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Review

The ergot alkaloid gene cluster: Functional analyses and evolutionary aspects

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ABSTRACT

Ergot alkaloids and their derivatives have been traditionally used as therapeutic agents in migraine, blood pressure regulation and help in childbirth and abortion. Their production in submerse culture is a long established biotechnological process. Ergot alkaloids are produced mainly by members of the genus *Claviceps*, with *Claviceps purpurea* as best investigated species concerning the biochemistry of ergot alkaloid synthesis (EAS). Genes encoding enzymes involved in EAS have been shown to be clustered; functional analyses of EAS cluster genes have allowed to assign specific functions to several gene products. Various *Claviceps* species differ with respect to their host specificity and their alkaloid content; comparison of the ergot alkaloid clusters in these species (and of clavine alkaloid clusters in other genera) yields interesting insights into the evolution of cluster structure. This review focuses on recently published and also yet unpublished data on the structure and evolution of the EAS gene cluster and on the function and regulation of cluster genes. These analyses have also significant biotechnological implications: the characterization of non-ribosomal peptide synthetases (NRPS) involved in the synthesis of the peptide moiety of ergopeptines opened interesting perspectives for the synthesis of ergot alkaloids; on the other hand, defined mutants could be generated producing interesting intermediates or only single peptide alkaloids (instead of the alkaloid mixtures usually produced by industrial strains).

Contents

1. Introduction

Ergot alkaloids: pharmacology and biochemistry

Members of the fungal ascomycetous genus *Claviceps* parasitize more than 600 monocotyledonous plants including economically important crop plants like rye, wheat, barley, rice, corn, millet and oat (Bové, 1970). Most of the almost 50 species in this group (Alderman, 2003; Pažoutová et al., 2008a) have a defined, narrow host range. *Claviceps purpurea* (Fries ex Fries) Tulasne, the most widely known species in Europe, is exceptional as it infects more than 400 plant species with a disease known as ergot. The common name "Ergot Fungus" is derived from the French word for spur ("argot") and refers to the dark sclerotia formed in the final disease stages. These structures contain the pharmaceutically interesting ergot alkaloids, tri – or tetracyclic derivatives of prenylated

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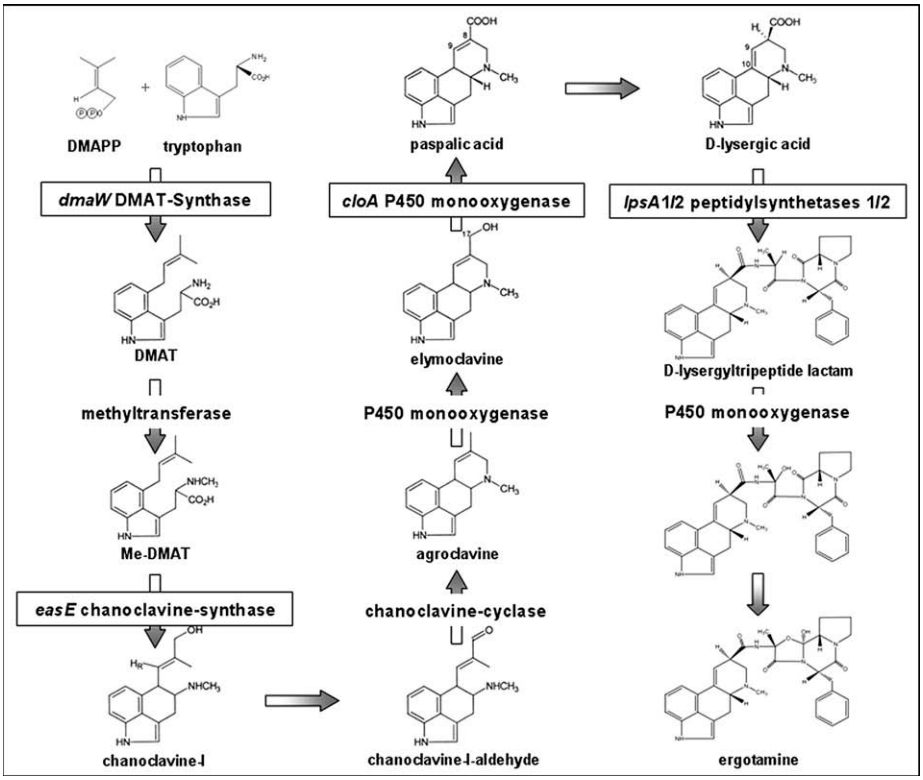

Fig. 1. Pathway of the ergot alkaloid biosynthesis of *C. purpurea*. Genes, whose role was verified by gene replacement followed by functional and biochemical analyses of the deletion mutants, are allocated together with the encoding protein at the corresponding position within the pathway. DMAPP = dimethylallylpyrophosphate; DMAT = dimethylallyltryptophan; Me-DMAT = N-methyl-DMAT. Modified after Haarmann et al. (2006).

tryptophan, that give the fungus its significance. In the Middle Ages consumption of ergot-contaminated rye bread caused vast epidemics of the so-called St. Anthony's Fire disease. On the other hand, ergot sclerotia were used by midwives as help in child birth. This dual role of ergot persisted over the centuries. The ergot disease is feared by farmers because ergot-contamination can render the grain too poisonous for use, whereas the seed loss (5–10%) is almost negligible (Alderman et al., 1998). But there has been considerable commercial interest in the biotechnological production of ergot alkaloids due to their pharmacological effects.

These pharmacological activities are mostly due to the structural similarity between D-lysergic acid derived compounds and neurotransmitters like noradrenalin, dopamine and serotonin; ergot alkaloids can interact with receptors for these neurotransmitters (Berde and Stürmer, 1978) either as agonist or antagonist, depending on the substituents attached to the carboxyl group of D-lysergic acid (Stadler and Giger, 1984). This broad specificity can provoke undesirable side effects and the approaches to improve natural ergot alkaloids as therapeutic agents involve narrowing the specificity of the compounds by chemical modification (Vendrell et al., 2007). The biosynthesis of ergot alkaloids has been studied in detail, mostly by feeding experiments with radiolabelled putative precursors or intermediates added to rye ears infected with *C. purpurea* or to fermentation cultures of *C. purpurea*, *Claviceps fusiformis* or *Claviceps paspali* (Keller and Tudzynski, 2002). As biosynthetic building blocks of the ergoline ring system tryptophan, the methyl group of S-adenosyl methionine, and an isoprene unit derived from mevalonic acid have been identified (recent review: Schardl et al., 2006).

The pathway leading to ergopeptines (see Fig. 1) starts with the isoprenylation of tryptophan yielding 4-dimethylallyltryptophan (DMAT). The enzyme catalysing this first pathway specific step, dimethylallyl diphosphate: L-tryptophan dimethylallylsynthase (DMATS) was the first to be characterized in detail (Gebler and Poulter, 1992). As it catalyses the determinant step, DMATS is subject to strict regulation: tryptophan serves as inducer, whereas elymoclavine or agroclavine cause feedback regulation of the enzyme (Cheng et al., 1980).

Based on oligonucleotides derived from a partial amino acid sequence of the purified enzyme, the gene *dmaW* encoding DMATS was cloned, originally from a *C. fusiformis* strain (Tsai et al., 1995), and later from a *C. purpurea* strain P1 (Tudzynski et al., 1999). A chromosome walking approach starting from *dmaW* led to the detection of a cluster of genes, shown (or predicted) to encode ergot alkaloid synthesis (EAS) enzymes (Tudzynski et al., 1999). The original EAS cluster region contained 11 genes/ORFs (see Correia et al., 2003); it was recently extended (see below) and does now cover a region of 14 genes (see Fig. 2). Besides *dmaW*, the first functionally characterized genes of the cluster were two genes encoding non-ribosomal peptide synthetases (NRPS), *cpps1* and *cpps2*: biochemical evidence had shown that the final steps of the ergopeptine synthesis in *C. purpurea* include a complex of two interacting NRPSs (a new finding in fungi): D-lysergylpeptide-synthetase (LPS) 2, catalysing the activation of D-lysergic acid, and

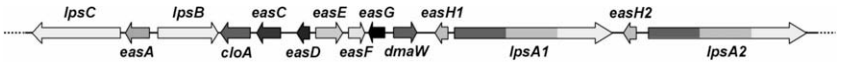

Fig. 2. Scheme of the alkaloid biosynthesis cluster of *C. purpurea*. Direction of transcription is indicated by orientation of the arrows. Modified after Haarmann et al. (2005).

LPS1 forming the tripeptide moiety (Fig. 1; Riederer et al., 1996). By partial sequencing of the protein and by a gene replacement approach, respectively, it could be shown that *cpps1* encodes LPS1 and *cpps2* codes for LPS2 (Tudzynski et al., 1999; Correia et al., 2003).

In the last years, our understanding of the function of genes within the cluster, the molecular basis of the existence of chemical races, and the evolution of the cluster region in the genus *Claviceps* (and beyond) has been significantly extended.

2. The ergot alkaloid synthesis (EAS) gene cluster: structure and functional analyses

The core EAS cluster region as described by Correia et al. (2003), stretching from the *easA* gene (left "border") to *lpsA1* (right "border"; see Fig. 2) was extended in both directions by chromosome walking using a genomic library of strain P1 (Haarmann et al., 2005). The new sequence revealed the presence of two additional NRPS genes (*lpsC* and *lpsA2*, the latter being highly homologous to *lpsA1*, probably a result of a recent duplication event), several unidentified reading frames (*easF-H*) and *cpimd*, probably encoding an enzyme of the primary metabolism, isopropylmalate-dehydratase. Northern analyses defined the borders of the cluster between *lpsC* and *lpsA2* (see Fig. 2): all genes between these borders were shown to be regulated, i.e. repressed by high phosphate levels (Haarmann et al., 2005).

Recently, in order to facilitate discussion of the genes and comparisons among ergot alkaloid producing fungi, a systematic set of names for the genes of the ergot alkaloid pathway has been agreed upon by the groups involved in the analysis (Schardl et al., 2006). *C. purpurea* alkaloid cluster genes that have not yet been functionally characterized are designated *easA* through *easH* (*e*rgot *a*lkaloid *s*ynthesis). Genes whose products have been biochemically characterized are named according to the enzyme activities of the encoded proteins. A list of the genes and their shown or predicted function is given in Table 1.

Several EAS cluster genes have been functionally analysed by gene disruption and analysis of intermediates in *C. purpurea* P1. These include three NRPS genes, $lpsA_1$; *lpsB* and *lpsC*. Functional analysis showed that the product of $lpsA_1$ (*cpps1*), LPSA-1, obviously is necessary for synthesis of the major alkaloid of strain P1, ergotamine, and that LPSA-2 most probably catalyses the synthesis of ergocryptine (Tudzynski et al., 1999; Haarmann et al., 2008). The gene *lpsB* encodes the lysergic acid activating enzyme LPS2 (see above), whereas *lpsC* probably encodes a monomodular NRPS enzyme that catalyses the formation of ergometrine, an ergopeptine with a single amino acid side chain (Ortel and Keller, 2009). Thus, this set of NRPS genes encodes a highly flexible natural combinatory system unique in eukaryotes: activated lysergic acid formed by LPS 2 can be used as recipient for the addition of several peptide moieties, explaining the high variability of the ergot peptide alkaloid spectrum in *C. purpurea* strains (see below).

Of the enzymes involved in the pathway from DMAT to lysergic acid, two have been identified so far by a knock out approach of the encoding genes. The gene product of *cloA* was shown to catalyse the conversion of elymoclavine to paspalic acid (see Fig. 1) (Haarmann et al., 2006). *CloA* encodes a cytochrome P450 monooxygenase; a deletion mutant accumulates agro-, elymo- and chanoclavine, but does not form any ergopeptines. Synthesis of ergopeptines could be restored by feeding the mutant with D-lysergic acid, proving that it is blocked in the step between elymoclavine and D-lysergic acid (see Fig. 1). Thus the enzyme encoded by *cloA* is a clavine oxidase, bridging the biosynthesis of the two major alkaloid classes, ergoclavines and ergopeptines (Haarmann et al., 2006).

The gene product of *easE*, annotated as an FAD-containing oxidoreductase, was recently shown to be probably involved in the "chanoclavine-I-synthase" acitity (N. Lorenz, J. Olšovská, M. Šulc, P. Tudzynski, unpublished data). In addition, Li and co-workers recently demonstrated that *easF* encodes a methyltransferase involved in synthesis of Me-DMAT (Rigbers and Li, 2008).

These functional analyses deepened our understanding of the molecular mechanisms of ergot alkaloid biosynthesis; still, several steps remain to be elucidated, e.g. the enzyme catalysing the cytochrome-P450-type reaction in the final step of the ergopeptine synthesis is not yet identified.

Table 1
Genes of the ergot alkaloid cluster of *C. purpurea*, former nomenclature and (putative) function. DMAT – dimethylallyltryptophan; LPS = lysergyl peptidyl synthetase.

Gene	Former nomenclature	(putative) Function
easA	*cpox3*	Reductase/dehydrogenase
lpsB	*cpps2*	LPS subunit 2
lpsC	*cpps3*	Monomodular NPPS
cloA	*cpP450-1*	Elymoclavine oxygenase
easC	*cpcat2*	Reductase/dehydrogenase
easD	*cpox2*	Reductase/dehydrogenase
easE	*cpox1*	Reductase/dehydrogenase
easF	*orfB*	Methyltransferase
easG	*orfA*	Reductase/dehydrogenase (NmrA-like protein)
dmaW	*cpd1*	DMAT synthase
easH1	*orfC*	Oxygenase/hydroxylase
lpsA1	*cpps1*	LPS subunit 1
easH2	*orfE*	Hydroxylase
lpsA2	*cpps4*	LPS subunit 1

3. Regulation of EAS cluster genes

Expression of the ergot alkaloid biosynthetic genes is regulated at least at two levels: in wild type strains normally the alkaloid biosynthesis is induced only *in planta*, in ripening sclerotia; they do not produce any alkaloids in axenic culture. Strains producing alkaloids in submerged culture have been generated by successive cycles of mutagenesis, e.g. strain P1 used throughout the molecular analyses of *C. purpurea* described above. In these strains, however, specific culture conditions are necessary for induction of alkaloid production: tryptophan is necessary as inducer (and precursor), a high osmotic value (perhaps in analogy to the situation *in planta* after tapping of the phloem), and a low phosphate level, suggesting involvement of the phosphate repression regulatory system. Apart from the phosphate effect, which has been demonstrated on the transcriptional level (Haarmann et al., 2005), so far the molecular mechanisms of cluster regulation in *C. purpurea* are unknown.

In contrast to most secondary metabolic clusters in fungi, there is no obvious transcription factor gene within the eas cluster region. A possible candidate for a gene encoding a regulatory factor, however, could be *easG*. Orthologs are also present in the *eas* clusters of *C. fusiformis* (ABV57825), *Neotyphodium lolii* (ABM91452) and *Aspergillus fumigatus* (XM_751041) and show homology to NmrA-like proteins which have been shown to have regulatory functions in other fungi. The involvement of Nmr in nitrogen regulation was at first investigated in *Aspergillus nidulans* (Andrianopoulos et al., 1998) and *Neurospora crassa* (Tomsett et al., 1981), but only the homologue of *Fusarium fujikuroi* (Mihlan et al., 2003) could be linked to secondary metabolite biosynthesis. It was previously shown that in *F. fujikuroi* Nmr interacts with the nitrogen regulator AreA leading to the expression of genes involved in gibberellin biosynthesis (Schönig et al., 2008). Preliminary data indicate that the gene product of *bik4* in the bikaverin cluster of *F. fujikuroi*, also showing homology to NmrA-like proteins has impact on bikaverin biosynthesis (Wiemann et al., 2009). So functional analysis of *easG* would be very interesting.

We have recently initiated research on regulation on two levels: we studied the impact of osmo-sensing/-regulation on alkaloid biosynthesis, and we had a first look on the role of chromatin structure; the preliminary results are briefly presented here.

3.1. Impact of osmo-sensing on alkaloid biosynthesis

In fungi osmo-regulation and response to osmotic stress are mediated by a mitogen-activated protein kinase (MAPK) cascade, a homologue of the Hog-cascade in yeast. Upstream of this cascade in filamentous fungi a histidin kinase acts as sensor. Histidine kinases (hk) are important components of signal transduction pathways being involved in the regulation of a variety of different physiological processes, e.g. cell cycle control, sporulation and stress response (reviewed e.g. in Stock et al., 2000). They activate/inactivate other proteins by transferring a phosphate which is bound temporarily to the kinase at a specific histidine residue located in the transmitter domain of the protein, more exactly being the essential part of the H-box. The transmitter domain further consists of the N-, the G1-, the F- and the G2-box required for binding and phosphate transfer from the co-factor ATP resulting in the phosphorylated substrate and ADP (Stock et al., 1988; Parkinson and Kofoid, 1992). The best investigated model of osmo-regulation is that of the budding yeast *Saccharomyces cerevisiae* which includes a membrane bound histidine kinase (SLN1; the only hk in yeast!) activated by low osmolarity. The signal transduction is mediated by several successive steps of phosphorelay via a histidine-containing phosphor-transfer protein (YPD1) and a response regulator (SSK1) which activates a MAP kinase cascade leading to the MAP kinase HOG1 which regulates several transcription factors controlling genes involved in the response to changes in osmolarity (Maeda et al., 1994).

In filamentous fungi, the histidine kinases can be subdivided into 11 groups (Catlett et al., 2003), defined by a characteristic set of domains. A special group of histidine kinases (group III) which is characterized by six HAMP domains (domain found in *h*istidine kinases, *a*denylate cyclases, *m*ethyl-accepting chemotaxis proteins and *p*hosphatases) additionally to the typical transmitter and acceptor domain, was shown to be involved in sensing and reacting to changes in the osmolaric status of the environment: Nik1 of *N. crassa* (Alex et al., 1996), Bos1 of *Botrytis cinerea* (Viaud et al., 2006), Hik1 of *Magnaporthe grisea* (Motoyama et al., 2005) and Fik1 of *Nectria haematococca*. Deletion of the corresponding genes led to an increased sensitivity to high osmolarity and mediated resistance to several fungicides although the detailed mechanism remains unclear.

Since ergot alkaloid production in *C. purpurea* is dependent on high osmotic pressure of the culture medium (see above), we were interested if the Hog cascade would be involved in EAS regulation. This idea was substantiated by observations that histidine kinases of group III are not only involved in osmo-regulation but could also be relevant for the investigation of secondary metabolite biosynthesis. Ochiai et al. could show that the production of aurofusarin of *Fusarium graminearum* is regulated by the group III histidine kinase FgOs1 (Ochiai et al., 2007). Therefore, we initiated functional analysis of the corresponding homologous hk gene in *C. purpurea*.

Screening of a genomic λ-library of *C. purpurea* revealed an ORF of 4278 bp with 87% identity to *nik1* of *N. crassa*. RT-PCR verified three introns of 89 bp, 74 bp, and 109 bp, respectively leading to a corresponding protein of 1336 aa. A domain search using the program PROSITE (http://www.expasy.ch/prosite) showed a transmitter as well as an acceptor domain and six additional HAMP domains characteristic for the histidine kinases of group III (Fig. 3).

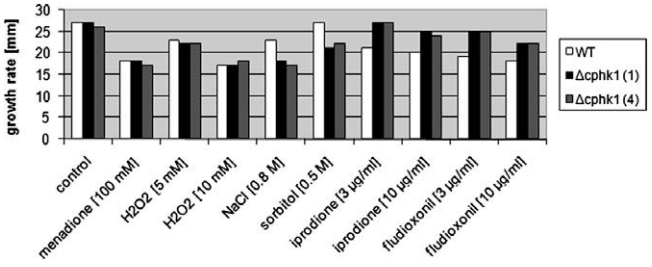

Fig. 4. Impact of CPHK1 on resistance against oxidative and osmotic stress and fungicides. Strain P1 (WT) and two independent Δcphk1 mutants were grown on media supplemented with NaCl (0.8 M), sorbitol (0.5 M), iprodione (3/10 µg/ml), fludioxonil (3/10 µg/ml), H_2O_2 (5/10 mM), menadione (100 mM). Growth rates (4 parallels) were measured after 7 d. For details see text.

Two independent knock out mutants of *cphk1* (Δcphk1-1 and -4) were generated in a *ku70* deficient strain of *C. purpurea* P1 which was confirmed by PCR and Southern analyses (data not shown).

Growth tests proved an influence of CpHK1 on osmo-regulation as well as on fungicide resistance which is characteristic for histidine kinases of group III (Fig. 4). The deletion mutants showed reduced growth rates when cultivated under osmotic stress, mediated by either 0.8 M NaCl or 0.5 M sorbitol. Furthermore, the mutants possessed an increased resistance to fungicides, (iprodione and fludioxonil). Additionally, sensitivity to oxidative stress was tested by the addition of H_2O_2 to an end concentration of 5 and 10 mM, respectively. No differences could be observed between the wild type strain and the mutants, ruling out an involvement of CpHK1 in the response to reactive oxygen species. This assumption was confirmed by addition of menadione to an end concentration of 100 µM, also revealing no significant differences. The results were in accordance with properties published for other histidine kinases of group III, verifying the affiliation of CpHK1 to this group.

Since these data showed that CpHK1 is indeed involved in osmo-regulation, phosphorylation assays were performed to verify whether CpHK1 is an activator of the Hog cascade, i.e. is acting upstream of the MAP kinase cascade as it is well documented for the yeast *S. cerevisiae* (Maeda et al., 1994). Therefore, the phosphorylation status of the MAP kinase CpHog1 was analysed by western blot analysis using cultures of *C. purpurea* wild type and the deletion mutants shifted to medium either supplemented with fungicides (5 µg/ml iprodione or 5 µg/ml fludioxonil) or osmotic stressors (0.8 M NaCl or 0.5 M sorbitol). Cultures incubated without any stressors acted as control. Total proteins were extracted, separated in a 10% SDS electrophoretic gel and blotted to a nitrocellulose membrane. The total amount of CpHog1 was visualized by the utilization of a Hog1 antibody and showed an equal amount of protein in each probe (Fig. 5). The phosphorylation status of CpHog1 could be measured by the antibody phospho-p38 which is specific for the phosphorylated form of Hog1. Interestingly, no significant differences could be observed between the different conditions, but between the wild type and the deletion mutants (Fig. 5). In all cases (including the control), the amount of phosphorylated CpHog1 is drastically reduced in the knock out mutants compared to the wild type, proving that the histidine kinase CpHK1 is indeed an upstream effector of the Hog cascade in *C. purpurea*.

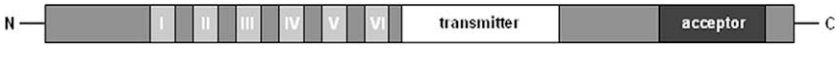

Fig. 3. Schematic structure of the histidine kinase CpHK1 of *C. purpurea*. Light grey: HAMP domains I–VI; white: transmitter domain; black: acceptor domain. Accession no: FN393561.

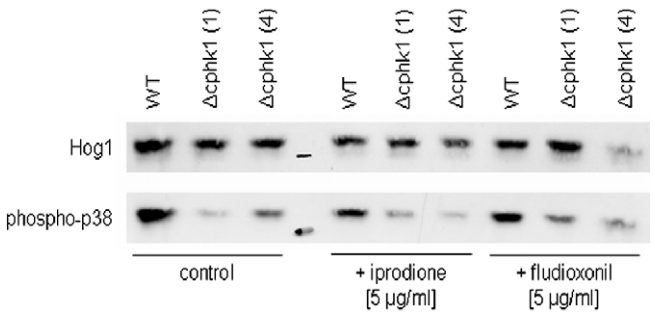

Fig. 5. Phosphorylation of the MAP kinase CpHog1 in the presence of fungicides. The cultures were shifted for 1 h to complete medium supplemented with either 5 µg/ml iprodione or 5 µg/ml fludioxonil. Phosphorylation of CpHog1 was monitored using dually phosphorylated p^{38} antibody (New England Biolabs) whereas the total amount of CpHog1 protein was determined with the "C-terminal anti-Hog1" antibody (Santa Cruz Biotechnology) as described in Segmüller et al. (2007).

Finally, the transformants were functionally analysed with regard to alkaloid biosynthetic capability. Unfortunately, no differences could be observed between the wild type and the deletion mutants concerning the alkaloid spectrum as well as the amount of ergot alkaloids synthesized under alkaloid producing conditions (data not shown). In addition, under non-producing conditions the strain did not synthesize any alkaloids, ruling out a deregulation effect in the mutant. Therefore, it can be concluded that CpHK1 (and hence the Hog cascade) is not involved in the regulation of alkaloid biosynthesis of *C. purpurea*.

3.2. Role of chromatin organization

In the last years the role of histone modifications in activation/repression of secondary metabolite gene clusters was well documented. N. Keller and co-workers showed that in *Aspergillus* species a histone methyltransferase (LaeA) plays a crucial role in global regulation of cluster genes. Deletion of *laeA* blocks the expression of several metabolic gene clusters whereas overexpression triggers increased gene transcription and product formation (Bok and Keller, 2004).

Based on fungal genome sequencing projects it appears that common locations for biosynthetic gene clusters are regions linked to the telomere (Galagan et al., 2005; Nierman et al., 2005; Rehmeyer et al., 2006). These regions are frequently sites of heterochromatin formation linked to gene expression in other eukaryotic organisms, e.g., in yeast (Millar and Grunstein, 2006; Merrick and Duraisingh, 2006). The LaeA protein of *A. nidulans* might initiate a process that converts heterochromatin to euchromatin, perhaps by interfering with methylases or deacetylases altering the histone structure. Therefore, we assumed that the overexpression of *laeA* from *A. nidulans* could lead to an activation of secondary metabolite genes, in particular ergot alkaloid genes, in *C. purpurea*.

To analyse this we expressed the *laeA* gene from *A. nidulans* in several strains of the ergot fungus: the wild type strain 20.1 which produces alkaloids exclusively on its host plant and not in axenic culture, the mutant strain P1 which is able to form alkaloids in axenic culture, and the mutant strain ECC93 which produces small amounts of alkaloids in axenic culture.

The expression of the *A. nidulans laeA* gene in the *C. purpurea* background and its impact on the expression of pathway specific genes (*dmaW*) of the ergot alkaloid gene cluster was tested by northern blot analysis (data not shown). *LaeA* was expressed in all transformants under all tested conditions. Expression of the gene *dmaW* could be observed in the *laeA* transformant of strain P1 as was expected under alkaloid producing conditions. In contrast, no expression of *dmaW* in the *laeA* overexpression mutant 20.1 could be detected. This corresponds to the fact that 20.1 lacks the ability to form ergot alkaloids in axenic culture. ECC93 wild type produces alkaloids in axenic culture in low yield and that accounts for the detection of little expression of *dmaW* in the *laeA* overexpression mutant of ECC93. However, there is no significant increase of expression of *dmaW* compared to ECC93 wild type strain.

Taken together we could show that the *laeA* gene of *A. nidulans* is highly expressed in the *C. purpurea* background but that it has no impact on the expression of ergot alkaloid cluster genes in different strains of *C. purpurea*. Concordantly, no enhanced alkaloid production in axenic cultures of the *laeA* overexpressing mutants could be measured (data not shown).

According to this it was observed in *A. nidulans* and *Aspergillus terreus* that although overexpression of *laeA* increased penicillin and lovastatin gene transcript and concomitant product formation, this could not be shown for sterigmatocystin gene transcription or production (Bok and Keller, 2004). This raises the question of a unique interaction between *laeA* and secondary metabolite gene clusters exclusively in *Aspergillus* species.

Our findings clarified that LaeA (at least LaeA from *A. nidulans*) is not the main switch of ergot alkaloid biosynthesis in *C. purpurea* but that there have to be other factors specifically regulating the expression of ergot alkaloid cluster genes.

Apart from methylation, acetylation/deacetylation of histones has been shown to affect gene expression. Acetylation of the ε-amino groups of specific lysine residues of especially histone 4, but also of the histones 3 and 2A by histone acetyltransferases (HATs) (Davie, 1998; Kornberg and Lorch, 1999) leads to a loose chromatin organization, referred to as euchromatin, where gene expression occurs, whereas expression is repressed when the chromatin is in a dense structure, designated as heterochromatin (reviewed in Razin et al., 2007). This antagonistic reaction is performed by histone deacetylases (HDACs) leading to repression of the corresponding DNA region (Grunstein, 1997).

As it was described above, the genes encoding enzymes belonging to the synthetic pathway of a secondary metabolite are mostly gathered in a cluster in the genome. In this context, it is interesting to mention that regulation of the biosynthesis by chromatin organization seems to be increased when the cluster is located in the subtelomeric region as it was shown by investigating biosynthesis gene clusters of *Aspergillus* spec. (Perrin et al., 2007). Comparison of the cluster sequences of two *Claviceps* species indicates that the alkaloid cluster could also be located in the subtelomeric region (Lorenz et al., 2007) rendering it more likely that the biosynthesis is regulated by chromatin remodeling.

The influence of the chromatin structure on the regulation of the alkaloid biosynthesis was tested by the application of inhibitors specific for either HDACs or HATs (see Fig. 7): use of HAT inhibitors (HATi) mimic the effect of HDACs and therefore, prefer heterochromatin formation leading to repression of the corresponding DNA region. The antagonistic effect could be initialized by addition of HDAC inhibitores (HDACi) resulting in activation of the corresponding DNA region, perhaps independent of a specific transcription factor.

Inhibitors of HDACs could be subdivided into four classes according to their chemical structure (Kim et al., 2003): (I) short chain fatty acids, e.g. butyrate, (II) hydroxamid acids with lipophilic linker regions, e.g. Trichostatin A (TSA) and related substances like suberoylanilide-hydroxyaminoacid (SAHA), (III) cyclic tetrapeptide compounds that have epoxy ketone or disulphite (releasing sulfohydryl functions) groups as functional groups, e.g. Trapoxin B, HC-toxin and FK228 and (IV) benzamide-bearing compounds, e.g. MS-27-275 and CI-994. The inhibitory effects are based on chelate reactions with a zinc ion essential for HDACs

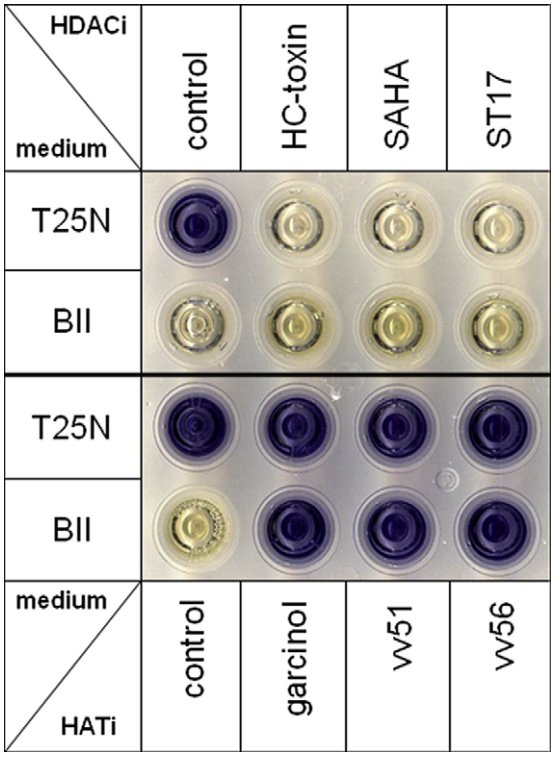

Fig. 6. Effect of histone acetyltransferase and histone deacetylase inhibitors on ergot alkaloid production. Strain P1 of *C. purpurea* was cultivated under either induced (T25N medium) or non-induced (BII medium) conditions. Addition of DMSO to the culture medium functions as control. HDACi = histone deacetylase inhibitor; HATi = histone acetyltransferase inhibitor. Inhibitors were added to culture medium at the following concentrations: ST 17: 300 μM; SAHA: 100 μM; HC-toxin: 0.65 μM; garcinol: 50 μM; vv51: 75 μM; vv56: 100 μM. Blue staining indicates alkaloid production (Van Urk reagent; Ehmann, 1977).

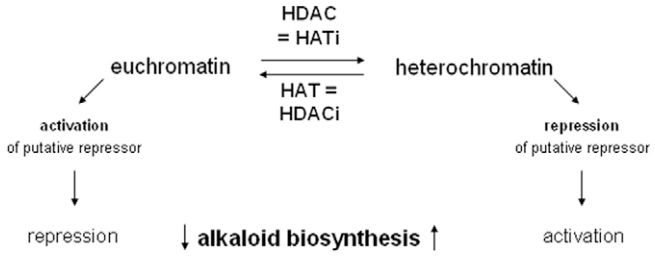

Fig. 7. Hypothetical scheme for the role of histone acetylation/deacetylation on regulation of alkaloid biosynthesis in *C. purpurea*. For details see text.

leading to temporary as well as irreversible covalent binding of HDACs (Kim et al., 2003). For this approach inhibitors of group II and group III are used: (I) the inhibitor SAHA, structurally related to TSA, a natural product isolated from *Streptomyces hygroscopicus* that was initially used as an antifungal antibiotic (Yoshida et al., 1995), (II) HC-toxin, naturally produced by *Cochliobolus carbonum* and essential for pathogenicity on maize (reviewed in Kawai et al., 1983; Walton, 2006) and (III) the synthetical HDAC inhibitor ST17 (Schäfer et al., 2008).

On the other hand, the inhibitors of HATs are to a considerably lesser extent investigated. Only a very limited number of current data are available and the widespread screening for putative inhibitors is still at the beginning (Wynne Aherne et al., 2002). Nevertheless, naturally as well as synthetically produced inhibitors of HATs are known and should be discussed in this paper, represented by the synthetical substances vv51 and vv56, (Valkov, 2007; Eliseeva et al., 2007) along with the naturally produced garcinol, a polyisoprenylated benzophenone derivative from *Garcinia indica* fruit rind (Bakana et al., 1987; Yamaguchi et al., 2000).

The production strain P1 of *C. purpurea* was cultivated under both alkaloid producing (T25N medium) and non-producing (BII medium) conditions. The cultures were supplemented with one of the inhibitors, harvested after 7 d and analysed with regard to alkaloid biosynthetic capability. Cultures incubated without any inhibitor acted as a control.

To measure the amount of ergot alkaloids produced the Van Urk test was performed (Ehmann, 1977), a "quick test" during which successive incubation of the culture medium with dimethylaminobenzaldehyde and sodium nitrite leads to blue staining of the filtrate caused by the aromatic ring system of indole derivatives.

The wild type incubated without any HDACi showed a deep blue staining under induced conditions, whereas the culture medium remained white when incubated under non-induced conditions (Fig. 6). In contrast to that, addition of each of the HDACis (the synthetical ST17 and SAHA and the naturally derived HC-toxin from *C. carbonum*) led to a complete repression of alkaloid biosynthesis independent of the utilized medium overruling the regulatory effects mediated by the different media.

Interestingly, the opposite effect could be observed by the application of HATis (synthetical inhibitors vv51 and vv56 as well as the naturally derived compound garcinol from *G. indica* fruit rind): all cultures analysed showed deep blue staining indicating massive production of ergot alkaloids (Fig. 6) also independent of the culture conditions (induced/non-induced).

To verify that the blue staining indeed correlates with alkaloid biosynthesis, extraction followed by TLC and HPLC analyses were performed and confirmed ergotamine production (data not shown). In addition, expression of *dmaW*, encoding the key enzyme of the pathway, was shown by northern analyses under these conditions (data not shown).

These preliminary data indicate a more indirect effect of chromatin remodeling on alkaloid biosynthesis, because in contrast to the previous findings, alkaloid biosynthesis is repressed instead of induced by the addition of HDACis and *vice versa*. One possible explanation would include a hypothetical repressor of alkaloid biosynthesis, as explained in the simple model in Fig. 7. Furthermore, it was previously shown that the HAT CBP of *Drosophila melanogaster* represses the transcription factor TCF (Waltzer and Bienz, 1998) which otherwise leads to colon carcinoma (Korinek et al., 1997) substantiating the postulated model.

Based on this model, one possibility for further investigations on the role of chromatin remodeling on the regulation of alkaloid biosynthesis would be knock down of putative HAT encoding genes. The second alternative would be the overexpression of at least one HDAC gene, to overrule the inhibiting effect mediated by HATs.

4. Evolution of the EAS cluster

Within the genus *Claviceps* the capacity to produce ergot alkaloids differs widely: whereas *C. purpurea* and e.g. *Claviceps africana* produce the "high-end" ergopeptines, other species like *C. fusiformis* produce only intermediates like the clavine alkaloids. Also different strains of *C. purpurea* vary in the types of alkaloids they produce ("chemical races"). To understand the genetic basis of this variation and to study the cluster evolution, we analysed the cluster regions of different strains.

4.1. Intra-species evolution: chemical races

Most of the commercially used *C. purpurea* strains produce mixtures of alkaloids. The different ergopeptines vary in their

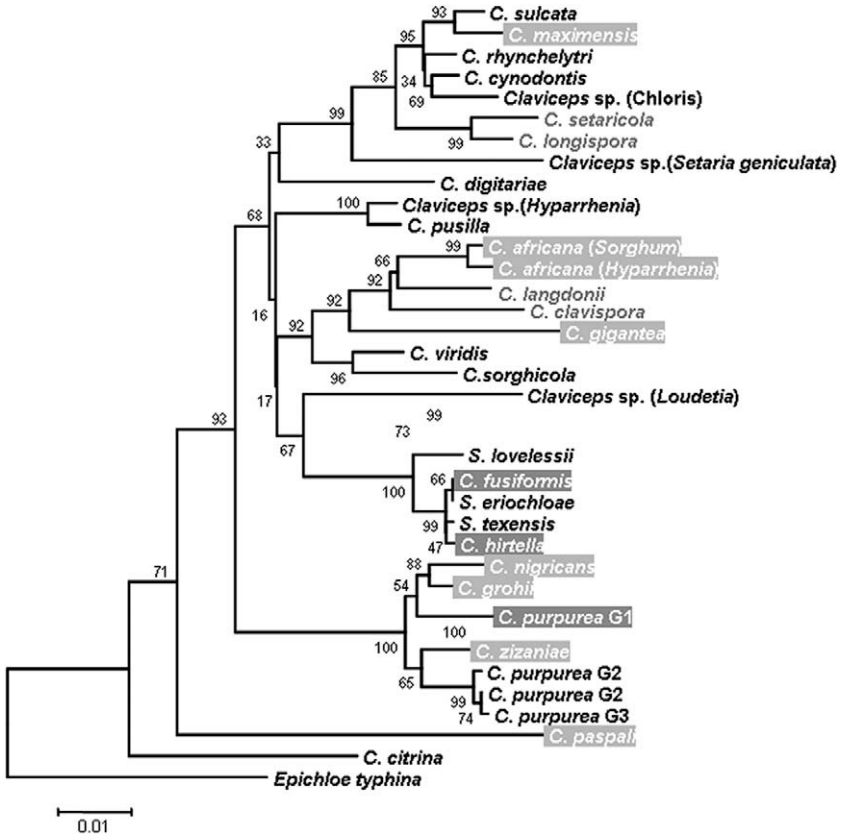

Fig. 8. Evolutionary relationships of 37 *Claviceps* species and isolates based on tubulin sequences. Tubulin sequences were amplified using T1 and T22 primers (O'Donnell and Cigelnik, 1997). Species of further interest are highlighted in light grey boxes, whereas species already investigated are marked in dark grey. Light grey colour indicates species not yet described. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.54277233 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 976 positions in the final dataset, 161 of these were parsimony-informative. The tree was rooted with *Epichloe typhina* as an outgroup. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Abbreviations of species names: C = *Claviceps*; S = *Sphacelia*.

amino acid composition (positions 1 and 2 of the tripeptide moiety can vary, the third is always proline). To analyse if the types of alkaloids produced depend mainly on the internal amino acid pools (as postulated by Riederer et al., 1996) or if there is a genetic basis for the variation (i.e. variability of the NRPS genes present in the different chemical races) we compared the alkaloid gene cluster of standard strain P1 (produces mainly ergotamine and traces of α-ergocryptine) with that of strain ECC93 (main alkaloid: ergocristine). Both strains have an identical cluster structure and possess two trimodular NRPS genes (*lpsA*$_1$/*A*$_2$). Sequence analysis showed that they differ in the sequence of the substrate specificity-determining aminoacid residues of the A domains of the first two modules, strongly suggesting that this difference accounts for the alkaloid types the two strains produce (Haarmann et al., 2005).

4.2. Inter-species evolution

Only very few *Claviceps* species produce ergopeptines, i.e. the end products of the full biosynthetic pathway; in several species only clavine alkaloids or simple lysergic acid amides are synthesized. Apart from the biotechnological relevance of this variability, from the evolutionary point of view it is interesting to study the genetic basis; the major question is if the alkaloid gene cluster in *C. purpurea* is the end point of the cluster evolution and the other species contain precursors. We chose for a comparison with *C. purpurea* (strain P1) two other species (Fig. 8): *C. fusiformis*, a parasite of pearl millet and buffel gras (*Penisetum glaucum* and *Penisetum ciliare*), producing mainly elymoclavine, and *Claviceps hirtella* (a closely related pathogen endemic in Australia, which has a broader host spectrum, colonizing *Urochloa* and *Paspalidium* and *Cenchrus* as well), producing 3–8% of ergometrine in addition to clavine alkaloids (Pažoutová et al., 2008a; see Fig. 9).

The EAS cluster of *C. fusiformis* was sequenced based on clones from a cosmid library (Lorenz et al., 2007). Apart from the three NRPS genes (*lpsC*, *lpsA*$_1$/*A*$_2$) homologues of all *C. purpurea* EAS genes were identified in *C. fusiformis*, including also homologues of *lpsB* and *cloA*, which would not be necessary for the production of clavine alkaloids (the end product in *C. fusiformis*). The *C. fusiformis* cluster was partly rearranged around *lpsB* (see Fig. 11), which (due to partial truncation and several frameshifts) obviously is a pseudogene. The homologue of *cloA*, *CfcloA*, however, showed no obvious inactivating mutations, though the product of *CloA* (D-lysergic acid) is not detectable in *C. fusiformis*. Cross-complementation experiments showed that *cfcloA* and *cflpsB* were expressed in *C. purpurea* but did not encode functional enzymes. On the other hand, CloA from *C. purpurea* catalysed lysergic acid biosynthesis in *C. fusiformis*, strongly suggesting that *C. fusiformis* terminates its EAS pathway at elymoclavine because the *cfcloA* gene product is inactive (Lorenz et al., 2007). These data indicate that the *C. fusiformis* EAS cluster evolved from a more complex cluster (like that of *C. purpurea*) by loss of some *lps* genes and by rearrangement and mutations inactivating genes encoding enzymes involved in pathway steps beyond elymoclavine.

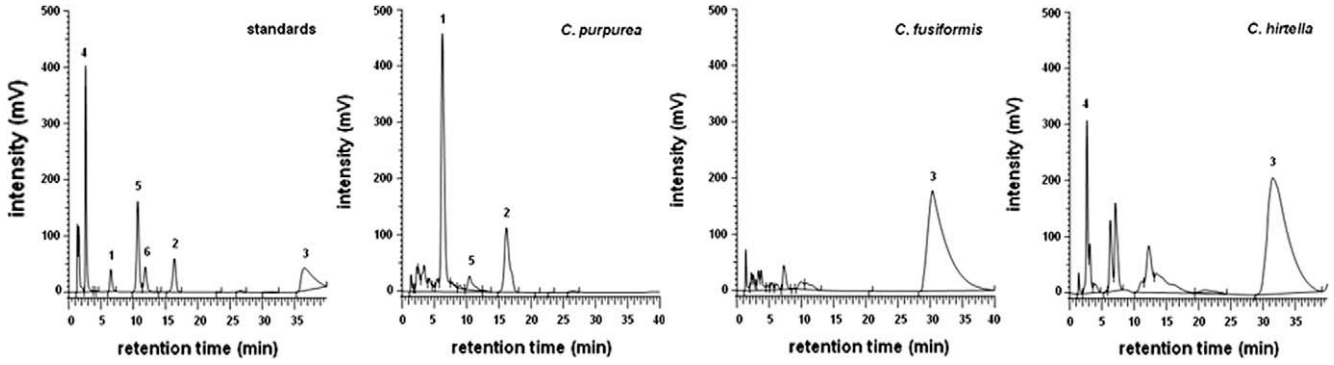

Fig. 9. HPLC analyses of the alkaloid spectra produced by *C. purpurea*, *C. fusiformis* and *C. hirtella*. *C. purpurea* is the only species producing ergopeptides, whereas the other two species have a truncated pathway, leading to the accumulation either exclusively of clavine alkaloids (*C. fusiformis*) or additionally of the D-lysergic acid amide ergometrine (*C. hirtella*). Ergot alkaloids were identified as described by Haarmann et al. (2006) by comparison to known standards: 1 = ergotamine; 2 = its isoform ergotaminine; 3 = agro- and elymoclavine; 4 = ergometrine; 5 = ergocryptine; 6 = ergocristine.

The comparison with the *C. hirtella* cluster was especially interesting, because of its ability to synthesize lysergic acid derivatives, despite its very close relatedness with *C. fusiformis*. The EAS cluster of *C. hirtella* was amplified using primers derived from the sequence of *C. fusiformis* (core EAS cluster) combined with the method of *t*hermal *a*symmetric *i*nter*l*aced (TAIL) PCR (Liu and Whittier, 1995; Terauchi and Kahl, 2000) leading to the sequence of a genomic region of nearly 30 kb containing ten open reading frames. As shown in Fig. 11, the cluster structure is very similar to that of *C. fusiformis*, including the lack of the "large" NRPS encoding genes *lpsA1* and *lpsA2* (which are needed for the production of the complex form of ergopeptides) and the rearrangement on the "left" cluster side. However, the rearrangement in this case does not affect the structure of the *lpsB* homologue. Furthermore, the cluster contains an *lpsC* homologue, explaining why EAS biosynthesis in this species proceeds beyond elymoclavine (including even production of ergometrine), although the *lpsC* is allocated when compared with *C. purpurea* terminating the "right" border in *C. hirtella* instead of the "left" border (see also Fig. 10). On the left border, the cluster is adjacent to an amino peptidase encoding gene, belonging to the primary metabolism. The identity of the *eas* genes varies from 97% to 80% when compared with *C. fusiformis* and between 78% and 65% in comparison to *C. purpurea* on amino acid level.

species / genes	*C. purpurea*	*C. fusiformis*	*C. hirtella*
dmaW	X	X	X
easF	X	X	X
easE	X	X	X
easA	X	X	X
easD	X	X	X
→ clavines		→ clavines	→ clavines
cloA → lysergic acid	X	X (non-functional)	X
lpsB activation of lysergic acid	X	X (non-functional)	X
lpsC activation of alanine → ergometrin	X → ergometrin	–	X → ergometrin
lpsA1(+*A2*) activation of 3 amino acids → ergopeptide	X → ergopeptides	– (neither TAIL-PCR nor cosmid clone)	–
easC	X	X	X
easG unknown functions	X	X	X

Fig. 10. Comparison of ergot alkaloid biosynthesis genes in three *Claviceps* species. The different shades of grey correspond to the position within the cluster (Fig. 11). In the first column all genes of the *eas* cluster of *C. purpurea* are listed in the order they are expected to be needed within the pathway; for example the first five genes are presumed to encode the enzymes essential for synthesis of clavine alkaloids which are the main end products of *C. fusiformis* and *C. hirtella*. The next two genes are present in all three species, but *C. fusiformis* codes for non-functional proteins. Copies of *lpsC* are only found in *C. purpurea* and *C. hirtella* resulting in ergometrine production, only a minor product synthesized in both species. The large NRPS encoding genes *lpsA1*/*2* are exclusively found in *C. purpurea*, the only species producing ergopeptides as main end product. The genes *easC* and *easG* are speculated to be involved in the regulation and were found in all three species. Accession no for the *C. hirtella* sequence: FN393422.

It is obvious that the overall organization of the cluster is highly conserved including also the length and position of the introns (Fig. 11). A detailed analysis was performed to compare the *cloA* copies including also *E. festucae*, especially focusing on the *cloA* genes of *C. hirtella* and *C. fusiformis*. A closer look on the nucleotide level revealed the same exon-intron structure of the genes (confirmed by RT-PCR; data not shown) as well as the same heme binding motif leaving the question unanswered why the gene product of *C. hirtella* remains functional, whereas the protein of *C. fusiformis* became unfunctional.

Comparison of the cluster structures of the three species strongly suggests that the clusters of *C. fusiformis* and *C. hirtella* are derived from the *C. purpurea* type. The rearrangement of the genes *easA* and *lpsB* has taken place before separation of the two species as well as the loss of the large NRPS genes at the right border preventing the production of the very complex ergopeptides. But whereas *C. fusiformis* possesses no functional NRPSs at all (only the presence of a *lpsB* pseudogene could be shown), the alkaloid cluster of *C. hirtella* includes homologues of the genes *lpsB* and *lpsC*, encoding the enzymes needed for activation of D-lysergic acid (LpsB) and incorporation of the amino acid alanine (LpsC) resulting in the production of ergometrine (Ortel and Keller, 2009). In contrast to that, the *lpsB* homologue of *C. fusiformis* became truncated, losing the complete third domain (condensation domain). Furthermore, the *lpsC* homologue "vanished" in *C. fusiformis*, whereas the homologous gene of *C. hirtella* is only relocated at the "right" instead of the "left" border compared to *C. purpurea*.

The most interesting difference between *C. fusiformis* and *C. hirtella* concerns the functionality of the monooxygenase CloA. Investigations on amino acid as well as nucleotide level achieved no obvious hints why this enzyme became non-functional in *C. fusiformis*. It was discussed previously that changes in the genomic environment of this gene could be responsible, especially the rearrangement in the terminator region of *cloA*. But the new results showed that in *C. hirtella* the rearrangement also occurred but did

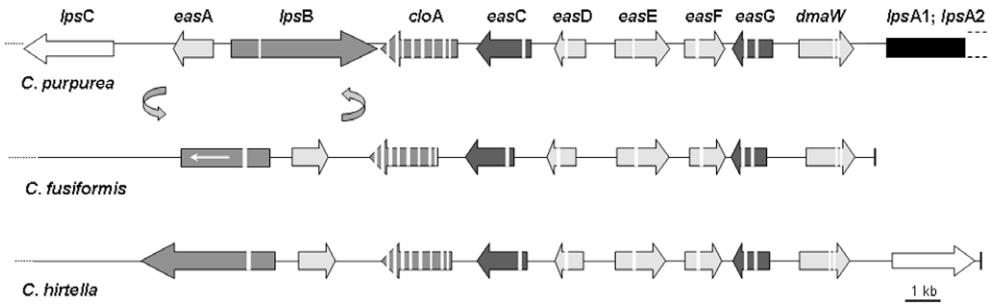

Fig. 11. Schematic overview of the gene composition and orientation of the alkaloid cluster sequences of three *Claviceps* species. Direction of transcription is indicated by arrows. The different shades of grey correspond to the gene groups in Fig. 10: light grey: early cluster genes; medium grey: genes needed for further production and activation of D-lysergic acid; dark grey: genes of unknown function; black: large NRPS genes for the incorporation of three amino acids; white: *lpsC* for the incorporation of alanine; white bars indicate the relative positions and lengths of introns.

not affect functionality of CloA. Therefore, it seems likely that the loss of functionality in *C. fusiformis* is a secondary effect occurring after the rearrangement and was not caused by that event.

Interestingly, all members of the genus *Claviceps* that synthesize ergot alkaloids produce either clavine alkaloids or ergopeptides. There is no species which accumulates paspalic or D-lysergic acid.

Since neither harmful nor toxic effects were observed by accumulation of D-lysergic acid neither in the *lpsB* deletion mutant of *C. purpurea* (Correia et al., 2003) or in the mutant of *C. fusiformis* complemented with the *cloA* gene of *C. purpurea* (Lorenz et al., 2007), it is unclear why ergot alkaloid biosynthesis is strictly limited to either clavine alkaloid or ergopepetide production. A possible explanation could be that the critical time point within the life cycle of *Claviceps* is the overwintering. The hibernation of the fungus is essential for a new round of colonization in the next year, so the ergot alkaloids could be discussed as some kind of a "grub protection" preventing the sclerotium from being eaten by herbivores as well as decomposed by microorganisms. Therefore, it is interesting to mention that clavine alkaloids as well as ergopeptides can be secreted, whereas D-lysergic acid remains in the mycelia, a fact that reduces the effectiveness of the substance produced (data not shown). Therefore, it seems more beneficial to reduce the pathway to the production of clavine alkaloids to enrich the titre of a product which could be secreted provoking much more drastic effects; this could explain the accumulation of mutations in *cloA* in *C. fusiformis* after the loss of *lpsB*.

The variations in the alkaloid spectra can also be discussed in light of fungus/host interaction considering that different classes of alkaloids show slightly varying effects on diverse organisms: nematodes and insects are affected by ergopeptides, whereas the application of clavine alkaloids gained weaker effects (Ball et al., 1997; Clay and Cheplick, 1989). Conversely, clavine alkaloids have been shown to confer antibacterial and cytostatic activity that is superior to that of ergopeptides and simple amides of D-lysergic acid (Eich et al., 1984; Eich and Pertz, 1999; Schwarz and Eich, 1983). Furthermore, it was shown that perennial ryegrass infected with *Epichloë*/*Neotyphodium* species protected its host (Bush et al., 1997). Rabbits for example indeed preferred *Lolium perenne* infected with *Neotyphodium* spec. to non-infected perennial ryegrass, but only if it is "contaminated" with knock out mutants lacking ergot alkaloid biosynthetic capability. On the contrary, production of ergovaline led to "distaste" of the grasses completely spurned by the rabbits (Panaccione et al., 2006).

In summary, it can be concluded that the cluster sequences of *C. hirtella* and *C. fusiformis* most likely share a common ancestor of the *C. purpurea* type and that measured in evolutionary time points the *C. fusiformis* cluster became truncated by loss resp. inactivation of some crucial genes focusing on the production of clavine alkaloids instead of ergopeptide or D-lysergic amide production as it could be observed for *C. hirtella*.

5. Perspectives

The data presented here have improved our understanding of the molecular mechanisms and the evolution of ergot alkaloid biosynthesis in the genus *Claviceps* significantly. From the evolutionary view analysis of other *Claviceps* species with interesting alkaloid content would be highly interesting, e.g. *C. paspali*, because it produces ergometrine as main end product (Arcamone et al., 1960). Therefore, it would be interesting to search for differences in comparison to *C. hirtella* which possesses the ability to synthesize ergometrine but produces mainly clavine alkaloids. Phylogenetic analyses based on the ITS sequence and the β-tubulin gene group *C. paspali* outside the common clades, implicating that this species is very special (Fig. 8).

Another crucial point within the evolution of the alkaloid cluster is the duplication and modification of the lysergyl peptidyl synthetase subunit 1 (*lpsA*). As outlined above, the two copies of *lpsA* of *C. purpurea* strain P1 vary in the specificity of the first module responsible for the incorporation of either alanine or valine leading in the first case to the production of ergotamine, whereas in the second case ergocryptine is synthesized (Haarmann et al., 2005, 2008). Analyses of the alkaloid spectra of ergopeptide producing *Claviceps* species revealed either the production of only one or at least two main end products. Therefore, it would be interesting to examine if the number of ergopeptides produced correlates with the quantity of copies of *lpsA* in the different fungi. Promising candidates would be *Claviceps zizaniae* (→ergocryptine; Kantorová et al., 2002), *Claviceps nigricans* (→ergocristine, ergosine and perhaps traces of ergocryptine and the lactam ergocristam; Pažoutová et al., 2008b) and *Claviceps grohii* (→ergocristine and the lactam ergocristam; Pažoutová et al., 2008b).

Last but not least, the investigation of a putative alkaloid cluster of *Claviceps gigantea* (Fuentes et al., 1964) or *C. africana* (Frederickson et al., 1991) could be very interesting, because both species produce dihydroclavine-derived alkaloids (→dihydroclavine) and in *C. africana*, dihydroergosine was found as the main alkaloid. These species do not belong to either the *C. fusiformis* or *C. purpurea* clade of related species (Fig. 8). It would be informative to examine if the clusters contain the same gene composition leading to the formation of clavine alkaloids or if clavine alkaloid producing capability evolved independently in several *Claviceps* species.

Also a detailed comparison with the closely related (and probably more ancient) endophytic species of the Clavicipitales (Fleetwood et al., 2007; Schardl et al., 2009) would help in understanding the evolution of the complex EAS cluster; in addition, clavine-clusters in not-closely related fungi like that in *A.*

fumigatus would help in identification of "building blocks" of the highly advanced clusters.

An important side-product of the functional analyses of EAS cluster genes is the perspective provided for biotechnological exploitation of the EAS system. Several fascinating new approaches are based on these recent data. The availability of the Δ*dmaW* mutant (N. Lorenz, P. Tudzymnski, unpublished data) allows feeding with DMAT derivatives for the design of alternative clavine alkaloids. Lysergic acid overproducing strains have been generated by deletion of *lpsB* in *C. purpurea* and overexpression of *cloA* in *C. fusiformis*. And the unraveling of the two-component NRPS system opens the possibility to generate (I) strains producing "pure" alkaloids (by deletion of either $lpsA_1$ or A_2) and (II) new alkaloids by using the possibility of combinatoric biosynthesis.

Biosynthesis of ergot alkaloids is a complex and from several standpoints highly interesting pathway. The understanding of the complex regulation and evolution of the EAS cluster will require (and stimulate) further intensive molecular and biochemical research in this area, and the biotechnological exploitation of the wealth of molecular data already available has only just begun.

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