The NADPH: cytochrome P450 reductase gene from *Gibberella fujikuroi* is essential for gibberellin biosynthesis

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Running title: The *Gibberella fujikuroi* cpr gene
Abstract

The fungus *Gibberella fujikuroi* is used for the commercial production of gibberellins (GAs), which it produces in very large quantities. Four of the seven GA-biosynthetic genes in this species encode cytochrome P450 monooxygenases, which function in association with NADPH: cytochrome P450 reductases (CPRs) that mediate the transfer of electrons from NADPH to the P450 monooxygenases. Only one *cpr* gene (*cpr-Gf*) was found in *G. fujikuroi* and cloned by a PCR approach. The encoded protein contains the conserved CPR functional domains, including the FAD-, FMN-, and NADPH-binding motifs. *cpr-Gf* disruption mutants were viable but showed a reduced growth rate. Furthermore, disruption resulted in total loss of GA$_3$, GA$_4$ and GA$_7$ production, but low levels of non-hydroxylated C$_{20}$-GAs (GA$_{15}$ and GA$_{24}$) were still detected. In addition, the knock-out mutants were much more sensitive to benzoate than the wild-type due to loss of activity of another P450 monooxygenase, the detoxifying enzyme, benzoate p-hydroxylase.

The UV-induced mutant of *G. fujikuroi*, SG138, which was shown to be blocked at most of the GA-biosynthetic steps catalyzed by P450 monooxygenases, displayed the same phenotype. Sequence analysis of the mutant *cpr* allele in SG138 revealed a nonsense mutation at amino acid position 627. The mutant was complemented with the *cpr-Gf* and the *Aspergillus niger cprA* genes, both genes fully restoring the ability to produce GAs.

Northern blot analysis revealed co-regulated expression of the *cpr-Gf* gene and the GA-biosynthetic genes *P450-1, P450-2, P450-4* under GA production conditions (nitrogen starvation). In addition, expression of *cpr-Gf* is induced by benzoate. These results indicate that CPR-Gf is the main, but not the only electron donor for several P450 monooxygenases from primary and secondary metabolism.
INTRODUCTION

The ascomycete *Gibberella fujikuroi* MP\(^1\)-C (1), recently renamed *Fusarium fujikuroi* (2), is well known as a rich source of gibberellins (GAs), which function as hormones in higher plants. The major GA in most strains of *G. fujikuroi* is gibberellic acid (GA\(_3\)), the biosynthesis of which requires seven genes, which are arranged in a gene cluster. Each of these genes and the function of the encoded enzymes have recently been fully characterized (3-8). As well as genes encoding a pathway-specific geranylgeranyl diphosphate synthase (*ggs2*), *ent*-copalylidiphosphate/*ent*-kaurene synthase (*cps/ks*) and GA\(_4\) desaturase (*des*), the cluster contains four cytochrome P450 monooxygenase genes (*P450-1* to *P450-4*), which, in most cases, encode multifunctional enzymes that catalyze several biosynthetic steps.

About 40 different P450 monooxygenases involved in diverse metabolic pathways have been identified in fungi (9). Many of the P450 genes form part of gene clusters that are responsible for the biosynthesis of metabolites, such as aflatoxins (10), trichothecenes (11), fumonisins (12) and paxillin (13), or for the metabolism of xenobiotics (14). Indeed a large group of the fungal cytochrome P450s have been shown to be involved in the metabolism of drugs and other foreign compounds (14-16), while others participate in the biosynthesis of intracellular compounds such as steroids (cited in 16). Metabolism of xenobiotics, including drugs and toxins, is also an important function of P450 monooxygenases in animals as is the formation of endogenous compounds, such as sterols and fatty acids (17). In plants, P450s are implicated in the formation of a broad range of metabolites, including the growth hormones, gibberellins and jasmonates, essential components such as lignin, pigments and fatty acids, and secondary metabolites, such as alkaloids, phytoalexins, glucosinolates, phenylpropanoids, and terpenoids. In addition, they are involved in the detoxification of herbicides and pesticides (18-20).

Eukaryotic non-mitochondrial cytochrome P450 monooxygenases are membrane proteins that require association with a NADPH: cytochrome P450 oxidoreductase (CPR) for activity. CPRs facilitate the transfer of electrons from NADPH via FAD and FMN to the prosthetic heme group of
the P450 monooxygenase. Interaction of both proteins in the microsomal membrane have been reported to occur through charge pairing as well as by hydrophobic interactions through the N-terminal region of CPR (21). In contrast to the many different cytochrome P450 monooxygenases that can be found in a single species, only one CPR-encoding gene is found in most organisms. Exceptionally, certain plants (22-24) and some zygomycetes (25) possess two or three CPRs, although the physiological relevance of the occurrence of multiple CPRs in these organisms is unknown. Fungal cpr genes have been isolated from yeasts (26-30) and some filamentous fungi, such as A. niger (31), Phanerochaete chrysosporium (32), Cunninghamella (33), Rhizopus nigricans (25) and Coriolus versicolor (34). Targeted cpr gene disruption to determine the function of these proteins has not yet been described for mycelial fungi.

Few data are available on the regulation of cpr gene expression in lower eukaryotes. In the yeasts Candida maltosa and C. tropicalis, the assimilation of n-alkanes is catalyzed by P450 monooxygenases. The addition of n-alkanes to the medium resulted in strong induction of P450 and cpr gene (28; 35), and co-regulation of the cpr and P450 genes has been reported for Saccharomyces cerevisiae (36). In A. niger, addition of benzoate increased the expression of the benzoate p-hydroxylase (bphA) and cprA gene (14).

The G. fujikuroi CPR would be expected to have a strong influence on GA biosynthesis since four P450 monooxygenases (P450-1 – P450-4) are known to be involved in this pathway. Thus, loss of CPR activity should affect the rates of several reactions in the pathway. Such an effect was found for the UV-induced G. fujikuroi mutant SG138, which has lost most of the oxidation steps catalyzed by P450 monooxygenases (37). Since it is unlikely that all structural P450 monooxygenase genes are mutated by one UV treatment it is possible that the mutant contains a lesion mutation in the cpr gene. This is confirmed in the present paper, in which we report the cloning, sequencing and targeted gene disruption of the G. fujikuroi cpr gene (cpr-Gf) and its effect on GA production. We show by sequence comparison that cpr-Gf contains a mutation in SG138 and we were able to restore GA production to the mutant by complementation, not only with the cpr-Gf gene but also
with the heterologous *A. niger cprA* gene. We have also compared the regulation of *cpr-Gf* with that of the four GA-biosynthetic monooxygenase genes.

**EXPERIMENTAL PROCEDURES**

*Fungal Strains*- Strains IMI58289 (Commonwealth Mycological Institute, Kew, UK) and m567 (Fungal Culture Collection, Weimar, Germany) are GA-producing wild-type strains of *G. fujikuroi* mating population (MP)-C (anamorph *F. fujikuroi*). The GA-deficient mutant *G. fujikuroi* SG138 (kindly provided by J. Avalos, University of Sevilla, Spain) strain was derived from IMI58289 via UV mutagenesis of spores (37).

*Bacterial strains and plasmids*- *Escherichia coli* strain Top10 (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Vector pUC19 was used to clone DNA fragments carrying the *G. fujikuroi cpr* gene and gene fragments. First, a 2.2 kb *Bgl*II-fragment of λ-clone 2-1 was cloned into *Bam*HI-restricted pUC19 (pCPR1A). Then a 3.8 kb *Xba*I-fragment was cloned into pUC19/*Xba*I (pCPR1B). pCPR1B was cut with *Nco*I/*Sph*I. The derived 3 kb-fragment was cloned into pCPR1A/*Nco*I/*Sph*I to produce vector pCPR-Gf, containing the entire *cpr-Gf* gene. The vector pNR1 was constructed by cloning the *Pst*I/*Bam*HI fragment of the *Streptomyces noursei* nat1 gene encoding the nourseothricin acetyltransferase (38), into pBluescript II KS. The gene was transcribed under control of the *A. nidulans oliC* promoter (39) and terminated by the *Botrytis cinerea tub1* terminator (van Kan, personal communication). For gene replacement experiments, fragments from the 5´- and 3´-noncoding regions of *cpr* were cloned into the vector pUCH2-8 (40), carrying the hygromycin B resistance marker.

*Media and culture conditions*- For DNA isolation, fungal strains were grown in 100 ml of liquid CM medium optimized for *Fusarium* spp. (41) for three days at 28°C on a rotary shaker set at 200 rpm. The mycelium was harvested by filtration through a sterile glass filter (G2, Schott Jena, Germany), washed with sterile distilled water, frozen in liquid nitrogen, and lyophilized for 24 h. The lyophilized mycelia were ground to a fine powder with a mortar and pestle. For RNA isolation,
fungal strains were grown in 100%, 20% or 0% ICI medium (42), containing 8% glucose, 0.5% MgSO₄, 0.1% KH₂PO₄, and 5.0, 1.0 or 0 g/l NH₄NO₃, respectively.

For analysis of cpr-Gf expression with and without benzoate, strain IMI58289 was cultivated for three days in 10% ICI medium on a rotary shaker at 28°C. The mycelium was washed and 1.5 g (wet weight) each were transferred to 50 ml of 0% or 100% ICI medium with or without (0.5 mM or 1mM) benzoate. For GA production, the strains were grown for 7-10 days on a rotary shaker (200 rpm) at 28°C in 300-ml Erlenmeyer flasks containing 100 ml of either 20% ICI or optimized production medium (OPM), containing 6% sunflower oil, 0.05% (NH₄)₂SO₄, 1.5% corn-steep solids (Sigma-Aldrich, Taufkirchen, Germany), and 0.1% KH₂PO₄. Benzoate plate tests were performed on CM and Czapek Dox (Sigma-Aldrich) agar with 1 mM benzoate or without benzoate.

DNA and RNA isolation—Genomic DNA was isolated from lyophilized mycelium as described by Doyle and Doyle (43). Lambda DNA from positive lambda clones was prepared according to Maniatis et al. (44). Plasmid DNA was extracted using Genomed columns following the manufacturer’s protocol (Genomed, Bad Oeynhausen, Germany). RNA was isolated using the RNAgents total RNA isolation kit (Promega, Mannheim, Germany).

PCR—Degenerate primers CPR1 and CPR2 were designed by C. Wasmann (University of Arizona, USA) on the basis of CLUSTAL alignment of fungal CPRs and kindly provided for cloning the G. fujikuroi cpr-Gf gene. PCR reactions contained 25 ng DNA, 10 ng of each primer, 0.2 mM dNTPs and 2 U Taq polymerase (Red Taq, Sigma-Aldrich, Deisenhofen, Germany) in 50 µl. PCR was carried out at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. The resulting 0.5 kb PCR-fragment was used as a probe for screening a genomic λ-DASH II library (Strategene Europe, Amsterdam, the Netherlands) of G. fujikuroi m567 at 65°C.

CPR1: 5'-AAG YTG CAG CCY CGC TAC TAY TCS ATC TC-3'

CPR2: 5'-CTT CCA YTC RTC CTT GTA SAR GAA RTC CTC-3'
For cloning *cpr-Gf* of SG138, four primer pairs were used in order to get overlapping fragments after amplification:

P138-1: 5'-GTG GCC AAA GTT CAT GAT TAG TGC-3'
- 2: 5'-TTG CGG ACC ATA GAG TTG TAG TGC-3'
- 3: 5'-TCG CCA AGG AGG GTA AGA-GTC-3'
- 4: 5'-GCT GCC AGG GCG GTT CAT-3'
- 5: 5'-AAC CCC TAC ATT GCC CCT ATC G-3'
- 6: 5'-TCG GCA ACC AAA GAA CAA GAG TG-3'
- 7: 5'-ACA GGC CCC CGC AAT AAG TA-3'
- 8: 5'-TGT CGG CAA GTC CAT GTC TAA GTG-3'

Each fragment is about 850 bp long.

For RT-PCR, primers CPR-RT1 and CPR-RT2 were used, to amplify fragments of about 330 bp including a putative intron.

Primer CPR-RT1 (1): 5'-CAA CCG AGG ATT TCA TGT ACC-3'

CPR-RT2 (2): 5'-CCC TTG GCC TCA GAC ACC-3'

For analysis of putative *cpr* knock-out transformants, the following diagnostic primers were used:

for integration at the 5' region of *cpr*:

CPR-DF1 (7): 5'-CGG GGA TGG AGG CAA GAG AAT GAA-3'

PUCH-P (8): 5'-CCC TTG GCC TCA GAC ACC-3'

for integration at the 3' region of *cpr*:

CPR-DF2 (9): 5'-GAT CTA CAG ACT TGC TTC TGT LGG-3'

PUCH-T (10): 5'-TCA ACG CAT ATA GCG CTA GC-3'

The gene replacement vector pΔcpr-Gf was constructed by cloning the PCR-amplified flanks into vector pUCH2-8 (45). For amplifying the 5' and 3' flanks the following primers with introduced restriction sites were used:

Dcpr-1 (3): 5'-CGG GTA CAA GGA GTTG ACC GTG CAA AT-3'
Screening the lambda DASH II library- About 35,000 recombinant phages from a lambda library prepared from genomic DNA of wild-type *G. fujikuroi* m567 (46) were plated and transferred to Nylon N+ membranes (Amersham Pharmacia, Freiburg, Germany). Hybridization was performed at high stringency (65°C). The blots were washed in 2 x SSC 0.1% SDS at 65°C followed by a wash with 0.1 x SSC 0.1% SDS at 65°C. Positive recombinant phages were used for a second round of plaque purification.

Southern and Northern blot analysis- After digestion with restriction endonucleases and electrophoresis, genomic or lambda DNA was transferred onto Hybond N+ filters (Amersham Pharmacia, Freiburg, Germany). ³²P-labelled probes were prepared using the random oligomer-primer method (44). Filters were hybridized at 65°C or 56°C in 5 x Denhardt’s solution containing 5% dextran sulfate (44). Filters were washed at the same temperature used for hybridization in 2 x SSPE 0.1% SDS and 1 x SSPE 0.1% SDS.

Northern blot hybridizations were accomplished by the method of Church and Gilbert (47). The *G. fujikuroi* rDNA gene was used as a control hybridization probe to confirm RNA transfer.

Sequencing-DNA sequencing of recombinant plasmid clones was accomplished with an automatic sequencer "LI-COR 4000" (MWG, München, Germany). The two strands of overlapping subclones obtained from the genomic DNA clones were sequenced using the universal and the reverse primers or specific oligonucleotides obtained from MWG Biotech (Munich, Germany).
DNA and protein sequence alignments were done with DNA Star (Madison, WI, USA). For phylogenetic analyses, gaps were not considered and corrections for multiple substitutions were applied. Trees were constructed with the program MegAlign (DNA Star Madison, WI, USA).

Transformation of G. fujikuroi - The preparation of protoplasts and the transformation procedure were as previously described (46). For gene replacement, $10^7$ protoplasts (50 µl) of strain IMI58289 were transformed with 10 µg of the KpnI/SacI fragment of the gene replacement vector pΔcpr. For complementation of the mutant strain SG138 with intact cpr genes, protoplasts were transformed with 10 µg of the circular complementation vector pcpr-Gf carrying the G. fujikuroi cpr gene or pcpra with the cpr gene from A. niger (31). Both plasmids were co-transformed with pAN7.1 (48), carrying the hygromycin resistance marker.

For complementation of the transformant Δcpr - T20 with the wild-type cpr-Gf gene, protoplasts were co-transformed with 10 µg of each the circular complementation vector pcpr-Gf and pNR1. Transformed protoplasts were regenerated at 28°C on complete regeneration agar (0.7 M sucrose, 0.05 % yeast extract, 0.1 % (NH$_4$)$_2$SO$_4$) containing 120 µg/ml hygromycin B (Calbiochem, Bad Soden, Germany) or 100 µg/ml nourseothricin (Werner BioAgents, Jena, Germany) for 6-7 days. For purification, single conidial cultures were obtained from hygromycin B- or nourseothricin-resistant transformants and used for DNA isolation and Southern blot analysis.

Gibberellin assays - GA$_3$ and GA$_{4/7}$ were analyzed by thin layer chromatography on silica gel eluted with ethyl acetate/ chloroform/ acetic acid (60:40:5). The complete GA complement produced by the different strains was determined by GC-MS analysis after extraction from the culture fluid as already described (8), except that compounds were separated on a 30m x 0.32 mm x 0.25 µm HP-5 WCOT column (Agilent Technologies) and analysed using a MAT95XP mass spectrometer (Thermo Electron Corporation) GC-MS conditions were as described previously (8). Compounds were identified by comparison of their mass spectra with those in a spectral library (49). For quantitative analysis of GAs aliquots of the extracts were spiked with [17-$^2$H$_2$]GA internal
standards and analyzing using a GCQ instrument (Thermo Electron Corporation) as described previously (50), except chromatogram peak areas were obtained from full scans.

Plate tests with benzoate-For analysis of benzoate tolerance, strains IMI58289, SG138 and transformants T20 and KT-1 were grown for 6 d at 28°C on CM and CD agar containing 1 mM benzoate.

RESULTS

Cloning and targeted gene disruption of cpr-Gf-A fragment of the cpr-Gf gene with the expected size of 500 bp was amplified by PCR using degenerate primers CPR1 and CPR2 derived from the FAD- and NADPH-binding domains, respectively (see Fig. 1A). The fragment exhibited a high degree of homology with CPRs from other fungi and served as a probe for screening the lambda DASH II genomic library of G. fujikuroi, MP-C. Of the three genomic lambda clones isolated, one was used for isolating the putative full-length gene (Fig. 1A). About 4000 bp sequence including 1000 bp of the 5’non-coding region was obtained by sequencing in both directions. A 273-bp cDNA fragment spanning a putative intron was generated by RT-PCR using primers CPR-RT1 and CPR-RT2 (Fig. 1A). Comparison of the genomic and cDNA sequences confirmed the expected intron of 52 bp. The cpr-Gf gene is deposited under the accession number AJ576025.

The deduced amino acid sequence of cpr-Gf was aligned with known CPRs from taxonomically diverse species and allowed the identification of all functional domains involved in the binding of the cofactors FMN, FAD and NADPH, and the P450 proteins (Fig. 1A). A phylogenetic tree comparing CPR-Gf with CPRs from other species indicates that it is most closely related to CPRA of Aspergillus niger (61% identity), followed by the yeast and the other fungal CPRs (Fig. 2).

In order to determine the importance of the cpr-Gf gene for GA production, a gene replacement vector was constructed as shown in Figure 1B. Two flanking sequences were amplified by PCR introducing KpnI and SalI (flank 1) and HindIII and SacI (flank 2) restriction sites into the oligonucleotides. The flanking sequences were cloned into the corresponding sites of vector
pUCH2-8 carrying the hygromycin resistance cassette producing vector pΔcpr-Gf (Fig. 1B). The 4.4 kb KpnI/SacI fragment of the vector was used for transforming the wild-type IMI58289. 72 transformants were analyzed by PCR for homologous integration using the primer pairs 7 (CPR-DF1) and 8 (PUCH-P), and primer pairs 9 (CPR-DF2) and 10 (PUCH-T) (Fig. 1B). Three transformants revealed the expected diagnostic bands. After purification by single-spore isolation, these transformants were analyzed by Southern blot analysis together with the wild-type IMI58289 and the putative cpr mutant SG138. The HindIII-digested DNA was hybridized to flank 2 as probe (Fig. 1B). For two transformants, T20 and T49, the hybridizing 2.5 kb wild-type band was replaced by a smaller (1.4 kb) and a bigger (4.4 kb) band (Fig. 3A) due to a tandem integration of two replacement cassettes into the cpr locus. The tandem integration was confirmed by PCR with primers 8 (PUCH-P) and 9 (CPR-DF2) (Fig. 1B) and sequencing of the fragment. Transformant T57 integrated the fragment ectopically and still contains the wild-type band.

The deletion of the wild-type copy of cpr in transformants T20 and T49 was confirmed by Northern blot analysis. The wild-type, transformant T57 and mutant SG138 contain a transcript of about 2.1 kb, whereas T20 and T49 have lost the entire coding region of cpr and do not show any cpr transcript (Fig. 3B).

**GA production and growth characteristics of deletion mutants and strain SG138**—In order to determine the effect of cpr-Gf deletion on GA production, the wild-type strain, both knock-out mutants and the putative cpr mutant SG138 were cultivated for 7 days in the synthetic 20% ICI medium. The culture fluids were then analyzed by TLC (Fig. 3C) and GC-MS (Table I). Transformants T20 and T49 as well as mutant SG138 do not produce GA₄, GA₇ and GA₃, the last three products of the pathway. Interestingly, the deletion mutants and SG138 show a reduction of growth rate on agar plates, which is more significant on minimal CD agar (Fig. 4C) than on CM medium (Fig. 4A). The similar characteristics for T20, T49 (data not shown) and SG138 as well as the described loss of P450-catalyzed oxidation steps (37) support our proposal that the latter strain is also affected in cpr-Gf.
Identification of the mutation in SG138-To confirm our proposal that the UV treatment affected the cpr gene in the mutant SG138, the cpr gene from the mutant was amplified by four primer pairs to give four overlapping fragments, which were cloned and three independent clones were sequenced in both directions. Comparison with the sequence of the wild-type gene copy confirmed a point mutation in the first position of the codon at aa position 627 from C to T resulting in a TGA stop codon instead of CGA for arginine. The truncated CPR peptide is therefore 83 aa shorter than the wild-type CPR enzyme. All CPR proteins analyzed so far contain a NADPH binding domain consisting of three segments (32). This is also the case for CPR-Gf (Fig. 1A). In the mutant SG138 a large part of the last segment of the NADPH binding domain is missing.

Complementation of a deletion mutant with the wild-type cpr-Gf gene-The deletion mutant T20 was co-transformed with the complementation vector pcpr-Gf carrying the wild-type cpr-Gf gene, and vector pNR1 with the nourseothricin resistance gene as selection marker (see Experimental procedures). Two nourseothricin-resistant transformants, KT-1 and KT-13, were analyzed for correct integration of the cpr-Gf gene. As shown in Southern blot (data not shown) and Northern blot analysis (Fig. 5), only KT-1 showed multiple copies of the hybridizing wild-type gene and a high transcript level of the correct size, whereas transformant KT-13 does not contain vector pcpr-Gf. Analysis of the GA concentrations showed almost full restoration of GA production. Analysis of the three final products, GA4, GA7 and GA3, by GC-MS (Table I) demonstrated that the activity of all four P450 monoxygenases was at least partially restored resulting in production of the normal GA pattern (Fig. 6) and formation of wild-type-like amounts (or even more) of the final product gibberellic acid (GA3). Furthermore, the growth rate of KT-1 on CM and CD agar was comparable to that of the wild-type (Fig. 4A, 4C).

Analysis of GA intermediates in the cpr mutants-The effect of cpr-Gf-deletion on each of the P450-catalyzed steps in the GA-biosynthetic pathway was investigated by determining the full spectrum of intermediates in the Δcpr mutants (Fig. 6) and quantifying selected intermediates by GC-MS (Table I). The cpr deletion mutants do not produce GA3, GA4 or GA7, the final products of the GA-
biosynthetic pathway, but only a very low amount of the non-hydroxylated intermediates GA$_{15}$ and GA$_{24}$ together with high levels of ent-kaurene (Fig. 7). The level of GAs in Δcpr is about 1% of that found in the wild-type strain IMI58289. These results indicate a very low activity of ent-kaurene oxidase (P450-4) and low but significant activities for GA 7-oxidase (one of the activities of P450-1) and GA 20-oxidase (P450-2) in the absence of the P450 reductase. In contrast, 3β-hydroxylation, another activity of the P450-1 monooxygenase, was absent in Δcpr and thus would appear to have an absolute requirement for the P450 reductase. The spectrum of GAs found in SG138 (Table I) was similar to that in Δcpr, consistent with our demonstration of a mutation in the P450 reductase in this strain. The requirement of the 13-hydroxylation reaction, catalyzed by P450-3, for the reductase was tested in the cpr mutants by incubating with [14C]GA$_4$ and analysing the products by HPLC and GC-MS. Formation of [14C]GA$_3$ was reduced in the mutants relative to the wild-type (20% compared with 84%) indicating that the 13-hydroxylase has a partial, but not absolute requirement for the P450 reductase. Complementation of SG138 with cpr-Gf increased [14C]GA$_3$ formation from [14C]GA$_4$ to 56%.

The GA profile and amounts in Δcpr and SG138 were restored to those in the wild-type strain by complementation with the reductase gene from G. fujikuroi (Table I and Fig. 6). The major products synthesized by the complementation mutants were the 3β-hydroxylated GAs GA$_3$ and GA$_1$ plus lower amounts of GA$_4$ and GA$_7$. Interestingly, the GA-producing ability of SG138 was also fully restored by complementation with the reductase gene from Aspergillus niger (cpr-A, see below) although A. niger is not able to produce any GAs (Table I).

The specificity of cpr-Gf for GA biosynthesis activity—As part of an enquiry into whether or not cpr-Gf is specific for the P450s involved in GA biosynthesis we investigated if the cpr and GA monooxygenase genes were co-regulated. Co-regulation has been reported, for example, for the A. niger cprA and benzoate p-hydroxylase (bphA) genes (14).
Three of the four GA-specific P450 monooxygenase genes (P450-1, P450-2 and P450-4) are known to be regulated by the general transcription factor AREA (51) and thus are highly expressed under nitrogen starvation conditions. Therefore, we compared the expression pattern of these three P450 genes with that of cpr-Gf. Interestingly, the cpr-Gf gene is co-regulated with P450-1, P450-2 and P450-4: high cpr-Gf transcription levels were found under nitrogen starvation conditions, but much less (though higher than for the monooxygenase genes) with high amounts of nitrogen (Fig. 8A). We investigated the specificity of the interaction between CPR-Gf and the GA-biosynthetic monooxygenases by transforming the mutant SG138 with the cpra gene of A. niger. Ten hygromycin-resistant transformants were cultivated under GA production conditions and analyzed for GA content. Three transformants, SG138-cpra-7, -8, and -19, were able to produce GA3 (e.g. SG138-cpra-7, Table I), demonstrating that CPRA from the GA-non-producing fungal species A. niger which is described as activator of the benzoate p-hydroxylase, is able to act as electron donor and activator of GA-biosynthetic enzymes P450-1 to P450-4 in G. fujikuroi. However, in contrast to cpr-Gf, cpra was expressed independently of nitrogen condition in G. fujikuroi (Fig. 8B).

On the basis of these results, we speculated that CPR-Gf may also act in much more than the GA-biosynthetic pathways. In order to show this, we determined if CPR-Gf is involved in detoxification of benzoate in a similar way to CPRA in A. niger. We grew the wild-type strain IMI58289, two cpr-Gf mutants, SG138 and T20, as well as the complemented strain KT-1 on CM and CD agar with or without benzoate. The growth patterns show very clearly that mutation or deletion of cpr-Gf led to an extreme sensitivity to this compound due to the lost activation of the benzoate p-hydroxylase by CPR-Gf. On the other hand, complementation of T20 with the wild-type cpr-Gf copy fully restored the high resistance level for benzoate (Fig. 4B,D).

These results led us to anticipate induction of cpr-Gf gene expression by benzoate, as is the case for cpra in A. niger. Addition of benzoate to the medium significantly induced the cpr-Gf transcription level in G. fujikuroi, especially when 1mM benzoate was added (Fig. 9). Interestingly, with benzoate in the medium, cpr-Gf expression is no longer repressed by high amounts of nitrogen.
DISCUSSION

Using degenerate PCR and genomic library screening we isolated a gene from *G. fujikuroi* with high homology with cytochrome P450 reductase (CPR) genes from other species, particularly the gene from *A. niger* (*cprA*) with which it is 61% identical at the amino acid level. The *G. fujikuroi* *cpr* gene (*cpr-Gf*) encodes a protein of 713 amino acids containing all expected domains for binding the prosthetic factors FAD, FMN and NADPH as well as for P450s (32). Targeted disruption of *cpr-Gf* led to a very substantial loss of GA production demonstrating that the reductase is required for normal activity of P450s involved in GA-biosynthesis. In *G. fujikuroi* GA biosynthesis requires four P450 monooxygenases, which are responsible for more than 10 enzymatic steps. P450-4 catalyzes the three oxidation steps from *ent*-kaurene to *ent*-kaurenoic acid (5). P450-1 catalyzes the oxidation of *ent*-kaurenoic acid to *ent*-7α-hydroxykaurenoic acid, followed by ring contraction with the production of GA12-aldehyde, its 3ß-hydroxylation to GA14-aldehyde, and oxidation to GA14, in addition to several side reactions (6). P450-2 converts GA14 to GA4 and GA12 to GA0 with the loss of carbon-20 (7). Finally, P450-3 is responsible for the 13-hydroxylation of GA7 to GA3, and, in a minor pathway, of GA4 to GA1 (8). Although not all P450-catalyzed steps are completely blocked in the deletion mutants, they are reduced in activity and some reactions, such as 3ß-hydroxylation and loss of C-20, are completely absent in Δcpr mutants. Thus, CPR-Gf may act as electron donor of all four P450 monooxygenases.

The small amounts of the non-hydroxylated GA15 and GA24, and the high level of *ent*-kaurene found in Δcpr and SG138 indicate low activities of *ent*-kaurene oxidase (P450-4), GA 7-oxidase (P450-1) and 20-oxidase (P450-2) in the absence of the reductase. The activity of 13-hydroxylase (P450-3), which could not be assessed from the GA profiles of the cpr mutants because its substrate is not produced, was assayed by incubating with [14C]GA4 and shown to have reduced activity in the absence of the reductase. The residual activities of these enzymes indicate the participation of a second electron transport protein in GA biosynthesis that would supply electrons
only to some of the reactions catalyzed by P450 monooxygenases and with less efficiency than the
P450 reductase.

Analysis of the GA content of the UV-induced mutant SG138 suggests that it contains slightly
higher P450 activities than the Δcpr lines and may thus possess low CPR activity. The GA levels in
SG138 are similar to Δcpr, but ent-kaurene does not accumulate and traces of GA₄, GA₃, GA₁₅, and
GA₂₄ are present. SG138 contains a point mutation in the cpr gene that gives a truncated protein that
lacks part of the NADPH binding domain. Mutation in SG138 would thus reduce considerably but
may not abolish CPR activity. Complementation of Δcpr with the cpr-Gf gene, fully restored GA
synthesis giving 3β-hydroxylated C₁₉-GAs (GA₃, GA₄, GA₁ and GA₇) at similar levels than in the
wild-type strain. This demonstrates that the P450 reductase is the main electron donor to the four
GA-biosynthetic P450s in G. fujikuroi. The second electron transport pathway is much less
effective and can only partially compensate for the absence of CPR.

Interestingly, the two activities of the multifunctional P450-1 monooxygenase (6) differ in their
dependence on CPR. While 3β-hydroxylase depends absolutely on CPR and is completely blocked
in Δcpr, the 7-oxidase activity can obtain electrons from an alternative source and is still
moderately active in the deletion mutant. Our results agree with previous findings about differences
in nucleotide co-factor requirements for the different activities of P450-1 (52). The GA 20-oxidase
(P450-2) (7) does not produce C₁₉-GAs by cleavage of C-20 in the absence of the CPR. Instead, the
C₂₀ tricarboxylic acid product GA₂₄ was found in Δcpr, together with GAs with intermediate
oxidation states (alcohol and aldehyde) at C-20 that do not accumulate in the wild-type. Production
of the C₁₉-GAs thus appears to be completely dependent on CPR in contrast to tricarboxylic acid
synthesis. If the rate of C-20 oxidation is considerably reduced in the absence of CPR this may
result in the accumulation of enzyme-bound intermediates, which can be hydrolyzed to give the
C₂₀-GAs detected (7).
Beside the dramatic effect on GA production, mutations in cpr in the deletion mutants and SG138 affected also the growth rate on synthetic medium and to a lesser extend on CM medium indicating that CPR probably acts as electron donor also for P450-related pathways in primary metabolism, e.g. for metabolism of sterols and fatty acids. However, the effect on primary metabolism, especially on CM medium, is not as strong as might be expected if CPR-Gf were the only electron donor associated with P450s in G. fujikuroi. It is also possible that the reduced growth rate is due to the accumulation of toxic intermediates of disrupted secondary metabolite pathways.

Although A. niger does not produce GAs and does not contain GA-related P450s, the cprA gene, involved in benzoate detoxification in A. niger, fully restored the GA production capacity of the cpr mutant SG138. This result indicates that CPRs act unspecifically as general electron donors for P450 monooxygenases from different pathways. The recently completed genome of the basidiomycete, Phanerochaete chrysosporium, revealed the presence of only one CPR-encoding gene (CPR:EC 1.6.2.4) and at least 123 cytochrome P450 monooxygenase genes (53). The genome sequences of Neurospora crassa (http://www-genome.wi.mit.edu/annotation/fungi/neurospora) and Fusarium graminearum (http://www-genome.wi.mit.edu/cgi-bin/annotation/fusarium), a species of the same genus (Fusarium) as G. fujikuroi, revealed 44 and 40 cytochrome P450 monooxygenases, respectively (9), but only one CPR-encoding gene, with a high degree of identity to CPR-Gf. Therefore, it is likely that G. fujikuroi contains a single cpr gene that interacts with each of the P450s.

We analyzed the GA-biosynthetic and cpr genes also in the closest related members of the G. fujikuroi species complex consisting of eight mating populations (MP-A to MP-H). Most of these species contain the complete GA gene cluster, but only members of MP-C (F. fujikuroi) are able to produce GAs. The loss of GA production capability is due to a set of mutations in the coding and 5′-noncoding regions of the GA-biosynthetic genes resulting in an overall amino acid sequence identity of only 84-94% in the case of P450-4. In contrast to the dispensable GA pathway genes, the level of sequence identity between the CPR enzymes is about 98% (Malonek and Tudzynski,
unpublished results). These results suggest the importance of CPR for essential functions of cell metabolism.

Here we report on the first deletion of a *cpr* gene in a filamentous fungus. So far, only in *S. cerevisiae* has the single *cpr* gene been successfully deleted without dramatic influence on viability (54), indicating that an alternative electron donor must exist. It was suggested that in *S. cerevisiae*, a cytochrome b5 (cyt b5) could act as a second important element in the electron donating system. Deletion of cyt b5 in the wild-type did not display a phenotype, whereas disruption of the gene in a Δcpr strain was lethal demonstrating that both enzymes can complement each other in mutants with single disruptions of *cpr* or *b5* (54). However, there is no additional electron donating system overcoming the double knock out.

The exact mechanism by which cyt b5 interacts with P450 reductase is not yet clear. Numerous studies have shown that P450 activity can be enhanced by addition of cyt b5 in some, but not all reactions (55-57). Human, but not yeast, cyt b5 can selectively augment the rate of steroid hormone hydroxylations by more than 10-fold, but this stimulation requires CPR and occurs without electron transfer to or from cyt b5 (55).

In petunia, the product of a cyt b5 gene, which is expressed exclusively in the flowers, regulates the activity of two P450s involved in the biosynthesis of anthocyanin pigments. Targeted inactivation of the *b5* gene resulted in a flower colour change caused by reduction in activity of these two P450s, but it did not affect other P450s (17). We suggest that in *G. fujikuroi* a cyt b5 might take over the function of CPR for activation of P450s involved in primary metabolism, and, to a much lesser extent, some of those functions in the dispensable GA-biosynthetic pathway.

A possible alternative to general CPRs and cyt b5 as electron donors was found in the fumonisin gene cluster of *G. fujikuroi* MP-A (*F. verticilloides*): the ORF of the fumonisin biosynthetic gene *FUM6* consists of a P450 gene that is fused to a *cpr* gene (12), in which the FMN, FAD, and NADPH binding domains are arranged in the same order as in other CPRs. This unusual enzyme belongs to a family consisting of another fungal and two bacterial enzymes, the *F. oxysporum* fatty...
acid ω-hydroxylase (58), the *Bacillus megaterium* fatty acid hydroxylase P450$_{\text{BM-3}}$ (59), and the *B. subtilis* *Yfn1* gene product (A69975). The fusion of a P450 and a CPR into one single enzyme (FUM6) is highly unusual for fungal secondary metabolite genes. In all other examples described so far, only typical P450 monooxygenase genes are present in gene clusters for dispensable metabolites; no CPRs, either as single genes or fused to P450s, are present in such clusters (3; 10; 45; 60). It is not yet clear if the fusion protein is specific for the P450s in the fumonisin pathway or could complement the functions of the general reductase in other pathways.

Major functions of P450s in numerous organisms, including fungi, are the metabolism of xenobiotic drugs and toxins, the assimilation of long chain alkanes, as well as the metabolism of endogenous compounds, such as sterols and fatty acids (cited in 16). For *F. moniliforme* it was shown that the fungus can oxidize propylbenzene, and that this reaction needs molecular oxygen and NADPH as the preferential coenzyme, suggesting a microsomal cytochrome P450 monooxygenase system that contained NADPH-cytochrome P450 reductase (61). Other *Fusarium* strains, like *F. solani*, are able detoxify plant phytoalexins, such as pisatin, by a cytochrome P450 enzyme system (62). The loss of CPR would have dramatic consequences for fungal survival in the environment and for its ability to infect plant tissue.

Many of the P450s are substrate-inducible. To become functionally active, they are dependent on the high electron donating activity of CPR under the same induction conditions. Thus phenobarbital and numerous other drugs and chemicals induce not only the expression of many drug- and steroid-metabolizing P450s, but also that of NADPH-dependent reductase gene in liver tissue (15). In the subtropical plant *Catharanthus roseus*, where so far only one *cpr* gene has been identified, expression of this gene is co-regulated with that of numerous P450 genes, involved in the biosynthesis of terpenoid indole alkaloids, phenylpropanoids and in other defence-related compounds (63). For *A. niger* it was shown that expression of both the P450 gene *bphA*, involved in benzoic acid hydroxylation, and *cprA* were induced by the substrate, benzoate. Interestingly, the majority of *cprA* transcripts after benzoate induction were of a larger size due to the use of four
alternative transcription start points. In addition, an upstream open reading frame (uORF) was found (14).

In *G. fujikuroi*, the GA-biosynthetic pathway is under control of nitrogen metabolite repression. Nitrogen starvation results in a many-fold increase in transcript level for three of the four P450s (*P450-1*, *P450-2* and *P450-4*) and induction of *cpr-Gf*. In order to demonstrate, that CPR-Gf acts as a general electron donor for many P450s, we analyzed the expression of *cpr-Gf* with and without benzoate. As in *A. niger*, the *cpr-Gf* gene was significantly induced by benzoate indicating the existence of a CPR-Gf-dependent benzoate p-hydroxylase in *G. fujikuroi*. This was supported by the finding that Δ*cpr* mutants are significantly less resistant to benzoate than the wild-type and the complemented mutant. Interestingly, the 5' non-coding region of *cpr-Gf* contains two n-alkane-inducible sequence elements, CACAT, suggesting a role for CPR also in n-alkane hydroxylation (see 64). In contrast to the *cprA* of *A. niger*, benzoate responsive regions (BRR) were not found in the *G. fujikuroi* gene. However, these sequence elements might be located at position –1400 to –1600 bp upstream of ATG (14).

NADPH-dependent oxidoreductase is the common electron donor to multiple P450 monooxygenases in all eukaryotic organisms. In most fungi and all animals tested so far, only a single *cpr* gene was identified, indicating involvement of single CPR in a multitude of P450-related reactions. In contrast to *F. verticilloides* (*G. fujikuroi* MP-A), where a catalytically self-sufficient P450 gene, *Fum6*, was found, the GA gene cluster in *G. fujikuroi* MP-C consists of four P450 monooxygenase genes not fused to *cpr* genes. Our results indicate that *cpr-Gf*, which is not located in the GA gene cluster, encodes the main electron donor responsible not only for activation of all four GA-related P450 monooxygenases, but also for benzoate p-hydroxylase and at least some P450s involved in primary metabolism. Cloning and characterization of the gene encoding the *G. fujikuroi* cytochrome b5 might provide insights into the possible role of this enzyme as an alternative electron donator and its participation in the functional complex of GA P450s.
Acknowledgements—Strain SG138 was kindly provided by J. Avalos (University of Sevilla, Spain). We gratefully acknowledge Catherine Wasmann (University of Arizona) for sharing the sequence of the CPR-specific degenerated primers. We also thank P. Punt (TNO Nutrition and Food Research Institute, Zeist, the Netherlands) for providing us the cprA gene and for critical reading of the manuscript. We thank B. Berns for typing the manuscript, and J. Schulte for technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft (DFG, Tu101/9-1) and Fondo Nacional de Desarrollo Cientifico y Tecnologico (Grant 1020140). Rothamstead Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

Table I: Concentration of GAs in culture filtrates of wild-type, cpr mutants and cpr-complemented strains.

Results are expressed in µg/ml ± S.E., n=3, except where indicated otherwise.

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<th>Strain</th>
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<td>13.4±0.3</td>
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^a<0.004 µg/ml.
^b^n=2
^c^n=6
^d^n=5
Legends to the Figures

Fig. 1
A Physical map and major subclones of gene cpr-Gf. The functional domains of the CPR-Gf enzyme and the position of PCR primers are marked.
(abbreviations: 1 - CPR-RT1; 2 - CPR-RT2; 3 - Dcpr-1; 4 – Dcpr-2; 5 – Dcpr-3; 6 – Dcpr-4)
(abbreviations: 7 – CPR-DF1; 8 – PUCH-P; 9 – CPR-DF2; 10 – PUCH-T)

Fig. 2 Phylogenetic tree of cytochrome P450 reductases from fungi, plants and animals, based on amino acid sequences.
(accession numbers: *Aspergillus niger* CprA S38427; *Arabidopsis thaliana* ATR1 S21530; *Arabidopsis thaliana* ATR2 S21531; *Bombyx mori* BAA95684; *Cunninghamella echinulata* AAF89959; *Candida maltosa* N_CPR P50126; *Cavia porcellus* P37039; *Coriolus versicolor* CPR BAB83588; *Candida tropicalis* N_CPR P37201; *Drosophila melanogaster* Cpr-P1 NP_477158; *Homo sapiens* P16435; *Musca domestica* Q07994; *Mus musculus* NP_032924 ; *Phanerochaete chrysosporium* CPR AAG31351; *Phanerochaete chrysosporium* CPR2 AAG31350; *Rattus norvegicus* AAA41683; *Saccharomyces cerevisiae* NCPRNP_011908; *Schizosaccharomyces pombe* CPR CAA2429; *Schizosaccharomyces pombe* CPR2 T40056;

Fig. 3 Analysis of gene replacement strains .
A Southern blot analysis of the wild-type strain IMI58289, UV-mutant SG138 and three transformants. Genomic DNA was digested with HindIII and hybridized with the right flanking sequence of the replacement vector pΔcpr-Gf (see Fig.1)
B Northern blot analysis. *G. fujikuroi* rDNA was used as control.
C Thin layer chromatography (TLC) of the wild-type IMI58289 and mutant strains after 7 days of cultivation in 20% ICI medium
Fig. 4 Growth of the cpr-deficient mutants is compromised. The wild-type IMI58289, mutants SG138 and T20 and the complemented strain KT-1 were grown on CM- and CD agar with and without benzoate.

A SG138
B IMI58289
C T20
D KT-1

Fig. 5 Northern blot analysis of the wild-type IMI58289 and two transformants KT-13 and KT-1 after transformation with the complementation vector pcpr-Gf.

Fig. 6 GC-MS analysis of culture filtrates of the wild-type (IMI58289), cpr-disruption mutant (∆CPR-T20) and line KT-1, in which T20 has been complemented with the G. fujikuroi CPR gene. Total ion currents are shown for extracts as methyl esters trimethylsilyl ethers. Components were identified by comparison of their mass spectra with published data (65) as follows: peak 1, ent-kaurene; peak 2, ent-kaurenoic acid; peak 3, GA9; peak 4, GA25; peak 5, GA24; peak 6, GA14 and 7β-hydroxykaurenolide; peak 7, GA4; peak 8, GA7; peak 9, fujenoic acid, peak 10 GA13; peak 11, GA36; peak 12, GA3 isolactone; peak 13, 7β, 18-dihydroxykaurenolide; peak 14, GA1; peak 15, GA3; peak 16, ent-kaurenol; peak 17, GA15. Unlabeled peaks are due to compounds unrelated to GA biosynthesis. The peak at the same retention time as ent-kaurene in the KT-1 extract contains no ent-kaurene.

Fig. 7 GA-biosynthetic pathways in G. fujikuroi indicating reactions affected in the cpr-Gf mutant.

Products detected in the culture are underlined, absent reactions are marked by a cross, undetected final products are in brackets, and proposed sequence of reactions are shown with pointed arrows. Participation of CPR in reactions after the block at GA12-aldehyde was not demonstrated experimentally.

Fig. 8

A Northern blot analysis of the wild-type IMI58289 and the mutant SG138 showing regulation of P450 and cpr gene expression by nitrogen. Strains were grown for three days in 10% ICI medium, washed and transferred to 0% ICI (no nitrogen) or 100% ICI (high amounts of nitrogen) medium for 5 or 10 hours.

B Northern blot analysis of transformant SG138-K1, carrying the A. niger cprA gene.
Fig. 9  Northern blot analysis of the wild-type IMI58289 showing benzoate induction of cpr gene expression. The strain was cultivated for three days in 10% ICI and then transferred to 0% or 100% ICI medium with 0.5mM, 1mM or without benzoate.
Footnotes

The abbreviations used are: GA, gibberellin; GC-MS, combined gas chromatography-mass spectrometry; HPLC, High performance liquid chromatography; ICI, Imperial Chemical Industries Ltd., UK; MP, mating population; CPR, NADPH:cytochrome P450 oxidoreductase; Δcpr, disruption mutant
Malonek et al. Fig. 1
Malonek et al. Fig. 2

The diagram illustrates a phylogenetic tree comparing various species, including:

- H. sapiens
- R. norvegicus
- C. porcellus
- B. mori
- M. domestica
- A. thaliana ATR2
- A. thaliana ATR1
- C. echinulata CPR
- S. pombe NCPR
- S. pombe NCPR2
- A. niger CPRA
- G. fujikuroi CPRGf
- C. tropicalis NCPR
- C. maltosa NCPR
- S. cerevisiae
- P. chrysosporium CPR
- P. chrysosporium CPR2
- C. versicolor CPR
- D. melanogaster Cpr-P1
- M. musculus

The tree branches out to show the evolutionary relationships and distances between these species.
Malonek et al. Fig. 6
Malonek et al. Fig. 8

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- cprGf
- P450-4
- P450-2
- P450-1
- rDNA

B

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Malonek et al. Fig. 9

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- benzoate
- cprGf
- rDNA
The NADPH: Cytochrome P450 reductase gene from Gibberella fujikuroi is essential for gibberellin biosynthesis

Stefan Malonek, Maria C. Rojas, Peter Hedden, Paul Gaskin, Paul Hopkins and Bettina Tudzynski

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