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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, 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*.

Keywords *Penicillium citrinum* · ML-236B · Secondary metabolite · LaeA · VeA · Global regulator

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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

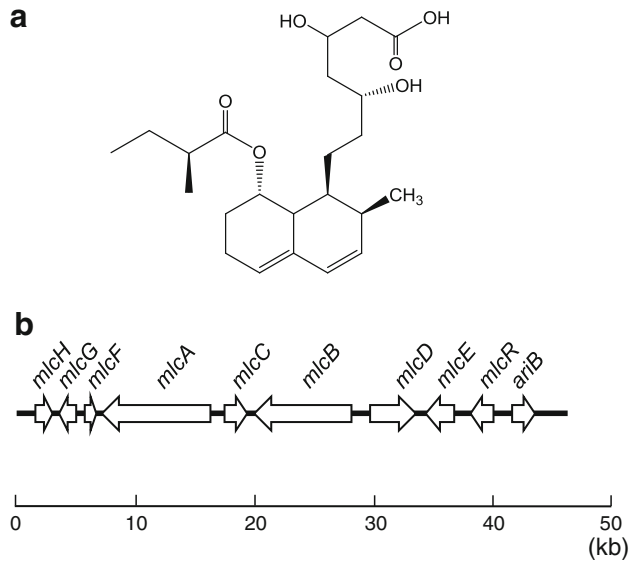

Fig. 1 A 40-kb region of the ML-236B biosynthetic gene cluster in *P. citrinum*. **a** Chemical structure of ML-236B. **b** The location and putative transcriptional direction of the genes responsible for ML-236B biosynthesis. *mlcA*, *mlcB* polyketide synthase; *mlcC* cytochrome P450; *mlcD* HMG-CoA 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

Table 1 Degenerate primers used for the isolation of *laeA* and *veA*

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* DH5α 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 *Bgl*II 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 *Mlu*I and *Not*I, and ligated with *Mlu*I/*Not*I-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 *Spe*I and inserted into the unique *Spe*I 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 *Mlu*I, and inserted into a unique *Mlu*I 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).

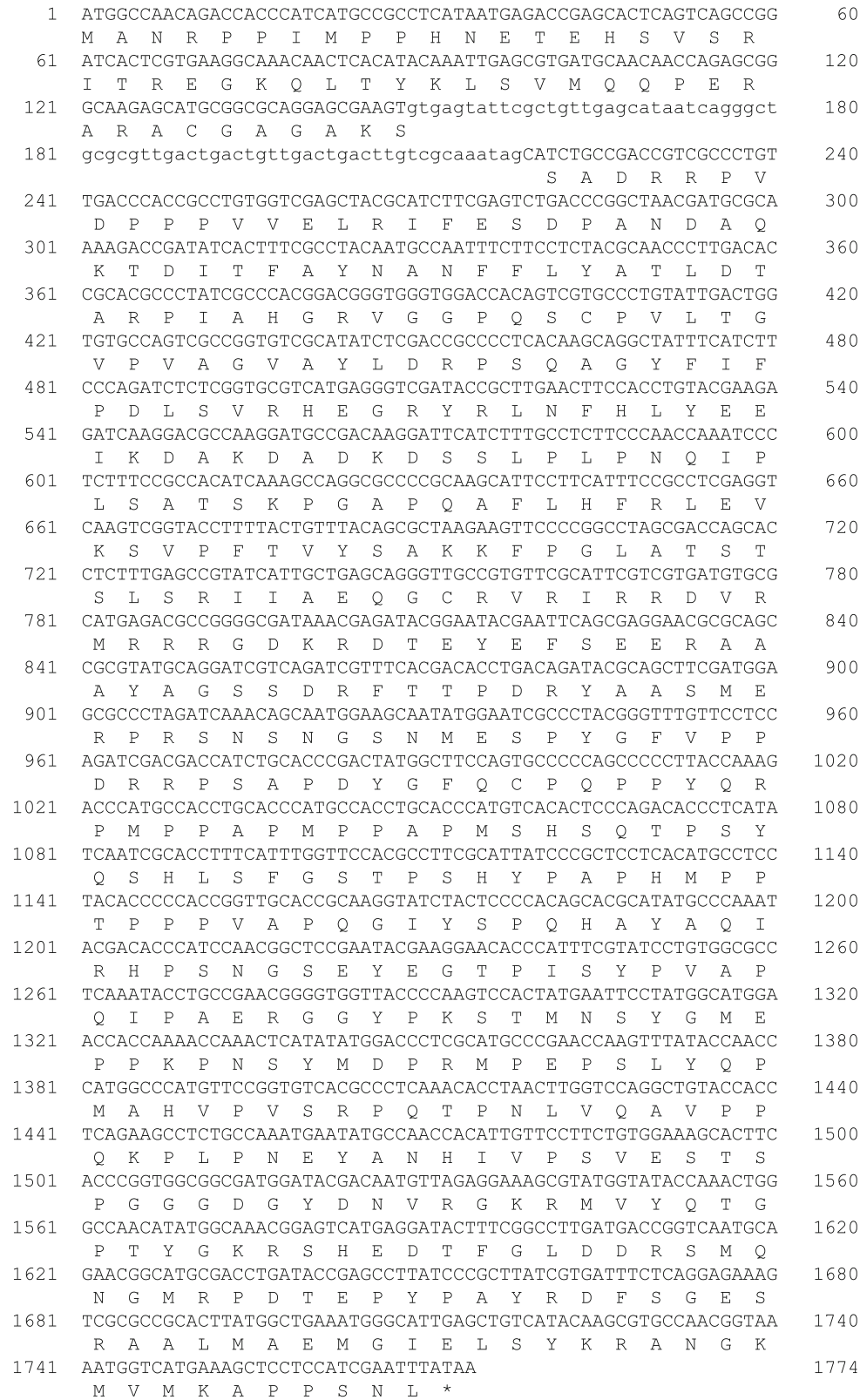

Fig. 2 Nucleotide and deduced amino acid sequences of *P. citrinum veA*. Intron sequences are written in *lower case letters*

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. fumigatus* 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 *veA*-complemented 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 pathway-specific 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

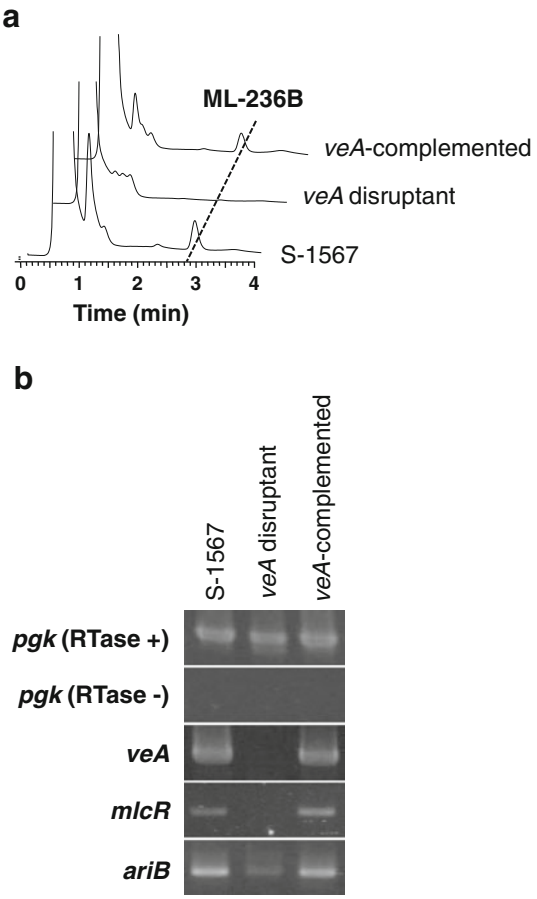

Fig. 3 Phenotypic analysis of the *veA* disruptant. **a** ML-236B production of the parental strain (S-1567), the *veA* disruptant, and the *veA*-complemented strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** Transcriptional analysis by RT-PCR. 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

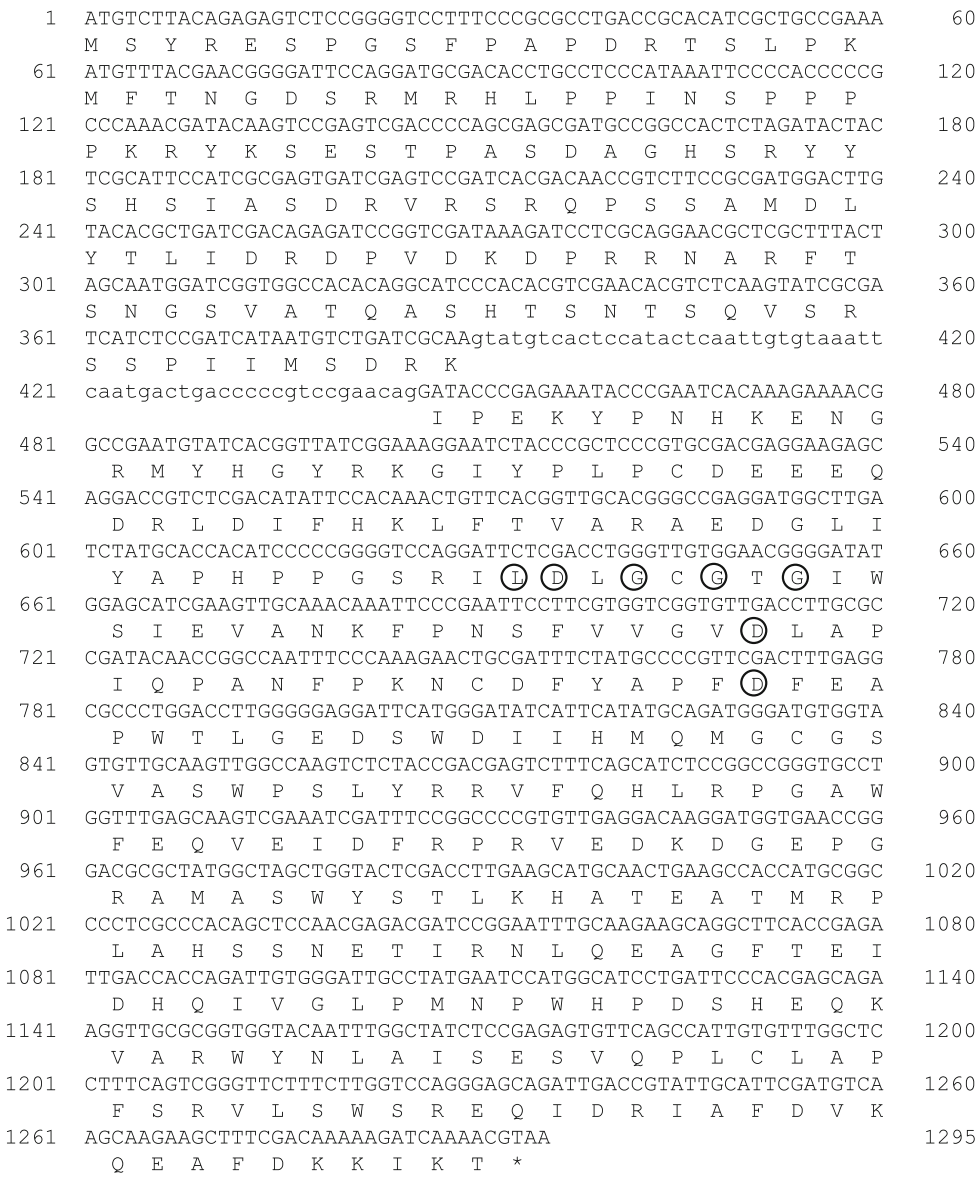

Fig. 4 Nucleotide and deduced amino acid sequences of *laeA1* (*P. citrinum* SANK18767 *laeA*). Intron sequences are indicated in *lower case letters*. The conserved SAM-binding sites in the protein methyltransferase family are *circled*

(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).

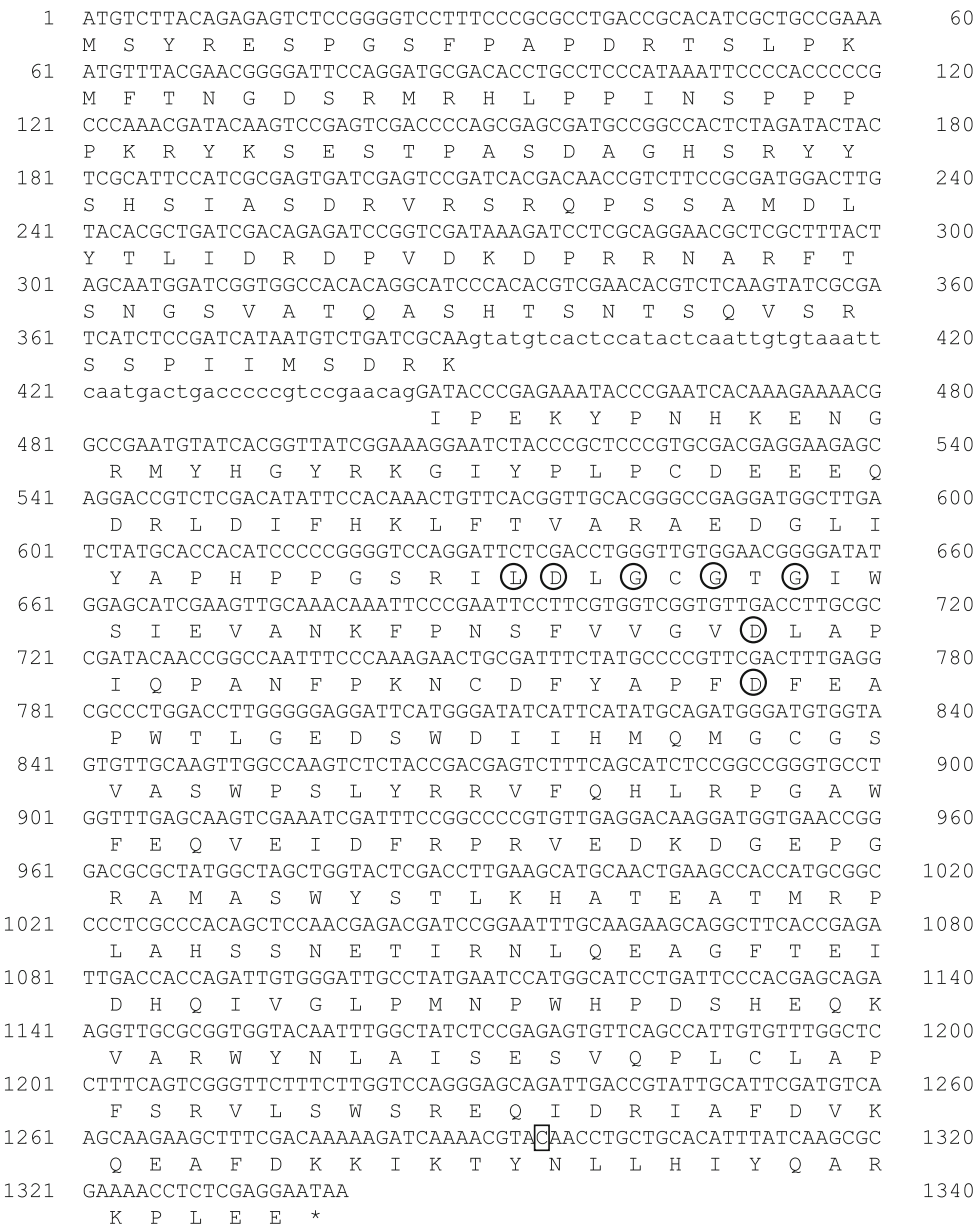

Fig. 5 Nucleotide and deduced amino acid sequences of *laeA2* (*P. citrinum* S-1567 *laeA*). Intron sequences are indicated in *lower case letters*. The conserved SAM-binding sites in the protein methyltransferase family are *circled*. Nucleotides different from their counterparts in *laeA1* are *boxed*

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 high-level production of ML-236B in S-1567, *laeA1* or *laeA2*

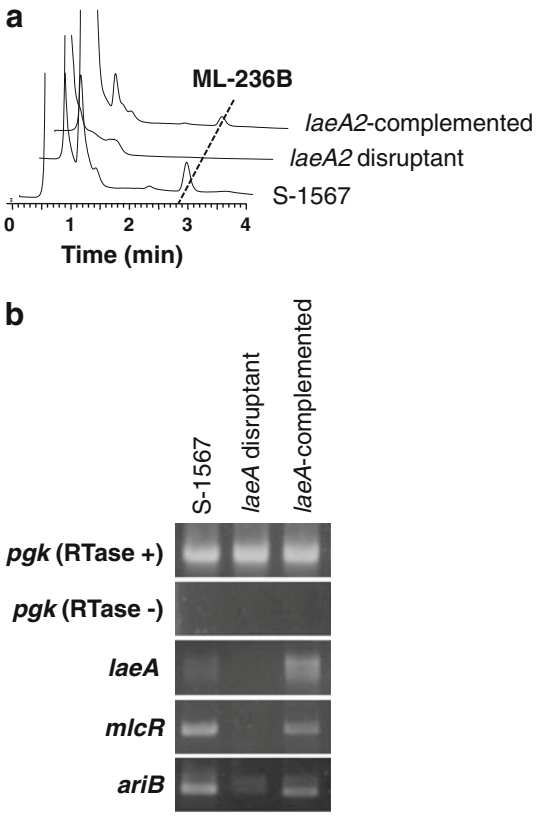

Fig. 6 Phenotypic analysis of the *laeA2* disruptant. **a** ML-236B production of the parental strain (S-1567), the *laeA2* disruptant, and the *laeA2*-complemented strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** Transcriptional analysis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

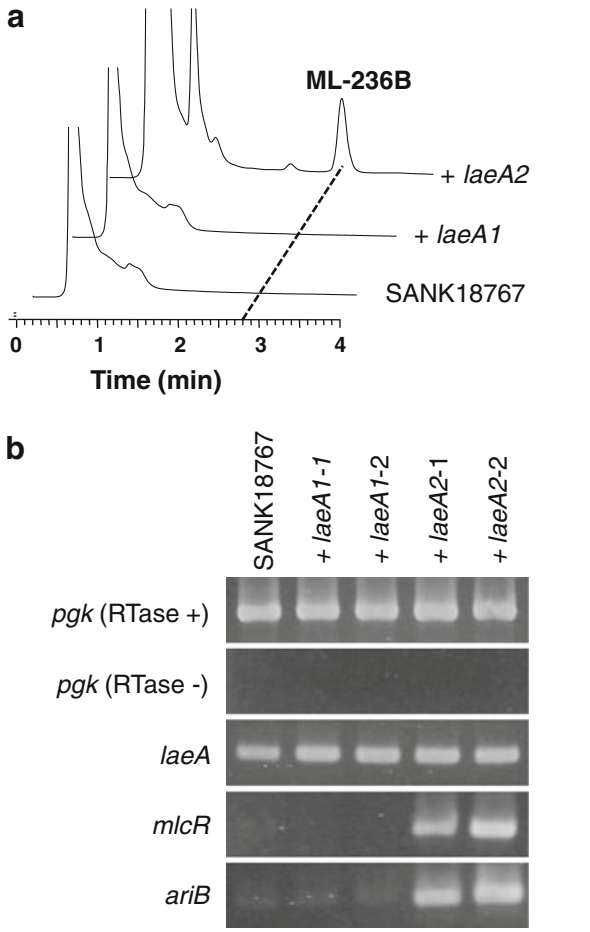

Fig. 7 Phenotypic analysis of *laeA1*- or *laeA2*-introduced strains. **a** ML-236B production of the parental strain (SANK18767), the *laeA1*-introduced strain, and the *laeA2*-introduced strain, after 9 days of cultivation. A *dotted line* indicates the elution position (2.8 min) of ML-236B. **b** 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

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 *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 methyltransferase 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 *velvet*-like 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 VeA/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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