Isolation and characterisation of five different hydrophobin-encoding cDNAs from the fungal tomato pathogen \textit{Cladosporium fulvum}

**Abstract** Five different hydrophobin-encoding cDNA clones from \textit{Cladosporium fulvum} were isolated from cDNA libraries, made from nutrient-depleted mycelium. One cDNA clone was identical to the previously isolated hydrophobin \textit{HCf}-1. The other clones were named \textit{HCf}-2, -3, -4 and -5. \textit{HCf}-1, -2, -3 and -4 show a high degree of identity, and are predicted to encode class I hydrophobins. \textit{HCf}-5 encodes a class II hydrophobin. The expression patterns of these hydrophobins at various stages of development, and in liquid media lacking either carbon or nitrogen, or both, showed clear differences. All hydrophobins were more strongly expressed during sporulation than before, with \textit{HCf}-4 and -5 showing the highest increase. Expression of \textit{HCf} genes in infected plants was also higher at 16 days than at 10 days after infection. The expression of \textit{HCf}-5 in sporulating mycelium was much lower in planta than in vitro. All \textit{HCf} genes were upregulated under conditions of nutrient deprivation. \textit{HCf}-1, -2, -3 and -4 showed highest levels of transcription in medium lacking both carbon and nitrogen. Expression of \textit{HCf}-5 was highest in medium lacking nitrogen but containing carbon. \textit{HCf}-1 was generally the most abundant hydrophobin. The introduction of multiple copies of \textit{HCf}-1, which caused cosuppression of the endogenous \textit{HCf}-1 gene, was shown to affect the expression of \textit{HCf}-2, -3 and -4 also. Expression of \textit{HCf}-4 was suppressed, but expression of \textit{HCf}-2 and -3 was upregulated. Expression of \textit{HCf}-5 was not changed.

**Key words** \textit{Cladosporium fulvum} · Cosuppression · Cross-cosuppression · Expression · Starvation

**Introduction**

Hydrophobins are abundant fungal proteins that have received considerable attention in recent years (reviewed by Wessels 1997 and Kershaw and Talbot 1998). Hydrophobins are small, moderately hydrophobic proteins which are characterised by eight cysteine residues in a conserved pattern, including two cysteine doublets. To date, more than 34 hydrophobin genes have been isolated from 16 fungal species belonging to the ascomycetes and basidiomycetes, and these have been divided into two classes, based on the solubility properties and the hydrophy patterns of the proteins. Hydrophobins aggregate readily to form insoluble complexes. Class I hydrophobin complexes can only be dissolved with 100% trifluoroacetic acid or formic acid, and the cysteine doublets are followed by a number of hydrophilic amino acids. Class II hydrophobin complexes dissolve readily in 60% aqueous ethanol, and the cysteine doublets are followed by a stretch of hydrophobic amino acids (Wessels 1994).

In most cases, hydrophobins were either identified from mRNAs which are abundantly transcribed during developmental processes, or as proteins which are characterised by their insolubility in hot SDS and solubility in trifluoroacetic acid. Hydrophobins are secreted and self-assemble into a hydrophobic rodlet layer on aerial hyphae, conidia and fruiting bodies. In a number of species, hydrophobin genes have been ablated and the mutants showed an easily wettable phenotype (Kershaw and Talbot 1998). Thus, hydrophobins contribute to surface hydrophobicity. MPG1, from \textit{Magnaporthe grisea}, appears to function in surface attachment and/or recognition (Beckerman and Ebbole 1996).

Expression of hydrophobin-encoding genes is regulated by a complex set of factors. For example, \textit{Sc}1, \textit{Sc}3,
Sc4 and Sc6 from the basidiomycete *Schizopyllum commune* are under the control of mating-type genes (Wessels 1997). The hydrophobin-encoding gene *Eas* from *Neurospora crassa* is expressed in a circadian pattern and regulated by nutrient starvation (Bell-Pedersen et al. 1996). Nutrient availability also plays a role in the expression of the hydrophobin-encoding genes *MPG1* from *Magnaporthe grisea*, and *hfb1* and *hfb2* from *Trichoderma reesei*, which are induced by starvation for either carbon or nitrogen (Talbot et al. 1993; Lau and Hamer 1996; Nakari-Setälä et al. 1997).

In order to study the role of hydrophobins in compatible plant/fungus interactions, a hydrophobin protein (HCf-1) from the tomato pathogen *Cladosporium fulvum* was isolated and characterised (Spanu 1997). The HCf-1 protein was purified from culture medium and was isolated and characterised (Spanu 1997). The *C. fulvum* HCf-1 protein was purified from culture medium and was isolated and characterised (Spanu 1997). The HCf-1 protein was purified from culture medium and was isolated and characterised (Spanu 1997). The HCf-1 protein was purified from culture medium and was isolated and characterised (Spanu 1997).

Here we describe the isolation and characterisation of four new hydrophobin-encoding cDNA clones in addition to *HCf-1*. The expression of the hydrophobin genes was monitored at different developmental stages and under different nutritional conditions. Expression of *HCf-1* can be reduced by the introduction of extra copies of this gene into the *C. fulvum* genome, a phenomenon known as co-suppression (Bollman et al. 1991; Brusslan et al. 1993; Hamada and Spanu 1998). In this work we show that in co-suppressed isolates, changes also occur in the expression of some, but not all, of the other hydrophobin genes present in *C. fulvum*.

### Materials and methods

**Fungal isolates and culture conditions**

*C. fulvum* race 4 was maintained on V8 agar, as described by Harling et al. (1988). Mycelium for DNA and RNA extractions was grown in liquid B5 medium, at 20°C in the dark with shaking at 130 rpm. For DNA extractions, the culture was grown for 5 days. For RNA extractions, the culture was grown for 2 days, then the mycelium was collected by filtration in a sintered glass filter, washed with the appropriate medium, and then transferred to the same medium (B5 medium without sucrose and/or without nitrogen, or complete B5) for 16 h. The mycelium was harvested by filtration, washed, frozen in liquid nitrogen and freeze-dried overnight.

For extraction of RNA from aerial mycelium, the conidial suspension was spread on a plate containing Modified Czapec Dox medium (0.5 g/l NaNO₃, 0.5 g/l KCl, 0.5 g/l MgSO₄, 1 g/l KH₂PO₄, 30 mg/l FeSO₄, 15 g/l sucrose, pH 6, 2% agar), which was overlaid with a cellophane membrane that had been boiled three times in distilled water and autoclaved. The mycelium was scraped off the cellophane after 3 days (pre-sporulation or vegetative mycelium) or after 7 days (sporulating mycelium) and frozen in liquid nitrogen.

### Nucleic acid manipulations

DNA manipulations were carried out by standard techniques (Sambrook et al. 1989). Fungal genomic DNA was prepared as described in Raeder and Broda (1985).

Southern blots were prepared by digesting 3 µg of genomic DNA with the appropriate restriction enzyme, fractionating the digested DNA on a 0.8% agarose/TBE gel and transferring it onto Zetablot GT membranes (Biorad) using a vacuum blotting system (VacuGene XL, Pharmacia).

PCR was performed on 50 ng of template DNA, in a final volume of 50 µl containing 0.2 mM of each dNTP (Pharmacia), 0.3 µM of each primer, and 2.5 U of *Taq* DNA polymerase (Promega) in the buffer supplied by the enzyme manufacturer. The reaction mix was overlaid with mineral oil and DNA amplification was carried out using a Perkin-Elmer/Cetus Thermocycler under the following conditions: 30 s at 94°C, 30 s at 58°C and 30 s at 72°C for 25 cycles. The primer sequences specific to the *HCf* probe were as follows: (HCf2-F1: 5'-GAGACCTTGCGCCGCAGTCG-3'; HCf4-F1: 5'-TGGCCTCCTTCTTGCTTGC-3') and primer R3 (5'-CGCTCTAGAACTATGTTGAC-3') which hybridises to the polylinker of the plasmid. The PCR product was purified by electrophoresis through a 1% agarose/TBE gel, excised and eluted using QIAEX (Qiagen).

RNA was extracted from fungal and plant tissue using the Hybrid RNA isolation kit, as recommended by the manufacturer, with 0.01% β-mercaptoethanol added to the extraction buffer.

mRNA was prepared using the Poly(A) Quik mRNA isolation kit (Stratagene) according to the manufacturer’s instructions.

Northern blots were prepared by denaturation of RNA samples, electrophoresis on denaturing formaldehyde-agarose gels as described by Sambrook et al. (1989) and transfer onto Zetablot GT membranes. Equal amounts of the RNA samples were loaded in six wells to produce six identical Northern blots, which were hybridised with the five different *HCf* probes and the *C. fulvum* actin probe which displays no cross-hybridisation to plant actin genes (Coleman et al. 1997). Hybridisations and washes were carried out at 65°C using the hybridisation and washing solutions recommended by the manufacturer of the membrane.

Probes were prepared by random-primer labelled DNA using the *Taq* polymerase. Primers HCf2 (HCf2-F1: 5'-GAGACCTTGCGCCGCAGTCG-3'; HCf4-F1: 5'-TGGCCTCCTTCTTGCTTGC-3') and HCf4 (HCf4-F1: 5'-TGGCCTCCTTCTTGCTTGC-3') were used to generate DNA fragments of approximately 250 bp using a PCR kit according to the manufacturer’s instructions (Boehringer-Mannheim). After hybridisation, blots were incubated with a phosphor screen which was monitored using a PhosphorImager Model 425E from Molecular Dynamics. The software package ImageQuant Version 3.3 from Molecular Dynamics was used to analyse and quantify the hybridisation signals. In order to determine the amount of fungal RNA in infected plants, and to correct for differences in the amounts of RNA in different lanes, the quantified hybridisation signals were normalised with reference to the hybridisation signal for the actin gene, which is expressed constitutively. In this way, the expression levels in the different samples could be accurately compared. The activity of the different probes was compared on dot blots with identical amounts of genomic DNA. The dot blots were hybridised with the same probes as used for the Northern blots. The absolute abundance of hydrophobin mRNAs was calculated by relating the hybridisation signal on Northern blots to the signal obtained from the DNA dot blots.

DNA sequencing was done using the Thermosequenase dye terminator cycle sequencing kit (Amersham) according to the manufacturer’s instructions. The reactions were analysed on an ABI 377 automated sequencer.