

Structural and functional analysis of an oligomeric hydrophobin gene from *Claviceps purpurea*

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SUMMARY

Fungal hydrophobins are small hydrophobic proteins containing eight cysteine residues at conserved positions which have the ability to form amphipathic polymers. We have characterized a gene from the phytopathogenic ascomycete *Claviceps purpurea*, *cpph1*, which encodes a modular-type hydrophobin. It consists of five units, each showing a significant homology to class II hydrophobins. The units are separated by GN-repeat regions, which could form amphipathic α -helices; the amino terminus contains a glycine-rich region which could be involved in attaching the protein to the cell wall. The presence of long direct repeats within *cpph1*, and the high homology of the three internal modules suggest a recent generation of this gene from a tripartite precursor. Although sequencing of cDNA clones indicated that recombination could be mediated via the direct repeats, the majority of the transcripts appear to be full-sized. This was confirmed by Northern blot analysis, which showed the presence of a full-sized transcript in axenic culture. The high molecular weight pentahydrophobin was detected by Western blot analysis, indicating that CPPH1 is not processed into monomeric subunits. Targeted deletion of *cpph1* did not lead to differences in morphology, growth rate, sporulation, or hydrophobicity of spores. Furthermore, the *cpph1* deletion mutants showed no reduction in virulence on rye.

INTRODUCTION

Hydrophobins are a class of small (\approx 120 amino acids) hydrophobic proteins which have been detected in several types of filamentous fungi and are characterized by their ability to form amphipathic layers at hydrophobic/hydrophilic interfaces

(Wessels, 1994). They are abundant in the aerial structures of fungi, such as aerial hyphae, conidia and fruiting bodies. Hydrophobins have also been implicated in mediation of the interaction between phytopathogenic fungi and their hosts, for example in *Magnaporthe grisea* and *Cryphonectria parasitica* (for review see Kershaw and Talbot, 1998). During a differential cDNA screening aimed at identifying genes involved in ergot alkaloid biosynthesis using a (non-pathogenic) production strain of *Claviceps fusiformis*, we isolated the gene *cfth1* which encodes a new type of hydrophobin: it contained three hydrophobin domains separated by glycine/asparagine (GN) repeats (Arntz and Tudzynski, 1997). Detailed biochemical analysis resulted in the identification of the *cfth1* gene product, a full-sized trimeric protein (not processed into single hydrophobin units) which showed properties of a typical class II hydrophobin (de Vries *et al.*, 1999). Screening of a cDNA library derived from a pathogenic strain of *Claviceps purpurea* established under conditions of C-catabolite derepression identified a comparable gene from this related species. *C. purpurea* is a common pathogen of cereals, infecting young ovaries and replacing the plant's ovarian tissue by its own dormant structures, called sclerotia (for review see Oeser *et al.*, 2002; Tenberge, 1999; Tudzynski *et al.*, 1995). As *in situ* hybridization using the cDNA of the putative trihydrophobin-homologue showed the corresponding transcript in early stages of *C. purpurea* infection *in planta* (Tenberge *et al.*, 1998), we were interested in the function of this hydrophobin gene during pathogenesis. Here we report the cloning and characterization of the *C. purpurea* homologue of *cfth1*, a pentahydrophobin gene called *cpph1*, and the result of a targeted gene replacement aimed at defining the gene product's role in fungal development and pathogenicity.

RESULTS

Characterization of *cpph1*, a pentahydrophobin gene from *C. purpurea*

Screening of a cDNA library of *C. purpurea* strain T5 grown under low glucose conditions for a β 1,3-glucanase gene (using a

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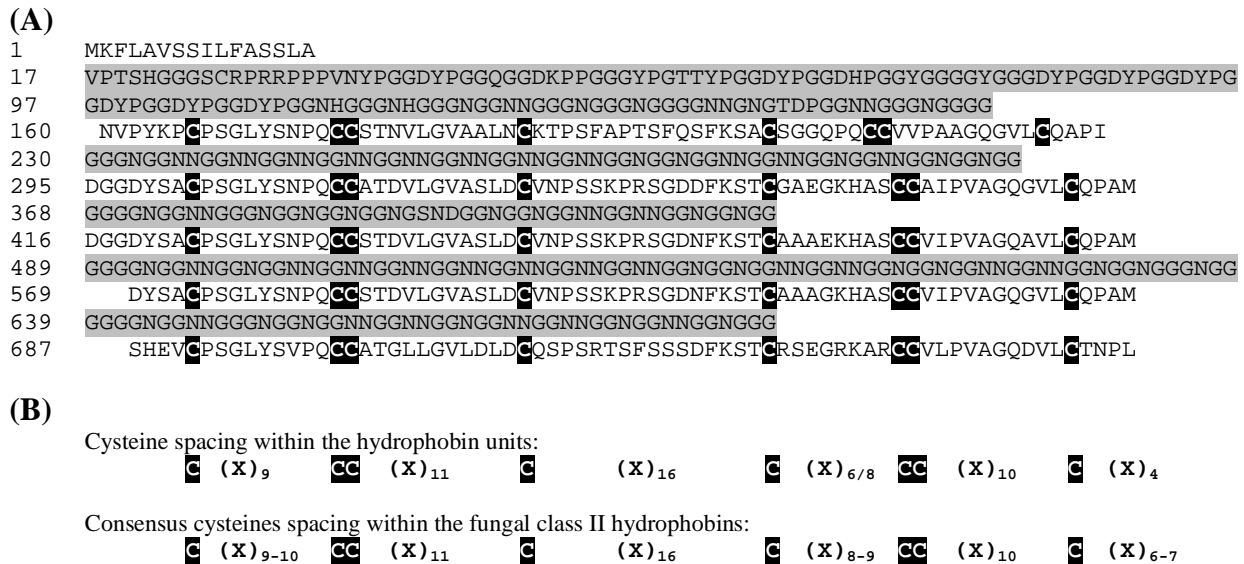


Fig. 1 CPPH1 modular structure. (A) *cph1* deduced peptide sequence. The first 16 amino acids correspond to the predicted peptide signal. The glycine-rich region and the glycine/asparagine repeats are highlighted in grey. The conserved cysteine residues of the hydrophobin domains are shown in black. (B) Spacing of the conserved cysteine residues within the five hydrophobin units from CPPH1 (top) and within all the fungal class II hydrophobins (bottom).

glucanase gene from *Trichoderma viride* as probe) by chance yielded a cDNA clone which showed significant homology to the *C. fusiformis* trihydrophobin gene *cfth1*. Like *cfth1*, this gene seemed to have at least three hydrophobin domains separated by GN-repeats. In order to obtain a full length copy of the gene, we probed a genomic λ EMBL3 library of strain T5 with a cDNA clone from the *cfth1* gene of *C. fusiformis*. A total of five genomic clones were selected which spanned a 4.2-kb genomic region containing a complete copy of a gene which showed a similar structure and significant homology to the *C. fusiformis* trihydrophobin gene *cfth1*, but which contained two additional hydrophobin-encoding modules. The gene was dubbed *cph1* (*C. purpurea* pentahydrophobin gene 1). The derived amino acid sequence of the *cph1* coding region and a schematic map of the gene are shown in Figs 1 and 3, respectively.

The putative *cph1* product (CPPH1) has a modular structure: five hydrophobin domains (of 70, 73, 73, 70 and 70 amino acids, respectively) are separated by long stretches of glycine/asparagine repeats (Fig. 1A). The first G/N repeat is preceded by a glycine-rich region, containing repeats of a PGGDY motif. The first 16 amino acids of CPPH1 probably represent a signal peptide, according to the criteria of von Heijne (1986), indicating that the protein is secreted (Fig. 1A). The coding sequence of *cph1* is interrupted by 10 introns, two in each of the hydrophobin domains (Fig. 3); their position has been postulated by the presence of intron-consensus sequences (Balance, 1991) and confirmed by sequencing cDNA clones (data not shown). Taking into account the putative signal peptide and the introns, the mature

CPPH1 protein should have a size of 756 amino acids, corresponding to a molecular weight of about 70 kDa.

An alignment of the five CPPH1 hydrophobin domains with other class II hydrophobins showed that they have the highest similarity with the domains of *C. fusiformis* CFTH1 (60–71% identity) but they also display significant homology to other conventional class II hydrophobins such as *Trichoderma harzianum* Qid 3 (47–55%), *Cryphonectria parasitica* cryparin (CRP, 47–52%), *Trichoderma reesei* HFB I (46–51%), HFB II (41–44%), and *Ophiostoma ulmi* cerato-ulmin (36–39%). No significant homology was found between the CPPH1 hydrophobin domains and class I hydrophobins.

Consistent with the sequence homology observed, the spacing of cysteine residues within the five hydrophobin units of CPPH1 corresponds to the consensus defined for the fungal class II hydrophobins (Fig. 1B) (Wessels, 1994). In addition, the hydrophobicity profiles of the hydrophobin domains of CPPH1 show significant similarity to class II hydrophobins, as the two cysteine doublets are followed by hydrophobic residues, instead of the hydrophilic residues found in the corresponding regions of class I hydrophobins (Segers *et al.*, 1999; Wessels, 1997). Taken together, these data suggest that *cph1* encodes a modular class II hydrophobin.

A comparison of the five hydrophobin domains shows that the three internal units are more than 90% identical at both peptide and nucleotide levels and have significantly higher homology to each other than to the first and the fifth one, implying that these internal domains may stem from a common ancestor (Fig. 2).

(A)

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PH 1  NVP-YKPCPSGLYSNPQCCSTNVLGVAAALNCKTPSEFAP
PH 2  DGGDYSAACPSGLYSNPQCCATDVLGVASLDCVNPSSKP
PH 3  DGGDYSAACPSGLYSNPQCCSTDVLGVASLDCVNPSSKP
PH 4  D---YSACPSGLYSNPQCCSTDVLGVASLDCVNPSSKP
PH 5  S---HEVCPSTGLYSVPQCCATGLLGVLDLDCQSPSRTS

PH 1  TSFQSFKSACSGG--QPQCCVVPA--AGQGVLCQAPI
PH 2  RSGDDFKSTCGAEGKHASCAIPV--AGQGVLCQPAM
PH 3  RSGDNFKSTCAAAEKHASCCVIPV--AGQAVLCQPAM
PH 4  RSGDNFKSTCAAAGKHASCCVIPV--AGQGVLCQPAM
PH 5  FSSSDFKSTCRSEGRRKARCCVLPVSQAGQDVLCTNPL

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(B)

PH2	PH3	PH4	PH5	
54.3	54.3	57.1	42.9	PH1
	90.4	92.9	52.8	PH2
		97.1	48.6	PH3
			52.4	PH4

Fig. 2 Identity between the different hydrophobin units from CPPH1. (A) Alignment of the deduced peptide sequences of the five hydrophobin units from CPPH1. Conserved amino acids are shown in black. The alignment was constructed using the MEGALIGN program (DNASTAR package). (B) Percentage of identity between the predicted peptidic sequences of the CPPH1 hydrophobin units (named PH1 to PH5).

Expression of *cpph1*

Interestingly, the first cDNA clone that we obtained corresponding to *cpph1* (see above) had a trimeric structure. As shown in Figs 2 and 3, the second, third and fourth hydrophobin units constitute three long direct repeats and recombination between these repeats could yield the trimeric cDNA (schematic representation in Fig. 3). In order to determine whether this recombination occurs with a high enough frequency to produce a trihydrophobin such as that found in *C. fusiformis*, several cDNA clones were isolated by screening a cDNA library of strain 20.1 (a haploid derivative of strain T5, showing identical parasitic properties and being used now as a standard strain for molecular analyses), using *cpph1* as a probe. Four out of five sequenced cDNAs were shown to encode the full-length pentahydrophobin protein (Fig. 3). Only one recombinant clone was isolated, which carries a small in-frame deletion in the GN repeat preceding the first hydrophobin unit. Furthermore, Northern analyses using mRNA extracted from axenic cultures of *C. purpurea* strain 20.1 and a complete cDNA as a probe showed that the main transcript size was 2.4 kb (Fig. 4, lane 1, a). This corresponds to the size expected for the full length mature transcript. In addition, there is a smaller (≈ 1.4 kb) transcript (Fig. 4, lane 1, b), the origin of

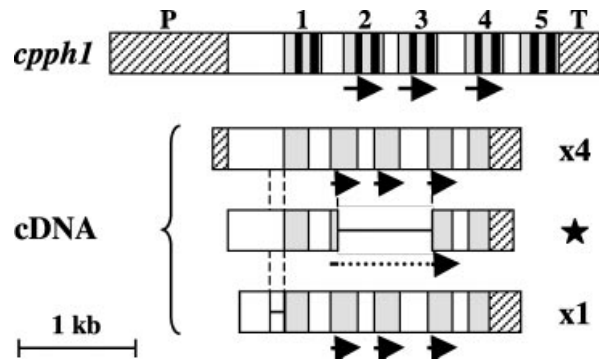


Fig. 3 Schematic representation of the *cpph1* gene and cDNAs. Promoter (P) and terminator regions (T) from the *cpph1* gene (top) are represented by hatched boxes, the coding sequence with a white box. The hydrophobin domains are shown in grey and the introns in black. The three internal hydrophobin units form direct repeats, which are indicated with black arrows. The structure of the cDNA clones is represented below with the same colour usage. The number of each type of clone isolated and further characterized is shown on the right. The first cDNA clone isolated, encoding a trihydrophobin, is marked with a black star. The parts of the exons deleted in the recombinant cDNA clones are indicated with a thin line.

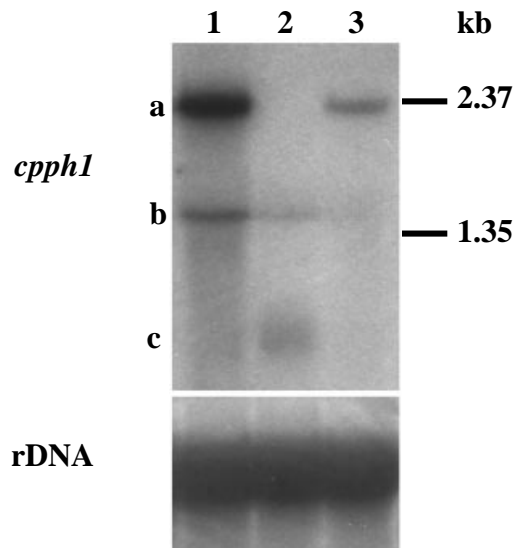


Fig. 4 The major *cpph1* transcript codes for the full-length protein. Northern blot analysis of the mRNA extracted from the wild-type strain (lane 1), the Δ *cpph1-1* mutant (lane 2) and the ectopic transformant P2.43 (lane 3) grown for 4 days in Mantle medium. 20 μ g RNA were loaded in each lane and hybridized with a *cpph1* complete cDNA clone. The blot was dehybridized and hybridized with a ribosomal DNA probe.

which is unknown; it is too small to represent the trimeric form. These results suggest that the recombination event leading to the trimeric cDNA clone is not of major significance and that the main product of *cpph1* is a pentahydrophobin.

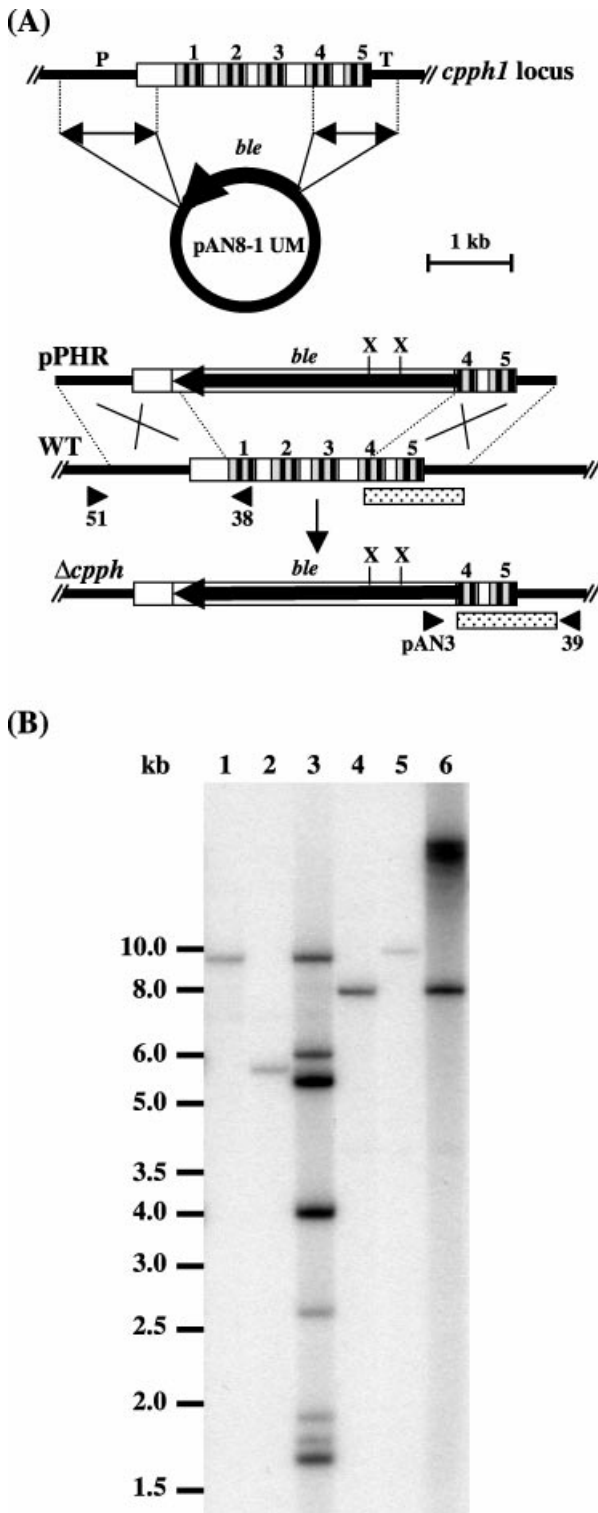


Fig. 5 Gene replacement approach used to inactivate *cph1*. (A) The replacement vector pPHR was constructed by cloning the 3' and 5' parts of *cph1* on each side of a phleomycin resistance cassette (*ble*) in the pAN8-1UM plasmid (top, see Experimental procedures for details). The resistance cassette, excised using a *Sac*II-*Kpn*I restriction, was used to transform the *C. purpurea*

Generation of *cph1*-deleted mutants

In order to unequivocally identify the main product of *cph1* and to assess its role in pathogenesis, deletion mutants were created using a gene replacement approach. A replacement vector, pPHR, was constructed which contains a phleomycin-resistance cassette flanked by 5' and 3' parts of the gene (see Experimental procedures and Fig. 5A). The replacement construct, excised using a *Sac*II/*Bam*HI restriction, was used to transform *C. purpurea* wild-type strain 20-1. Phleomycin-resistant transformants were selected and two primary transformants P2.35 and P2.29, named $\Delta cph1-1$ and $\Delta cph1-2$, respectively, were identified by PCR using primers 39 and pAN3 (Fig. 5A) as containing nuclei with the desired replacement event. The heterokaryotic strains were genetically purified by single spore isolation and progeny were analysed for loss of *cph1* (Fig. 5A).

To confirm the gene replacement event, genomic DNA from the wild-type strain, an ectopic transformant (P2.43) and the deletion mutant $\Delta cph1-1$ were subjected to Southern blot analysis. As shown in Fig. 5B, a 9.5 kb *Xho*I fragment and a 8 kb *Hind*III fragment which hybridize with *cph1* are present in both the wild-type strain and the transformant P2.43; the latter carries several ectopic insertions of the replacement construct as indicated by additional hybridizing bands (Fig. 5B). Neither of these wild-type fragments could be detected in the $\Delta cph1-1$ strain. Instead, *Xho*I and *Hind*III fragments of 5.7 kb and 9.7 kb, respectively, were revealed which are characteristic of a gene replacement. No additional fragment was detected in the $\Delta cph1-1$ transformant, indicating that no additional insertion of the replacement vector occurred.

Northern blot analysis of axenically grown cultures showed that the deletion mutant $\Delta cph1-1$ lacks the full-sized *cph1* transcript (Fig. 4, lane 2,a). The 1.4 kb transcript (b) is still present in the mutant, ruling out that it could represent the (recombinant) trimeric form. In the mutant, a unique small transcript (c) was

wild-type strain 20-1. $\Delta cph1$ mutants were generated following the deletion of the wild-type gene (WT) by homologous recombination through a double crossover event. The *cph1* coding sequence and introns are represented by white and hatched boxes, respectively. The hatched arrow indicates the orientation of the phleomycin resistance cassette within the replacement construct. The positions of primers 51/38 and pAN3/39, used for the detection of *cph1* wild-type copy and for the identification of the $\Delta cph1$ mutants, respectively, are indicated with black triangles. X: *Xho*I restriction sites. There is no *Hind*III restriction site within the sequenced genomic region carrying *cph1*. (B) Southern blot analysis of the genomic DNA extracted from the wild-type strain 20-1 (lanes 1 and 4), the $\Delta cph1-1$ mutant (lanes 2 and 5) and the transformant P2.43 carrying ectopic integrations of the replacement construct (lanes 3 and 6). The DNA was digested with *Xho*I (lanes 1-3) and *Hind*III (lanes 4-6). The bp *Eco*RV-*Kpn*I fragment from *cph1*, corresponding to the 3' flank of the replacement vector (dotted box shown in A), was used as a probe.

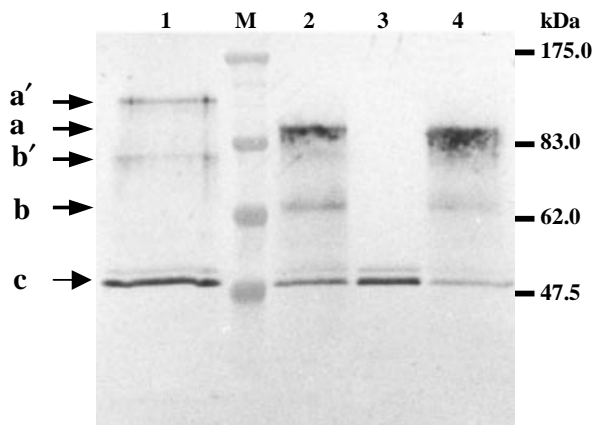


Fig. 6 *cpph1* encodes a high molecular weight hydrophobin. Western blot analysis of the cell-wall associated proteins from the wild-type strain 20-1 (lanes 1 and 2), mutant $\Delta c p p h 1-1$ (lane 3) and transformant P2.43 carrying ectopic integrations of the replacement construct (lane 4). The mycelium was grown for 4 days in Mantle medium. The ethanol soluble fraction of the cell-wall proteins was then purified, separated in a SDS-PAGE gel containing 8.5% acrylamid, blotted on to nitrocellulose and probed with the antibodies raised against the trihydrophobin from *C. fusiformis*. Loading buffer containing β -mercaptoethanol was used in lane no. 1, no β -mercaptoethanol was used in the sample loaded on lane 2.

detected by the probe. According to the sequence of the four full-length cDNA clones, the transcription of *cpph1* is initiated 137 bp upstream of the predicted start codon. The deletion of *cpph1* following the replacement event is predicted to interrupt the coding region 157 bp downstream of the translation initiation site. Thus, the truncated 5' part of the deleted gene copy could be at least 300 bp long and could be responsible for the small transcript detected in the $\Delta c p p h 1-1$ mutant. This mRNA does not code for a full-length hydrophobin unit.

Characterization of the *cpph1* translation product

Western blot analysis was performed to identify the *cpph1* gene product and to determine whether CPPH1 is post-translationally processed to yield monomeric hydrophobin subunits. Class II hydrophobins were isolated from the cell-walls of the axenically cultivated strains 20.1, $\Delta c p p h 1-1$ and P2.43, and subjected to Western analysis using polyclonal antibodies raised against *C. fusiformis* CFTH1. A prominent protein band with a molecular size of about 85 kDa (a), and a faint band of about 65 kDa (b), detected in protein extracts from strains 20.1 and P2.43 (Fig. 6, lanes 2 and 4) were missing in the $\Delta c p p h 1-1$ strain (Fig. 6, lane 3), whereas a double band of about 50 kDa (c) is present in all four lanes. Full denaturation of cell-wall associated proteins from the wild-type strain and the P2.43 transformant with β -mercaptoethanol decreased the migration distance of the high molecular weight cross-reacting proteins, whose apparent

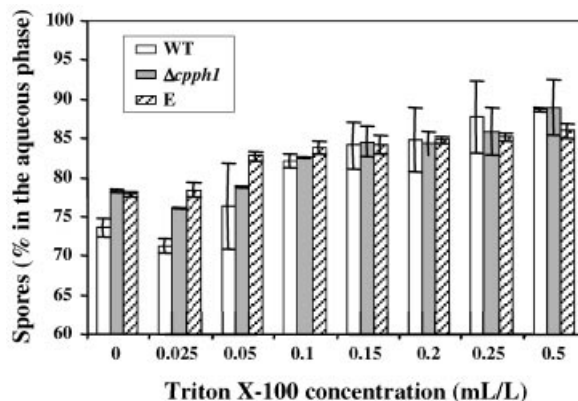


Fig. 7 Hydrophobicity assay. Conidia from the wild-type strain 20-1 (WT), the $\Delta c p p h 1-1$ mutant ($\Delta c p p h 1$), and the transformant P2.43 carrying an ectopic integration of the replacement construct (E) were resuspended in PBS buffer and mixed with hexadecane and various concentrations of Triton X-100. After separation of the aqueous and the solvent phase, the concentration of the conidia in the aqueous phase was determined by measuring the absorbance at 600 nm.

molecular mass increased to about 120 kDa and 80 kDa, respectively (Fig. 6, lane 1, a' and b', and data not shown). This shift to a higher apparent molecular weight of the prominent band after cleavage of cysteine bridges is typical for hydrophobins (Wessels *et al.*, 1991). It should be noted that the observed apparent molecular weight of band a is much larger than that reported for the trihydrophobin CFTH1 (55–65 kDa). We conclude that this high molecular weight protein present in the wild-type and absent in the deletion mutant is the full-sized product of *cpph1*, i.e. a pentahydrophobin.

Role of CPPH1 in the pathogenicity of *C. purpurea* and hydrophobicity of conidia

The deletion mutant showed no specific phenotype in axenic culture with respect to morphology, growth rate and sporulation efficiency. Because hydrophobins are known to influence the hydrophobicity of fungal hyphae and spores, we performed the microbial adhesion to hydrocarbons (MATH) assay described by Rosenberg and Doyle (1990) and adapted by Whiteford and Spanu (2001) to characterize hydrophobin-deficient mutants of *Cladosporium fulvum*. Hexadecane was added to a suspension of conidia from the wild-type strain, the deletion mutant $\Delta c p p h 1-1$ and an ectopic transformant P2.43. The mixture was shaken and left to separate into aqueous and non-aqueous layers. Conidia tend to attach to the oil–buffer interface and are released into the aqueous phase by addition of a detergent, such as Triton X-100. Titration with the detergent may reveal differences in hydrophobicity between different spore samples, based on the amount of spores present in the aqueous phase at a given detergent concentration. As shown in Fig. 7, the amount of conidia in the

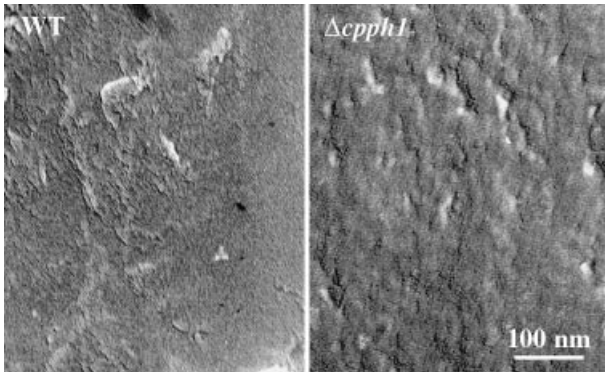


Fig. 8 Surface analysis of conidia. conidia of the wild strain 20.1 (WT) and the $\Delta cpph1$ mutant were viewed as replicas made after freeze fracture.

aqueous phase is already rather high without Triton X-100 (74–78% of the total amount). Although the proportion of spores in the aqueous phase rose slightly with increasing amounts of detergent, no significant difference in the hydrophobicity of the three strains tested was observed (Fig. 7). This indicates that the conidia of *C. purpurea* have a relatively hydrophilic surface compared to other previously examined species, but that the pentahydrophobin does not contribute significantly to its surface activity. Similarly, no differences in the contact angles of water droplets placed on the surface of sporulating mycelium from the wild-type strain and the transformants $\Delta cpph1-1$ and P2.43 were observed.

Rodlet proteins have been shown to be the products of Class 1 hydrophobins by their absence in null mutants (Stringer *et al.*, 1991; Bell-Pederson *et al.*, 1992; Talbot *et al.*, 1996). To date no rodlet structures have been reported as the products of Class II hydrophobins. We examined the surface of conidia of both the *C. purpurea* wild-type 20.1 and the mutant strain $\Delta cpph1-1$ using transmission electron microscopy of replicas of freeze-fractured conidia. However, no rodlet layer was observed on the conidia of either strain (Fig. 8).

In order to test whether deletion of the pentahydrophobin gene affects the pathogenicity of *C. purpurea*, *in planta* infection tests were performed. Rye florets were inoculated with conidial suspensions of the mutant strain $\Delta cpph1-1$, the wild-type strain 20.1 and the ectopic transformant P2.43 (10–12 ears with 30–40 individual florets for each strain). The development of disease symptoms was monitored over 4 weeks. The ears inoculated with spores of the $\Delta cpph1-1$ mutant did not differ significantly from those infected with the wild strain or an ectopic mutant with respect to the amount and the time of appearance of honeydew (about 7 dpi) and sclerotia (about 20 dpi) (see Fig. 9). These observations indicate that under the conditions of the test system, the *cpph1* gene product is not essential for the pathogenicity of *C. purpurea*.



Fig. 9 Pathogenicity assay on rye plants. Conidial suspensions of the wild-type strain 20.1 (WT), the $\Delta cpph1-1$ mutant ($\Delta cpph1$), and the transformant P2.43 carrying an ectopic integration of the replacement construct (E) were used to infect rye florets. The pictures were taken 4 weeks post-inoculation. The white arrows indicate the position of the sclerotia.

Thus, examination of $\Delta cpph1$ -deletion mutants revealed no phenotype in both axenic and parasitic culture, and as of yet, no specific function can be attributed to this unique oligomeric hydrophobin.

DISCUSSION

Here we have reported the isolation and characterization of a gene encoding a modular protein composed of five class II hydrophobin units separated by glycine/asparagine repeats. This kind of modular structure has until now only been described for the trihydrophobin CFTH1 in *C. fusiformis* (de Vries *et al.*, 1999) and seems unique to the genus *Claviceps*. The nearly identical three internal hydrophobin units within CPPH1 constitute three direct repeats. Together with the existence of a recombinant clone encoding a trihydrophobin-encoding version of CPPH1, this observation has interesting implications for the generation of such an oligomeric gene. The available data suggest that the central domain of a tripartite ancestor duplicated, subsequently generating the pentameric form.

Computer predictions indicate that the glycine/asparagine repeats may form amphipathic α -helices, with a bi-facial

distribution of the glycine and asparagine residues, comparable to *cft1* (de Vries *et al.*, 1999). The class II hydrophobins QID3 from *T. harzianum* (Lora *et al.*, 1994) and Hcf6 from *C. fulvum* (Nielsen *et al.* 2001) possess similar N-terminal GN repeats. In addition, CPPH1 is characterized by a long N-terminal extension, including a glycine-rich region with (partially modified) repeats of a PGGDY motif upstream of the first GN repeat. Interestingly, this domain is not present in the corresponding protein CFTH1 from *C. fusiformis*. The presence of such a long N-terminal region has also been reported for the class II hydrophobins Hcf4 from *C. fulvum* (Segers *et al.*, 1999) and CRP from *C. parasitica* (Zhang *et al.* 1994), although only the latter is rich in glycine residues. The CPPH1 hydrophobin units have significant homology to the class II hydrophobins QID3, CRP and Hcf6; the major difference is the oligomeric structure of CPPH1.

The isolation of a cDNA clone encoding a tripartite version of CPPH1 raised the possibility that recombination between the internal direct repeats may occur naturally, leading to the synthesis of truncated versions of the protein. This hypothesis was supported by the further identification of a recombinant cDNA clone carrying an in-frame deletion in the GN repeat preceding the first unit. However, most of the cDNA clones thus far sequenced encode the complete pentameric protein, and the major band detected in Northern analysis corresponds to the full-length transcript (Fig. 4, lane 1,a), suggesting that recombination constitutes a rare event. The identity of the smaller transcript observed in the wild-type RNA sample (Fig. 4, lane 1,b) is open; it represents a minor species. Its absence in lane 3 (ectopic transformant) is most likely due to the overall lower hybridization in this lane. Since it is also present in the mutant, it cannot be derived from *cpph1*.

In order to determine whether CPPH1 could be post-translationally processed to yield single hydrophobin units, SDS-PAGE analysis was undertaken. Western blots of cell-wall associated proteins separated by SDS-PAGE and then probed with polyclonal antibodies raised against *C. fusiformis* CFTH1 revealed two protein bands of high molecular weight which could be shifted by the addition of β -mercaptoethanol (Fig. 4, a/a', b/b'). No degradation products of small size were detected in Western blot experiments, suggesting that CPPH1 is not processed into single units. However, the occurrence of such small degradation products cannot be ruled out completely, because they might behave differently in the extraction procedure, or they might not react with the antibodies. In *U. maydis*, the surface-active proteins named repellents are processed from a single precursor protein, Rep1. These peptides are produced in abundance during filamentous growth; only the proteolysis products could be detected by Coomassie Blue staining after SDS-PAGE as well as by HPLC (Wösten *et al.*, 1996). In contrast to Rep1, the CPPH1 sequence does not contain any obvious cleavage site for a Kex2-like protease, although the presence of other protease cleavage sites

cannot be excluded. The hypothesis that CPPH1 is not processed to yield single hydrophobin units is further supported by the observation that it can be resolved as a single protein with an apparent molecular weight that is significantly higher than that of its homologue, CFTH1 from *C. fusiformis*, which is definitely not cleaved into single hydrophobin units, as revealed by the N-terminal sequencing of the mature protein and of a fragment obtained by trypsin digestion (de Vries *et al.*, 1999).

Moreover, the 65 kDa protein detected in the Western blot analysis (Fig. 4b) could be the pentameric form of CPH1; corresponding observations have been published for other hydrophobins (e.g. de Vries *et al.*, 1993); alternatively, it could correspond to the trimeric form. The protein is also shifted by treatment with β -mercaptoethanol and it is obviously a translation product of *cpph1*, as it is lacking in the Δ *cpph1* mutant. On the other hand, the observation that over-expression of a truncated version of one hydrophobin gene in *C. fulvum* (Hamada and Spanu, 1998; Whiteford and Spanu, 2001) can lead to the silencing of other hydrophobin genes showing almost no sequence homology, raises the possibility that the anti-CFTH1 antibodies bind another hydrophobin whose expression is repressed in the Δ *cpph1* mutant, perhaps due to the synthesis of a short *cpph1* transcript.

The prominent double band at about 50 kDa, which is also present in the mutant, could represent a cross-reacting cell wall protein; it is probably not a hydrophobin, because it is not shifted with β -mercapto-ethanol. *Cpph1* was previously shown to be expressed during both vegetative growth and rye infection (Tenberge *et al.*, 1998; T. Correia, unpublished observations). We developed a gene replacement approach in order to investigate the role of CPPH1 during these different developmental processes. The characterized Δ *cpph1* mutant did not show any phenotype, either in axenic culture with respect to conidiation, hydrophobicity of conidia and of sporulating mycelium, or during the parasitic stage. In a number of Basidiomycetes, hydrophobins were shown to be expressed during the dikaryotic phase and were found in fruiting bodies (see Kershaw and Talbot, 1998 for review; Ando *et al.*, 2001; Ng *et al.*, 2000; Santos and Labarère, 1999). Until now, no indication of the involvement of hydrophobins in the sexual reproduction of ascomycetes has been documented, and it would be interesting to determine whether *C. purpurea* CPPH1 could be involved in such developmental processes. On the other hand, the expression of *cpph1* during the whole infection process, as shown by RT-PCR (data not shown) points to a more general role of these proteins.

A distinct monomeric class II hydrophobin named CPH1, showing significant homology to CPPH1, was recently identified in *C. purpurea* (S. Moore, M. Mihlan, P. Tudzynski, unpublished data). If CPH1 and CPPH1 have comparable functions, the lack of CPPH1 in the generated deletion mutants could be compensated by CPH1. The further analysis of the role of class II hydrophobins

in *C. purpurea* could require the generation of transformants in which both genes are simultaneously deleted.

A typical property of class I hydrophobins is the formation of rodlet layers at the surface of fungal structures exposed to the atmosphere. Up to now, no rodlet structure could be attributed to class II hydrophobins; the purified class II hydrophobins CRP from *C. parasitica* and CFTH1 from *C. fusiformis* do not form rodlets upon self-assembly (de Vries *et al.*, 1999; Wösten and de Vocht, 2000). Rodlet structures formed by class I hydrophobins at the fungus/plant interface have already been observed within the lichen thallus formed by the ascomycetes *Xanthoria parietina* and *X. ectaneoides* (Scherrer *et al.*, 2000). Interestingly, a rodlet layer was observed neither on the conidia from *C. purpurea* in axenic culture nor on the different structures (hyphae, conidiophores, phyalids and conidia) developed during parasitic growth. Thus, no class I hydrophobins seem to be synthesized by *C. purpurea* in axenic culture or during plant infection. The observation reported here, that the spores of *C. purpurea* are obviously relatively hydrophilic compared with those of other species, is consistent with the lack of an observable rodlet layer.

It is interesting to note that thus far no function has been attributed to a class II hydrophobin. CPPH1, with its unique oligomeric structure, appears to be no exception. This is significant, because class II hydrophobins are widespread and their specific cysteine pattern is highly conserved in different classes of fungi. It is possible that the role of class II hydrophobins in filamentous fungi involves interactions with other proteins or is not immediately apparent under the narrow range of conditions in which fungi are cultivated in the laboratory. Further investigations will require an assessment of the relative fitness of strains lacking class II hydrophobins if the function of this elusive class of proteins is to be determined.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The wild-type *Claviceps purpurea* strain used in these experiments was 20.1, a putative haploid derivative of a standard field isolate T5 (Fr.Fr.) Tul., isolated from rye (*Secale cereale* L.; Hohenheim, Germany), and obtained by benomyl treatment (Hüsken *et al.*, 1999). For conidial harvesting and DNA isolation, mycelia were cultivated on Mantle agar (16 g/L agar) with 100 g/L sucrose (Mantle and Nisbet, 1976) at 28 °C for 12–14 days. For the expression analyses, 10⁶ conidia were inoculated into 100 mL Mantle medium. The cultures were incubated for 4 days at 28 °C and 180 r.p.m.

Escherichia coli strain TOP10F' (Invitrogen) was used for all the subcloning experiments. *E. coli* strains LE392 and XL1Blue (Stratagene) were employed for the propagation of *C. purpurea* genomic and cDNA lambda clones, respectively.

Nucleic acid extraction and analysis

Standard recombinant DNA methods were performed according to Sambrook *et al.* (1989) and Ausubel *et al.* (1987). Genomic DNA from *C. purpurea* was prepared from lyophilized mycelium according to Cenis (1992). For Southern blot analysis, 5–10 µg of restricted chromosomal DNA or PCR products were electrophoresed in 0.8–1.6% agarose gels with salt free buffer (Sambrook *et al.*, 1989), blotted on to positively charged nylon filters (Hybond N+, Amersham, Braunschweig, Germany), and hybridized with radio-labelled DNA probes in Denhardt's hybridization solution (Sambrook *et al.*, 1989) at 65 °C. Filters were washed for 10 min in 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulphate (SDS) and 10 min in 1 × SSC 0.1% SDS at 65 °C.

DNA sequencing was carried out as described by Moore *et al.* (2002). Protein and DNA sequence alignment, editing and organization was done with the help of DNASTAR (Madison, WI). Sequence analysis was done using BLAST at NCBI (Altschul *et al.*, 1990).

The polymerase chain reaction (PCR) was carried out as described by Saiki *et al.* (1988), using Red Taq Polymerase (Sigma). All primers were synthesized by MWG-Biotech (München, Germany). The amplification products were cloned using the PCR 2.1 TOPO-Cloning Kit (Invitrogen).

Cloning of *cpph1*

Isolation of the genomic copy of *cpph1* was performed by probing a genomic λEMBL3 library of *C. purpurea* strain T5 (Smit and Tudzynski, 1992), with a 1.8-kb cDNA fragment of the *C. fusiformis cpth1* gene (Arntz and Tudzynski, 1997) using the plaque filter hybridization technique according to Sambrook *et al.* (1989). Of 50 000 screened plaques, 16 hybridizing λ clones were obtained from them; two were purified and shown to contain an overlapping genomic region which hybridized to the probe. A 3.8 kb *EcoRI/SalI* fragment common to both phages was subcloned, completely sequenced and shown to contain the complete *cpph1* coding sequence. *Cpph1*-cDNA clones were obtained by screening a λ ZAP express cDNA library constructed from copper-induced cDNA of strain T5 (Moore *et al.*, 2002).

Gene expression analysis

Total RNA was extracted from lyophilized mycelium using the RNeasy total RNA Isolation System (Promega, Madison, WI). 20 µg of total RNA were loaded per lane in a 1.5% (w/v) formaldehyde agarose gel, blotted on to a positively charged nylon filter (Hybond N+, Amersham, Braunschweig, Germany), and hybridized to ³²P-labelled DNA probes in Denhardt's hybridization solution (Sambrook *et al.*, 1989) at 65 °C. The filter was washed

for 10 min in 2 × SSC 0.1% SDS and 10 min in 1 × SSC 0.1% SDS at 65 °C. The membrane was stripped between hybridizations with different probes by washing for 10 min at 100 °C in 0.1% SDS.

Construction of the replacement vector

For construction of the *cpph1* replacement vector, the genomic region located between –326/+312 bp relative to the stop codon, was amplified by PCR using primers 53 (5'-GTCAACCG-TAAGTTACC-3') and 35 (5'-CCAGCGCTGCCTTGGTAGC-3') and cloned into the PCR2.1 TOPO vector (Invitrogen). After excision of the fragment with *EcoRV*–*KpnI* restriction, it was subcloned into the corresponding restriction sites of the pAN8–1UM vector (Müller, 1997) upstream of the phleomycin resistance cassette (*ble*) to create the pPHT1 vector. The genomic region located between –924/+156 bp relative to the predicted start codon was amplified by PCR using primers 54 (5'-CCACCACCACTGT-GACAGC-3') and 43 (5'-CGCCAGGAGTTTATACC-3') and cloned into the PCR2.1 TOPO vector to yield the vector p43. The replacement vector pPHR was obtained by subcloning the *SacII*–*NotI* insert from p43 into the corresponding restriction sites of the pPHT1 vector. The linear replacement construct was excised using a *SacII*–*KpnI* restriction and used to transform the *C. purpurea* wild-type strain 20-1.

Transformation and analysis of fungal transformants

Transformation of *C. purpurea* was performed as described by Mey *et al.* (2002). Phleomycin-resistant transformants carrying a homologous integration of the replacement construct were identified by PCR, using primers 39 (5'-GCAGGGTAGGCCGCTTT-TCTGG-3') and pAN3 (5'-GGTCACCAGTCGCTGGCTTCCCG-3') and 10–100 ng of genomic DNA. The predicted 1080 bp fragment was amplified from strains P2.29 and P2.35. The lack of the wild-type gene copy in the Δ *cpph1* mutants was checked using primers 51 (5'-GGACATGGACGAAACAACGG-3') and 38 (5'-GCTCTGAAAGCTCGTAGGAGC-3'), which amplified a fragment of 1600 bp with the wild-type gene copy. The transformants were subjected to at least one round of single spore isolation to obtain homokaryotic transformed strains.

Western blot analysis of the cell-wall associated proteins

The cell-wall associated proteins were isolated as described by de Vries *et al.* (1999). The protein concentration was determined according to Lowry *et al.* (1951). 20 µg of proteins were fractionated in a SDS-PAGE gel (Laemmli, 1970), containing 8.5% or 12% acrylamide in the separating gel. The proteins were electrotransferred on to a nitrocellulose membrane and immunoblotted using

the polyclonal antibodies raised against *C. fusiformis* CFTH1 (de Vries *et al.*, 1999) (final concentration 1 : 5000) and goat anti-rabbit IgG conjugated to alkaline phosphatase (Fluka) 1 : 10 000.

C. purpurea pathogenicity assays

Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (1992). Florets of blooming ears (60–80/ear) were inoculated with 5 µL of a suspension containing 2×10^6 /mL conidia collected from Mantle agar, as described by Tenberge *et al.* (1996). In order to avoid cross-contamination, the ears were covered with paper bags equipped with cellophane windows directly after inoculation. Results were recorded 4 weeks after inoculation.

Hydrophobicity tests

The hydrophobicity of *C. purpurea* sporulating mycelium was assayed on 2 cm large agar discs cut from sporulating mycelium grown for 12 days on Mantle agar at 28 °C. A 50 µL water droplet was added to the top of the disc and photographed from the side. The contact angle was measured on the pictures obtained. The hydrophobicity of the conidia was assayed using the MATH assay described by Whiteford and Spanu (2001).

Electron microscopy

The surface of *C. purpurea* conidia were viewed as replicas made after freeze fracture. For freeze etching, conidia were fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) for 1 h and then washed three times in buffer. Samples were then cryoprotected by infiltration with 10% glycerol (1 h) and 20% glycerol (overnight). Conidia were then frozen in Freon 22 (ICI, Runcorn, UK) and nitrogen slush. Freeze-fracturing was carried out in a Baltzer's BA 301 (Baltzer Pfeiffer GmbH, Leichenstein) and conidia were then shadowed with carbon and platinum at 45 °C. A backing layer of pure carbon was added at 90 °C and the replicas floated on to distilled water. Replicas were cleaned overnight in 50% chromic acid and washed several times in distilled water before being picked up on to copper grids and viewed with a Joel 100C transmission electron microscope (Jeol, Tokyo, Japan).

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