SCIENCE CHINA
Life Sciences

SPECIAL TOPIC: Biosynthesis and molecular regulation of secondary metabolites in microorganisms September 2017 Vol.60 No.9: 958–967
• RESEARCH PAPER • doi: 10.1007/s11427-017-9118-9

A GATA-type transcription factor AcAREB for nitrogen metabolism is involved in regulation of cephalosporin biosynthesis in *Acremonium chrysogenum*

Feifei Guan[1,2], Yuanyuan Pan[1*], Jinyang Li[1] & Gang Liu[1,2*]

[1]*State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;*
[2]*University of Chinese Academy of Sciences, Beijing 100049, China*

Received April 19, 2017; accepted May 11, 2017; published online August 14, 2017

In filamentous fungi, nitrogen metabolism is repressed by GATA-type zinc finger transcription factors. Nitrogen metabolite repression has been found to affect antibiotic production, but the mechanism is still poorly understood. *AcareB*, encoding a homologue of fungal GATA-type regulatory protein, was cloned from *Acremonium chrysogenum*. Gene disruption and genetic complementation demonstrated that *AcareB* plays a key role in utilization of ammonium, glutamine and urea. In addition, significant reduction of cephalosporin production in the *AcareB* disruption mutant indicated that *AcareB* is important for cephalosporin production. In consistence with it, the transcriptional level of cephalosporin biosynthetic genes was significantly decreased in the *AcareB* disruption mutant. Electrophoretic mobility shift assay showed that AcAREB directly bound to the intergenic regions of *pcbAB-pcbC*, *cefD1-cefD2* and *cefEF-cefG*. Sequence analysis showed that all the AcAREB binding sites contained the consensus GATA elements. *AcareB* is negatively autoregulated during cephalosporin production. Moreover, another GATA zinc-finger protein encoded by *AcareA* positively regulates the transcription of *AcareB*. However, *AcareB* does not regulate the transcription of *AcareA*. These results indicated that AcAREB plays an important role in both regulation of nitrogen metabolism and cephalosporin production in *A. chrysogenum*.

***AcareB*, *Acremonium chrysogenum*, cephalosporin biosynthesis, GATA-type transcription factor, regulation**

Citation: Guan, F., Pan, Y., Li, J., and Liu, G. (2017). A GATA-type transcription factor AcAREB for nitrogen metabolism is involved in regulation of cephalosporin biosynthesis in *Acremonium chrysogenum*. Sci China Life Sci 60, 958–967. doi: 10.1007/s11427-017-9118-9

INTRODUCTION

Acremonium chrysogenum is famous due to its ability to produce different derivatives of pharmaceutically relevant β-lactam antibiotics isopenicillin N and cephalosporin C (Brakhage et al., 2009). Cephalosporins have been widely used for treatment of the diseases caused by pathogenic bacteria in humans and animals. The key enzymes involved in cephalosporin biosynthesis have been identified and the cephalosporin biosynthetic pathway has been well studied in *A. chrysogenum* (Dreyer et al., 2007; Lee et al., 2017; Liu et al., 2015; Martin et al., 2004; Martín et al., 2010; Martín et al., 2012). However, the regulatory mechanism of cephalosporin biosynthesis is still poorly understood.

Cephalosporin production is affected by carbon and nitrogen sources, ambient pH and oxidative stress, and regulated at multiple levels such as pathway-specific regulation, global regulation and pleiotropic control in *A. chrysogenum*. So far, many regulators have been identified, such as nitro-

*Corresponding authors (Gang Liu, email: liug@im.ac.cn; Yuanyuan Pan, email: panyy@im.ac.cn)

© Science China Press and Springer-Verlag Berlin Heidelberg 2017

gen metabolic activator AreA, carbon catabolite repressor CRE1, the pH-dependent regulator PACC, CPCR1, AcStuA involved in conidiation and cell wall integrity, as well as AcVEA involved in hyphal fragmentation (Dreyer et al., 2007; Hu et al., 2015; Jekosch and Kück, 2000). In filamentous fungi, nitrogen metabolite repression is mainly mediated by GATA-type zinc finger transcription factors. Of them, AreA is responsible for the activation of genes that allow utilization of less-favored nitrogen sources in a similar manner to the yeast homologs Gln3p and Gat1p, and AreB is suggested to repress the corresponding genes as the yeast GATA factors Dal80p and Gzf3p (Wong et al., 2009). AreB and its orthologs have been found in a number of filamentous fungi, and their function involved in the nitrogen metabolite regulation has been studied extensively (Wiemann and Tudzynski, 2013). The first functional analyses of AreB confirmed its proposed role as repressor through DNA-binding competition in *Aspergillus nidulans* and *Penicillium chrysogenum*. Over-expression of *areB* resulted in the loss of AreA-dependent gene expression (Wong et al., 2009). However, the impact of *areB* disruption on secondary metabolism remains elusive. As the positive regulator of secondary metabolism, AreB was described only in *Fusarium fujikuroi* and it was essential for expression of the GA biosynthetic genes under conditions of nitrogen starvation. In addition, AreA and AreB were shown to be co-localized in the nucleus and interact with each other (Michielse et al., 2014). Interestingly, AreB is involved in both nitrogen starvation-induced secondary metabolic biosynthesis and nitrogen sufficient-induced secondary metabolic biosynthesis. For example, fusaric acid and apicidin F are not produced in the *areB* disruption mutant no matter under the conditions of nitrogen starvation or sufficient. However, the nitrogen-induced formation of fusarin C is not affected by the *areB* disruption in *F. fujikuroi* (Niehaus et al., 2013). So far, nothing has been known about the relationship between *areB* homologue and cephalosporin production in *A. chrysogenum*. Here we cloned an *areB* homologue (*AcareB*) from *A. chrysogenum* and studied its function in nitrogen metabolism and cephalosporin biosynthesis. These results provide an insight into better understanding of global regulation exerted by GATA factor AREB during cephalosporin biosynthesis.

RESULTS

AcareB encodes a putative GATA-type transcription factor

Based on the similarity of AreB and its orthologs, a 1,793 bp DNA fragment was amplified from *A. chrysogenum* CGMCC 3.3795. Sequence analysis revealed that this fragment contains an open reading frame (named *AcareB*, GenBank accession No. KY494643) and encodes a putative GATA-type transcription factor. *AcareB* contains three introns (extending from positions +156 to +219, +341 to +742 and +938 to +1100 with respect to the *AcareB* translation start point). The deduced AcAREB consists of 387 amino acids with the theoretical molecular mass of 41.85 kD and contains one Cys_2/Cys_2-type zinc finger DNA-binding domain. The sequence of AcAREB showed 51% identity with AreB (CAJ27265.1) from *F. fujikuroi*, 49% identity with ASD4 (AAG45180.1) from *Neurospora crassa*, 48% identity with AreB (XP_014581160.1) from *Metarhizium majus* ARSEF 297, 45% identity with NREB (AAC09045.1) from *P. chrysogenum* and 42% identity with AREB (AAG49353.1) from *A. nidulans*. The DNA-binding domain with a leucine amino residue at the central loop is highly conserved in these proteins, indicating that the AcAREB might recognize the similar elements as other GATA zinc-finger proteins (Figure 1).

Effect of *AcareB* disruption on the utilization of ammonium, glutamine and urea in *A. chrysogenum*

To reveal the function of *AcareB*, homologous recombination was employed (Figure 2A). The constructed pAgHBLR was introduced into the *A. chrysogenum* wild-type strain (WT) through ATMT. The putative *AcareB* disruption mutant (ΔAcareB) was selected and validated by PCR analysis (Figure 2B). With the outside primers, a 2,104 DNA fragment was amplified from WT but a 2,509 bp fragment was amplified from ΔAcareB. When using the inside primers, a 218 bp DNA fragment was amplified from WT, while no fragment was amplified from ΔAcareB. These results confirmed that *AcareB* has been replaced by the hygromycin phosphotransferase gene (*hph*) in ΔAcareB. One of the validated ΔAcareB was chosen randomly for the following experiments. The reverse transcription-PCR (RT-PCR) analysis further indicated that expression of *AcareB* was completely suppressed in ΔAcareB, and the complemented strain (AcareBC) could restore its expression (Figure 2C).

The Czapek-N medium supplemented with different nitrogen sources was used to determine the growth of WT, ΔAcareB and AcareBC. When ammonium, glutamine or urea was used as the sole nitrogen source at the concentration of 5, 10 and 50 mmol L^{-1}, ΔAcareB grew poorly, but WT and AcareBC grew well. The growth of ΔAcareB remained poorly even when increasing the nitrogen concentration (Figure 3). When sodium nitrate was used, ΔAcareB exhibited the same growth as WT and AcareBC. When amino acids rather than glutamine were used as the sole nitrogen sources, the growth of ΔAcareB was almost the same as WT and AcareBC (data not shown). These results indicated that *AcareB* is a key regulatory gene for utilization of ammonium, glutamine and urea in the Czapek-N medium.

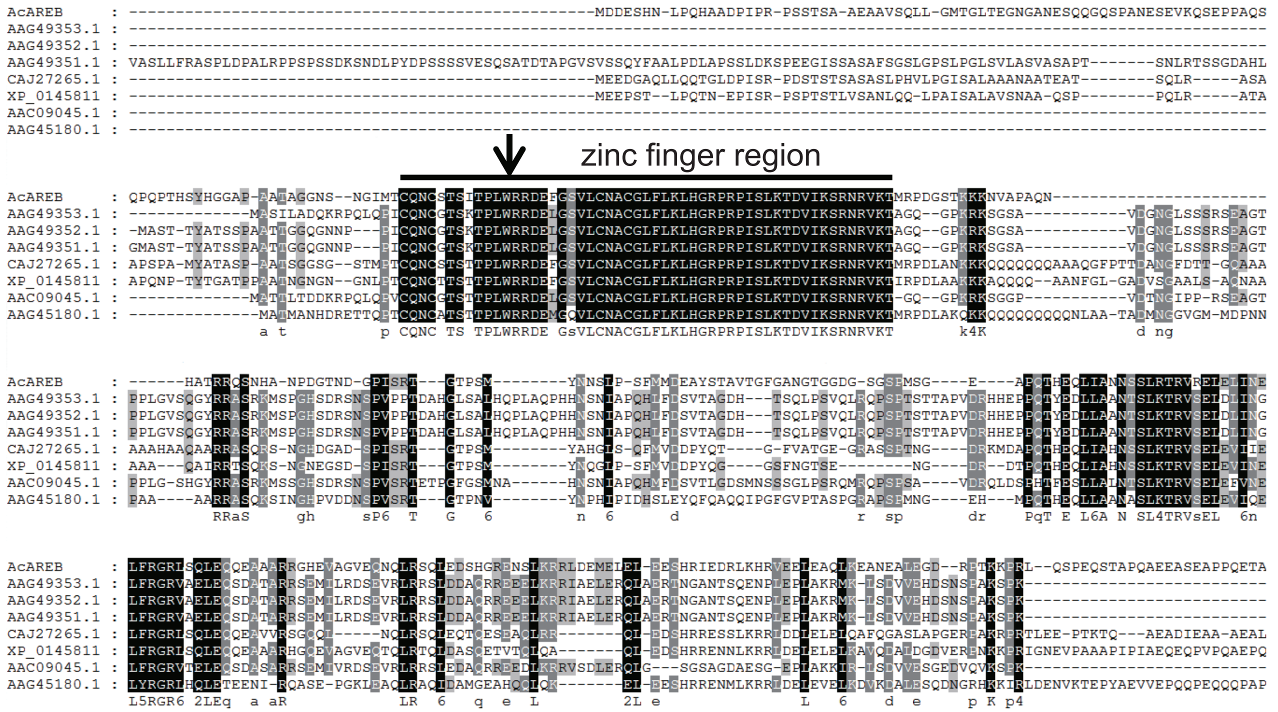

Figure 1 Partial sequence alignment of GATA zinc-finger proteins. The GATA zinc-finger proteins include AcAREB with AREB alpha, beta, gamma (AAG49353.1, AAG49352.1 and AAG49351.1) from *A. nidulans*, AreB (CAJ27265.1) from *F. fujikuroi*, AreB (XP_014581160.1) from *M. majus* ARSEF 297, NreB (AAC09045.1) from *P. chrysogenum*, and ASD4 (AAG45180.1) from *N. crassa*. The consensus sequences containing zinc finger region are shaded and overlined. The arrow indicates the leucine residue involved in promoter recognition.

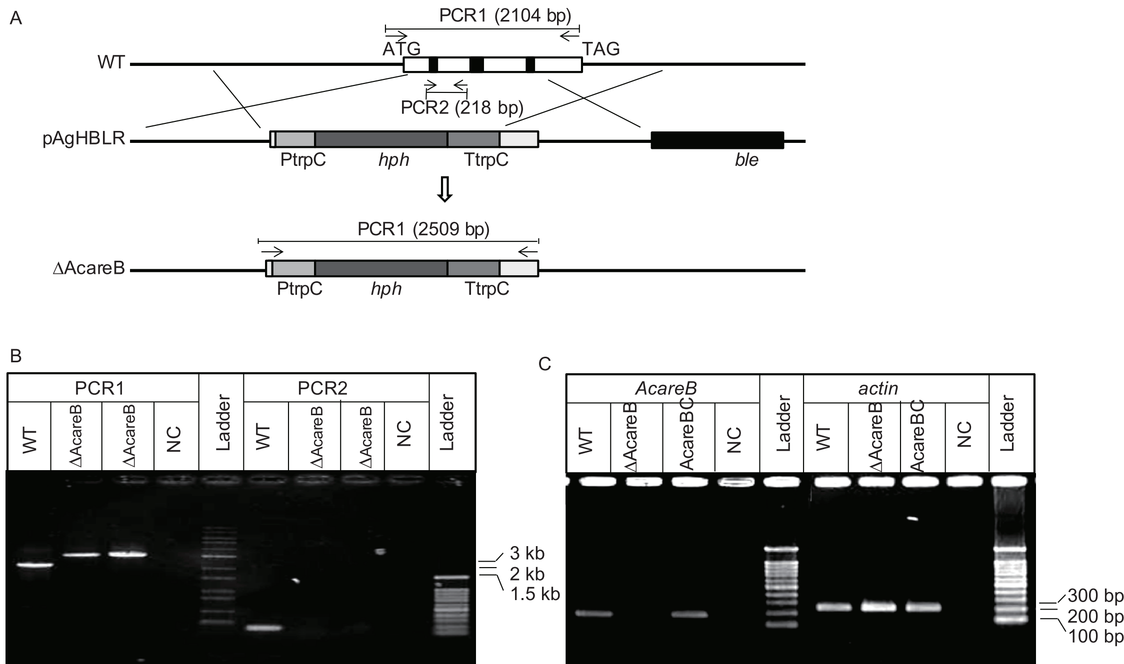

Figure 2 Construction and confirmation of the *AcareB* disruption mutant. A, Strategy for construction of ΔAcareB via homologous recombination. kb, kilobase pairs; bp, base pairs; *hph*, hygromycin phosphotransferase gene. B, Confirmation of ΔAcareB by PCR. PCR1 and PCR2 were performed with the gene-outside primers (YareB1-F/YareB1-R) and the gene-inside primers (YareB2-F/YareB2-R), respectively. WT, the wild-type strain; NC, negative control. C, Detection of *AcareB* transcription in WT, ΔAcareB and AcareBC by RT-PCR. All of the strains were grown in TSA liquid medium at 28°C for 2 days. Total RNA isolation and cDNA synthesis were performed as described in Materials and methods. Transcription of both *AcareB* and *actin* was detected with primers RTareB-F/RTareB-R and Actin-F/Actin-R, respectively.

AcAREB positively regulates the production of cephalosporin

Although GATA-type transcription factors have been shown to influence secondary metabolite biosynthesis in filamentous fungi (Brakhage, 2012; Wiemann and Tudzynski, 2013; Yin and Keller, 2011), the mechanism is still poorly understood. To investigate it, we examined the transcriptional profile of *AcareB* in the modified MDFA medium which was used for cephalosporin production. Quantitative real-time RT-PCR results showed that the transcriptional level of *AcareB* in WT was enhanced from 48 h and reached the maximum level at 96 h of cultivation when cephalosporin production was sharply increased. The transcriptional level of *AcareB* decreased from 96 to 168 h gradually (Figure 4A). It indicated that the expression of *AcareB* is related to cephalosporin production in time course. To further address the role of *AcareB* in cephalosporin biosynthesis, fermentation experiment was performed. Compared with WT, a 20% decrease of cephalosporin production in the fermentation culture of ΔAcareB was detected (Figure 4B). Complementation of ΔAcareB with a functional copy of *AcareB* in pAgHB almost restored the production of cephalosporin to the wild-type level (Figure 4B). All the strains had the comparable biomass and growth rate (Figure 4C), indicating that decrease of cephalosporin production was mainly due to the *AcareB* disruption. Therefore, *AcareB* positively regulates cephalosporin production in *A. chrysogenum*. However, overexpression of *AcareB* in AcareBOE did not produce more cephalosporin than WT (data not shown). It could be understood since cephalosporin biosynthesis is controlled by many factors besides AcAREB. It also implies that the regulation of cephalosporin biosynthesis is complicated in *A. chrysogenum*.

Consistent with the production of cephalosporin, the transcripts of *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* and *cefG* were decreased in ΔAcareB in comparison with that in WT (Figure 5). These results revealed that *AcareB* positively affects the cephalosporin production by controlling the transcription of key structural genes and AcAREB is one of the key transcription regulators for cephalosporin biosynthesis in *A. chrysogenum*.

AcAREB binds to the promoter regions of cephalosporin biosynthetic genes

EMSA was used to determine whether AcAREB directly regulates cephalosporin biosynthesis. For EMSA, *AcareB* was expressed in *E. coli* BL21 (DE3) and the recombinant AcAREB-His$_6$ was purified by chromatography on nickel-nitrilotriacetic acid resin. DNA probes of *pcbAB-pcbC* (Epcb-

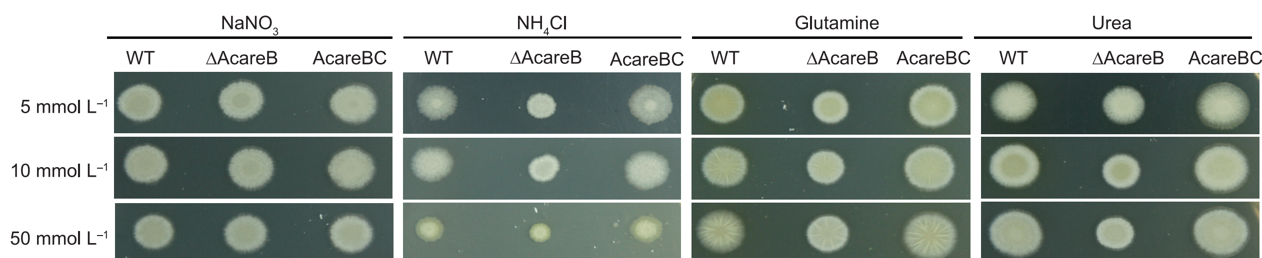

Figure 3 (Color online) Phenotype of the wild-type strain (WT), *AcareB* disruption mutant (ΔAcareB) and the complemented strain (AcareBC). Phenotype of WT, ΔAcareB and AcareBC on the Czapek-N medium using $NaNO_3$, NH_4Cl, glutamine or urea as sole nitrogen source at the concentration of 5 mmol L^{-1},10 mmol L^{-1} and 50 mmol L^{-1}. 1×10^4 fungal spores were spread on the Czapek-N medium. Growth was observed after incubation at 28°C for 5 days.

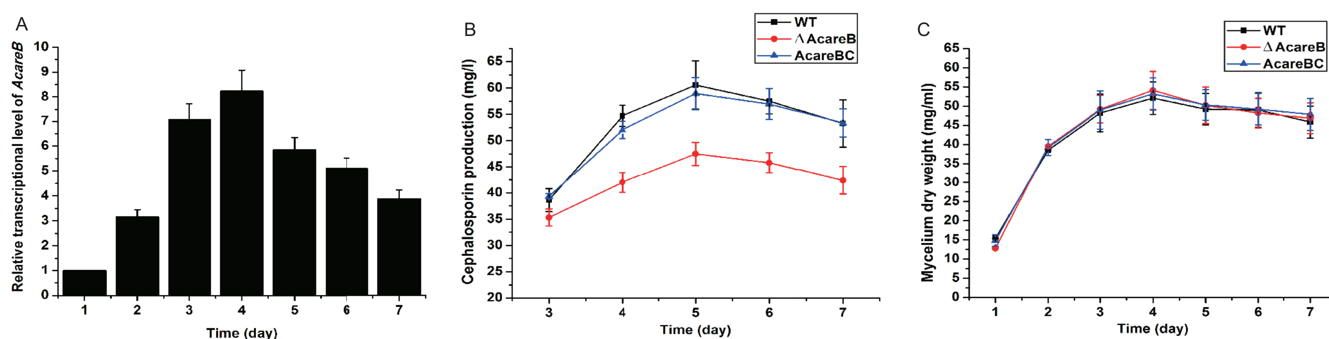

Figure 4 (Color online) Effect of *AcareB* disruption on cephalosporin production. A, Transcriptional profile of *AcareB* in WT. The total RNAs of *A. chrysogenum* grown in LPE medium were isolated after incubation for 24, 48, 72, 96, 120, 144 and 168 h, respectively. An arbitrary transcription of 1 was assigned to *AcareB* based on the signal detected from the total RNA collected at 24 h. B, Cephalosporin production of WT, ΔAcareB and AcareBC grown in the modified MDFA medium was detected. C, Biomass of WT, ΔAcareB and AcareBC. Dry cell weight was determined after drying the fungal mycelia at 42°C in a hot air oven until a constant weight. Error bars represent standard deviations from three independent experiments.

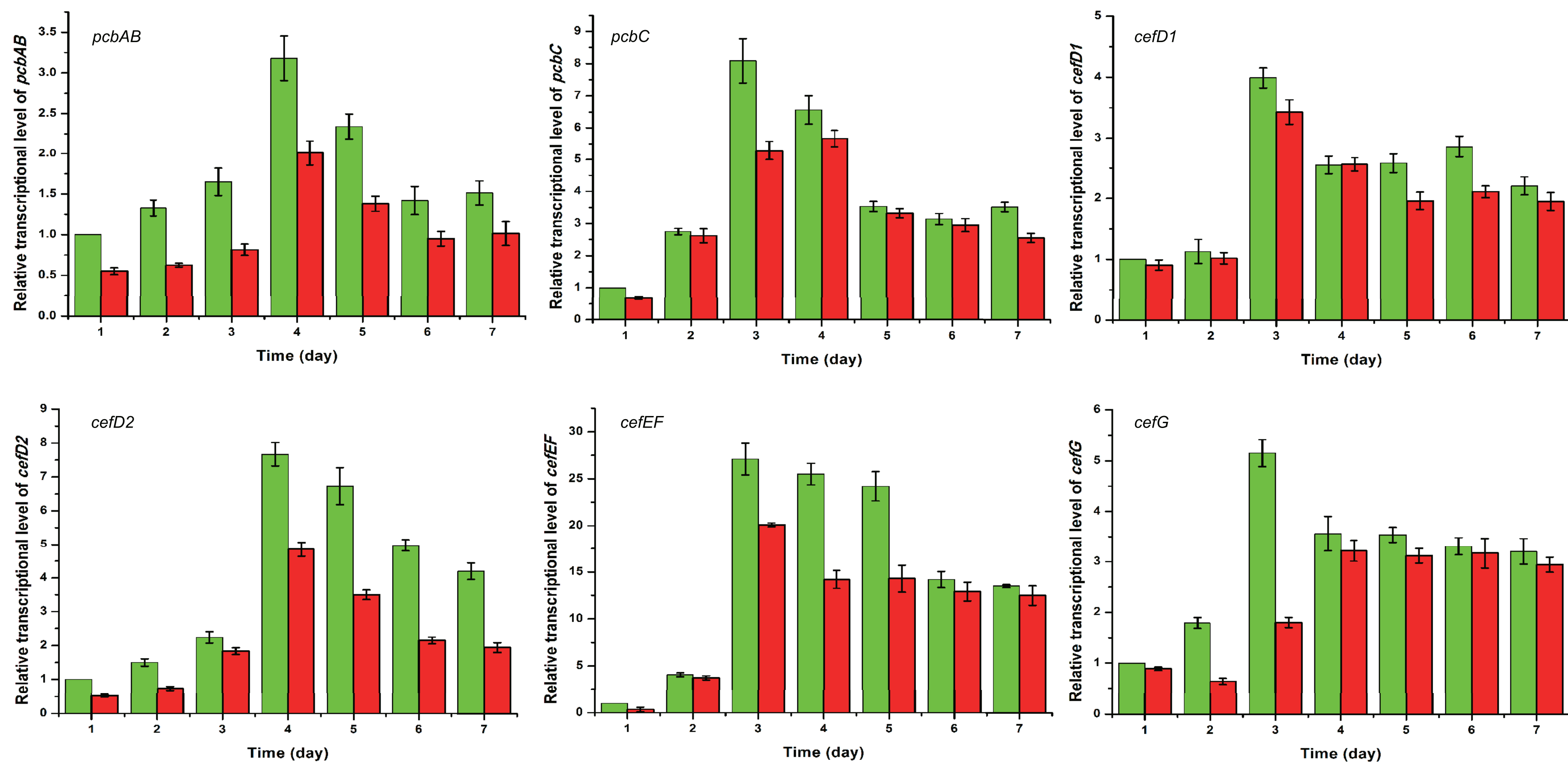

Figure 5 Transcriptional analysis of the cephalosporin biosynthetic genes. The relative abundance of mRNAs was standardized against the levels of *actin* gene. The gene transcriptional level in WT is indicated by green column, and that in ΔAcareB is indicated by red column. Error bars represent standard deviations from three independent experiments.

ABC1 and EpcbABC2), *cefD1-cefD2* (EcefD1, EcefD2 and EcefD3) and *cefEF-cefG* (EcefEFG1 and EcefEFG2) were designed and used (Figure 6A). EMSA results showed that AcAREB directly bound to the upstream regions of *pcbAB-pcbC*, *cefD1-cefD2* and *cefEF-cefG* (Figure 6B). In contrast, no shift was found for the non-specific DNA (Eactin). Thus, AcAREB regulates cephalosporin biosynthesis directly through interaction with the upstream regions of cephalosporin biosynthetic genes in *A. chrysogenum*.

Sequence analysis demonstrated that all the binding probes of AcAREB contain a conserved sequence "5′-HGATAR-3′" which is the same as the DNA binding motif of AcAREA. The previous studies on AcAREA binding sites revealed that numbers and flanking sequences of GATA are important for its binding (Li et al., 2013). Therefore, it is possible that AcAREB and AcAREA coordinate the regulation of cephalosporin biosynthesis through the DNA binding motifs.

AcareA regulates the expression of *AcareB* during cephalosporin production

The transcriptional level of *AcareB* dramatically increased in ΔAcareB from 72 h to 144 h fermentation (Figure 7A), indicating that AcAREB represses its own transcription during cephalosporin production. Sequence analysis revealed a GATA motif in *AcareB* encoding region, providing the possible evidence of *AcareB* autoregulation. To study the regulatory relationship of GATA-type zinc finger regulators encoded by *AcareA* and *AcareB* in *A. chrysogenum*, the transcript of *AcareB* was detected in WT and ΔAcareA by quantitative real-time RT-PCR with primers Real-areB-F/Real-areB-R. Meanwhile, the transcript of *AcareA* was also detected in WT and ΔAcareB with primers Real-areA-F/Real-areA-R. The transcription results showed that the transcript of *AcareB* is significantly reduced in ΔAcareA compared with that of WT, indicating that *AcareA* positively regulates *AcareB* transcription (Figure 7B). However, sequence analysis of the *AcareB* promoter region did not show any possible GATA motif, implying that *AcareA* regulates the expression of *AcareB* indirectly. In addition, the transcriptional level of *AcareA* in the ΔAcareB was almost the same as that in WT, suggesting that *AcareB* did not affect *AcareA* expression in *A. chrysogenum* (Figure 7C).

DISCUSSION

In this study, *AcareB* was cloned and its function was studied in the industrial fungus *A. chrysogenum*. The deduced protein AcAREB shares extensive regions of homology with the well-known fungal GATA-type nitrogen regulatory proteins NreB from *P. chrysogenum*, ASD4 from *N. crassa*, and AreB of *F. fujikuroi*. Disruption of *AcareB* exhibited weak growth when ammonium, glutamine or urea was used as the sole nitrogen source. It is likely that the weak growth of ΔAcareB on ammonium, glutamine or urea is partially due to effects on

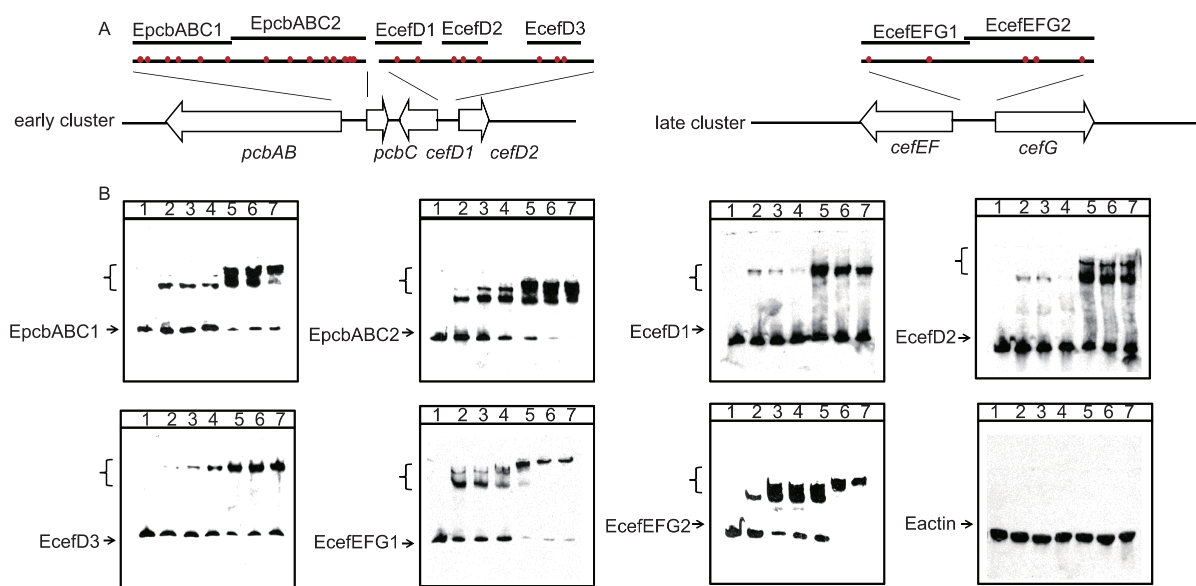

Figure 6 Electrophoretic mobility shift assay (EMSA) of AcAREB to the upstream regions of the cephalosporin biosynthetic genes. A, Organization of the cephalosporin biosynthetic gene cluster in *A. chrysogenum* and schematic representation of the relative positions of probes used for EMSAs. Red dots represent the conserved GATA sequences. EpcbABC1 and EpcbABC2 extend from nucleotide positions 595 to 13 and 1188 to 577 relative to the translation start point of *pcbC* respectively; EcefD1, EcefD2 and EcefD3 extend from nucleotide positions 1151 to 852, 678 to 374 and 411 to 143 relative to the translation start point of *cefD2* respectively; EcefEFG1 and EcefEFG2 extend from nucleotide positions 1081 to 647 and 666 to 165 relative to the translation start point of *cefG* respectively. B, AcAREB bound to the upstream regions of *pcbAB-pcbC*, *cefD1-cefD2* and *cefEF-cefG*. Varying amount of the purified AcAREB with 0.038 nmol L^{-1} DIG-labelled probes was added into the reaction solution. Lanes 1−7 contain 0, 12, 24, 48, 120, 240 and 360 ng AcAREB, respectively. The probe (Eactin) containing the upstream region of *actin* was used as the negative control. The free probes and DNA-protein complexes are indicated by arrows and parentheses, respectively.

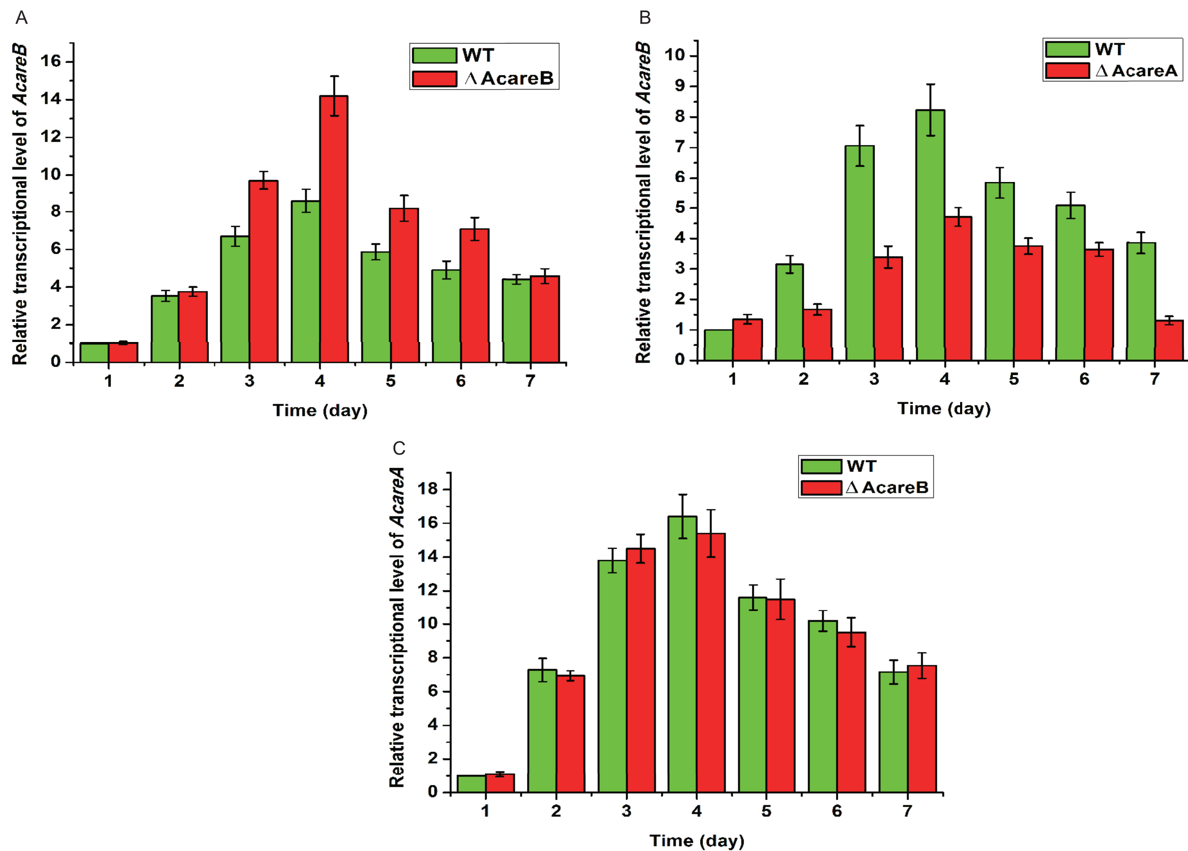

Figure 7 The regulatory relationship of GATA-type transcription factors encoded by *AcareA* and *AcareB* during cephalosporin production in the modified MDFA medium. A, *AcareB* negatively regulates its own transcription during cephalosporin production. B, Transcriptional level of *AcareB* was determined in WT and ΔAcareA during cephalosporin production. C, Transcriptional level of *AcareA* was determined in WT and ΔAcareB during cephalosporin production. Total RNA was isolated from the fungal mycelia grown for 1, 2, 3, 4, 5, 6 and 7 days, followed by cDNA synthesis and quantitative real-time RT-PCR. The actin gene was used to normalize the transcriptional data. Error bars represent standard deviations from three independent experiments.

transport system as AcAREA in *A. chrysogenum* (Li et al., 2013). Our results also demonstrated that AcAREB regulates cephalosporin production directly through interaction with the bidirectional promoter regions of *pcbAB*-*pcbC*, *cefD1*-*cefD2* and *cefEF*-*cefG*. As we know, this is the first report about the detailed mechanism of AreB homologs regulating the secondary metabolic biosynthesis in filamentous fungi.

The GATA transcription factor AreB regulates nitrogen utilization in many filamentous fungi. For example, AREB negatively regulates the expression of arginine catabolism genes *agaA* and *otaA* under nitrogen-repressing conditions in *A. nidulans*. The expression of *areB* is repressed by nitrogen and induced by nitrogen starvation in *F. fujikuroi*. While Asd4 has no impact on the expression of nitrogen regulated genes under carbon-limiting conditions, but plays a role in development of asci and ascospores in *N. crassa* (Macios et al., 2012). Here, we showed that AcAREB did not regulate morphological differentiation, but affect some nitrogen utilization of the *A. chrysogenum*, indicating the multiple biological role of AreB in different filamentous fungi.

One of the most interesting findings in this study is that *AcareB* encodes one protein containing the GATA zinc finger and a leucine zipper motif, which is different from the situation in *A. nidulans* and *F. fujikuroi* (Conlon et al., 2001; Michielse et al., 2014). Moreover, another interesting finding in this study is that the transcript of *AcareB* depends on *AcareA*, but the transcript of *AcareA* does not depend on *AcareB*. The similar result has also been found in *F. fujikuroi* (Michielse et al., 2014). The *AcareB* promoter region does not contain potential GATA factor binding sites, suggesting that the *AcareA* indirectly regulates expression of *AcareB*. In addition, a GATA element was found in the coding region of *AcareB*, which probably regulates its own expression through binding to GATA motifs as Dal80p in yeast and NreB in *P. chrysogenum*.

MATERIALS AND METHODS

Strains, plasmids, media and growth conditions

Strains and plasmids used in this study are listed in Table 1. All used media including LPE, TSA, modified MDFA, Czapek-N, LB, minimal medium (MM), induction medium (IM) and co-cultivation medium (CM) medium were the same as described previously (Li et al., 2013). *E. coli* BL21 (DE3)

Table 1 Strains and plasmids used in this study[a)]

Strains or plasmids	Characteristics	Source
Strains		
Acremonium chrysogenum 3.3795	Wild-type strain	CGMCC*
ΔAcareB	*AcareB* disruption mutant	This study
AcareBC	complemented strain of ΔAcareB	This study
ΔAcareA	*AcareA* disruption mutant	(Li et al., 2013)
AcareBOE	*AcareB* overexpressed strain	This study
Agrobacterium tumefaciens AGL-1	Strain used for fungal transformation	(Khang et al., 2006)
Escherichia coli DH5	Strain used for routine cloning	Gibco BRL
E. coli BL21 (DE3)	Strain used for gene expression	Novagen
Bacillus subtilis 1.1630	Indicator strain for cephalosporin production	CGMCC*
Plasmids		
pEASY-Blunt	Routine cloning vector	Transgen
pEBB	DNA of *AcareB* was inserted into pEASY-Blunt	This study
pEBEB	cDNA of *AcareB* was inserted into pEASY-Blunt	This study
pEBU	DNA fragment containing the upstream flanking sequence of *AcareB* was inserted into pEASY-Blunt	This study
pEBD	DNA fragment containing the downstream flanking sequence of *AcarB* was inserted into pEASY-Blunt	This study
pAg1-H3	The vector used for ATMT	(Khang et al., 2006)
pJL43-RNAi	A plasmid containing the bleomycin resistant gene	(Ullán et al., 2008)
pAgHB	The bleomycin resistant gene was insert into pAg1-H3	(Li et al., 2013)
pAgHBL	One DNA fragment containing the upstream flanking sequence of *AcareB* was inserted into pAgHB	This study
pAgHBLR	One DNA fragment containing the downstream flanking sequence of *AcareB* was inserted into pAgHBL	This study
pEBCareB	The fragment containing *AcareB*, its putative promoter and terminator was inserted into pEASY-Blunt	This study
pAgHBCareB	The plasmid used for complementation	This study
pET28a	Expression vector	Novagen
pET28a::*AcareB*	Plasmid for expression of *AcareB*	This study

a) *, CGMCC indicates China General Microbiological Culture Collection Center.

and DH5α were cultured at 37°C. *Agrobacterium tumefaciens*, *A. chrysogenum* and its derivatives were grown at 28°C. Co-culture of fungal spores and *A. tumefaciens* was incubated at 24°C for 3 days.

Disruption, complementation and overexpression of *AcareB*

For construction of ΔAcareB, a 2,725 bp DNA fragment corresponding to the upstream region of *AcareB* (extending from positions −2705 to +19with respect to the *AcareB* translation start point) was amplified with the primers UareB-F/UareB-R and ligated with the vector pEASY-Blunt to give pEBU. After verified by sequencing, pEBU was digested with *Kpn* I and ligated into the corresponding site of pAgHB (Li et al., 2013), giving pAgHBL. A 2946 bp DNA fragment (extending from positions +1745 to +4690 with respect to the *AcareB* translation start point) was amplified with the primers DareB-F/DareB-R and ligated into pEASY-Blunt to give pEBD. After verified by sequencing, the resulting pEBD was digested with *Asc* I/*Pac* I and ligated into the corresponding sites of pAgHBL to generate pAgHBLR. After confirmed by PCR and restriction enzyme digestion analysis, pAgHBLR was transferred into *A. chrysogenum* through ATMT. Subsequently, screening of the double cross-over transformants was performed as described previously (Wang et al., 2014). The hygromycin resistant and bleomycin sensitive strains were chosen and finally verified by PCR analysis with the inside primers YareB2-F/YareB2-R and the outside primers YareB1-F/YareB1-R.

For complementation of ΔAcareB, a DNA fragment covering the coding region of *AcareB*, its upstream region (1,841 bp) and its downstream region (1,001 bp) was amplified from *A. chrysogenum* with the primers CareB-F/CareB-R and inserted into pEASY-Blunt to generate pEBCareB. The pEBCareB was digested with *Asc* I/*Pac* I and inserted into the corresponding sites of pAgHB to generate pAgHBCareB.

Table 2 Primers used in this study[a)]

Primers	Sequence (5′–3′)
areB-F	ATGGATGACGAGTCGCACAA
areB-R	CTACGCCACCCGGGCGCCGA
UareB-F	AGTGAATTCGAGCTCTTTTGTCACTTGCGTTGG
UareB-R	GCCAGCCCGGGCCTTTTGTGCGACTCGTCATCC
DareB-F	GGCGCGCCACCGCTGCGCCTGAACAA
DareB-R	TTAATTAATCCGCCGTCCGTCTCACCAA
YareB1-F	CACGACCCTCAGCACGACT
YareB1-R	TGCCCGCCTAAACCAACA
YareB2-F	GTCTCACCGAGGGCAATG
YareB2-R	TTCCCACCAGCAGTAGCG
CareB-F	TTAATTAAATTATCGGTGTCCTGCTTG
CareB-R	GGCGCGCCTCGTTTCGCTTCTTTCCT
EareB-F	CATATGGATGACGAGTCGCACAATC
EareB-R	AAGCTTCGCCACCCGGGCGCCGACAT
YCareB-F	ATTATCGGTGTCCTGCTTG
YCareB-R	TCGTTTCGCTTCTTTCCT
Ble-F	GCCAAGTTGACCAGTGCC
Ble-R	CAGTCCTGCTCCTCGGCC
Real-areA-F	GCAGACGGCAATGCTCAAC
Real-areA-R	GCGGGACTTGATGGGGAT
Real-areB-F	CAAACGAATCGCAACAAGGTC
Real-areB-R	GCGTCGCCATAGTGGTGTT
RTareB-F	CAAACGAATCGCAACAAGGTC
RTareB-R	GCGTCGCCATAGTGGTGTT
RTpcbAB-F	ACCAGTCCGACGTGCAGAAT
RTpcbAB-R	TCGGTGATATGGGCCATGTAG
RTpcbC-F	ACCAGTCCGACGTGCAGAAT
RTpcbC-R	TCGGTGATATGGGCCATGTAG
RTcefEF-F	CCGTAACCACCAAGGGTATCT
RTcefEF-R	CTCCTCGCTTCCGTTCTTGA
RTcefG-F	AAGAGCAAACCTGCGATGGA
RTcefG-R	TCTGTGCCGTTGATTTCCTTCT
RTcefD1-F	TGCTGCTCCTGCCCTCAT
RTcefD1-R	CGAAGCCGCTCACCAACT
RTcefD2-F	AGGAACAAGTCGTCCATCTGC
RTcefD2-R	CTTGAGAAGGACCTCTGTGGG
AutoB-F	CGACTCCACTGTCCCCGAA
AutoB-R	CGACTCGTCATCCATGGCTAC
Actin-F	AGTCCAAGCGTGGTATCC
Actin-R	TAGAAGGCAGGGGCGTTG

a) The underlined nucleotide sequences indicate restriction enzyme.

The plasmid pAgHBCareB was introduced into ΔAcareB to obtain the complemented strain AcareBC and AcareBC was confirmed by PCR with the primers YCareB-F/YCareB-R or Ble-F/Ble-R.

RNA isolation and quantitative real-time RT-PCR

RNA isolation, synthesis of cDNA and quantitative real-time RT-PCR were carried out as described previously (Li et al., 2013). All primers used in this study are listed in Table 2.

Overexpression of *AcareB* and protein purification

The coding region of *AcareB* was amplified from *A. chrysogenum* by PCR with the primers EareB-F/EareB-R. The PCR product was ligated with pEASY-Blunt to generate pEBEB. After verified by sequencing, pEBEB was digested with *Nde* I and *Hin*d III, and the fragment containing *AcareB*

coding region (GenBank accession No. KY494643) was inserted into the corresponding sites of pET28a to generate pET28a::*AcareB*. The recombinant plasmid was introduced into *E. coli* BL21 (DE3) for expression. His-tagged protein AcAREB-His_6 was purified, concentrated and stored as described previously (Wang et al., 2014).

Electrophoretic mobility shift assays (EMSAs)

For EMSAs, probes EpcbABC1, EpcbABC2, EcefD1, EcefD2, EcefD3, EcefEFG1 and EcefEFG2 covering the upstream regions of *pcbAB-pcbC*, *cefD1-cefD2* and *cefEF-cefG* were amplified by using the primers described previously (Li et al., 2013). The EMSAs were performed as described previously (Yu et al., 2015).

Cephalosporin production and detection

For cephalosporin production, the fungal spore suspensions of 3×10^7 were inoculated in the shake flask containing 40 mL of the modified MDFA medium. The cultures were incubated at 28°C. For cephalosporin detection, culture broths were centrifuged to remove the mycelia and the yield of cephalosporin was determined using agar-diffusion method with the sensitive strain *Bacillus subtilis* CGMCC 1.1630. The detailed manipulation was adopted as described previously (Li et al., 2013).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *We are grateful to Prof. Juan F. Martín (Universidad de León, Spain) for providing the plasmid pJL43-RNAi. We thank Prof. Xingzhong Liu (Institute of Microbiology, Chinese Academy of Sciences) and Prof. Seogchan Kang (Penn State University, USA) for providing the pAg1-H3 plasmid. This work was supported by the National Natural Science Foundation of China (31670091 and 31470177).*

Brakhage, A.A. (2012). Regulation of fungal secondary metabolism. Nat Rev Micro 11, 21–32.

Brakhage, A.A., Thön, M., Spröte, P., Scharf, D.H., Al-Abdallah, Q., Wolke, S.M., and Hortschansky, P. (2009). Aspects on evolution of fungal β-lactam biosynthesis gene clusters and recruitment of trans-acting factors. Phytochemistry 70, 1801–1811.

Conlon, H., Zadra, I., Haas, H., Arst, H.N., Jones, M.G., and Caddick, M.X. (2001). The *Aspergillus nidulans* GATA transcription factor gene *areB* encodes at least three proteins and features three classes of mutation. Mol Microbiol 40, 361–375.

Dreyer, J., Eichhorn, H., Friedlin, E., Kürnsteiner, H., and Kück, U. (2007). A homologue of the *Aspergillus* velvet gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum*. Appl Environ Microbiol 73, 3412–3422.

Hu, P., Wang, Y., Zhou, J., Pan, Y., and Liu, G. (2015). AcstuA, which encodes an APSES transcription regulator, is involved in conidiation, cephalosporin biosynthesis and cell wall integrity of *Acremonium chrysogenum*. Fungal Genets Biol 83, 26–40.

Jekosch, K., and Kück, U. (2000). Loss of glucose repression in an *Acremonium chrysogenum* β-lactam producer strain and its restoration by multiple copies of the *cre1* gene. Appl Microbiol Biotech 54, 556–563.

Khang, C.H., Park, S.Y., Rho, H.S., Lee, Y.H., and Kang, S. (2006). Filamentous fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). Methods Mol Biol 344, 403−420.

Lee, J.H., Yoo, H.Y., Yang, X., Kim, D.S., Lee, J.H., Lee, S.K., Han, S.O., and Kim, S.W. (2017). Utilization of algal sugars and glycerol for enhanced cephalosporin C production by*Acremonium chrysogenum* M35. Lett Appl Microbiol 64, 66–72.

Li, J., Pan, Y., and Liu, G. (2013). Disruption of the nitrogen regulatory gene *AcareA* in *Acremonium chrysogenum* leads to reduction of cephalosporin production and repression of nitrogen metabolism. Fungal Genets Biol 61, 69–79.

Liu, Y., Zhang, W., Xie, L., Liu, H., Gong, G., Zhu, B., and Hu, Y. (2015). Acthi, a thiazole biosynthesis enzyme, is essential for thiamine biosynthesis and CPC production in *Acremonium chrysogenum*. Microb Cell Fact 14, 50.

Macios, M., Caddick, M.X., Weglenski, P., Scazzocchio, C., and Dzikowska, A. (2012). The GATA factors AREA and AREB together with the co-repressor NMRA, negatively regulate arginine catabolism in *Aspergillus nidulans* in response to nitrogen and carbon source. Fungal Genets Biol 49, 189–198.

Martin, J.F., Ullan, R.V., and Casqueiro, J. (2004). Novel genes involved in cephalosporin biosynthesis: the three-component isopenicillin N epimerase system. Adv Biochem Eng Biotechnol 88, 91−109.

Martín, J.F., Ullán, R.V., and García-Estrada, C. (2010). Regulation and compartmentalization of β-lactam biosynthesis. Microbial Biotech 3, 285–299.

Martín, J.F., Ullán, R.V., and García-Estrada, C. (2012). Role of peroxisomes in the biosynthesis and secretion of β-lactams and other secondary metabolites. J Ind Microbiol Biotechnol 39, 367–382.

Michielse, C.B., Pfannmüller, A., Macios, M., Rengers, P., Dzikowska, A., and Tudzynski, B. (2014). The interplay between the GATA transcription factors AreA, the global nitrogen regulator and AreB in *Fusarium fujikuroi*. Mol Microbiol 91, 472–493.

Niehaus, E.M., Kleigrewe, K., Wiemann, P., Studt, L., Sieber, C.M.K., Connolly, L.R., Freitag, M., Güldener, U., Tudzynski, B., and Humpf, H.U. (2013). Genetic manipulation of the *Fusarium fujikuroi* fusarin gene cluster yields insight into the complex regulation and fusarin biosynthetic pathway. Chem Biol 20, 1055–1066.

Ullán, R.V., Godio, R.P., Teijeira, F., Vaca, I., García-Estrada, C., Feltrer, R., Kosalkova, K., and Martín, J.F. (2008). RNA-silencing in *Penicillium chrysogenum* and *Acremonium chrysogenum*: Validation studies using β-lactam genes expression. J Microbiol Methods 75, 209–218.

Wang, H., Pan, Y., Hu, P., Zhu, Y., Li, J., Jiang, X., and Liu, G. (2014). The autophagy-related gene *Acatg1* is involved in conidiation and cephalosporin production in *Acremonium chrysogenum*. Fungal Genets Biol 69, 65–74.

Wiemann, P., and Tudzynski, B. (2013). The nitrogen regulation network and its impact on secondary metabolism and pathogenicity. In: Brown D.W., and Proctor R.H, ed. Fusarium: Genomics, Molecular and Cellular Biology. (Norwich, UK: Caister Academic Press).

Wong, K.H., Hynes, M.J., Todd, R.B., and Davis, M.A. (2009). Deletion and overexpression of the *Aspergillus nidulans* GATA factor AreB reveals unexpected pleiotropy. Microbiology 155, 3868–3880.

Yin, W., and Keller, N.P. (2011). Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol 49, 329–339.

Yu, L., Li, S., Gao, W., Pan, Y., Tan, H., and Liu, G. (2015). Regulation of myo-inositol catabolism by a GntR-type repressor SCO6974 in *Streptomyces coelicolor*. Appl Microbiol Biotechnol 99, 3141–3153.