Genomic Wake-Up Call

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CHAPTER 2

Impact of the Regulator LaeA on Secondary Metabolite Production in Industrial Strains of *Penicillium chrysogenum*

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Abstract

The nuclear protein, LaeA, is highly conserved in filamentous fungi and functions as a global regulator of secondary metabolite production and sexual development. Here, we examined the function of LaeA in industrial strains of Penicillium chrysogenum. *LaeA was overexpressed in the penicillin biosynthetic gene cluster-free strain DS54555 which is derived from the high yielding β-lactam producing strain DS17690. This resulted in the up-regulation of several nonribosomal peptide synthethase (NRPS) and polyketide synthase (PKS) genes concomitantly with the presence of novel metabolites in the growth medium. The overexpression and deletion of the LaeA gene in strain DS17690 caused a change in the expression of key genes involved in β-lactam biosynthesis but had little effect on penicillin production. It is concluded that industrial strains of* P. chrysogenum *remain responsive to the regulatory action of despite the classical strain improvement, but that other factors limit β-lactam production at high biosynthetic enzyme levels.*

2.1 Introduction

The sequencing of the genome of *Penicillium chrysogenum* Wisconsin54-1255 has revealed a greater potential for secondary metabolite formation than previously anticipated (Berg et al. [2008]). In addition to the known penicillin biosynthetic gene cluster and three nonribosomal peptide synthetases (NRPS) specifying siderophores, *P. chrysogenum* contains the genes for 20 polyketide synthetases (PKS), 2 hybrids NRPS-PKS, and 6 NRPS. For all these enzymes, it neither is known what products they form nor is it clear what activity they specify. Transcriptome data revealed that many of these potential secondary metabolite forming genes are clustered together with a transcription factor that most likely acts specifically on the genes within that cluster (Berg et al. [2008]). However, others secondary metabolite genes do not seem to include such a local regulator, or are not organized in a gene cluster. Transcriptome analysis revealed that most of the secondary metabolite gene clusters remain silent under standard laboratory conditions and are thus the nature of their product formed remains unknown. It is possible that these genes respond only to very specific environmental factors. In order to define what kind of secondary metabolites emerge from these gene clusters, it is important to understand the natural interactions the fungus undergoes in its original ecological niche (Brakhage and Schroeckh [2011]; Fox and Howlett [2008]).

One of the most successful strategies for the elucidation of cryptic secondary metabolite pathways is based on genetic engineering techniques. In particular, the deletion or overexpression of local transcriptional regulators has proven to be an effective method to activate the expression of such secondary metabolite pathway genes. Another, but less dependable tool is the epigenetic manipulation via the chromatin structure transition, which can lead to gene activation (Kück and Hoff [2010]; Strauss and Reyes-Dominguez [2011]; Brakhage and Schroeckh [2011]). In filamentous fungi, LaeA (Loss of aflR expression A) is a major transcriptional regulator of secondary metabolite formation and sexual development (Bok and Keller [2004]). LaeA carries an S-adenosyl-L-methionine (SAM) domain which might be involved in methylation of histones (Bok and Keller [2004]). The VelB-VeA-LaeA velvet complex coordinates light signal with fungal development and secondary metabolism (Bayram et al. [2008a]), as well as differentiation processes such as sclerotia and fruiting body formation (Bayram et al. [2012]). This heterotrimeric complex was established in recent years as highly conserved in numerous filamentous fungi (Bayram et al. [2008b]; Wiemann et al. [2010]; Bayram and Braus [2012]; Yang et al. [2013]; Karimi-Aghcheh et al. [2013]), including *P. chrysogenum* (Hoff et al. [2010a]; Veiga et al. [2012]). Members of the velvet family share the common velvet domain and interact with each other, and with the non-velvet LaeA protein. They localize within the nucleus. Under dark conditions, VeA (velvet protein) (Kafer [1965]) bridges VelB (velvet - like B) with LaeA to form the VelB-VeA-LaeA complex (Bayram et al.

[2008a]). VelB is part of a second complex, which includes VosA (viability of spores A), coordinated by LaeA in light dependent manner (Sarikaya Bayram et al. [2010]). LaeA controls the protein levels of members of the velvet family and the complex allocation between VelB-VeA, VelB-VeA-LaeA, or VosA-VelB complexes within the fungal cell (Sarikaya Bayram et al. [2010]).

The precise mechanism by which LaeA alters the chromatin structure and how it enables the expression of a wide range of natural products genes is essentially unknown (Cichewicz [2009]; Strauss and Reyes-Dominguez [2011]). LaeA was identified as a global regulator of secondary metabolism in Aspergillus species including *A. nidulans*, *A. terreus*, and *A. fumigatus* (Bok and Keller [2004]). It controls the formation of products such penicillin, lovastatin, and gliotoxin (Bok and Keller [2004]; Sugui et al. [2007]). These molecules show a broad range of bioactivities, and function as antibiotics, toxins, and immunosuppressant agents. In *Aspergillus* species, secondary metabolite formation can be enhanced by the overexpression of the LaeA protein.

A gene homologous to *laeA* is present as a single copy in the genome of *P. chrysogenum* (Kosalková et al. [2009]). Velvet family proteins; PcVelA, PcVelB, and PcVosA directly interact with each other in *P. chrysogenum* (Hoff et al. [2010a]; Kopke et al. [2013]). LaeA interacts only with PcVelA and not with other velvet components (Kopke et al. [2013]).

Overexpression of LaeA had only a marginal effect on penicillin production (20-25% increase) in the low penicillin yielding strain Wis54-1255 (Kosalková et al. [2009]). On the other hand, deletion of *laeA* in one out of ten transformants caused a 2-fold reduction in β-lactam production (Kosalková et al. [2009]), what led to the conclusion that LaeA acts as positive regulator of penicillin production in *P. chrysogenum* even though the phenomenon appears a low frequency observation as the majority of the transformants did not respond. A similar decrease in penicillin titers was also observed in the high penicillin producer *P. chrysogenum* P2niaD 18 strain (Hoff et al. [2010b]).

Industrial strains of *P. chrysogenum* have evolved through a classical strain improvement (CSI) program of mutagenesis and selection. This has led to the amplification of the entire penicillin biosynthesis gene cluster (Fierro et al. [1995]; Newbert et al. [1997]; Berg et al. [2008]). Three of the key genes for penicillin production which are localized in this amplified region are *pcbAB*, encoding α-aminoadipoyl-D-cysteinyl-D-valine synthetases; *pcbC*, encoding isopenicillin N synthase; and *penDE*, encoding acetyl-CoA:isopepenicillin N-acyltransferase (Barredo et al. [1989a]; Barredo et al. [1989b]; Smith et al. [1990]; van den Berg et al. [2008]). In addition, the CSI resulted in strains that are geared towards a high level supply of the precursor amino acids of penicillin pathway, i.e. the amino acids α-aminoadipate, cysteine, and valine; the inactivation of side chain metabolism; and the proliferations of microbodies, organelles that harbor the last two enzymatic steps of the biosynthetic

Table 2.1: *P. chrysogenum* strains used in this study

Strain	Genotype	Reference
DS17690	Reference strain with 8 copies of the penicillin gene cluster	Harris et al. [2009]
DS54465	Deletion of *ku70* in DS17690	Snoek et al. [2009]
DS54555	Deletion of penicillin gene clusters in DS17690	Harris et al. [2009]
AL4	Overexpression of LaeA in DS54555	This study
AL6	Overexpression of LaeA in DS54555	This study
DL4	Overexpression of LaeA in DS17690	This study
DS67261	Deletion of *laeA* in DS54465	Veiga et al. [2012]

pathway. Interestingly, in the industrial strain *P. chrysogenum* DS17690, most of the secondary metabolite gene clusters are silent or exhibit low expression levels. It appears that the CSI has led to the down-regulation of many of these genes. Overall, little is known on how secondary metabolite formation is regulated in such strains and what has been altered in the regulatory networks during the course of the CSI.

Here, we have examined the role of LaeA in *P. chrysogenum* DS17690, a high yielding β-lactam strain that harbors multiple copies of the β-lactam biosynthesis pathway. In addition, we have used a penicillin biosynthetic gene cluster-free derivative, strain DS54555 that has maintained the fermentation characteristics of the high yielding strain but in which amino acid precursor supply is no longer connected to β-lactam biosynthesis. Our data demonstrate that the LaeA regulatory network in these strains is functional. Despite a marked increase of the expression levels of the β-lactam biosynthesis genes upon the overexpression of *laeA*, only a minor increase in β-lactam production was achieved. Moreover, we provide evidence of transcriptional control of LaeA over various secondary metabolite gene clusters, including the formation of distinct compounds.

2.2 Materials and Methods

2.2.1 Fungal Stains, Media and Culture Conditions

Penicillium chrysogenum strain DS17690, its derivative strain DS54555, which lacks the amplified β-lactam gene cluster, and strain DS54465 Δ*ku70* (Snoek et al. [2009]) were kindly provided by DSM Biotechnology Center (Table 2.1).

Spores were inoculated in YGG-medium containing: 134 mM KCl, 100 mM glucose, 7.1 mM citric acid, 34.4 mM K_2HPO_4, and Yeast Extract YeastNitrogenBase (YNB). Cells were grown for 24 hrs in a shaking incubator at 220 rpm and 25°C. At day 0, the mycelium was diluted 8-times in Penicillin V production medium (PPM)

containing: 25 mM Glucose. 208.1 mM Lactose, 66.6 mM Urea, 28.2 mM Na_2SO_4, 64.9 mM CH_3COONH_4, 12.2 mM K_2HPO_4 and 37.5 mM KH_2PO_4 and trace elements. The pH was adjusted to pH 6.3. Where indicated, the medium was supplemented with 16.4 mM phenoxyacetic acid (POA) or 18.4 mM phenylacetic acid (PAA). Cells were grown for 120 hrs in a shaking incubator at 220 rpm and 25°C.

2.2.2 Vectors and Cloning Procedures

Genetic constructs were prepared using the Multisite Gateway system (Invitrogen). All oligonucleotides used in this cloning procedure are listed in Suppl. Tab. S2.1. The *laeA* overexpression cassette was constructed as follow: BP-recombinase reactions with the promoter of the *pcbAB* gene of *P. chrysogenum* as a left (L-) flank was cloned into pDONRP4-P1R, the *laeA* gene was cloned into pDONR221, and the terminator of the *penDE* gene of *P. chrysogenum* was cloned into pDONRP2R-P3 as described (Kovalchuk et al. [2012]). Both flanking regions and the *laeA* gene were recombined by a single LR-clonase reaction into pDEST-R4R3-amds with the additional incorporation of the acetamidase-gene (*amdS*) gene as a selection marker under control of the *gpdA* promotor of *A. nidulans*. The *amdS* gene was introduced into pDEST-R4R3 as follow: A fragment of 666 base pairs was amplified between the EcoRI and NdeI sites of the pDest43 with additional SpeI and KpnI sites in the reverse primer. The amplified fragment was replaced in the original pDest43 vector to introduce the additional SpeI and KpnI sites. The *amdS* gene with the *gpdA* promotor and AT terminator was cut from plasmid pBlue-amds with SpeI and KpnI and cloned into the modified pDEST-R4R3 yielding plasmid pDEST-R4R3-amds. After LR reaction the pDlaeAOE vector for the overexpression of *laeA* gene was obtained. The inactivation of the *laeA* gene (Pc16g14010) has been described elsewhere (Veiga et al. [2012]).

2.2.3 Transformation of *P. chrysogenum laeA* Overexpression and Deletion Vector

For the overexpression of *laeA*, protoplasts of *P. chrysogenum* DS17690 were isolated (Alvarez et al. [1987]) and cells were transformed with the pDlaeAOE vector. This resulted in the random integration of three additional copies of the *laeA* gene into the genome. The same vector pDlaeAOE was linearized with the restriction enzyme Bsp68I (NruI) and used for overexpression of *laeA* in DS54555 strain. For the deletion of the *laeA* gene, the pDest43-KO LaeA was digested with HindIII and purified with the High Pure PCR Purification Kit from Roche. The fragment was transformed into protoplasts isolated from *P. chrysogenum* DS54465. Transformants were selected on plates with acetamide as sole nitrogen source (Kolar et al. [1988]).

2.2.4 Southern Blotting and DIG Detection

Southern blotting was performed by standard methods (Southern [1975]). Genomic DNA isolated from wild type and transformant mycelium was digested overnight with Hind III. The digestion mixture was concentrated (Concentrator plus, Eppendorf) and loaded on an agarose gel. A hybridization probe for *laeA* was prepared by PCR with Phusion using the primers listed in Suppl. Tab. S2.1, yielding probes with a size of 955 base pairs. The probe was denaturated and labeled with the DIG-High Prime Probe (Roche). Hybridization was done by standard techniques and detection was carried out by a Luminescent Image Analyzer (LAS 4000, Fujifilm) after the addition of CDP-Star as a chemiluminescene substrate.

2.2.5 Expression Analyses by qPCR

Genomic DNA was isolated after 96 hours of growth of the indicated *P. chrysogenum* strains in penicillin producing medium using a modified Yeast genomic DNA isolation protocol (Harju et al. [2004]). The fungal mycelium was broken in a Fastprep FP120 (Qbiogene). Isolated gDNA was measured with a Nanodrop ND-1000 (Thermo Scientific) and set at a concentration of 40 ng per qPCR reaction of 25 μl. Total RNA of the transformants was isolated after 168 hours of growth in penicillin producing medium using TRIzol® (Invitrogen) with additional DNAse treatment by the Turbo DNA-free kit (Ambion). Total RNA was measured in a Nanodrop ND-1000 and set at a concentration of 500 ng per cDNA reaction. cDNA was synthesized using iScript™ cDNA synthesis kit (Bio Rad) in 10 μl end volume. The primers used for the qPCR reaction are listed in the Suppl. Tab. S2.1 and designed around introns in order to enable the separate amplification on gDNA and cDNA. Expression levels were analyzed in duple using the Miniopticon™ system (Bio Rad) and Bio Rad CFX manager software. The SensiMix™ SYBR mix (Bioline) was used as a master mix for qPCR with 0.4 μM primers. The program was: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Subsequently a melting curve was performed to determine the specificity of the qPCR reaction. γ-*actin* (*actA*) was used for normalization.

Gene Copy Number Determination

The number of copies of the *laeA* and *pcbAB*, *pcbC* genes was determined by qPCR as described by (Nijland et al. [2010]).

2.2.6 Protein Analysis

Cell free extracts of *P. chrysogenum* were isolated as follows: After 144 hours of growth in penicillin producing medium, 1 ml culture was mixed with 1 ml 25%

trichloroacetic acid (TCA) and frozen at $-20°C$. After defrosting, the cells were pelleted by centrifugation (10.000 g for 10 min) and washed twice with 80% acetone. The pellets were air-dried overnight and solubilized in 250 μl buffer containing 1% SDS and 0.1 M NaOH. Subsequently, 50 μl of 5×SDS-PAGE loading buffer was added. Samples were boiled for 5 min and centrifuged for 10 min at 10.000 g. The protein concentration was determined by the RC DC Protein Assay (BioRad) and samples were normalized after Coomassie Blue staining of SDS-PAGE. Samples containing an equal amount of protein were loaded to SDS-PAGE gel and analyzed by Western blotting using polyclonal antibodies against IPNS and AT as indicated.

2.2.7 HPLC-UV Analysis of PAA, POA, Penicillin V and G

P. chrysogenum strains were grown for 168 hours and samples were collected every 24 hours. The cultures ware supplemented with an additional amount of side chain precursor 20 μg after 120 hrs. The extracellular titers of PAA, POA, penicillin V and G were determined by high-pressure liquid chromatography (HPLC-UV) using an isocratic flow of acetonitrile at 245 g/l, 640 mg/l KH_2PO_4, and 340 mg/l H_3PO_4. Separation was performed on Shim-pack XR-ODS C18 column (3.0 × 75 mm, 2.2 μm, Shimadzu) (Japan) at a flow rate of 0.5 ml/min at 40°C. Chromatograms were acquired at a wavelength of 254 nm (Harris et al. [2006]).

2.2.8 Comparative Metabolite Profiling

A. Sample Preparation

The three groups of LaeA altered strains and their corresponding wild type strains: [AL6 (OE::PcLaeA DS54555) and WT (DS54555)] ten replicates of each, and seven replicates of each [DL4 (OE::PcLaeA DS17690) and WT (DS17690)] and [DS67261 (PclaeAΔ DS54465) and WT (DS54465)] were grown according to the procedure described in Sec. 2.2.1. To 50 μl of thawed fermentation broth 230 μl of methanol was added for protein precipitation and vortexed for 10 min. The sample was then centrifuged at 14,000 g for 10 min at 10°C. Supernatant (100 μ) was transferred to an Eppendorf vial and evaporated for 30 min in a Thermo-Speedvac (Thermo Scientific, San Jose, CA). The dried sample was resolved in 100 μl water containing 2% acetonitrile, vortexed for 10 min and transferred to an autosampler vial.

B. HPLC-MS Analysis

For separation, a Thermo Scientific Accela 1250 pump coupled to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) was used. A sample of 1 μL was injected onto a reversed-phase Shim-pack XR-ODS C18 column (3.0 × 75 mm, 2.2 μm) (Shimadzu, Japan). The elution was performed starting with 95% of solvent

A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) for 5 min at a flow rate of 500 μl/min followed by two linear gradients. The first gradient reached 60% B at 30 minutes, the second 95% B at 35 minutes. The column was flushed for 10 minutes at 95% B followed by equilibration for 15 minutes at 5% B. The column effluent was directed to the ESI-Orbitrap MS, operated in full scan (*m/z* 150-2000) in pos/neg switching mode with following settings: Positive ion mode (4.2 kV source voltage, 88 V capillary voltage, 120 V tube lens), negative ion mode (3 kV source voltage, -86.5 V capillary voltage, -172.5 V tube lens) with capillary temperature 275°C, sheath gas flow 60 and auxiliary gas flow 5.

C. Data Processing

For differential analysis of LC-MS data Sieve software (Thermo Scientific, San Jose, CA) was used with following settings: 0.02 Daltons for *m/z* isolation width, 2.5 minutes for the retention time width and $480\ 000$ counts intensity threshold. From differential analysis obtained features were reintegrated using LCQuan (Thermo Scientific, San Jose, CA). Identified metabolites from the roquefortine/meleagrin and chrysogine pathway were compared to their standards.

2.3 Results

2.3.1 Sequencing and Expression of the *laeA* Gene in the High β-lactam Yielding *P. chrysogenum* Strain DS17690

P. chrysogenum strain DS17690 was derived from the Wisconsin54-1255 strain by classical strain improvement Newbert et al. [1997]), which ultimately yielded a high β-lactam producing strain. To examine the role of LaeA in this industrial strain, the *laeA* gene was first cloned by PCR and sequenced. The sequence analysis revealed a single mutation (G1068A) in the *laeA* gene of strain DS17690. It generates a single amino acid substitution Gly338Ser. Previous transcriptome analysis revealed that the expression of *laeA* is identical in the DS17690 and Wis54-1255 strain (Berg et al. [2008]). This suggests that no major alterations in the *laeA* gene occurred during the classical strain improvement (CSI) program that affected expression, although the mutation may affect the properties of LaeA. Therefore, for the overexpression experiments, the mutation was corrected by mutagenesis (Table 2.1) and restored to the sequence of the Wis54-1255 strain.

There already was a single mutation (C850T) in the *laeA* sequence of the NRRL strain, which resulted in an amino acid substitution Glu284Lys (O. Salo, unpublished data). This amino acid replacement does not influence the activity of LaeA, as described for the 2PniaD18 strain (Hoff et al. [2008]; Hoff et al. [2010a]; Kopke et al. [2013]).

Apart from LaeA, the VeA protein of the velvet complex was also affected by a mutation (C943T), which generated a stop codon (Gln315.) in the DS17690 strain (O. Salo, unpublished data).

2.3.2 Overexpression and Inactivation of the *laeA* Gene in *P. chrysogenum*

Strain DS17690 was transformed with the pDlaeAOE vector for overexpression of the *laeA* gene under control of the *ipns* promoter (Figure 2.1A). This resulted in the random integration of the *laeA* gene into the *P. chrysogenum* genome. Positive clones were selected by growth on plates with acetamide as sole nitrogen source. qPCR on gDNA was employed to determine the number of *laeA* gene copies in the transformants. Transformant DL4 contained three additional copies of *laeA* gene (Suppl. Fig. S2.1). qPCR analysis on the RNA isolated from cells grown for 7 days on penicillin production medium (PPM), indicated a 20-fold higher expression of *laeA* gene as compared to the parental strain (Figure 2.1C).

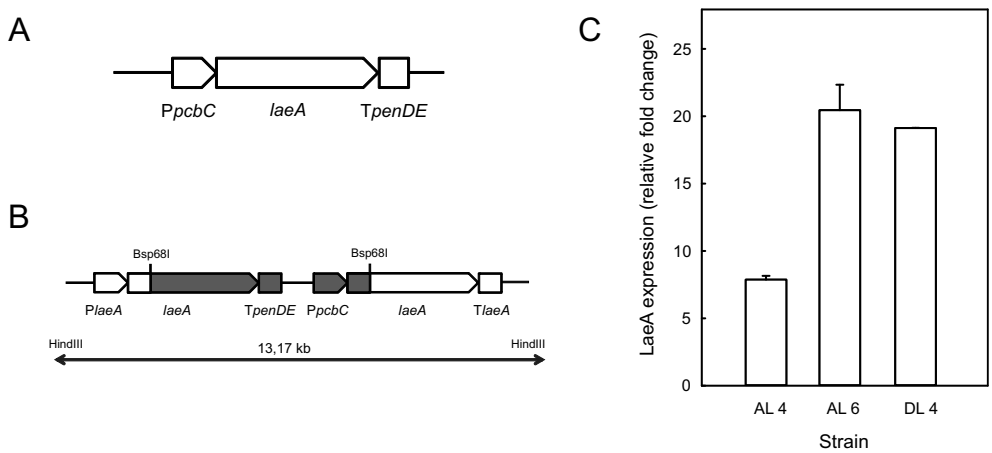

Figure 2.1: Overexpression of LaeA in *P. chrysogenum*. **A)** Scheme of over-expression cassette of the *laeA* gene. The *pcbC* promoter and *penDE* terminator were used for overexpression of *laeA*. The pDlaeAOE was transformed to DS17690 and DS54555 strains of *P. chrysogenum*. **B)** Insertion of additional *laeA* gene copies under *pcbC* promoter via single cross-over recombination in the DS54555 strain. **C)** Quantitative RT-PCR analysis of the expression of *laeA* in AL4, AL6 and DL4 strains. Transcript levels were analyzed after 7 days of growth in shaken flask cultures in the strains with additional copies of *laeA* under control of the *pcbC* promoter: AL4 (DS54555; 1 additional *laeA* copy), AL6 (DS54555; 2 additional copies of *laeA*) and in DL4 (DS17690; 3 additional copies of *laeA*).

Strain DS17690 was also used to obtain a *laeA* deletion mutant. For this pur-

pose, the derivative strain DS54465 was used in which the fungal homolog of human *ku70* gene involved in the non-homologous end-joining was inactivated by a gene deletion (Snoek et al. [2009]). The *laeA* deletion mutant was obtained as previously described (Veiga et al. [2012]). Negative transformants were further analyzed by Southern blotting showing the successful removal of *laeA* in strain DS54465 (Figure 2.2A).

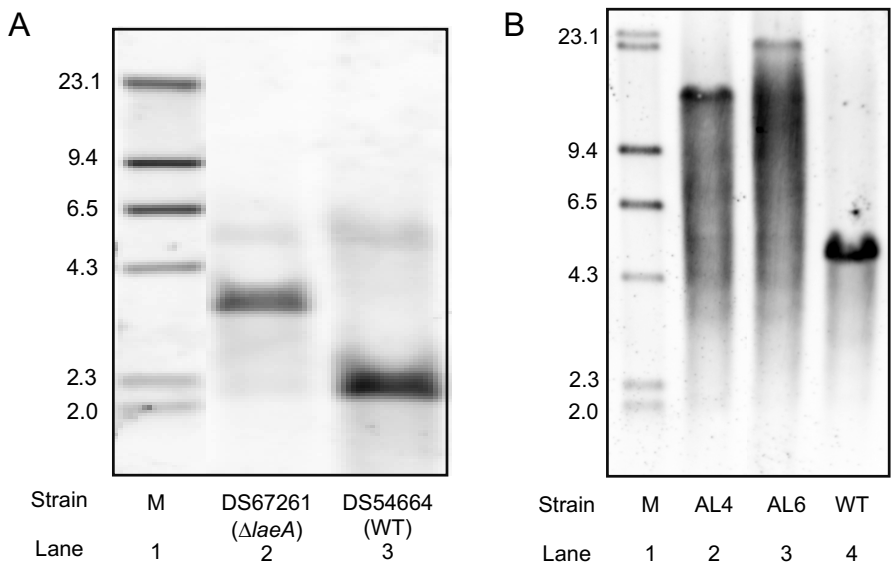

Figure 2.2: Southern blot analysis of the deletion and overexpression of *laeA*. A) Southern blot of the *laeA* deletion strain DS67261 and the parental DS54465 strain. The fragment of 5.6 kb confirms the presence of the deletion cassette. **B)** Southern blot of the *laeA* overproducing strains AL4 and AL6. Genomic DNA was digested by HindIII, yielding a fragment of 4.7 kb in wild type strain DS54555, and a 13.17 kb fragment for transformant AL4. A 26 kb fragment was obtained for AL6, in which the overexpression cassette was double inserted.

The *laeA* gene was also overexpressed in the penicillin gene cluster free strain DS54555 in order to examine its effect on secondary metabolite gene clusters in general, i.e., without interference the penicillin biosynthetic enzymes that may present a major drain on precursor supply. The DS54555 strain lacks the *ku70* gene and was transformed with the Bsp68I linearized pDlaeAOE vector that integrated into the genome via homologous recombination (Figure 2.1B). The recombination by single cross-over with the *laeA* gene resulted in two LaeA overexpression strains: AL4 and AL6. The integration of the recombination cassette by a single cross-over was confirmed by Southern blot analysis for AL4 transformant yielding the expected 13.2 kb fragment (Figure 2.2). The Southern blot results for AL4 corresponded to an expected *laeA* gene copy number of 2 (Suppl. Fig. S2.1). In the case of the transformant

AL6, a double integration must have occurred as the Southern blot yielded a DNA fragment of 23 kb (Figure 2.2B), which indicated the presence of three copies of the *laeA* gene (Suppl. Fig. S2.1). The transcript level of *laeA* after 7 days of growth was increased 8-fold in strain AL4, and 20-fold in strain AL6 as compared to the parental strain (Figure 2.1C). The resultant strains, in which the *laeA* gene was overexpressed or inactivated were used for further analysis in the remainder of this study.

2.3.3 Expression of Secondary Metabolite Genes in *P. chrysogenum* Strains Harboring Additional Copies of the *laeA* Gene

Analysis of *P. chrysogenum* Wisconsin54-1255 genome using the SMURF software led to the identification of 33 putative secondary metabolism genes (Berg et al. [2008]). The expression of these genes was examined by qPCR in the LaeA overexpressing strains that were derived from the penicillin cluster-free strain DS54555. Herein, primers were designed for 10 nonribosomal peptide synthetases (NRPS), 20 polyketide synthases (PKS) and 2 hybrid NRPS/PKSs (Suppl. Tab. S2.1). In the AL6 strain, two of the PKS genes, i.e., PKS12 (Pc21g05070) and PKS13 (Pc21g05080) were upregulated by 8 and 26-fold, respectively (Figure 2.3A). These genes were also upregulated in the AL4 strain (data not shown) but not in transformant DL4 (Figure 2.3B), whereas the Δ*laeA* strain only marginal changes in expression occurred (Figure 2.3C) in line with previous observations (Veiga et al. [2012]).

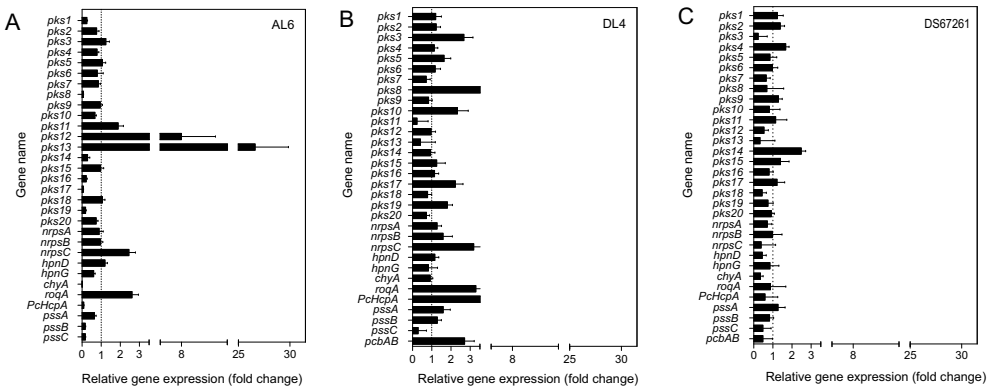

Figure 2.3: Effect of *laeA* overexpression and deletion on the expression of secondary metabolite genes. A) LaeA overproducing strain AL6 relative to the wild type DS54555. **B)** LaeA overproducing strain DL4 relative to the wild type DS17690. **C)** *LaeA* deletion strain DS67261 relative to the wild type. Data shown are duplicates obtained from two biological duplicates. The bars represent the fold change of transcript level relative to corresponding wild type. Error bars represent standard error of the mean. The samples were collected after 7 day of growth. Gene names correspond to the gene numbers listed in Suppl. Tab. S2.1

The highly expressed Pc21g15480 (RoqA) gene that has been linked to the production of roquefortine (Ali et al. [2013]) was up-regulated in the both AL6 and DL4 (Figure 2.3A,B). Examination of the expression of selected genes that belong to the roquefortine biosynthesis cluster showed that they are all up-regulated (Suppl. Fig. S2.2). Overall, the effects of LaeA overexpression were much weaker in the strain that still contained the penicillin biosynthetic gene cluster as compared to the cluster free strain.

Next the effect of LaeA overexpression was examined on the penicillin biosynthesis cluster genes (pcbAB, *pcbC*, *penDE*). The *pcbAB* and *penDE* transcript levels in strain DL4 increased up to 7-fold when cultures were supplied with the penicillin V precursor POA (Figure 2.4A). The LaeA regulation effect was smaller under penicillin producing condition (Figure 2.4A). Importantly, the expression of the isopenicillin N synthase gene *pcbC* was the least affected. In tested conditions the expression of *penDE* was slightly lower and the other β-lactam biosynthetic genes remained unaltered in LaeA deletion strain (Figure 2.4B).

2.3.4 Overexpression of LaeA Affects Metabolites Formation

The culture supernatant fractions of the LaeA overproducing and deletion strains were analyzed for metabolite levels and compared with the parental strains. Differential analysis of the HPLC-MS data using statistical t-tests showed that the concentration of several compounds in the supernatant fraction of the LaeA overexpressing strain AL6 had changed (Table 2.2). These compounds belong to three major groups. The most striking compounds (PcL1-PcL8) with a 10 to 26 increase in concentration had similar chemical formulas and might thus very likely originate from the same biological pathway. Further structure elucidation to confirm their identity is currently on-going. Other notable features are that metabolites from the chrysogine pathway that are produced at a slightly lower level, while the concentration of metabolites in meleagrin biosynthesis varied with only for roquefortine C a marked increased (Suppl. Fig. S2.3A, Table 2.2). Similar results on the roquefortine and chrysogine related metabolites were obtained with strain DL4 (Suppl. Fig. S2.3B) while the *laeA* deletion strain DS54555 showed in particular a somewhat shifted pattern of roquefortine-related metabolites (Suppl. Fig. S2.3C), except that the novel compounds mentioned before could not be detected (data not shown). Notably, the LaeA overproducing strain AL6 showed a distinct pigment formation (Suppl. Fig. S2.4) absent in the wild type strain.

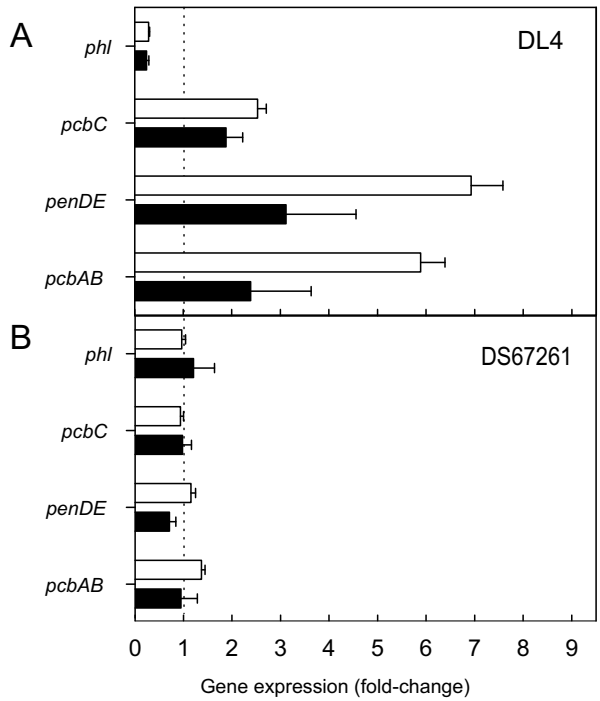

Figure 2.4: Effect of LaeA overexpression and deletion on the expression of penicillin biosynthesis cluster genes and the *phl* gene. A) LaeA overproducting strain DL4 relative to the wild type DS17690. **B)** *LaeA* deletion strain DS67261 relative to the wild type. Transcript levels of *pcbAB*, *penDE*, *pcbC*, and *phl* were measured after 7 days of growth in shaking flasks in the presence and absence of the phenoxyacetic acid. Data shown are duplicates obtained from two biological duplicates. The bars represent the fold change of transcript level relative to corresponding wild type. Error bars represent standard error of the mean.

2.3.5 Effect of *laeA* Deletion and Overexpression on β-lactam Formation in the High β-lactam Yielding *P. chrysogenum* Strain

Overexpression of LaeA in the high yielding *P. chrysogenum* DS17690 strain (DL4) resulted only in a modest 30% increase penicillin G production (Figure 2.5A), but the production of penicillin V remained unaltered. The protein levels of two of the key enzymes of penicillin biosynthesis, i.e., IPNS and AT were determined by Western blotting (Figure 2.5B). The IPNS protein levels did not differ between DS17690 and the derived DL4 strain irrespective of the presence of PAA or POA in the growth medium. In contrast, the AT protein level was approximately 30% higher in DL4 mutant (Figure 2.5B). In the case of the *laeA* deletion strain DS67261 no changes in

Table 2.2: Metabolites of *P. chrysogenum* DS17690 derivatives characterized in this study by LC-MS/MS.

No.	Name	Pathway	Formula $[MH]^+$	Retention time [min]	Fold change [a]	$[MH]^+$ *m/z* calculated	Accuracy [ppm]
1	PcL1	unknown	C14H21N2O4	5.9	26.5	281.1496	13.29
2	PcL2	unknown	C11H14N3O2	10.2	12.2	220.1081	14.07
3	PcL3	unknown	C14H21N2O3	13.6	7.6	265.1547	13.43
4	PcL4	unknown	C14H21N2O3	14.0	15.7	265.1547	12.64
5	PcL5	unknown	C18H24N3O4	20.5	16.0	346.1761	12.73
6	PcL6	unknown	C16H23N2O4	20.8	18.9	307.1652	12.20
7	PcL7	unknown	C17H25N2O4	23.3	13.6	321.1809	13.10
8	PcL8	unknown	C28H39N4O6	19.0	4.5	527.2864	12.55
9		chrysogine	C10H11N2O3	9.2	-2.3	207.0764	14.01
10		chrysogine	C12H16N3O3	11.6	-2.1	250.1186	13.44
11		chrysogine	C13H16N3O5	11.0	-2.0	294.1085	11.98
12	chrysogine	chrysogine	C10H11N2O2	12.9	-1.2	191.0815	12.80
13	HTD	meleagrin	C17H18N5O2	3.6	2.7	324.1455	11.60
14	DHTD	meleagrin	C17H16N5O2	5.1	2.1	324.1299	11.85
15	glandicoline A	meleagrin	C22H22N5O3	15.7	-1.2	404.1717	10.67
16	neoxaline	meleagrin	C23H26N5O4	16.8	1.0	436.1979	12.61
17	glandicoline B	meleagrin	C22H22N5O4	17.0	1.5	420.1666	12.57
18	roquefortine D	meleagrin	C22H26N5O2	16.9	3.8	392.2081	12.13
19	meleagrin	meleagrin	C23H24N5O4	18.2	1.0	434.1823	10.20
20	roquefortine C	meleagrin	C22H24N5O2	20.1	1.1	390.1925	12.89
21	roquefortine F	meleagrin	C23H26N5O3	21.5	-1.0	420.2030	12.31

[a]The fold change was calculated from the arithmetic mean values of the concentration of compounds present in the supernatant fraction of the LaeA overexpression strain AL6 compared to the parental strain DS54555. A positive value indicates a higher concentration in over-expression while a negative value indicates a lower concentration compared to host strain. The PcL1-PcL8 compounds have similar chemical formulas but unknown identity. The compounds numbered 9-12 belongs to chrysogine pathway and those numbered 13-21 are intermediates in biosynthesis of meleagrin. HTD, histidyltryptophanyldiketopiperazine; DHTD, dehydrohistidyl-tryptophanyldiketopiperazine.

penicillin titer were observed compared to parental strain. These results show that although LaeA overexpression has a marked elevating effect on the transcriptional levels of some of the penicillin biosynthetic genes, this is not accompanied with a major increase in β-lactam production.

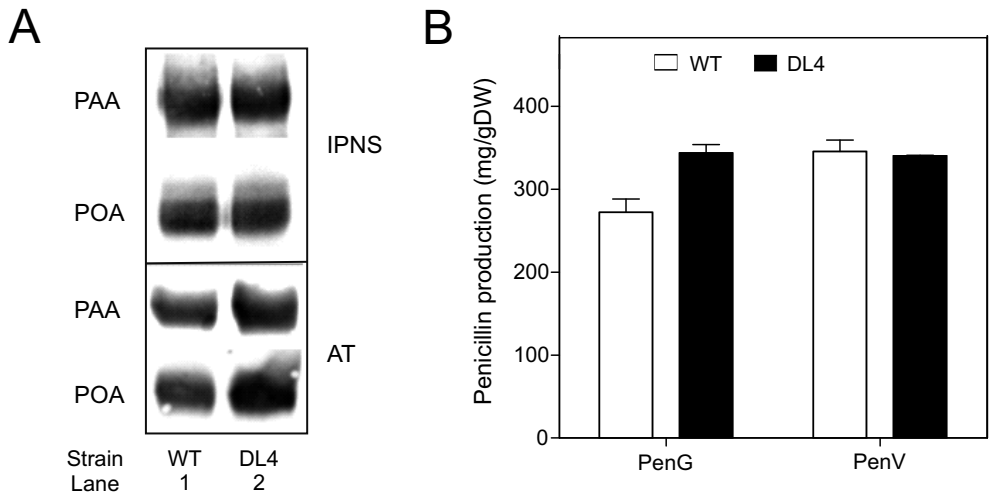

Figure 2.5: Effect of LaeA overexpression on β-lactam production and the IPNS and AT protein levels. A) HPLC analysis of penicillin G, penicillin V, PAA and POA levels in the culture broth of the DL4 and DS17690 strains grown for 7 days in shaking flasks. Error bars represent the standard deviation from two biological replicates. **B)** Protein levels of IPNS and AT in DL4 and DS17690 mycellium as determined by Western blotting and immunostaining using specific polyclonal antibodies.

2.3.6 Expression Levels of CoA Ligases Genes upon LaeA Overexpression.

The last step of penicillin biosynthesis requires the activation of side-chain precursors PAA or POA into a CoA derivative (Lamas-Maceiras et al. [2006]). This process occurs in microbodies and CoA ligases involved in this process typically contain a PTS1 targeting sequence. Gene inactivation studies indicated that the *phl* gene in a major contributor to penicillin G production (Lamas-Maceiras et al. [2006]). In addition, *P. chrysogenum* contains 7 proteins with high similarity to fatty acid CoA ligases and strong similarity to Pcl, of which 6 are equipped with a PTS1 peroxisome targeting sequence (Koetsier et al. [2009]; Koetsier et al. [2010]). Three of these genes, i.e. *aclA* (Pc22g20270), *acs2* (Pc13g12270), and *phl* (Pc22g14900) are expressed in the Wis54-1255 and DS17690 strains when grown in a glucose-limited chemostat under penicillin production conditions (Berg et al. [2008]). When tested in shaken flask cul-

tures, expression of *aclA*, *acs2*, *phl*, and to a lesser degree Pc21g20650 and Pc22g24780 responded to the presence of PAA or POA in the medium (Suppl. Fig. S2.5A, B). Only the *phl* and aclA genes were slightly up-regulated in the LaeA overexpression DL4 strain (Suppl. Fig. S2.5B), but this minor fold change suggests that they are not under the direct control of LaeA.

2.4 Discussion

Here, we have analyzed the role of the transcriptional regulator LaeA on the expression of secondary metabolite genes in an industrial strain of *Penicillium chrysogenum*. The data indicate that the overexpression of LaeA results in the up- and down-regulation of a number of distinct secondary metabolite gene clusters amongst which several the penicillin biosynthetic genes. Analysis of LaeA sequence in the *P. chrysogenum* DS17690 strain revealed a point mutation when compared to Wis54-1255, which seems to have occurred during conventional strain improvement programs (Newbert et al. [1997]). To minimize the effect of this mutation, for the LaeA overproduction, the mutation was corrected by mutagenesis.

2.4.1 Control Secondary Metabolites Formation by LaeA

The *laeA* gene was shown to encode a key protein required for the expression of genes of secondary metabolite formation in *A. nidulans* and controls for instance the biosynthesis of sterigmatocystin (Bok and Keller [2004]), kojic acid (Oda et al. [2011]), terrequinone A (Bok et al. [2006]) and aflatoxin (Kale et al. [2008]). Overexpression of LaeA in the industrial *P. chrysogenum* DS54555 strain resulted in significantly altered expression profile of the secondary metabolite genes. Most notably, two putative polyketide synthetases PKS12 and PKS13 were strongly up-regulated by 7 and 25 fold, respectively. Analysis of the supernatant fraction by LC-MS revealed eight compounds with unknown identity, whose concentration was more than 10-fold higher than in the parental strain. Possibly, these compounds cause the pigmentation observed in the LaeA overproducing strain. In contrast, in the DS17690 strain, PKS12/13 was expressed at only low levels (Figure 2.3B) and the aforementioned unknown compounds could be detected only in very low amounts (data not shown). This strongly suggests that PKS12/13 are responsible for the formation these unknown compounds. The structures of these compounds are currently under investigation. In addition, LaeA overproduction resulted in increased levels of roquefortin-related metabolites and decreased levels of chrysogine-related compounds. However, no regulation of roquefortine was found in LaeA deletion mutant in Wisconsin54-1255, where no changes could be observed in metabolite production (Kosalková et al. [2009]; García-Estrada et al. [2011]).

Remarkably, LaeA overexpression showed significant differences in the secondary metabolite gene expression profiles in the penicillin cluster free strain DS54555 and its parental strain DS17690. In the latter strain, several NRPS and PKS genes are up-regulated (2-5 fold), whereas in the DS54555 strain some genes are strongly down-regulated in addition to the strong up-regulation of PKS12/13. This suggests that the effect of LaeA is strain specific. LaeA contains S-adenosylmethionine-dependent (SAM), and the methyltransferase domains (MTD) (Bayram and Braus [2012]). The presence of these domains has led to speculations that LaeA is a methyltransferase involved in histone methylation thereby affecting the chromatin remodeling remodeling (Bok et al. [2009]; Strauss and Reyes-Dominguez [2011]). LaeA was shown to act through a network in conjunction with local transcription factors (Bok and Keller [2004]), or through sequences similar to the transposable elements required for efficient expression of the penicillin cluster (Shaaban et al. [2010]). The latter might be relevant for the LaeA overproducing strain. However, the precise epigenetic function of LaeA remains enigmatic. LaeA regulation of gene clusters was found to be location dependent (Palmer and Keller [2010]), though the detailed mechanism of its function is still unresolved. This may possibly explain the differences observed with LaeA overexpression in DS17690 high-producing strain, and the related penicillin cluster free strain DS54555. The DS17690 strain contains eight repeats of the penicillin gene clusters that likely have originated from recombination events (Fierro et al. [1995]; Nijland et al. [2010]a). The Non-Homologous End Joining (NHEJ) pathway present in DS17690 strain may have promoted the random integration of additional leaA copies into the genome at unknown loci. In the DS54555 strain, which is derived from DS54465 (Δ*hdf* DS17690), the two additional *laeA* copies recombined at the original *laeA* gene locus. Apparently, this can lead to a much stronger effect on the expression of various secondary metabolism genes.

2.4.2 Transcriptional Regulation of Penicillin Gene Cluster by LaeA.

In *A. nidulans*, LaeA significantly affected the expression of the penicillin gene cluster with a corresponding effect on antibiotic production (Bok and Keller [2004]). LaeA has a similar impact on β-lactam production in *P. chrysogenum* (Kosalková et al. [2009]; Hoff et al. [2010a]; Veiga et al. [2012]). Previously, it was shown that deletion of *laeA* causes more than 50% reduction of penicillin titer in the single penicillin biosynthetic cluster containing Wis54-1255 strain (Kosalková et al. [2009]) and its improved derivative P2niaD18 (Hoff et al. [2008]; Hoff et al. [2010a]). To examine the function of LaeA in the high industrial penicillin production strain DS17690 (and its ΔKU70 derivative DS54465), we have examined the expression level of penicillin biosynthetic genes upon *laeA* overexpression and deletion. In Δ*laeA* strain, the expression of the β-lactam biosynthetic genes was only marginally altered, which confirms earlier observations with chemostat grown cells (Veiga et al. [2012]). Lack

of transcriptional control between members of velvet complex (Veiga et al. [2012]) and the insignificant impact on the penicillin clustered genes expression shown under chemostat-relevant conditions (Veiga et al. [2012]), might indicate a potentially high extend velvet-independent penicillin biosynthesis. In contrast, overexpression of LaeA resulted in strong transcriptional response on the *penDE* (AT) and *pcbAB* (ACVS) genes, in particular when cells were grown in the presence of POA. The increased *penDE* transcript levels also resulted in higher AT protein but not to the extent as observed for the transcripts. The lack of a quantitative correlation between the AT transcript and protein levels was also noted previously (Nijland et al. [2010]). On the other hand, the transcript (and protein) level of *pcbC* (IPNS) was not elevated in the LaeA overproducing strain. Consistently, also only a small increase in penicillin G production (about 30%) was noted compared to DS17690, which matches observations in other *P. chrysogenum* strains (Kosalková et al. [2009]). . Moreover, the production of penicillin V even remained the same. To understand the latter discrepancy, the expression of the eight CoA-ligases was studied. Pcl is the main, but likely not the only CoA ligase involved in phenylacetic acid activation (Koetsier et al. [2009]). However, the expression of *phl* and the other characterized genes *aclA* and *acs2* was only marginally affected by the LaeA overproduction and it is uncertain if this can account for the small increase in penicillin G production.

Taken together, our data suggests that LaeA is involved in a regulatory network that remained operational in industrial strains of *P. chrysogenum* despite the classical strain improvement. Furthermore, although LaeA affects the transcription of the penicillin biosynthetic genes, it has little impact on production indicating that other LaeA-independent factor limit β-lactam production at high biosynthetic enzyme levels. The precise epigenetic function of LaeA in these strains remains enigmatic.

2.5 Supplemental Data

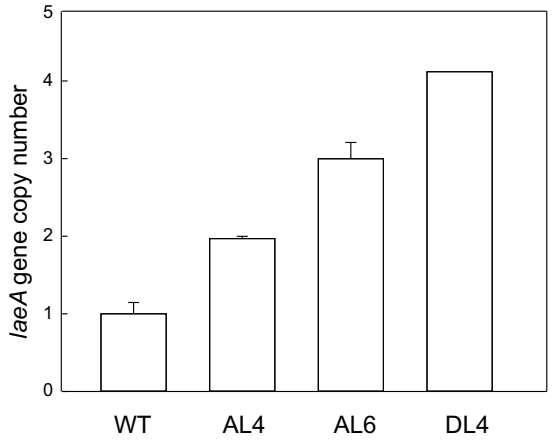

Figure S2.1: Gene copy number of *laeA* in the strains used in this study. The copy number of the *laeA* gene was determined by quantitative PCR on genomic DNA as described (Nijland et al. [2010]). Error bars presented standard deviation from duplicate samples.

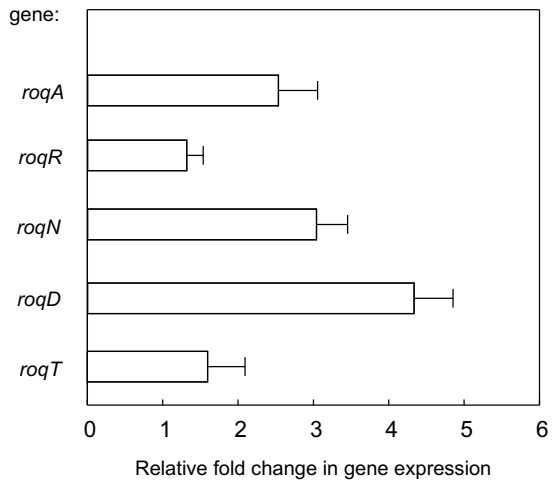

Figure S2.2: Effect of LaeA overexpression on the expression of selected genes in the meleagrin cluster. LaeA overproducing strain AL6 was analyzed relative to the wild type DS54555. Data shown are duplicates obtained from two biological duplicates. The bars represent the fold change of transcript level relative to corresponding wild type. Error bars represent standard error of the mean. The samples were collected after 7 day of growth.

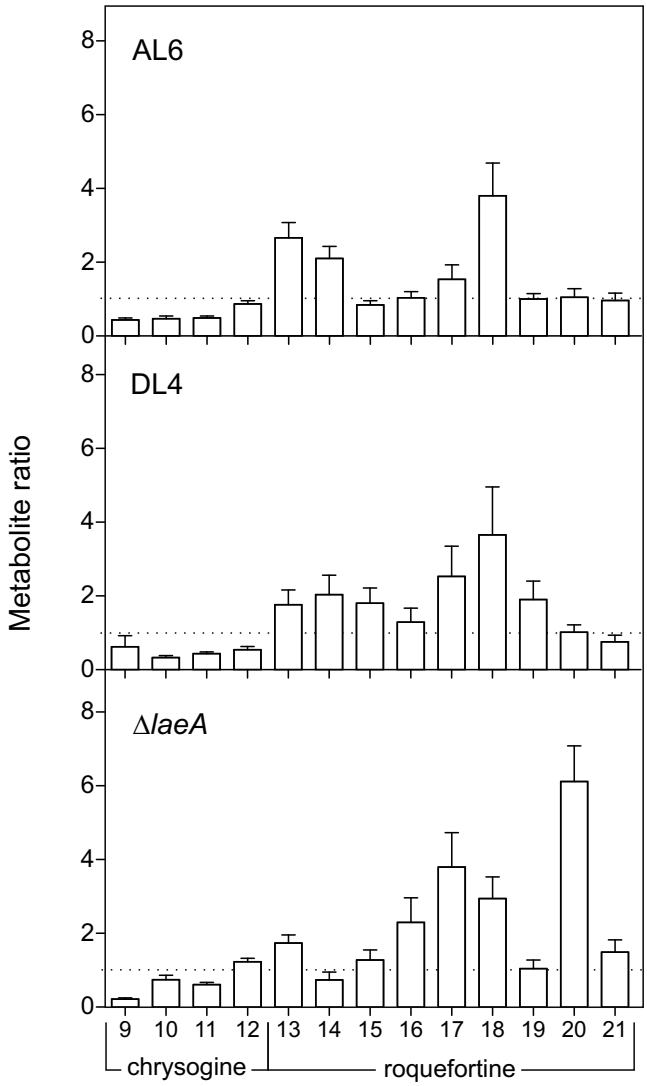

Figure S2.3: Effect of LaeA overexpression and deletion on the metabolite levels of the chrysogine and meleagrin pathways. A) LaeA overproducing strain AL6 relative to the wild type DS54555. **B)** LaeA overproducing strain DL4 relative to the wild type DS17690. **C)** *LaeA* deletion strain DS67261 relative to the wild type. Strains were cultivated for 7 days in shaken flask culture in three independent experiments. Ten biological replicates per strain were cultivated for the first group, and seven replicates per strain were cultivated for the second and third group. The metabolites are depicted as the ratio of abundance in the modified strain vs the abundance of the parental strain. The chrysogine compounds numbered 9-12 were slightly decreased in all analyzed groups. Intermediates of meleagrin biosynthesis numbered 13-21 were moderately increased.

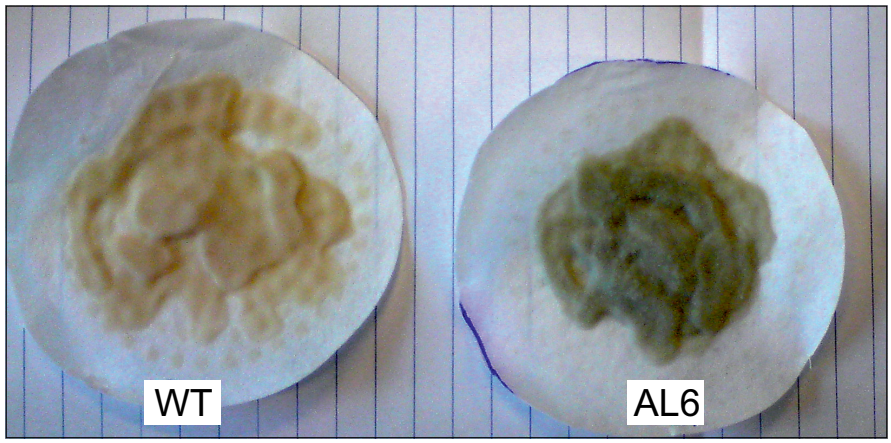

Figure S2.4: Effect of LaeA overexpression of pigment formation. The LaeA overproducing strain AL6 and wild type DS54555 were grown for 7 days in shaken flasks. The mycelium was filtered through a Büchner funnel with a sintered glass disc.

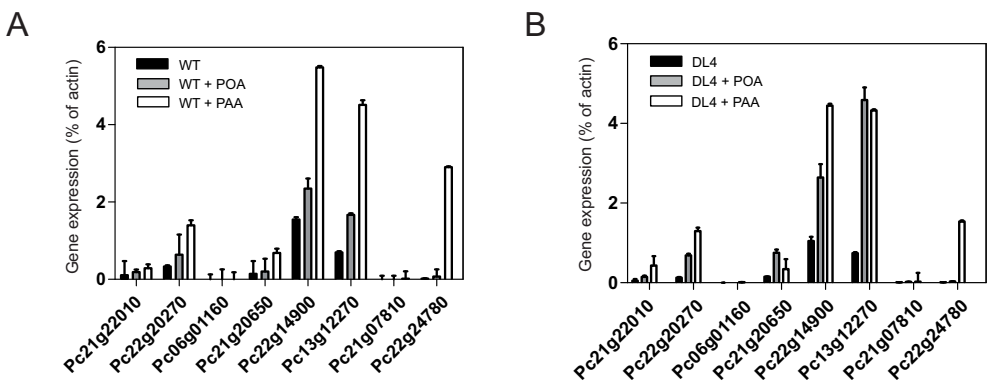

Figure S2.5: Effect of LaeA overexpression on the expression of putative acyl-CoA ligase genes. The expression levels of the indicated CoA ligase gene were determined for the LaeA overproducing strain DL4 and the wild type DS17690 in the absence or presence of POA or PAA in the culture medium. The bars represent the transcript levels were measured relative to actin (actin set as 100%). Data shown are duplicates obtained from two biological duplicates. Error bars represent standard deviation. The samples were collected after 7 day of growth.

Table S2.1: Oligo sequences used in quantitative PCR.

Oligo	gene number	Forward (5′ → 3′)	Reverse (5′ → 3′)
pks1	Pc12g05590	CGATATGCTGGACAAATTGTACGCCG	CTGGCTGTTTGCTTCTTTCTCCATATCG
pks2	Pc13g04470	CCGAAGATGCCGGCGACGG	CGCTGGTCTGCGATGTGGCC
pks3	Pc13g08690	CGAGAGACCAGGATAAGGTTCTTGGC	GGTGGTCTGTCACCACTCTTCCC
pks4	Pc16g00370	CATGGTCAGCACCCTCAGTGCC	CCAGGTCAGGCGTCGTACGC
pks5	Pc16g03800	CGGGTGCTGCATAGATGTACTACGC	GCTGGCCACGGAAGACAACGC
pks6	Pc16g04890	CATCCTATGTCAAGCCCGGTATTGTTG	GTGAGGTTTGGTTATGCGCTGAGAAGG
pks7	Pc16g11480	CACGATTTTAGCAAGTCAACCAGCGCG	CTCGCTCTCCCAGAATGTCAAGGC
pks8	Pc21g00960	GCCACACTCATCGGCACCACG	GCTCCACAGAGCAACCAACCCG
pks9	Pc21g03930	GACGTGGCCGGTGATGCCG	GCGATGTTGCGGACGAGGCC
pks10	Pc21g03990	CCAGGAGTTCCCAGGATACCCG	CGAAGGAATGGGCGTAACCTGCC
pks11	Pc21g04840	CTGTCCGCGATTGCCGATGACG	CGGAGTGCGGATATTCGTCAAGGC
pks12	Pc21g05070	GTCGGAGGCAATTCGGGAAGGC	GCAAAGTTCCACCACAATGCCGCG
pks13	Pc21g05080	CCGAGGATCTCCGCCAGGC	GGTTGTGCAGGTTCCAGGTGCC
pks14	Pc21g12440	GCACCACCATCAGCCAAAGCATACC	CCGAGGTCCATTGGAACTATGCGC
pks15	Pc21g12450	CCAGTTGTCTGCAGCCGGCC	GCCCAGATCACCGCCGTACG
pks16	Pc21g15160	CAGCCGCGTAGTTTGCCTGGC	GCACAGTGTGCTGAGGTTACGGC
pks17	Pc21g16000	CTTGTCATCAGCAGCCCAGAGG	CAATTTGCGGTGGCTGAGACGC
pks18	Pc22g08170	GGTTGATACTCCTGGGACTGAATACAG	GCTGCTGTGGATCCATCTGCTCG
pks19	Pc22g22850	CGGTCAACCAGGGATCCAACTGC	CTGAAGCGGTCTCTGTGTGGCC
pks20	Pc22g23750	CCAGGGTGACGTCTCCAAGATGG	CGTAGTTGGACTGCGAGATGTGGC
nrpsH	Pc21g12630	GAGCCAACTCTGTTGTCTACG	CAGGGCAATTTGCCTCATTCTG
roqA	Pc21g15480	CTTGGTGGATGCAGCGAAGG	CTGTGAGAGAGGCTCTTGAGTA
nrpsJ	Pc16g04690	CTTTCCAGAACAGTTGGCTGGT	GCTGCATCTTACCCAGGTAATTG
pssA	Pc16g03850	TGGTTGAAAGAGGGCAGTCTC	CGCGAACATACACAACACCAC
pssB	Pc22g20400	TTCGCGAACATCCGAAGAAGC	TCGGGCGAAGACACTGTTCA
pssC	Pc13g05250	GCAGACCTGTATCCATCGCAA	GGAGGCAAGTGAAGGTGTGTT
nrpsA	Pc21g01710	GCTATCTCGGTGGAGGATCTTCTGTCC	GTGCTGCTGAGAACACGGGATTGT
nrpsB	Pc13g14330	GCGACAGCCGCCGGAGTAACTATGG	GAGAGACGGGGACACGCGTGATG
nrpsC	Pc21g10790	GTGAGGCAGCTTTGTTCAACACCATT	TTCTGCAGCAGGCTGTCGGCCTGAG
hpn D	Pc16g13930	CCACCCTTGTTCAGCCGCTGAATTCC	GGACGAGGCGAACAACATCGGAC

Oligo	gene number	Forward (5′ → 3′)	Reverse (5′ → 3′)
hpnG	Pc14g00080	ACGTACGCTCGAGCTGGACT	GCCGTCGCGTTGATAATTGG
pcbC	Pc21g21380	AGGGTTACCTCGATATCGAGGCG	GTCGCCGTACGAGATTGGCCG
pcbAB	Pc21g21390	CACTTGACGTTGCGCACCGGTC	CTGGTGGGTGAGAACCTGACAG
penDE	Pc21g21370	CATCCTCTGTCAAGGCACTCC	CCATCTTTCCTCGATCACGC
phl	Pc22g14900	CACACGTCTTGAGTGCCCAGC	TGCTCTTGGTCTCCTTCCGCAG
acs2	Pc13g12270	CGCCCGCCGAGTTAGAGGCTC	TGGCGGTGGCGGATTGGCCG
aclA	Pc22g20270	CCCAGAAGTGGATCTTTGGACC	CTAGCGAGGCATCACGTAGCG
CoA-ligase 4	Pc21g22010	GTTACGGGGTTCCCTTGGTCG	AAGGTCCCTGTCCGCCAGGCA
CoA-ligase 5	Pc22g24780	CAGATTCCCGTAGACCGGCGA	GTCCTGCTTCCAACAAAGGACC
CoA-ligase 6	Pc06g01160	GATAGACTCCAAGTCGGCTGG	TCAAATGACGCGTTTATCGACG
CoA-ligase 7	Pc21g20650	GCCCCTGGACCGGCCTATGTA	GCCGAAGCCCGTTGGACAGAGAG
CoA-ligase 8	Pc21g07810	ACATAGCGTATGTCAAGCAGGGCCAG	GTGTTGGAGGAGGACGCCTTCAAC
actA	Pc20g11630	CTGGCGGTATCCACGTCACC	AGGCCAGAATGGATCCACCG
laeA	Pc16g14010	GTGCTATGGCTAACTGGTACTCG	TTTCGCGCTTGATAGATGTGCAG
pDONR221	Pc16g14010	GGGGACAAGTTTGTACAAAAAAGCAGG CTTGATGTCTTACCGAGAGTCATCTGG	GGGGACCACTTTGTACAAGAAAGCTGG GTTTCCTCGACTGGTTTTCGC
MUTpDlaeAOE	Pc16g14010	GAATTTGCAAGAAGCGGGCTTCACCGA GATTG	CAATCTCGGTGAAGCCCGCTTCTTGCAA ATTC
pDONR P4-P1R	att B4+F primer GW IPNS ipns strep attB1R	GGGGACAACTTTGTATAGAAAAGTTGTC CTTATACTGGGCCTGCTGCATTGG	GGGGACTGCTTTTTTGTACAAACTTGTTT TTTCGAACTGCGGGTGGCTCCACATGCG TCTAGAAAAATAATGGTGAAAACTTGA AGGC
pDEST with *amdS*	For R4R3 EcoR1 Rev R4R3 Nde1 + Spe/Kpn	GTCTTTCATTGCCATACGGAATTCCGGA	ATATCATATGACTAGTCCGCGGGGTACC CGGTGTGAAATACCGCACAGATGCGT
5′flank KO	attB4F5-LaeA HindIII attB1R4-1 LaeA	GGGGACAACTTTGTATAGAAAAGTTGA AGCTTCGATCTAGCTCGGAGTTCTGATT CG	GGGGACTGCTTTTTTGTACAAACTTGGC GTTCGAGGCGTGGGATGCCTG
3′flank KO	attB2F LaeA attB3R LaeA H3	GGGGACAGCTTTCTTGTACAAAGTGGGT GAAGCATAGCAATCGACCGCC	GGGGACAACTTTGTATAATAAAGTTGGT TGGTCTACAATCCGGCGTTGGG
DIG labeled probe	For and Rev 2 NruI	CCCATCATAATTTCCGACCGCAA	TCGCGCTTGATAGATGTGCAG
pDONR221 AMDS	attB1F AMDS attB2R AMDS	GGGGACAAGTTTGTACAAAAAAGCAGG CTCGCAGGAATTCGAGCTCTGTAC	GGGGACCACTTTGTACAAGAAAGCTGG GTCTCGCTCGTACCATGGGTTGAG

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