 (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl), EnCyclon (http://www.encyclon.net), and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) search engines. Each DNA fragment including the entire *nppR* gene was amplified by polymerase chain reaction (PCR) using genomic DNA from the wild-type NPP-producing strain as a template. PCR was performed in final volume of 20 μl containing 0.4 μM of each primer, 0.25 mM of each of the four deoxynucleoside triphosphates (dNTPs, Roche), 1 μl extracted DNA, 1 U Ex Taq polymerase (TaKaRa, Japan) in its recommended reaction buffer, and 10% dimethyl sulfoxide (DMSO). Amplifications were performed in a Thermal Cycler (Bio-Rad, USA) according to the following profile: 30 cycles of 60 s at 95°C, 60 s at 55°C, and 70 s at 75°C. The amplified PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel and purified via a DNA extraction kit (COSMO, Korea). This was ligated into pMD18-T (TaKaRa), followed by complete nucleotide sequencing confirmation by COSMO Genetech, Korea. Each of the PCR-amplified *nppR* genes was cloned into the *Bam*HI and *Xba*I double-digested pSET152 derivative integrative plasmid [4] containing the strong constitutive promoter *ermE**.

HPLC quantification for NPP production

P. autotrophica strains were cultivated at 28°C in YEME media for NPP production. For time course of NPP production, each seed culture was first grown for 72 h in TSB medium, and then 2 ml seed culture (1% v/v) was transferred to inoculate a 500-ml triple-baffled flask containing 200 ml production media. Each culture was grown at 28°C in a rotary shaker at 210 rpm for 7 days. Extracts were prepared by extraction with equal volume of butanol, followed by concentration and methanol resuspension. A Shimadzu SPD M10A (Shimadzu, Japan) with reversed-phase C-18 column (5 μm particles, 4.6 × 150 mm, Phenomenex, USA) was used for the assay. The sample injection volume was 20 μl, and the run time was fixed at 20 min. The column was kept at 25°C, and an isocratic elution system was maintained with 0.05 M ammonium

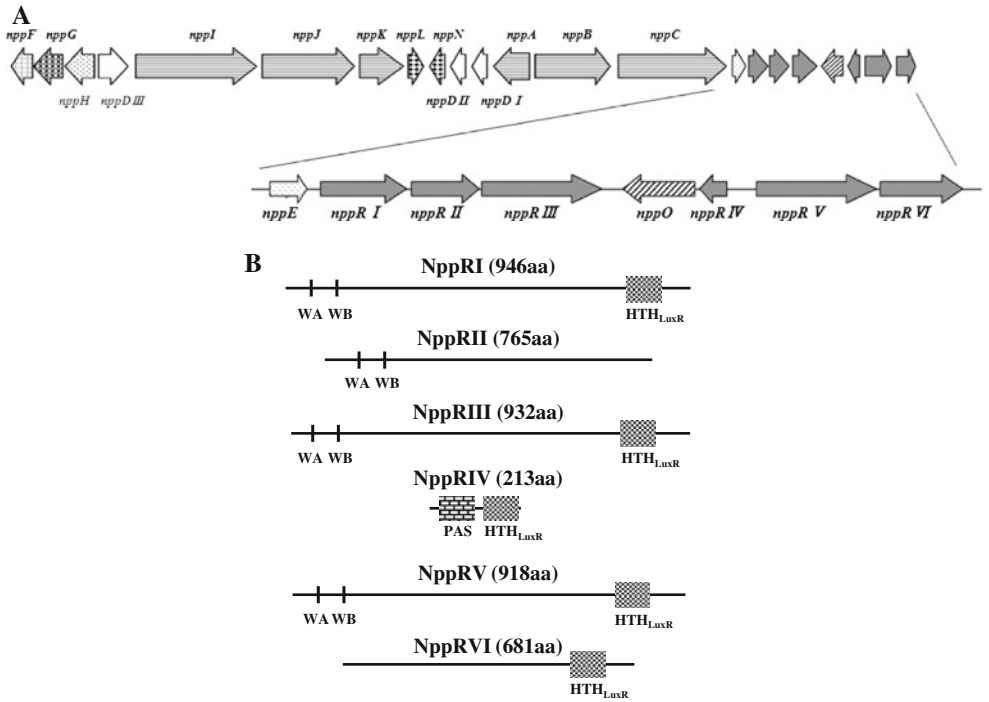

Fig. 1 **a** NPP biosynthetic pathway gene cluster. **b** Functional features of the NppRI, NppRII, NppRIII, NppRIV, NppRV, and NppRVI proteins. *WA* and *WB* Walker A and B NTP binding motifs, *PAS* PAS-like domain

acetate (pH 3.8) mixed with acetonitrile at ratio of 65:35. The flow rate was kept constant at 1.0 µl/min, and the polyene compounds were detected using a photodiode array (PDA) detector [13].

Antifungal bioassay against *Candida albicans*

NPP production of empty vector-containing *P. autotrophica* wild type and *nppRIII*-overexpressing *P. autotrophica* exconjugant was compared using a paper disc agar diffusion method using *C. albicans* as indicator [13]. The agar medium was composed of two separate layers. In the first layer, 15 ml YM medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1.0%) formed a base layer in the Petri dish. After solidification, 0.03 ml suspension of *C. albicans* mixed with 15 ml sterile YM medium at 35–40°C was poured immediately onto the base layer to constitute the upper layer. Four pieces of 6-mm-diameter paper disk were then placed onto a dish. Two paper disks contained 15 µl methanol-extracted NPP produced by each recombinant *P. autotrophica*, with two paper disks as control with 15 µl methanol as negative and 15 µl nystatin (Sigma) as positive standards. To observe diffusion of NPP in the methanol extract into the medium of the Petri dish, the plates were incubated for 1 day at 37°C, and the inhibitory zone was then observed.

Construction of *nppRIII* disruption via chromosomal gene disruption

A putative regulatory gene, *nppRIII*, was inactivated using a PCR-targeted gene-disruption system [8]. An apramycin resistance gene/oriT cassette for replacement of *nppRIII* was amplified using pIJ773 as template [8], using the following primers: forward (5′-gagacccacgaggagcaggacc gggtgcgcgagctgcag ATTCCGGGGATCCGTCGACC-3′) and reverse (5′-aacagggccgggttggaccagccgaggtgctccaggtgt TGTAGGCTGGAGCTGCTTC-3′), where lower-case type represents 39 nt homologous extensions to the DNA regions inside *nppRIII*. This cassette was introduced into *E. coli* BW25113/pIJ790 containing the cosmid which has the *nppRIII* group. It was introduced into *P. autotrophica* by conjugation from *E. coli* ET12567/pUZ8002. After incubation at 28°C for 18 h, each plate was flooded with 1 ml sterile water containing apramycin at final concentration of 1.5 mg/ml and trimethoprim at final concentration of 1.5 mg/ml. Incubation continued at 28°C until conjugants appeared. The double crossover exconjugants were selected as a standard Apr^r/Kan^s method [12]. Two different PCR primer pairs were used for confirmation of the double crossover recombination. Two pairs of primers (#1-1′ and #2-2′) were used for confirmation of the replacement of the apramycin resistance gene/oriT cassette

(forward primer 1, 5′-GACTTCGAGTTCGGCGTC-3′; reverse primer 1′, 5′-GGTCAGCCGTTTCTCCAC-3′; forward primer 2, 5′-ACGAAGAAGAACGGAAACGC-3′; reverse primer 2′, 5′-TTTCCACACCTGGTTGCTGA-3′).

Isolation of total RNA and gene expression analysis by RT-PCR

To investigate transcription of *nppRIII* and its effect, total RNA was isolated from strains grown in YEME medium for 48 h. The mycelia were harvested by centrifugation and immediately frozen by liquid nitrogen. The frozen mycelia were ground using a mortar into a fine white powder, and RLT buffer (Qiagen) in the presence of 1% β-mercaptoethanol was added. RNeasy mini spin columns were used for RNA isolation according to the manufacturer's protocol. RNA preparations were treated with DNase I (Qiagen) to prevent possible chromosomal DNA contamination. DNase I-treated RNA (7 μg) was used as a template for reverse transcription at 50°C with an AVM Reverse Transcriptase XL (TaKaRa) and random hexamers. The conditions for complementary DNA (cDNA) synthesis were as follows: 30°C for 10 min, 50°C for 1 h, 99°C for 5 min, 5°C for 5 min. The synthesized cDNA was used for PCR amplification under the following conditions: 34 cycles of denaturation at 95°C for 45 s, annealing at 61°C for 45 s, and extension at 72°C for 1 min. Each primer pair of NPP biosynthetic genes was carefully designed to generate a PCR product of approximately 150–200 bp using the genscript site (http://www.genscript.com/ssl-bin/app/primer).

Results and discussion

Previously, a total of six putative regulatory genes (*nppRI*, *nppRII*, *nppRIII*, *nppRIV*, *nppV*, and *nppVI*), located just downstream of *nppE* at the right fringe of the cluster (Fig. 1a), were suggested to be involved in NPP biosynthesis, on the basis of high amino acid similarities and previously characterized regulatory functions in both nystatin and FR008/candicidin biosynthesis (Fig. 2) [4, 21]. Unlike a typical single pathway-specific gene for a secondary metabolite regulatory system present in *Streptomyces* species, interestingly, six putative pathway-specific regulatory genes were found to be clustered in the NPP biosynthetic cluster in *P. autotrophica* [13]. Using database-assisted *in silico* analysis, three putative NPP regulators (NppRI, NppRIII, and NppRV) were revealed to belong to a typical LAL (large ATP-binding regulators of the LuxR) family (Fig. 1b). Notably, the predicted amino acid sequences of these three proteins showed two highly conserved domains: a putative HTH motif in the C-terminal region typically found in various bacterial DNA-binding proteins [11], and Walker A and B nucleoside triphosphate (NTP) binding motifs at the N-terminal region [6, 23]. Since the polyketide pathway-specific regulatory proteins including PikD for pikromycin from *S. venezuelae*, RapH for rapamycin from *S. hygroscopicus*, and NysRI/RIII for nystatin from *S. nouresi* were previously assigned to the LAL family, NppRI, NppRIII, and NppRV are also presumed to encode a LAL-family pathway-specific regulatory protein involved in NPP biosynthesis from *P. autotrophica*. Meanwhile, NppRIV was found to

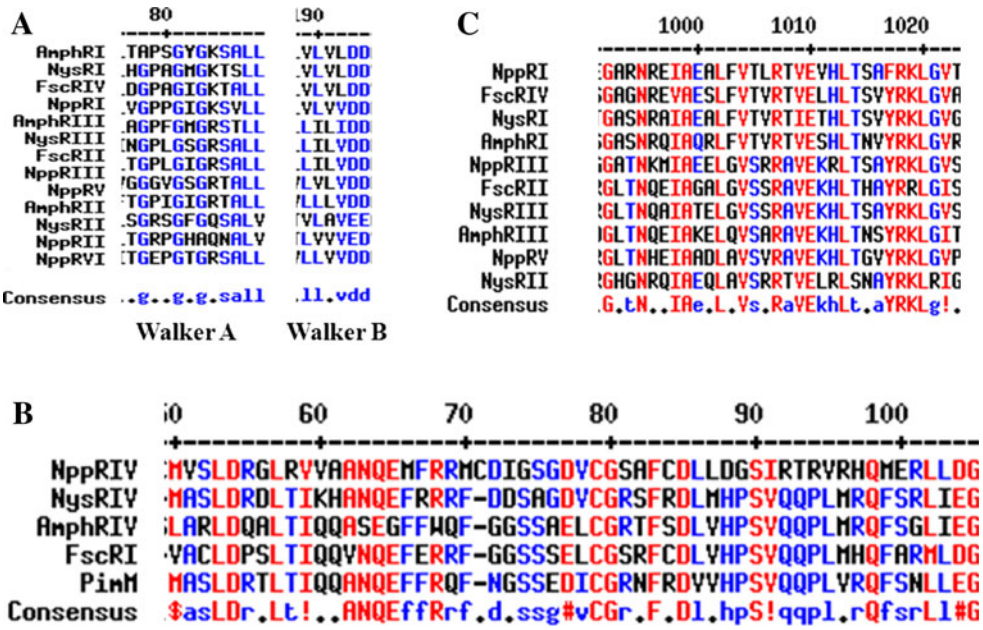

Fig. 2 **a** Amino acid sequence alignment to compare NppR regulatory functions with their ortholog; NPP (Npp), amphotericin (Amph), candicidin (Fsc), nystatins (Nys), pimaricin (Pim). Sequence comparison of the N-terminal Walker A and B NTP binding motifs of the NppR group with that of LAL-family regulators. **b** PAS domains of NppRIV and other regulators of polyene biosynthetic gene clusters. **c** Sequence alignment of the C-terminal LuxR-type HTH DNA-binding domains of NppR group orthologs

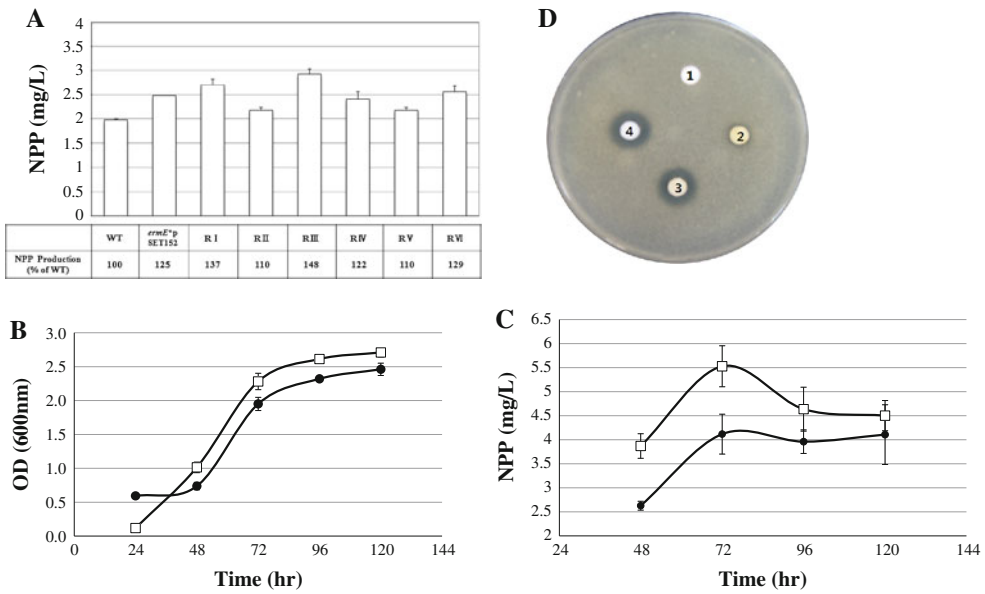

Fig. 3 **a** NPP volumetric productivity in derivative strains of *P. autotrophica*. WT, wild type; *ermE**pSET152, *P. autotrophica/ermE**pSET152; RI, *P. autotrophica/ermE*nppRI*; RII, *P. autotrophica/ermE*nppRII*; RIII, *P. autotrophica/ermE*nppRIII*; RIV, *P. autotrophica/ermE*nppRIV*; RV, *P. autotrophica/ermE*nppRV*; RVI, *P. autotrophica/ermE*nppRVI*. Data are means form three independent triplicates experiments. **b** Comparison of growth in derivative strains of *P. autotrophica*: growth curves of the strain containing an empty vector (*P. autotrophica/ermE**pSET152, *solid circles*) and the *nppRIII*-overexpression strain (*P. autotrophica/ermE*nppRIII*, *open squares*). **c** Time course of NPP production measured by quantitative high-performance liquid chromatography (HPLC) analyses from the strain containing an empty vector (*P. autotrophica/ermE**pSET152, *solid circles*) and the strain with an extra copy of *nppRIII* (*P. autotrophica/ermE*nppRIII*, *open squares*). Data are the means of two triplicates flasks. **d** Comparison of NPP production in derivative strains of *P. autotrophica* by paper disc agar diffusion method using *C. albicans* as the indicator; 1—methanol; 2—*P. autotrophica/ermE**pSET152 extract (19.5 μg); 3—*P. autotrophica/ermE*nppRIII* extract (37.5 μg); 4—Nystatin (15 μg)

contain a signal sensor PAS domain at the N-terminal and a HTH DNA binding motif at the C-terminal [10, 19, 22]. Since NysRIV and PimM, which show significantly high amino acid identities (45.6% and 45.0%) to NppRIV, were verified to control the expression of their biosynthetic genes via targeted gene disruption and complementation [1, 21], NppRIV is also proposed to be a pathway-specific regulator. Since the remaining two putative regulatory gene products, NppRII and NppRVI, lack a C-terminal HTH motif and an N-terminal Walker A and B NTP binding motif, respectively, it is not clear whether they are functional regulators for NPP biosynthesis.

To demonstrate that these six genes are responsible for stimulation of NPP biosynthesis, we engineered the wild-type strain of *P. autotrophica* by expressing each of these six genes under the influence of the strong constitutive *ermE** promoter in the integrative expression pSET152 vector. This was followed by conjugation into NPP-producing *P. autotrophica*. Comparing NPP production of wild type and the *nppR*-containing exconjugant showed that *nppRI*, *nppRIII*, and *nppRVI* stimulated NPP production by approximately 37%, 48%, and 29%, respectively, in *P. autotrophica* (Fig 3a). Interestingly, the *P. autotrophica* with the empty vector integration also showed about 25% increase of NPP production, the detailed mechanism for which is not clear at the moment. Moreover, culture-time-dependent analysis revealed that the *nppRIII*-stimulated NPP biosynthesis was most significant in the late exponential growth stage (72 h) and then gradually decreased (Fig. 3b, c). NPP antifungal bioassay against *C. albicans* was also performed to visually confirm the enhanced production of NPP by the *nppRIII* overexpression (Fig. 3d).

Although the *in silico* sequence analysis and functional overexpression of *nppRIII* are consistent with its key regulatory role in NPP biosynthesis, we sought to confirm the in vivo function of *nppRIII* using reverse-transcription (RT)-PCR analyses of the *nppRIII*-overexpression strain as well as the *nppRIII*-disruption mutant strain. Inactivation of *nppRIII* was performed using a PCR-targeted gene disruption system (Fig. 4a). The expected size of 2.6 kb for the PCR-amplified band was observed in genomic DNA samples isolated from *P. autotrophica*, while a band of the expected size (2.1 kb) was observed in genomic DNA samples isolated from the *P. autotrophica nppRIII*-disruption mutant (Fig. 4b). The expected size (1.8 kb) PCR-amplified fragment using the alternative PCR primer pair #2-2′ was observed in the *P. autotrophica nppRIII* diruption mutant which contain cosmid (single crossover); whereas, there is no PCR amplified fragment in double crossovered *P. autotrophica nppRIII* diruption mutant (Fig. 4c). As

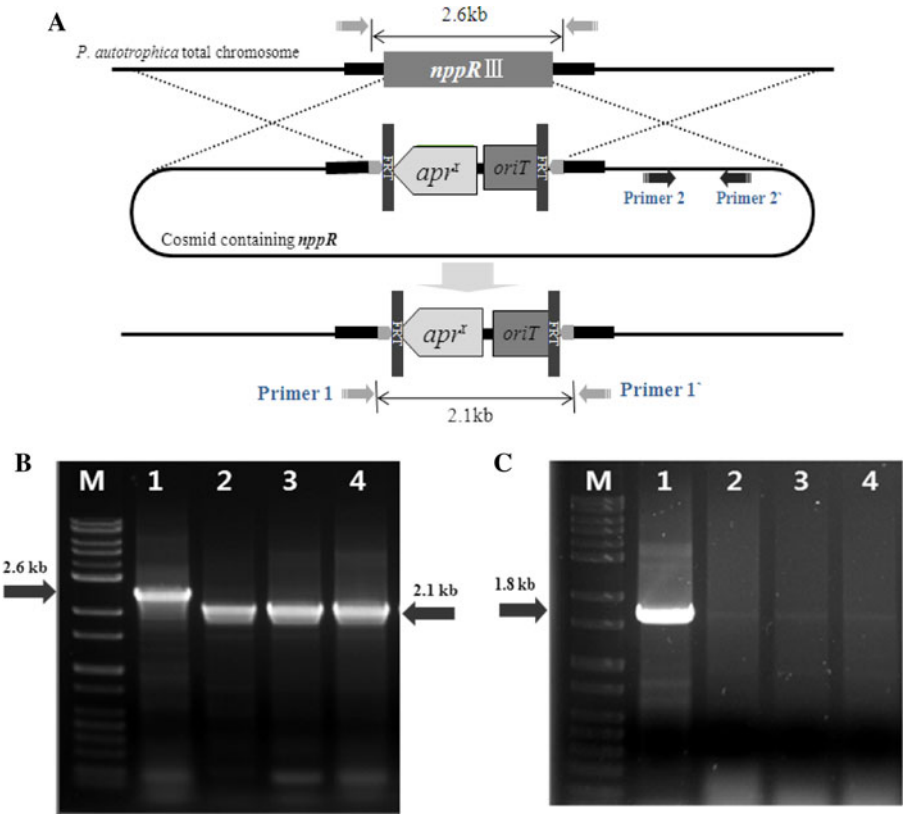

Fig. 4 **a** Schematic representation of PCR-targeted gene disruption of *nppRIII*. **b** Gene replacement was confirmed by PCR. The PCR products were amplified by using the primer pair #1-1′; *lane M*, 1-kb DNA ladder; *lane 1*, *P. autotrophica*; *lane 2*, cosmid DNA (*nppRIII*::apr-oriT); *lanes 3–4*, *P. autotrophica nppRIII*-disruption mutant strains. **c** The presence of cosmid in *P. autotrophica* total chromosomal DNA was confirmed by PCR. The PCR products were amplified by using the primer pair #2-2′; *lane M*, 1-kb DNA ladder; *lane 1*, cosmid DNA (*nppRIII*::apr-oriT); *lane 2*, *P. autotrophica*; *lanes 3–4*, *P. autotrophica nppRIII*-disruption mutant strains

expected, the *P. autotrophica nppRIII*-disruption mutant failed to produce NPP and exhibited no antifungal activity (data not shown). As shown in Fig. 5a, most of the NPP biosynthetic gene transcripts were detected at higher (or at least similar) levels in the *nppRIII*-overexpressing strain compared with the wild-type strain (Fig. 5a). Moreover, significantly reduced or no transcripts were detected in all the NPP biosynthetic genes from the *P. autotrophica nppRIII*-disruption mutant (Fig. 5b). These results strongly suggest that *nppRIII* could be probably the most important key regulatory gene among the six putative pathway-specific regulatory genes in the cluster in *P. autotrophica*, whose detailed regulatory mechanism should be further elaborated.

In conclusion, the results described suggest that identification and overexpression of key pathway-specific regulatory gene, followed by optimum harvest timing, should be critical factors to maximize the productivity of an

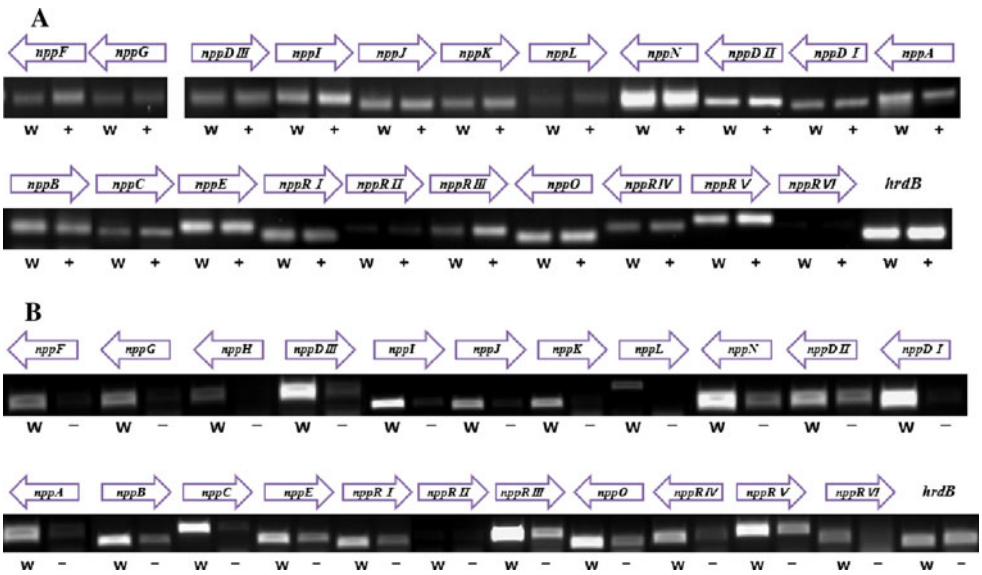

Fig. 5 **a** Gene expression analysis of the NPP gene cluster by RT-PCR was carried out on *P. autotrophica* wild-type (W) and *P. autotrophica nppRIII*-overexpressing strains (+). **b** Gene expression analysis of the NPP gene cluster by RT-PCR was carried out on *P. autotrophica* wild-type (W) and *P. autotrophica nppRIII*-disruption mutant strains (–). Transcription of the *hrdB* gene was also assessed as an internal control

intrinsically low-level metabolite such as NPP produced by rare actinomycetes species.

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