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The ergot alkaloid gene cluster in *Claviceps purpurea*: Extension of the cluster sequence and intra species evolution

Thomas Haarmann [a], Caroline Machado [b], Yvonne Lübbe [a], Telmo Correia [a], Christopher L. Schardl [b], Daniel G. Panaccione [c], Paul Tudzynski [a,*]

[a] *Institut für Botanik, Westf. Wilhelms-Universität Münster, Schlossgarten 3, D-48149 Münster, Germany*
[b] *Department of Plant Pathology, University of Kentucky, 201 F Plant Science Building, Lexington, KY 40546-0312, USA*
[c] *Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV 26506-6058, USA*

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Abstract

The genomic region of *Claviceps purpurea* strain P1 containing the ergot alkaloid gene cluster [Tudzynski, P., Hölter, K., Correia, T., Arntz, C., Grammel, N., Keller, U., 1999. Evidence for an ergot alkaloid gene cluster in Claviceps purpurea. Mol. Gen. Genet. 261, 133–141] was explored by chromosome walking, and additional genes probably involved in the ergot alkaloid biosynthesis have been identified. The putative cluster sequence (extending over 68.5 kb) contains 4 different nonribosomal peptide synthetase (NRPS) genes and several putative oxidases. Northern analysis showed that most of the genes were co-regulated (repressed by high phosphate), and identified probable flanking genes by lack of co-regulation. Comparison of the cluster sequences of strain P1, an ergotamine producer, with that of strain ECC93, an ergocristine producer, showed high conservation of most of the cluster genes, but significant variation in the NRPS modules, strongly suggesting that evolution of these chemical races of *C. purpurea* is determined by evolution of NRPS module specificity.

Keywords: *Claviceps purpurea*; Ergot fungus; Ergot alkaloids; Gene cluster; Phosphate effect; Nonribosomal peptide synthetases; Chemical races; Ergotamine; Ergocristine

1. Introduction

The ergot disease of grasses and cereals caused by the ergot fungus *Claviceps purpurea* is characterized by the formation of overwintering structures, so-called sclerotia, containing variable amounts of mycotoxins, the ergopeptines. This is a class of cyclol-structured alkaloid peptides containing D-lysergic acid. Since some of these substances have considerable pharmacological importance, physiology, biochemistry and genetics of their formation have been studied in detail (Stadler and Giger, 1984; Tudzynski et al., 2001). Tsai et al. (1995) identified the gene (*dmaW*) for the first specific enzyme of the pathway, dimethylallyltryptophan synthase (see Fig. 1), in *C. fusiformis*, and Tudzynski et al. (1999) described a putative ergot alkaloid gene cluster in the *C. purpurea* strain P1; the putative cluster included a homologue of *dmaW*, *cpd1*, and a trimodular NRPS gene, *cpps1*, encoding lysergylpeptidyl synthetase 1 (LPS1) involved in the attachment of the tripeptide moiety to dehydrolysergic acid (Riederer et al., 1996). Recently, a second NRPS gene in the cluster region, *cpps2*, was shown to encode LPS2, the D-lysergic acid activating NRPS module (Correia et al., 2003; see Fig. 1). The function of the other genes identified in the neighborhood of *cpd1*, *cpps1* and *cpps2* –

* Corresponding author. Tel.: +49 251 83 2 49 98; fax: +49 251 83 2 16 01.
E-mail address: tudzyns@uni-muenster.de (P. Tudzynski).

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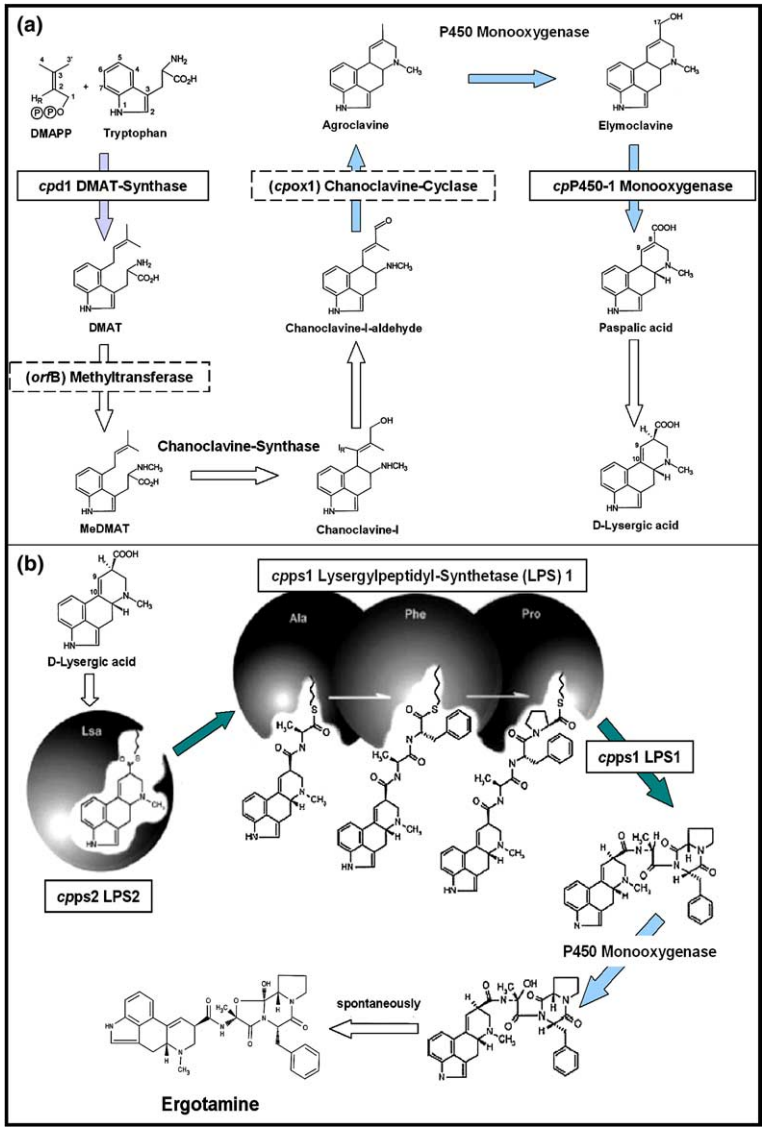

Fig. 1. Biochemical pathway of ergot alkaloids: (a) synthesis of D-lysergic acid; (b) synthesis of ergopeptines (example: ergotamine). Genes with known or suspected functions are indicated (for details see, e.g., Tudzynski et al., 2001).

among them several oxidase/oxygenase genes – remains to be elucidated. Here, we show that the putative cluster region extends on both sides of the originally described sequence, covering at least 68.5 kb. It contains two additional NRPS genes, *cpps3* and *cpps4*, the functions of which remain to be determined. A second major aspect of the present paper is the comparison of the putative ergot alkaloid cluster in two chemical races: strain P1 (major alkaloid: ergotamine) and ECC93 (ergocristine) to study variability/evolution of the cluster sequence within the species *C. purpurea* and to determine if the difference in the primary ergopeptine produced is mainly due to internal amino acid pools (as postulated by Riederer et al., 1996) or to differences in critical substrate specificity conferring amino acids of the NRPS modules.

2. Results and discussion

2.1. Defining the ergot alkaloid gene cluster in strain P1

The core region of the ergot alkaloid gene cluster (Correia et al., 2003) stretches from a putative oxidase gene (*cpox3*) to the LPS1 encoding gene *cpps1* over 40 kb. Chromosome walking using clones of an EMBL3 genomic library of strain P1 extended the available genomic sequence downstream of *cpox3* (“left” side) over 15 kb (see Fig. 2). This region contains an additional NRPS gene, *cpps3*, and a gene probably involved in primary metabolism, *cpimd* (encoding an isopropylmalate-dehydratase). *Cpps3* encodes a monomodular NRPS of 1634 amino acids with high similarity to CPPS2 (22.1% identity) and the first module of CPPS1

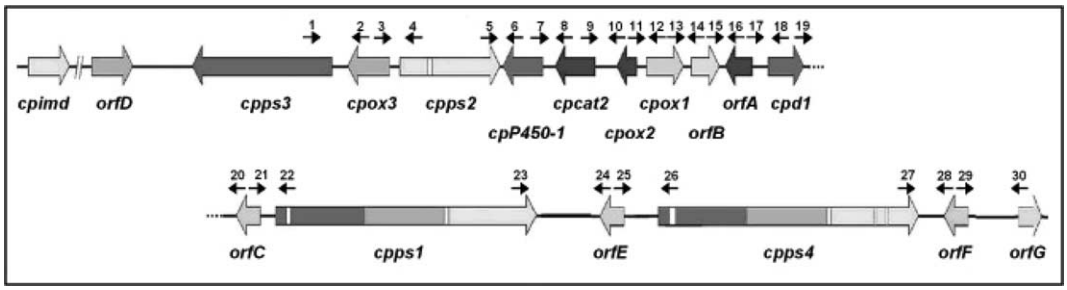

Fig. 2. Schematic map of the ergot alkaloid cluster region in *C. purpurea* strain P1. For gene descriptions, see Table 1. Primer binding sites for comparative analysis of the homologous genes in strain ECC93 are indicated by arrows (see Section 3 for primer sequences).

(32.7% identity), but is unusual in that it appears to contain a C-terminal reductase domain (data not shown); its function is open. The "right" end of the core cluster region was extended by screening and sequencing genomic lambda clones and a cosmid clone.

As shown in Fig. 2, downstream of *cpps1* an open reading frame (154 amino acids) with similarity to dioxygenases (orfE) is followed by an additional NRPS gene, *cpps4*, and two small open reading frames (orfF and orfG). A compilation of the cluster genes is given in Table 1. For some of the genes (in addition to *cpd1*, *cpps1* and *cpps4*) possible functions in the biochemical pathway (see Fig. 1) can be proposed because of their annotation: the product of *cpP450-1*, a putative cytochrome-P450-monooxygenase, could be the enzyme catalyzing the oxidative steps from agroclavine to lysergic acid. Indeed, a recent knock-out of the *cpP450-1* gene led to loss of ergotamine production in the mutant and accumulation of elymoclavine, whereas feeding of the mutant with lysergic acid yielded ergotamine, proving that *Cp-P450-1* encodes the elymoclavine oxidase (Haarmann et al., unpublished). The gene product of

Table 1
Compilation of genes/orfs in the ergot alkaloid cluster region of *C. purpurea* strain P1

ORF	Number of exons	Size of the product (aa)	Accession No.	Similar entity/entities	Identity with similar entity (%)	Origin	Reference
orfD	5	575	–	–	–	–	–
cpps3	1	1634	AJ884677	Nonribosomal peptide synthase	30	*Alternaria brassicae*	Guillemette et al. (2004)
cpox3	1	369	CAG28312	Probable NADPH2 dehydrogenase (CAF05994)	52	*Neurospora crassa*	GenBank
cpps2	2	1308	AJ439610	Nonribosomal peptide synthase	31	*Alternaria brassicae*	Guillemette et al. (2004)
cpP450-1	9	507	AJ884676	CND5p (cytochrome P450)	27	*Botryotinia fuckeliana*	Viaud et al. (2003)
cpcat2	2	473	AJ703808	AN5918.2 (hypothetical catalase)	43	*Aspergillus nidulans*	GenBank
cpox2	2	261	CAB39316	MG02225.4 (short chain dehydrogenase)	38	*Magnaporthe grisea*	GenBank
cpox1	2	483	AJ703809	Isoamyl alcohol oxidase	26	*Aspergillus oryzae*	Yamashita et al. (2000)
orfB	2	319	–	AN7620.2 (hypothetical protein)	31	*Aspergillus nidulans*	GenBank
orfA	3	173	–	CAE14644 (hypothetical protein)	34	*Photorhabdus luminescens*	GenBank
cpd1	3	441	CAC37397	Dimethylallyltryptophan synthase	68	*Balansia obtecta*	Wang et al. (2004)
orfC	1	314	–	nfa12720 (phytanoyl-CoA dioxygenase)	36	*Nocardia farcinica*	Ishikawa et al. (2004)
cpps1	3	3585	CAB39315	Peptide synthetase	56	*Neotyphodium lolii*	Panaccione et al. (2001)
orfE	1	154	–	AN9246.2 (phytanoyl-CoA dioxygenase)	27	*Aspergillus nidulans*	GenBank
cpps4	5	3524	AJ884678	Peptide synthetase	54	*Neotyphodium lolii*	Panaccione et al. (2001)
orfF	1		–	–	–	–	–
orfG	1		–	–	–	–	–

cpox1, a putative FAD-dependent oxidase, could be the chanoclavine cyclase (see Fig. 1(a)). It is interesting to note that the two orfs linked to *cpps1* and *cpps4* – namely, *orfC* and *orfE* – both have BLAST similarities to dioxygenases.

An unequivocal assignment for functions of all genes identified in the cluster region will require combined gene inactivation and heterologous expression studies. However, a first criterion for the genuine cluster genes should be their co-regulation (which has been shown in several other fungal secondary metabolite clusters, e.g., for the gibberellin cluster in *Gibberella fujikuroi*, Mihlan et al., 2003). Ergot alkaloid biosynthesis is regulated, e.g., by phosphate levels in the culture medium (the "phosphate effect": Pažoutová and Rehacek, 1984; Robbers et al., 1972). In keeping with the phosphate effect the genes *cpd1*, *cpps1* and *cpps2* are transcribed under low phosphate conditions, whereas at high phosphate concentrations transcription of these genes is almost undetectable (Correia et al., 2003). As shown in Fig. 3, almost all genes of the cluster region show a differential expression pattern under low/high phosphate conditions, i.e., they are co-regulated, supporting the hypothesis that their products could be involved in alkaloid biosynthesis. In contrast, the *cpimd* gene – probably encoding an enzyme involved in amino acid biosynthesis – is not co-regulated with the known ergot alkaloid biosynthesis genes, i.e., it is expressed independently of the phosphate concentration. Therefore, it is reasonable to suppose that *cpimd* lies beyond the "left" border of the cluster. The "rightmost" genes of the sequenced region – namely, *orfF* (data not shown) and *orfG* – are not expressed at detectable levels under alkaloid-production conditions, indicating that they probably lie beyond the right border of the cluster. This does not prove that all the genes within the "borders" are indeed involved in alkaloid biosynthesis, nor does it necessarily mean that this region contains all genes necessary for ergot alkaloid biosynthesis; e.g., in *Fusarium sporotrichioides* three genes involved in biosynthesis of trichothecenes are not included in the gene cluster (Brown et al., 2004). However, the number of genes in the putative cluster region (15) would be sufficient for the steps of the pathway characterized so far, and in theory could direct the entire pathway from common precursor amino acids and dimethylallyl diphosphate to the ergopeptines. Evaluation of the roles of these genes in the biosynthesis will take some time; especially interesting is the function of the two additional NRPS genes identified in this study.

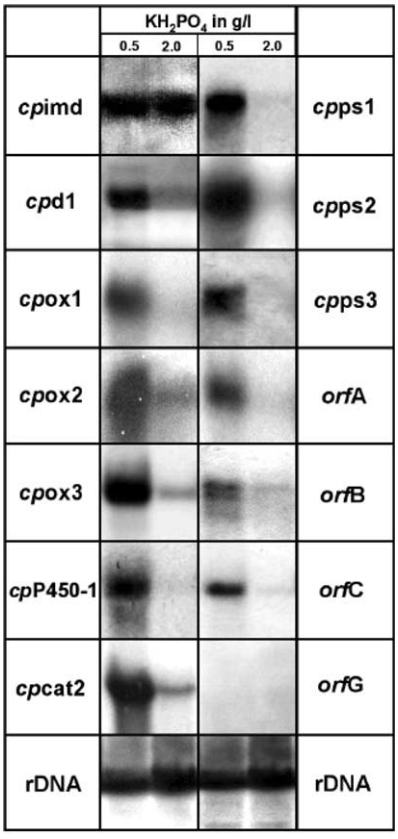

Fig. 3. Expression analysis of genes of the ergot alkaloid cluster region in strain P1. Mycelia were grown under low and high phosphate conditions, RNA was extracted and blotted on nylon membranes, and ^{32}P-labelled PCR fragments of the orfs/genes were used as probes (for designation of genes, see Table 1 and Fig. 2).

The nonribosomal peptide synthetase (NRPS) responsible for synthesis of ergotamine lactam, comprises two subunits. The LPS1 subunit is trifunctional and activates the three amino acids of the lactam ring, and LPS2 activates lysergic acid (Riederer et al., 1996). The predicted *cpps1* gene product included sequences previously identified in purified LPS1 (Tudzynski et al., 1999). The LPS2 subunit is encoded by *cpps2* (Correia et al., 2003).

The putative NRPS gene *cpps4* has a structure similar to *cpps1* and could have arisen from a gene duplication event (also the high similarity of *orfC* to *orfE* indicate such a duplication, see above). The *cpps4* ORF of 10,852 bp is interrupted by 4 introns of 79, 110, 46, 40 bp, respectively, and is predicted to encode a polypeptide of 3525 amino acids. The overall similarity of the putative gene product to CPPS1 is high (average identity of 85%), but there are differences, even in the specificity-determining putative substrate binding pockets (see below). CPPS4, like LPS1, lacks an N-terminal condensation-domain and hence probably also cooperates with LPS2 encoded by *cpps2*; a possible product of the concerted action of LPS2 and *CPPS4* (or *CPPS1*? see below) could be the minor peptide alkaloid produced by strain P1, α-ergocryptine (Fig. 4). As shown in Table 2, ergotamine and α-ergocryptine differ in 2 of the 3 amino acids of the peptide part: ergotamine contains alanine, phenylalanine and proline, whereas ergocryptine contains valine and leucine in the first 2 positions. A comparison

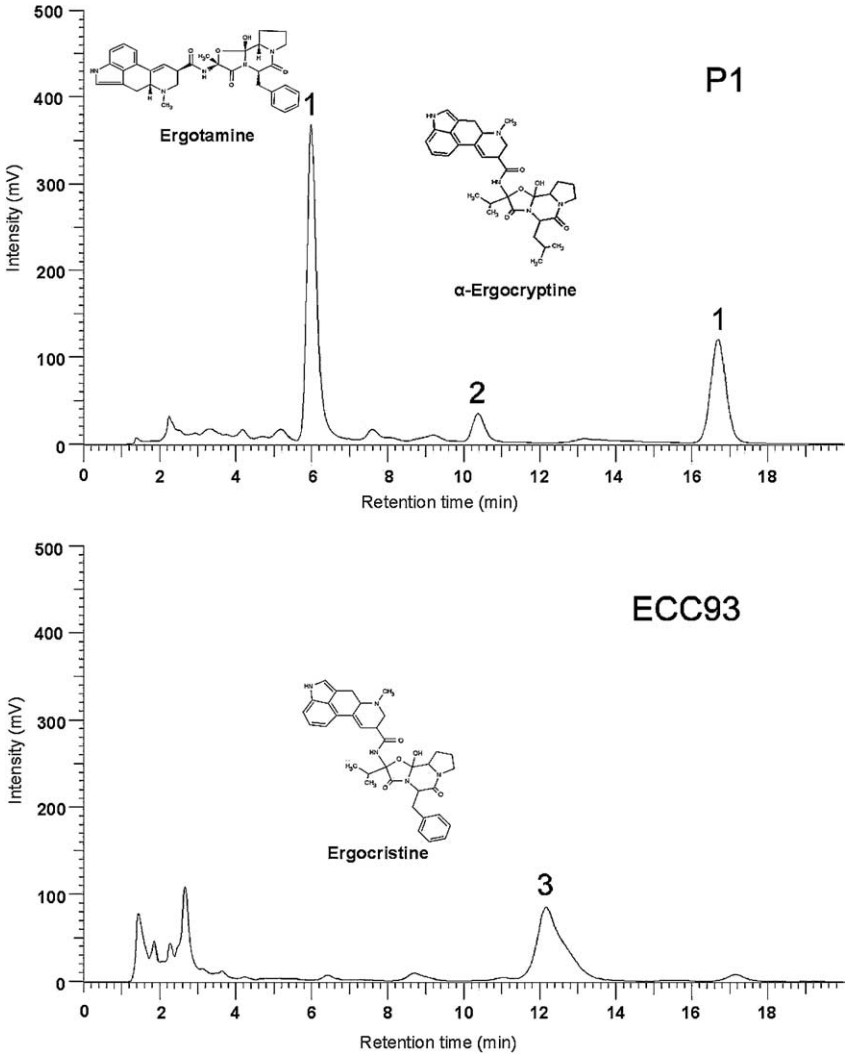

Fig. 4. HPLC analysis of culture filtrates of strains P1 and ECC93. The identification of peaks (relative to standards) is indicated: 1, ergotamine and its later-eluting stereoisomer ergotaminine; 2, α-ergocryptine; 3, ergocristine.

Table 2
Amino acid components of the peptide moiety of ergopeptines; the third amino acid is always proline in the ergopeptines listed (from Keller and Tudzynski, 2001)

Position II	Position I		
	Alanine	α-Amino butyric acid	Valine
Phenylalanine	Ergotamine	Ergostine	Ergocristine
Leucine	α-Ergosine	α-Ergoptine	α-Ergocryptine
Isoleucine	β-Ergosine	β-Ergoptine	β-Ergocryptine
Valine	Ergovaline	Ergonine	Ergocornine
α-Amino butyric acid	Ergobine	Ergobutine	Ergobutyrine

of the putative specificity-determining amino acids within the A-domains of CPPS1 and CPPS4 (Table 3) supports the view that the enzymes each produce a different ergopeptine: the modules 1 and 2 of both enzymes differ in their substrate specificity-determining amino acids as expected, whereas the corresponding sequences of the third modules (they both should activate proline) are identical (data not shown). Moreover, determination of peptide sequences obtained from LPS1 enzyme preparations (Keller, personal communication) showed that most of them match sequences of CPPS1, several both, CPPS1 and CPPS4, but one exclusively CPPS4. This suggests that the band initially believed to represent LPS1 alone, actually is a mixture of both, LPS1 and LPS4. From the relative abundancies of peptide sequences of the two proteins, it can be concluded that CPPS1 might be the major component. Possibly, therefore, CPPS1 is responsible for formation of the major ergopeptine, ergotamine, and CPPS4 for α-ergocryptine, the minor ergopeptine of the total alkaloid mixture elaborated by *C. purpurea* strain P1. For a final proof of the specificity of the enzymes two approaches could be taken: inactivation of the genes by gene replacement, and biochemical characterization of the substrate binding properties of the isolated domains obtained by heterologous expression.

Table 3
Alignment of substrate specificity-determining amino acid residues of CPPS1 and CPPS4 (strain P1) and of CPPS1-EC and CPPS4-EC (strain ECC93); shown are extracted residues demonstrated to be important in the PheA domain of gramicidin synthetase 1 (Stachelhaus et al., 1999)

NRPS	Module 1	Substrate	Module 2	Substrate	Product
P1					
cpps1	DLFFCGGP	Alanine	DLVGMAAV	Phenylalanine	Ergotamine
cpps4	DAIFCGGP	Valine?	DLAGMGAV	Leucine?	α-Ergocryptine?
ECC93					
cpps1-EC	DAIFCGGC	Valine?	DLVGMAAV	Phenylalanine?	Ergocristine?
cpps4-EC	DAISCGGC	?	DLAGMGAV	Leucine?	?

Since the third module always has to be specific for proline, only the first two modules each were compared. The assignment of probable substrates is based on the assumption that CPPS1 catalyzes ergotamine formation (see text).

2.2. Comparison of two chemical races of C. purpurea

Several "chemical races" of *C. purpurea* differing in their ergopeptine spectrum have been described (Pažoutová and Parbery, 1999). Since the relative proportion of alkaloids within a chemical race can be influenced by amino acid feeding (Németh, 1999), and since U. Keller and collaborators (Riederer et al., 1996) found that purified LPS1 shows some inaccuracy of amino acid activation depending on the amount of available amino acids (which could of course at last partially be due to "mixed" LPS-preparations, see above), the chemical races of *C. purpurea* could differ in the intracellular amino acid pools available for the ergopeptine assembly system. An alternative reason for the different alkaloid spectra could be variability of the NRPS genes present in the different chemical races, i.e., an intraspecies evolution of the alkaloid cluster. To address this question, we characterized the alkaloid gene cluster region of a second *C. purpurea* strain, ECC93. As shown in Fig. 4, this strain produces ergocristine as major ergopeptine, i.e., it represents a different chemical race.

Instead of sequencing the whole cluster region of ECC93, we performed a PCR approach to get an impression of the cluster organization. Primer pairs spanning the intergenic regions of the P1 cluster were designed (see Fig. 2). In most cases PCR products were obtained (Fig. 5), and the sequences showed high similarity to the P1 sequence, e.g., primers 3/4 (*cpox3*/*cpps2*): 92.2% identity; primers 5/6 (*cpps2*/*cp450-1*): 94.9% identity; primers 19/20 (*cpd1*/*orfC*): 95.3% identity. This indicates the existence of a highly conserved alkaloid gene cluster in ECC93. However, primers covering the region downstream of *orfC* (i.e., the trimodular NRPS genes) did not yield amplification products (Fig. 5), indicating sequence heterogeneity of the NRPS genes in this cluster region. In contrast, the genes downstream of *cpps4* – namely, *orfF*, *orfG* – were sufficiently conserved that the respective primer combinations based on the genes from P1 yielded amplification products from DNA of ECC93.

To characterize the heterogeneity of the NRPS genes, in more detail, a genomic lambda library of strain

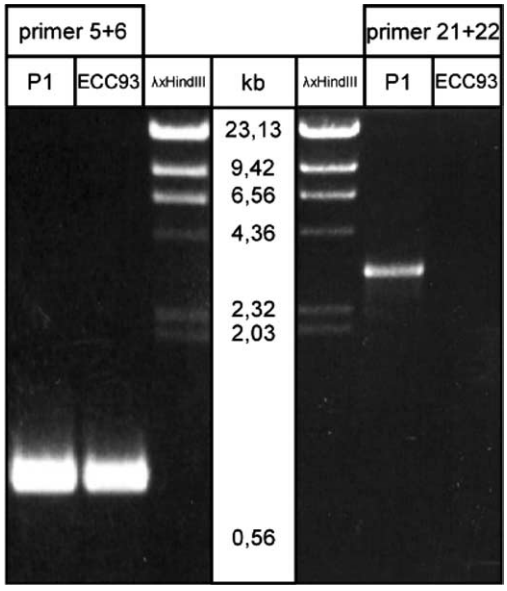

Fig. 5. Characterization of the ergot alkaloid cluster region in ECC93. PCR primers designed based on the corresponding P1 sequence (see Fig. 2) were used to amplify corresponding DNA fragments from strain ECC93. As examples, the results for primer pairs 5 and 6 (intergenic region of *cpps2* and *cpP450-1*), and 21 and 22 (intergenic region of *orfC* and *cpps1*) are shown.

ECC93 was established and screened for clones covering this region. Sequence analysis showed that *cpps1*- and *cpps4*-homologous NRPS genes are also present in the homologous cluster in ECC93, but they showed sequence differences that resulted in the lack of PCR amplification: e.g., the region covered by primers 25/26 (*orfE*/*cpps4*) showed only 43.2% identity. In contrast, comparison of the coding sequences of the "core" genes of the cluster based on genomic clones of P1 and ECC93 showed high identity (on amino acid basis): e.g., 90.9% for *cp450-1*, 97.6% for *cpcat2*, and 97.6% for *cpd1*.

The overall organization of the two NRPS genes of ECC93 is similar to that in P1, but a comparison of the substrate binding pockets (Table 3) shows significant differences. Interestingly, the comparison revealed that the substrate binding pockets of the first modules of CPPS4 and CPPS1-EC have high similarity, which fits

to the composition of their putative products: both, α-ergocryptine and ergocristine have a valine in the first position. In addition, the binding pockets of the second modules of CPPS1 and CPPS1-EC are identical, which is exactly what would be expected if CPPS1-EC catalyzes the biosynthesis of ergocristine: both alkaloids, ergotamin and ergocristine, contain phenylalanin in their second position!

These data strongly suggest that indeed the specificity of the LPS1 encoding NRPS genes determines the type of ergopeptine produced, though of course an additional impact of the amino acid pool cannot be excluded. An unequivocal proof could be obtained by targeted inactivation of the respective NRPS genes and by ectopic heterologous integration, e.g., will the transfer of *cpps1* into ECC93 induce ergotamine biosynthesis in the recipient strain? Because of the sizes of the respective genes this approach is not easy: though full-length copies of *cpps1* and *cpps4* are available from the cosmid clones, integration of the vectors in most cases would lead to incomplete copies, and ectopic expression might cause additional problems.

In conclusion, the data presented indicate that within the species *C. purpurea* the NRPS genes of the ergot alkaloid cluster have evolved substantially, whereas the genes in the central part of the cluster have remained highly conserved. It is intriguing to consider whether this may reflect diversifying selection for variation in alkaloid structures.

3. Experimental

3.1. Strains and culture conditions

Claviceps purpurea strains P1 and ECC93, which produce mainly ergotamine and ergocristine, respectively, have been described previously (Tudzynski et al., 1999; Keller et al., 1988), as were the standard media and culture conditions (Tudzynski et al., 1999).

For northern analyses, mycelia were cultivated in Mantle A medium with low (0.5 g/l KH_2PO_4) and high (2.0 g/l KH_2PO_4) amounts of phosphate, respectively. After 5 d mycelia were harvested and RNA was prepared using the *RNAgents® Total RNA Isolation System* (Promega).

3.2. HPLC-analyses

The HPLC system consisted of a D-7000 interface, L-7400 UV detector, L-7200 autosampler and a L-7100 pump (Merck/Hitachi). The HPLC separation was carried out on LiChrospher 100 RP-18 (5 μm) 250 × 4 mm column (Merck) at a flow rate of 1 ml/min operated at 25 °C. Compounds were eluted using a 1:1 mixture of 10 mM $(NH_4)_2CO_3$ and CH_3CN for 20 min. Standards used: 1 mg/ml ergotamine (Sigma), 1 mg/ml ergocryptine (Sigma) and 1 mg/ml ergocristine (Sigma).

3.3. Nucleic acid cloning and analysis

Standard cloning and DNA analysis techniques were performed according to Sambrook et al. (1989). *Escherichia coli* strains used for cloning (mostly in plasmid pUC19, Amersham Pharmacia Biotec) and propagation of λ clones were TOP10F' (Invitrogen) and XL1-Blue (Stratagene), respectively. Extraction of genomic DNA and RNA, southern and northern analyses, and DNA sequencing were performed as described (Mey et al., 2002).

A genomic library of strain ECC93 was established using the lambda DASH vector kit (Stratagene), following the manufacturer's manual. Genomic DNA was partially digested with *Sau*3AI, size fractionated (15 kb) in a sucrose gradient before ligation to the *Bam*HI-digested vector arms. The primary library had a phage titer of 2.36×10^{10} pfu/ml. For chromosome walking, the library was screened using the standard plaque filter hybridization technique as described (Tudzynski et al., 1999).

The cosmid library of genomic DNA from *C. purpurea* ATCC 20102 (which was derived from strain P1) was described previously, as was identification of three cosmids containing the *dmaW* homolog (Wang et al., 2004). Southern analysis indicated that one of these cosmids contained both *cpps1* and *cpps4* in their entirety. Random transposon insertions were generated by using the TnsABC transposase/Tn7 transposon-based genome-priming system (GPS-1; New England Biolabs; Beverly, MA), following the manufacturer's instructions. GPS-tagged cosmids were electroporated into *E. coli* XL1-Blue, and colonies were selected on kanamycin and ampicillin. Cosmid DNAs were purified by standard techniques, and sequenced with the GPS-1 North and South primers, by using DTCS sequencing kits (Beckman-Coulter) and analyzing products with a Beckman-Coulter CEQ8000. Sequences were assembled using Phrap, then viewed and edited using Consed (David Gordon; gordon@genome.washington.edu). Relative positions of contigs were determined by designing primers based on their end-sequences and conducting PCR tests. The PCR products were then sequenced. Contig sequences were used as queries in BLAST searches (www.ncbi.nlm.nih.gov/BLAST/) against the predicted protein database (blastx). In addition, contigs were submitted to FGENESH (www.softberry.com/berry.phtml) to predict the potential genes based on parameters for *Aspergillus* spp. and *Neurospora crassa* genomes. The predicted open reading frames (ORFs) were then further submitted to blastx and rpsblast.

For sequence comparisons, multiple sequence alignments and identity scores, Megalign (DNA STAR), Best FIT (HUSAR), or CLUSTALX (Thompson et al.,

1997) was used. For PCR analyses, BioTherm (Genecraft) polymerase was used according to the manufacturer's instructions.

PCR primers used (numbers in brackets refer to primer numbering in Fig. 2): (1) 5′-TTGCGACAG GAATCCATCTACAGCG-3′; (2) 5′-CGCACCGGG GGAGCCTACG-3′; (3) GCGGCGATTGCAGGAAG ATTG-3′; (4) CCCCAGGCGACATTAAGCACTA CC-3′; (5) 5′-CGGATGCGACGTATGCAATCAACT TAT-3′; (6) 5′-GCGGCCGCAGAACCTCGCCAAT GGATTG-3′; (7) 5′-GGCCATTGAAATACTTATA GAACGACTTATCC-3′; (8) 5′-TCAGGCGAACAGA TTGGAGCAGAC-3′; (9) 5′-AGATCTCCCAATTTC GATCCTGC-3′; (10) 5′-CACCACCTTGCAGAAA GCCTCCTACT-3′; (11) 5′-GGGAGGTGAAATTGG TGCTACTGACG-3′; (12) 5′-CACCACCTTGCAGA AAGCCTCCTAC-3′; (13) 5′-ACCGTGGGTGCAG TAGGAGGC-3′; (14) 5′-GGGTCGGAGGAAA GGGTGGTTAG-3′; (15) 5′-TGAGACCCGAACACT GGCATTACC-3′; (16) 5′-CCTATTTCCGTGGTCTG GCTATGTCC-3′; (17) 5′-ACCACGGAAATGAACG GAATCTTG-3′; (18) 5′-CTGGAACGCGGGCTG TATGC-3′; (19) 5′-GCCAACTATACACTGCACCA CAAC-3′; (20) 5′-CCTAATTCTCAACAGCGGA TAAAG-3′; (21) 5′-ATCCTATCTGCTCTCCTGC CTCTCG-3′; (22) 5′-TCTCCGAATACAAATCGT CGTCTAACAG-3′; (23) 5′-CGGGATTCGGAGTGC CTGTTACC-3′; (24) 5′-CCATGGGGTCTGCGTT TGTGATTT-3′; (25) 5′-GACGCACCAACCTCGCTA TGAATGT-3′; (26) 5′-GCCGCCGGCCTTCA TAACG-3′; (27) 5′-CTGCGGCGCGACTGGAA GAT-3′; (28) 5′-GAGGCGCTTCACGGGGAGAC-3′; (29) 5′-GCAAGCGGCCAGTAACCAGAATG-3′; (30) 5′-CGCCTGCCGCACCCAACAA-3′.

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