Two Components of a velvet-Like Complex Control Hyphal Morphogenesis, Conidiophore Development, and Penicillin Biosynthesis in Penicillium chrysogenum\textsuperscript{V}†

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Penicillium chrysogenum is the industrial producer of the antibiotic penicillin, whose biosynthetic regulation is barely understood. Here, we provide a functional analysis of two major homologues of the velvet complex in P. chrysogenum, which we have named \textit{P. chrysogenum} velA (PcvelA) and PclaeA. Data from array analysis using a ΔPcvelA deletion strain indicate a significant role of PcVeA on the expression of biosynthesis and developmental genes, including PclaeA. Northern hybridization and high-performance liquid chromatography quantifications of penicillin titers clearly show that both PcVeA and PclaeA play a major role in penicillin biosynthesis in a producer strain that underwent several rounds of UV mutagenesis during a strain improvement program. Both regulators are further involved in different developmental processes. While PcvelA deletion leads to light-independent conidial formation, dichotomous branching of hyphae, and pellet formation in shaking cultures, a ΔPclaeA strain shows a severe impairment in conidiophore formation under both light and dark conditions. Bimolecular fluorescence complementation assays provide evidence for a velvet-like complex in \textit{P. chrysogenum}, with structurally conserved components that have distinct developmental roles, illustrating the functional plasticity of these regulators in genera other than \textit{Aspergillus}.

Bacteria and fungi share a clustered chromosomal organization of genes encoding secondary metabolism (11, 34). Moreover, biosynthesis genes in prokaryotes and eukaryotes encoding identical products, such as \beta-lactam antibiotics, support the hypothesis that fungi have acquired these genes from bacteria through horizontal gene transfers (12, 62). Despite their highly similar structures, there are distinct differences in the modes of regulation of these biosynthetic pathways. In bacteria, pathway-specific regulators generally control expression of biosynthesis genes. Conversely, in fungi the coordinated regulation of gene clusters is controlled by a complex network of global regulators that act on different metabolic pathways (13, 27, 64, 70). A well-studied global regulator is Velvet (VeA), which was originally discovered in \textit{Aspergillus nidulans} as an inhibitor of light-dependent conidiation (36), and was later shown to affect the biosynthesis of a large number of metabolites (38, 65). In the meantime, studies of diverse filamentous ascomycetes have identified structural homologues of VeA with a regulatory role in secondary metabolism and development (7, 23–24, 45). Very recent, thorough genetic, molecular, and biochemical work has shown that Velvet is part of a high-molecular-weight complex containing at least 10 different proteins, some of which have been assigned distinct regulatory roles (6, 16, 26, 57). Another characterized component of this complex is LaeA, a second global regulator of secondary metabolism that was first discovered through complementation of an \textit{A. nidulans} mutant deficient in secondary metabolism (9). LaeA is supposed to have methyltransferase activity; deletion of the \textit{laeA} gene results in the loss of secondary metabolite production in many members of the aspergilli (2, 37, 46, 55, 68). Both VeA and LaeA are conserved fungal proteins that link secondary metabolism with fungal development. Despite their structural conservation, both regulators have a marked functional plasticity in different species, thus reflecting the diversity of fungal lifestyles within individual environments (16).

Recently, the genome sequence of \textit{Penicillium chrysogenum}, the main industrial producer of the pharmaceutically relevant \beta-lactam antibiotic penicillin, was published (71). This new resource provides many options for the investigation of conserved regulators of secondary metabolism, particularly in production strains. Penicillin biosynthesis is catalyzed by three enzymes, namely, 8-(L-α-aminoacidipyl)-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (IPNS), acylcoenzyme A (CoA):isopenicillin N acyltransferase (IAT) (reviewed in references 12 and 47), and the corresponding genes \textit{pchaB} (synonym, \textit{acvaA}), \textit{pchaC} (synonym, \textit{ipnaA}), and \textit{penDE} (synonym, \textit{auaA}) are found in a single cluster which is conserved in bacteria and other fungi such as \textit{A. nidulans} (48, 74, 76). So far, only a few factors controlling penicillin biosynthesis have been functionally characterized in \textit{P. chrysogenum}. Examples include the pH-dependent transcription factor PACC, the nitrogen-dependent factor NRE, and the CCAAT-box binding complex AnCF (reviewed in reference 12). Thus, our understanding of the network regulating penicillin biosynthesis is still
far from complete. For targeted strain improvement programs, however, a detailed knowledge of determinants regulating secondary metabolism is very important as it extends options to manipulate biotechnological processes for optimal production yields.

This study provides a detailed functional characterization of homologues of the global regulators VeA and LaeA from an industrial strain of the penicillin producer *P. chrysogenum*. The genes for both regulators were deleted to construct knockout strains, and rescue vectors containing the native genes were used to complement these knockouts. Functional studies of components from predicted *velvet*-like protein complexes are of major value since recent analysis of diverse ascomycetes has shown that homologous proteins (e.g., *aatB* and *ial* from the model and industrial species *A. nidulans* and *P. chrysogenum*, respectively) have very different roles in the biosynthesis of secondary metabolites (28, 66). So far, the roles of *VeA* and *LaeA* homologues have not been investigated in strains subjected to several rounds of strain improvement programs. Here, we provide the first extensive analysis of both regulators, including data from array hybridizations, high-performance liquid chromatography (HPLC) quantification of penicillin titers, and microscopic investigations. Our results clearly demonstrate that both global regulators have conserved as well as novel roles in secondary metabolism and morphogenesis.

**Materials and Methods**

Strains and culture conditions. *Escherichia coli* strain K-12 XL1-Blue was used for general plasmid construction and maintenance (14). Cloning and propagation of recombinant plasmids were performed using standard protocols (61). All *P. chrysogenum* strains used in this study are listed in Table 1. *P. chrysogenum* P2niaD18 (33) was previously received from the sequenced strain Wisconsin 54-1255 (71) and served as a recipient to construct the *Peca70* strain lacking the *Peca70* gene (31). All *P. chrysogenum* strains were cultivated in liquid complete culture medium (CCM), minimal medium (MM), and production medium at 25°C and 130 rpm or on solid medium as already described (30, 51). In all cases, fresh spores collected from 5-day-old cultures grown on CCM plates were used to inoculate shake flasks and static liquid medium. Conidiospores for quantification assays were collected from cultures grown for 168 h on solid medium. Transformation of *P. chrysogenum* and determination of penicillin titers were done as described recently (31).

Preparation of nucleic acids, hybridization protocols, quantitative real-time PCR, and PCRs were done as recently described (31). Quantification of autoradiograms was done with Scion Image.

**Identification of *P. chrysogenum* genes and sequence analysis.** The genomic sequence of *P. chrysogenum* ATCC 28898 (71) was the source of the sequences used in this study to identify Protein sequence similarity tools such as the NCBI BLAST program (69) and displayed using GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html). Conserved protein domains and motifs were predicted using the SUERFAMILY database (http://sufam.mrc-lmb.cam.ac.uk SUERFAMILY) (29), the PEST finder Internet tool (http://emb1.bcc.univie.ac.at/toolbar/pestdif/pestdif-analysis-webtool.htm), the computer program WoLF PSORT (35), and the NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES) (44).

**Construction of the ΔPclaeA knockout strain.** The sequences of all oligonucleotides and plasmids used in this study are listed in Tables S2 and S3 in the supplemental material. For construction of the *PclaeA* replacement vector pKOPcLAEA, 1,551 bp of the 5’ and 1,588 bp of the 3’ regions of the gene were amplified by PCR with primer pair PclaeA-5s and PclaeA-3a and the pair PclaeA-3s and PclaeA-3a, respectively, and the resulting amplicons were cloned into plasmid pDrive (Qiagen, Hilden, Germany). Subsequently, the SplI-BamHI 5’ fragment and the NheI 3’ fragment were ligated into the corresponding sites of the vector pD-Phleo. In this plasmid, expression of the phleomycin-resistance gene *ble* is controlled by the trpC promoter of *A. nidulans*. The resulting plasmid pKOPcLAEA (see Table S3) was then used as a template to amplify the *PclaeA* open reading frame under the control of the promoter and terminator of the *P. chrysogenum* 3-phosphoglycerate kinase gene *pgkA* and the *ateA* gene from *Aspergillus oryzae* as a selectable marker. To generate ΔPclaeA::PclaeA replacement strains, this plasmid was linearized by digestion with BamHI. Transformed *P. chrysogenum* and determination of penicillin titer were done as described recently (31).

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Fflael gene was fused to the N-terminal part (73). Both constructs were co-transformed with pPTRII (42), and the resulting retransformant ΔPcvelA:Ffvel1 and ΔPcvelA:Fflael strains were selected on minimal medium containing pyrithiamine, nourseothricin, and phleomycin. The presence of Ffvel1 or Fflael in the P. chrysogenum knockout mutants was confirmed by PCR experiments.

Construction of bimolecular fluorescence complementation (BiFC) plasmids. As described previously (32), vectors pEYFPN and pHYFPC were used for construction of recombinant plasmids carrying gene fusions encoding either the C or N terminus of enhanced yellow fluorescent protein (EYFP) together with the full-length regulatory proteins. Both vectors were further modified by replacement of the hph resistance cassette by the nat1 selectable marker gene (43). The resulting plasmids were named pEYFP-Nnat and pEYFPNat-c and used for further constructions. The sequences encoding the entire open reading frames of the full-length regulatory proteins. Both vectors were further modified by replacement of the NotI and NcoI sites in the homologues from Aspergillus nidulans with the NotI or NcoI sequences encoding the entire open reading frames of the full-length regulatory proteins. Both vectors were further modified by replacement of the NotI and NcoI sites. After subcloning, the NotI or NcoI amplified using the primers detailed in Table S2 in the supplemental material to introduce flanking NotI and NcoI sites. After subcloning, the NotI or NcoI fragments were ligated into the corresponding site of either pEYFPN-nat or pEYFPN-nat to generate the expression vectors described in Table S3. All recombinant plasmids carried the nat1 resistance gene and were used in pairs for the transformation of P2niaD18. Successful cotransformation was confirmed by PCR analysis and Southern hybridization. The resulting strains P2Bi-1 to P2Bi-3 are listed in Table 1. In addition, another four strains (P2Bi-4 to P2Bi-7) carrying only one recombinant plasmid and one strain producing only the two split EYFPs (P2Bi-8) were used as controls (Table 1).

Microscopy. Hyphal morphology at different growth stages was analyzed using a Zeiss Axioskop microscope (Zeiss, Jena, Germany). Differential interference contrast (DIC) images were captured with an Axiosvision digital imaging system including a Zeiss AxioCam camera. Fluorescence microscopic investigations were carried out with an Axiosimager microscope (Zeiss, Jena, Germany) using a XN XB075 Xe lamp for fluorescence excitation. Fluorescence was studied using Chroma filter set 41028 (Chroma Technology Corp., Rockingham, VT) for detection of EYFP. Calcofluor white and DAPI (4′,6′-diamidino-2-phenylindole) staining was observed using Chroma filter set 31000v2. Images were captured with a Photometric CoolSnap camera (Roper Scientific) and Chroma filter set 31000v2. Fluorescence microscopic investigations were inoculated on solid CCM for 3 days, and small pieces of the resulting colonies (0.5 × 1.0 cm) were cut out and placed on a nylon membrane. The samples were then fixed with 2% osmium tetroxide for 24 h and air dried for a further 24 h. The dried samples were mounted on metal stubs, coated with gold by using a scanning electron microscope (SEM) coating unit (E 51000 Polarion Equipment, Ltd.), and then examined using a Zeiss DSM 950 scanning electron microscope operating at 10 to 20 kV. All recorded pictures were processed with Adobe Photoshop CS2 software.

Microarray analysis. Detailed protocols for microarray sample preparation and hybridization have been described previously by Hoff et al. (31).

Microarray data accession number. The microarray data are available from the NCBI Gene Expression Omnibus (GEO) under the series accession number GSE18585.

RESULTS

Comparison of PcvelA to other Velvet homologues. The P. chrysogenum Velvet protein PcvelA exhibits an overall sequence identity of 45% with the well-characterized A. nidulans VeA protein and 55% identity to its homologue in Aspergillus fumigatus (data not shown). Comparison of the predicted amino acid sequence to sequences of other ascomycete homologues shows significant N-terminal conservation, indicating a specific function for this part of the polypeptide. Despite these high levels of sequence conservation, there are two notable differences between PcvelA and Velvet homologues from Aspergillus species, Neurospora crassa, or Acremonium chrysogenum (16): (i) PcvelA does not appear to comprise any predictable PEST motif, which is known to be responsible for protein degradation (39, 59), and (ii) no classical nuclear localization (NLS) or nuclear export signal (NES) can be predicted by in silico analysis for PcvelA. While the A. nidulans VeA protein contains a functional bipartite NLS in its N-terminal part and a predicted NES in the middle part of the protein sequence (67), clearly different localizations of predicted NLSs were described in the homologues from A. chrysogenum and N. crassa (7, 23).

Microarray analysis of the ΔPcvelA strain. In order to gain insight into the molecular changes associated with the deletion of PcvelA, we subjected the ΔPcvelA strain to extensive microarray analysis. For comparison we used microarray data from the Δpek70 strain, a high penicillin producer and the recipient of the ΔPcvelA strain (31). In a time course study RNA for array hybridization was isolated from cultures grown on solid medium for 48, 60, and 96 h, respectively. Surface cultures were used to both investigate genes specifically involved in conidioaphore formation and measure transcripts from biosynthesis genes. At this point, it is worth mentioning that, at the time points investigated, the ΔPcvelA mutant showed normal colony growth with biomass production comparable to that of the wild type, thus excluding the possibility that any observed effects are due to growth differences.

The expression of 858 genes, representing 6.6% of all nuclear P. chrysogenum genes, was determined to be downregulated in the ΔPcvelA strain when a 2-fold threshold was applied. Similarly, the expression of 913 genes, corresponding to 7% of the nuclear genome, was found to be upregulated at least one of the three time points (Fig. 1A). An exclusive differential expression was observed for the majority of genes after 96 h of growth, while a minority appears to be regulated at 60 h. Interestingly, a subset of 154 sequences exhibit lower expression levels across all time points, while only 47 genes can be found in the common subset of upregulated sequences at 48, 60, and 96 h (Fig. 1A). Because of the lack of a publicly accessible Penicillium or Aspergillus MIPS (Munich Information Center for Protein Sequences) classification, the Fusarium graminearum homologues of the P. chrysogenum gene products were determined using the BLASTX algorithm for functional characterization of the differentially expressed genes (1, 22). The F. graminearum sequences were then used for FunCat classification (Fig. 1B) (50, 60). For 858 P. chrysogenum sequences with reduced expression levels, 780 F. graminearum homologues were detected and categorized. Because of multifunctional proteins, at least 879 were assigned to distinct functional categories (Fig. 1B). Furthermore, from 913 upregulated transcripts, 897 homologous genes were found in F. graminearum and assigned to 926 different functions (Fig. 1B). The most-represented functional class comprises gene products involved in primary and secondary metabolism, with a total of 190 (21.6%) downregulated and 243 (26.2%) upregulated genes. About 2,300 genes (18%) of P. chrysogenum are thought to be involved in metabolism (71), and this rather high number of differentially expressed genes is strong evidence that PcvelA is a key regulator of metabolism in P. chrysogenum. With respect to secondary metabolism, 65 sequences (7%) are positively regulated, and 42 sequences (4.8%) are negatively regulated in the ΔPcvelA strain; thus, PcvelA appears to act both as an activator and repressor of secondary metabolism. The second-largest group consists of genes coding for components of the cellular transport machinery, with 90 (10.2%) downregulated and 127 (15.7%) upregulated transcripts. In contrast, only nine genes with predicted functions in growth and development were determined to be differentially ex-
pressed. However, 453 genes (51.5%) exhibiting reduced expression levels could not be assigned to any function, while 368 sequences (39.7%) showing elevated expression lack functional classification (Fig. 1B).

To gain further insight into the involvement of PcVelA in development and metabolism, we also performed a detailed analysis of the expression of genes coding for key components of both processes. Only genes which in a pairwise comparison between the ΔPcku70 and P2niaD18 recipient strain showed no deviant expression levels were considered. In agreement with the results of the FunCat analysis, we were able to identify only a few regulated sequences with known functions in conidiophore development. While PcbrlA (Pc06g00470), a homologue of the essential brlA gene for conidiospore development in A. nidulans (52), is not differentially expressed in the ΔPvelA strain, PcabA (Pc16g09610) and PphiA (Pc22g00190), two homologues of the A. nidulans developmental regulators abaA and phiA (49, 52), respectively, exhibit reduced expression levels in the mutant strain, in particular, after 96 h of growth (Fig. 2A; see also Table S1 in the supplemental material). Interestingly, upregulation was seen when we measured the expression of the PclaA gene (Pc16g14010), a global regulator of secondary metabolism and asexual sporulation in P. chrysogenum. A significant value was obtained after 60 h of growth (Fig. 2A; see also Table S1), a time point which is characterized by the morphological shift from vegetative growth to asexual differentiation and the peak in penicillin biosynthesis. Furthermore, investigation of the genes involved in secondary metabolism revealed that the genes of the penicillin cluster, pcbAB (Pc21g21390), pcbC (Pc21g21380), and penDE (Pc21g21370), had reduced expression levels after 60 and 96 h of growth, which corresponds to the time points of increased penicillin production in wild-type and production strains (Fig. 2B; see also Table S1). Further verification of this result is provided by Northern hybridization data described in the next paragraph.

In addition to this, expression levels of genes coding for key enzymes of secondary metabolism, like polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), are not consistent with each other. From a total of 48 genes within these classes, only 7 are significantly differentially regulated in the ΔPvelA strain, indicating biosynthesis-specific regulation by PcVelA (Fig. 2B; see also Table S1 in the supplemental material). A more consistent picture was observed for genes encoding enzymes of amino sugar metabolism (www.genome.jp/kegg; map00520). As shown in Fig. 2B, genes involved in chitin catabolism are downregulated in the ΔPvelA strain, including transcripts for N-acetyl-β-D-glucosaminidase (NagA), N-acetyl glucosamine kinase, class V chitinase (ChiB1), N-acetylglucosamine-6-phos-
phate deacetylase, glucosamine-6-phosphate deaminase, and β-N-hexosaminidase. These array data were further confirmed by quantitative reverse transcription-PCR (qRT-PCR), which showed that there was a minimum 3-fold reduction in transcript levels (see Fig. S1 in the supplemental material). As we show in the results, hyphal morphogenesis is severely impaired in the ΔPcvelA strain, with the strain producing an increased amount of chitin in its cell walls.

Control of penicillin biosynthesis by global regulators. The microarray analysis revealed that the penicillin biosynthesis genes pcbAB, pcbC, and penDE are substantially downregulated in the ΔPcvelA strain (Fig. 2B). Another remarkable result is the upregulation of PclaA, the homologue of the global regulator laeA from A. nidulans, which is known to be involved in the control of secondary metabolism (9). In order to characterize the involvement of both regulators in penicillin biosynthesis in a producer strain, we also analyzed a strain carrying a deletion of the PclaA gene in addition to the ΔPcvelA strain. Conventional strategies were applied, and the corresponding knockout strain was generated with the ΔPku70 strain as the recipient. Two independent knockout isolates were further characterized in detail and showed identical phenotypes in all of the following experiments.

For Northern hybridizations, RNA from liquid cultures was isolated after 72 h of growth since the largest penicillin V titers are produced at this time point. As can be seen from the hybridization and quantification results shown in Fig. 3A and B, the transcript levels of both the pcbC and penDE genes are highly reduced in the two knockout strains compared to levels in the ΔPku70 strain. This result was confirmed by investigating the transcript levels in retransformants carrying the corresponding wild-type copy of either the PcvelA or PclaA gene using the self-replicating plasmids pMBF3112 and pMBF3115 (Fig. 3A and B; see also Table S3 in the supplemental material). The reduction in transcript levels was also consistent with data from HPLC analysis to quantify the penicillin V titer. While the recipient P2niaD18 and ΔPku70 strains produced a titer of ca. 2.5 g/liter penicillin V in liquid shaking cultures, the penicillin V yields for both deletion strains were drastically lower at ca. 0.5 g/liter, an 80% reduction (Fig. 3C). In order to confirm these results and to characterize complemented strains with an intact PcvelA or PclaA gene, we conducted bioassays,

FIG. 2. Microarray analysis of selected Pku70- and PcvelA-dependent genes. Heat maps of developmental genes (A) and genes of secondary and amino sugar metabolisms (B) are shown. Red, upregulation in the ΔPcvelA mutant compared to the ΔPku70 strain; green, downregulation in the ΔPcvelA mutant compared to the ΔPku70 strain. The color of each square represents the log2-fold change in expression at the given time point.
as shown in Fig. 3D. In contrast to the HPLC analysis, the bioassay is a semiquantitative measurement to detect any penicillin production. We therefore have used it for a rough estimate to demonstrate the restoration of penicillin biosynthesis in complemented strains. The two knockouts are clearly unable to inhibit the growth of *Staphylococcus aureus* biofilms while all other tested strains produced a clear halo, confirming that the lack of the wild-type genes was responsible for the decreased
penicillin titers. Taken together, these results conclusively show that both PcVelA and PcLaeA act as positive regulators of penicillin biosynthesis by activating gene expression.

Impact of PcVelA and PcLaeA on asexual development. Besides their described roles as key regulators of secondary metabolism, components of the velvet complex are also known to affect fungal morphogenesis (16). Their effects on morphological development vary substantially in different species, as studies of diverse ascomycetes have shown. Consequently, we characterized the PcvelA and PclaeA deletion strains by measuring light-dependent spore formation and by investigating mycelial morphology and conidiophore formation by light and scanning electron microscopy.

VeA was originally identified in A. nidulans as an inhibitor of light-dependent conidiation (36). To test whether either PcVelA or PcLaeA affects conidiation in P. chrysogenum, the corresponding knockout strains and recipients were grown under light and dark conditions. The total rate of spore formation in the recipient P2niaD18 and ΔPcku70 strains was rather variable (14 × 10⁷/cm² versus 6 × 10⁷/cm²); however, both showed a ca. 50% reduction in conidial formation in the dark. In contrast, the ΔPcvelA strain produced an almost equal number of conidia independent of light conditions (10 × 10⁷/cm² versus 9.5 × 10⁷/cm²), with light-dependent conidiation restored following reinsertion of the wild-type PcvelA copy (Fig. 4A). A completely different phenotype was seen in the mutant ΔpclaeA strain, with drastically reduced conidiation (<10⁷/cm²) that was light independent (Fig. 4A).

This reduced spore formation in the ΔpclaeA strain is illustrated by the scanning electron micrographs shown in Fig. 4B. While the parental strain P2niaD18 produced long chains of conidia, the ΔpclaeA strain formed only a single conidium from a terminal vesicle of each phialide. This, the number of conidia, but not of conidiophores, is significantly reduced in the ΔpclaeA strain, as indicated by white arrows in the overview (Fig. 4B). This effect was confirmed by complementation of the knockout strain with the wild-type pclaeA gene, which rescued light-dependent conidiospore formation (Fig. 4A). In summary, these results clearly show that PcVelA and PcLaeA have very different roles in asexual development. PcVelA negatively regulates light-dependent sporulation but has no effect on conidiophore morphogenesis. On the other hand, PcLaeA is involved in the regulation of both conidiophore differentiation and the rate of conidial production in P. chrysogenum.

Impact of PcVelA and PcLaeA on hyphal morphogenesis. For detailed microscopic investigations of the ΔpvelA and ΔpclaeA strains, as well as the corresponding recombinant strains and recipients, two different types of culturing techniques were used. All strains were grown (i) in surface-liquid culture for 24 to 120 h and (ii) for 72 to 168 h in submerged-liquid shaking culture. In surface-liquid culture, the mycelial growth of the recipient P2niaD18 and ΔPcku70 strains typically consists of slender hyphal filaments that almost exclusively display polarized growth and lateral branching (Fig. 4C). In contrast, the PvelA disruption strain shows a hyperbranching phenotype, altered hyphal tip morphology, and frequent dichotomous branching, even after 24 h of growth. Importantly, the wild-type phenotype was restored when a copy of the PvelA gene was reintroduced into the ΔpvelA deletion strain (Fig. 4C). As indicated by our array and qRT-PCR data, genes involved in chitin catabolism are downregulated in the ΔpvelA strain. This led us to investigate chitin distribution in fungal cells by calcofluor white staining. As can be seen in Fig. 4D, calcofluor fluorescence is enhanced in hyphal tips of the ΔpvelA strain while the complemented strain shows a similar fluorescence pattern to the recipients.

In the case of submerged cultivation, two opposing types of morphology, pellets and freely dispersed filaments, could be distinguished. The usual structure of fungal pellets is a core of highly entangled, densely packed hyphae surrounded to a greater or lesser extent by more loosely packed radiating hyphae (20–21). As can be seen in Fig. 4E, after 72 h of growth the PvelA deletion strain underwent pellet formation. These pellets were characterized by a smooth surface with very short radiating hyphae. Interestingly, this phenotype was retained for at least 168 h of growth. The strain also produced a significantly higher dry weight between 120 to 168 h of cultivation than the wild type, suggesting a delay in the autolytic process (data not shown). A clearly different morphology was seen in the parental and complemented ΔpvelA::PvelA strains after 72 h. They exhibited fewer and smaller pellets, with a lower core density, and generally had a significantly larger amount of freely dispersed hyphae. After 168 h, the mycelial clumps had disappeared, and only short hyphal fragments were visible by light microscopy (Fig. 4E), indicating autolysis of hyphae which is accompanied by a significant decrease in biomass (data not shown).

Compared with the ΔpvelA strain, the deletion of the PclaeA gene had a more moderate effect on hyphal morphology. However, hyphae in the ΔpclaeA knockout strain displayed a “curly” phenotype together with hyperbranching when grown for 72 h in surface-liquid medium. Retransformation of the wild-type copy restored the phenotype of the parental strain back to normal hyphae with typical branching patterns (Fig. 4F). From the sum of these results we can conclude that both global regulators PcVelA and PcLaeA affect conidiospore formation and hyphal differentiation in P. chrysogenum.

Interaction studies with components of the velvet-like protein complex. Interactions within protein complexes can be studied using a diverse range of methods. To confirm different interactions in vivo in the homologous system, we used bimolecular fluorescence complementation analysis (BiFC), which was recently established for fungi (32). The functional plasticity of PcVelA and PcLaeA appeared to suggest the existence of a velvet-like complex in P. chrysogenum that is of major importance. In addition to PcVelA and PcLaeA, the interactions of two further putative components of this presumptive complex, namely, the importin α homologue PcKapA and the velvet-like protein PcVelB, were investigated. Both possess an amino acid identity of 50% and 63%, respectively, to their A. nidulans homologues (see Fig. S2 in the supplemental material). A set of plasmids based on the two modified BiFC vectors pEYFP-N1 and pEYFPC-N1 was used for the analysis. The genes encoding PcVelA, PcLaeA, PcKapA, or PcVelB were fused with eYFP fragments encoding either the N or the C terminus of the yellow fluorescent protein. Recombinant strains carrying two BiFC plasmids were generated as described in the Materials and Methods section, and after incubation in the dark they were further analyzed by fluorescence microscopy. As
controls, we investigated strains producing only split EYFPs. As can be seen in Fig. 5A (see also Fig. S3 in the supplemental material), no fluorescence was visible. In addition, the nuclei were stained with DAPI in order to verify in subsequent analyses that interactions of individual components of the velvet-like complex occur in these organelles.

From the results shown in Fig. 5, we can draw the following conclusions. Similar to findings with *A. nidulans*, we detected an

FIG. 4. Morphogenesis of the ΔPvelA and ΔPclaeA strains as well as the corresponding recipients and complemented strains. (A) Quantification of light- and dark-dependent conidial formation in strains as indicated. All strains were grown for 168 h under constant light or dark conditions. The experiment was performed in triplicate; bars show standard deviations. (B) Scanning electron microscopy of conidiophores from the wild-type P2niaD18 and the ΔPclaeA mutant strains. While P2niaD18 produces long chains of conidia (indicated by arrows), the ΔPclaeA strain consistently forms only a single conidium from a terminal vesicle of each phialide (indicated by arrows). Scale bars are valid for either both top or three bottom figures. (C) PvelA-dependent hyphal morphology after 72 h of cultivation in surface liquid culture. White arrows indicate dichotomous hyphal branching sites. (D) Calcofluor white staining of hyphae from strains as indicated. Hyphae were stained after 48, 72, and 96 h of growth in surface-liquid cultures. (E) PvelA-dependent pellet formation after 72, 120, and 168 h in submerged-liquid shaking cultures. (F) PclaeA-dependent hyphal morphology after 72 h of cultivation in surface-liquid culture. White arrows indicate dichotomous hyphal branching sites.
FIG. 4—Continued.
interaction of PcVelA with PcLaeA, PcVelB, and PcKapA in the nucleus. In our BiFC analysis we found another interaction for PcVelB. A nuclear fluorescence was observed when PcVelB was tested together with PcKapA in the wild-type strain. However, this interaction could not be confirmed by yeast two-hybrid analysis, suggesting that PcVelA acts as a bridge protein, bringing PcVelB and PcKapA into close proximity (data not shown). In summary, the demonstrated interactions provide evidence for the presence of a velvet-like complex in *P. chrysogenum*.

**Heterologous complementation studies.** Our data presented so far indicate that both PcVelA and PcLaeA have conserved as well as distinct roles in secondary metabolism and fungal development. In order to test whether a functional substitution between global regulators from different filamentous fungi is possible, the *Fflae1* and *Ffvel1* genes from *F. fujikuroi* were used in heterologous complementation experiments to complement the *PcvelA* and *PclaeA* deletions in *P. chrysogenum*. *F. fujikuroi* and *P. chrysogenum* are distantly related ascomycetes which belong to the *Hypocreales* and *Eurotiales*, respectively. In both species, the proteins encoded by the homologous genes show an amino acid identity of 38% (VeA) and 33% (LaeA) (73). The obtained retransformant ΔPcVelA::Ffvel1 and ΔPclaeA::Fflae1 strains were selected on pyrithiamine-containing medium, and four complemented strains each from both experiments were further characterized.

Most importantly, a restoration of the wild-type phenotype could be achieved when we measured secondary metabolism in both types of complemented strains, as shown in Fig. 6A and B. Similar to the parental ΔPcku70 strain, the complemented strains from both experiments produced a clear halo, confirming that introduction of *Ffvel1* or *Fflae1* results in the restoration of the penicillin titer (Fig. 6B). An important morphogenic restoration was observed in the complemented ΔPclaeA::Fflae1 strains. While ΔPclaeA showed a drastic reduction in conidial formation leading to a white appearance on solid medium, indicating only hyphal growth, the ΔPclaeA::Fflae1 strain again formed conidiospores (Fig. 6C). However, the number of conidia is lower in this strain than in the recipient ΔPcku70 strain, suggesting only a partial restoration of conidiophore development by the Fflae1 protein.

**DISCUSSION**

Recent molecular and biochemical data from the model organism *A. nidulans* have provided evidence that VeA and...
expression in 

115 out of 136 differentially expressed sequences had reduced

PcvelA revealed that deletion of 
development and secondary metabolism. Microarray analysis re-

Aspergillus plex in a fungus outside the 
expression of nuclear genes in the 
strain revealed that 21.6% of the sequences could be assigned 

functions in growth and development were determined as dif-
erentially expressed in the ΔPcvelA strain. This rather low 
number is remarkable because it was previously shown for A. nidulans that VeA is a positive regulator of sexual development and also a negative regulator of asexual conidial formation (39).

Both PcVelA and PcLaeA regulate secondary metabolism, in particular, penicillin biosynthesis. Our functional analysis using a disruption strain clearly demonstrates the regulatory role of PcVelA on genes of the penicillin cluster. These data are also comparable to those from A. chrysogenum, where disruption of the velvet-like gene causes a drastic decrease in the expression of all biosynthesis genes and subsequently in production of the β-lactam antibiotic cephalosporin C (23). Furthermore, it is known that penicillin production in the model organism A. nidulans is also dependent on proper veA expression. It was observed that VeA represses the transcription of the isopenicillin synthase gene pcbC and is simultaneously neces-

sors as well as repressors of secondary metabolism in fila-

metabolites (16). This hypothesis is supported by several investi-
gations characterizing Velvet homologues as activa-
tors as well as repressors of secondary metabolism in fila-
mentous fungi (2, 17, 24, 38, 54).

In conclusion, our data clearly define a specific role for PcVelA as a transcriptional activator of the penicillin biosyn-
thetic cluster in P. chrysogenum. A broader role of VeA in the regulation of secondary metabolism has been observed in A. flavus. In this fungus, VeA is not only essential for controlling the expression of the sterigmatocystin cluster but also responsible for the expression of genes involved in cyclopiazonic acid and aflatrem synthesis (24). Similarly, in F. fujikuroi, the PcVeA homologue FfVel1 regulates the expression of genes involved in the biosynthetic pathways of gibberellins, fumonisins, and bikaverin (73). It seems, therefore, that in some fungi the Velvet protein has a more global regulatory role in secondary metabolism than in P. chrysogenum. What is most striking in the ΔPcvelA strain, however, is the reduced expres-
sion of several genes involved in amino sugar (cell wall) me-
tabolism, as discussed below.

In contrast to VeA and its homologues, the global regulator and Velvet interaction partner LaeA is known to be an acti-
vator of only secondary metabolite production in all filament-
tous fungi investigated to date. Several reports from Aspergillus species revealed that deletion of laeA, which encodes a puta-
tive methyltransferase, results in reduced production of sec-

FIG. 6. Heterologous complementation experiments with P. chry-
sogenum mutants using homologues from F. fujikuroi. Penicillin bio-
assays of complemented strains obtained after transformation of the 
ΔPcvelA strain with the homologous gene Ffv1l (A) or the ΔPclaeA 
strain with Fflae1 (B). As reference, the recipient ΔPcku70 strain as 
well as the knockout ΔPcvelA and ΔPclaeA mutant strains are shown. 
From four different complemented strains, two are given as examples. 
In all cases, S. aureus served as a Gram-positive indicator. (C) Photo-
graphs of the recipient ΔPcvelA strain, the ΔPclaeA knockout mutant, 
and the complemented ΔPclaeA::Fflae1 T2 strain. Green pigmentation 
indicates proper conidial formation.

LaeA, two global regulators of secondary metabolism, are 
components of the multisubunit velvet complex (6). So far, 
however, no evidence exists as to whether a comparable com-
plex occurs in any other filamentous fungus and, in particular, 
in an industrial producer strain. The data provided in this study 
clearly indicate distinct roles for homologues of both regu-

PcVelA controls a distinct subset of genes involved in devel-

tment and secondary metabolism. Microarray analysis re-

vealed that deletion of PcvelA induced a major change in the 
expression of nuclear genes in the ΔPcvelA strain compared to 
expression in the recipient ΔPcku70 strain. Around 7% of all 
nuclear genes were either up- or downregulated under the 
conditions investigated. In a time course analysis with a veA 
deletion mutant of Aspergillus flavus, Cary et al. (18) deter-

mined that the mutant had a significantly smaller amount of 
differentially expressed sequences than the wild type. In their 
microarray analysis the expression of 136 out of 5,002 (2.7%) 
investigated genes was found to be veA dependent. A total of 
115 out of 136 differentially expressed sequences had reduced 
expression in a ΔveA strain, illustrating that VeA predomi-
nantly functions as a global activator of gene expression in this 
fungus (18). In contrast, deletion of PcvelA in P. chrysogenum is 
responsible for a nearly equal number of up- and downregu-
lated sequences; therefore, PcVeA can be considered to func-
tion as both an activator and repressor of a broad spectrum of 
genes. Analysis of the downregulated sequences in the ΔPcvelA 
strain revealed that 21.6% of the sequences could be assigned 
to the protein functional class metabolism. A slightly higher 
percentage (26%) of all genes were upregulated in the ΔPcvelA strain, suggesting that PcVeA is a general repressor of metab-
lism. According to FunCat, only nine genes with predicted 
functions in growth and development were determined as dif-

related to the construction of a 

in the absence of a 
velvet 

PcVelA as a transcriptional activator of the penicillin biosyn-
thetic cluster in P. chrysogenum. A broader role of VeA in the regulation of secondary metabolism has been observed in A. flavus. In this fungus, VeA is not only essential for controlling the expression of the sterigmatocystin cluster but also responsible for the expression of genes involved in cyclopiazonic acid and aflatrem synthesis (24). Similarly, in F. fujikuroi, the PcVeA homologue FfVel1 regulates the expression of genes involved in the biosynthetic pathways of gibberellins, fumonisins, and bikaverin (73). It seems, therefore, that in some fungi the Velvet protein has a more global regulatory role in secondary metabolism than in P. chrysogenum. What is most striking in the ΔPcvelA strain, however, is the reduced expression of several genes involved in amino sugar (cell wall) metabolism, as discussed below.

In contrast to VeA and its homologues, the global regulator and Velvet interaction partner LaeA is known to be an acti-
vator of only secondary metabolite production in all filament-
tous fungi investigated to date. Several reports from Aspergillus species revealed that deletion of laeA, which encodes a puta-
tive methyltransferase, results in reduced production of sec-

secondary metabolites such as aflatoxin, sterigmatocystin, lova-

statin, and protostadienol (6, 9–10, 46, 55). In addition,
microarray data from *A. fumigatus* comparing a ΔlaeA mutant to the wild type demonstrated that LaeA positively controls the expression of 20 to 40% of the major classes of secondary metabolite biosynthesis genes such as nonribosomal peptide synthetases, polyketide synthases, and P450 monoxygenases (55). Very recently, Kosalková and coworkers (40) investigated laeA knockdown strains from *P. chrysogenum*. One out of 10 transformants showed a ca. 50% reduction in its benzylpenicillin production, thus confirming the drastic effect we observed in the ΔpvelA strain.

**PcVelA controls hyphal branching, pellet formation, and light-dependent sporulation.** Aside from its effect on secondary metabolism, PcVelA is also involved in the regulation of growth, morphology, and asexual development, similar to Velvet homologues from different ascomycetes (16).

The light-dependent phenotype observed in the ΔpvelA knockout mutant is similar to what has been observed in *A. nidulans* velvet mutants (19, 36, 75). In *A. nidulans* strains with the veA wild-type allele, light promotes the formation of conidia, similar to the situation found in our study of *P. chrysogenum*. Therefore, the Velvet protein was proposed to be a negative regulator of asexual development whose function could be suppressed by light not only in *A. nidulans* but also, as shown here, in *P. chrysogenum*. However, in contrast to our results, *A. nidulans* VeA is also required for proper asexual sporulation in light, since veA deletion mutants are also defective in light-induced asexual conidiation (39). Additionally, recent studies on several veA homologues have demonstrated that proper expression of *velvet* is in general necessary for normal asexual conidial formation. While deletion of the *N. crassa* ve-1 locus causes a significant increase in asexual conidiation (7), veA mutants of, for example, *A. fumigatus*, *Aspergillus parasiticus*, and *A. flavus* exhibit reduced conidial formation (17, 25, 41). Interestingly, the reduced conidiation in these four species is supposedly not light dependent, in contrast to *A. nidulans* and *P. chrysogenum*. The altered hyphal morphology seen in the ΔpvelA strain correlates well with the downregulation of genes involved in amino sugar (cell wall) metabolism. As discussed previously for *Aspergillus* species, an increased branching frequency as well as a delay in autolytic processes can be the consequence of cell wall defects and modifications (53). Interestingly, our microarray analysis has identified a set of genes that are significantly downregulated at all time points investigated. These genes encode enzymes involved in amino sugar metabolism, in particular, chitin degradation, for example, chitinase ChiB1 (Pc22g01100), N-acetylglucosaminidase NagA (Pc20g10360), and an N-acetyl-glucosamine kinase (Pc21g04830). This is consistent with the increased chitin content observed in the hyphal tips of the ΔpvelA strain.

Chitin together with mannoproteins and β-1,3-glucans are essential components of the cell walls of filamentous fungi. Usually chitin exists in a highly rigid, crystalline state, but it can become plastic in the hyphal apex to allow for extension and hyphal branching. Recent analyses have revealed that the fungal cell wall is a dynamic organelle in which components are continuously synthesized, degraded, and chemically modified, with their structure constantly subject to rearrangement (8, 56). Thus, fungal cell wall biosynthesis and regeneration necessarily imply chitin biosynthesis by chitin synthases and degradation by chitinases and N-acetyl-glucosaminidases. Bartnicki-García (5) presented a model showing that a delicate balance exists between synthesis and lysis and is important for proper fungal polarized growth. Considering this model, it can be envisioned that deletion of *PcvelA* and, thus, downregulation of genes specifically involved in chitin degradation result in an imbalance which is responsible for the hyperbranching phenotype, pellet formation, and delayed fragmentation and autolysis in *P. chrysogenum*. In *A. oryzae*, an imbalance in chitin synthesis and lysis caused by disruption of the chitin synthase gene ChsB leads to more densely branched hyphae with significantly larger diameters (53).

A similar effect of a Velvet homologue on hyphal morphology was observed in *Fusarium verticilloides* (45). In surface-liquid cultures, Δfve1 mutants proliferated mainly by forming hyperbranched hyphae and undergoing yeast-like growth, which was suggestive of alterations in their hyphal polarity. Furthermore, the mutant had reduced aerial hyphae. Since addition of osmotic stabilizers restored the wild-type phenotype in the knockout strain, it was suggested that the alterations caused by Fve1 deletion were due to cell wall defects. Investigations of cell wall composition revealed a statistically significant reduction in the mannoprotein content of the mutant while glucan and chitin levels did not differ significantly. These results support an important role for FvVe1 in cell wall integrity and hyphal polarity (45). Similarly, deletion of Velvet homologues in *A. chrysogenum* and *N. crassa* causes an altered hyphal branching pattern on solid medium, which suggests that Velvet homologues also have a conserved role in these fungi in the regulation of hyphal growth and polarity (7, 23). However, the cell wall defect-related phenotypes exhibited by the *P. chrysogenum* ΔpvelA mutant were not observed in velvet deletion mutants of *Aspergillus* species, suggesting that the role of cell wall proteins regulated by veA homologous genes is species specific.

**PcLaeA has a conserved as well as a specific developmental function, specific to *P. chrysogenum*.** In addition to its conserved role in the regulation of secondary metabolism, PcLaeA also possesses a *P. chrysogenum*-specific function in conidioaphore development and hyphal differentiation. The *PcLaeA* deletion mutant exhibits a hyperbranching phenotype similar to that of the ΔpvelA strain. This change in hyphal morphogenesis associated with the ΔpvelA mutant has so far not been observed in any other filamentous fungi, suggesting a distinct regulatory role of PcLaeA in *P. chrysogenum*. Therefore, future experiments will be directed toward identifying target genes in order to elucidate the contribution of PcLaeA in morphogenetic signaling pathways in *P. chrysogenum*.

Our studies further revealed a drastic reduction in conidiospore formation on solid medium in the *PclaeA* deletion strain. This is consistent with the above-mentioned report of a slight reduction in conidiospore formation in a single laeA-silenced strain of *P. chrysogenum* (40). The observed generation of a terminal vesicle with one single conidium in the ΔpvelA strain is reminiscent of the phenotype of the abaA mutant from *A. nidulans* and further supports the control of LaeA homologues on developmental genes (3, 63).

A reduction in spore production is also described for *A. flavus*, *A. fumigatus*, and *F. fujikuroi* ΔlaeA deletion mutants (37, 75). At this point it is worth mentioning that the LaeA
effect on sporulation in these organisms is less severe than its effect on \textit{P. chrysogenum}. The \textit{A. fumigatus} \textDelta \textit{laeA} mutants were impaired only in conidial production when grown in liquid shaking cultures. On solid medium, however, conidial production levels were similar to the level of the wild type, and in \textit{A. nidulans} a time-dependent impairment of conidiophore development was observed (N. P. Keller, personal communication). A remarkable conservation of function was seen when we complemented the \textit{P. chrysogenum} mutant with \textit{Fflae1} from \textit{F. fujikuroi}. It appears that the \textit{Fusarium} homologue is able to restore protein-protein interaction within this multisubunit complex, as discussed later. These results clearly show, and further support, the idea that \textit{LaeA} homologues also have conserved as well as species-specific regulatory roles in fungal development.

**Evidence for a velvet-like protein complex in an ascomycete other than \textit{A. nidulans}.** Recently Bayram and coworkers (6) identified the so-called \textit{velvet} complex in \textit{A. nidulans} consisting of three core components, VeA, \textit{LaeA}, and the \textit{velvet}-like protein \textit{VelB}. Further biochemical as well as yeast two-hybrid data have shown that the importin \textit{a} homologue KapA interacts with \textit{VeA}, and it is therefore purported to be responsible for its transport into the nucleus (4, 6, 67). Based on these observations, a model for the role of \textit{VeA} was developed. It was proposed that in the dark an increased amount of \textit{VeA}, together with \textit{VelB}, enters the nucleus via KapA. In the nucleus \textit{VeA} and \textit{VelB} interact with each other and with other as yet unknown factors to control sexual development. For the activation of secondary metabolism, however, \textit{LaeA} forms a heterotrimeric complex with \textit{VeA}/\textit{VelB} heterodimers. Different studies indicate that \textit{VeA} is a bridge protein of the \textit{velvet} complex, connecting regulation of light-dependent morphogenesis with secondary metabolism in \textit{A. nidulans} (6, 26, 57).

We have used a BiFC interaction analysis to show that \textit{PcVeA} forms a core complex with \textit{PcKapA}, \textit{PcLaeA}, and \textit{PcVelB} in the nucleus of \textit{P. chrysogenum} hyphae, very similar to the system in \textit{A. nidulans}. To the best of our knowledge, this is the first report demonstrating the interaction of components from a \textit{velvet}-like complex in an organism other than \textit{A. nidulans}. This view of a \textit{velvet}-like complex in \textit{P. chrysogenum} is further supported by our array data showing a clear upregulation in the case of the global regulator \textit{PelacA} which thus indicates that both factors are part of a common regulatory network. The fact that the \textit{Fusarium} \textit{Fve1l} and \textit{Flae1} genes are able to partially restore the function of the missing homologues in the \textit{P. chrysogenum} mutants further indicates that the \textit{velvet} complex is conserved within the \textit{Ascomycetes}. Future work will therefore focus on the identification and characterization of novel subunits of the \textit{velvet} complex as it is a large protein complex which might display high structural and functional dynamics (16). A multisubunit \textit{velvet} complex could be hypothesized to function as a depot for regulatory proteins performing inducible and context-dependent cellular functions (58). Other examples of candidates for macromolecular complexes acting as depots for regulatory proteins are the proteosome and the COP9 signalosome, both of which are known to control major aspects of development (15, 72).

In summary, the finding that \textit{PcVeA} and \textit{PcLaeA} are members of a protein complex in \textit{P. chrysogenum} points to the observed similarities in \textit{VeA} and \textit{LaeA} function in different fungi. However, we have detected notable distinctions concerning, for example, conidial development. These differences might be due to the fact that deletion of single components leads to conformational changes in the complex and thus to the loss of certain functions. Our results confirm and extend the current picture of regulatory networks controlling both fungal secondary metabolism and morphogenesis. This is not only significant for a better understanding of model fungal systems but also of relevance for the genetic manipulation of fungal metabolism as part of industrial strain improvement programs.

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