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A newly constructed *Agrobacterium*-mediated transformation system revealed the influence of nitrogen sources on the function of the LaeA regulator in *Penicillium chrysogenum*

Tao Xuan Vu [a, b], Ha Hong Vu [a, b], Giang Thu Nguyen [a, b], Hien Thu Vu [a, b], Linh Thi Dam Mai [a], Duc-Ngoc Pham [a], Diep Hong Le [c], Huy Quang Nguyen [c], Van-Tuan Tran [a, b, *]

[a] Department of Microbiology, Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam
[b] Genomics Unit, National Key Laboratory of Enzyme and Protein Technology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam
[c] Department of Biochemistry and Molecular Biology, Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam

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ABSTRACT

Penicillium chrysogenum is not only an industrially important filamentous fungus for penicillin production, but it also represents as a promising cell factory for production of natural products. Development of efficient transformation systems with suitable selection markers is essential for genetic manipulations in *P. chrysogenum*. In this study, we have constructed a new and efficient *Agrobacterium tumefaciens*-mediated transformation (ATMT) system with two different selection markers conferring the resistance to nourseothricin and phleomycin for *P. chrysogenum*. Under the optimized conditions for co-cultivation at 22 °C for 60 h with acetosyringone concentration of 200 μM, the transformation efficiency of the ATMT system could reach 5009 ± 96 transformants per 10^6 spores. The obtained transformants could be exploited as the T-DNA insertion mutants for screening genes involved in morphogenesis and secondary metabolism. Especially, the constructed ATMT system was applied successfully to generate a knockout mutant of the *laeA* regulatory gene and relevant complementation strains in a wild strain of *P. chrysogenum*. Our results indicated that the LaeA regulator controls growth, sporulation, osmotic stress response and antibiotic production in *P. chrysogenum*, but its function is reliant on nitrogen sources. Furthermore, we showed that the *laeA* orthologous genes from the citrus postharvest pathogen *P. digitatum* and from the industrial fungus *Aspergillus niger* could recover the phenotypic defects in the *P. chrysogenum laeA* deletion mutant. Conclusively, this work provides a new ATMT system, which can be employed for T-DNA insertional mutagenesis, heterologous gene expression or for molecular inspections of potential genes related to secondary metabolism in *P. chrysogenum*.

1. Introduction

The filamentous fungus *Penicillium chrysogenum* is well-known for industrial production of the first β-lactam antibiotic, penicillin, used for medical treatment of bacterial infectious diseases (van den Berg, 2010). This fungus has been recently employed as a host for production of different natural products such as the pigment chrysogine, antifungal proteins, extracellular enzymes and semi-synthetic cephalosporin (Guzman-Chavez et al., 2018b; Jami et al., 2010; Sonderegger et al., 2016; Veiga et al., 2012a). *P. chrysogenum* has been sequenced for the whole genome (van den Berg et al., 2008), therefore engineering of this fungus to improve its capacity for biosynthesis of natural products becomes more convenient.

Engineering of fungi usually requires genetic tools including efficient transformation methods (Martins-Santana et al., 2018; Nora et al., 2019a,b). Recently, the new genome editing tool CRISPR/Cas9 has been successfully developed in fungi including *P. chrysogenum* (Nødvig et al., 2015; Pohl et al., 2016). CRISPR/Cas9 systems for genetic engineering of filamentous fungi also require suitable delivery methods, in which protoplast-mediated

* Corresponding author. Department of Microbiology, Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam.
E-mail address: tuantran@vnu.edu.vn (V.-T. Tran).

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transformation (PMT) and *Agrobacterium tumefaciens*-mediated transformation (ATMT) are the commonly used methods (Song et al., 2019). ATMT has been used for gene transfer in numerous filamentous fungi since 1998 (de Groot et al., 1998; Idnurm et al., 2017; Michielse et al., 2005; Sun et al., 2019). Although ATMT was applied successfully to *P. chrysogenum* (de Boer et al., 2013; Sun et al., 2002), it has not been significantly exploited for genetic manipulations in this fungus. Currently, PMT is still the preferred method employed for *P. chrysogenum* (Guzman-Chavez et al., 2018a; Hoff et al., 2010; Li et al., 2017). However, PMT usually requires protoplasts, which are generated by using an enzyme cocktail and a complicated protocol (de Bekker et al., 2009). In comparison to the PMT method, the advantage of the ATMT method is that fungal spores can be directly used as material for transformation without any further treatment (Nguyen et al., 2016; Vu et al., 2018). Therefore, development of efficient ATMT systems with more options for selection markers in *P. chrysogenum* will provide additional platforms for engineering of this industrial fungus.

Up to date, several genes associated with penicillin biosynthesis in *P. chrysogenum* have been investigated for their functions by genetic engineering approaches (Guzman-Chavez et al., 2018a; Liu et al., 2013; Sigl et al., 2011; Weber et al., 2012). Among them, the genes encoding regulatory proteins of velvet complex play vital roles in fungal development and penicillin biosynthesis in *P. chrysogenum* (Kopke et al., 2013). A key member of this protein complex as the global regulator LaeA was indicated to control fungal growth, sporulation and secondary metabolism (Hoff et al., 2010; Kosalkova et al., 2009). In other filamentous fungi, LaeA control of fungal development and secondary metabolism is coordinated by environmental factors such as nutrition and abiotic stresses (Sarikaya Bayram et al., 2010; Wiemann et al., 2010). However, the influence of these factors on the function of LaeA in *P. chrysogenum* remains to be clarified.

In this study, we have constructed a highly efficient ATMT system for genetic manipulation in *P. chrysogenum*. By employing the developed ATMT system, we further showed the first time that the function of the LaeA regulator in a wild strain of *P. chrysogenum* in regulation of fungal development, osmotic stress response and secondary metabolism is strongly affected by nitrogen sources, especially when nitrate is used as sole nitrogen source.

2. Materials and methods

2.1. Microbial strains and cultivation media

Escherichia coli DH5α and *A. tumefaciens* AGL1 were used for plasmid propagation and fungal transformation, respectively. *Staphylococcus aureus* ATCC 25923 was employed as the indicator strain for antibacterial activity assay (Treangen et al., 2014). These bacterial strains were cultivated in Luria-Bertani (LB) medium. A wild strain of *P. chrysogenum* (code: VTCC 31172) isolated from fungi-contaminated rice was provided by Vietnam Type Culture Collection (http://vtcc.info), Vietnam National University – Hanoi. This fungal strain was grown and maintained on the potato dextrose agar (PDA) medium.

2.2. Preparation of fungal spore suspension and genomic DNA extraction

For spore preparation, the fungal strains were grown on the PDA plates at 25–28 °C for 3–6 d. Sterile distilled water was added to the agar plate surface and spores were liberated from fungal mycelium by scraping with a sterile glass spreader. The liquid mixture was collected and filtered through Miracloth (Calbiochem, Darmstadt, Germany) before a centrifugation at 4000 rpm for 10 min. The spore pellet was washed twice with sterile distilled water and resuspended in sterile distilled water again to obtain a final spore suspension. Fungal spore concentration was quantified under microscopy using a hemocytometer (HEINZ HERENZ Medizinalbedarf GmbH, Hamburg, Germany). The obtained spore suspension was adjusted to the concentration of 10^6 spores/ml. The spore suspension was directly used after preparation or stored at 4 °C for later use.

For fungal genomic DNA extraction, the fungal strains were grown in the potato dextrose broth (PDB) medium at the temperature of 25–28 °C, 200 rpm for 3 d and the mycelia were harvested by filtration of the cultures through Miracloth (Calbiochem, Darmstadt, Germany). Extraction of genomic DNA from the fungal mycelia was conducted as previously described (Tran et al., 2017).

2.3. Assays of fungal sensitivity towards antifungal substances

A fresh 4-mm diameter agar plug containing the 3-day-old fungal mycelium or 10 μl of each spore suspension (10^6 spores/ml) of the tested fungal strain was placed on the PDA medium supplemented with different antibiotic concentrations of hygromycin (100–800 μg/ml), nourseothricin (25–100 μg/ml) or phleomycin (50–200 μg/ml). The plates were incubated at the temperature of 25 °C for 3–4 d to examine fungal growth.

2.4. PCR amplification

Target DNA sequences were amplified by PCR with specific primer pairs (Table 1). Phusion high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA) was used for DNA cloning, while the GoTaq® Green Master Mix (Promega, Madison, USA) was used for PCR screening, instead. The PCR procedure includes 94 °C (3 min); 30 cycles of 94 °C (30 s), 58–60 °C (30 s), 72 °C (1–2 min); 72 °C (7 min). The PCR products were analyzed on 0.7 % agarose gels. Target DNA bands were excised and purified with MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (Intron Biotechnology, Gyeonggi-do, Korea).

2.5. Plasmid construction

The binary vector pPK2-phleo was constructed by a replacement of the hygromycin resistance cassette of pPK2 (Covert et al., 2001) with the phleomycin resistance cassette, which was isolated from pAN8-1 (Mattern et al., 1988) by using the restriction enzymes *Eco*RI and *Xba*I. The *DsRed* expression cassette including the *Aspergillus nidulans gpdA* promoter, the *DsRed* gene and the *A. nidulans trpC* terminator was isolated from pEX2 (Nguyen et al., 2016) by digestion with *Spe*I and *Hin*dIII. This expression cassette was purified and ligated to pPK2-phleo at the compatible sticky ends generated by digestion with *Xba*I and *Hin*dIII. The recombinant plasmid pPK2-Red2 harbors the phleomycin resistance marker for the expression of the *DsRed* reporter gene under the control of the *A. nidulans gpdA* promoter.

The binary vector for deletion of *laeA* in *P. chrysogenum* VTCC 31172 was constructed as follows. The 5′ and 3′ flanking regions of the *laeA* gene corresponding to the scaffold_4:2207350-2211500 in the genome database of the filamentous fungus *P. chrysogenum* (https://genome.jgi.doe.gov/Pench1/Pench1.home.html) were amplified by PCR using the specific primer pairs P1/P2 and P3/P4, respectively (Table 1). The obtained PCR products were digested with *Eco*RV/*Sac*I or *Xba*I/*Hin*dIII and purified for ligations to the binary vector pKO211 at the compatible restriction sites. The recombinant binary vector pLaeAΔ harbors the *laeA* deletion cassette including the *laeA* 5′ flanking sequence (1.499 kb), the

Table 1
The primers used in this study.

Primer name	Sequence (5′-3′)	Product size (bp)	Source
GFP-F	ATGGTGAGCAAGGGCGAG	720	Nguyen et al. (2017)
GFP-R	TCACTTGTACAGCTCGTCCATGC		
NAT-F	GGTTAATTAACAACTGATATTGAAGGAGCA	970	Vu et al. (2018)
NAT-R	GGACTAGTGCTTTGGTTTAGGGTTAGG		
DsRed-F	AACTCGAGCACGTGCTTAAGGATATCATGGCCTCCTCCGAGG	729	Nguyen et al. (2017)
DsRed-R	AAGGATCCCCGCGGGAGCTCGATATCCTACAGGAACAGGTGGTGGC		
Phleo-F	GGGCTCGAGAGGCCTCCGGTGACTCTTTCTGGC	1250	This study
Phleo-R	TCGGTCAGTCCTGCTCCT		
ORF-F	GCGACACCTACCTCCCATAA	1254	This study
ORF-R	TTATTCCTCGACTGGTTTTCG		
P1	GG**GATATC**AATTACTGAGCCTGGGCCTAC (*Eco*RV)	1499	This study
P2	GG**GAGCTC**GAAGGCGGTTGTGAATTGAT (*Sac*I)		
P3	GG**TCTAGA**GGGACGTGCTATGGCTAACT (*Xba*I)	1359	This study
P4	GG**AAGCTT**CAAGAATGGCTCAGGTGCTA (*Hin*dIII)		
P5	CCTGCTGCTAATGGGACTTC		This study
P6	TCCTATGACTGGCATGATCG	2697	This study
P7	GGGAGGATGTTGTTGCTGTT		
PdlaeA-F	GG**CTCGAG**ATGGACTTGTATACGCTGATCG (*Xho*I)	1496	This study
PdlaeA-R	GG**TCTAGA**CGTCAGCAGTTGGGGTCTAT (*Xba*I)		
AnlaeA-F	GGG**TACGTA**TTGATTGAACGAACCCCTGT (*Sna*BI)	1526	This study
AnlaeA-R	GGG**AAGCTT**CGTCAAGGAGATGGAATTGG (*Hin*dIII)		

nourseothricin resistant gene under the control of the *A. nidulans trpC* promoter (0.93 kb), and the *laeA* 3′ flanking sequence (1.359 kb). This vector was verified for the correctness by digestion with *Eco*RI.

The binary vectors harboring the intact *laeA* gene from *P. chrysogenum* or its orthologs from *Penicillium digitatum* and *Aspergillus niger* under the regulation of the *A. nidulans gpdA* promoter or the *Aspergillus oryzae amyB* promoter were constructed as follows for complementation of the Δ*laeA* mutant. The open reading frame (ORF) of *laeA* with its terminator was amplified from *P. chrysogenum* genome with the primer pair P6/P7 (Table 1) using Phusion high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA) to generate a blunt-end PCR product of 2.697 kb. This product was digested with *Hin*dIII prior to being purified for a ligation to the binary vector pPK2-Red2 at the restriction sites *Eco*RV and *Hin*dIII. The corresponding plasmid pPclaeA was confirmed by digestion with *Eco*RI. Similarly, the primer pair PdlaeA-F/PdlaeA-R (Table 1) provided a 1.480 kb– PCR product of the *laeA* gene with its 3′ terminal sequence from *P. digitatum* PdVN1 (Vu et al., 2018). The obtained blunt-end product was digested with *Xba*I and ligated into pPK2-Red2 at the restriction sites *Eco*RV and *Xba*I. The resultant binary vector pPdlaeA for expression of *PdlaeA* under the regulation of the *gpdA* promoter was verified by digestion with *Eco*RI and *Bam*HI. For expression of the *laeA* gene from *A. niger* N402 (Bos et al., 1988), the sequence of this gene with its terminal sequence was amplified with the primer pair AnlaeA-F/AnlaeA-R (Table 1). A 2.317 kb–PCR product was digested with *Sna*BI and *Hin*dIII prior to being fused to the *amyB* promoter at the compatible sites *Pml*I and *Hin*dIII in pEX2B (Nguyen et al., 2017). The whole *AnlaeA* expression cassette was isolated and ligated to a modified version of the binary vector pPK2-phleo at the restriction sites *Spe*I and *Hin*dIII. The recombinant vector pAnlaeA was confirmed by digested with *Eco*RI.

2.6. Agrobacterium tumefaciens-*mediated transformation of* P. chrysogenum

Genetic transformation of *P. chrysogenum* VTCC 31172 using *A. tumefaciens* was performed as previously reported for the citrus postharvest pathogen *P. digitatum* (Vu et al., 2018) with some minor modifications. The binary vector was transformed into the *A. tumefaciens* AGL1 competent cells by electroporation. Positive bacterial colonies were confirmed by PCR using a gene-specific primer pair (Table 1). *A. tumefaciens* AGL1 carrying the binary vector was then grown in LB liquid medium and subsequently pre-induced in the induction medium (IM) containing 200 μM acetosyringone (AS). A mixture including 100 μl of the induced *A. tumefaciens* culture and 100 μl of the fungal spore suspension (10^4, 10^5 or 10^6 spores/ml) was spread on the filter paper, code: FT-3-303-090 (Sartorius, Göttingen, Germany), laid on the IM agar plates containing AS (100, 200, 300 μM). Different time intervals (48, 60, 72 h) and different temperatures (20, 22, 25 °C) for co-cultivation were tested. The filter membranes were transferred to the PDA plates supplemented with nourseothricin (50 μg/ml) or phleomycin (200 μg/ml) for selection of fungal transformants and cefotaxime (300 μg/ml) for elimination of the *Agrobacterium* cells. The plates were incubated at the temperature of 25–28 °C for 4–5 d.

2.7. Analysis of fungal transformants

The obtained fungal transformants were grown on the PDA medium supplemented with nourseothricin (50 μg/ml) or phleomycin (200 μg/ml) to confirm their antifungal compound resistance ability. These transformants were then purified by single spore isolation and their mitotic stability was examined for at least three successive generations on the PDA medium without the antifungal compounds. The purified transformants were cultivated in the PDB medium and the obtained respective mycelia were used for genomic DNA extraction. Successful integrations of T-DNA fragments carrying respective target cassettes from the binary vectors into fungal genome were confirmed by PCR using the specific primer pairs (Table 1).

Expression of the *GFP* or *DsRed* reporter gene in the transformants was examined by fluorescence microscopy. The tested transformants were cultivated separately on sterile microscopic slides with the PDA medium as previously described (Vu et al., 2018). The samples were then detected for the green fluorescent or red fluorescent signal under the Axioplan fluorescence microscope (Carl Zeiss, Germany).

Examination of T-DNA insertion transformants was performed by cultivating them on the PDA medium for 3–5 d, at 28 °C for morphological observations of fungal colonies and fungal hyphae under microscopy. Further, these mutants were evaluated for

antibiotic biosynthesis ability, which exhibits the antibacterial activity against *S. aureus*.

2.8. Assays of carbon sources and osmotic stress agents on fungal growth

Fungal strains including the wild strain *P. chrysogenum* VTCC 31172, Δ*laeA* mutant, and complementation strain were cultivated on the Czapek-Dox agar (CDA) medium (2 % sucrose, 0.2 % $NaNO_3$, 0.1 % KH_2PO_4, 0.05 % $MgSO_4$, 0.05 % KCl, 0.05 % NaCl, 0.002 % $FeSO_4$, 1.6 % agar, pH 7), in which sucrose was replaced with different carbon sources (glucose, lactose, maltose, galactose, starch, cellulose, xylan). For osmotic stress assays, spore suspensions (10^6 spores/ml) of examined strains were grown on PDA, CDA plates supplemented with different concentrations of sorbitol (1–3 M) and sodium chloride (0.5–2 M). The minimal CDA medium containing sodium nitrate or ammonium acetate as the sole nitrogen source for comparison. The plates were incubated at 28 °C for 5 d.

2.9. Assays of antibiotic production in fungal strains

The antibiotic production medium (APM) for *P. chrysogenum* comprises (per liter) 22.5 g lactose, 7.5 g glucose, 3 g CH_3COONH_4, 3 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$, 0.005 g $CuSO_4.5H_2O$, 0.02 g $ZnSO_4.7H_2O$, 0.5 g Na_2SO_4, 0.02 g $MnSO_4.H_2O$, 0.05 g $CaCl_2.2H_2O$, pH 7.3. The wild strain *P. chrysogenum* VTCC 31172 and transgenic strains (T-DNA insertion mutants, the Δ*laeA* mutant and the complementation strains) were grown on the PDA plates at 28 °C for 7 d for harvesting spores. Afterwards, 1 ml of each spore suspension (10^6 spores/ml) was inoculated in a conical flask containing 50 ml of the liquid APM. The flasks were incubated in a shaking incubator at 200 rpm, 28 °C for 7 d. The obtained cultures were centrifuged at 6000 rpm for 10 min and the supernatants were collected for antibacterial activity assays.

For the antibacterial activity assay, the indicator bacterium *S. aureus* ATCC 25923 was grown in the LB liquid medium at 30 °C for 24 h and 50 μl of the bacterial culture was spread on a PDA plate by using sterile glass beads. Afterwards, four agar holes were made on the PDA plate by using a sterile 9 mm–diameter plastic tube and 50 μl of each supernatant collected from the respective fungal culture was added to the agar holes. The plate was kept at 4 °C for 4–6 h prior to being incubated at 37 °C for 24 h.

2.10. Assays for effects of nitrogen sources and acetate on fungal growth and antibiotic production

Ammonium salts including $(NH_4)_2SO_4$, NH_4Cl, and CH_3COONH_4 were used to replace $NaNO_3$ present in the media (CDA, APM) with the equal amounts (3 g/l) to examine their roles in fungal growth and antibiotic production. For evaluating effects of acetate, sodium acetate or acetic acid (3 g/l) was added to the media. The pH values of the modified CDA were adjusted to pH 7, while the pH values of the modified APM were set at pH 7.3. Fungal cultivation on agar plates was maintained at 28 °C for 5–7 d. Fungal growth on the media was captured with a digital camera and quantification of antibiotic production was performed as described above.

3. Results

3.1. P. chrysogenum is highly resistant to hygromycin, but sensitive to nourseothricin and phleomycin

The dominant selection markers conferring the resistance to hygromycin, nourseothricin and phleomycin have been widely used for genetic transformation of different fungi (Alshahni et al., 2010; de Groot et al., 1998; Janus et al., 2007; Punt et al., 1987; Tran et al., 2014; Vu et al., 2018). In this study, we examined the sensitivity of the wild strain *P. chrysogenum* VTCC 31172 to these antifungal compounds. The results revealed that this fungal strain is inherently resistant to high concentrations of hygromycin (up to 800 μg/ml), but is sensitive to nourseothricin and phleomycin. Fungal spores appear to be more susceptible towards nourseothricin and phleomycin than fungal mycelia. The concentration of 50 μg/ml for nourseothricin or 200 μg/ml for phleomycin inhibits completely fungal growth from both the inoculation material types (mycelia and spores) (Figs. S1 and S2). Therefore, these concentrations of the selection agents can be used for genetic transformation of *P. chrysogenum* VTCC 31172 to suppress the growth of untransformed fungal cells.

3.2. ATMT is a powerful method for genetic transformation of P. chrysogenum

The ATMT method has been demonstrated to be highly efficient for genetic transformation of filamentous fungi (de Groot et al., 1998; Idnurm et al., 2017; Michielse et al., 2005; Mullins et al., 2001; Nguyen et al., 2017; Vu et al., 2018). Although the ATMT method has been successfully utilized for the penicillin-producing fungus *P. chrysogenum*, the PMT method remains to be the most commonly used method for this industrial fungus (Cantoral et al., 1987; de Boer et al., 2010, 2013; Opalinski et al., 2010; Pohl et al., 2016; Sonderegger et al., 2016; Sun et al., 2002). In this study, we employed the binary vector pGreen3 (Vu et al., 2018) harboring the nourseothricin acetyltransferase gene under the control of the *A. nidulans trpC* promoter (P*trpC*) (Fig. 1A) for evaluating genetic transformation of *P. chrysogenum* VTCC 31172 by using the ATMT method with optimized transformation parameters. The results showed that ATMT is a very effective method for genetic transformation of *P. chrysogenum* VTCC 31172. We showed that changes in the parameters of the co-cultivation step strongly influenced on the transformation efficiencies. Under the optimized conditions for the ATMT method including the co-cultivation temperature of 22 °C, the co-cultivation time of 60 h, the AS concentration of 200 μM and the spore concentration of 10^6 spores/ml, the transformation efficiency of *P. chrysogenum* VTCC 31172 could reach a very high yield of 5009 ± 96 transformants per 10^6 spores (Fig. 1B). Additionally, five transformants were randomly selected for cultivation on the PDA medium without addition of nourseothricin as the selection agent for three successive generations. These transformants were then examined by PCR using the specific primer pairs (Table 1) and by fluorescence microscopy. The results revealed that the integration of the T-DNA fragment carrying the cassettes for nourseothricin resistance and *GFP* expression in all five transformants was mitotically stable through several generations. Examination under fluorescence microscopy confirmed the expression of the *GFP* reporter gene in fungal mycelium, in which the clear green signal was homogenously distributed across fungal cells (data not shown).

The phleomycin resistance gene was shown the first time as a selection marker for the protoplast-mediated transformation of *Aspergillus* species (Mattern et al., 1988). In present study, we indicated that this selection marker can be used effectively for the ATMT of *P. chrysogenum*. Two newly constructed binary vectors pPK2-phleo and pPK2-Red2 (Fig. S2) were used successfully for genetic transformation of the wild strain *P. chrysogenum* VTCC 31172 with the phleomycin concentration of 200 μg/ml for fungal selection. All tested transformants could maintain T-DNA fragments in their genomes and express stably the *DsRed* fluorescent reporter gene through several mitotic generations (Fig. 2).

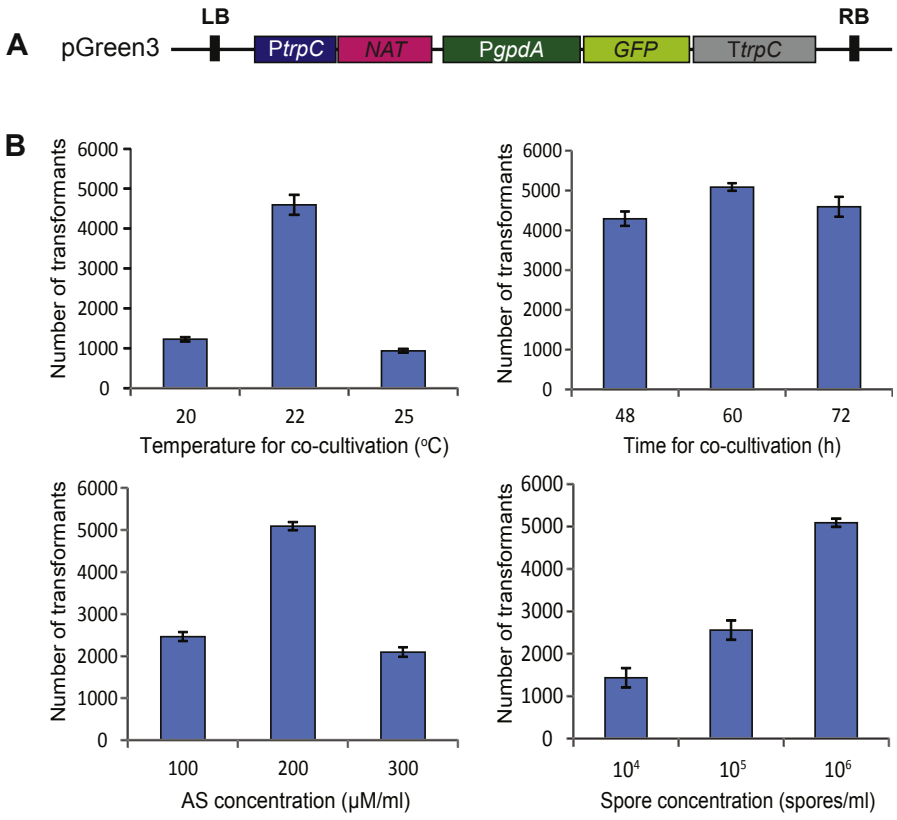

Fig. 1. Optimization of the ATMT method in *P. chrysogenum* VTCC 31172. (**A**) The scheme representing the T-DNA fragment of the binary vector pGreen3, which carries the cassettes for expression of the nourseothricin resistance gene (*NAT*) and the green fluorescent protein gene (*GFP*). The T-DNA fragment is restricted by two short specific sequences named left border (LB) and right border (RB). (**B**) Optimization of the ATMT method with different parameters of transformation including temperature (22–25 °C) and time (48–72 h) for co-cultivation, spore concentration (10^4–10^6 spores/ml) and acetosyringone (AS) concentration (100–300 μM/ml). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. *ATMT represents an effective tool for generation of T-DNA insertion mutants in* P. chrysogenum

ATMT has been demonstrated to be efficient for genetic transformation in numerous filamentous fungal species (de Groot et al., 1998; Idnurm et al., 2017; Michielse et al., 2005). Additionally, this method was also broadly employed as a tool for construction of libraries of T-DNA insertion mutants in filamentous fungi. The T-DNA insertion mutants can be used for identification of target genes involved in cellular differentiation, metabolic processes or fungal virulence by thermal asymmetric interlaced PCR (TAIL-PCR), inverse PCR or next-generation sequencing (NGS) analyses (Chambers et al., 2014; Maruthachalam et al., 2011; Mullins et al., 2001; Wang et al., 2014). Although ATMT was exploited successfully for transformation of *P. chrysogenum* (de Boer et al., 2013; Sun et al., 2002), there is no report on its applications in T-DNA insertional mutagenesis in this industrial fungus. Here, by using the optimized ATMT method we could generate a large number of transformants in *P. chrysogenum* (Figs. 1 and 2). Based on the phenotypic changes of the transformants by the T-DNA insertion events, we selected three mutants including Pc72-XN, Pc72-T and Pc72-V for further examinations. All three strains have defects in their phenotypes, especially in sporulation and pigmentation (Fig. 3A). Assays of antibiotic production for three mutant strains and the wild strain indicated that biosynthesis of penicillin in the fungal strains was increased by cultivation time. At the time point of 24 h of cultivation, all the tested strains represented no antibiotic activity against the indicator bacterial strain *S. aureus*. However, by the time periods of 48–96 h of cultivation, the supernatants from three mutants and the wild strain displayed strong activity in suppression of the growth of *S. aureus*. Interestingly, we found that the mutants Pc72-XN and Pc72-T displayed a significant reduction of antibacterial activity when compared to the wild strain (Fig. 3B).

3.4. *Successful construction of the* laeA *deletion mutant and the respective complemented strain in* P. chrysogenum *using the developed ATMT system*

In the filamentous fungus *P. chrysogenum*, LaeA was reported to control penicillin biosynthesis and sporulation by using RNA silencing technology (Kosalkova et al., 2009) or gene knockout via the PEG-mediated protoplast transformation method based on the *P. chrysogenum* ΔPcku70 strain lacking the *Pcku70* gene (Hoff et al., 2010). In this study, we successfully constructed a deletion mutant of the *laeA* gene (Δ*laeA* mutant) directly from the wild strain *P. chrysogenum* VTCC 31172. The *laeA* deletion construct harboring the nourseothricin resistance marker flanked by the 5′ and 3′ sequences of the *laeA* gene was used to delete this gene in *P. chrysogenum* VTCC 31172 by homologous recombination mediated by ATMT (Figs. S3, 4). Successful deletion of the *laeA* gene from the *P. chrysogenum* VTCC 31172 genome was confirmed with three specific primer pairs (Table 1). Firstly, all tested transformants including the wild strain, the Δ*laeA* mutant and two ectopic transformants were confirmed for the presence of the nourseothricin resistance cassette in their genomes. The respective primer pair NAT-F/NAT-R amplified a DNA band of 0.97 kb, which confers the nourseothricin resistance ability, only in the *laeA* mutant and ectopic strains. Further confirmations using two other primer pairs

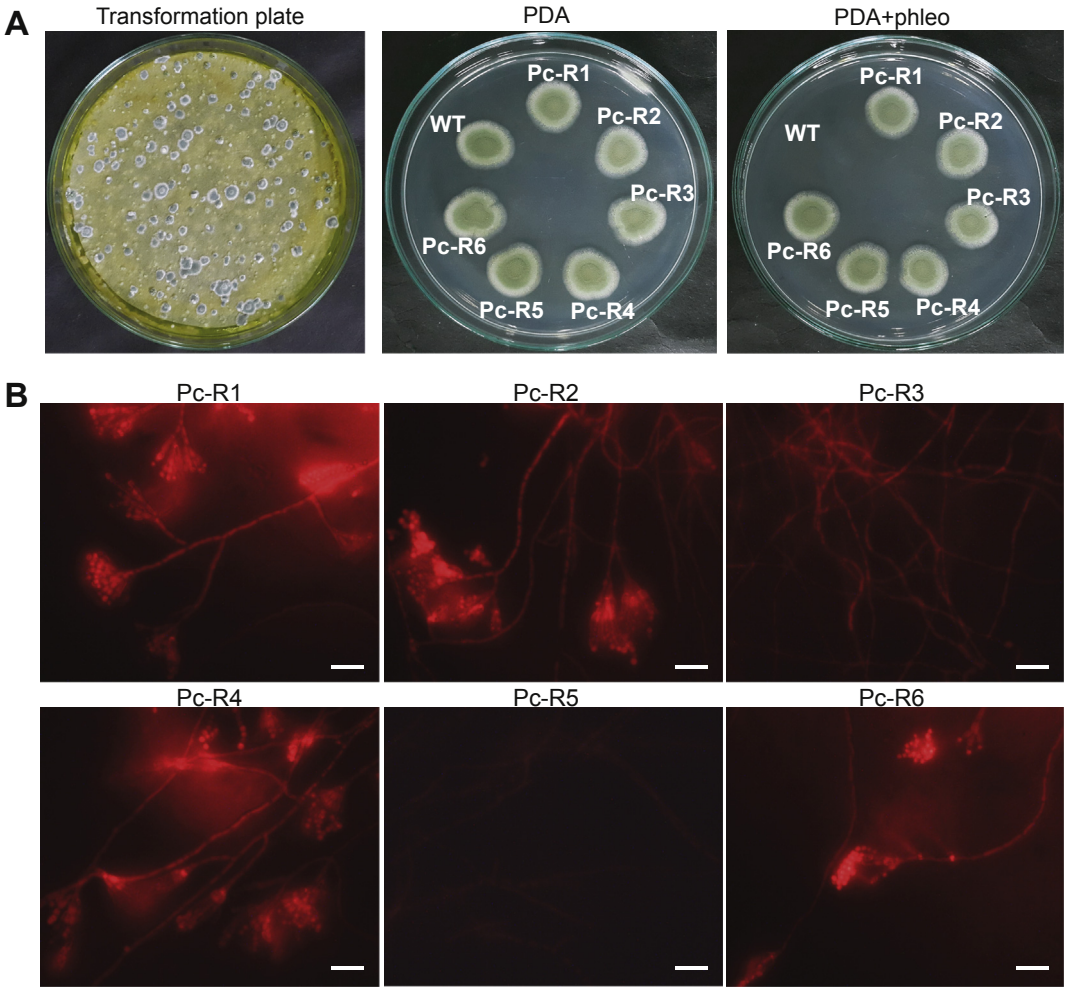

Fig. 2. Genetic transformation of *P. chrysogenum* VTCC 31172 using the optimized ATMT method and a newly constructed binary vector harboring the phleomycin resistance marker. (**A**) The transformation plate with phleomycin-resistant transformants. Six randomly selected transformants were cultivated in parallel on PDA and PDA supplemented with 200 μg/ml of phleomycin (PDA + phleo). The wild strain (WT) was used as control for comparison. (**B**) The selected transformants were confirmed by PCR using the primer pairs specific for the phleomycin resistance marker and the *DsRed* fluorescent reporter gene. Genomic DNA extracted from the wild strain and the binary vector pPK2-Red2 were used as template for negative control and positive control (+), respectively. (**C**) Expression of the *DsRed* gene in the transformants were examined and images were captured with a fluorescence microscope. The scale bars represent the same sizes of the images. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(ORF-F/ORF-R, NAT-F/P5), which bind specifically to the *laeA* locus, indicated the loss of this gene in the deletion mutant strain (Fig. 4A–B). Surprisingly, the results revealed that the *laeA* mutant could grow normally like the wild strain and the ectopic strains when cultivated on the rich medium (PDA). However, when cultivated on the minimal medium (CDA), growth and spore formation of the deletion mutant appeared to be remarkably retarded (Fig. 4C). We further confirmed that the defects in the Δ*laeA* mutant were recovered by complementation of the mutant with the intact *P. chrysogenum laeA* gene using the ATMT system with the phleomycin resistance marker (Fig. S4).

3.5. *LaeA controls fungal growth, sporulation, antibiotic production and osmotic stress response in a nitrogen source-dependent manner*

Fungal LaeA regulators have been demonstrated to play important roles in controlling fungal development and secondary metabolism in numerous filamentous fungi (Bok and Keller, 2004; Kumar et al., 2017; Martin, 2017; Sarikaya Bayram et al., 2010). In *P. chrysogenum*, LaeA was shown to be required for fungal sporulation and antibiotic production (Hoff et al., 2010; Kosalkova et al., 2009). However, effects of nutrition sources on these characteristics of the *laeA* gene in *P. chrysogenum* have not been reported so far. This study revealed that the deletion of *laeA* in the wild strain *P. chrysogenum* VTCC 31172 resulted in the delayed growth of the fungus only on the minimal CDA medium, but not on the nutrition-rich PDA medium (Fig. 4C). We examined if nitrogen sources could influence on the Δ*laeA* mutant. Two common nitrogen sources as salts of nitrate and ammonium including sodium nitrate ($NaNO_3$), ammonium sulfate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl), and ammonium acetate (CH_3COONH_4) were examined for their impacts on the growth and antibiotic production of the *laeA* mutant. We found that the growth of the *laeA* mutant was strongly reduced on CDA with nitrate as the sole nitrogen source. Conversely, this mutant could grow almost like the wild strain and the complemented strain on CDA when the nitrogen source was used as ammonium salts, especially with ammonium acetate (Fig. 5A). In correlation with the delayed growth, sporulation in the *laeA* mutant was significantly decreased in the CDA medium containing nitrate as the sole nitrogen source. We further compared sporulation of the *laeA* mutant grown on the rich-nutrition PDA medium with that on the minimal CDA medium containing sodium nitrate or ammonium

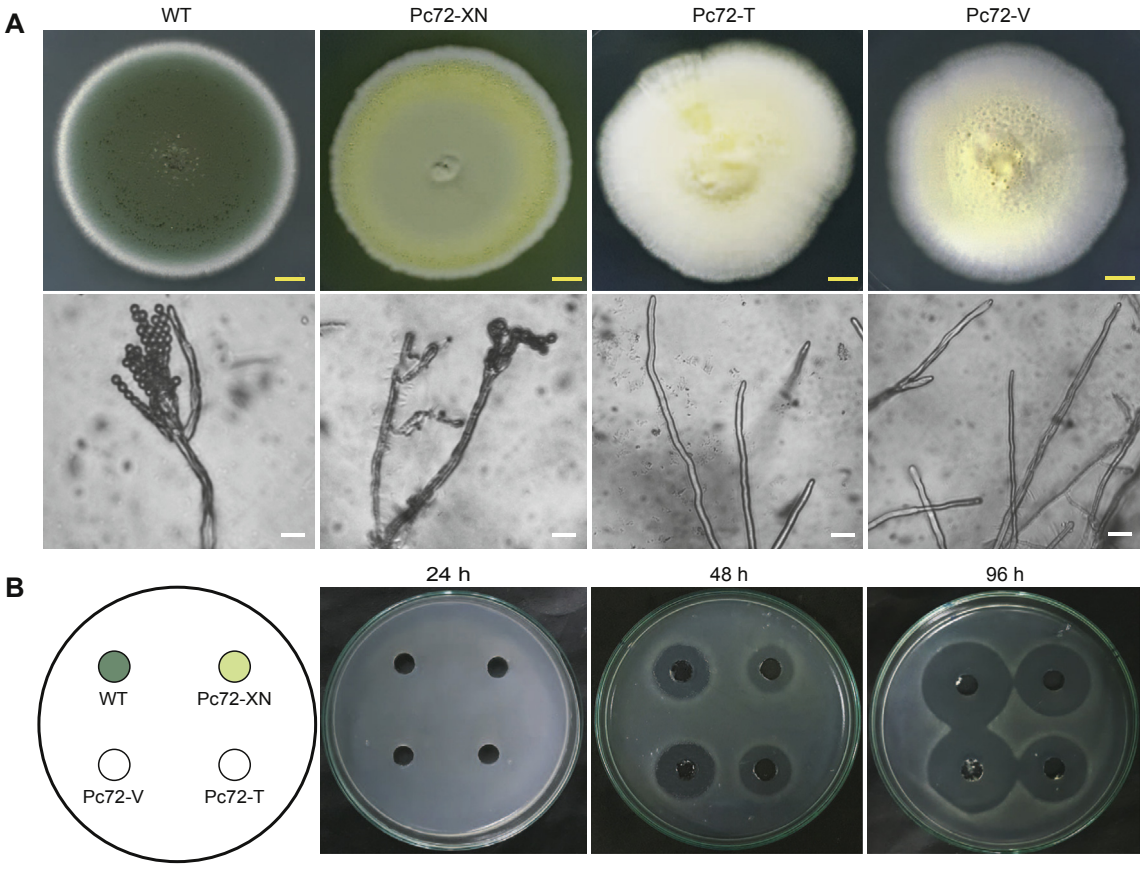

Fig. 3. Examination of some selected T-DNA insertion mutants for fungal development and antibiotic production. (**A**) Morphology of three selected insertion mutants in comparison to the wild strain (WT). All strains were cultivated on the PDA medium at 28 °C for 3–5 d. Fungal colonies were captured with a digital camera and fungal hyphae were observed under microscopy. (**B**) Antibiotic biosynthesis ability of the tested strains. These strains were grown in APM at 28 °C for time intervals of 24–96 h and respective supernatants were collected for testing the antibacterial activity against the indicator bacterium *S. aureus*.

acetate as the sole nitrogen source, which was referred as CDA ($NaNO_3$) and CDA (CH_3COONH_4), respectively. Our results revealed that sporulation of the *laeA* mutant did not occur on CDA ($NaNO_3$) and significantly reduced on CDA (CH_3COONH_4) as well as on PDA at 3 d of cultivation. At the time point of 6 d, the impact of sodium nitrate on the decrease of sporulation in the Δ*laeA* mutant became more obvious than that of ammonium acetate (Fig. 5B). The *laeA* complementation recovered most of the wild strain-like characteristics in the complementation strain (Figs. S4–S6). However, sporulation in the complementation strain was a bit delayed on CDA ($NaNO_3$) in comparison to the wild strain *P. chrysogenum* VTCC 31172 (Fig. 5B). It could be that the *laeA* complementation cassette was randomly integrated in the genome and its expression was not good enough for full recovery of sporulation in the complementation strain.

The influence of the nitrogen sources on penicillin biosynthesis in the Δ*laeA* mutant was also evaluated. The antibiotic production medium (APM) was used to promote penicillin production with exchange of the above nitrogen sources. The results showed that *P. chrysogenum* VTCC 31172 only exhibits high antibacterial activity when cultivated in APM containing sodium nitrate or ammonium acetate as the sole nitrogen source. In correlation with the delayed growth, the Δ*laeA* mutant lost completely the antibiotic production when grown in the medium containing sodium nitrate. However, surprisingly this mutant still maintained the antibiotic biosynthesis ability like the wild strain and the complemented strain when cultivated in the minimal medium with ammonium acetate. Our study revealed that among three tested ammonium salts, only ammonium acetate with acetate anion could improve significantly antibiotic production in both *P. chrysogenum* VTCC 31172 and the Δ*laeA* mutant (Fig. 6A). In fact, acetate had been reported to enhance penicillin production in *P. chrysogenum* by promoting fungal primary metabolism (Jensen et al., 1981). Therefore, we examined if acetate has an impact on growth and antibiotic production in the Δ*laeA* mutant. After addition of acetate, the pH values for CDA or APM were adjusted to 7 or 7.3, respectively. The results showed that additions of acetate to CDA and APM containing the nitrogen source as ammonium chloride, ammonium sulfate, sodium nitrate/ammonium chloride or sodium nitrate/ammonium sulfate could recover growth and antibiotic production in the Δ*laeA* mutant, which were resemble in the wild strain and the complemented strains. However, addition of acetate to CDA or APM containing only sodium nitrate as nitrogen source did not rescue the defects of growth and antibiotic production in the *laeA* mutant (Fig. 6B).

Furthermore, we also figured out that carbon sources (glucose, lactose, maltose, galactose, starch, cellulose, or xylan) did not impact on the retarded growth of the *laeA* mutant (Fig. S5), and the Δ*laeA* mutant became more sensitive to osmotic stresses (sorbitol, sodium chloride) when grown on the minimal CDA medium containing sodium nitrate than on other media (Fig. S6).

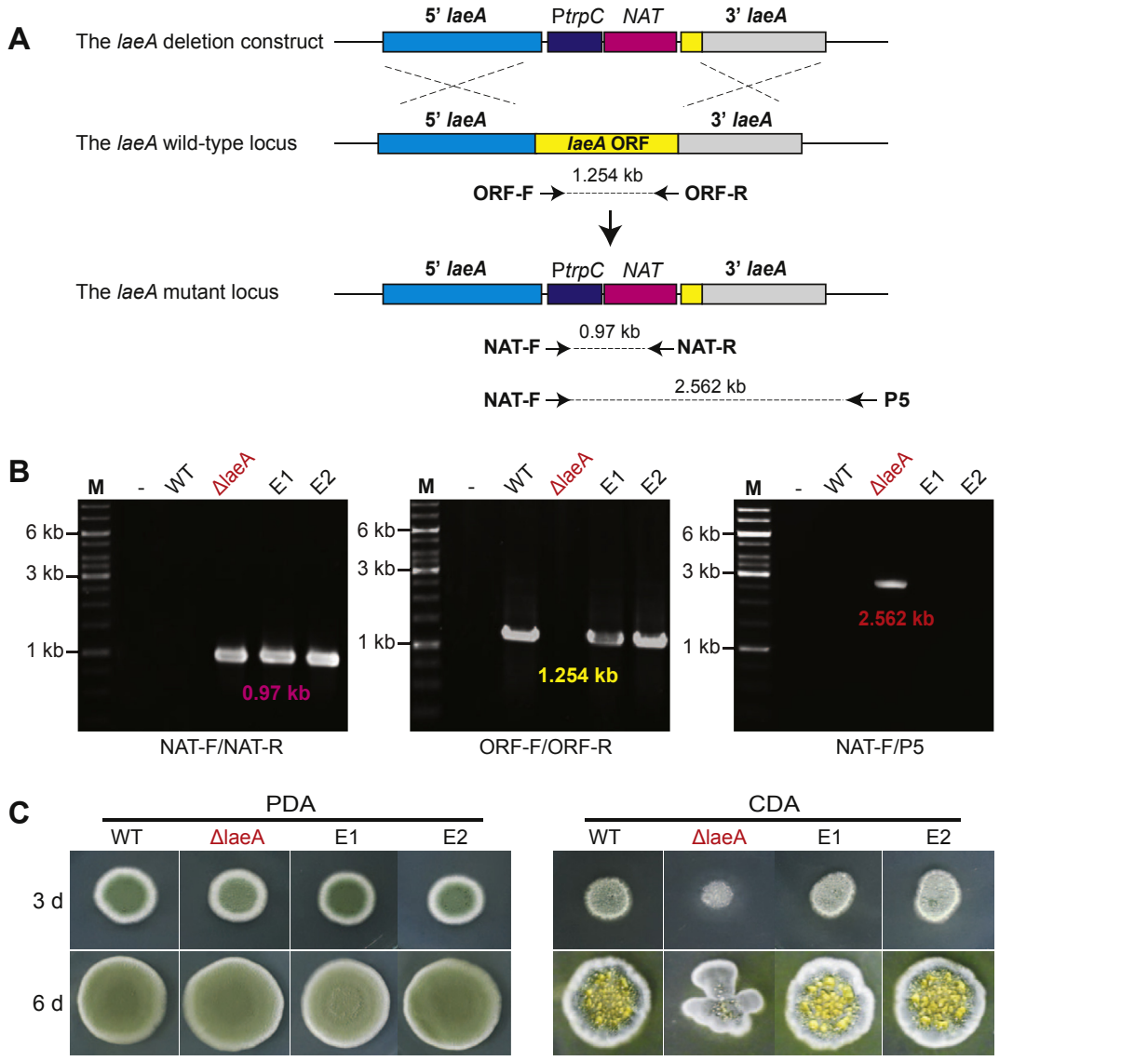

Fig. 4. Deletion of the *laeA* gene in *P. chrysogenum* using the ATMT system. (**A**) The scheme for *laeA* deletion by homologous recombination. (**B**) Confirmation of the Δ*laeA* mutant using PCR with three specific primer pairs. The wild strain (WT) and the ectopic strains (E1, E2) were used as controls. (**C**) The fungal strains were cultivated simultaneously on PDA and CDA for examining morphological changes. Spore suspensions were spotted on the agar surface and the plates were incubated at 28 °C for 3–6 d.

3.6. *The conserved* laeA *orthologous genes from* P. digitatum *and* A. niger *could rescue the defects in the* P. chrysogenum *Δ*laeA *mutant*

Roles of LaeA regulators in fungal development and secondary metabolism have been well characterized in different species of *Aspergillus* and *Penicillium* (Bayram et al., 2008; Bok and Keller, 2004; Kumar et al., 2017; Sarikaya-Bayram et al., 2015). However, little is known about the functional conservation of LaeA orthologs in two these fungal genera. The LaeA regulator in the industrial fungus *A. niger* plays a vital role in citric acid production and secondary metabolism (Niu et al., 2015; Wang et al., 2018), while potential roles of a putative LaeA ortholog in the postharvest citrus pathogen *P. digitatum* has not been reported yet. In this study, we performed a phylogenetic analysis for some LaeA orthologs extracted from the GenBank database and showed that the LaeA orthologs from *P. digitatum* and *A. niger* share amino acid similarities of 95.14 % and 60.05 % with the LaeA regulator of *P. chrysogenum*, respectively (Fig. 7A). We performed successfully gene complementation in the *P. chrysogenum* VTCC 31172 Δ*laeA* mutant by employing newly constructed binary vectors carrying the phleomycin resistance marker and the expression cassettes for the *laeA* orthologous genes from *P. digitatum* and *A. niger* (Fig. S7). The results showed that conserved functions of the *laeA* genes from *P. digitatum* and *A. niger* could recover the defective features such as sporulation and antibiotic production for the *P. chrysogenum* Δ*laeA* mutant (Fig. 7B–C).

4. Discussion

In 1998, the first paper reported the success of the ATMT method in filamentous fungi (de Groot et al., 1998). Up to date, this transformation method has been applied effectively to a large number of fungal species from different genera (Idnurm et al., 2017; Michielse et al., 2005). In comparison to the commonly used PMT method, which requires protoplasts as material for transformation, the advantages of ATMT are that fungal spores can be used directly as transformation material and T-DNA of a binary vector tends to be

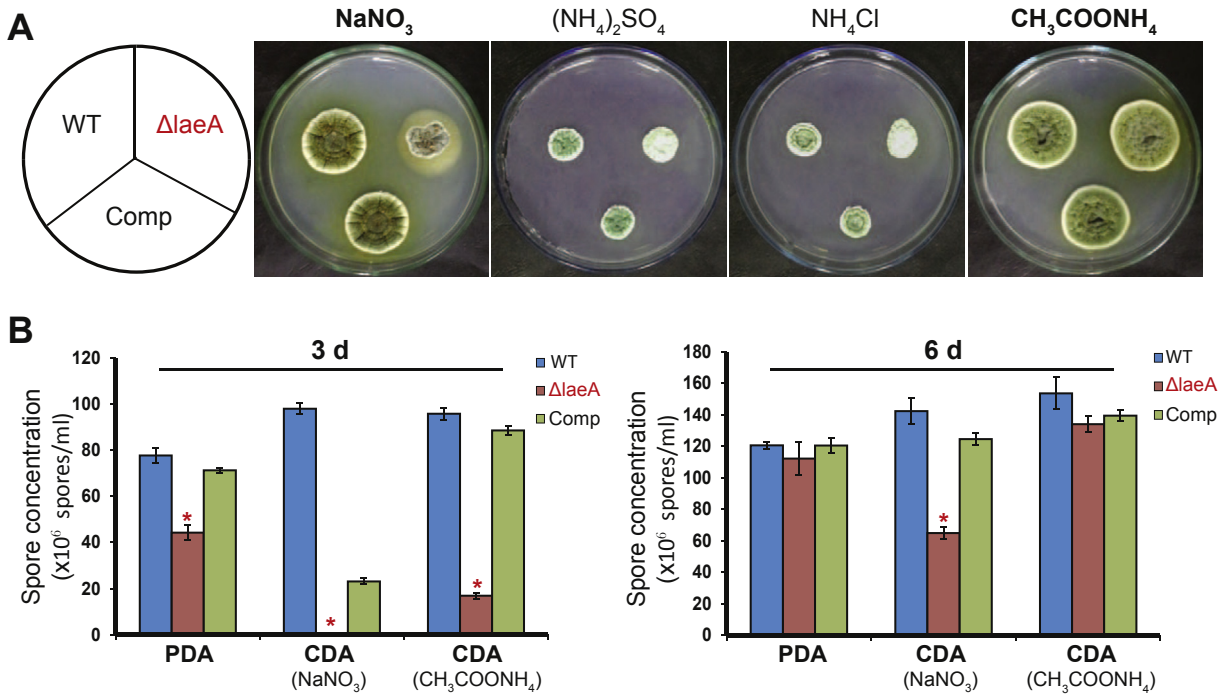

Fig. 5. The influence of nitrogen sources on growth and sporulation of the *laeA* deletion mutant. The wild strain (WT), *laeA* deletion mutant (ΔlaeA) and ectopic strains (E1, E2) were grown on the rich PDA medium and the minimal CDA medium to examine their growth (**A**). Nitrogen sources were tested for effects on fungal growth (**B**) and sporulation (**C**) of the *laeA* mutant in comparison to the wild strain and the complemented strain (Comp). These strains were grown on CDA containing one of the nitrogen sources for 3–6 d at 28 °C. The asterisks indicate significant differences from three independent replicates (Student's t-test, $p < 0.01$).

integrated with a higher rate of a single copy into fungal genome. This facilitates construction of random mutant libraries for identification of potential genes responsible for important cellular processes in fungi (Kemski et al., 2013; Maruthachalam et al., 2011; Mullins et al., 2001). In 2002, ATMT was reported for *P. chrysogenum* (Sun et al., 2002). However, this method is still less used for transformation of *P. chrysogenum*. Until now, there are only three publications exploited the ATMT method for genetic manipulations of this industrially important fungus (de Boer et al., 2013; Sun et al., 2002; Wang et al., 2019). Our study aimed to establish a new ATMT system for facilitating molecular studies on T-DNA insertion mutagenesis and genetic manipulation in *P. chrysogenum*. We first tested the sensitivity of the wild strain *P. chrysogenum* VTCC 31172 isolated from fungi-contaminated rice towards three commonly used antifungal agents including hygromycin, nourseothricin and phleomycin. The results showed that only nourseothricin and phleomycin could totally inhibit this fungal strain at the concentrations of 50 μg/ml and 200 μg/ml, respectively (Figs. S1 and S2). By employing the binary vector pGreen3 harboring the nourseothricin resistance marker, which had been used successfully for ATMT in the citrus postharvest pathogen *P. digitatum* (Vu et al., 2018), we showed in this study that the ATMT of *P. chrysogenum* VTCC 31172 with this binary vector was also very efficient. Under the optimized conditions for co-cultivation at 22 °C for 60 h with the concentration of 200 μM of AS, the transformation efficiency could reach over 5000 transformants per 10^6 spores (Fig. 1), which is approximately 2–10 times higher than those of the previous reports (de Boer et al., 2013; Sun et al., 2002). Additionally, we recommend that the filter paper (code: FT-3-303-090, Sartorius) and the freshly prepared spore suspension should be used for the ATMT of *P. chrysogenum* to obtain the optimal transformation efficiency. In the previous reports for ATMT in *A. oryzae* and *P. digitatum*, we had indicated that transformation efficiencies of this method are also reliant on the types of the filter membranes and fungal strains used for transformation (Nguyen et al., 2017; Vu et al., 2018).

Furthermore, we constructed a new binary vector pPK2-Red2 carrying the phleomycin resistance marker and showed that this vector works well in *P. chrysogenum* for heterologous expression of the *DsRed* fluorescent reporter gene via the ATMT method (Fig. 2, S2). Additionally, the fungal transformants generated by our ATMT system could be used as T-DNA insertion mutants for screening potential genes involved in fungal development and secondary metabolism in *P. chrysogenum*. We selected randomly three transformants (Pc72-XN, Pc72-T, Pc72-V) with phenotypes of distorted sporulation for evaluation of antibiotic production. The results showed that changes in morphology in these T-DNA insertion mutants could also be accompanied with different antibiotic biosynthesis capacities (Fig. 3). The phenotypic changes and the penicillin biosynthesis in the mutants might be caused by T-DNA insertion events into related genes and these potential genes may be identified by TAIL-PCR, inverse PCR or NGS analyses (Chambers et al., 2014; Maruthachalam et al., 2011; Mullins et al., 2001; Wang et al., 2014). Even some insertion mutants have more than one T-DNA copy in the genome, disrupted target DNA sequences by T-DNA insertions may be still identified by Illumina next-generation sequencing approach (Chambers et al., 2014).

The ATMT system constructed in this study was further employed for gene deletion and gene complementation in *P. chrysogenum* VTCC 31172. We constructed a binary vector for a successful deletion of the *laeA* regulatory gene by homologous recombination in *P. chrysogenum* using the nourseothricin resistance marker (Figs. S3 and S4). In fungi, LaeA is a master regulator of various cellular processes including vegetative growth, sporulation, metabolism and environmental stress responses. Especially, this regulator is well known for its role in biosynthesis of secondary metabolites such as mycotoxins and antibiotics (Bayram and Braus, 2012; Bok and Keller, 2004; Kumar et al., 2018; Martin, 2017;

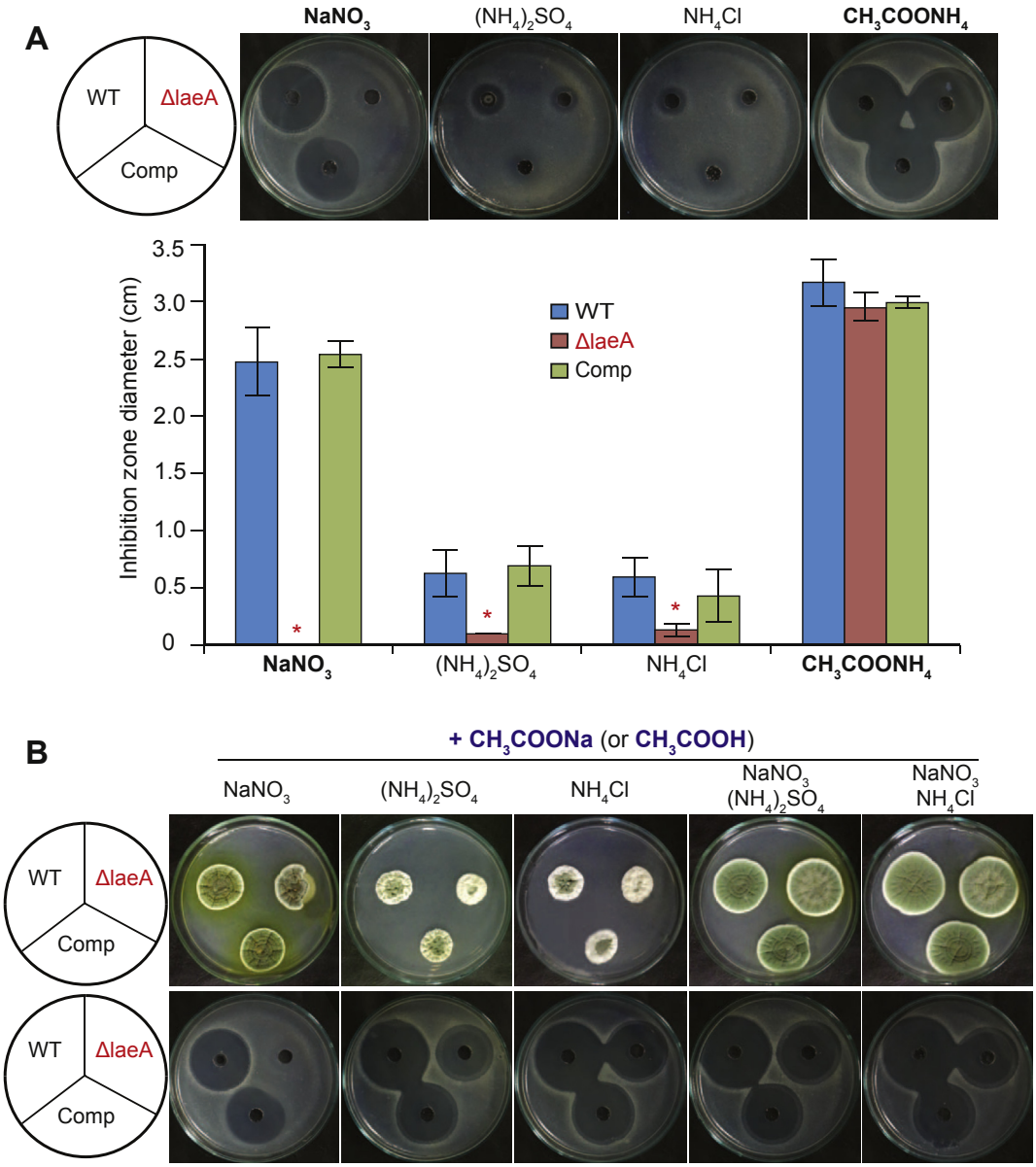

Fig. 6. The influence of nitrogen sources and acetate on antibiotic production in the *laeA* deletion mutant. The fungal strains were cultivated on CDA or in APM containing different nitrogen sources without (**A**) or with (**B**) additions of acetate (sodium acetate or acetic acid) for examination of fungal growth and antibiotic production, respectively. Fungal cultivation was performed at 28 °C for 5–7 d. Fungal growth on the agar plates were captured with a digital camera, while culture supernatants were collected by centrifugation for antibacterial activity assays against the indicator bacterium *S. aureus*. The asterisks indicate significant differences from three independent replicates (Student's t-test, $p < 0.01$).

Wiemann et al., 2010). In *P. chrysogenum*, LaeA plays a key role in regulation of sporulation and penicillin production (Hoff et al., 2010; Kamerewerd et al., 2011; Kosalkova et al., 2009; Veiga et al., 2012b). In present work, we further indicated that the LaeA control of sporulation and penicillin production is reliant on the type of nitrogen sources. To confirm the obtained results, we brought the intact *laeA* gene back to the *P. chrysogenum* VTCC 31172 Δ*laeA* strain using a binary vector harboring the phleomycin resistance marker and the results showed that the defects caused by the *laeA* loss were recovered in the complemented strain (Figs. 4 and 5, S5, S6, S7). Although nitrogen regulation was reported to be required for secondary metabolism in fungi (Lopez-Berges et al., 2014; Tudzynski, 2014), it has not been investigated yet in *P. chrysogenum*. Our data revealed that nitrate as the sole nitrogen source displayed a strong impact on growth, sporulation, osmotic stress response and antibiotic production in the *P. chrysogenum* VTCC 31172 Δ*laeA* mutant. In contrast, the nitrogen source as ammonium salts, especially ammonium acetate, did not show substantial differences in growth, sporulation, stress response and penicillin production between the Δ*laeA* mutant and the wild strain (Figs. 5 and 6, S5, S6). Acetate was reported to enhance penicillin production by promoting primary metabolism in *P. chrysogenum* (Jensen et al., 1981). Our results further indicated that acetate co-ordinates with nitrogen sources to promote growth, sporulation and antibiotic biosynthesis in *P. chrysogenum* VTCC 31172 (Fig. 6). Taken together, this study revealed that LaeA regulator appears to be required for assimilation of nitrate, which subsequently controls fungal development and secondary metabolism in *P. chrysogenum*.

As previously reported, the global LaeA regulator belongs to the SAM-dependent methyltransferase family and is conserved for its

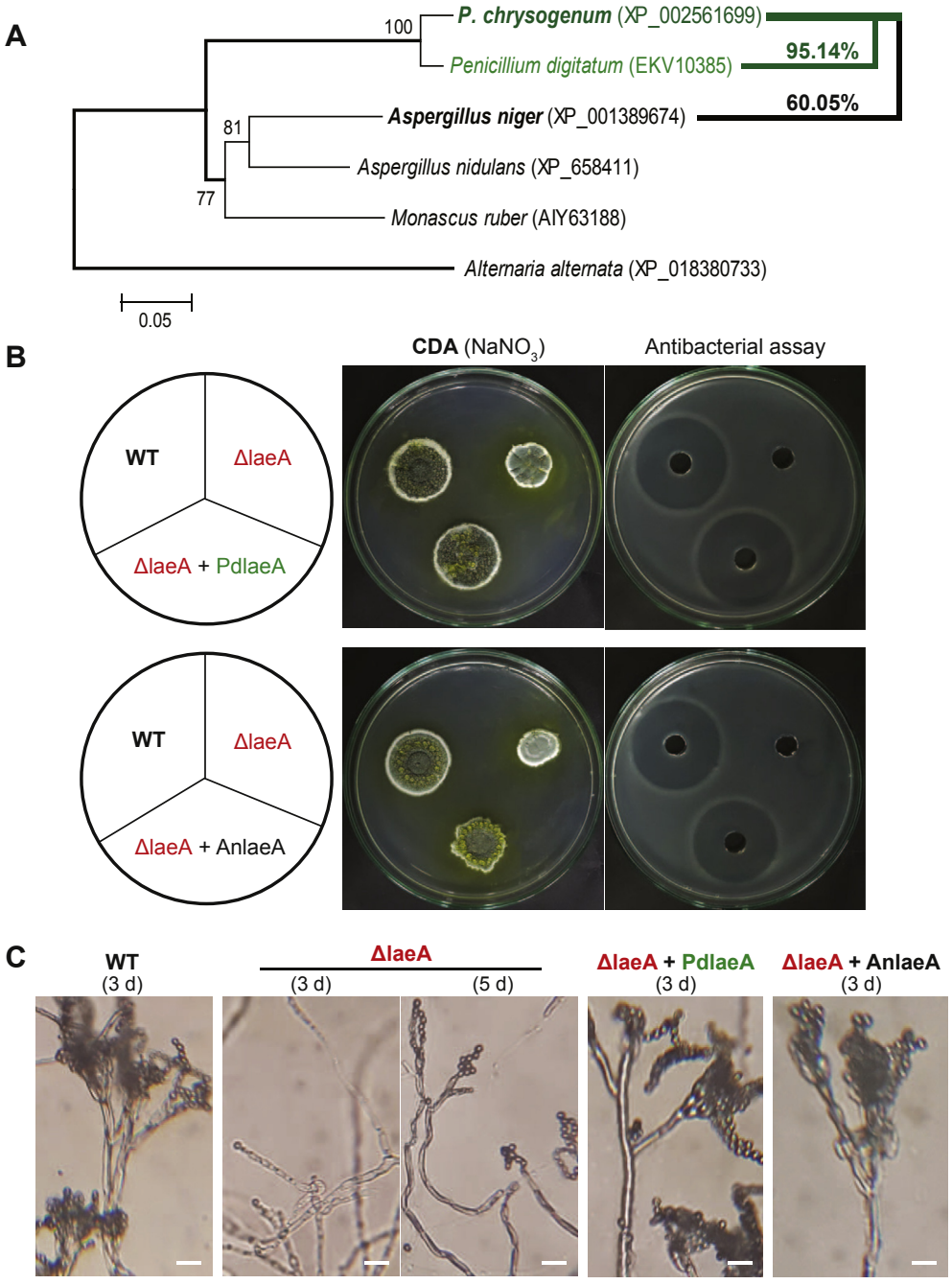

Fig. 7. Complementation of the *P. chrysogenum laeA* deletion mutant with the *laeA* orthologous genes from the citrus postharvest pathogen *P. digitatum* and the industrial fungus *A. niger*. (**A**) Phylogenetic analysis of LaeA (accession number: XP_002561699) from *P. chrysogenum* and its orthologs from other filamentous fungi. The phylogenetic tree was constructed with the MEGA6 software using neighbor-joining method and 1000 bootstrap replicates. The statistical support values at nodes of the tree branches, genetic distance scale and the respective accession numbers extracted from GenBank are indicated. (**B**) Assays for growth and antibiotic production of the *P. chrysogenum* VTCC 31172 Δ*laeA* mutant expressing the *laeA* orthologous genes from *P. digitatum* (ΔlaeA + PdlaeA) and *A. niger* (ΔlaeA + AnlaeA). These cross-complementation strains were cultivated on CDA for fungal growth or in APM for antibiotic production. To induce the expression of the *AnlaeA* gene under control of the *amyB* promoter, glucose in APM was replaced with soluble starch. Fungal cultivation was maintained at 28 °C for 5–7 d. (**C**) Analysis of morphological recovery in the cross-complemented strains under microscopy using slide culture method. The wild strain (WT) and the *laeA* deletion mutant (ΔlaeA) of *P. chrysogenum* were used as positive control and negative control for the comparison, respectively. Fungal strains were examined at 3 d and 5 d after inoculation. The scale bars indicate the same sizes of images.

function across different filamentous fungi (Bayram and Braus, 2012; Sarikaya-Bayram et al., 2015). The phylogenetic analysis showed that the *P. chrysogenum* LaeA shares high similarities of amino acids (60–95 %) with its orthologs from the citrus postharvest pathogen *P. digitatum* and the industrial fungus *A. niger* (Fig. 7A). Our data demonstrated that heterologous expression of the conserved *laeA* orthologous genes from *P. digitatum* and *A. niger* could rescue the defects for sporulation and antibiotic production in the *P. chrysogenum* VTCC 31172 Δ*laeA* mutant (Fig. 7B–C).

Additionally, some advances in developing small high-yielding binary vectors for ATMT in plants and fungi have been reported recently. Small binary vectors usually increase the cloning efficiency and plasmid yield in both *E. coli* and *A. tumefaciens* for transformation (Lee et al., 2012; Nora et al., 2019a,b). Future work

will focus on improving the constructed ATMT system based on a smaller binary plasmid backbone to increase its efficiency.

5. Conclusions

In this study, we have constructed a new and highly efficient ATMT system for genetic manipulation in *P. chrysogenum* using two different dominant selection markers conferring the resistance to nourseothricin and phleomycin. The transformation efficiency of the constructed ATMT system could reach over 5000 transformants per 10^6 fungal spores under the optimized conditions for the co-cultivation step at 22 °C for 60 h and the concentration of 200 μM of AS in the induction medium. This ATMT system can be exploited for heterologous gene expression, gene targeting or for constructing large collections of random mutants by T-DNA insertion mutagenesis. Especially, the developed ATMT system was employed successfully to inspect the LaeA regulator by gene deletion and gene complementation in a wild strain of *P. chrysogenum*. LaeA controls growth, sporulation, osmotic stress response and antibiotic production in *P. chrysogenum*, but its function is reliant on nitrogen sources.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.08.010.

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