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The NADPH-cytochrome P450 Reductase Gene from *Gibberella fujikuroi* Is Essential for Gibberellin Biosynthesis*

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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.

The Ascomycetes *Gibberella fujikuroi* mating population (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 that 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*-copalyldiphosphate/*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), whereas others participate in the biosynthesis of intracellular compounds such as steroids (cited in Ref. 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 physiologi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ576025.

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[1] The abbreviations used are: MP, mating population; GA, gibberellin; GC-MS, combined gas chromatography-mass spectrometry; ICI, Imperial Chemical Industries Ltd.; CPR, NADPH-cytochrome P450 oxidoreductase; Δ*cpr,* disruption mutant; cyt b_5, cytochrome b_5; kb, kilobase(s); CM, culture medium; CD, Czapek Dox medium.

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cal 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 *Aspergillus 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 *C. maltosa* and *Candida 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*, the 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). Because 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* 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) 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 BglII fragment of λ-clone 2–1 was cloned into BamHI-restricted pUCI9 (pCPR1A). Then a 3.8-kb XbaI fragment was cloned into pUCI9/XbaI (pCPR1B). pCPR1B was cut with NcoI/SphI. The derived 3-kb fragment was cloned into pCPR1A/NcoI/SphI to produce vector p*cpr-Gf*, containing the entire *cpr-Gf* gene. The vector pNR1 was constructed by cloning the PstI/BamHI fragment of the *Streptomyces noursei nat1* gene encoding the nourseothricin acetyltransferase (38) into pBluescript II KS. The gene was transcribed under the control of the *Aspergillus nidulans oliC* promoter (39) and terminated by the *Botrytis cinerea tub1* terminator.[2] 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 optimized for *Fusarium* spp. (41) for 3 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% $MgSO_4$, 0.1% KH_2PO_4, and 5.0, 1.0, or 0 g/liter NH_4NO_3, respectively.

For analysis of *cpr-Gf* expression with and without benzoate, strain IMI58289 was cultivated for 3 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 or 1 mM) 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 containing 6% sunflower oil, 0.05% $(NH_4)_2SO_4$, 1.5% corn-steep solids (Sigma-Aldrich), and 0.1% KH_2PO_4. Benzoate plate tests were performed on CM and Czapek Dox (CD)[2] (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). λ DNA from positive λ 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) 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 of DNA, 10 ng of each primer, 0.2 mM dNTPs and 2 units of *Taq* polymerase (Red Taq, Sigma-Aldrich) 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 (Stratagene Europe, Amsterdam, The Netherlands) of *G. fujikuroi* m567 at 65 °C. Sequences are: 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 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′, and 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′, and 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 GAA GTA CAA GGT ACC GTG CAA AT-3′ (underlines indicate the KpnI site); Dcpr-2 (4), 5′-TCA ACG AGA TGT CGA CGT TTT TGT CC-3′ (underlines indicate the SalI site); Dcpr-3 (5), 5′-TCC AAC TTC AAG CTT CCC TCG GAC-3′ (underlines indicate the HindIII site); Dcpr-4 (6), 5′-GAT AAC CAA AGA GCT CGT GGA CAG GT-3′ (underlines indicate the SacI site).

Screening the λ-DASH II Library—About 35,000 recombinant phages from a λ library prepared from genomic DNA of wild-type *G. fujikuroi* m567 (46) were plated and transferred to Nylon N^+ membranes (Amersham Biosciences). Hybridization was performed at high stringency (65 °C). The blots were washed in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 65 °C followed by a wash with 0.1× 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 λ DNA was transferred onto Hybond N^+ filters (Amersham Biosciences). ^{32}P-Labeled probes were prepared using the random oligomer-primer method (44). Filters were hybridized at 65 or 56 °C in 5× Denhardt's solution containing 5% dextran sulfate (44). Filters were washed at the same temperature used for hybridization in 2× saline/sodium phosphate/EDTA (SSPE), 0.1% SDS and 1× 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.

[2] J. van Kan, personal communication.

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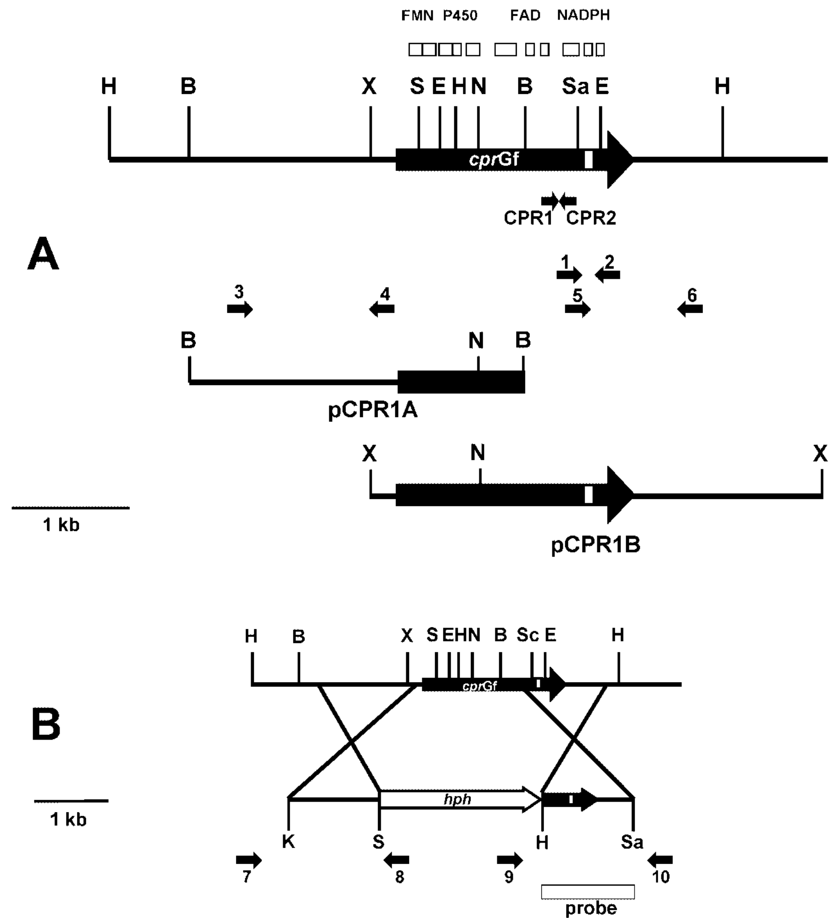

FIG. 1. *A*, physical map and major subclones of gene *cpr-Gf*. The functional domains of the CPR-Gf enzyme and the position of PCR primers are marked. *1*, CPR-RT1; *2*, CPR-RT2; *3*, Dcpr-1; *4*, Dcpr-2; *5*, Dcpr-3; *6*, Dcpr-4. *B*, strategy for construction of the gene replacement vector pΔ*cpr-Gf*. *7*, CPR-DF1; *8*, PUCH-P; *9*, CPR-DF2; *10*, PUCH-T. *H*, HindIII; *B*, BamHI; *X*, XbaI; *S*, SalI; *E*, EcoRI; *N*, NcoI; *Sa*, SacII.

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). 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).

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 p*cpr-Gf* carrying the *G. fujikuroi cpr* gene or p*cprA* 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 p*cpr-Gf* and pNR1. Transformed protoplasts were regenerated at 28 °C on complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$) containing 120 μg/ml hygromycin B (Calbiochem) 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 30-m × 0.32-mm × 0.25-μm HP-5 WCOT column (Agilent Technologies) and analyzed using a MAT95XP mass spectrometer (Thermo Electron Corp.) 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-$^2H_2$]GA internal standards and analyzing using a GCQ instrument (Thermo Electron Corp.) 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 days 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. 1*A*). The fragment exhibited a high degree of homology with CPRs from other fungi and served as a probe for screening the λ-DASH II genomic library of *G. fujikuroi,* MP-C. Of the three genomic λ clones isolated, one was used for isolating the putative full-length gene (Fig. 1*A*). About a 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. 1*A*). Comparison of the genomic and cDNA sequences confirmed the expected intron of 52 bp. The *cpr-Gf* gene has been 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. 1*A*). A phylogenetic tree comparing CPR-Gf with CPRs from other species indicates that it is most closely related

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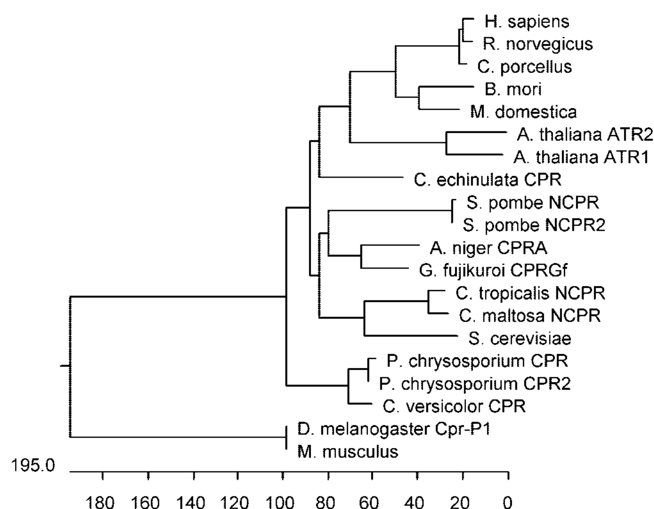

FIG. 2. **Phylogenetic tree of cytochrome P450 reductases from fungi, plants, and animals, based on amino acid sequences.** Accession numbers: *A. niger* CprA S38427; *Arabidopsis thaliana* ATR1 S21530; *A. thaliana* ATR2 S21531; *Bombyx mori* BAA95684; *Cunninghamella echinulata* AAF89959; *C. maltosa*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

to CPRA of *A.niger* (61% identity) followed by the yeast and the other fungal CPRs (Fig. 2).

To determine the importance of the *cpr-Gf* gene for GA production, a gene replacement vector was constructed as shown in Fig. 1*B*. 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. 1*B*). 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. 1*B*). 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. 1*B*). 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. 3*A*) 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. 1*B*) and sequencing of the fragment. Transformant T57 integrated the fragment ectopically and still contained 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 contained a transcript of about 2.1 kb, whereas T20 and T49 lost the entire coding region of *cpr* and did not show any *cpr* transcript (Fig. 3*B*).

GA Production and Growth Characteristics of Deletion Mutants and Strain SG138—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. 3*C*) and GC-MS (Table I). Transformants T20 and T49 as well as mutant SG138 do not produce GA_4, GA_7, and GA_3, 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. 4*C*) than on CM (Fig. 4*A*). 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 amino acid position 627 from C to T resulting in a TGA stop codon instead of CGA for arginine. The truncated CPR peptide is, therefore, 83 amino acids 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. 1*A*). 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 p*cpr-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 p*cpr-Gf*. Analysis of the GA concentrations showed almost full restoration of GA production. Analysis of the three final products, GA_4, GA_7, and GA_3, by GC-MS (Table I) demonstrated that the activity of all four P450 monooxygenases 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 (GA_3). Furthermore, the growth rate of KT-1 on CM and CD agar was comparable with that of the wild-type (Fig. 4, *A* and *C*).

Analysis of GA Intermediates in the cpr Mutants—The effect of the *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 Δ*cpr* mutants (Fig. 6) and quantifying selected intermediates by GC-MS (Table I). The *cpr* deletion mutants do not produce GA_3, GA_4, or GA_7, 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 Δ*cpr* 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 Δ*cpr* 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 Δ*cpr*, consistent with our demonstration of a mutation in the P450 reductase in this strain. The requirement of the 13-

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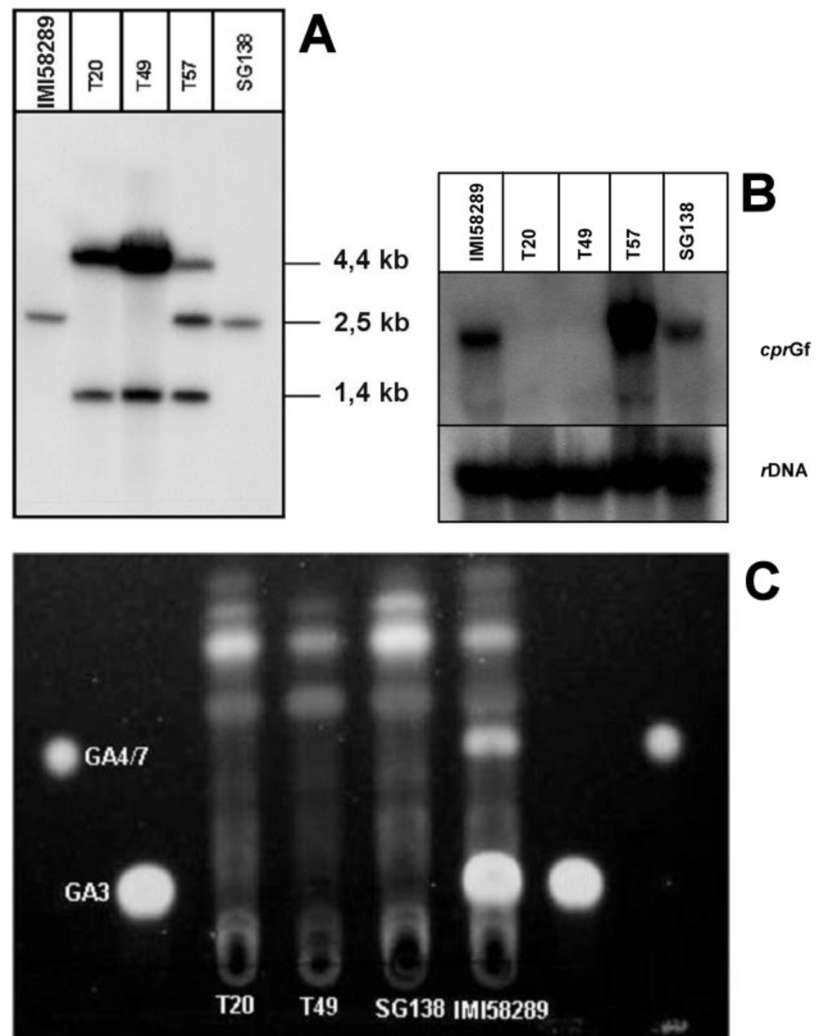

FIG. 3. **Analysis of gene replacement strains.** *A*, Southern blot analysis of the wild-type strain IMI58289, UV mutant SG138, and three transformants. Genomic DNA was digested with HindIII and hybridized with the right flanking sequence of the replacement vector pΔ*cpr-Gf* (see Fig. 1). *B*, Northern blot analysis. *G. fujikuroi* rDNA was used as the control. *C*, thin layer chromatography (TLC) of the wild-type IMI58289 and mutant strains after 7 days of cultivation in 20% ICI medium.

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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.

Strain	GA_3	GA_1	GA_7	GA_4	GA_9	GA_{15}	GA_{24}	GA_{25}
IMI58289 (wild-type)	13.4 ± 0.3	5.75 ± 0.25	1.34 ± 0.09	3.23 ± 0.11	1.26 ± 0.05	0.35 ± 0.02	0.80 ± 0.07	0.50 ± 0.03
Δ*cpr-T20*	0[a]	0	0.004 ± 0.001	0.017 ± 0.004	0	0.64 ± 0.02	0.17 ± 0.01	0
Δ*cpr-cprGf* (KT-1)	19.5 ± 0.5	15.1 ± 0.5	1.57 ± 0.07	5.95 ± 0.13	2.21 ± 0.14	0.44 ± 0.03	1.15 ± 0.03	1.32 ± 0.17
SG138	0	0	0.012 ± 0.0005[b]	0.014 ± 0.002	0	0.37 ± 0.02	0.14 ± 0.004	0
SG138-*cprGf*	11.5 ± 0.4	1.50 ± 0.02	1.01 ± 0.06	1.15 ± 0.08	0.54 ± 0.03	0.06 ± 0.007[b]	0.20 ± 0.02[b]	0.39 ± 0.01
SG138-*cprA*-7	29.5 ± 0.2	6.29 ± 0.13[c]	3.10 ± 0.05[d]	0.56 ± 0.05[c]	0.42 ± 0.03[c]	0.15 ± 0.04[b]	0.35 ± 0.01	1.03 ± 0.06[c]

[a] <0.004 μg/ml.
[b] n = 2.
[c] n = 6.
[d] n = 5.

hydroxylation reaction, catalyzed by P450-3, for the reductase was tested in the *cpr* mutants by incubating with [^{14}C]GA_4 and analyzing the products by high performance liquid chromatography and GC-MS. Formation of [^{14}C]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 [^{14}C]GA_3 formation from [^{14}C]GA_4 to 56%.

The GA profile and amounts in Δ*cpr* 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 *A. niger* (*cpr-A*), 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 attempted to determine 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-regu-

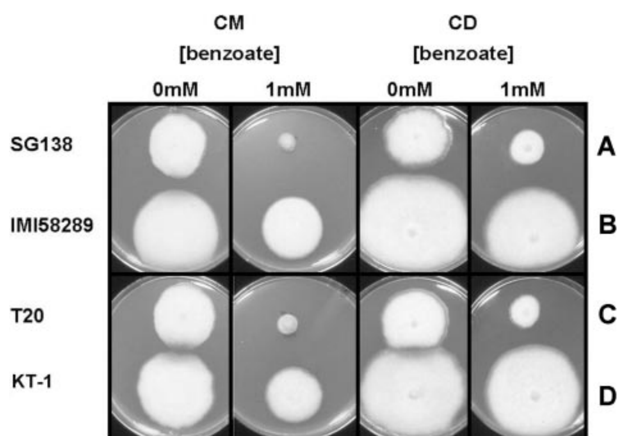

FIG. 4. **Growth of the *cpr*-deficient mutants is compromised.** The wild-type IMI58289, mutants SG138 and T20, and the complemented strain KT-1 were grown on CM and CD agar with and without benzoate. *A*, SG138. *B*, IMI58289. *C*, T20. *D*, KT-1

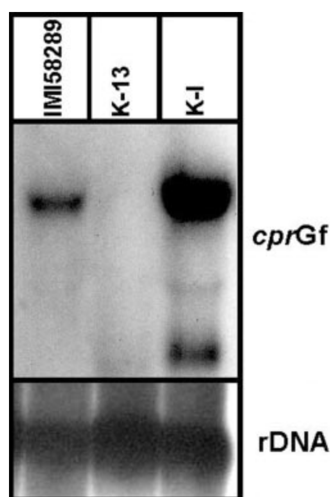

FIG. 5. **Northern blot analysis of the wild-type IMI58289 and two transformants, KT-13 and KT-1, after transformation with the complementation vector p*cpr-Gf*.**

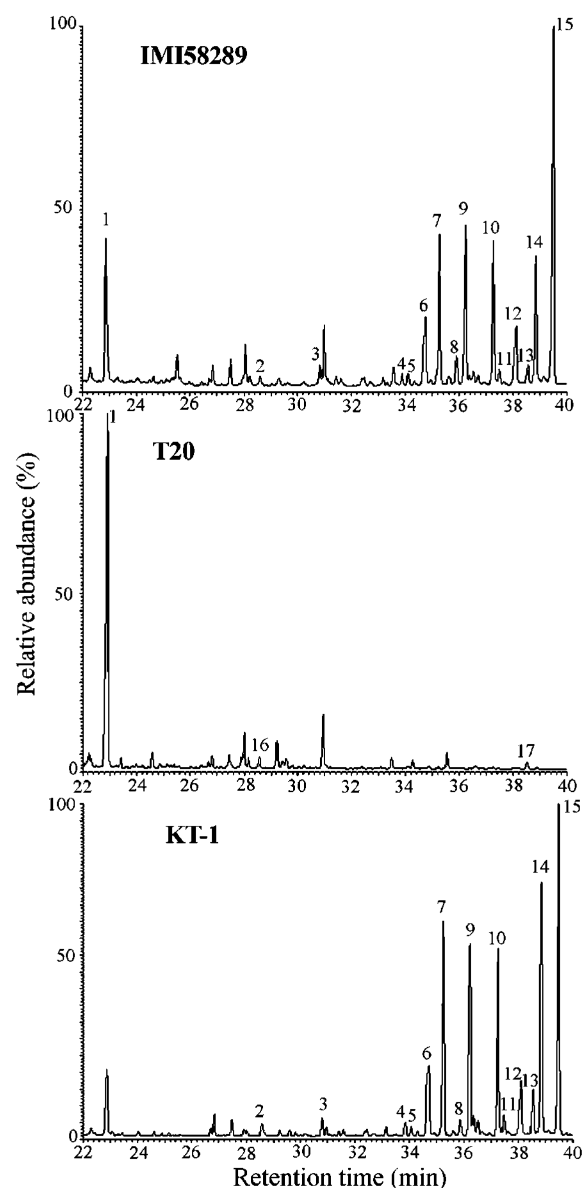

FIG. 6. **GC-MS analysis of culture filtrates of the wild-type (IMI58289), *cpr* disruption mutant (ΔCPR-T20), and line KT-1, in which T20 has been complemented with the *G. fujikuroi CPR* gene.** Total ion currents are shown for extracts as methyl esters trimethylsilyl ethers. Components were identified by comparison of their mass spectra with published data (49) as follows: *peak 1*, *ent*-kaurene; *peak 2*, *ent*-kaurenoic acid; *peak 3*, GA_9; *peak 4*, GA_{25}; *peak 5*, GA_{24}; *peak 6*, GA_{14} and 7β-hydroxykaurenolide; *peak 7*, GA_4; *peak 8*, GA_7; *peak 9*, fujenoic acid, *peak 10* GA_{13}; *peak 11*, GA_{36}; *peak 12*; GA_3 isolactone; *peak 13*, 7β, 18-dihydroxykaurenolide; *peak 14*, GA_1; *peak 15*, GA_3; *peak 16*, *ent*-kaurenol; *peak 17*, GA_{15}. 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.

lated 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. 8*A*). 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 GA_3 (*e.g.* SG138-*cprA*-7, Table I), demonstrating that CPRA from the GA-non-producing fungal species *A. niger*, which is described as an activator of the benzoate *p*-hydroxylase, is able to act as electron donor and activator of GA biosynthetic enzymes *P450-1-P450-4* in *G. fujikuroi*. However, in contrast to *cpr-Gf*, *cprA* was expressed independently of the nitrogen condition in *G. fujikuroi* (Fig. 8*B*).

On the basis of these results we speculated that CPR-Gf may also act in much more than the GA biosynthetic pathways. 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. 4, *B* and *D*).

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

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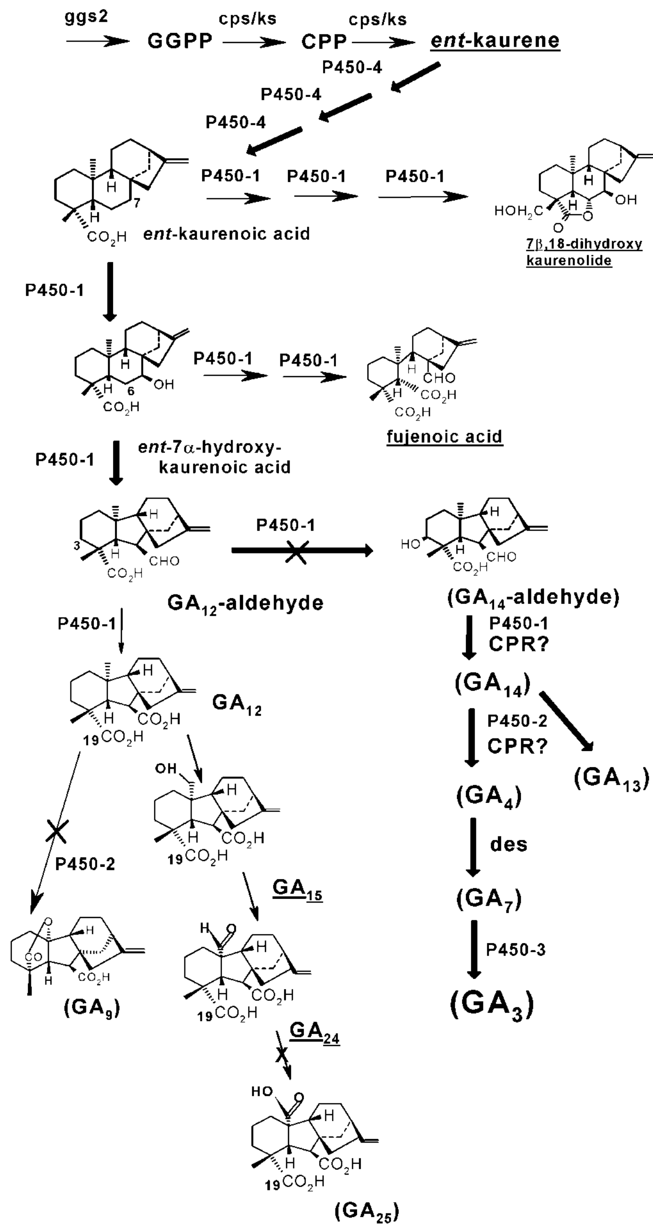

FIG. 7. **GA biosynthetic pathways in *G. fujikuroi* indicating reactions affected in the *cpr-Gf* mutant.** Products detected in the culture are *underlined*, absent reactions are marked by an *X*, undetected final products are in *parentheses*, and the proposed sequence of reactions are shown with *pointed arrows*. Participation of CPR in reactions after the block at GA_{12}-aldehyde was not demonstrated experimentally.

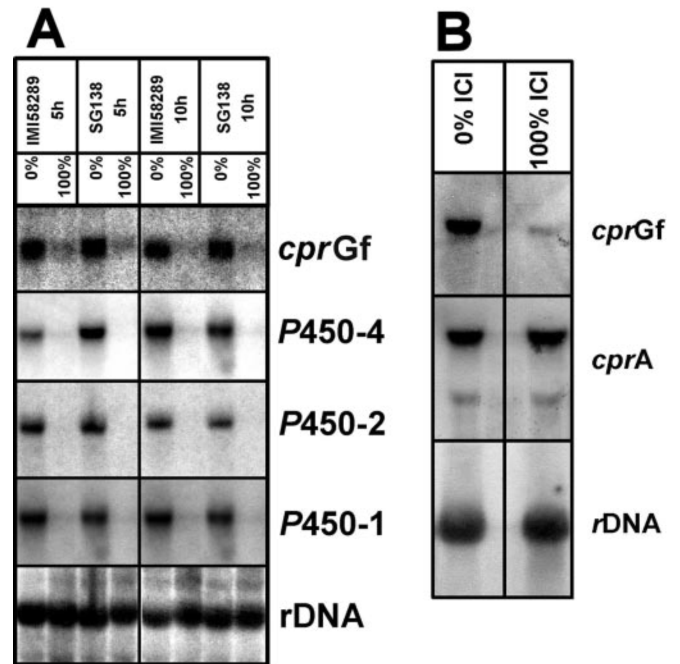

FIG. 8. *A*,**Northern blot analysis of the wild-type IMI58289 and the mutant SG138 showing regulation of P450 and *cpr* gene expression by nitrogen.** Strains were grown for 3 days in 10% ICI medium, washed, and transferred to 0% ICI (no nitrogen) or 100% ICI (high amounts of nitrogen) medium for 5 or 10 h. *B*, Northern blot analysis of transformant SG138-K1, carrying the *A. niger cprA* gene.

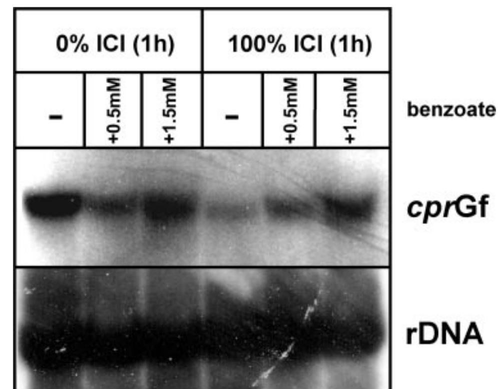

FIG. 9. **Northern blot analysis of the wild-type IMI58289 showing benzoate induction of *cpr* gene expression.** The strain was cultivated for 3 days in 10% ICI and then transferred to 0 or 100% ICI medium with 0.5 or 1 mM benzoate or without benzoate.

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 the 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 GA_{12}-aldehyde, its 3β-hydroxylation to GA_{14}-aldehyde, and oxidation to GA_{14}, in addition to several side reactions (6). P450-2 converts GA_{14} to GA_4 and GA_{12} to GA_9 with the loss of carbon-20 (7). Finally, P450-3 is responsible for the 13-hydroxylation of GA_7 to GA_3, and, in a minor pathway, of GA_4 to GA_1 (8). Although not all P450-catalyzed steps are completely blocked in the deletion mutants, they are reduced in activity and some reactions, such as 3β-hydroxylation and loss of C-20, are completely absent in Δ*cpr* mutants. Thus, CPR-Gf may act as electron donor of all four P450 monooxygenases.

The small amounts of the non-hydroxylated GA_{15} and GA_{24}, and the high level of *ent*-kaurene found in Δ*cpr* 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

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because its substrate is not produced, was assayed by incubating with [^{14}C]GA_4 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 Δ*cpr* lines and may, thus, possess low CPR activity. The GA levels in SG138 are similar to Δ*cpr*, but *ent*-kaurene does not accumulate, and traces of GA_4, GA_7, GA_{15}, and GA_{24} are present. SG138 contains a point mutation in the *cpr* gene that gives a truncated protein that lacks part of the NADPH binding domain. This mutation in SG138 would, thus, reduce considerably but may not abolish CPR activity. Complementation of Δ*cpr* with the *cpr-Gf* gene fully restored GA synthesis, giving 3β-hydroxylated C_{19}-GAs (GA_3, GA_4, GA_1, and GA_7) 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. Although 3β-hydroxylase depends absolutely on CPR and is completely blocked in Δ*cpr*, 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 C_{19}-GAs by cleavage of C-20 in the absence of the CPR. Instead, the C_{20} tricarboxylic acid product GA_{24} was found in Δ*cpr* together with GAs with intermediate oxidation states (alcohol and aldehyde) at C-20 that do not accumulate in the wild type. Production of the C_{19}-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 C_{20}-GAs detected (7).

Besides the dramatic effect on GA production, mutations in *cpr* in the deletion mutants and SG138 also affected the growth rate on synthetic medium and to a lesser extent on CM, 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, 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 nonspecifically as general electron donors for P450 monooxygenases from different pathways. The recently completed genome of the Basidiomycetes, *P. 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* (www-genome.wi.mit.edu/annotation/fungi/neurospora) and *Fusarium graminearum* (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%.[3] 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 b_5 (cyt b_5) could act as a second important element in the electron donating system. Deletion of cyt b_5 in the wild type did not display a phenotype, whereas disruption of the gene in a Δ*cpr* strain was lethal, demonstrating that both enzymes can complement each other in mutants with single disruptions of *cpr* or b_5 (54). However, there is no additional electron-donating system overcoming the double knock out.

The exact mechanism by which cyt b_5 interacts with P450 reductase is not yet clear. Numerous studies show that P450 activity can be enhanced by the addition of cyt b_5 in some, but not all reactions (55–57). Human, but not yeast cyt b_5 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 b_5 (55).

In petunia, the product of a cyt b_5 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 b_5 gene resulted in a flower color 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 b_5 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 b_5 as electron donors was found in the fumonisin gene cluster of *G. fujikuroi* MP-A (*Fusarium verticilloides*); the open reading frame 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 *Fusarium oxysporum* fatty acid ω-hydroxylase (58), the *Bacillus megaterium* fatty acid hydroxylase P450 $_{BM\text{-}3}$ (59), and the *Bacillus 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 the 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

[3] S. Malonek and B. Tudzynski, unpublished results.

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fungi, are the metabolism of xenobiotic drugs and toxins and the assimilation of long chain alkanes as well as the metabolism of endogenous compounds, such as sterols and fatty acids (cited in Ref. 16). For *Fusarium 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 to 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 defense-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 was found (14).

In *G. fujikuroi* the GA biosynthetic pathway is under control of nitrogen metabolite repression. Nitrogen starvation results in a manyfold increase in transcript level for three of the four P450s (*P450-1*, *P450-2*, and *P450-4*) and induction of *cpr-Gf*. 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 Ref. 64). In contrast to the *cprA* of *A. niger*, benzoate-responsive regions 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 b_5 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.

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