RESEARCH ARTICLE

Identification and characterization of *Penicillium citrinum* VeA and LaeA as global regulators for ML-236B production

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Abstract In filamentous fungi, production of multiple secondary metabolites is controlled by so-called global regulators. In this study, two genes encoding homologs of VeA and LaeA, representative fungal global regulators, were identified in ML-236B-producing *Penicillium citrinum*. Disruption of VeA and/or LaeA and complementation clearly demonstrated that both of them played critical roles in ML-236B production by controlling the expression of *mlcR*, the pathway-specific activator gene for ML-236B biosynthesis. Moreover, sequence analysis revealed that *laeA* in a mutant strain producing high levels of ML-236B (strain S-1567) possessed a single nucleotide alteration,

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Faculty of Science, Mahidol University–Osaka University Collaborative Research Center for Bioscience and Biotechnology, Mahidol University, 5th Fl., Chalermphrakeit Bld., Rama VI Rd., Bangkok 10400, Thailand which resulted in 15 surplus amino acids at the carboxyl terminus of LaeA compared to the LaeA in the wild-type strain (strain SANK18767). Introduction of the mutated *laeA* into SANK18767 proved that the extended carboxyl region plays a crucial role in the higher production of ML-236B. These results indicated that VeA and LaeA dominantly control the biosynthesis of ML-236B, and the enhanced production in the strain S-1567 is attributable to the mutation in *laeA*.

Introduction

Filamentous fungi produce a wide variety of secondary metabolites which include antibiotic, antiviral, antitumor, and antihypercholesterolemic agents as well as toxic substances (Demain and Fang 2000). *Penicillium citrinum* produces a polyketide ML-236B (compactin; Fig. 1a) which exhibits a potent inhibitory effect on the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Brown et al. 1976; Endo et al. 1976). Further exploratory researches generated a more tissue-selective and more potent inhibitor of HMG-CoA reductase, pravastatin, which was produced by microbial conversion of ML-236B (Serizawa et al. 1983). Currently, pravastatin has been widely used as a pharmaceutical drug for the treatment of hypercholesterolemia.

The molecular mechanism of ML-236B biosynthesis has been investigated to improve ML-236B production. The genes responsible for ML-236B biosynthesis are clustered in an adjacent 40-kb region on the genome of *P. citrinum*. The gene cluster is composed of two genes (*mlcA* and



Fig. 1 A 40-kb region of the ML-236B biosynthetic gene cluster in *P. citrinum.* **a** Chemical structure of ML-236B. **b** The location and putative transcriptional direction of the genes responsible for ML-236B biosynthesis. *mlcA*, *mlcB* polyketide synthase; *mlcC* cytochrome P450; *mlcD* HMG-CoA reductase; *mlcE* efflux pump; *mlcF* oxidoreductase; *mlcG* dehydrogenase; *mlcH* transesterase; *mlcR*, *ariB* transcriptional regulator

mlcB) encoding polyketide synthases (PKSs) responsible for synthesizing a nonaketide and a diketide moiety, respectively, and four genes encoding modification enzymes, such as cytochrome P450 monooxygenase (mlcC), enoyl reductase (mlcG), and transesterase (mlcH), together with the mlcF gene, the function of which is still unclear (Abe et al. 2002a) (Fig. 1b). In addition to these structural genes, it has been clarified that the cluster contains two self-resistance genes (mlcD and mlcE) and one regulatory gene (mlcR) that is necessary for transcriptional activation of the gene cluster (Abe et al. 2002b; Baba et al. 2006). Hence, mlcR has been utilized to create strains producing high levels of ML-236B (Abe et al. 2002c; Baba et al. 2009).

Since the discovery of the original ML-236B producer (strain SANK18767), classical random mutagenesis has been applied to improve the production, because the initial productivity of ML-236B in the strain SANK18767 was quite low for industrial purposes (Endo et al. 1976; Hosobuchi et al. 1993a, b). Consequently, we have succeeded in generating a variety of mutants that produce hundreds of times more ML-236B compared with the strain SANK18767 (Hosobuchi et al. 1993a, b). A previous study by Abe et al. (2004) characterized a series of mutants producing high levels of ML-236B, such as S-1567, S-5808, and No. 41520, and revealed that the expression of ML-236B biosynthetic genes was dramatically enhanced in Strain No. 41520. However, neither nucleotide alteration nor gene-dosage amplification was observed in any of the genes of the ML-236B biosynthetic gene cluster, even in Strain No. 41520, suggesting that favorable mutations should have occurred on a factor upstream of the regulatory cascade for the ML-236B biosynthesis.

A recent study revealed that several factors called "global regulators" control the production of many secondary metabolites in filamentous fungi (Keller et al. 2005). Among them, LaeA has been proven to play an essential role in the expression of many genes in the biosynthetic gene clusters, such as for sterigmatocystin, penicillin, and lovastatin in Aspergillus (Bok and Keller 2004; Bok et al. 2006a, b), and penicillin in P. chrysogenum (Kosalkova et al. 2009). Another factor, VeA, which was initially isolated as a light-dependent sexual development factor, has also been reported to affect the expression of these genes (Calvo 2008). Although the detailed mechanism by which LaeA and VeA control the biosynthetic genes for secondary metabolism has not been clarified, these two proteins are considered to activate secondary metabolism as a heterotrimeric complex with VelB (Bayram et al. 2008). Therefore, it is plausible that an LaeA homolog and/or VeA homolog in *P. citrinum* control the ML-236B biosynthesis, and that mutations in these genes would result in increased production of ML-236B in the strains already producing high levels of ML-236B.

In this work, we identified and characterized genes encoding LaeA and VeA homologs from both *P. citrinum* SANK18767 and S-1567, one of the strains producing high levels of ML-236B. Disruption of these genes in S-1567 demonstrated that LaeA and VeA were essential factors for ML-236B biosynthesis, and also that both proteins were related to morphological development both in the dark/light conditions, as previously reported for *Aspergillus* and *Penicillum* (Kale et al. 2008; Kosalkova et al. 2009). Furthermore, sequence comparison of the *laeA* gene between SANK18767 and S-1567, together with a heterologous expression experiment, clarified that a single mutation in the *laeA* gene caused the significant increase in the ML-236B production.

Materials and methods

Strains, media and culture conditions

Penicillium citrinum strains SANK18767 and S-1567 (Abe et al. 2004) were used in this study. To obtain conidia, spores were incubated for 2 weeks on potato dextrose agar (Difco) at 24°C. YPL-10 liquid medium (10% lactose, 0.1% yeast extract, 0.5% polypeptone, pH 5.0) and Czapek Dox agar supplemented by 21.8% (w/v) sucrose were used for the fungal transformation. MBG3-8 liquid medium was used for nucleic acid isolation and ML-236B production (Hosobuchi et al. 1993b). Cultivation was carried out under

Primer	Sequence $(5'-3')$
dLae-C-F1	GCGAATTCGATGAGCAGGARCARGAYMG
dLae-C-F2	GCGAATTCGGGACCGGCATHTGGGCNAT
dLae-C-R1	GCGAAGCTTACATCAATTGCCCARCTNCC
dLae-E-R1	GCGAAGCTTTCCACCTGYTCRAACCANGC
dVe-A-F1	AGGAATTCGCAAGAAGATTACNTAYAARYT
dVe-C-F1	AGGAATTCGCCTACAACGCCAAYTTYTTCC
dVe-E-R1	AGCAAGCTTAGTCCAGGGAAYTTCTTGGC
dVe-F-R1	AGCAAGCTTCCGCACTCGRCANCCYTGCTC

R A/G, Y C/T, M A/C, H A/T/C, N A/C/G/T

the same conditions as described previously (Baba et al. 2006). *Escherichia coli* DH5 α was used for the construction, propagation and amplification of plasmids. *E. coli* XL-1 Blue MR (Stratagene) was used as a host for the construction of a cosmid DNA library. LB medium containing suitable antibiotics was used for the cultivations.

Cloning of laeA and veA from P. citrinum

laeA and *veA* genes were amplified by PCR using degenerate primers designed by CODEHOP (Rose et al. 1998) (Table 1). Degenerated PCR was performed with Go Taq (Promega) under the following condition: 3 min at 94°C and 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, followed by a final elongation step at 72°C for 7 min. The amplified fragments were cloned into pGEM-T Easy Vector (Promega), sequenced, and used as probes for Southern and colony hybridizations after DIG-labeling using DIG High Prime (Roche Diagnostics).

Fungal transformation and nucleic acid manipulation

Transformation of P. citrinum was performed as described previously with some modifications (Nara et al. 1993; Baba et al. 2006). An enzyme mixture [10 mg/ml each of Yatalase (Takara Bio Inc.), Usukizyme (Wako Pure Chemical Industries), and lysing enzymes (Sigma)] was used for protoplast formation. Colony direct PCR was carried out using an FTA classic card (Whatman) according to the supplier's instructions. Fungal chromosomal DNA was isolated according to the method developed by Yelton et al. (1984) and the total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen). Southern and colony hybridizations were performed using a DIG system (Roche Diagnostics). RT-PCR analysis, 5'-RACE, and 3'-RACE were carried out with a One Step RNA PCR Kit (AMV) (Takara Bio Inc.) and GeneRacer Kit (Invitrogen). The primers used in RT-PCR are listed in Tables S1 and S2.

Construction of vectors for the fungal transformation

All the primer sequences are shown in Table S3. The plasmid vector pSAK1002 was constructed by deleting the unique BglII site in pSAK1000 (Baba et al. 2008). The P. citrinum pgk promoter-terminator cassette was constructed by overlap extension PCR with PfuTurbo (Stratagene) and the primers PcPGKp-01 M, PcPGKpt-01Spe, PcPGKpt-02Spe, and PcPGKt-01N8. The amplified fragment was digested with MluI and NotI, and ligated with MluI/NotI-digested pSAK1002, resulting in the plasmid pSAK1100. A primer set (PcLae-TOP-Spe, PcLae-END-Spe, and PcLae-END02-Spe), and another set (PcVe-TOP-Spe and PcVe-END-Spe) were used to amplify the coding region of laeA and veA, respectively. The amplified fragments were digested with SpeI and inserted into the unique SpeI site on pSAK1100, yielding the plasmids pSAK-explaeA1, pSAK-explaeA2, and pSAK-expve01 for the constitutive expression of laeA1 (from SANK18767), laeA2 (from S-1567), and veA, respectively. To construct plasmids for gene disruption, each internal region of laeA or veA was amplified by PCR, digested with MluI, and inserted into a unique MluI site on pSAK1002, yielding the plasmids pSAK-Dislae01 and pSAK-Disve01, respectively.

Measurement of ML-236B production

ML-236B was measured as described previously (Baba et al. 2006). To confirm the identity of ML-236B, a UV absorption spectrum was analyzed. ML-236B production by Strain No. 41520 was set at 100 U/ml (Baba et al. 2009).

Results

Isolation of a veA homolog from P. citrinum

PCR was conducted to amplify a part of *veA* from SANK18767 with four different combinations of the degenerated primers designed from a multiple amino acid sequence alignment of VeA from *Aspergillus clavatus* (Accession No. XP_001269004), *A. flavus* (ABF61220), *A. fumigatus* (CAE47975), *A. parasiticus* (AAS07022), *Emericella nidulans* (AAD42946), and *Neosartorya fischeri* (XP_001264513) (Table 1). A band of the predicted size (400-bp) was successfully obtained, which showed a highest identity of 74% (blastx) with *veA* from *A. clavatus* (data not shown). Subsequently, a genomic cosmid library of SANK18767 was screened with the amplified *veA* fragment as the probe. A positive cosmid was isolated, and sequencing part of the insert gave a 5.4-kb nucleotide sequence including *veA* (Fig. 2).

case letters

Fig. 2 Nucleotide and deduced amino acid sequences of *P. citrinum veA*. Intron sequences are written in *lower*

1	ATGGCCAACAGACCACCATCATGCCGCCTCATAATGAGACCGAGCACTCAGTCAG	60
61	ATCACTCGTGAAGGCAAACAACTCACATACAAATTGAGCGTGATGCAACAACCAGAGCGG I T R E G K Q L T Y K L S V M Q Q P E R	120
121	GCAAGAGCATGCGGCGCAGGAGCGAAGTgtgagtattcgctgttgagcataatcagggct A R A C G A G A K S	180
181	gcgcgttgactgactgactgactgtcgcaaatagCATCTGCCGACCGTCGCCCTGT S A D R R P V	240
241	TGACCCACCGCCTGTGGTCGAGCTACGCATCTTCGAGTCTGACCCGCGCTAACGATGCGCA D P P P V V E L R I F E S D P A N D A Q	300
301	AAAGACCGATATCACTTTCGCCTACAATGCCAATTTCTTCCTCTCGCAACCCTTGACAC K T D I T F A Y N A N F F L Y A T L D T	360
361	$\begin{array}{cccc} CGCACGCCCTATCGCCCACGGACGGGTGGGTGGACCACAGTCGTGCCCTGTATTGACTGG\\ A & R & P & I & A & H & G & R & V & G & G & P & Q & S & C & P & V & L & T & G \end{array}$	420
421	TGTGCCAGTCGCCGGTGTCGCATATCTCGACCGCCCCTCACAAGCAGGCTATTTCATCTT V P V A G V A Y L D R P S Q A G Y F I F	480
481	CCCAGATCTCTCGGTGCGTCATGAGGGTCGATACCGCTTGAACTTCCACCTGTACGAAGA P D L S V R H E G R Y R L N F H L Y E E	540
541	GATCAAGGACGCCAAGGATGCCGACAAGGATTCATCTTTGCCTCTTCCCAACCAA	600
601	TCTTTCCGCCACATCAAAGCCAGGCGCCCCGCAAGCATTCCTTCATTTCCGCCTCGAGGT L S A T S K P G A P Q A F L H F R L E V	660
661	CAAGTCGGTACCTTTTACTGTTTACAGCGCTAAGAAGTTCCCCGGCCTAGCGACCAGCAC K S V P F T V Y S A K K F P G L A T S T	720
721	CTCTTTGAGCCGTATCATTGCTGAGCAGGGTTGCCGTGTTCGCATTCGTCGTGATGTGCG S L S R I I A E Q G C R V R I R R D V R	780
781	CATGAGACGCCGGGGCGATAAACGAGATACGGAATACGAATTCAGCGAGGAACGCGCAGC M R R R G D K R D T E Y E F S E E R A A	840
841	CGCGTATGCAGGATCGTCAGATCGTTTCACGACACCTGACAGATACGCAGCTTCGATGGA A Y A G S S D R F T T P D R Y A A S M E	900
901	GCGCCCTAGATCAAACAGCAATGGAAGCAATATGGAATCGCCCTACGGGTTTGTTCCTCC R P R S N S N G S N M E S P Y G F V P P	960
961	AGATCGACCATCTGCACCCGACTATGGCTTCCAGTGCCCCCAGCCCCCTTACCAAAG D R R P S A P D Y G F Q C P Q P P Y Q R	1020
1021	$ \begin{array}{cccc} ACCCATGCCACCTGCACCCATGCCACCCTGCACCTGCCACCTGCCACCTGCCACCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCCTGCACCTGCACCCTGCACCCTGCACCTGCCACGCCACGTGCCACCTGCACCCTGCACCCTGCACCCCTGCACCCTGCACCCTGCACCCTGCACTGCACCCTGGCACCTGCACCTGCACCTGCACCTGCACCTGCACCTGCACCTGCACCTGCACTGCACCTGCACTGCACTGCACTGCACTGCACTGCACTGCACTGCACTGCCACTGCAC$	1080
1081	TCAATCGCACCTTTCATTTGGTTCCACGCCTTCGCATTATCCCGCTCCTCACATGCCTCC Q S H L S F G S T P S H Y P A P H M P P	1140
1141	TACACCCCCACCGGTTGCACCGCAAGGTATCTACTCCCCACAGCACGCATATGCCCAAAT T P P P V A P Q G I Y S P Q H A Y A Q I	1200
1201	ACGACACCCATCCAACGGCTCCGAATACGAAGGAACACCCATTTCGTATCCTGTGGCGCC R H P S N G S E Y E G T P I S Y P V A P	1260
1261	TCAAATACCTGCCGAACGGGGTGGTTACCCCAAGTCCACTATGAATTCCTATGGCATGGA Q I P A E R G G Y P K S T M N S Y G M E	1320
1321	ACCACCAAAACCCAAACTCATATATGGACCCTCGCATGCCCGAACCAAGTTTATACCAACC P P K P N S Y M D P R M P E P S L Y Q P	1380
1381	CATGGCCCATGTTCCGGTGTCACGCCCTCAAACACCTAACTTGGTCCAGGCTGTACCACC M A H V P V S R P Q T P N L V Q A V P P	1440
1441	TCAGAAGCCTCTGCCAAATGAATATGCCAACCACATTGTTCCTTCTGTGGAAAGCACTTC Q K P L P N E Y A N H I V P S V E S T S	1500
1501	ACCCGGTGGCGGCGATGGATACGACAATGTTAGAGGAAAGCGTATGGTATACCAAACTGG P G G D G Y D N V R G K R M V Y Q T G	1560
1561	$ \begin{array}{ccccc} {\rm GCCAACATATGGCAAACGGAGTCATGAGGATACTTTCGGCCTTGATGACCGGTCAATGCA} \\ {\rm P} & {\rm T} & {\rm Y} & {\rm G} & {\rm K} & {\rm R} & {\rm S} & {\rm H} & {\rm E} & {\rm D} & {\rm T} & {\rm F} & {\rm G} & {\rm L} & {\rm D} & {\rm D} & {\rm R} & {\rm S} & {\rm M} & {\rm Q} \\ \end{array} $	1620
1621	GAACGGCATGCGACCTGATACCGAGCCTTATCCCGCTTATCGTGATTTCTCAGGAGAAAG N G M R P D T E P Y P A Y R D F S G E S	1680
1681	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1740
1741	AATGGTCATGAAAGCTCCTCCATCGAATTTATAA M V M K A P P S N L *	1774

Since transcriptional analysis revealed that veA in SANK18767 is expressed after 5 days of cultivation, the transcript was characterized by the 5'- and 3'-RACE method and was compared to the determined genomic

nucleotide sequence. Two 5'-ends (at positions -340 and -311 relative to the putative start codon) and two 3'-ends (at positions +228 and +342 relative to the putative stop codon) were observed, and one 70-bp intron was found in

the coding region. The predicted *P. citrinum* VeA is comprised of 567 amino acids and shows 60% identity with *A. funigatus* VeA and 91% with *P. chrysogenum* VeA registered during this study (Fig. 2). The sequence has been deposited under DDBJ Accession No. AB548655.

The orthologue in strain S-1567, a third generation mutant through the strain improvement program (Abe et al. 2004), was obtained by PCR. No sequence difference was observed between *veA* from SANK18767 and *veA* from S-1567, and *veA* was expressed at quite similar levels in the two strains, implying that VeA is not the cause of the ML-236B overproduction in strain S-1567 (data not shown).

P. citrinum VeA is essential for ML-236B biosynthesis

To clarify the influence of VeA on ML-236B production, *veA* was disrupted in strain S-1567, because the wild-type strain SANK18767 produces too small an amount of ML-236B to detect reproducibly under the normal HPLC conditions. Two transformants showed the expected genotype of truncated *veA* as a result of single homologous crossover (the 5.3 and 4.1-kb bands in Southern hybridization; Fig. S1). The *veA*-complemented strain was also created by reintroducing the *veA*-constitutive expression plasmid pSAK-Expve01 into the *veA* disruptant.

After 9 days of liquid cultivation, strain S-1567 produced 3 U/ml of ML-236B. The veA disruptants, on the other hand, produced only a negligible amount of ML-236B, while the ML-236B production was restored in the veAcomplemented strain to 2.3 U/ml, indicating that VeA was essential for ML-236B production (Fig. 3a). Since VeA in Aspergillus species controls the production of secondary metabolites by regulating the expression of a pathwayspecific transcriptional activator, such as aflR for aflatoxin biosynthesis, the *mlcR* expression was examined in the *veA* disruptant by RT-PCR. As shown in Fig. 3b, while the expression of mlcR as well as veA was observed after 5 days of cultivation both in the parental strain S-1567 and the veA-complemented strain, the mlcR transcript was missing in the veA disruptant. In addition, ariB, which is located next to *mlcR* and is assumed to be a regulator of putative aristolochene biosynthesis (Baba et al. 2006), also showed significantly reduced expression only in the veA disruptant. These results implied that VeA in P. citrinum was responsible for the biosynthesis of multiple secondary metabolites, including ML-236B.

As many previous reports demonstrated that fungal VeA homologs were involved in the sensing of the light for their development and metabolisms (Hoff et al. 2010; Wiemann et al. 2010), we analyzed the function of *P. citrinum* VeA under the light/dark conditions by cultivating S-1567 and its derivative strains on the potato dextrose agar plate. As shown in Fig. S1C, S-1567 did not produce yellow pigment



Fig. 3 Phenotypic analysis of the *veA* disruptant. **a** ML-236B production of the parental strain (S-1567), the *veA* disruptant, and the *veA*-complemented strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** Transcriptional analysis by RT-PCR. Total RNA isolated after 5 days of cultivation was used as the template. The *pgk* gene was used as a control

under the light conditions, indicating that *P. citrinum* equipped sensing system to the light. Under the dark conditions, the *veA* disruptant produced reduced amount of yellow pigment and showed faster hyphal growth, whereas it elongated less aerial mycelia under the light, resulting in the thin colony layer on the plate. These data strongly indicate that *P. citrinum* VeA affects not only secondary metabolisms but also morphological development under the dark/ light conditions.

The P. citrinum laeA gene was altered in strain S-1567

Similar to the case of *veA*, based on multiple sequence alignment with LaeA from *A. clavatus* (Accession No. XP_001268793), *A. fumigatus* (XP_747013), *A. niger* (XP_001390491), *A. oryzae* (BAF74528), and *A. sojae* (AAX68413), degenerate primers were designed for partial cloning of *laeA* from *P. citrinum*, for the region including the conserved *S*-adenosyl methionine (SAM)-binding motif Fig. 4 Nucleotide and deduced amino acid sequences of laeA1 (P. citrinum SANK18767 laeA). Intron sequences are indicated in lower case letters. The conserved SAM-binding sites in the protein methyltransferase family are circled

A TOTAL DOTATION OF THE ALL OF ALL
 M F T N G D S R M R H L P P I N S P P P 121 CCCAAACGATACAAGTCCGAGTCGAGCCCAGCGAGCGGCGGCCGCCCTCTAGATACTAC 181 TCGCATTCCATCGCGAGTGATCGAGTCCGATCACGACACCGTCTTCCGCGATGGACTTG 242 S H S I A S D R V R S R Q P S S A M D L 241 TACACGCTGATCGACAGAGATCCGGTCGATAAAGATCCTCGCAGGAACGCTCGCT
<pre>121 CCCAAACGATACAAGTCCGAGTCGACCCCAGCGAGGCGAGCGCGGCCACTCTAGATACTAC P K R Y K S E S T P A S D A G H S R Y Y 181 TCGCATTCCATCGCGAGTGATCGAGTCGACTCACGACACCGTCTTCCGCGAGTGGACTG S H S I A S D R V R S R Q P S S A M D L 241 TACACGCTGATCGACAGAGATCCGGTCGATAAAGATCCTCGCAGGAACGCTCGCT</pre>
 121 CCCARACGATACAAGTCCGAGCGAGCGAGCGAGGGAGGCCACTCTAGAGTACTAC P K R Y K S E S T P A S D A G H S R Y Y 181 TCGCATTCCATCGGGAGTACGAGTCCGATCACGACACCGTCTTCCGCGGAGAGCTTG 241 TACACGCTGATCGACAGAGATCCGGTCGATAAAGATCCTCGCAGAACGCTCGCT
<pre>181 TCGCATTCCATCGCGAGGATCGAGTCCGATCACGACAACCGTCTTCCGCGGATGGACTG S H S I A S D R V R S R Q P S S A M D L 241 TACACGCTGATCGACAGAGATCCGGCTCGATAAAGATCCTCGCAGGAACGGTCGCTTTACT Y T L I D R D P V D K D P R R N A R F T 301 AGCAATGGATCGGTGGCCACACAGGCATCCCACACGTCGAACACGTCTCAAGTATCGCGA S N G S V A T Q A S H T S N T S Q V S R 361 TCATCTCCGATCATAATGTCTGACGAAGTATCGCGAAGACCGTCTCAAGTATCGCGA S S P I I M S D R K 421 caatgactgaccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG S S P I I M S D R K 421 caatgactgaccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG R M Y H G Y R K G I Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCGGTCGCGGGCGAGGAAGAGC D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGGTCCAGGATTCTCGACCTGGGGCGAGGATAT GGAGCCGTCTCGACATATTCCCCGGGTCCGGGTGTGGACGGGGGATAT GGAGCCGTCTCGACAAACTACTCCCGAATTCCTCGGGTCGGGGGGAGGAGAGGC Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAAGTGCCAAAATTCCCGGATTCCTGGGCGGTGTTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTTCCCAAAGAACTGCGGATTCTCTATGCCCCGTTCGACTTTGAGG I Q P A N F P K N C D F Y A P F D F E A</pre>
 181 TEGEATTECATEGEGAGTEGAGTEGAGTEGAGTEGAGTEG
 S H S I A S D K V K S K Q P S S A M D L 241 TACACGCTGATCGACAGAGATCCGGTCGATAAAGATCCTCGCAGGAACGCTCGCT
<pre>241 TACACGCTGATCGACAGAGATCCGGTCGATAAAGATCCTCGCAGGAACGCTCGCT</pre>
Y T L I D R D P V D K D P R R N A R F T 301 AGCAATGGATCGGTGGCCACACAGGCATCCCACAGGTCGAACACGTCTCAAGTATCGCGA S N G S V A T Q A S H T S N T S Q V S R 361 TCATCTCCGATCATAATGTCTGATCGCAAgtatgtcactccatactcaattgtgtaaatt 42 S S P I I M S D R K 421 caatgactgacccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG 48 I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 54 R M Y H G Y R K G I Y P L P C D E E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGGTCCAGGATTCTCGGACCTGGGTGTGGAACGGGGATAT GGAGCCACTCGAAAGTACAAATTCCCGAATTCCTCGACCTGGGTTGTGAACGGGGATAT 661 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCTATGCCCCGTTCGACTTTGAGG I Q P A N F P K N C D F Y A P F D F A
301 AGCAATGGATCGGTGGCCACACAGGCATCCCACACGTCGAACACGTCTCAAGTATCGCGA S N G S V A T Q A S H T S N T S Q V S R 361 TCATCTCCGATCATAATGTCTGATCGCAAgtatgtcactccatactcaattgtgtaaatt S S P I I M S D R K 421 caatgactgacccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAAATCACCGGTCCCGTGCGACGAGGAAGAGC R M Y H G Y R K G I Y P L P C D E E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGTCCAGGATTCTCGGACCGGGGTGGTGGAACGGGGATAT Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAGTTCCCCGAATCCTTCGTGGTCGGTGGTGGACCGGGGATAT CGATACAACCGGCCAATTCCCCAAAACTGCTCTTCGTGGTCGGTGTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGG I Q P A N F P K N C D F Y A P F D F A A 201 COATGCACTGGCGAATTCCCAAAGAACTGCGATTCCTATGCCCCGTTCGACTTGAGG 78 1 Q P A N F P K N C D F Y A P F D F A A 36 36 36 36 36 36 36 36 36 36 36 36 36
S N G S V A T Q A S H T S N T S Q V S R 361 TCATCTCCGATCATAATGTCTGATCGCAAgtatgtcactccatactcaattgtgtaaatt 42 S S P I I M S D R K 421 caatgactgacccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG 48 I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 54 R M Y H G Y R K G I Y P L P C D E E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA 60 D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGTCCAGGATTCTCGACCTGGGTGGTGGAACGGGGATAT 66 Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTCGTGGTCGGTGTGACCTTGCGC 72 S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGG 78 I Q P A N F P K N C D F Y A P F F F F A
361 TCATCTCCGATCATAATGTCTGATCGCAAgtatgtcactccatactcaattgtgtaaatt S S P I I M S D R K 421 caatgactgacccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG 48 I CCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 48 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 54 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA 60 R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGGTCCAGGATTCTCGACCTGGGGTGGGACGGGGATAT 61 GGAGCATCGAAGTTGCAAAAATTCCCGAATTCCTCGACCTGGGTCGGGGGGATAT 62 I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGG 78 I Q P A N F P K N C D F Y A P F F F F A
S S P I I M S D R K 421 caatgactgacccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG 48 I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 54 R M Y H G Y R K G I Y P L P C D E E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCCGGGGTCCAGGATTCTCGACCTGGGTGTGGAACGGGGATAT Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAGTTGCCAAAATTCCCGAATTCCTCGTGGTCGGTGTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGG I Q P A N F P K N C D F Y A P F D F E A
 421 caatgactgaccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCGGGGACGAGGAAGAGC 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCGGGGACGAGGAAGAGC 481 GCCGAATGTATCACGGTTATCCGAAAGGAATCTACCCGCTCCGGGGGGAGGAGGAGGC 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGGCCGAGGATGGCTTGA 541 AGGACCGTCTCGACATATTCCCCCGAGGATCCTCGGCCGGGGCCGAGGATGGCTTGA 541 AGGACCGTCCCCCCGGGGTCCAGGATTCCTCGACCTGGGCCGAGGATGGCTTGA 541 CTATGCACCACATCCCCCGGGGTCCAGGATTCCTCGACCTGGGACCGGGGATAT 541 CTATGCACCACACCCCCGGGGTCCAGGATTCCTCGACCTGGGACCGGGGATAT 541 CTATGCACCACACCAAATTCCCGAATTCCTCGTGGTCGGTGGACCGGGGATAT 541 CTATGCACCGGCCAATTCCCCAAAGAACTGCGATTCCTTCGTGGTCGGTGTGACCTTGCGC 542 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTTCGACTTTGAGG 543 CGATCAACCGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTCGACTTTGAGG 544 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTTCGACTTTGAGG 545 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTTCGACTTTGAGG 546 CGACACGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTCGACTTTGAGG 547 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTCCTATGCCCCGTCGACTTTGAGG 548 CA N F P K N C D F Y A P F F F F A
I P E K Y P N H K E N G 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCGGTCCGGTGGACGAGGAAGAGC R M Y H G Y R K G I Y P L P C D E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGGTCCAGGATTCTCGACCTGGGTGGG
 481 GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC R M Y H G Y R K G I Y P L P C D E E E Q 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCCGGGGTCCAGGATTCTCGACCTGGGTGTGGAACGGGGATAT 61 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTCGTGGTCGGTGTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTTCCCAAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGGG 78 A P A F F K N C D F Y A P F F F F A
$\begin{array}{cccccccccccccccccccccccccccccccccccc$
 541 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA D R L D I F H K L F T V A R A E D G L I 601 TCTATGCACCACATCCCCGGGGTCCAGGATTCTCGACCTGGGTTGTGGAACGGGGATAT 61 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTGACCTTGCGC 72 S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTTCCCAAAGAACTGCGATTCTATGCCCCGTTCGACTTGAGG 78 I Q P A N F P K N C D F Y A P F D F E A
D R L D I F H K L F T V A R A E D G L I 101 TCTATGCACCACATCCCCCGGGGTCCAGGATTCTCGACCTGGGTTGTGGAACGGGGATAT Y A P H P P G S R I D D L G C G T G I W 101 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 102 F A N F P K N C D F Y A P F F F F A
 601 TCTATGCACCACATCCCCCGGGGTCCAGGATTCTCGACCTGGGTTGTGGAACGGGGATAT Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG 78 I Q P A N F P K N C D F Y A P F F F F A
Y A P H P P G S R I D D L G C G T G I W 661 GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTTGACCTTGCGC S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG I Q P A N F P K N C D F Y A P F D F E A
 661 GGAGCATCGAAGTTGCAAACAAATTCCCGGAATTCCTTCGTGGTCGGTGTTGACCTTGCGC 72 S I E V A N K F P N S F V V G V D L A P 721 CGATACAACCGGCCAATTCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG 78 I Q P A N F P K N C D F Y A P F D F E A
S I E V A N K F P N S F V V G V \bigcirc L A P 721 CGATACAACCGGCCAATTTCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG 78 I Q P A N F P K N C D F Y A P F \bigcirc F E A
721 CGATACAACCGGCCAATTTCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG 78 I Q P A N F P K N C D F Y A P F \bigcirc F E A
I Q P A N F P K N C D F Y A P F 🛈 F E A
781 CCCCCTCGACCTTCGCGCGACGATTCATCGCGATATCATTCAT
$\mathbf{F} = \mathbf{Q} \mathbf{V} = \mathbf{I} \mathbf{D} \mathbf{F} \mathbf{K} \mathbf{P} \mathbf{K} \mathbf{V} = \mathbf{D} \mathbf{K} \mathbf{D} \mathbf{G} = \mathbf{P} \mathbf{G}$
961 GACGCGCTATGGCTAGCTGGTACTCGACCTTGAGCAACTGAAGCCACCCATGCGGC 102
RAMASWYSTLKHATEATMRP
1021 CCCTCGCCACAGCTCCAACGAGACGATCCGGAATTTGCAAGAAGCAGGCTTCACCGAGA 108
L A H S S N E T I R N L Q E A G F T E I
1081 TTGACCACCAGATTGTGGGATTGCCTATGAATCCATGGCATCCTGATTCCCACGAGCAGA 114
D H Q I V G L P M N P W H P D S H E Q K
1141 AGGTTGCGCGGTGGTACAATTTGGCTATCTCCGAGAGTGTTCAGCCATTGTGTTTGGCTC 120
V A R W Y N L A I S E S V Q P L C L A P
1201 CTTTCAGTCGGGTTCTTTCTTGGTCCAGGGAGCAGATTGACCGTATTGCATTCGATGTCA 126
F S R V L S W S R E Q I D R I A F D V K
1261 AGCAAGAAGCTTTCGACAAAAAGATCAAAACGTAA 129
QEAFDKKIKT*

(Bok and Keller 2004). PCR using the genomic DNA of SANK18767 as template amplified a fragment of predicted size (390-bp) with 79% (blastx) identities to the corresponding region of A. fumigatus LaeA (data not shown). A cosmid was obtained from a genomic library of SANK18767 and the presence of laeA on its insert was confirmed by sequencing the 4.5-kb region.

The whole ORF of laeA from SANK18767 (laeA1) was compared with its cDNA sequence, resulting in the identification of two 5'-ends (at positions -171 and -146 relative to the putative start codon), one 3'-end (at +750 relative to the putative stop codon), and one 56-bp intron as commonly observed in Aspergillus laeA. LaeA1 in strain SANK18767 was predicted to have 392 amino acids, to contain a conserved SAM motif and to have 61 and 95% identity with A. clavatus LaeA and P. schrysogenum LaeA, respectively (DDBJ Accession No. AB548656; Fig. 4).

Based on the determined *laeA1* sequence, the *laeA* gene from strain S-1567 (laeA2) was cloned by PCR, together with the flanking region, and sequenced. A comparison between laeA1 and laeA2, including the 5'-flanking and 3'-flanking regions, revealed that there is only one point mutation. The stop codon "TAA" at the carboxyl terminus of *laeA1* was substituted to "TAC" in *laeA2*, and the stop codon at 45-bp downstream became the new stop codon. As a result, in the comparison with LaeA1, LaeA2 possessed 15 surplus amino acids (YNLLHIYQARKPLEE) at its carboxyl terminus and the total length of LaeA2 became 407 amino acids (DDBJ Accession No. AB548657; Fig. 5).

Fig. 5 Nucleotide and deduced amino acid sequences of *laeA2* (P. citrinum S-1567 laeA). Intron sequences are indicated in lower case letters. The conserved SAM-binding sites in the protein methyltransferase family are circled. Nucleotides different from their counterparts in laeA1 are boxed

241

301

361

421

481

541

601

661

721

781

841

901

961

1021

1141

1201

1261

1 ATGTCTTACAGAGAGTCTCCCGGGGTCCTTTCCCGCGCCTGACCGCACATCGCTGCCGAAA 60 M S Y R E S P G S F P A P D R T S T. PK ATGTTTACGAACGGGGATTCCAGGATGCGACACCTGCCTCCCATAAATTCCCCACCCCG 61 120 M F T N G D S R M R H L P P I N S P P Р 121 CCCAAACGATACAAGTCCGAGTCGACCCCAGCGAGCGATGCCGGCCACTCTAGATACTAC 180 P K R Y K S E S T P A S D A G H S R Y Y 181 TCGCATTCCATCGCGAGTGATCGAGTCCGATCACGACAACCGTCTTCCGCGATGGACTTG 240 H S I A S D R V R S R Q P S S A M S D T. 300 Y T L I D R D P V D K D P R R N A R AGCAATGGATCGGTGGCCACACAGGCATCCCACACGTCGAACACGTCTCAAGTATCGCGA 360 S N G S V A T O A S H T S N T S O V S R TCATCTCCGATCATAATGTCTGATCGCAAgtatgtcactccatactcaattgtgtaaatt 420 SSPIIMSDRK caatgactgaccccgtccgaacagGATACCCGAGAAATACCCGAATCACAAAGAAAACG 480 IPEKYPNHKENG GCCGAATGTATCACGGTTATCGGAAAGGAATCTACCCGCTCCCGTGCGACGAGGAAGAGC 540 R M Y H G Y R K G I Y P L P C D E E E 0 AGGACCGTCTCGACATATTCCACAAACTGTTCACGGTTGCACGGGCCGAGGATGGCTTGA 600 D R L D I F H K L F T V A R A E D G L I TCTATGCACCACATCCCCCGGGGTCCAGGATTCTCGACCTGGGTTGTGGAACGGGGATAT 660 Y A P H P P G S R I D D L G C G T G I W GGAGCATCGAAGTTGCAAACAAATTCCCGAATTCCTTCGTGGTCGGTGTTGACCTTGCGC 720 SIEVANKFPNSFVVGV(D)LA Ρ CGATACAACCGGCCAATTTCCCAAAGAACTGCGATTTCTATGCCCCGTTCGACTTTGAGG 780 TOPANFPKNCDFYAP F (D) F E Α 840 Ρ W Т LGEDSWDIIHMQMG С G S 900 V A S W P S L Y R R V F O H L R P G A W GGTTTGAGCAAGTCGAAATCGATTTCCGGCCCCGTGTTGAGGACAAGGATGGTGAACCGG 960 FEQVEIDFRPRVEDKDGE G Ρ GACGCGCTATGGCTAGCTGGTACTCGACCTTGAAGCATGCAACTGAAGCCACCATGCGGC 1020 RAMASWYSTLKHATEA Т М R P CCCTCGCCCACAGCTCCAACGAGACGATCCGGAATTTGCAAGAAGCAGGCTTCACCGAGA 1080 T. A H S S N E T T R N L O E A G F Т E Т 1081 TTGACCACCAGATTGTGGGATTGCCTATGAATCCATGGCATCCTGATTCCCACGAGCAGA 1140 Η 0 Ι VGLPMNPWHP D S Η Ε K AGGTTGCGCGGTGGTACAATTTGGCTATCTCCGAGAGTGTTCAGCCATTGTGTTTGGCTC 1200 V A R W Y N L A I S E S V Q P L C L A P CTTTCAGTCGGGTTCTTTCTTGGTCCAGGGAGCAGATTGACCGTATTGCATTCGATGTCA 1260 F S R V L S W S R E Q I D R I A F D V K AGCAAGAAGCTTTCGACAAAAAGATCAAAACGTACAACCTGCTGCACATTTATCAAGCGC 1320 Q E A F D K K I K T Y N L L H I Y Q A R GAAAACCTCTCGAGGAATAA 1340 1321 KPLEE

Functional analysis of P. citrinum LaeA

To examine the relation of LaeA to ML-236B production, laeA2 was disrupted in strain S-1567 by inserting the laeA disruption plasmid pSAK-Dislae01 via homologous recombination (Fig. S2A). Two out of 30 hygromycin-resistant strains were selected as *laeA2* disruptants based on the bands of expected size (3.5- and 2.4-kb) in Southern hybridization (Fig. S2B).

The laeA2 disruptant showed much weaker pigmentation on the bottom side of the plate and faster hyphal growth than the parental strain on the PDA plate under the dark conditions, indicating that LaeA2 was responsible for both secondary metabolism and the morphological growth in P. citrinum (Fig. S2C). Furthermore, the laeA2 disruptant scarcely showed aerial mycelial growth, similar to the case of the veA disruptant, demonstrating that P. citrinum LaeA2 is also involved in sensing the light condition.

As for ML-236B, the production was missing in the *laeA2* disruptant at 9 days of liquid cultivation, whereas it was restored in the laeA2-complemented strain (1.4 U/ml), indicating that LaeA2 is required for the ML-236B production (Fig. 6a). As expected, not only the expression of mlcR but also that of ariB was dependent on the presence of functional laeA2, similar to the case of veA (Fig. 6b). These data demonstrated that LaeA2 in P. citrinum also regulated the biosynthesis for multiple secondary metabolites by controlling the expression of pathway-specific transcriptional regulators.

To clarify whether LaeA2 is responsible for the highlevel production of ML-236B in S-1567, laeA1 or laeA2



Fig. 6 Phenotypic analysis of the *laeA2* disruptant. **a** ML-236B production of the parental strain (S-1567), the *laeA2* disruptant, and the *laeA2*-complemented strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** Transcriptional analysis by RT-PCR. Total RNA isolated after 5 days of cultivation was used as the template. The *pgk* gene was used as a control

was overexpressed in strain SANK18767. Transformants which contained one extra copy of the *laeA* gene (*laeA1* or *laeA2*) in addition to the native *laeA1* were selected and used for phenotypic analyses (Fig. S3). The *laeA2*-introduced strain showed obvious poor growth on PDA medium, whereas the *laeA1*-introduced strain showed the same morphology as that of the parental strain SANK18767 (Fig. S3C). This phenotypic change seemed to coincide with the faster hyphal growth of *laeA2*-disrupted S-1567 (Fig. S2C). Furthermore, in the case of submerged cultivation, the *laeA2*-introduced strain showed an increased amount of green spores on the wall-grown hyphae after 9 days of cultivation (Fig. S3D).

Regarding ML-236B biosynthesis, the *laeA2*-introduced strain produced dramatically higher ML-236B levels (6.7 U/ml), which coincided with enhanced expression of the regulator gene *mlcR*, while the *laeA1*-introduced strain hardly produced any ML-236B and showed almost the same level of *mlcR* transcription as that in strain SANK18767 (Fig. 7). This finding, together with the fact that expression of the *ariB* gene was also induced in the



Fig. 7 Phenotypic analysis of *laeA1*- or *laeA2*-introduced strains. **a** ML-236B production of the parental strain (SANK18767), the *laeA1*-introduced strain, and the *laeA2*-introduced strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** Transcriptional analysis by RT-PCR. Two independent transformants were analyzed, respectively. Total RNA isolated after 5 days of cultivation was used as the template. The *pgk* gene was used as a control

laeA2-introduced strain, suggests that alteration in LaeA was the major cause of the high-level production of ML-236B in strain S-1567.

Discussion

Filamentous fungi produce many compounds which are beneficial or detrimental for humans as secondary metabolites (Demain and Fang 2000). Therefore, increasing attempts have been made to rationally control the production of secondary metabolites, especially for the purposes of improving the yield of desirable products and avoiding the production of harmful fungal toxins. Pathway-specific transcriptional regulators have been the primary targets of this research, and have been well investigated. Most of them are located inside the biosynthetic gene clusters and belong to the $Zn(II)_2Cys_6$ zinc binuclear cluster protein family (Todd and Andrianopoulos 1997), as represented by AflR, which induces the expression of whole genes in the biosynthetic clusters for aflatoxin and sterigmatocystin in *Aspergillus* (Woloshuk et al. 1994; Chang et al. 1995; Yu et al. 1996; Cary et al. 2000). In the case of ML-236B production in *P. citrinum*, MlcR is the regulator responsible for the synchronized expression of ML-236B biosynthetic genes, and thus the multicopy of *mlcR* enhanced ML-236B production even in mutants producing high levels of ML-236B (Abe et al. 2002b; Baba et al. 2006, 2009).

The discovery of a novel type of global regulator LaeA from *A. nidulans* has provided a new approach to improving the productivity of secondary metabolites in filamentous fungi (Bok and Keller 2004). LaeA is considered to regulate the expression of genes located in heterochromatin by modifying the chromatin structure through its methyl-transferase activity (Bok and Keller 2004; Bok et al. 2006a, b). Genes encoding LaeA or its homologs have been proven necessary for the production of secondary metabolites, including sterigmatocystin and penicillin in *A. nidulans*, lovastatin in *A. terreus*, gliotoxin in *A. fumigatus*, aflatoxin in *A. flavus*, and penicillin in *P. chrysogenum* (Bok and Keller 2004; Bok et al. 2006a, b; Perrin et al. 2007; Kale et al. 2008; Kosalkova et al. 2009).

Although another global regulator, veA, was initially identified as a velvet gene and was demonstrated to be required for the control of asexual and sexual development, further research clarified that VeA also regulates the secondary metabolism, as in the case of sterigmatocystin and penicillin production in A. nidulans, and aflatoxin production in A. parasiticus and A. flavus (Kato et al. 2003; Calvo et al. 2004; Duran et al. 2007; Cary et al. 2007; Calvo 2008). Since VeA in Aspergillus is involved in the control of both the morphological differentiation and biosynthesis of secondary metabolites, it had been hypothesized that there should be a branching point downstream of VeA, where two corresponding signal cascades for the fungal development and the secondary metabolism, respectively, diverged. Bayram et al. (2008) reported that LaeA and VeA form a heterotrimeric complex with VelB, another velvetlike protein. According to their assumption, VeA supports the nuclear localization of VelB by forming the VelB/VeA complex, and the interaction between VelB and VeA in the nucleus also controls the activity of LaeA to induce secondary metabolism in response to the light/dark environment. Recently, some papers demonstrated that the *velvet*-like complex plays a crucial role on controlling differentiation and secondary metabolism in different fungi (Hoff et al. 2010; Wiemann et al. 2010). Because the velvet-like complex is mentioned to have functional plasticity in different species, here the velvet-like complex genes were cloned from *P. citrinum* and their roles were investigated. As a result, we successfully cloned two of the *velvet*-like complex genes, *veA* and *laeA*, and demonstrated that they are pivotal members in the regulation of biosynthetic genes for secondary metabolites, including ML-236B. Although the function of VelB is not fully understood and the *velB* gene of *P. citrinum* has not been identified yet, it is probable that VelB plays the same role in regulating the function of LaeA by interacting with VeA, and thus that secondary metabolism in *P. citrinum* is under the control of VelB/LaeA system.

Although classical random mutagenesis followed by selection of higher producers has been used for breeding of a variety of overproducing mutants, in most cases, the details of the mutations conferring the overproduction are not known. Perhaps the single exception is the case of P. chrysogenum, in which penicillin-overproducers were revealed to contain multiple sets of the structural genes (up to 50 copies; Smith et al. 1989; Barredo et al. 1989). Likewise, the mutations conferring the ML-236B overproduction in the strain S-1567 have not been adequately examined and remain unclear. Our previous study revealed that, despite the dramatic increase in the expression of mlcR and ML-236B biosynthetic genes in strain S-1567, no difference of copy number or mutations was detected in the ML-236B biosynthetic gene cluster (Abe et al. 2004). Since the expression of *ariB*, which localizes next to *mlcR* and is assumed to be the regulator of putative aristolochene biosynthesis, increased in the mutant as well, we predicted that the mutations responsible for ML-236B overproduction are in a component of the global regulatory network for secondary metabolism. During our study, Hoff et al. (2010) reported that, even in the industrial strain, the VelA and LaeA of the penicillin producer P. chrysogenum regulated not only the penicillin production but also the development, suggesting that the fungal velvet-like complex has an essential role on increasing the secondary metabolite production during the strain improvement program. As expected, the mutation was identified within the laeA loci of strain S-1567, which generated mutated LaeA (LaeA2) containing 15 additional amino acids at the carboxyl terminus. The essential nature of LaeA2 in causing overproduction of ML-236B was demonstrated by the heterologous expression of the LaeA2 in the strain SANK18767, which resulted in a remarkable and simultaneous enhancement of both the mlcR expression and ML-236B production (Fig. 7). As the N-terminal part of LaeA is considered to interact with VeA (Bayram et al. 2008), it is plausible that the C-terminal region of LaeA plays an essential role in the ability of LaeA to exert its own activity as a global regulator. We will need to produce various C-terminally truncated or point-mutated LaeA to clarify the actual function of the region further. Interestingly, introduction of laeA2 into the strain

SANK18767 strongly enhanced the ML-236B production (6.7 U/ml), which was twice higher than the case of the strain S-1567 (3 U/ml). It is possible that not only the mutations positive to ML-236B production but also the negative ones were introduced during the strain improvement program from SANK18767 to S-1567, although it has not been confirmed.

The strain S-1567 harboring LaeA2 shows reduced hyphal growth on PDA medium, compared to SANK18767 containing LaeA1. This study confirmed that the phenotypic difference is caused by the mutation in *laeA*, because *laeA* disruption in S-1567 resulted in faster hyphal growth, less sporulation, and a thicker hyphal layer on the PDA plate as in the case of the wild-type strain SANK18767 (Fig. S2C). Moreover, introduction of *laeA2* into the strain SANK18767 clearly reduced the amount of mycelia on the plate (Fig. 7). Judging from the fact that the disruption of both *veA* and *laeA* affected hyphal growth under the light condition, *P. citrinum* velvet-like complex is involved in the development and light-sensing, and it is possible that LaeA is the key factor of the development of industrial *P. citrinum* strain.

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