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Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*

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Abstract A gene (*cpd1*) coding for the dimethylallyltryptophan synthase (DMATS) that catalyzes the first specific step in the biosynthesis of ergot alkaloids, was cloned from a strain of *Claviceps purpurea* that produces alkaloids in axenic culture. The derived gene product (CPD1) shows only 70% similarity to the corresponding gene previously isolated from *Claviceps* strain ATCC 26245, which is likely to be an isolate of *C. fusiformis*. Therefore, the related *cpd1* most probably represents the first *C. purpurea* gene coding for an enzymatic step of the alkaloid biosynthetic pathway to be cloned. Analysis of the 3′-flanking region of *cpd1* revealed a second, closely linked ergot alkaloid biosynthetic gene named *cpps1*, which codes for a 356-kDa polypeptide showing significant similiarity to fungal modular peptide synthetases. The protein contains three amino acid-activating modules, and in the second module a sequence is found which matches that of an internal peptide (17 amino acids in length) obtained from a tryptic digest of lysergyl peptide synthetase 1 (LPS1) of *C. purpurea*, thus confirming that *cpps1* encodes LPS1. LPS1 activates the three amino acids of the peptide portion of ergot peptide alkaloids during D-lysergyl peptide assembly. Chromosome walking revealed the presence of additional genes upstream of *cpd1* which are probably also involved in ergot alkaloid biosynthesis: *cpox1* probably codes for an FAD-dependent oxidoreductase (which could represent the chanoclavine cyclase), and a second putative oxidoreductase gene, *cpox2*, is closely linked to it in inverse orientation. RT-PCR experiments confirm that all four genes are expressed under conditions of peptide alkaloid biosynthesis. These results strongly suggest that at least some genes of ergot alkaloid biosynthesis in *C. purpurea* are clustered, opening the way for a detailed molecular genetic analysis of the pathway.

Key words Peptide alkaloids · D-lysergyl-peptide synthetase · Dimethylallyltryptophan synthase · FAD oxidoreductase

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Introduction

Claviceps purpurea is a common pathogen of grasses, which attacks young ovaries and forms the typical ergot sclerotia (Tudzynski et al. 1995). These dormant structures contain ergot alkaloids, pharmaceutically important mycotoxins which have a wide range of therapeutical applications and are produced world-wide on a large scale. Under natural conditions, alkaloid biosynthesis is restricted to sclerotial tissue formed during parasitic culture; however, several deregulated strains have been described which produce significant amounts of alkaloids under axenic culture conditions (Lohmeyer and Tudzynski 1997).

Although the physiology and biochemistry of ergot alkaloid biosynthesis have been studied in detail, knowledge of the enzymes involved is limited (Socic and Gaberc-Porekar 1992). As shown in Fig. 1, biosynthesis begins with dimethylallyltryptophan which is converted, via several intermediates, into lysergic acid (Fig. 1A). Successive condensation of the D-lysergic acid moiety with three amino acids and modification and cyclization of the resultant D-lysergyl tripeptide yields the peptide alkaloids (ergopeptines) (Fig. 1B). So far only a few enzymes of the pathway have been characterized in detail, among them the dimethylallyltryptophan synthase (DMATS) (Gebler and Poulter 1992), which, as the catalyst of the first pathway-specific step, is probably a

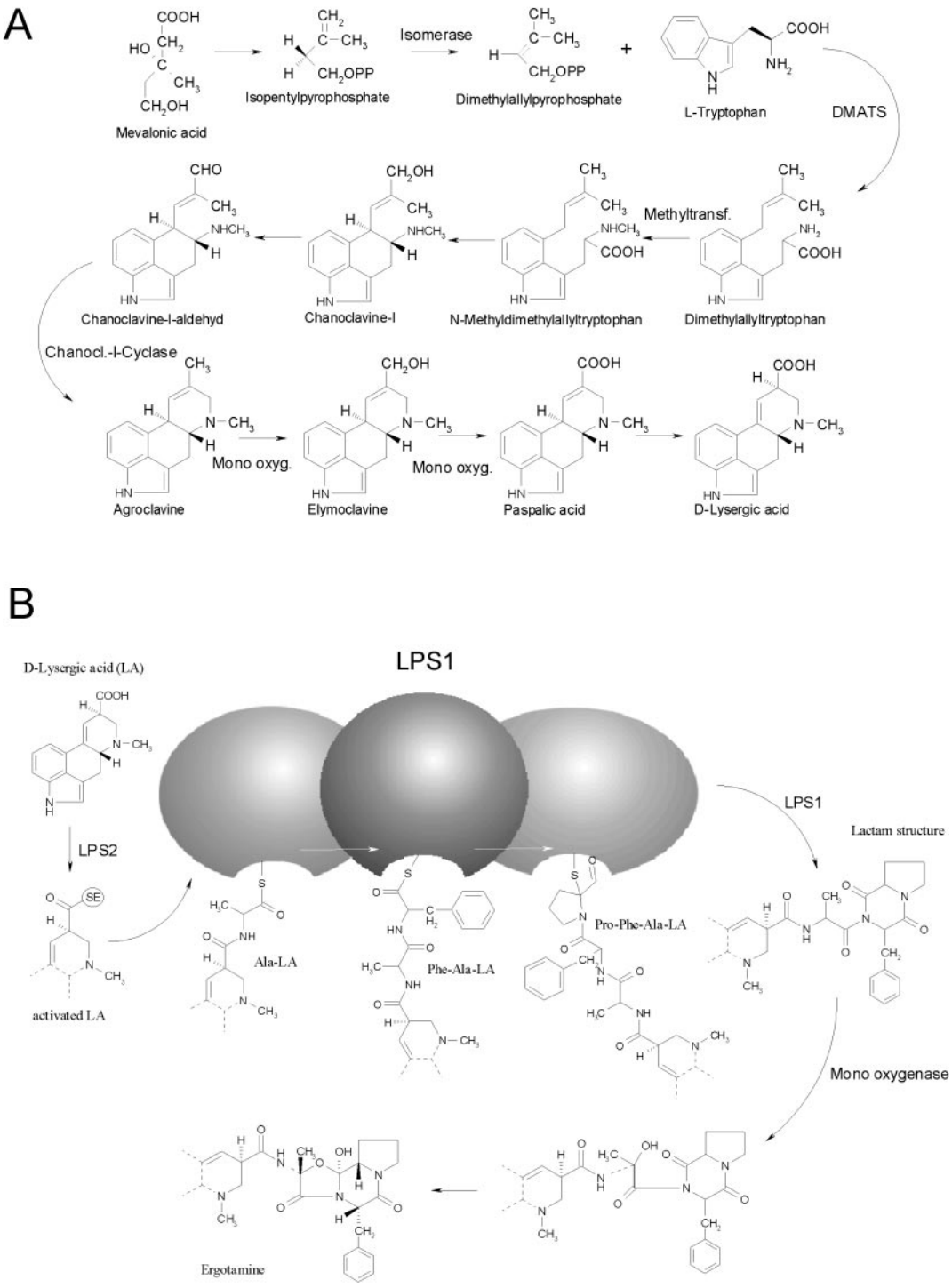

Fig. 1A, B The ergot alkaloid biosynthetic pathway. **A** Biosynthesis of lysergic acid. **B** Biosynthesis of ergopeptines, as exemplified by ergotamin (modified from Lohmeyer and Tudzynski 1997 and Walzel et al. 1997). LPS, D-Lysergyl peptide synthetase; DMATS, dimethylallyltryptophan synthase; SE, thioesterified LPS2

strictly regulated enzyme; chanoclavine cyclase has been preliminarily characterized as an FAD-containing oxidoreductase (Socic and Gaberc-Porekar 1992). Furthermore the D-lysergyl peptide synthetases (LPS1 and 2) catalyze the attachment of D-lysergic acid to three amino acids of the peptide portion of ergot peptide alkaloids by a non-ribosomal mechanism (Riederer et al. 1996; Walzel et al. 1997).

Alkaloid biosynthesis in axenic culture is strictly regulated in most strains: tryptophan acts as precursor and inducer, while phosphate, glucose and ammonium repress synthesis (Socic and Gaberc-Porekar 1992). As a potential target for these regulatory circuits, the

DMATS is of special interest. Recently a gene for DMATS (*dmaW*) was cloned from strain ATCC 26245 via reverse genetics (Tsai et al. 1995); we identified the same gene (using the same strain) by a differential cDNA screening approach and could show that it is induced in alkaloid-producing cultures, i.e. it is transcriptionally regulated (Arntz and Tudzynski 1997). The strain used in these first molecular studies, ATCC 26245, has turned out to be a *C. fusiformis* isolate rather than a *C. purpurea* strain (Pazoutova and Tudzynski 1998); it produces no peptide alkaloids, only clavines. Therefore we decided to clone and characterize the corresponding gene from a "genuine" *C. purpurea* isolate, P1, a (mutant) strain that produces peptide alkaloids in axenic culture. Since there are now several examples for the clustering of genes involved in secondary metabolite pathways in fungi (Keller and Hohn 1997), we initiated a chromosome walking approach starting from the DMATS gene. The results presented here provide strong evidence for clustering of ergot alkaloid genes in *C. purpurea*.

Materials and methods

Strains and culture conditions

C. purpurea strain P1 [= 1029N5, a derivative of strain 1029 (Keller 1983), which produces peptide alkaloids in axenic culture], was grown in the dark at 24° C on a rotary shaker (200 rpm), either for 5 days in BII medium (Esser and Tudzynski 1978) for DNA preparation, or for the induction of alkaloid biosynthesis (and RNA isolation) by a three-step culture method according to M. Lohmeyer (personal communication): mycelia were cultivated for 4 days in pre-culture medium (PCM, 100 g/l glucose, 0.5 g/l KH_2PO_4, 10 g/l citric acid, 1.0 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4 \times 7H_2O$, 0.12 g/l KCl, 0.006 g/l $ZnSO_4 \times 7H_2O$, 0.007 g/l $FeSO_4 \times 7H_2O$, 0.75 g/l nicotinic acid amide, pH 5.2), then transferred for 3 days to fresh medium, and then to the main culture medium (as PCM, but cotaining 300 g/l sucrose instead of glucose, 15 g/l citric acid, and KH_2PO_4 as indicated). Every 24 h the total alkaloid content of the culture medium was determined according to Lohmeyer et al. (1990).

Techniques of protein analysis

LPS1 was purified from *C. purpurea* strain D1 as described by Riederer et al. (1996) with slight modifications: protein precipitate (35–55% ammonium sulfate, w/v) was size fractionated on an Ultragel AcA34 column and further FPLC purified on an anion exchange matrix (Resource Q, Pharmacia). LPS1 was eluted with buffer B containing 160 mM NaCl and finally purified by preparative 5% SDS-PAGE. Coomassie stained LPS1 was in-gel digested with trypsin as described (Stone and Williams 1993). Tryptic peptides were separated by HPLC on a μRPC-C2/C18-PC 3.2/3 column (Pharmacia) with an acetonitrile/H2O gradient. Peptides were further purified on a Sephasil-C18-SC-2.1/10 column (Pharmacia). Sequence analysis of peptides was performed by automated Edman-degradation on an Applied Biosysthem (Procise) sequencer.

Molecular techniques

Agarose gel electrophoresis, preparation of *E. coli* DNA and bacteriophage lambda DNA, and standard cloning procedures were performed according to Sambrook et al. (1989). Preparation of fungal DNA and RNA was done as described by Cenis (1992) and Chambers and Russo (1986); Southern and Northern analysis was performed according to Ausubel et al. (1987) and Church and Gilbert (1984), respectively.

DNA sequencing was performed on a Licor 4000 (MWG, Ebersberg) automated sequencer, using the cycle sequencing protocol of the manufacturer, with either pUC19 or pBluescript based primers or – for longer clones – with insert-based primers. For evaluation of the nucleotide and derived amino-acid sequence HUSAR (EMBL, Heidelberg) was used.

A genomic library of strain P1 in EMBL3 was established according to Sambrook et al. (1989): *Sau*3A digested genomic DNA of strain P1 was size-fractionated using two successive sucrose gradients; fragments of 14–15 kb were ligated to *Bam*HI-digested EMBL3 arms (Stratagene), and packaged in vitro (Packagene extract, Promega). Library screening was performed by plaque filter hybridization according to Sambrook et al. (1989). Positive plaques were replated and purified as single plaques. DNA was extracted and characterized by restriction analysis and Southern hybridization.

RT-PCR was performed as described by Tenberge et al. (1996), using the primer pairs indicated in the text. Primer sequences were the following; for *cpd1*: P1 (5′-TACATTGGCAATTATGAC-3′), P2 (5′-CCTGTAGGCGTCGAAAG-3′); for *cpps1*: P3 (5′-CAACACCCTGATCACGGTC-3′) and P4 (5′-GTTTGCCGAGTACCTTGCTG-3′); for *cpox1*: P5 (5′-GGAAACCGAGGCACGAACC-3′) and P6 (5′-GCGTGTCTTGGTAGTGATACC-3′); and for *cpox2*: P7 (5′-GCATTGCTGCC GACGTCTTC-3′) and P8 (5′-GCACGATGACTGCTTCAATC-3′).

Accession numbers

The sequences reported in this paper have been assigned the following Genbank accession numbers: *cpd1*, AJ 011963; *cpps1* AJ 011964; *cpox1*, AJ 011965; *cpox2*, AJ 011966.

Results

Cloning and characterization of a DMATS gene from *C. purpurea*

To study DMATS expression during alkaloid biosynthesis by *C. purpurea* in axenic culture, we decided to clone a DMATS gene from a production strain. We chose the strain P1, a derivative of *C. purpurea* 1029, which shows improved productivity for peptide alkaloids (mainly ergotamin) in axenic culture. We established a genomic library from strain P1 in lambda EMBL3 (see Materials and methods) and probed it with the cDNA clone of *dmaW*. From among the 40,000 plaques screened, we obtained 11 positive phages after two rounds of screening. Positive phages were characterized by restriction analysis, subcloning and sequencing. Two different copies of putative DMATS genes were obtained: four of the positive lambda clones contained a version of the gene which was inactivated by a frameshift caused by an insertion of 7 bp (Arntz 1998; C. Arntz, T. Correia and P. Tudzynski, unpublished); the other seven clones, which derive from a different genomic region, carried an active version (as judged from primary sequence data) of the putative DMATS gene, which was named *cpd1*. The gene contains an ORF of 1344 bp, which codes for a polypeptide of 448 amino acids. The coding region is interrupted by two introns of

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                .         .         .         .         .
dmaW  MMT.KAPATAVYDTLSLLFDFPNQEQRLWWHSIAPMFAAMLDTAGHNVHDQYR 52
      | |.|.|:.:||:.|||:|||||..||||||||.|||||||||.||.|:|||||
cpd1  MSTAKDPGNGVYEILSLIFDFPSNEQRLWWHSTAPMFAAMLDNAGYNIHDQYR 53
                .         .         .         .         .
       53 HLGIFKKHIIPFLGVYPAQGKHTWPSVLTRYGIPFELSLNCLDSVVRYTF 102
          |||||||||||||||||..:|. | |:|||:|:|:|||||| |||||||:
       54 HLGIFKKHIIPFLGVYPTKDKERWLSILTRCGLPLELSLNCTDSVVRYTY 103
                .         .         .         .         .
      103 EPTTEHTGTGDDSYNAFAILECIQKLVRIQPGIDMEWFSYFRNELVLNAT 152
          ||..| |||:.|.:|.:||:.::|||..||:|||:||||||::||.|:..
      104 EPINEVTGTEKDTFNTLAIMTSVQKLAQIQAGIDLEWFSYFKDELTLDES 153
                .         .         .         .         .
      153 ESARLGRNDSVNQQPIRTQNKLALDLKGDRFALKVYLYPHLKSIATGVSS 202
          ||| |..|: |.:| |:||||||||||:..||||||:|||||||||| |.
      154 ESATLQSNELVKEQ.IKTQNKLALDLKESQFALKVYFYPHLKSIATGKST 202
                .         .         .         .         .
      203 HDLIFNSVRKLSQKHTSIQPSFNVLCDYVASRNDPDSNAAEAEAGVPASA 252
          |||||:|| ||||||.||||.|.||||||..||    :.||.:   . :|
      203 HDLIFDSVLKLSQKHDSIQPAFQVLCDYVSRRN....HSAEVD...QHGA 245
                .         .         .         .         .
      253 LRARLLSCDLVDPSKSRIKIYLLEQTVSLTAMEDLWTLGGRRTDSSTLNG 302
          |:|||||||:||.|||:||||||.||||..|||||||||.|.|.|||::|
      246 LHARLLSCDLIDPAKSRVKIYLLEKTVSLSVMEDLWTLGGQRVDASTMDG 295
                .         .         .         .         .
      303 LDMMRELWHLLQIPSGFMKYPESDLKLGEVPDEQLPSMVHYALHPDQPMP 352
          |||:|||| ||.:|.| :.||.: |.|||:|:|||||.:|.||.::|||
      296 LDMLRELWSLLKVPTGHLEYPKGYLELGEIPNEQLPSMANYTLHHNDPMP 345
                .         .         .         .    ↓    .
      353 EPQVYFTVFGMSDAGITNALATFFSRHGWYEMAKKYRVFLEGSFPNHDFE 402
          ||||||||||.||:|.|||..|| |||: :|||.|||||::|:| ||||
      346 EPQVYFTVFGMNDAEISNALTIFFQRHGFDDMAKNYRVFLQDSYPYHDFE 395
                .         .         .    ↓    .         .
      403 SLNYLHTYVSFSYRKNKPYLSVYLHSFETGQWPAFSDDPTAFNAFKRCDL 452
          ||||||.|:|||||:||||||||||.||||.||.|.|.|..|:|::||:|
      396 SLNYLHAYISFSYRRNKPYLSVYLHTFETGRWPVFADSPISFDAYRRCEL 445

      453 SLT* 455
          | .
      446 STK* 448
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Fig. 2 Alignment of the amino acid sequences derived from *cpd1* of *C. purpurea* P1 and *dmaW* from *C. fusiformis* (Tsai et al. 1995). The major variable region and a putative prenyltransferase domain are indicated in *bold face*

65 and 54 bp. The presence of the introns (and transcription of the gene) was confirmed by the sequencing of an RT-PCR product using primers P1 and P2 and mRNA from an alkaloid-producing culture of *C. purpurea* P1 as a template (data not shown). The derived gene product (CPD1) shows significant homology (68% identity) to the *dmaW* gene from ATCC 26245 (Fig. 2). However, this value is lower than would be expected for two closely related strains, this supporting the classification of ATCC 26245 as another species (*C. fusiformis*, see above). The two introns in *cpd1* are in the same positions as those in *dmaW*; the derived gene product is smaller (seven amino acids shorter than DMAW), due to deletions of amino acids at position 167 and in the region of residues 232–252 (see Fig. 2). In addition, the putative "crippled" prenyl transferase domain identified by Tsai et al. (1995) at residues 113–117 (DDSYN) differs significantly from that in the CPD1 sequence. The promoter region is completely different from the published portion of *dmaW* and also from that of the inactive copy in strain P1 (data not shown). It contains putative binding sites for (homologues of) several regulatory proteins of *Aspergillus nidulans*: for AREA, the major positive regulatory element of nitrogen metabolism (Caddick et al. 1986), for CREA, involved in catabolite repression (Felenbok and Kelly 1996), for PACC (pH control, Tilburn et al. 1995) and – especially interesting – for NUC1, a positively acting regulatory protein which mediates gene activation under starvation for phosphate in *Neurospora crassa* (Peleg and Metzenberg 1994). In addition, there are putative binding sites for homologues of the *A. nidulans* developmental regulatory proteins ABAA and STUAP (Andrianapoulos and Timberlake 1994; Dutton et al. 1997).

Chromosome walking in the region downstream of *cpd1* identifies *cpps1*

Since evidence is accumulating that fungal genes involved in secondary metabolism are often arranged in clusters (Keller and Hohn 1997), we sequenced parts of the genomic region (contained in three overlapping lambda clones) around *cpd1* (see Fig. 3). Downstream of the gene we found an ORF with no similarity to any published sequence (data not shown), and immediately adjacent to it a large ORF showing significant similarity to bacterial and fungal peptide synthetases, e.g. to the HC-toxin synthetase of *Cochliobolus carbonum* (Scott-Craig et al. 1992).The complete gene (named *cpps1*), which encompasses more than 9 kb, encodes a protein of

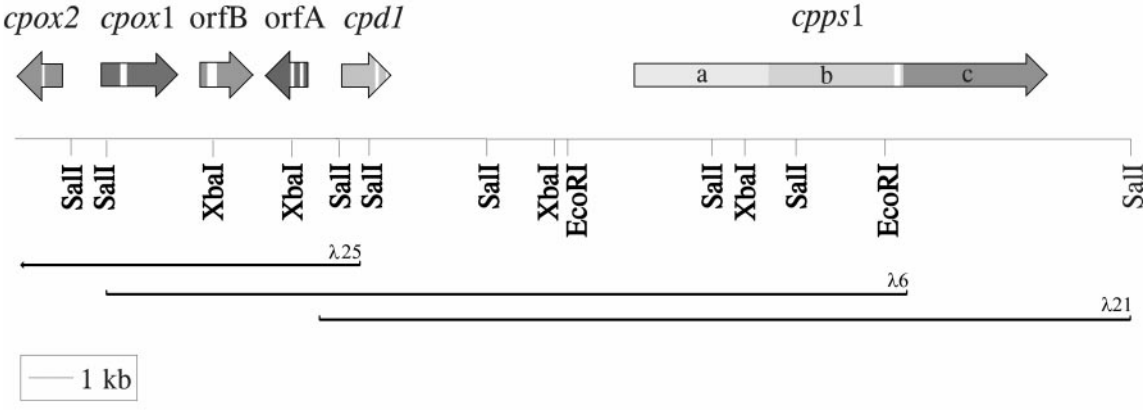

Fig. 3 Schematic map of the genomic region including *cpd1*. The orientation of the genes is indicated by *arrowheads*, the three modules of *cpps1* are indicated by different shading, positions of introns by *open boxes*. The arrangement of the three overlapping lambda clones which form the basis for this map are shown by the lines *below* the map

3233 amino acids containing three repeats of the conserved domains described by Marahiel et al. (1997) as characteristic for bacterial and fungal peptide synthetases (Figs. 3, 4). Obviously *cpps1* encodes a trimodular peptidesynthetase, comparable to the alkaloid biosynthetic enzyme LPS1 described by Riederer et al. (1996). To prove this, purified LPS1 was digested with trypsin and several of the resulting peptides were purified and subjected to microsequencing. As shown in Fig. 4, the sequence of a 17-residue peptide shows a nearly perfect match to a sequence in the second module of CPPS1. Analysis of additional (shorter) peptides gave similar results (not shown).

Within the terminal part of the second module a frameshift was observed which is flanked by typical fungal intron consensus sequences. This was surprising, since – with only one exception (Bailey at al. 1996) – all fungal peptide synthetase genes characterized until now do not contain introns. We therefore performed RT-PCR to confirm the presence of an intron in this module. Using primer P4 (see Materials and methods) for reverse transcription of mRNA isolated from an alkaloid-producing culture and primers P3 and P4 for PCR, we obtained a single PCR product of 753 bp, 99 bp smaller than the corresponding control PCR product from genomic DNA (see Fig. 5a). Sequence analysis showed that the mRNA-derived PCR product lacks exactly the DNA region flanked by the intron consensus sequences (Fig. 5b). Thus the presence of an intron in the second module of *cpps1* is confirmed; no evidence for any additional intron in *cpps1* was found.

Taking the intron into account, the derived gene product of *cpps1* has a calculated molecular weight of 356 kDa and a pI of 6.04. A computer-based evaluation of the upstream non-coding sequences of *cpps1* revealed several putative binding sites for regulatory proteins, e.g. CREA, AREA, PACC and STUAP (data not shown).

Chromosome walking reveals two putative oxidoreductase genes upstream of *cpd1*

Sequence analysis of the region upstream of *cpd1* (see Fig. 3) revealed two ORFs which show similarity to data bank entries of unknown function [ORFA: YesF of *Saccharomyces cerevisiae* (A69580); ORFB: hypothetical protein Rv3701c of *Mycobacterium tuberculosis* (AL 022121)]. A larger ORF, containing one intron, was detected upstream of ORFB. The derived gene product of 493 amino acids shows significant similarity to several FAD-containing oxidoreductases, e.g. to the 6-hydroxy-D-nicotine oxidase of *Arthrobacter oxidans* (Brandsch et al. 1993) and to the putative berberine bridge enzyme of *Arabidopsis thaliana* (an enzyme involved in alkaloid

Fig. 4 Alignment of the three amino acid-activating modules of CPPS1 with the second module of the HC-toxin-synthetase of *Cochliobolus carbonum* (Hts; Scott-Craig et al. 1992) and a partial peptide sequence derived from LPS1 (in *bold face*). Residues conserved within the three modules of CPPS1 are printed in *bold face*, residues that are identical in all sequences are marked by *asterisks*. The locations of the conserved domains described by Marahiel et al. (1997) are indicated by *overlining* (A1-A10, T)

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                             A1                                         A2
CPPS 1a   CCSRPTCQAVASWDGCLTYDEMSILSSHLAQRLRAAGVKPGVFVALCLDRCKWAVIGILAVLKAGGAFCALDSSYPVSRLQDMCRDLEITIVLTVKTN-----IQHASPLAD   182
CPPS 1b   CQAQPDATAIDAWDGCVTYGELERRAEKLAGLVRSHVSKPDQVVVLYFSKSWLTVVAQLAVLKAGAAFITLEISQPVHYLQRVISALGPVLVLTSEDL-----FSAAEDLQE   1274
CPPS 1c   CLRCPDSPSVCAWDGNFTYRQLDDLSSALSEEIVRKGAGPEVTIPIVLEKTRWTPVAILAVLKSGSSFVLMDSTHPAARVGSIVQAIGPPVIIVSAQT-----RSKVATFST   2384
HTS1      VVEHPQREAICSWDGSLTYAELSDLSQRLSIHLVSLGIKVGTKIPICFEKSMWTIVTILAVVQAGGVFVLLEPGHPESRLSGIIKQVQAELLLCSPATSRMGALQNISTQMG   1942
              *       ***  **         *                             ***   *  *       *

                                                  A3                                        A4
CPPS 1a   ---TVILLDDDLYSESALSSAQKCASRSSLSPHDPVYAVFTSGSTGKPKGIIMEHASFSACALSSMEP---LHIGPQDRVLHFASYAFDLSLFEILAPLIAGATVVIPSEKA   288
CPPS 1b   NAVPVMAVDKDDLSDATARTSQASSS-ACTVECDLMYIIATSGTTGMPKIVMTDHQAFMTNASPLMNG---LGITSDSRVFQFCGYSFDLLIVEHFLTLLAGGCICIPSLHN   1382
CPPS 1c   ---DVVEVGDWLAREVPFEKQQGTRQTGLLKATNAAYLVFTSGSTGKPKGAIVEHASLSTAAKYMASR---LHIDSASRVLQFSSHAWDIPVTEVLVTLRMGGCVCVPSEEE   2490
HTS1      TEFKIVELEPEFIRSLPLPPKPNHQP--MVGLNDDLYVVFTSGSTGVPKGAVATHQAYATGIYEHAVACGMTSLGAPPRSLQFASYSFDASIGDIFTTLAVGGCLCIPREED   2052
                                              *   *** ** **     *                       *   *     *         *  *     *

                                                                          A5
CPPS 1a   RL-ENLPCAMTDLGATWAFLTPTVARLYRPTQMPTLKTLCLGGEAVNASDIKSWSS-KNLISGYNPAECCPLGIS-GLLNDHMPRALGSTFPS-QMAWIVDPEDHEKLLPV-   396
CPPS 1b   RN-NRFAASIVELEANWVGSPSSVLQLLDPQTVPTVKTIMQAGERLQQGLVDRWASHVRLINAYGPAECSVGALARDTVRPDTDDVQNLGFATGSVCWIVNAETSEKLLPVP   1493
CPPS 1c   RT-GNLAKASERMKVNWALWTPTVARLFKPEEFPHLKTLVFAGEALSATDLETWCDRVRLVQGYGPAECSLISTVTDPLTRSDN-PRCIGLPSGCVAWVVNRDNHELLAPP-   2600
HTS1      RNPAGITTFINRYGVTWAGITPSLALHLDPDAVPTLKALCVAGEPLSMSVVTVWSKRLNLINMYGPTEATVACIA-NQVTCTTTTVSDIGRGYRATTWVVQPDNHNSLVPI-   2163
          *               *            *   *        **         *     *   * * *                             * *       * *

                  A6                                             A7                      A8
CPPS 1a   -GAIGELAIEGPVVARGYVHDLKSSDSSTPFVVKTPTWLCRFRSNINRS--NRIYLTGDLARQDCDDGSVHYLGRKDDQVKIHGQRVELAEIEQHIEQHFSSLATKAV-VML   504
CPPS 1b   IGAEGELIIEGHTLSRGYLGDADKTNAS---FLRLPNWLRDFRADRGQSQGHRVYLTGDIVRQNSD-GSMSFVRRKDAQVKIRGQRVELTDVEHQVERCFIGAHQ-VVTDIV   1600
CPPS 1c   -GATGELVLEGPIVGRGYLGDPGRAASA---FISPPAWLMRLRGS-GSS--NRLYKTGDLVRQHVSSGLLTFVGRNDDQVKVRGQRVEPGEVEGQVAQVFPGSQ----VIVL   2700
HTS1      -GAVGELIIEGSILCRGYLNDPERTAEV---FIRSPSWLHDLRPN---S---TLYKTGDLVRYSAD-GKIIFIGRKDTQVKMNGQRFELGEVEHALQLQLDPSDGPIIVDLL   2263
           ** ***  **    ***  *              * **   *     *     * ***  *     *      * * ***  *** *    *

                                                                            A9                  A10
CPPS 1a   LRPISGRTVLTALVMPHQRLENGEKTSNSLL-------MELADINQDFRATLALAASKLR--LALPSHMVPSVYLPIR-HFPTTKGGKIDRGHLQSLLLSLPPEYLYGSEES   605
CPPS 1b   QIPNSQSSILVALVLTKDAMTN-HKQQESLLDQKSAGGLSILAPTSSFTANANAAETALQ--DRMPAYMVPDLFVPVS-DLPREASGKIGRKAIKQYLASLTQQDWS--RY-   1707
CPPS 1c   VVKKSAGAVLAALVLQNGEDRSSAGETANLFP--------P--PSLAFAALAKAAFSKLR--ETMPTYMIPSIMLPIS-YLPKAATGKADRNLLRDRVASLSDGEIEAYVAY   2799
HTS1      KRTQSGEPDLLIAFLFVGRANTGTGNSDEIFIA------TSTSSLSEFSTVIKKLQDAQRAMEVLPLFMVPQAYIPIEGGIPLTAAGKIDRRMLRKLCEPFNRNDLISFTSF   2369
              *    *                                     *                    * *    *     *    **  *
                                                                          MPAYMVPDLFVPVS DDP

                                                       T
CPPS 1a   EEATTHQGEEPKSDREKLLQGCFAQALDLPRTRIDLDSN--FFQLGGDSLSAM   653
CPPS 1b   RYSSTRKVP-PSNATEHEISAIWARVLQIEPHTFGVHDS--FFRLGGDSISGM   1753
CPPS 1c   VAASVSHRP-ASTAMEAELQRLVGQVLQRPLHSISLDED--LFRLGMDSLTAM   2846
HTS1      TSKALSTSVKDAETTDTVEDRLARIWEKVLGVK-GVGRESDFFSSGGNSMAAI   2418
                                                    *   * *
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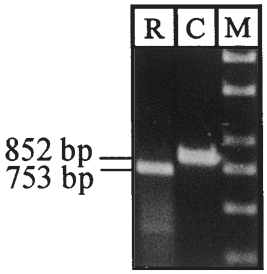


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                PS3
              ------>

a   CAACACCCTGATCACGGTCGTCAATGACCAGCCCGAGGACCATGTCTCTCAATCTGCACT
b   CAACACCCTGATCACGGTCGTCAATGACCAGCCCGAGGACCATGTCTCTCAATCTGCACT
c    N  T  L  I  T  V  V  N  D  Q  P  E  D  H  V  S  Q  S  A  L   2177

a   GAGGCTGGTCGGTGATTCAGTCCAGAGTCGATCCGAGgtaggtcattcctatattgctat
b   GAGGCTGGTCGGTGATTCAGTCCAGAGTCGATCCGAG.....................
c    R  L  V  G  D  S  V  Q  S  R  S  E                        2189

a   tccccaacgcgtcatgacttcgtgtattctaatcgcgtaacaaattcggtttgtgggttc
b   ...........................................................
c

a   ttttttactactacagTACCCGATCACCTTGAATATTCTCAACCATGCGGACAAGATCAA
b   ...............TACCCGATCACCTTGAATATTCTCAACCATGCGGACAAGATCAA
c                  Y  P  I  T  L  N  I  L  N  H  A  D  K  I  K   2204

a   GATGCAGCTATCGTACCACACGTCGTTGCTAAGCGGCGTCTCTGCCAACACCATTGCCAA
b   GATGCAGCTATCGTACCACACGTCGTTGCTAAGCGGCGTCTCTGCCAACACCATTGCCAA
c    M  Q  L  S  Y  H  T  S  L  L  S  G  V  S  A  N  T  I  A  K   2224
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Fig. 5A, B Evidence for the presence of an intron in cpps1. **A** RT-PCR experiment using total RNA from an alkaloid-producing culture of strain P1 (R) or genomic DNA from P1 (C) as template, primer P4 for reverse transcription and primers P3 and P4 for PCR. *M*, Molecular weight marker (1 kb ladder, GIBCO-BRL) **B** Alignment of part of the genomic *cpps1* sequence (a) with the corresponding part of the RT-PCR product obtained with primers P3 and P4 (b) and the derived amino acid sequence (c). The position of primer P3 is indicated by the *arrow*, primer P4 binds further downstream (see Materials and methods)

metabolism; Dittrich and Kutchan 1991). The putative oxidase gene was named *cpox1*. Figure 6 shows a comparison of CPOX1 and these two sequences in the region of the site of covalent binding of FAD. This polypeptide could very well correspond to the chanoclavine cyclase which catalyses the ring D cyclization, yielding agroclavine; this enzyme is probably an FAD-containing oxidoreductase (Floss 1976; Socic and Gaberc-Porecar 1992). Further upstream, a second putative oxidoreductase gene, termed *cpox2*, was detected (see Fig. 3). Its derived product (261 amino acids) shows similarity to several bacterial enzymes of the short-chain dehydrogenase/reductase (SDR) family, e.g. a dehydrogenase from the rapamycin gene cluster of *Streptomyces hygroscopicus* (Schwecke et al. 1995). The common promoter-region of *cpox1* and *cpox2*, like that of *cpd1*, contains several putative binding sites for regulatory proteins. RT-PCR experiments confirmed that both genes, *cpox1* and *cpox2*, are expressed in ergot alkaloid-producing mycelia, and that they both contain one intron (*cpox1*: 52 bp; *cpox2*: 63 bp) (data not shown).

Fig. 6 Alignment of parts of the amino acid sequences derived from *cpox1* (a) with two oxidoreductases containing covalently bound FAD, the berberine bridge enzyme of *Arabidopsis thaliana* (AC004238; b) and the 6-hydroxy-D-nicotine oxidase of *Arthobacter oxidans* (Brandsch et al. 1987; c). The histidine residue to which the FAD is covalently linked in the bacterial enzyme is *boxed*; residues common in the CPOX1 sequence and at least one of the two other sequences are printed in *bold face*; *asterisks* indicate residues conserved in all three sequences

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a   LPLYSAVVRSTSDIQASVRFASRHNLRLVIKNTGHDSAGRSSAPHSFQIHTSLLQNISLHKNF   75
b   KPEVIVAAVTETHIRATISCCKLLNLELRIRSGGHDYEGFSYTSPVPFVILDMYNFNKIDIN-   143
c   RPSLIARCLSAGDVAKSVRYACDNGLEISVRSGGHNPNGYATNDGGIVLDLRLMNSIHIDT--   97
     *                          *       **   *

a   IARGSTTGRGPAVTLGAGVMQWQAYVHGAKNG--YTILGGECPTVGAVGGFLQGGGVSSIHSF   136
b   -------MKDETVWIQSGASLGQLYYNIASKSKVHAFPAGVCPKVGAGGHFSGGG--FGNLMR   197
c   --------AGSRARIGGGVISGDLVKEAAKFG--LAAVTGMHPKVGFCGLALNGG--VGFLTP   158
                      *          *          *  * **  *    **

a   TRGLAVDQVLEYQVVSANGDLITANEDNNQDLFWALKGGGGGTFGVVTEATVRVFSDDPVTVT   199
b   KYGLSIDHIIDAQIMDANGKVYRNRQAMGEDVFWAIRGGGGGSYGVILAWKIKLVRVPEKVTV   260
c   KYGLASDNILGATLVTATGDVIYCSDDERPELFWAVRGAG-PNFGVVTEVEVQLYELP-----   205
      **  *          * *              ***  * *    **
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Discussion

Characterization of *cpd1*, a putative DMATS gene

We have cloned and characterized a putative DMATS gene, *cpd1*, from the *C. purpurea* strain P1, using a cDNA clone of *dmaW*, the DMATS gene from *Claviceps* spec. ATCC 26248 as a probe. The gene is nearly identical to the corresponding gene from the field isolate T5 (Arntz 1998; C. Arntz, T. Correia and P. Tudzynski, unpublished). However, the *cpd1* gene product has only limited homology (67% identity) to DMAW, confirming that ATCC 26245 is indeed not closely related to *C. purpurea* P1; it is most probably a *C. fusiformis* isolate (Pazoutova and Tudzynski 1998). Nevertheless, the degree of amino acid sequence identity is highly significant (as are the conserved positions of introns), strongly supporting the idea that *cpd1* codes for a DMATS. Both the estimated molecular weight (51.7 kDa) and the pI (5.82) correspond well to the enzymatic data published, for example, by Gebler and Poulter (1992). Interestingly, apart from a short deletion in the *C. purpurea* sequence (at positions 245–250 of the *dmaW* sequence), there is a further modification of the already reduced general prenyltransferase domain DDXXD (see Fig. 2), which is hardly recognizable in Cpd1 (XDXXN). The function of *dmaW* has been proved by expression in yeast (Tsai et al. 1995). Targeted gene inactivation can be achieved in *C. purpurea* (e.g. Garre et al. 1998), so disruption of *cpd1*

will provide an excellent means of confirming its function there, because the second DMATS gene detected in strain P1 will not interfere with the disruption, since sequence analysis and RT-PCR have revealed this copy to be inactive (C. Arntz, T. Correia and P. Tudzynski, unpublished).

The *cpd1* promoter contains several putative binding sites for homologs of the major regulatory proteins CREA (catabolite repression), and AREA/NIT2 (N metabolism), although only a few of them fulfill the criteria for spatial arrangement [e.g. for AREA/NIT2 there have to be short-distance pairwise arrangements (Chiang and Marzluf 1994)]. With respect to the physiology of alkaloid formation the putative binding site for a NUC1 homologue is especially interesting. NUC1 is a positive regulatory element of phosphate metabolism in *Neurospora crassa*, activating several genes under conditions of starvation for phosphate (Peleg and Metzenberg 1994). A comparable system could be operative in *C. purpurea*, triggering the induction of the alkaloid pathway under low-phosphate conditions. It had been postulated that the induction of alkaloid biosynthesis by lowering the phosphate level operates at the post-translational level (Krupinski et al. 1976); preliminary Northern experiments (Arntz 1998) and the presence of a putative NUC1 binding site indeed suggest that the DMATS gene is induced under conditions of low phosphate, concomitantly with the production of alkaloids.

The presence of putative binding sites for homologs of the *A. nidulans* regulatory proteins AbaA and StuaP is interesting, since these proteins are involved in the regulation of development in *A. nidulans* (sexual and asexual reproduction). Though these computer-derived data have to be interpreted very cautiously, it is tempting to speculate that the presence of these binding sites is connected with the strict developmental specificity of induction of this alkaloid pathway in natural isolates of *C. purpurea*: production occurs only in ripening sclerotia in planta. The ability of mutants such as P1 to produce alkaloids in axenic culture could be due to an absence of this developmental control, caused either by inactivation/modification of the regulatory gene (this alternative is supported by the fact that productive strains usually have abnormal morphology, no or significantly reduced asexual and sexual propagation and loss of pathogencity) or by modifications of the promoter(s) of genes coding for the key enzyme(s) of the pathway.

Evidence for an ergot alkaloid gene cluster

A chromosome walking approach led to the isolation of three overlapping lambda-clones and revealed, on both sides of *cpd1*, genes, which – with a very high probability – also code for enzymes of the alkaloid biosynthetic pathway. Cpps1 encodes a trimodular peptide synthetase and comparison of its derived amino acid sequence with internal peptide sequences of lysergyl peptide synthetase 1 (LPS1) from *C. purpurea* leave no doubt that CPPS1 is LPS1. LPS1 activates the three amino acids of the peptide portion of the ergopeptines (Riederer et al. 1996). Moreover, in close proximity on the upstream side of *cpd1* there are two further genes with possible (but not yet proven) functions in the alkaloid biosynthesis pathway (*cpox1* and *cpox2*). These findings add further weight to observations that the organization of genes encoding biosynthetic pathways for secondary metabolites in cluster is the rule rather than the exception in fungi (see review by Keller and Hohn 1997). Well characterized examples are the aflatoxin cluster in *Aspergillus parasiticus* (Trail et al. 1995), the penicillin cluster in *Penicillium chrysogenum* (Smith et al. 1990), the trichothecene cluster in *Fusarium sporotrichoides* (Hohn et al. 1993) and the recently characterized gibberellin cluster in *Gibberella fujikuroi* (Tudzynski and Hölter 1998). In all these cases the available evidence also suggests that these genes are coordinately regulated; this is not yet clear in the case of the putative *C. purpurea* alkaloid biosynthesis gene cluster. Data published by Riederer et al. (1996) on the *C. purpurea* LPS peptide synthetase indicate that LPS1 is constitutively synthesized, whereas its promoter structure indicates that *cpps1* is transcriptionally regulated. This discrepancy must be studied in more detail in the future, especially with respect to the turnover rate of the enzyme.

cpps1 has several interesting features; one is the presence of an intron in the second module – the first example of an intron in a gene for a multimodular peptide synthetase. In the case of a PCR-amplified partial sequence of a peptide-synthetase gene from the entomopathogen *Metarhizium anisopliae* an intron-like sequence has also been found (Bailey et al. 1996). However, the function of this gene in a specific peptide synthesis system is still unknown. The previously anticipated lack of introns in these giant fungal genes and their similarity to bacterial genes had even led to the idea of horizontal gene transfer (Marahiel et al. 1997). We found no evidence for any further introns, and we believe that the lack of any additional frame shift or fungal intron consensus sequences, as well as the colinearity of the derived amino acid sequence between the three modules and in comparison to the HTS strongly indicate that *cpps1* has only a single intron. Furthermore, the apparent dissection of the LPS system into two distinct peptide synthetases (i.e. LPS1 and LPS2) is a novel finding because all fungal peptide synthetases described so far consist of one single polypeptide chain (Zocher and Keller 1997). It is also interesting to note that the second module of CPPS1 shows some similarity to one of the putative peptide synthetase gene fragments previously cloned by a PCR-based approach (Cp 605; Panaccione 1996).

Upstream of *cpd1* there are two further genes (*cpox1* and *cpox2*) with possible functions in the ergot alkaloid pathway. The *cpox1* gene could code for an FAD-dependent oxidoreductase. As outlined above, such an enzyme, the chanoclavin oxidase or cyclase, is involved in the formation of agroclavine, i.e. in the formation of

the fourth ring of the ergoline backbone (Floss 1976). The product of *cpox2*, probably a short chain dehydrogenase, could also be involved in one of the less well characterized oxidation steps (see Fig. 1).

The RT-PCR experiments confirm that all four genes are expressed in mycelia which produce ergot alkaloids; this is a prerequisite, but not final proof for their involvement in alkaloid biosynthesis. A detailed Northern analysis will reveal if (and to what extent) they are co-regulated during ergot alkaloid biosynthesis.

Taken together, these data strongly suggest that at least some of the genes coding for enzymes of the alkaloid pathway in *C. purpurea* are clustered. Targeted inactivation of *cpps1*, *cpox1* and *cpox2* by a gene disruption approach (combined with a detailed biochemical characterization of the mutants) will clarify their roles in alkaloid biosynthesis; these analyses will also be extended to the ORFs immediately surrounding *cpd1*. Since the pathway requires probably more than 10 enzymes, including a one-module "peptide synthetase" (LPS2, lysergic acid-activating enzyme) and at least one P450 monooxygenase (Socic and Gaberc-Porekar 1992; Riederer et al. 1996), the chromosome walking approach has to be extended on both sides of the region characterized here. The data presented here form a good basis for a detailed molecular analysis of the ergot alkaloid pathway (especially of the regulatory circuits involved), which is not only highly interesting for basic research, but also has considerable practical implications, considering the growing therapeutic importance of ergot peptide alkaloids, e.g. with respect to degenerative diseases of the CNS.

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References

Andrianopoulos A, Timberlake WE (1994) The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol Cell Biol 14:2503–2515

Arntz C (1998) Molekularbiologische Untersuchungen zur Alkaloidsynthese bei *Claviceps*. PhD thesis, Westfälische Wilhelms-Universität Münster

Arntz C, Tudzynski P (1997) Identification of genes induced in alkaloid producing cultures of *Claviceps* sp. Curr Genet 31:357–360

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current protocols in molecular biology. Wiley, New York

Bailey AM, Kerchow MJ, Hunt BA, Perterson IC, Chamley AK, Reynolds SE, Clarkson JM (1996) Cloning and sequence analysis of an intron-containing domain from a peptide synthetase-encoding gene of the entomopathogenic fungus *M. anisopliae*. Gene 173:195–197

Brandsch R, Bichler V, Mauch L, Decker K (1993) Cysteine to serine replacements in 6-hydroxy-D-nicotine oxidase. Consequences for enzyme activity, cofactor incorporation, and formation of high molecular weight protein complexes with molecular chaperones. J Biol Chem 268:12724–12729

Caddick MX, Arst HN, Taylor LH, Johnson RI, Brownlee AG (1986) Cloning of the regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. EMBO J 5:1087–1090

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res 20:2380

Chambers AA, Russo VEA (1986) Isolating RNA is easy and fun. Fungal Genet Newslett 33:22–23

Chiang TY, Marzluf GA (1994) DNA recognition by the NIT2 nitrogen regulatory protein: importance of the number, spacing and orientation of GATA core elements and their flanking sequences upon NIT2-binding. Biochemistry 33:576–582

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Dittrich H, Kutchan TM (1991) Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. Proc Natl Acad Sci USA 66:9969–9973

Dutton JR, John S, Miller BL (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J 16:5710–5721

Esser K, Tudzynski P (1978) Genetics of the ergot fungus *Claviceps purpurea*. I. Proof of a monoecious life cycle and segregation patterns for mycelial morphology and alkaloid production. Theor Appl Genet 53:145–149

Felenbok B, Kelly JM (1996) Regulation of carbon metabolism in mycelial fungi. In: Brambl Marzluf GA (ed) The Mycota III: biochemistry and molecular biology. Springer, Berlin Heidelberg New York, pp 369–380

Floss HG (1976) Biosynthesis of ergot alkaloids and related compounds. Tetrahedron Lett 32:873–912

Garre V, Müller, U, Tudzynski P (1998) Cloning, characterization and targeted disruption of *cpcat1*, coding for an in planta secreted catalase of *Claviceps purpurea*. Mol Plant-Microbe Interact 11:772–783

Gebler JC, Poulter CD (1992) Purification and charakterization of dimethylallyl tryptophan synthase from *Claviceps purpurea*. Arch Biochem Biophys 296:308–313

Hohn TM, McCormick SP, Desjardins AE (1993) Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichoides*. Curr Genet 24:291–295

Keller U (1983) Highly efficient mutagenesis of *Claviceps purpurea* by using protoplasts. Appl Environ Microbiol 46:580–584

Keller NP, Hohn TM (1997) Metabolic pathway gene cluster in filamentous fungi. Fungal Genet Biol 21:17–29

Krupinski VW, Robbers JE, Floss HG (1976) Physiological study of ergot: induction of alkaloid synthesis by tryptophan at the enzymatic level. J Bacteriol 125:158–165

Lohmeyer M, Tudzynski P (1997) *Claviceps* alkaloids. In: Anke T (ed) Fungal biotechnology. Chapman and Hall, Weinheim, pp173–185

Lohmeyer M, Dierkes W, Rehm HJ (1990) Influence of inorganic phosphate and immobilization on *Claviceps purpurea*. Appl Microbiol Biotechnol 33:196–201

Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2673

Panaccione DG (1996) Multiple families of peptide synthetase genes from ergopeptine-producing fungi. Mycol Res 100:429–436

Pazoutova S, Tudzynski P (1998) *Claviceps* sp. PRL 1980 (ATCC 26245), 59 and Pepty 695/ch-1: their true story. Mycol Res, in press

Peleg Y, Metzenberg RL (1994) Analysis of the DNA-binding and dimerization activities of *Neurospora crassa* transcription factor NUC-1. Mol Cell Biol 14:7816–7826

Riederer B, Han M, Keller U (1996) D-lysergyl peptide synthetase from the ergot fungus *Claviceps purpurea*. J Biol Chem 271:27524–27530

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Scott-Craig JS, Panaccione DG, Pocard J-A, Walton JD (1992) The cyclic peptide synthetase catalysing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-kilobase open reading frame. J Biol Chem 267:26044–26049

Schwecke T, Aparicio JF, Molnar I, Koenig A, Khaw LE, Haydock SF, Olinyk M, Caffrey P, Cortes J, Lester JB, Boehem GA, Staunton J, Leadlay PF (1995) The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc Natl Acad Sci USA 92:7839–7843

Smith DJ, Earl AJ, Turner G (1990) The multifunctional peptide synthetase performing the first step of penicillin biosynthesis in *Penicillium chrysogenum* is a protein similiar to *Bacillus brevis* peptide antibiotic synthetases. EMBO J 9:2743–2750

Socic H, Gaberc-Porekar V (1992) Biosynthesis and physiology of ergot alkaloids. In: Arora DK, Elander RP, Mukerji KG (eds) Handbook of applied mycology, vol 4. Fungal biotechnology. Dekker, New York, pp 475–515

Stone KL, Williams KR (1993) Enzymatic digestion of proteins and HPLC peptide isolation. In: Matsudira P (ed) A practical guide to protein and peptide purification for microsequencing. Academic Press, San Diego, pp 123–155

Tenberge KB, Homann V, Oeser B, Tudzynski P (1996) Structure and expression of two polygalacturonase genes of *Claviceps purpurea* orientated in tandem and cytological evidence for pectinolytic enzyme activity during infection of rye. Phytopathol 86:1084–1097

Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Penalva MA, Arst HN (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 14:779–790

Trail F, Mahanti N, Rarick M, Mehigh R, Liang SH, Zhou R, Linz JE (1998) Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. Appl Environ Microbiol 61:2665–2673

Tsai H-F, Wang H, Gebler JC, Poulter D, Schardl CL (1995) The *Claviceps purpurea* gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. Biochem Biophys Res Commun 216:119–125

Tudzynski B, Hölter K (1998) The gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. Fungal Genet Biol, in press

Tudzynski P, Tenberge KB, Oeser B (1995) *Claviceps purpurea*. In: Kohmoto D, Singh US, Singh RP (eds) Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic and molecular bases; vol. 2: Eukaryotes. Pergamon Press-Elsevier Science, Oxford, pp161–187

Walzel B, Riederer B, Keller U (1997) Mechanism of alkaloid cyclopeptide formation in the ergot fungus *Claviceps purpurea*. Chem Biol 4:223–230

Zocher R, Keller U (1997) Thioltemplate peptide synthesis systems in bacteria and fungi. Adv Microb Physiol 38:85–131