Trichoderma G protein-coupled receptors: functional characterisation of a cAMP receptor-like protein from *Trichoderma atroviride*

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Abstract

Gα subunits act to regulate vegetative growth, conidiation, and the mycoparasitic response in *Trichoderma atroviride*. To extend our knowledge on G protein signalling, we analysed G protein-coupled receptors (GPCRs). As the genome sequence of *T. atroviride* is not publicly available yet, we carried out an in silico exploration of the genome database of the close relative *T. reesei*. Twenty genes encoding putative GPCRs distributed over eight classes and additional 35 proteins similar to the *Magnaporthe grisea* PTH11 receptor were identified. Subsequently, four *T. atroviride* GPCR-encoding genes were isolated and affliated to the cAMP receptor-like family by phylogenetic and topological analyses. All four genes showed lowest expression on glycerol and highest mRNA levels upon carbon starvation. Transcription of *gpr3* and *gpr4* responded to exogenously added cAMP and the shift from liquid to solid media. *gpr3* mRNA levels also responded to the presence of fungal hyphae or cellulose membranes. Further characterisation of mutants bearing a *gpr1*-silencing construct revealed that Gpr1 is essential for vegetative growth, conidiation and conidial germination. Four genes encoding the first GPCRs described in *Trichoderma* were isolated and their expression characterized. At least one of these GPCRs is important for several cellular processes, supporting the fundamental role of G protein signalling in this fungus.

Keywords

Trichoderma; G protein signalling; GPCR; Gene expression

Introduction

Heterotrimeric G protein signalling is basically comprised of three parts: a G protein-coupled receptor (GPCR), a heterotrimeric G protein (α, β, γ subunits), and an effector (Neer 1995). GPCRs constitute the vast majority of receptors, thereby forming one of the

largest protein families found in nature with more than 600 members in the human genome (Lander et al. 2001; Venter et al. 2001). GPCRs do not have significant sequence similarity but share a common domain structure containing seven transmembrane helices that are connected by intra- and extracellular loops (Dohlman et al. 1991). Ligand binding to the receptor results in a conformational change leading to release of the G protein and exchange of GDP for GTP on the Gα subunit. GTP-bound α dissociates from its $\beta\gamma$ partner, allowing both signalling units to regulate the activities of downstream effectors (Kaziro et al. 1991; Birnbaumer et al. 1992; Neer 1995; Gutkind 1998).

In fungi, two signalling branches defined by cAMP-dependent protein kinase (PKA) or MAPK cascades primarily relay G protein-mediated signals to elicit cellular responses such as growth, mating, cell division, cell–cell fusion, morphogenesis, chemotaxis, and pathogenic development (Bölker 1998; Versele et al. 2001). Fungal Gα subunits are highly conserved and can be divided into three subgroups (Bölker 1998). As heterotrimeric G proteins act as signal transducers that connect cell surface receptors to cytoplasmic effectors, GPCRs are expected to play essential roles in the transduction of extracellular signals. Nevertheless, little information is available on the characteristics and functions of fungal GPCRs other than pheromone receptors which have been examined in fungi such as *Saccharomyces cerevisiae* (Hagen et al. 1986; Blumer et al. 1988), *Schizosaccharomyces pombe* (Tanaka et al. 1993), *Cryptococcus neoformans* (Chang et al. 2003), *Aspergillus nidulans* (Seo et al. 2004), *Ustilago maydis* (Bölker et al. 1992), *Neurospora crassa* (Kim and Borkovich 2004), and *Coprinus cinereus* (Olesnicky et al. 1999).

The *N. crassa* genome sequence was the first available database for a filamentous fungus, and during its initial annotation 10 predicted seven-transmembrane helix GPCRs were found and divided into five families (Borkovich et al. 2004). Subsequent to the release of the genome of *Magnaporthe grisea*, Kulkarni et al. (2005) annotated 76 GPCR-like proteins of which 61 represented a large novel class related to PTH11, a receptor required for development of the appressorium (DeZwaan et al. 1999). Recently, Lafon et al. (2006) reported on in silico exploration of G protein signalling in the genomes of *A. nidulans, A. fumigatus*, and *A. oryzae*. The investigations resulted in classification of GPCRs into nine classes: classes I and II: pheromone receptors; classes III and V: putative carbon and cAMP sensors; class IV: nitrogen sensors; class VI: a new class of GPCRs with an RGS (regulator of G protein signalling) domain; classes VII and VIII: members of class VII like MG00532.4 of *M. grisea*, and GprM and GprN of *A. nidulans* are related to different mammalian GPCRs including the rat growth hormone releasing factor receptor, and class VIII comprises fungal GPCRs that are homologous to the steroid receptor mPR; and class IX: fungal opsins. In the meantime, a more detailed analysis of the *N. crassa* proteome resulted in additional 29 putative GPCRs, 25 of them representing homologues of *M. grisea* PTH11 (Li et al. 2007).

Generally, analyses of fungal genomes confirmed the high diversity of GPCRs compared to the little diversity in G proteins and components of the cAMP-signalling pathway such as PKA and adenylate cyclase. This may reflect the ability of fungi to integrate a broad spectrum of environmental cues into highly conserved cellular processes.

Analysis of G protein signalling in *T. atroviride* revealed that subgroup I and III Gα proteins play crucial roles during vegetative growth and conidiation and the attack of phytopathogenic host fungi (Rocha-Ramirez et al. 2002; Reithner et al. 2005; Zeilinger et al. 2005). Until now, no GPCRs have been isolated or studied from any *Trichoderma* spp. although their characterisation should provide a deeper insight into the interrelation between the signal recognizing receptor and the triggered cellular response. Clarifying the role of

receptors would be a fundamental step toward understanding the role of G protein signalling in *Trichoderma*.

Recently, the first genome sequence of a member of the genus *Trichoderma, T. reesei*, became publicly available. Here we present a genomic exploration of the *T. reesei* database resulting in the identification of 20 GPCRs distributed over classes I to VIII according to Lafon et al. (2006) and additional 35 GPCRs related to the *M. grisea* PTH11 protein. Based on this analysis, we isolated four GPCR-encoding genes from the mycoparasite *T. atroviride* and analysed their expression under different growth conditions. Further characterization of the isolated *T. atroviride* GPCR-encoding genes showed that at least gpr1 plays a major role during vegetative growth and conidiation in *T. atroviride*. To our knowledge, this is the first report on GPCRs from a biocontrol fungus.

Materials and methods

Genome mining for the identification of genes encoding GPCR-like proteins

The genomic sequence of *T. reesei* is available at http://genome.jgi-psf.org/Trire2/ Trire2.home.html. The following genomic sequences and deduced proteomes were searched as basis for identifying GPCR-like proteins from *T. reesei*: *A. nidulans* (http:// www.broad.mit.edu/annotation/fungi/aspergillus/index.html), *A. fumigatus* (http:// www.tigr.org/tdb/e2k1/afu), *A. oryzae* (http://www.bio.nite.go.jp/dogan/MicroTop? GENOME_ID=ao), *N. crassa* (http://www.broad.mit.edu/annotation/genome/neurospora/ Home.html), *M. grisea* (http://www.broad.mit.edu/annotation/fungi/magnaporthe), *Podospora anserina* (http://podospora.igmors.u-psud.fr), *Chaetomium globosum* (http:// www.broad.mit.edu/annotation/genome/chaetomium_globosum/Home.html), *Fusarium graminearum* (http://mips.gsf.de/genre/proj/fusarium), and *Nectria haematococca* (http:// genome.jgi-psf.org/Necha2/Necha2.home.html).

Besides BLAST searches, a more sensitive database searching using hidden Markov models (HMM) was performed to identify putative GPCRs within the *T. reesei* proteome that lack significant sequence similarity to known GPCR-like proteins. To this end, the program HMMER (http://hmmer.wustl.edu; Eddy 1998) was applied.

For manual gene prediction the FGENESH algorithm (Softberry, Mount Kisco, NY) was applied to the raw sequence data and the predicted proteins were tested with TMHMM (Krogh et al. 2001) for transmembrane helices. ClustalW (http://www.ebi.ac.uk/clustalw) was used for alignment and phylogenetic analysis.

Strains and media

Trichoderma atroviride P1 (ATCC 74058; teleomorph *Hypocrea atroviridis*) was used throughout this study and maintained on potato dextrose agar (PDA). To monitor the transcription levels of GPCR-encoding genes in liquid medium augmented with different carbon sources the fungus was grown in synthetic medium (SM) containing [g/l]: $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, $CaCl_2 \cdot 2H_2O$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot H_2O$ 0.0028, $CoCl_2 \cdot 6H_2O$ 0.0032; with 2% glycerol as carbon source. After 36 h the mycelia were transferred to SM with 1% (w/v) of glucose, glycerol, N-acetyl-glucosamine, or colloidal chitin. For the determination of growth rates, fungi were inoculated at the centre of PDA plates and the colony diameter recorded every 24 h. *Rhizoctonia solani* strain 1450 (strain collection of the Institute of Plant Pathology, University of Naples Federico II, Italy) was used as a plant pathogenic host and was maintained on PDA. *Escherichia coli* JM109 served as a host for plasmid amplification and was grown as described by Sambrook et al. (1989).

For plate confrontation assays, 5-mm disks of *T. atroviride* and *T. atroviride*, or *T. atroviride* and *R. solani*, were placed on cellophane-covered PDA plates at a distance from each other of 4 cm. The plates were incubated at 25°C for 3 days in the absence of light. For dual-plate assays avoiding physical contact the two opposite organisms were separated by a tape of cellophane or a dialysis membrane (cutoff 12 kDa) embedded in the agar in the middle of the Petri dish in a way forming a barrier between the fungi as described previously by Zeilinger et al. (1999).

Transmitting light microscopy

Microscopic studies were mainly performed as previously described by Lu et al. (2004). Glass slides, on which 500 μl of PDA was spread onto, were inoculated and incubated on a moistened filter paper (Gel-blotting paper; Roth, Karlsruhe, Germany) at 28°C in a petri dish sealed with Parafilm. After 48–72 h, the fungal hyphae were viewed under a Leitz Aristoplan (Wetzlar, Germany) microscope, and pictures were taken using an Olympus DP 10 (Olympus America Inc., Melville, NY) camera.

DNA and RNA procedures

Standard molecular techniques were performed according to Sambrook et al. (1989); DNA and RNA isolation was carried out as described by Peterbauer et al. (1996). For standard PCR amplication, recombinant *Taq* Polymerase (Fermentas, Vilnius, Lithuania) was used according to the manufacturer's recommendations.

Cloning and sequencing of *T. atroviride* reference genes

Diagnostic fragments of the following genes were cloned and sequenced for their subsequent use as reference for relative-quantitative real-time PCR: *act1* (actin-encoding), *gpd1* (glyceraldehyde 3-phosphate-dehydrogenase-encoding), *tef1* (translation elongation factor-encoding), *sar1* (small GTP binding protein-encoding), and a fragment of the 28S rDNA. Primer pairs for amplification of the respective fragments from genomic DNA are given in Table 1a. The PCR amplicons were cloned into pGEM-T (Promega, Madison, WI) and sequenced (MWG Biotech, Ebersberg, Germany) using the standard sequencing primers SP6 and T7. All nucleotide sequences have been submitted to GenBank (GenBank accession numbers: EF581847, EF581848, EF581849, EF581850, EF591763). Primers for real-time PCR monitoring of these genes were designed with Vector-NTI (Invitrogen, Carlsbad, CA).

Cloning of four *T. atroviride* genes coding for putative GPCRs

Two pairs of degenerate primers were used for PCR on genomic *T. atroviride* DNA: Gpr-I-f: 5′-AAYMGRCTGGTCTTTTATGCKTCGTTYGGC-3′, Gpr-I-r: 5′-TCGGTTGGCGCTCGASGGMATCC-3′, and Gpr-II-f: 5′-ATGTTYATGCARWIGAYCCITGGTGG-3′, Gpr-II-r: 5′-CKIARRTAIGCIMDYTTDATIGG-3′. For amplification, the following PCR program was applied: 3 min initial denaturation at 95°C followed by 35 cycles with 30 s at 95°C, 30 s at 56.5°C for Gpr-I or 30 s at 53°C for Gpr-II, and 1 min elongation at 72°C.

A LambdaBlueStar genomic library (Novagen/Merck, Darmstadt, Germany) of *T. atroviride* P1 was screened with the respective PCR fragments to obtain full length clones of the putative GPCR-encoding genes. The screening was performed according to the manufacturer's manual. The nucleotide and putative protein sequences were deposited at GenBank (accession numbers: DQ284755, DQ384865, EF534712, EU391628).

Real-time RT-PCR

The sequences for the respective primer pairs for cDNA amplification of the reference genes and the four receptors are given in Table 1b. All quantifications were performed with the same PCR program: initial denaturation for 90 s at 95°C, 50 cycles with 95°C for 20 s, 60°C for 20 s and 72°C for 30 s. For cDNA synthesis the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) was used according to the manufacturer's instructions with a combination of the provided oligo-dT and random primers. All real-time PCR experiments were conducted on a Bio-Rad (Hercules, CA) iCycler IQ. For iCycler reaction the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was prepared for 25 µl assays with standard $MgCl_2$ concentration (3 mM) and with final primer concentrations of 100 nM each. All assays were carried out in 96-well plates which were covered with optical tape. PCR efficiency was determined from a single tube reaction set-up as described by Tichopad et al. (2003) and expression ratio was calculated according to the equation published by Pfaffl (2001). All samples were analyzed in three independent experiments with three replicates in each run.

Gene silencing procedure and construction of pSilent2

The method used to silence *gpr1* and *gpr3* gene expression in *T. atroviride* P1 was based on the construction of self-complementary RNAs (inverted repeats). These should activate the short interfering (si)RNA pathway mediated by the action of the Dicer complex resulting at the end in the endonucleolytic cleavage of the target mRNAs (Dykxhoorn et al. 2003).

An 850-bp fragment of the *A. nidulans gpdA* promoter was cloned between the *Xho*I and the *Cla*I sites of the vector pBluescriptSK+ (Stratagene, Cedar Creek, TX) and a 720-bp fragment of the *A. nidulans trpC* terminator was inserted between *Bam*HI and *Xba*I. The hygromycin resistance-conferring cassette of pRLMex30 (Mach et al. 1994) was inserted at the *Not*I site of pBluescriptSK+, resulting in vector pSilent2.

Fragments of the *gpr1* and *gpr3* target genes were amplified with the following modified primers (a *Hin*dIII site was inserted into the forward primer and a *Xba*I site into the reverse primer): gpr1-fw, 5′-AAGCTTCCCATATCAATCGACGAGGTCACC-3′ and gpr1-rev, 5′-TCTAGATTGTCTCGCTCGTGATTTCGC-3′ (amplicon length 359-bp); gpr3-fw, 5′-AAGCTTGGATCTACTGCCTAATTTGTTACG-3′ and gpr3-rev, 5′-TCTAGAGAACTTCGGTAGTGACGGTGC-3′ (amplicon length 464-bp). All PCR reactions were performed at an annealing temperature of 56°C. Subsequently, the amplicons were digested with *Xba*I and self-ligated to obtain inverted repeats flanked by *Hin*dIII sites. 5 µl of the ligation mixture were used for a 35-cycle single-primer PCR with the forward primers previously used to amplify the respective fragments. The amplified inverted repeats were digested with *Hin*dIII and inserted into pSilent2 resulting in pSilent2-gpr1 and pSilent2-gpr3, respectively.

Transformation of *T. atroviride*

Trichoderma atroviride protoplasts were transformed with 10 µg of each silencing vector as described by Peterbauer et al. (2002). HygromycinB-resistant strains were transferred to PDA for sporulation and colonies obtained from three subsequent single spore isolations were used for further analysis. The integration of the respective dsRNA expressing constructs was tested by PCR with primers binding to the *gpdA* and *trpC* regulatory sequences: SilTest-f: 5′-AATGTGAAGCCAGGGGTGTATAGC-3′ and SilTest-r: 5′-GTCTCCCGAAAATGAAAATAGCTC-3′.

Biolog phenotype array analysis

The Biolog FF MicroPlate assay (Biolog Inc., Hayward, CA) which comprises 95 wells with different carbon-containing compounds and one well with water was used to investigate growth rates on selected carbon sources. Nutrients and test reagents are prefilled and dried into the 96 wells of the microplate. Conidia were collected from the *Trichoderma* strains and used as inoculums as described (Druzhinina et al. 2006). Inoculated microplates were incubated in darkness at 25°C, and OD_{750} readings determined after 18, 24, 42, 48, 66, 72, 96 and 168 h using a microplate reader (Biolog), which measures the turbidity and reflects mycelial production on the tested substrate. Analyses were performed in triplicate.

Results

Identification of genes predicted to encode GPCRs in the genome of *T. reesei*

At the beginning of our study, seven proteins were annotated as GPCRs in the *T. reesei* genome database. To get a more complete picture, we searched the *T. reesei* proteome database for additional GPCR-like proteins based on their similarity to known fungal receptors. To this end, GPCR sequences GprA—GprQ and NopA from *A. nidulans, A. fumigatus*, an *A. oryzae* (Lafon et al. 2006) were used as query in a BLASTP search against the predicted proteomes of species of the Sordariomycetes (*N. crassa, M. grisea, P. anserina, C. globosum, F. graminearum, N. haematococca*, and *T. reesei*), a subgroup within the Pezizomycotina/Ascomycota, to find putative orthologues in these species. The retrieved proteins from each species were subsequently used as query in similar BLAST searches of the proteomes from the other species to end up with each possible combination.

All obtained putative GPCR sequences were evaluated for seven transmembrane regions with the TMHMM algorithm (Krogh et al. 2001). For nearly all retrieved proteins the algorithm predicted the seven-span helix topology. For those not predicted to contain seven transmembrane regions, the respective encoding gene and flanking sequences were retrieved from the genome and inspected. In individual cases, mistakes at the intron-exon boundaries were found and manual correction resulted in the detection of the missing membrane-spanning domain(s). Only those proteins fulfilling the topological characteristics of GPCRs (seven transmembrane-spanning domains, amino-terminal domain predicted to face outside the cell, and the C-terminus predicted to face the cytoplasm) were included in further analyses.

BLASTP searches using the *M. grisea* PTH11 receptor as query against the predicted proteomes of the above listed species of the Sordariomycetes resulted in a number of proteins with seven transmembrane regions. Again, the retrieved proteins from each species were subsequently used to BLAST in a second round the proteomes from the other species to end up with each possible combination.

In total, these combinatorial BLAST searches resulted in the identification of 52 *T. reesei* GPCR-like proteins including 32 PTH11-like receptors. To also identify putative GPCRs that lack significant sequence similarity to known GPCR-like proteins and therefore may escape detection by homology search, the *T. reesei* genome database was again queried using a HMM method. Applying the program HMMER, three additional PTH11-like receptors (Tr_69904, Tr_105224, Tr_122795) could be identified (Table 2). Phylogenetic analysis based on an alignment of all identified putative *T. reesei* GPCRs other than PTH11-like receptors with those identified in *Aspergillus* spp. was performed. The resulting tree (Fig. 1) exhibits the characteristic nine classes previously described for *Aspergillus* spp. (Lafon et al. 2006), grouping the 20 *T. reesei* GPCRs into classes I to VIII: (I and II) Tr_57526 and Tr_64018 (numbers indicate protein ID in the genome database) similar to pheromone receptors GprA (Ste2-like) and GprB (Ste3-like) from *Aspergillus* sp.,

respectively; (III) Tr_59778 similar to the putative carbon sensors GprC, GprD and GprE from *Aspergillus* sp.; (IV) Tr_4508, Tr_80125 and Tr_111600 similar to the putative *Aspergillus* nitrogen sensors GprF, GprG and GprJ; (V) Tr_28731, Tr_38672, Tr_72605, and Tr_123806 similar to the putative *Aspergillus* cAMP sensors GprH, GprI and GprL; (VI) Tr_23297, Tr_37525, Tr_63981, and Tr_81383 similar to the RGS domain-containing receptor GprK from *Aspergillus* sp.; (VII) Tr_53238 similar to *Aspergillus* GprM and GprN; (VIII) Tr_56426, Tr_68212, Tr_70139, Tr_82246, and Tr_119819 similar to the mPR_dom domain-containing proteins GprO, GprP, and GprQ from *Aspergillus* sp. Interestingly, we could not identify any member of class IX which contains receptors similar to bacterial opsins.

Cloning and in silico analysis of putative GPCR-encoding genes from *T. atroviride*

A PCR-based approach was followed for the isolation of GPCR-encoding genes from *T. atroviride*. Until now, only GPCRs from groups I, II (Kim and Borkovich 2004; Seo et al. 2004), III (Han et al. 2004; Li and Borkovich 2006), V (Krystofova and Borkovich 2006) and IX (Bieszke et al.2007) and the *M. grisea* PTH11 receptor (DeZwaan et al.1999; Kulkarni et al. 2005) have been analysed in more detail in filamentous fungi. Members of the other groups have only been characterised in silico and it is still unknown if they are actually expressed. Because of these considerations, we focused on class V GPCRs from *T. atroviride*. Members of this class exhibit similarity to cAMP receptor-like (CRL) proteins from *Dictyostelium discoideum* and are absent from the genomes of the ascomycete yeasts *S. cerevisiae* and *S. pombe*.

To amplify fragments of putative *T. atroviride* CRL-encoding genes, two degenerate primer pairs were designed based on protein alignments of putative class V receptors obtained from the genome databases of the close relatives *T. reesei* (four CRL proteins) and *F. graminearum* (five CRL proteins). Using genomic *T. atroviride* DNA in a PCR approach, primer pair Gpr-I yielded two fragments of ~800 and ~1,000-bp, respectively; the Gpr-II primers produced amplicons of ~900 and ~1,000-bp, respectively. Screening of a genomic library of *T. atroviride* with these fragments as probes resulted in the isolation of four different clones, each corresponding to one probe. The inserts were sequenced and the derived nucleotide sequences were scanned with FGENESH (Softberry, Mount Kisco, NY) resulting in detection of seven transmembrane helices in all four predicted proteins with a distribution typical for the CRL class: five domains at the N-terminal end followed by a long intracellular loop and by two helices close to the C-terminus (Fig. 2).

Alignment of the isolated putative *T. atroviride* GPCRs Gpr1, Gpr2, Gpr3, and Gpr4 with *N. crassa* GPR-1, the only CRL protein characterized in ascomycete fungi until now (Krystofova and Borkovich 2006), and predicted CRL proteins from *T. reesei* and *F. graminearum* (Fig. 2) and subsequent phylogenetic analysis resulted in two branches with Gpr1 and Gpr2 in one branch and Gpr3 and Gpr4 in the other branch (Fig. 3). As shown in Fig. 3, the CRL proteins from *T. atroviride* and *T. reesei* are closely related, which confirms and validates the approach used.

To prove that the isolated *T. atroviride* GPCR-encoding genes are actually transcribed and do not represent pseudogenes, transcriptional analyses were performed. As *gpr1, gpr2, gpr3* and *gpr4* mRNA levels were too low to be detected by Northern analysis, we performed quantitative real-time RT-PCR to determine their expression.

Cloning and evaluation of putative reference genes for real-time PCR

To allow accurate real-time PCR analysis of transcription rates, suitable reference genes with only minimal regulation of gene expression are indispensable. As only few sequences

of putative reference genes were available from *T. atroviride* P1, fragments from the following five genes were cloned by PCR with degenerate primers (Table 1a) and sequenced: the actin-encoding gene *act1*, the glyceral-dehyde-3-phosphate-dehydrogenase-encoding gene *gpd1*, the translation elongation factor-encoding gene *tef1*, the GTP-binding protein-encoding gene *sar1* and the gene for 28S rDNA. Primers for real-time PCR monitoring of the transcription of the respective gene were all tested for annealing at a temperature of 60°C to allow a combined use of these references for any run. All reference genes were evaluated for their constant expression under the following cultivation conditions of the fungus: (1) in liquid medium [potato dextrose broth (PDB)] and on solid agar (PDA) to allow monitoring of surface/cultivation-dependent gene expression differences, (2) after replacement of *Trichoderma* to liquid medium with different carbon sources (glucose, glycerol, ɴ-acetylglucosamine, colloidal chitin, or carbon-starvation), and (3) in dual plate assays when directly interacting with different confrontation partners or when the two fungi were separated by membrane barriers. Three independent real-time PCR runs with three replicates per sample were performed for each experiment and the data were evaluated with genNorm (Vandesompele et al. 2002) to identify the most stable internal control genes for each condition. Although this algorithm leads to two winner genes, the gene that performed best during the exclusion procedure was taken for normalization during the further experiments. To compare gene transcription upon growth on PDB and PDA, *tef1* turned out to be the best reference gene. To investigate mRNA levels in liquid media with different carbon sources *act1* was chosen for data normalization, and mRNA levels of in vitro biocontrol conditions are best to be referenced to *sar1* (Table 3).

Real-time PCR quantification of receptor gene expression under different cultivation conditions

As all four putative *T. atroviride* receptors, designated Gpr1, Gpr2, Gpr3, and Gpr4, grouped to the CRL class V which was suggested to be involved in cAMP sensing in *N. crassa* (Galagan et al. 2003), we investigated the influence of extracellular cAMP added to PDA plates in a concentration of 5 mM on their gene transcription using real-time RT-PCR with *sar1* as reference gene. *gpr1* and *gpr2* did not show significantly altered transcription in the presence of cAMP compared to growth on un-supplemented PDA, whereas expression of *gpr3* was reduced by 84% and that of *gpr4* was reduced by 70% on plates augmented with cAMP (Fig. 4a).

To test for a surface-dependent expression (i.e. differential expression between growth in liquid and on solid media) of the isolated receptor-encoding genes, *T. atroviride* was cultivated in potato dextrose broth (PDB) or on PDA. The compared growth conditions had no influence on *gpr2* transcription, whereas *gpr1* and *gpr4* mRNA levels were reduced to approx. 50% on PDB compared to growth on PDA. The most striking expression decrease was observed for *gpr3*: its transcription was reduced by 88% when the fungus was cultivated in PDB compared to growth on PDA (Fig. 4b).

Furthermore, the effect of different carbon sources on the expression of the four receptor genes was investigated using cDNA obtained from replacement cultivations. To this end, pre-grown mycelia of the fungus were transferred to liquid media with glucose, glycerol, colloidal chitin or ɴ-acetyl-glucosamine as sole carbon sources, the latter two representing fungal cell wall components or cell wall-derived degradation products. Additionally, cDNA from carbon-starved mycelium was integrated into this experiment. *gpr1, gpr2, gpr3* and *gpr4* all followed the same tendency showing lowest expression on glycerol and the highest mRNA levels when *T. atroviride* was transferred to a medium without carbon source (Fig. 5a) indicating that transcription of these GPCR-encoding genes is induced during carbon starvation.

To investigate the expression patterns of the receptor-encoding genes under in vitro biocontrol conditions, RNA was isolated from dual plate assays with various confrontation partners. *T. atroviride* grown alone served as the control condition and was compared with a self-confrontation (*T. atroviride–T. atroviride*) and with a confrontation against *R. solani*. *gpr1*, *gpr2* and *gpr4* showed no significant transcriptional regulation, whereas *gpr3* was significantly down-regulated upon contact of *T.atroviride* with itself or with *R. solani* (Fig. 5b). To investigate if this decrease in expression of *gpr3* is specific for a direct physical contact of the hyphae, we separated the confrontation partners by either a stripe of cellophane or by a dialysis membrane. As control, *Trichoderma* was grown against a membrane without another fungus behind this barrier. However, all experimental setups led to the same result namely a significant reduction of *gpr3* transcription after contact with the membrane. Therefore, the observed effect seems not to be specific for fungal–fungal interactions but to be more general occurring upon physical contact with any object.

Silencing of *gpr1* gene expression results in mutants with alterations in vegetative growth, conidiation, and conidial germination

To get further insights into putative functions of the isolated *T. atroviride* GPCR-encoding genes, one member of each branch (Fig. 3) was selected for functional studies. Vectors for silencing *gpr1* and *gpr3* were constructed and transformed into protoplasts of *T. atroviride* P1. Four strains transformed with the *gpr1*- silencing construct and five strains transformed with the *gpr3*- silencing construct were randomly selected for further studies. Integration of the silencing vectors was positively tested by PCR amplification of a fragment containing the *gpdA* promoter, the *trpC* terminator and the inverted repeats in-between. The PCR reaction produced diagnostic fragments of 718-bp for *gpr1*-silenced strains and 928-bp for *gpr3*-silenced transformants (data not shown).

Transformants bearing the *gpr3* silencing constructs exhibited no aberrant phenotype when grown on PDA and real-time RT-PCR analysis using primers gpr3forw and gpr3rev (Table 1b) showed that they, although bearing the *gpr3* silencing construct, exhibited unaltered *gpr3* mRNA levels.

In contrast, real-time RT-PCR analysis using primers gpr1forw and gpr1rev (Table 1b) revealed levels of *gpr1* silencing of ~60–80% in transformants bearing the *gpr1*-silencing construct (Fig. 6a). *gpr1*-silenced transformants showed an ~50% reduced growth rate compared to the parental strain upon cultivation on PDA plates (Fig. 6b). They sporulated continuously on solid medium and formed flat colonies with only little aerial mycelium and with a significant number of hyphae growing submerged in the agar. In liquid cultures in PDB or SM with 1% glycerol for 36 h, no conidiation occurred. Analysis of conidial germination after 9 h of incubation in PDB at 28°C revealed that 17% of the wild type conidia, but only 5% of the conidia of *gpr1*-silenced mutants had formed germ tubes; after 11 h, 67% of the wild type conidia and 34% of the gpr1-sil conidia had germinated.

To further analyze the role of Gpr1, we used Biolog Phenotype Arrays to find specific carbon utilization properties. The carbon-source utilization profile of *T. atroviride* P1 has been characterized previously (Seidl et al. 2006). The authors divided the tested carbon sources into four groups by joining cluster analysis where clusters I, II, and III contain carbon sources which allow fast, moderate, and slow growth, respectively, while cluster IV contains carbon sources where the fungus grows very poorly or does not grow at all.

The *gpr1*-silenced mutant exhibited reduced growth on the majority of the tested carbon sources (Fig. 7) and determination of the respective growth rates between 18 and 66 h of incubation revealed a 30–70% reduction. From the carbon sources belonging to clusters I, II, and III and therefore allowing explicit growth of the parental strain, a-ᴅ-lactose and

lactulose did not allow any detectable growth of the *gpr1*-silenced mutant during this period. On D-raffinose and maltose the mutant only grew poorly (8 and 17% of the parental strain, respectively), whereas on D-mannitol, D-arabitol, L-serine, salicin, and Tween 80 the mutant showed a similar growth rate as the parental strain.

Discussion

Analysis of G protein signalling in *T. atroviride* revealed that the Tga1 and Tga3 Gα subunits play important roles during vegetative growth and conidiation and in regulating mycoparasitism-related genes involved in infection structure formation and chitinase as well as antifungal metabolite production, and for both an interaction with the cAMP pathway was found (Rocha-Ramirez et al. 2002; Reithner et al. 2005; Zeilinger et al. 2005). Based on these findings and to gain further insights into the complex biochemical processes that allow *Trichoderma* to sense numerous environmental signals, we aimed to identify upstream GPCRs from *T. atroviride*.

As the genome sequence of *T. atroviride* was not available, we searched the proteome of its close relative *T. reesei* for GPCR-like proteins and identified a total of 55 sequences in its genome database (Table 2). The identified proteins include putative orthologues of fungal Ste2- and Ste3-like pheromone receptors previously reported to be required for the mating responses in *S. cerevisiae*, a homologue of the previously characterized carbon sensors GprD from *A. nidulans* (Han et al. 2004) and Gpr-4 from *N. crassa* (Li and Borkovich 2006), three putative nitrogen sensors, four CRL proteins grouping with the previously characterized *N. crassa* GPR-1 receptor (Krystofova and Borkovich 2006), and four RGS domain-containing GPCRs. Furthermore, we found six proteins grouping to classes VII and VIII, respectively, which were first described by Kulkarni et al. (2005) and consist of fungal GPCRs related to different mammalian GPCRs including the rat growth hormone releasing factor and the steroid receptor mPR. Interestingly, we did not detect a member of class IX, which includes the previously characterized *N. crassa* NOP-1 opsin (Bieszke et al. 1999; Bieszke et al.2007), in the *T. reesei* genome.

Based on the results on GPCRs present in the *T. reesei* genome, putative GPCR-encoding genes from the mycoparasite *T. atroviride* were isolated. As the information on functional fungal GPCRs other than pheromone receptors is very limited, the class of CRL proteins was chosen as it is not present in the genomes of the ascomycete yeasts *S. cerevisiae* and *S. pombe* but it includes proteins from ascomycete filamentous fungi like *N. crassa* GPR-1 which was shown to be actually expressed and functional. Furthermore, it is a rather big class represented by four members in the *T. reesei* genome.

Phylogenetic and topological analyses confirmed that all four encoded proteins from *T. atroviride* contain a distribution of the seven transmembrane helices characteristic for members of the CRL class and contain a Dicty_CAR domain (pfam05462). Furthermore, they share sequence homology to CRL proteins of other ascomycetes primarily in the seven transmembrane domains as well as the three extracellular loops which are important for ligand binding, whereas sequence similarity is minor in the third intracellular loop and the C-terminal tail which are interacting with the Gα subunits. These findings are in accordance to those from Krystofova and Borkovich (2006), who speculated that these GPCRs may therefore share functional similarity e.g. by responding to similar ligands. Although the related protein GprH of *A. nidulans* has already been described some years ago (Han et al. 2004), no function has yet been assigned to it. Until now, the only CRL protein of a filamentous fungus has been functionally characterized in *N. crassa*, where GPR-1 is required for female sexual development and is suggested by epistatic analysis to couple to the GNA-1 Gα subunit. Interestingly, in contrast to *T. atroviride* gpr1-sil mutants, no

obvious defects during asexual growth were found in Δ*gpr-1* strains (Krystofova and Borkovich 2006). In the basidiomycete *C. neoformans*, Gpr4, a GPCR with homology to *A. nidulans* GprH and the *D. discoideum* cAR1 cAMP receptor, was found to act upstream of the G*a* subunit Gpa1 in sensing amino acids to activate the cAMP-PKA signalling pathway. Furthermore, Gpr4 was reported to be rapidly internalized in response to methionine, a mechanism leading to GPCR signalling desensitization (Xue et al.2006). Nevertheless, recent analysis showed that *C. neoformans* Gpr4 is more similar to carbon-sensing GPCRs like Gpr1p from *S. cerevisiae* (Li et al. 2007).

GPCR-encoding genes like those coding for pheromone receptors were reported to be transcribed at low to undetectable levels in e.g. *N. crassa, Sordaria macrospora* (Pöggeler and Kück 2001), *U. maydis* and *U. hordei* (Urban et al. 1996; Anderson et al. 1999). This weak transcription of GPCR-encoding genes was also confirmed by analyses of *N. crassa* GPR-1 and the predicted carbon-sensing receptor GPR-4, both could not be detected using Northern analysis (Krystofova and Borkovich 2006; Li and Borkovich 2006).

Because of these findings, we decided to employ highly sensitive real-time RT-PCR for the transcriptional characterization of the *T. atroviride gpr1, gpr2, gpr3*, and *gpr4* genes.

For transcriptional analyses in *T. atroviride*, the actin-encoding gene *act1* was frequently used as reference during the last decades. Nevertheless, preliminary results from real-time RT-PCR analyses showed significant variation of *act1* gene transcription (Kratochwill and Brunner, unpublished), which is in accordance with a report from Gomez et al. (1997) on the regulation of actin gene transcription during interaction of different *T. harzianum* isolates. For these reasons, we tested four additional "housekeeping" genes for their constant transcription under varying growth conditions of *T. atroviride* to exclude carbon source- and surface-dependent differences in their expression. As their cDNA abundances again showed growth-dependent variations, reflecting previous findings (e.g. Zhang and Snyder 1992; Bhatia et al. 1994; Thellin et al. 1999; Bustin 2000; Schmittgen and Zakrajsek 2000; Suzuki et al. 2000), the most stable internal control gene for each condition was identified as described by Vandesompele et al. (2002). *act1* turned out to be the best suited reference for real-time investigations of samples from liquid media, whereas the use of *tef1* provided stable and reproducible results when comparing samples from growth on PDA plates with growth in liquid PBD. For analysing samples derived from in vitro biocontrol conditions, *sar1* was chosen.

The *gpr1, gpr2, gpr3* and *gpr4* genes of *T. atroviride* all encode putative GPCRs belonging to the class of CRL proteins. In *Trichoderma* spp. exogenous cAMP was described to promote conidiation even in the dark (Nemcovic and Farkas 1998; Berrocal-Tito et al., 2000) and to promote mycoparasitic behaviour by increasing coiling (Omero et al. 1999). In *T. virens*, Δ*tac1* mutants missing the gene encoding adenylate cyclase were unable to overgrow plant pathogens like *Sclerotium rolfsii, R. solani* or *Pythium* sp., and cAMP signalling was shown to also be essential for growth, conidial germination and secondary metabolite production (Mukherjee et al. 2007). *M. grisea* was reported to respond to exogenously added cAMP by developing appressoria although the authors noted that the cell wall and cell membrane should be relatively impermeable to cAMP and thus any responses to extracellular cAMP seem to be due to cAMP receptors (Lee and Dean 1993). Furthermore, Galagan et al. (2003) hypothesized that class V receptors of *N. crassa* might be involved in cAMP sensing. In addition, by studying the response of *T. atroviride pkr-1* antisense and over-expressing mutants to cAMP, Casas-Flores et al. (2006) obtained results showing that triggering of conidiation by cAMP is at least in part independent of PKA. In *pkr-1* antisense transformants, which showed elevated levels of PKA activity and were unable to conidiate even with light, addition of cAMP to the medium provoked conidiation.

pkr-1 overexpressing mutants, which had nearly undetectable PKA activity levels but nevertheless hypers-porulated in response to light and conidiated even in the dark, produced more conidia in the presence of cAMP. The finding that both the extracellular addition of dB-cAMP as well as sustained low PKA activity provokes sporulation encouraged the authors to suggest a second, alternate signalling pathway that uses a membrane receptor for exogenous cAMP. This cAMP receptor is supposed to exist beside the classical pathway of PKA activation by cAMP.

Analysis of cAMP-dependent transcription of the *T. atroviride* GPCR-encoding genes showed that expression of *gpr3* and *gpr4* was significantly reduced in the presence of exogenously added cAMP, whereas *gpr1* and *gpr2* mRNA levels were only slightly influenced.

When comparing growth on solid (PDA) to growth in liquid (PDB) potato-dextrose medium, again transcription of *gpr3* and *gpr4* showed the most noticeable responses in that mRNA levels were significantly lower upon growth of *T. atroviride* in liquid PDB compared to growth on PDA plates. Similar results were described for a pheromone receptor of *C. neoformans: CPR*a gene expression markedly increased when the cultures were shifted from liquid to solid media (Chang et al. 2003).

During plate confrontation assays, transcription of *gpr1, gpr2* and *gpr4* basically remained unaltered in the presence of a host fungus, whereas *gpr3* transcription significantly decreased upon contact with *R. solani*. Interestingly, this down-regulation of *gpr3* transcription could also be observed when *T. atroviride* was confronted with itself and when it touched a cellulose membrane, suggesting that the observed transcriptional regulation is the result of any physical contact and not of fungal–fungal interaction.

When testing the effect of different carbon sources and carbon starvation, all four *T. atroviride* GPCR-encoding genes showed a similar behaviour with the lowest expression on glycerol and highest mRNA levels when the fungus was cultivated without any carbon source followed by growth in the presence of the hardly utilisable chitin. These results resemble those reported for *C. neoformans CPR*a whose expression increased upon transfer from rich to poor medium (Chang et al. 2003), but are in contrast to *N. crassa gpr-4*, whose mRNA levels were highest in glycerol-grown cultures. *N. crassa gpr4* was demonstrated to encode a GPCR required for carbon source-dependent asexual growth and development and it was shown to be coupled to the Gα subunit GNA-1 in a cAMP-signalling pathway (Li and Borkovich 2006).

In general, *gpr3* and *gpr4* showed significantly lower transcript levels compared to *gpr1* and *gpr2*, being about 10-fold lower when *T. atroviride* was grown in PDB versus cultivation on PDA and under in vitro biocontrol conditions, or even about 100-fold lower upon cultivation in liquid medium with different carbon sources. Interestingly, these findings reflect the phylogenetic analysis grouping Gpr1 and Gpr2 into one branch and Gpr3 and Gpr4 in the other one (Fig. 3).

For further analyses of the *T. atroviride* GPCR-encoding *gpr1* and *gpr3* genes, a silencing method based on the synthesis of hairpin RNA was applied. Silencing of *gpr1* resulted in transformants with silencing levels of 70–80%, whereas transformants with the integrated *gpr3* silencing construct exhibited unaltered *gpr3* mRNA levels. Low-abundant transcripts were suggested to be less susceptible to siRNA-mediated degradation than medium- and high-abundant transcripts (Hu et al. 2004). The failure to obtain silencing of *gpr3* transcription may therefore be due to its low native mRNA levels. In addition, Fraser et al. (2000) reported that some genes are resistant to RNAi and in *C. cinereus* a lack of silencing in some transformants despite the presence of a complete silencing cassette was observed.

The authors suggested that this effect could be due to a lack of expression caused by the surrounding DNA of the ectopic insertion point (Wälti et al. 2006).

gpr1-silenced mutants grow slowly with only little aerial mycelium, but instead the hyphae invade the agar and grow submerged in it. In addition, the mutants continuously produce conidia when they are cultivated on solid media and the conidia have reduced and delayed germination. The finding that silencing of *gpr1* promotes constitutive sporulation even in the dark combined with the fact that exogenous cAMP induces sporulation (Nemcovic and Farkas 1998) makes it unlikely that cAMP is indeed the ligand of Gpr1. The phenotypes of the *gpr1*-silenced mutants are similar, although less pronounced (concerning reduction of growth rate and ratio of aerial hyphae to hyphae invading the agar), to the phenotype observed in $\Delta tga3$ mutants missing the subgroup III Gα subunit (Zeilinger et al., 2005). Whether Tga3 acts downstream of Gpr1 in the same signalling pathway needs further investigation, e.g. by applying the yeast 2-Hybrid-System and by genetic epistasis analysis.

When analysed in the Biolog system, *gpr1*-silenced mutants exhibited reduced growth on a variety of different carbon sources. For example, on glucose and glycerol, which are among the best carbon sources for the parental strain *T. atroviride* P1, the tested mutant exhibited an about 70% reduced growth rate during the first 66 h of incubation (Fig. 7). Furthermore, the mutant was not able to grow at all on the disaccharide lactose and its isomerization product lactulose, and also the disaccharide maltose and the trisaccharide raffinose did only allow sparse growth, although the sugars are well metabolized by the parental strain. Interestingly, on D-mannitol, D-arabitol, L-serine, salicin, and Tween 80 the mutant showed a similar growth rate as the parental strain suggesting that its reduced growth is carbon source-dependent and not a general effect.

Summarizing, we isolated and characterized four genes of *T. atroviride* P1 encoding GPCRs grouping to the class of CRL proteins. In the closely related ascomycete *N. crassa* this class of receptors consists of three proteins (Krystofova and Borkovich 2006), but with the exception of GPR-1 no information is available on their function. Furthermore, it is still unclear if these GPCRs respond to the same ligands or couple to the same downstream signalling components. Further characterization of *T. atroviride* Gpr1 showed that this GPCR affects conidiation, conidial germination and vegetative growth on a multitude of carbon sources, supporting the fundamental role of G protein signalling in *Trichoderma*.

Acknowledgments

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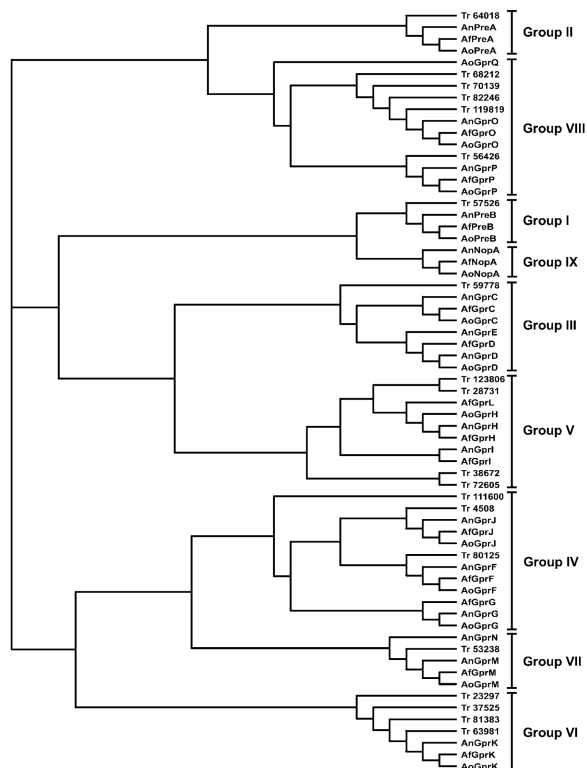
Fig. 1.
Cladogram of the phylogenetic relationship between putative GPCRs identified in the *T. reesei* predicted proteome (Table 2) and those previously identified in *A. nidulans, A. fumigatus* and *A. oryzae* (Lafon et al. 2006). The tree was generated using the CLUSTALX alignment
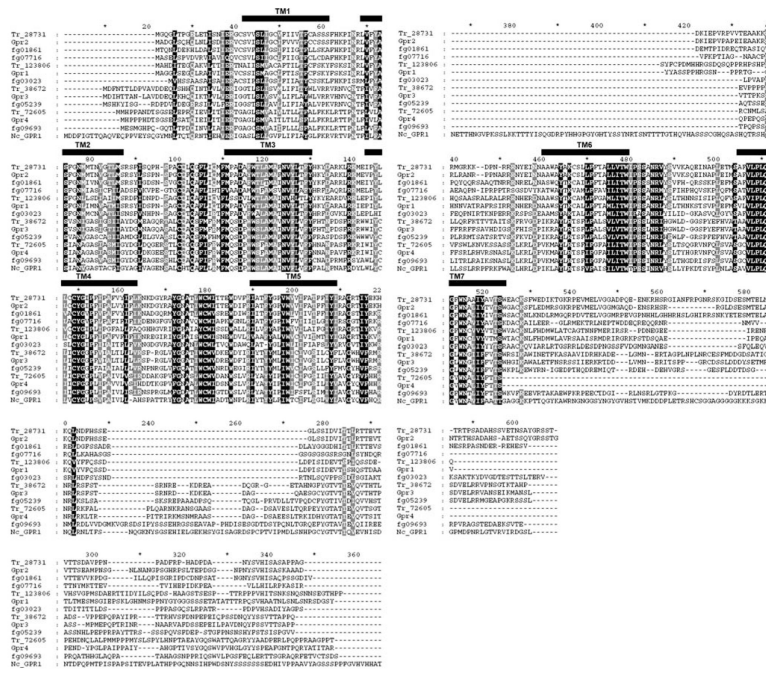
Fig. 2.
Alignment of *T. atroviride* Gpr1–4 with predicted fungal CRL proteins from other filamentous fungi. CLUSTALX was used to align GPCR sequences from *T. atroviride* (Gpr1, Gpr2, Gpr3, Gpr4) with predicted proteins from *T. reesei* (Tr_72605, Tr_38672, Tr_28731, Tr_123806), *F. graminearum* (fg01861, fg07716, fg03023, fg05239, fg09693), and the *N. crassa* GPR–1 (Nc_GPR1) receptor. Conserved residues are shaded in *black* (identical) or in *grey* (similar). The predicted seven transmembrane domains are marked by the bars above the sequence and are numbered (TM1–TM7)
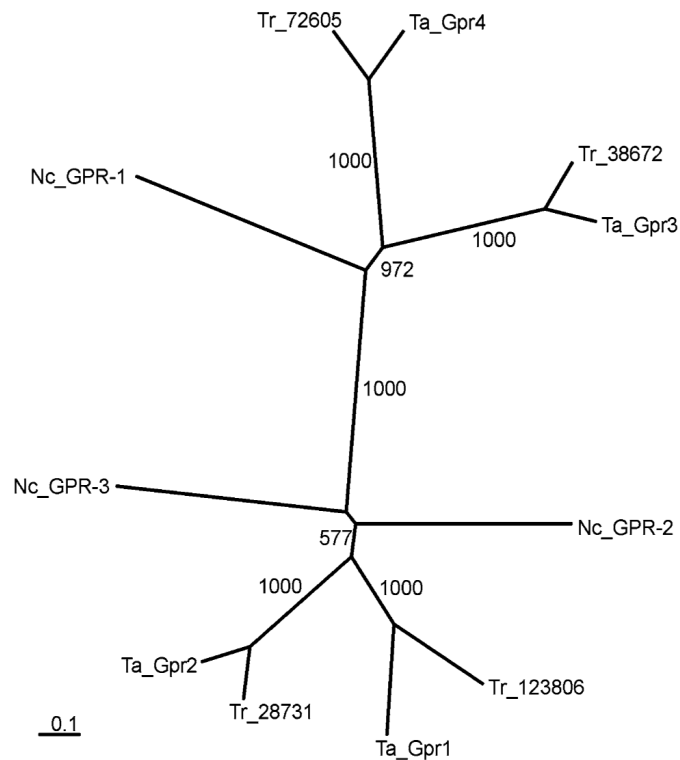
Fig. 3.
Phylogeny of class V GPCRs isolated from *T. atroviride* (Ta_Gpr1, Ta_Gpr2, Ta_Gpr3, Ta_Gpr4) and those identified in the *T. reesei* (Tr_72605, Tr_38672, Tr_28731, Tr_123806) and *N. crassa* (Nc_GPR-1 = NCU00786.3, Nc_GPR-2 = NCU04626.3, Nc_GPR-3 = NCU9427.3) genome sequences. The sequences were aligned and a tree was generated using CLUSTALX and neighbour-joining algorithm with 1,000 bootstraps
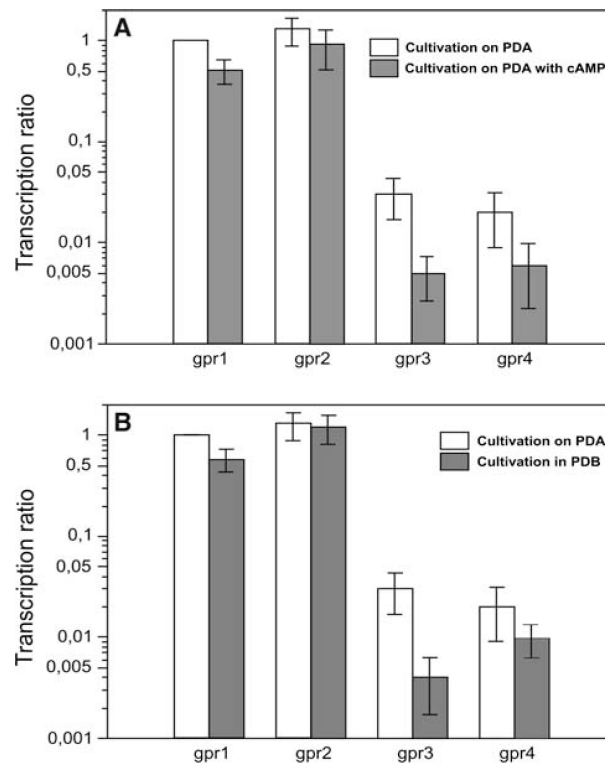
Fig. 4.
Expression of *gpr1, gpr2, gpr3*, and *gpr4* determined by real-time RT-PCR using *tef1* as a reference gene upon cultivation of *T. atroviride* on PDA compared to growth on PDA augmented with 5 mM cAMP (**a**) and on PDA compared to growth in PDB (**b**). To allow comparison of mRNA levels of the four GPCR-encoding genes, mRNA levels of *gpr1* on PDA were arbitrarily assigned the factor 1. The transcription ratio is presented in a logarithmic scale
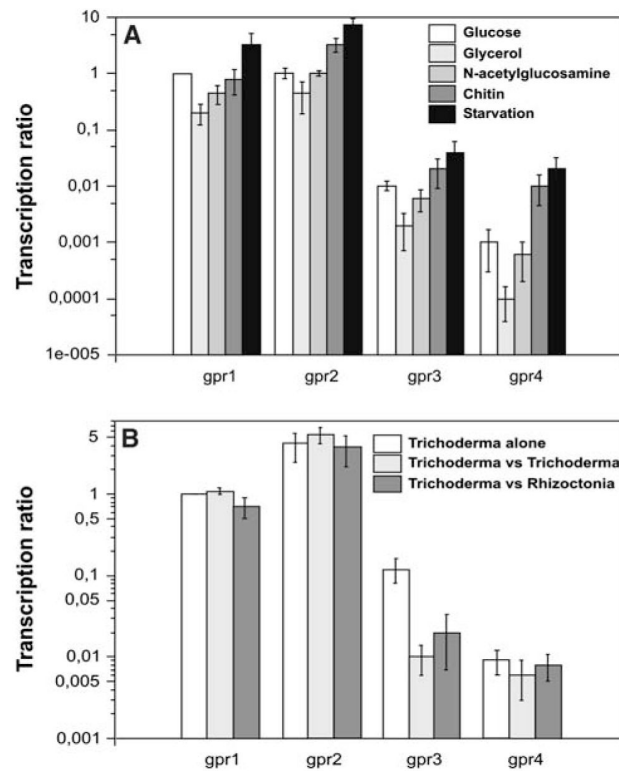
Fig. 5.
Real-time RT-PCR analyses of *gpr1, gpr2, gpr3* and *gpr4* mRNA levels upon cultivation of *T. atroviride* on different carbon sources using *act1* as a reference gene (**a**) and during in vitro biocontrol using *sar1* as reference gene (**b**). The samples from cultures with glucose, glycerol and N-acetyl-glucosamine as sole carbon sources were harvested 12 h after transfer, those from cultures with chitin as sole carbon source or from cultures grown without carbon source were harvested 24 h after transfer. For monitoring *gpr* expression during in vitro biocontrol, *T. atroviride* P1 was either grown alone, or confronted with itself, or confronted with *R. solani* as host fungus. To allow comparison of mRNA levels of the four GPCR-encoding genes, mRNA levels of *gpr1* on glucose (**a**) or PDA (**b**) were arbitrarily assigned the factor 1. The transcription ratio is presented in a logarithmic scale
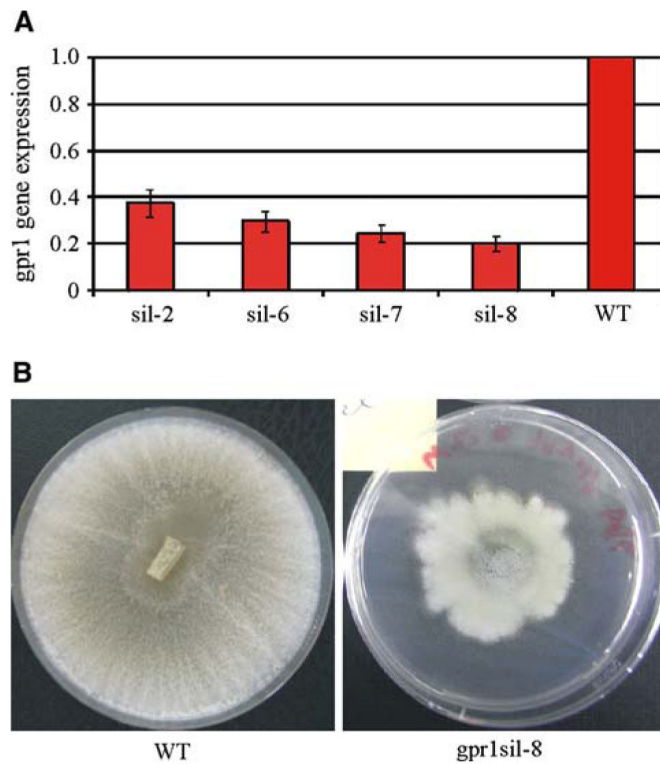
Fig. 6.
(**a**) Real-time RT-PCR analyses of *gpr1* gene expression in four selected *gpr1*-silenced transformants. mRNA levels of *gpr1* in the parental strain were assigned the factor 1. (**b**) Colony morphology of the *gpr1*-silenced transformant gpr1sil-8 compared to the parental stain *T. atroviride* P1 upon growth on solid medium (PDA) in the dark after 4 days at 28°C
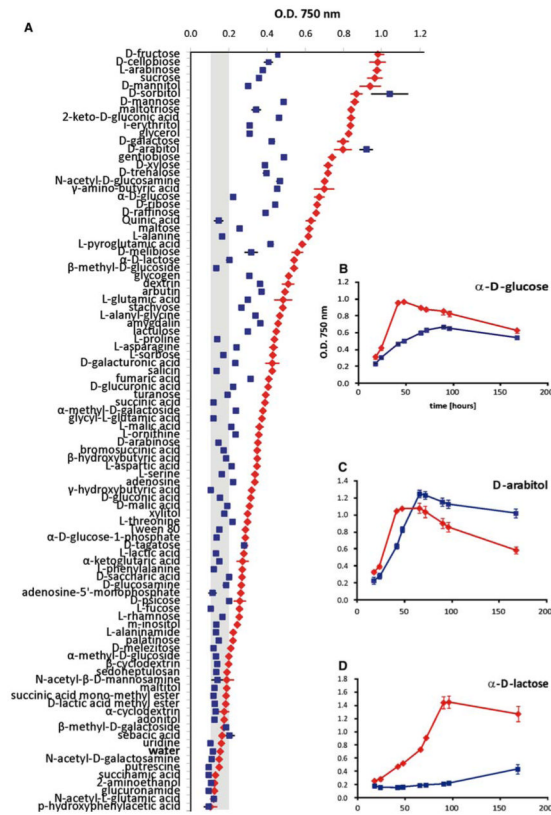
Fig. 7.
(a) Analysis of biomass formation of the *T. atroviride gpr1*-silenced mutant gpr1sil-8 (*filled square*) in comparison to the parental strain (*filled diamond*) after incubation for 66 h on 95 carbon sources and water using the BIOLOG microplate assay. (**b**–**d**) Growth curves of *T. atroviride* parental strain (*filled diamond*) and the *gpr1*-silenced mutant (*filled square*) on some individual carbon sources determined after 18, 24, 42, 48, 66, 72, 96 and 168 h of incubation. The values given represent the average of three analyses

Table 1

Oligonucleotides used in this study

Gene	Forward primer[a] (5′ to 3′)	Reverse primer (5′ to 3′)	Product (bp)
(a) Degenerate primer pairs used to amplify fragments of reference genes			
act1	GTIGCICCIGARGARCAYCCI	ACRTCIACRTCRCAYTTCAT	548
gpd1	GTIAARATHGTIAAYGAYAAYTT	GCIACIACISWTRYTCIGGYTT	873
tef1	GTCCTCAGTCTTGTCATTTTTTTCCT	GGAGGGGCCTGTCTGTGGGAC	906
sar1	CTCGACAAYGCCGGAAAGAC	TTGCCAAGGATGACAAAGGGG	833
28S	GGAACCTTTCCCCACTTC	AGTACCCGCTGAACTTAA	1,461
(b) Primer pairs used for quantification of transcripts by real-time PCR			
gpr1	TTGATCCAGACCTTCATGCCAGC	CATAAAAGGCCGCGACACGAA	
gpr2	CTGCCCTCGTGTACATCTTCC	GCCTTCAGATGAGTGAAAGTCG	
gpr3	GCCAGCATTGGAACCATCATCG	CCCAACACCAGATCGTAGCTCCA	
gpr4	ATTCTAACTGGAGCCTGGTC	GTACTTTGTGGCTCTGGTTG	
act1	GCACGGAATCGCTCGTTG	TTCTCCACCCCGCCAAGC	
gpd1	CGTCGTAGTCCTTGTGGTTGACACC	TCCTCCCACGGTCTCTTCAAGG	
sar1	CTCGACAATGCCGGAAAGACCA	TTGCCAAGGATGACAAAGGGG	
tef1	TACTTCCCAGGCTGACTGCGCTAT	GGAGGGGCCTGTCTGTGGGAC	
28S	TTTGAGTAAGAGCATACGGGGCC	GTTGATACATTCGAATGCCCACGT	

[a]R = A or G, Y = C or T, I = inosine (panel A)

Table 2

GPCRs identified in the *T. reesei* genome database

Receptor class	T. reesei	Class	T. reesei	T. reesei
I Pheromone	Tr_57526	PTH11-like	Tr_5647	Tr_82041
II Pheromone	Tr_64018		Tr_27983	Tr_103694
III Carbon sensor	Tr_59778		Tr_27992	Tr_105224
IV Nitrogen sensor	Tr_4508		Tr_39587	Tr_106082
	Tr_80125		Tr_40156	Tr_107042
	Tr_111600		Tr_41260	Tr_109146
V cAMP receptor-like	Tr_72605		Tr_41425	Tr_110339
	Tr_38672		Tr_45573	Tr_110744
	Tr_28731		Tr_53452	Tr_111861
	Tr_123806		Tr_55561	Tr_121990
VI RGS domain-containing	Tr_63981		Tr_57101	Tr_122795
	Tr_81383		Tr_58767	Tr_122824
	Tr_37525		Tr_61354	Tr_124113
	Tr_23297		Tr_62462	
VII	Tr_53238		Tr_66673	
VIII Similar to mPR	Tr_119819		Tr_66786	
	Tr_68212		Tr_67334	
	Tr_70139		Tr_69500	
	Tr_82246		Tr_69904	
	Tr_56426		Tr_70967	
IX Fungal opsins	nd		Tr_76763	
			Tr_78499	

Table 3

Stability of reference genes according to the output of geNorm (Vandesompele et al. 2002)

Ranking	Cultivation condition		
	PDB/PDA	**Various C-sources**	**Plate confrontation assays**
1/2	*act1/tef1*	*act1/sar1*	*sar1/gpd1*
3	*28S rRNA*	*tef1*	*28S rRNA*
4	*sar1*	*gpd1*	*act1*
5	*gpd1*	*28S rRNA*	*tef1*