

Restoration of Gibberellin Production in *Fusarium proliferatum* by Functional Complementation of Enzymatic Blocks

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Nine biological species, or mating populations (MPs), denoted by letters A to I, and at least 29 anamorphic *Fusarium* species have been identified within the *Gibberella fujikuroi* species complex. Members of this species complex are the only species of the genus *Fusarium* that contain the gibberellin (GA) biosynthetic gene cluster or at least parts of it. However, the ability of fusaria to produce GAs is so far restricted to *Fusarium fujikuroi*, although at least six other MPs contain all the genes of the GA biosynthetic gene cluster. Members of *Fusarium proliferatum*, the closest related species, have lost the ability to produce GAs as a result of the accumulation of several mutations in the coding and 5' noncoding regions of genes *P450-4* and *P450-1*, both encoding cytochrome P450 monooxygenases, resulting in metabolic blocks at the early stages of GA biosynthesis. In this study, we have determined additional enzymatic blocks at the first specific steps in the GA biosynthesis pathway of *F. proliferatum*: the synthesis of geranylgeranyl diphosphate and the synthesis of *ent*-kaurene. Complementation of these enzymatic blocks by transferring the corresponding genes from GA-producing *F. fujikuroi* to *F. proliferatum* resulted in the restoration of GA production. We discuss the reasons for *Fusarium* species outside the *G. fujikuroi* species complex having no GA biosynthetic genes, whereas species distantly related to *Fusarium*, e.g., *Sphaceloma* spp. and *Phaeosphaeria* spp., produce GAs.

Gibberella fujikuroi is a species complex of at least nine different biological *Fusarium* species, frequently named mating populations (MPs) (20, 70), and at least 29 closely related *Fusarium* species mostly corresponding to *Fusarium* section *Liseola* (38). These *Fusarium* species have been isolated from different host plants (24) and are the causative agents of numerous serious plant diseases. Furthermore, these species have been demonstrated to produce a broad variety of secondary metabolites, such as gibberellins (GAs) (58), the pigment bikaverin (21, 29), and the mycotoxins moniliformin (11, 26), fumonisin (9, 26, 42), fusaric acid (2), fusarin C (52), and beauvericin (11, 30), that in some cases may affect human and animal health. Among these mycotoxins, fumonisins are the most prominent toxins, primarily produced by members of the maize pathogens *Fusarium verticillioides* (MP-A) and *Fusarium proliferatum* (MP-D) but also by some *Fusarium fujikuroi* (MP-C) strains (9, 25, 42). In contrast, production of gibberellic acid (GA₃) is so far restricted to *F. fujikuroi* (33), which is responsible for the well-known bakanae disease of rice, which causes the characteristic overgrowth symptom. *F. fujikuroi* is used commercially for the large-scale production of GAs, mainly GA₃ and its precursors, GA₄ and GA₇, which have applications in agriculture and horticulture as plant growth regulators (43).

We have previously characterized the seven GA biosynthesis genes and the encoded enzymes in *F. fujikuroi* (46, 58, 62–64). Besides four cytochrome P450 monooxygenase genes (*P450-1* to *P450-4*), which in most cases encode multifunctional enzymes catalyzing several biosynthetic steps, the GA gene cluster contains genes encoding a pathway-specific geranylgeranyl diphosphate (GGDP) synthase (*ggs2*), the *ent*-copalyl diphosphate/*ent*-kaurene synthase (*cps/ks*), and GA₄-desaturase (*des*), responsible for early and late steps of the GA biosynthesis pathway (Fig. 1). GGDP is a common precursor for carotenoids, ubiquinones, and GAs (Fig. 1). In *F. fujikuroi*, two GGDP synthase-encoding genes, *ggs1* (36) and *ggs2* (58), the latter located in the GA gene cluster, have been found. Interestingly, GGDP for GA biosynthesis is specifically produced by GGS2, as disruption of the corresponding gene, *ggs2*, resulted in the total loss of GA production, while *ggs1*, which is responsible for general terpenoid biosynthesis, was unable to complement the block at GGS2 (B. Tudzynski, unpublished data).

A single-copy cytochrome P450 reductase gene, *cpr-Gf*, is required as an electron donor for the activity of the GA-specific as well as all the other NADPH-dependent cytochrome P450 monooxygenases of *F. fujikuroi*. Deletion of the *cpr-Gf* gene resulted in substantial loss of GA production (32). Previous expression studies revealed that all GA cluster genes except for *P450-3* and *cpr-Gf* are coregulated; they are repressed by high amounts of nitrogen and up-regulated under nitrogen-limiting conditions. Nitrogen regulation in *Gibberella* is mediated by AREA-Gf, a general transcription factor, which is involved in both derepression of several nitrogen utilization genes and GA biosynthesis genes under nitrogen starvation (37, 60, 61).

Recently, many extended phylogenetic studies of *Fusarium*

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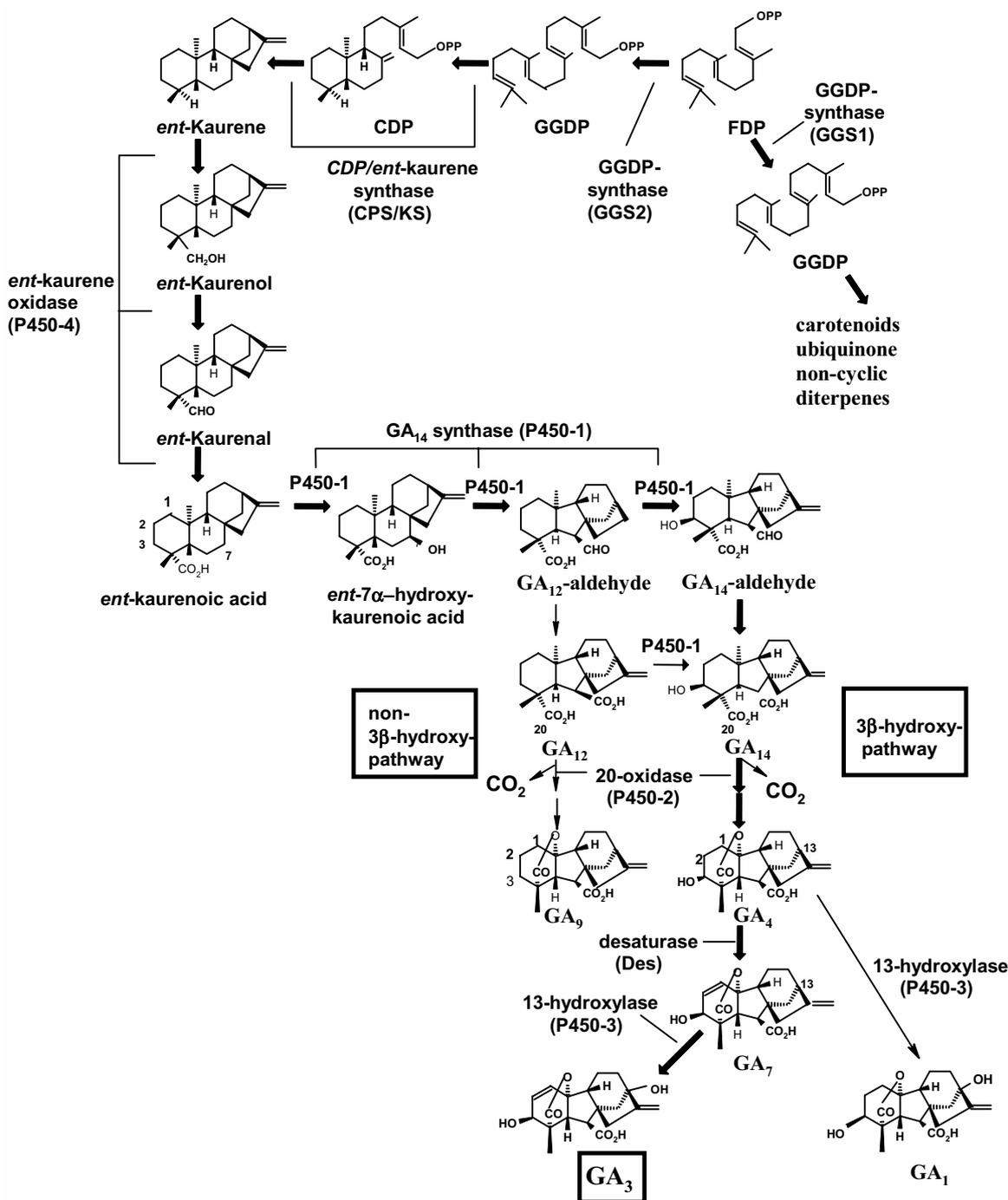


FIG. 1. Gibberellin biosynthesis pathway indicating the genes, enzymes, and final products. The major pathway is denoted by enlarged arrows and letters.

species based on the sequences of several protein-encoding nuclear genes clearly demonstrated that the species belonging to the *G. fujikuroi* species complex are closely related and have a monophyletic origin (39, 40, 42). Our own recent data on the distribution of GA biosynthetic genes and GA production inside and outside the *G. fujikuroi* species complex clearly demonstrated that only the nine sexually fertile species and the anamorphic *Fusarium* species inside the species complex con-

tain the entire GA biosynthetic gene cluster, or at least parts of it (33). Surprisingly, none of the other species of the genus *Fusarium*, such as *F. graminearum* (<http://www.broad.mit.edu/annotation/fungi/fusarium/>), *F. oxysporum*, *F. sporotrichoides*, *F. avenaceum*, or *F. culmorum*, contain any of these genes (33). All phylogenetic studies to date, including our own based on the sequences of *P450-4* from the GA gene cluster and the *cpr* gene, revealed that *F. fujikuroi* is most closely related to *F.*

proliferatum (33, 39, 40, 42). In addition, some isolates of these two biological species can still interbreed and produce viable progeny, suggesting that the genetic isolation between them is not complete (27). Despite the close relatedness between these two species, strains of *F. proliferatum* do not produce GAs, although they contain all seven GA biosynthetic genes (33, 34). Moreover, the GA biosynthetic genes are organized in the same order and orientation in the gene cluster and have a high degree of overall sequence identity (about 90%) with the corresponding *F. fujikuroi* genes (34). Therefore, we started a detailed molecular analysis of *F. proliferatum* to identify the reasons for the loss of GA production associated with the divergent evolution of these two species. The recent functional analysis of two cytochrome P450 monooxygenases, P450-1 (D) and P450-4 (D) (where D indicates derivation from *F. proliferatum* strains), in *F. proliferatum* revealed a block in *ent*-kaurene oxidase activity (P450-4 [D]), whereas GA₁₄ synthase (P450-1 [D]) was active in both strains, although the *F. proliferatum* enzyme is much less active than that in *F. fujikuroi* (34). Incubations with radiolabeled precursors, such as *ent*-kaurenoic acid and GA₁₂-aldehyde, revealed genetic and functional diversity between different *F. proliferatum* strains in some of the GA biosynthetic enzymes, as one strain (D00502) was able to produce GA₁ and GA₄ as final products, whereas the other strain (D02945) failed to produce any C₁₉ GAs. β-Glucuronidase reporter assays showed that mutations in the GATA-binding elements in the bidirectional promoter of *P450-1* and *P450-4*, which are recognized by AREA-Gf, resulted in low expression levels of both genes in comparison with the corresponding genes in *F. fujikuroi* (34).

Here, we describe the identification of additional enzymatic blocks in the GA biosynthesis pathway in *F. proliferatum* causing the loss of the initial steps that result in the formation of GGDP and *ent*-kaurene. Several mutations in the *F. proliferatum* *ggs2* and *cps/ks* genes resulted in nonfunctional proteins, as these genes were unable to complement *F. fujikuroi* *ggs2* and *cps/ks* disruption mutants. Moreover, we could show that the desaturase (DES) that catalyzes the step from GA₄ to GA₇ is functional in *F. proliferatum* strain D02945, in contrast to the homologous enzyme in strain D00502, indicating genetic and functional diversity of genes and enzymes within this species. The results indicate that the inability of strains of *F. proliferatum* to produce GAs results from several mutations in the coding and noncoding regions of three genes (*ggs2* [D], *cps/ks* [D], and *P450-4* [D]) that encode the enzymes that catalyze the first steps of the GA-specific pathway rather than from mutations in the regulation network. We demonstrate that complementation of *F. proliferatum* strains with the three homologous genes from *F. fujikuroi* (*ggs2* [C], *cps/ks* [C], and *P450-4* [C], where C indicates derivation from *F. fujikuroi* strains) restored the ability to produce GAs.

MATERIALS AND METHODS

Fungal strains and nomenclature. In association with their classification into the different mating populations, genes and encoded proteins from different *Fusarium* strains are indicated with the following affixes: C if they were derived from *F. fujikuroi* strains and D if they were derived from *F. proliferatum* strains (see also reference 34). Strains IMI58289 (CABI Biosciences, Kew, United Kingdom) and m567 (Fungal Culture Collection, Weimar, Germany) are GA-producing wild-type strains of *F. fujikuroi* (belonging to *G. fujikuroi* mating population C[MP-C]). *F. proliferatum* strains D02945 and D00502 were kindly

provided by J. F. Leslie (Kansas State University). Strain Δ*des* (C) is a *des* deletion mutant of strain IMI58289, which lacks the production of GA₃ and GA₇ (64). Strain Δ*ggs2* (C) (B. Tudzynski, unpublished) and strain Δ*cps/ks* (C) T22 (59) are disruption mutants that produce no GAs or precursors.

Bacterial strains and plasmids. *Escherichia coli* strain TOP10 (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Vector pUCBM20 (Boehringer, Mannheim, Germany) was used to clone DNA fragments carrying the *F. fujikuroi* cluster genes and gene fragments from *F. fujikuroi* and *F. proliferatum*. Plasmid pP450-4-GK (62, 63) was used as the complementation vector. To obtain plasmid pGGS2D, carrying the entire *ggs2* (D) gene, a PCR approach with primers *ggs2*-D-F1 and *ggs2*-D-R1, which were based on the *ggs2* (C) sequence, was performed. The 2.3-kb PCR fragment was then cloned into vector pCR2.1-TOPO (Invitrogen). Plasmid pCKSD, containing the entire *cps/ks* (D) gene copy, was obtained by PCR with primers *cps*-D-F1 and P450-3-Fus8. The 5.2-kb fragment was cloned into vector pCR-Blunt II-TOPO (Invitrogen). For complementation of the Δ*des* mutant (MP-C), vector p*des*GKD was used. This plasmid was produced by cloning a 3-kb *S*alI fragment of λ-clone DIII-1 into pUCBM20/*S*alI. In cotransformation experiments, either pAN7.1 (hygromycin resistance) or pNR1 (nourseothricin resistance) (34) was used for selection of transformants. The vector pNR1 was constructed by cloning the *Pst*I/*Bam*HI fragment of the *Streptomyces noursei* *nat1* gene encoding the nourseothricin acetyltransferase (22) into pBluescript II KS. The gene was transcribed under the control of the *Aspergillus nidulans* *oliC* promoter (67) and terminated by the *Botrytis cinerea* *tub1* terminator (J. A. L. van Kan, personal communication). Cosmid pGKScos1, containing both genes *ggs2* and *cps/ks* of MP-C strain m567, was constructed by P. Linnemannstons (unpublished data).

Media and culture conditions. For DNA isolation, fungal strains were grown in 100 ml of liquid CM medium optimized for *Fusarium* spp. (41) for 3 days at 28°C on a rotary shaker 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. *F. proliferatum* strains were cultivated on V8 juice agar for sporulation. For RNA isolation, fungal strains were grown in 100%, 20%, or 0% ICI medium (13) containing 8% glucose, 0.5% MgSO₄, 0.1% KH₂PO₄, and 5.0, 1.0, or 0 g/liter NH₄NO₃, respectively.

For analysis of gene expression and nitrogen regulation, strains were cultivated for 3 days in 10 or 20% ICI medium on a rotary shaker at 28°C. To elucidate nitrogen regulation, mycelium was washed, and 1.5 g (wet weight) each was transferred to 50 ml of 0% or 100% ICI medium. For GA production, the strains were grown for 7 to 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% (NH₄)₂SO₄, 1.5% corn-steep solids (Sigma-Aldrich, Taufkirchen, Germany), and 0.1% KH₂PO₄.

DNA and RNA isolation. Genomic DNA was isolated from lyophilized mycelium as described previously by Doyle and Doyle (10). Lambda DNA from positive lambda clones was prepared according to a method described previously by Sambrook et al. (48). Plasmid DNA was extracted using Genomed (Bad Oeynhausen, Germany) columns according to the manufacturer's protocol. RNA was isolated using the RNeasy total RNA isolation kit (Promega, Mannheim, Germany).

PCR. The reaction mixtures contained 25 ng DNA, 10 ng of each primer, 0.2 mM deoxynucleoside triphosphates, 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 35 cycles of 94°C for 1 min, 55 to 65°C for 1 to 3 min, and 72°C for 1 min. Annealing and elongation times were applied differently, depending on the annealing temperatures of each primer and the amplified fragment. For amplification of *cps/ks* (D), a special high-fidelity proofreading DNA polymerase was used (Phusion; Finnzymes, Finland).

For amplification of *ggs2* (D) and analysis of complementation of Δ*ggs2* (C), primers *ggs2*-D-F1 (5'-CTAGAAAGCGAGGTAATCAATAAAGT-3') and *ggs2*-D-R1 (5'-GGAAACAAGACCTAGCATAAAAAGAAT-3') were used.

For amplification of *cps/ks* (D) and analysis of the complementation of Δ*cps/ks* (C), primers *cps*-D-F1 (5'-ATGATAATGTTGTGATGTTCCGTT-3') and P450-3-Fus-8 (5'-CCGAACGGACGCTGGGTAAAAA-3') were used.

For analysis of full-length integration of vector pP450-4-GK after retransformation into strains D02945 and D00502, transformants were analyzed using primers REV (5'-GAGCGGATAACAATTTACACAGG-3') and P450-4-GD2 (5'-CGTGGTCTTCCTTTCCCATCTGGC-3').

For analysis of complementation of Δ*des* (C), primers ORF3-1 (5'-GCCAGTGCGCAAGAGTGTCACTGC-3') and ORF3-2 (5'-TCTCACTTCCTCTTCTCAGTTC-3') were used.

Screening the lambda DASH II library. About 35,000 recombinant phages from a lambda DASH II library (Stratagene) prepared from genomic DNA of *G. fujikuroi* strain D02945 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× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (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 analyses. After digestion with restriction endonucleases and electrophoresis, genomic or lambda DNA was transferred onto Hybond N⁺ filters (Amersham Pharmacia, Freiburg, Germany). ³²P-labeled probes were prepared using the random oligomer-primer method (48). Filters were hybridized at 65°C or 56°C in 5× Denhardt's solution containing 5% dextran sulfate (48). Filters were washed at the same temperature used for hybridization in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])–0.1% SDS and 1× SSPE–0.1% SDS.

Northern blot hybridizations were accomplished according to the method described previously by Church and Gilbert (8). The *G. fujikuroi* rRNA gene was used as a control hybridization probe to confirm RNA transfer. For quantification of expression levels, the method of densitometry was performed by using TotalLAB 1 D software, version 1.00 (Amersham, Pharmacia). Hybridizing bands were each normalized against the corresponding band of the wild-type (MP-C) gene copy.

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 (München, Germany). DNA and protein sequence alignments were made with DNA Star (Madison, WI).

Transformation of *Fusarium* strains. The preparation of protoplasts and the transformation procedure were performed as previously described (32, 34). For complementation experiments, 10⁷ protoplasts (50 µl) of strain D00502, D02945, Δ des-MP-C, Δ ggs2 (C), or Δ cps/ks (C) were transformed with up to 10 µg of the circular complementation vectors pP450-4-GK and pGKScos1. Plasmids pdes-GKD, pP450-4GK, pGGS2D, and pCKSD were cotransformed with pNR1 (32) carrying the nourseothricin resistance marker.

Transformed protoplasts were regenerated at 28°C on complete regeneration agar [0.7 M sucrose, 0.05% yeast extract, 0.1% (NH₄)₂SO₄] containing 120 µg/ml hygromycin B (Calbiochem, Bad Soden, Germany) or 80 µg/ml nourseothricin (Werner BioAgents, Jena, Germany) for 6 to 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 analysis. GA₃, GA₄, and GA₇ in the culture fluids of wild-type strain IMI58289, MD-D02945, and different transformants were analyzed by high-performance liquid chromatography (HPLC) according to a method described previously by Barendse et al. (3) using a Merck HPLC system with a UV detector and a Lichrospher 100 RP18 column (5 µm, 250 by 4 mm). These GAs were also analyzed by thin-layer chromatography (TLC) 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 gas chromatography-mass spectrometry (GC-MS) analysis after extraction from the culture fluid as previously described (64).

Nucleotide sequence accession numbers. The gene sequences for *des* (D), *ggs2* (D), and *cps/ks* (D) of *F. proliferatum* have been deposited in the GenBank database under accession numbers AJ628021, AJ 810803, and AJ810802, respectively.

RESULTS

Biochemical analysis of the initial steps of GA biosynthesis in strains of *F. proliferatum*. Previously, we reported that *F. proliferatum* strains D02945 and D00502 do not produce any biologically active C₁₉ GAs, including GA₃, GA₄, GA₇, and GA₁ (33, 34). Moreover, we could demonstrate an enzymatic block at *F. proliferatum* *ent*-kaurene oxidase (P450-4 [D]), whereas *F. proliferatum* GA₁₄ synthase (P450-1 [D]) shows low but significant enzymatic activity when the corresponding precursors were incubated with these strains (34).

In order to investigate whether there are also enzymatic

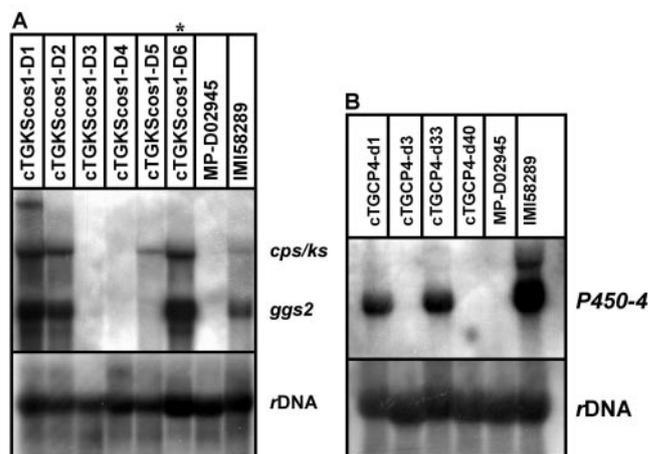


FIG. 2. Restoration of GA production in *F. proliferatum* strain D02945. (A) Northern blot analysis after transformation of strain D02945 with pGKScos1. (B) Northern blot analysis after transformation of strain cTGKScos-D6 with pP450-4-GK. Strains were grown for 3 days in 20% ICI medium. *, used for further transformation experiments. rRNA (rDNA) was used as a loading control.

blocks at the earliest steps of the GA biosynthesis pathway, the synthesis of GGDP by GGS2 and the two-step cyclization of GGDP to *ent*-kaurene via *ent*-copalyl diphosphate (Fig. 1), GC-MS analyses of culture filtrates and mycelial extracts were carried out. No GAs or the kaurenoic precursors *ent*-kaurene and *ent*-kaurenoic acid were detected in the culture fluids or the respective mycelial extracts of strains D00502 and D02945 (data not shown). These results indicate a putative block in the GA biosynthetic pathway in *F. proliferatum* at the stage of geranylgeranyl diphosphate synthase (GGS2 [D]) and/or *ent*-copalyl diphosphate/*ent*-kaurene synthase (CPS/KS [D]).

GA biosynthesis in *F. proliferatum* is blocked at the initial steps of the pathway. To confirm the proposed block in GGS2 and/or CPS/KS activities in *F. proliferatum*, we transformed the cosmid pGKScos1, carrying *ggs2* (C) and *cps/ks* (C) genes of *F. fujikuroi* (strain m567), into *F. proliferatum* (D02945). Four positive transformants were confirmed by Southern blot analysis and shown to contain one to three copies of the entire genomic fragment (data not shown). Northern blot analysis clearly demonstrated high transcript levels for both genes, comparable to those in *F. fujikuroi* wild-type strain IMI58289 (Fig. 2A). Analysis of transformants cTGKScos1-D2 and -D6 revealed high levels of *ent*-kaurene in the mycelia, whereas the recipient strain, D02945, as well as strain D00502 did not accumulate *ent*-kaurene, as expected (data not shown). Therefore, we were able to restore the initial enzymatic steps of GA biosynthesis in *F. proliferatum*.

In order to identify the mutations in *ggs2* (D) and/or *cps/ks* (D) that cause the enzymatic block, we cloned the *ggs2* gene of strain D02945 (GenBank accession number AJ810803) and analyzed its expression and functionality in the *F. fujikuroi* background. Sequence alignment of the promoter regions of *ggs2* from *F. fujikuroi* and *F. proliferatum* indicated 91% sequence identity. The *ggs2* promoters from both species contained 10 GATA sequence elements, which are putative binding sites for the nitrogen regulator AreA-Gf (37). Sequence analysis of the coding regions revealed 94% and 92% identity

A

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1                               50
MP-C GGS2 MAEQQISNLL SMFDASHASQ KLEITVQVMD TYHYRETPPD SSSSEGGSL
MP-D GGS2 MAEQQISNLL SMFDASHASQ KLEITVQVMD TFHYRDTPTL LFLFGRCSLS
          *           *           *           *           *           *
          *           *           *           *           *           *
51                               100
MP-C GGS2 RYDERRVSLP LSHNAASPDV VSQLCFSTAM SSELNHRWKS QRLKVDSPY
MP-D GGS2 RYDERRVSLP LSHNAASPDV VSQLCFLTAM SSELNHRWKS QRLKVDSPY
          *
          *
101          domain I          domain II 150
MP-C GGS2 NYILTLPSKG IRGAFIDSLN VWLEVPEDET SVIKEVIGML HNSSLIIDDF
MP-D GGS2 NYILTLPSKG IRGAFIDSLN VWLEVPEDKT SVIKEVIGML HNSSLIIDDF
          *
          *
151          domain I          domain II 200
MP-C GGS2 QDNSPLRRGK PSTHTVFGPA QAINATYVI VKAIEKIQDI VGHDALADVT
MP-D GGS2 QDNSPLRRGK PSTHTVFGPA QAINATYVI VKAIEKIQDI VSHDALADVT
          *
          *
201          domain III          domain IV 250
MP-C GGS2 GTITTFIQGQ AMDLWWTANA IVPSIQEYLL MVNDKTGALF RLSLELLALN
MP-D GGS2 GMTSIFQGQ AMDLWWTANG IVPSIQEYLL MVNDKTGALF RLSLELLALN
          *           *
          *           *
251          domain V          300
MP-C GGS2 SEASISDSAL ESLSSAVSLL GQYFQIRDDY MNLIDNKYTD QKGFCELDDE
MP-D GGS2 SEASISDSAL ESLSSAVSLL GQYFQIRDDY MNLIDNKYTD QKGFCELDDE
          *           *
          *           *
301                               343
MP-C GGS2 GKYSLTLIHA LQTDSSDLLT NILSMRRVQG KLTAQKRCWF WK.
MP-D GGS2 GKYSLTLIHA LQTDSSDLLT NILSMRRVQG KLTAQKMLI LEV.....
          *           *           *           *           *           *
          *           *           *           *           *           *

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B

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1                               70
CPS/KS MP-C MPGKIENGTP KDLKTGNDFV SAAKSLLDRA FKHSHSYGGL CSTSCQVYDT AWMAMPKTR DNVKQWLFPE
CPS/KS MP-D MPGKIENGSP KDLKTGNDLV SAAKSLLDRA FKHSHSYGGL CSTSCQVYDT AWMAMPKTT DNVKQWLFPE
          *           *           *           *           *           *
          *           *           *           *           *           *
71                               140
CPS/KS MP-C CFHYLLKTQA ADGSWGS LPT TQTAGILDTA SAVLALLCHA QEPLQILDVS PDEMGLRIEH GVTSLKRQLA
CPS/KS MP-D CFHYLLKTQA ADGSWGS LPT TQTAGILDTA SALLALLCHV QEPLQILDVS PDEMGFRIEH GVASLKRQLA
          *           *           *           *           *           *
          *           *           *           *           *           *
141          domain I          domain II 210
CPS/KS MP-C VVNDVDDTNH IGVEFIIPAL LSMLEKELDV PSFEFPCRSI LERMHGKGLG HFDLEQVYGK PSSLLHSLEA
CPS/KS MP-D VVNDVDDTNH IGVELIIPAL LSMLEKELDV PSFEFPCRDV LERMHEKGLG HFDLEQVYGK PSSLLHSLEA
          *           *           *           *           *           *
          *           *           *           *           *           *
211          domain I          domain II 280
CPS/KS MP-C FLGKLDLDFRL SHHLYHGSM SPSSSTAAYL IGATKWDEEA EDYLRHVMRN GAGHGNGGIS GTFPTTHFEC
CPS/KS MP-D FLGKLDLDFRL SRHLYRGSMM SPSSSTAAYL IGATKWDEEA EDYLRHVMRN GAGHGNGGIS GTFPTTHFEC
          *           *           *           *           *           *
          *           *           *           *           *           *
281          domain I          domain II 350
CPS/KS MP-C SWIATLLKQ GFTLQKIDG GLRGLSTILL EALRDENGVI GFAPRTADV DTAKALLALS LVNQPVSPDI
CPS/KS MP-D SWIATLLKQ GFTLQKIEG GLRGLSTILV DALRDDNGVI GFAPRTADV DTAKALLALS LINQPVSPDI
          *           *           *           *           *           *
          *           *           *           *           *           *
351          domain I          domain II 420
CPS/KS MP-C MIKGFEGKDH FTFGSRDP SLTSLNLHVLV SLPQKQSNLS QYHPQILKTT LFTCRWWWSG DHCVKDKWNL
CPS/KS MP-D MVKVFEGKDH FTFGSRDP SLTSLNLHVLV CLL-KQPKVS QYYPQILKTA LFIQWWSG DHYVKDKWNL
          *           *           *           *           *           *
          *           *           *           *           *           *
421          domain I          domain II 490
CPS/KS MP-C SHLYPTMLLV EAFTEVLHLI DGGELSSLFD ESFKCKIGLS IFQAVLRIL TQDNDGWSRG YREQTCYAIL
CPS/KS MP-D SHLYPTMLLV EAFTEVLHLI DAGELSSLFD KSLKCKIGLS VFQAVLRIL TQDNDGWSRG YREQTCYAIL
          *           *           *           *           *           *
          *           *           *           *           *           *
491          domain I          domain II 560
CPS/KS MP-C ALVQARHVCF FTHMVDRLQS CVDRGVSWLK SCSFHSQDLT WTSKTAYEVG FVAEAYKLA LQSASLEVPA
CPS/KS MP-D ALAQARHVCF FTHMVDKLQG CIDRGVSWLK SCRLHSQDLT WTSKTAYEVG FVAEAYKLA LQSASLEVPA
          *           *           *           *           *           *
          *           *           *           *           *           *
561          domain I          domain II 630
CPS/KS MP-C ATIGHSVTSA VPSSDLEKYM RLVKRTALFS PLDEWGLMAS IIESSFFVPL LQAQRVEIYP RDNKIVDEDK
CPS/KS MP-D ATIGHSVTSA VPSSDLEKYM RLVKRTALFS PLDEWELRAS VIESSFFMPL LQAQRVEIYP RDNKIVDEDK
          *           *           *           *           *           *
          *           *           *           *           *           *
631          domain I          domain II 700
CPS/KS MP-C YLSIIPFTWV GCNNSRSTFA SNRWLYDMY LSLLYQTDE YMEAVAGPVF GDVSLHQT I DKVIDNTMGN
CPS/KS MP-D YLSIIPFTWV GCNNSRSTFA SNRWLYDMY LSLLYQTDE YMEAVAGPAF SDISLLRLAI DKIIDNTRVN
          *           *           *           *           *           *
          *           *           *           *           *           *
701          domain I          domain II 770
CPS/KS MP-C LARANGTVHS GNGHQHESP IGVQVEDTLTR FTNSVLN HKD VLNSSSDQD TLRREFRFTM HAHITQIEDN
CPS/KS MP-D SSGTNGTVRN GNGHQYEPSP IRQVEDTLTR FANSVLN HKD VLRSSSDQD TLRQEFRAEM HAHATQIEDN
          *           *           *           *           *           *
          *           *           *           *           *           *
771          domain I          domain II 840
CPS/KS MP-C SRFSKQASSD AFSSPEQSYF QWVNSTGGSH VACAYSFAFS NCLMSANLLQ GKDAFPSTGT KYLISSVMRH
CPS/KS MP-D SRFSKQASGD VFSSPEQSYF QWVNSTGGSH VACAYSFAFS NCLLSANLHQ GKDAFPSTGT KYLISSVMRH
          *           *           *           *           *           *
          *           *           *           *           *           *
841          domain I          domain II 910
CPS/KS MP-C ATNMCRCMYND FGSIARDNAE RNVNSIHFFE FTLCNGTSQN LDERKERLLK IATYEQGYLD RALEALERQS
CPS/KS MP-D ATNMCRCMYND FGSIARDNAE RNLNSIHFFR VAV-QGTSQG LDERKERLSQ IATYEQVCLD SALGALERQS
          *           *           *           *           *           *
          *           *           *           *           *           *
911          domain I          domain II 953
CPS/KS MP-C RDDAGDRAGS KDMRKLKIVK LFCDVTDLYD QLYVIKDLSS SMK
CPS/KS MP-D RDDAGDCAGS MEVRKLKIVR LFCDVTDLYD QLYVIKDLSS SMK
          *           *           *           *           *           *
          *           *           *           *           *           *

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at the nucleotide and amino acid levels, respectively. The positions of the introns and of five characteristic domains, representing the catalytic center (5), were found to be highly conserved in both species (Fig. 3A).

Mutations resulting in amino acid substitutions were identified particularly in the N-terminal region of the protein in front of domain I and between the catalytic domains (Fig. 3A). Interestingly, insertion of an additional cytosine at the 3' coding region of the *F. proliferatum ggs2* (D) led to a translational termination different from that in the *F. fujikuroi ggs2* gene (Fig. 3A). In addition, only a faint band was detected for *F. proliferatum ggs2* in Northern blot analysis, indicating low levels of expression (Fig. 2A).

In order to investigate the functionality of *ggs2* (D), we transformed vector pGGS2D into the *F. fujikuroi* disruption mutant $\Delta ggs2$ (C), which fails to produce GAs. Transformants were screened for correct integration of the entire *ggs2* (D) gene by PCR with primers *ggs2*-D-F1 and *ggs2*-D-R1 and by Southern blot analysis (data not shown). Ten transformants, all carrying two or three entire gene copies, were chosen for Northern blot analysis. Interestingly, only a faint, hardly detectable transcript was found for *ggs2* (D) in the transformants, and a smear of hybridizing RNA fragments appeared, starting from the postulated size of *ggs2* (data not shown). Analysis of transformants by TLC and HPLC revealed no GA production.

Similarly, *F. proliferatum cps/ks* (D) was cloned by PCR. The nucleotide sequence of the *cps/ks* (D) gene can be accessed as GenBank accession number AJ810802. Sequencing of three independent clones indicated 89% identity with the *F. fujikuroi cps/ks* (C), at both the nucleotide and the amino acid levels. Typical aspartate-rich domains (see references 1, 68, and 69) are conserved between CPS/KS from *F. fujikuroi* and that from *F. proliferatum* (Fig. 3B). Amino acid substitutions were distributed over the entire protein. As *cps/ks* (D) expression was not detectable in Northern blot analyses of strain D02945 (Fig. 2A), functionality of the corresponding CPS/KS (D) enzyme was investigated by transformation of the corresponding gene into *F. fujikuroi* $\Delta cps/ks$ deletion strain T22 (58). Putative transformants were checked for full-length integration by PCR and Southern blot analysis. *cps/ks* (D) expression levels in 11 transformants were determined by Northern blot analysis. In contrast to the *F. proliferatum ggs2* gene, significant expression was detected for some high-copy transformants, but in each case, the level was only about 50% of that of *cps/ks* in *F. fujikuroi*. Analysis of extracts of culture filtrates by TLC and HPLC revealed that there was no GA production in these transformants.

In summary, neither *ggs2* (D) nor *cps/ks* (D) was able to restore GA production in the corresponding *F. fujikuroi* $\Delta ggs2$ and $\Delta cps/ks$ deletion strains. Therefore, GGS2 (D) and CPS/KS (D) were not functional. Additionally, RNA decay in the case of *ggs2* (D) and the generally low expression levels of both genes in the *F. proliferatum* background could also contribute to the failure to produce GAs.

Restoration of GA production in *F. proliferatum* by complementation of two strains with *ggs2* (C), *cps/ks* (C), and *P450-4* (C). In order to restore GA production in both *F. proliferatum* strains, we first transformed plasmid pP450-4-GK, carrying the entire *F. fujikuroi P450-4* gene copy, into D02945 transformant cTGKScos-D6, which was shown to accumulate *ent*-kaurene after complementation with the *F. fujikuroi ggs2* (C) and *cps/ks* (C) genes (see above and Fig. 2A). Twenty positive transformants were confirmed by PCR and Southern blot analysis and shown to carry one to three entire *P450-4* (C) gene copies. However, Northern blot analysis revealed a significant expression level of *P450-4* (C) in only 10 transformants (shown for cTGCP4-d1 and -33 in Fig. 2B), whereas most transformants did not show any expression by Northern analysis, despite carrying multiple gene copies. Analysis of several independent transformants by TLC, HPLC, and GC-MS showed that GA production capacity was restored in these lines. Interestingly, GA amounts corresponded closely with the gene expression levels, although total GA amounts in the transformants were significantly less than those in *F. fujikuroi* wild-type strain m567 (Table 1 and Fig. 4A and C). For example, as shown for transformant KT24 (D02945), significant amounts of GA₁ and GA₃ and several other unidentified compounds were detected in the culture filtrate. In addition, high levels of the intermediate *ent*-kaurenoic acid were found in the mycelial extract from KT24, whereas the wild-type strains of *F. fujikuroi* and *F. proliferatum* contain only traces of this compound (Fig. 4C).

Strain D00502 was shown to metabolize *ent*-[¹⁴C]kaurenoic acid to [¹⁴C]GA₁, demonstrating that enzymes P450-1, P450-2, and P450-3 are active in this strain but indicating a block at DES (see reference 34 and Fig. 1). Therefore, in a second experiment, we transformed pGKScos1 and pP450-4-GK into this strain in one step, producing 12 transformants, all carrying one to three entire copies of *ggs2* (C), *cps/ks* (C), and *P450-4* (C) (data not shown). Northern blot analysis revealed that *ggs2* (C) and *cps/ks* (C) were expressed in all the transformants but at different levels. Three transformants, D11, D13, and D34, also showed a transcript for *P450-4* (C) by Northern blot analysis (Fig. 5).

Analysis of these transformants by GC-MS revealed significant levels of GA₁ in the culture fluids, whereas recipient strain D00502 accumulated only traces of this compound (Table 1 and Fig. 4B). Moreover, no GA₃ was found in any of the D00502 transformants (shown for transformant KT11 [D00502] in Fig. 4B and D), in contrast to the D02945 transformants discussed above.

Thus, complementation of blocks in the first three enzymes of the pathway restored GA production to both *F. proliferatum* strains, although strain D00502 appears to lack DES activity since no GA₃ was found in any transformant from this strain.

Functional analysis of *des* (D), encoding the GA₄ desaturase. The *des* gene encodes the GA₄ desaturase, which catalyzes the conversion of GA₄ to GA₇. As mentioned above, strain D00502 accumulates [¹⁴C]GA₁ and [¹⁴C]GA₄ rather

FIG. 3. Comparison of GGS2 and CPS/KS from *F. fujikuroi* and *F. proliferatum* (D02945). (A) Alignment of GGS2 (C) and GGS2 (D). Conserved amino acid residues of the five catalytic domains (5) are marked in boldface type and are overlined. Amino acid substitutions are indicated by asterisks. (B) Alignment of CPS/KS (C) and CPS/KS (D). Conserved amino acid residues of the aspartate-rich domains (54, 68, 69) are indicated in boldface type and are overlined. Amino acid substitutions are indicated by asterisks.

TABLE 1. Qualitative analysis of GAs and related metabolites in *F. fujikuroi* wild-type strain m567, *F. proliferatum* strains D02945 and D00502, and transformants of both *F. proliferatum* strains after complementation of the blocks in the first three enzymes of the pathway^a

Strain	Products	
	Culture filtrate	Mycelial extract
m567 (MP-C)	GA ₃ ; GA ₁₃ ; fujenoic acid; GA ₃ isolactone; 7β,18-dihydroxykaurenolide; GA ₁₆ ; gibberellenic acid; GA ₇ ; GA ₂₅ ; GA ₁ ; GA ₉ ; GA ₄	<i>ent</i> -kaurenoic acid; fujenoic acid; GA ₂₅ ; GA ₉ ; <i>ent</i> -kaurene; <i>ent</i> -kaurenal; <i>ent</i> -kaurenol
D02945 (MP-D)		<i>ent</i> -kaurenoic acid (trace)
KT24 (D02945)	GA ₃ ; GA ₁ ; GA ₄ ; GA ₃ isolactone; fujenoic acid; <i>ent</i> -kaurenoic acid; GA ₁₃	<i>ent</i> -kaurenoic acid; <i>ent</i> -kaurenal; <i>ent</i> -kaurenol; <i>ent</i> -kaurene
D00502 (MP-D)	GA ₁ (trace)	<i>ent</i> -kaurenoic acid (trace)
KT11 (D00502)	GA ₁ ; GA ₁₃ ; fujenoic acid	<i>ent</i> -kaurenoic acid; <i>ent</i> -kaurene; <i>ent</i> -kaurenal (trace)

^a See the text for details. Strains were cultivated for 10 days in 20% ICI. Substances are listed in decreasing order of amounts. Experiments were repeated with similar results.

than [¹⁴C]GA₃ when incubated with precursors such as [¹⁴C]GA₁₂-aldehyde, suggesting a defect in the GA₄ DES (34). For strain D02945, incubations with *ent*-[¹⁴C]kaurenoic acid or [¹⁴C]GA₁₂-aldehyde did not result in the production of any C₁₉ GAs. We obtained the corresponding *des* (D) gene from *F. proliferatum* after screening the lambda DASH II genomic library of strain D02945 with a cDNA clone of *F. fujikuroi des* (C) as a probe. Sequence analysis of *des* from D02945 (GenBank accession number AJ628021) shows 92% and 96% identity at the nucleotide and amino acid levels, respectively, with *des* of *F. fujikuroi*. As for *P450-1* (D) and *P450-4* (D) (34), *des* expression levels were very low in *F. proliferatum*. In order to analyze the functionality of the encoded GA₄ desaturase in detail, we transformed the entire *des* (D) gene into a *F. fujikuroi* Δ *des* deletion strain of IMI58289, which is unable to produce GA₇ and GA₃ and, like strain D00502, accumulates GA₁ and GA₄ instead (64; Fig. 1). The gene was integrated with different copy numbers into the genome of strain Δ *des* (C) and was highly expressed in some of the transformants (Fig. 6). To investigate the functionality of DES (D), the concentrations of C₁₉ GAs in culture filtrates, determined by HPLC, were compared in strains Δ *des* (C) and three of the transformants (dKT Δ *des*-C1, -C2, and -C10) (Table 2). As expected, *F. fujikuroi* mutant strain Δ *des* (C) accumulated GA₄, which is partially metabolized to GA₁ by the 13-hydroxylase *P450-3* (64) (data not shown; Table 2). In contrast, transformants dKT Δ *des*-C1 and -C2 showed the wild-type GA pattern: the main component was GA₃ rather than GA₁ and GA₄. Thus, *des* (D) fully restored the ability to produce GA₃ via GA₇, overcoming the block between GA₄ and GA₇. Therefore, *des* (D) of strain D02945 encodes a functional protein, in contrast to the corresponding gene of D00502 (see above).

DISCUSSION

Recent results demonstrated the highest phylogenetic relatedness between *F. proliferatum* and *F. fujikuroi* within the *G. fujikuroi* species complex (27, 33, 40, 42). Moreover, *F. proliferatum* contains all of the GA biosynthesis genes, arranged in the same pattern as found in *F. fujikuroi*, but does not produce any GAs (33, 34). Functional analysis of two of the cytochrome P450 monooxygenase genes, *P450-4* (D) and *P450-1* (D), and the encoded enzymes in two *F. proliferatum* strains revealed a block at *ent*-kaurene oxidase, *P450-4* (D), which catalyzes the

conversion of *ent*-kaurene to *ent*-kaurenoic acid (34) (Fig. 1). Further biochemical analysis of the initial steps of GA biosynthesis in both strains D00502 and D02945 clearly indicated additional blocks at *ggs2* (D) and *cps/ks* (D), encoding GGS2 and CPS/KS, respectively, as no *ent*-kaurene accumulated in these strains. Complementation of strain D02945 with a cosmid carrying both *ggs2* (C) and *cps/ks* (C) from *F. fujikuroi* resulted in *ent*-kaurene accumulation, confirming the conclusion that there are additional blocks in one or both of these genes.

Prenyltransferases such as GGS2 are key enzymes in many essential biosynthetic pathways in which they are necessary for the formation of chlorophyll (49) and ubiquinones (55) in plants as well as carotenoids in plants (16) and fungi (28). In *F. fujikuroi*, two GGDP synthase-encoding genes, *ggs1* (36) and *ggs2* (58), have been characterized. One of them, *ggs2*, is located in the GA biosynthetic gene cluster and is specifically involved in GA production, whereas no pathway-specific *ggs* gene has been found so far in the *F. fujikuroi* carotenoid gene cluster (J. Avalos, personal communication).

In the present study, we demonstrated that the GA-specific enzyme GGS2 is not functional in *F. proliferatum* since complementation of an *F. fujikuroi ggs2* (C) mutant with the *F. proliferatum ggs2* (D) gene did not restore GA production. There are several reasons for this. Despite the fact that the amino acid sequence of GGS2 (D) is unchanged in the five highly conserved prenyltransferase domains that are important for catalytic activity (1, 5, 6, 35, 51), several mutations between the catalytic domains were found. Furthermore, a substitution of nine consecutive amino acid residues (Fig. 3) was found in the N terminus, which may affect protein folding and, consequently, enzyme activity. In addition to these mutations within the coding region, the low expression level of the *ggs2* (D) gene can also contribute to the loss of GGS2 activity. Sequence analysis of the promoter region revealed a 7-nucleotide deletion in front of a GATA element, the binding site of the general transcription factor AREA-Gf (37). Mutations in the region of GATA-binding elements may affect the expression level of AreA-regulated genes, as was shown previously for the bidirectional promoter of *P450-1/P450-4* genes in *F. fujikuroi* (37) and *F. proliferatum* (34) and for the avirulence gene *Avr9* of *Cladosporium fulvum* (50).

A third reason for loss of enzyme activity could be the nonsense mutation we observed in the *F. proliferatum ggs2* (D)

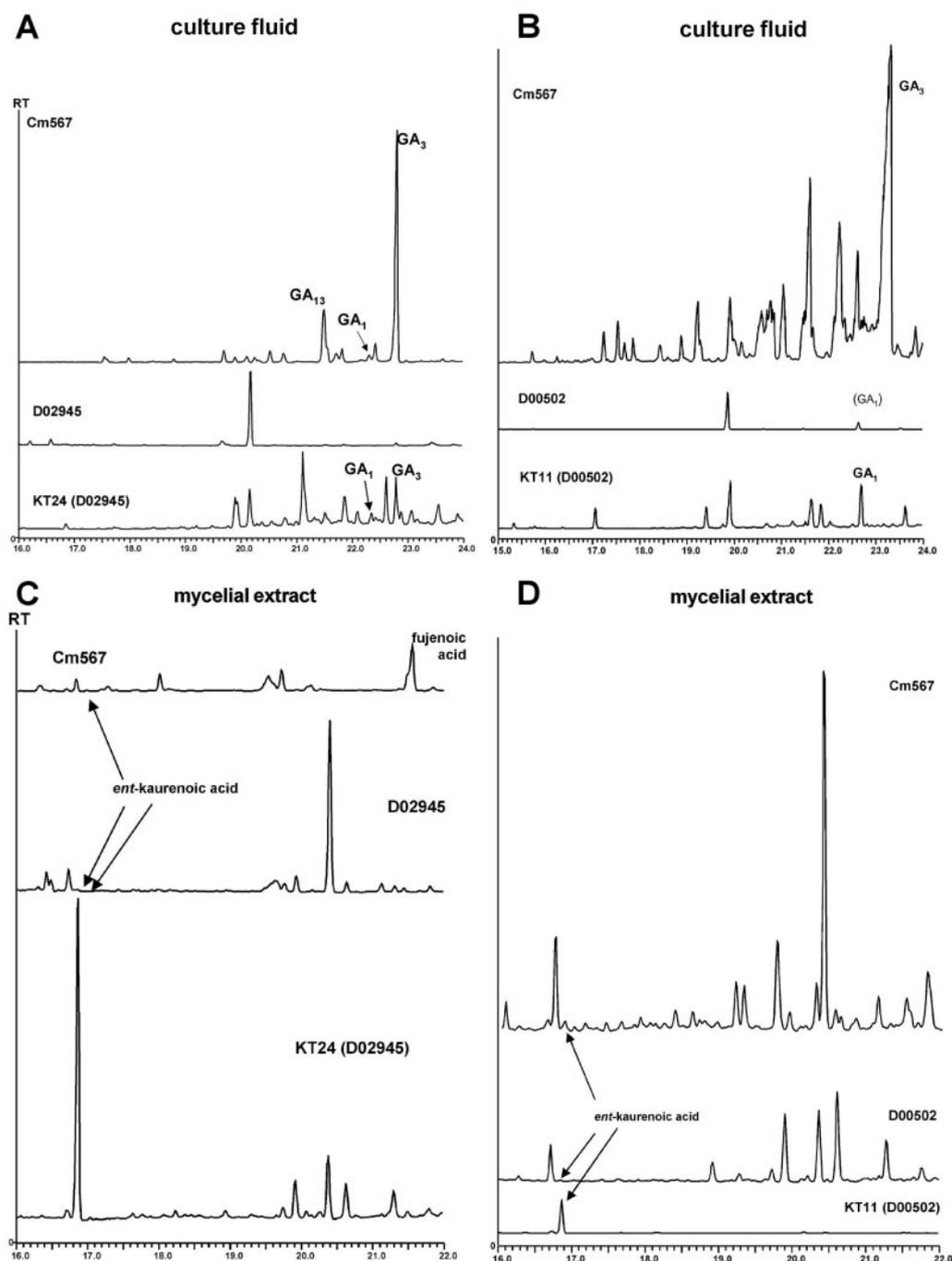


FIG. 4. Qualitative GC-MS analysis of *F. proliferatum* strains D02945 and D00502 before and after complementation with *ggs2* (C) and *cps/ks* (C) in comparison with MP-C wild-type strain m567. Strains were incubated for 10 days in 20% ICI. For full details, see Materials and Methods. (A) Culture filtrate of m567, D02945, and transformant KT24; (B) culture filtrate of m567, D00502, and transformant KT11; (C) mycelial extract of m567, D02945, and transformant KT24; (D) mycelial extract of m567, D00502, and transformant KT11.

gene (Fig. 3A). This mutation is probably the reason for the decay of the corresponding mRNA demonstrated in Northern blot analysis. In filamentous fungi, not much is known about mechanisms and reasons for targeted RNA degradation. Nonsense-mediated RNA decay, the loss of mRNA carrying premature stop codons, is a process by which cells recognize and degrade nonsense mRNAs to prevent possible toxic effects of

truncated peptides (44). This process was also found, e.g., for the plant flavonoid 3' hydroxylase gene in *Ipomoea* (17) and human cancer cells (18).

Besides P450-4 (D) and GGS2 (D), CPS/KS (D) was also found to be blocked in *F. proliferatum*. This last enzyme is responsible for *ent*-kaurene synthesis in the GA biosynthesis pathway (Fig. 1). Domain analyses of CPS/KS (D) clearly

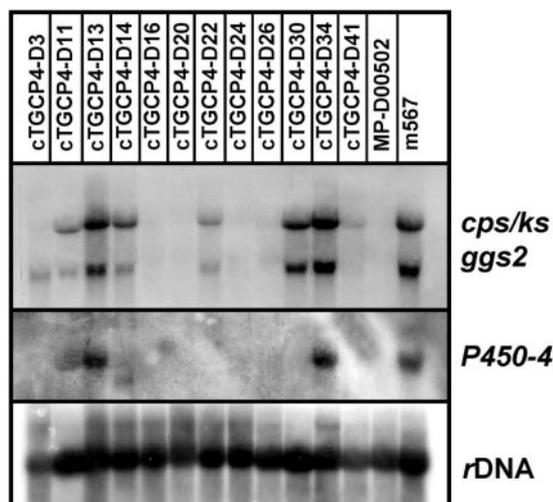


FIG. 5. Northern blot analysis of *ggs2*, *cps/ks*, and *P450-4* gene expression in *F. fujikuroi* (m567) and *F. proliferatum* (D00502) strains and in cTGKScos-D2 after transformation with pP450-4-GK. Strains were grown for 3 days in 20% ICI medium. rRNA (rDNA) was used as a loading control.

showed the existence of several aspartate-rich regions, which were also found to be typical for plant terpene cyclases, including the plant *ent*-kaurene synthases (54, 58, 68, 69). Thus, CPS/KS (D), like CPS/KS (C) from *F. fujikuroi*, has the characteristics of a bifunctional enzyme. Besides these two CPS/KS enzymes in different fusaria and FCPS/KS in *Phaeosphaeria* spp. (19, 56), only the aphidicolin-16 β -ol synthase in *Phoma betae* (57) and abietadiene synthase from *Abies grandis* (66) have been shown to be bifunctional diterpene cyclases. The terpene cyclase motif D(I/V)DDTA, which is missing in most plant *ent*-kaurene synthases, was shown to be particularly important for ring closure reactions of terpenoid compounds (53). On the other hand, the DDXXD motif, which is not found in plant CPS enzymes, mediates the binding of the divalent metal ion phosphate complex of the prenyl substrate (1). Attempts to complement the *cps/ks* (C) deletion mutant of *F. fujikuroi* with *cps/ks* (D) failed to restore GA production in the mutant, indicating that *cps/ks* (D) does not encode a functional enzyme. As shown in Fig. 3B, there are several amino acid substitutions relative to CPS/KS (C) throughout the CPS/KS (D) protein sequence, which may produce incorrect folding or affect substrate binding to the enzyme active site.

One of the aims of this work was to determine if it is possible to overcome all enzymatic blocks and thus restore GA biosynthesis in both *F. proliferatum* strains. Indeed, complementation of all three enzymatic blocks (at GGS2, CPS/KS, and P450-4) found in these strains resulted in the restoration of GA production, although the nature of the end products differed in the two complemented *F. proliferatum* strains (Fig. 4). Recently, it was shown for strain D00502 that incubations with radiolabeled substrates for the P450-1 monooxygenase (GA₁₄ synthase) led to the formation of significant but low amounts of the C₁₉ GAs GA₁ and GA₄, whereas incubation of the same precursors with cultures of strain D02945 resulted in the formation of unidentified oxidation products but no C₁₉ GAs (34). However, significant P450-1 (D) activity was observed in

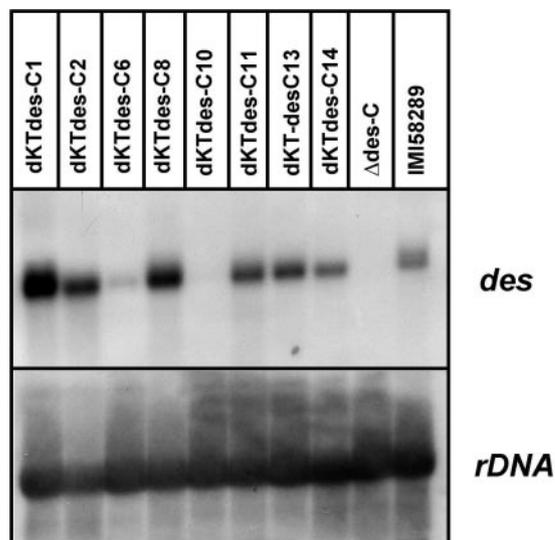


FIG. 6. Northern blot analysis of *des* gene expression in *F. fujikuroi* IMI58289 (wild type), the deletion mutant Δdes , and transformants of strain Δdes complemented with the *F. proliferatum des* gene. Strains were grown for 3 days in 20% ICI medium. rRNA (rDNA) was used as a loading control.

microsomal fractions from strain D02945, which partially converted the precursor [¹⁴C]GA₁₂-aldehyde to [¹⁴C]GA₁₄ (34). These results indicated that the strains, particularly D02945, contained only low P450-1 activities. The lower P450-1 activity in D02945 compared with D00502 would also explain the failure of strain D02945 to produce GAs after 3 days of incubation with [¹⁴C]GA₁₂-aldehyde, although significant amounts of GAs were detected in the strain after genetic complementation of the enzymatic blocks and a longer incubation period of 10 days. The much higher amounts of *ent*-kaurenoic acid that accumulate in the mycelia of the complemented D02945 strain compared with complemented D00502 (Fig. 4C and D) also suggest very low P450-1 activity in D02945. The activity of P450-1 (D) seems to be rate limiting for the formation of C₁₉ GAs, such as GA₁, GA₃, or GA₄, in the complemented *F. proliferatum* mutants. It is noteworthy that after 10 days, in contrast to shorter cultivation periods, traces of GA₁ were detected in strain D00502, indicating a residual activity for all the GA biosynthetic enzymes in this strain. However, a cross-contam-

TABLE 2. Production of GAs in *F. fujikuroi* strains IMI58289, Δdes , and different transformants of Δdes complemented with the *F. proliferatum des* gene^b

Strain	Concn (mg/liter) ^c		
	GA ₃	GA ₄	GA ₇
IMI58289	100	45	78
Δdes (C)	— ^a	340	—
dKT Δdes -C1	175	7	35
dKT Δdes -C2	85	9	130
dKT Δdes -C10	— ^a	365	—

^a GA₁, rather than GA₃, is the final product.

^b Strains were harvested after cultivation for 6 days in optimized production medium.

^c Given values are the averages of two independent measurements. Experiments were repeated three times with similar results. —, less than 1 mg/liter.

ination of corresponding D00502 culture filtrates cannot be excluded, as it seems unlikely that the *F. proliferatum* P450-4 or CPS/KS has any activity. In complementation transformants of both *F. proliferatum* strains, high amounts of unidentified *ent*-kaurenoid-like compounds were detected in addition to the GAs and *ent*-kaurenoids that are present in the *F. fujikuroi* strains (Fig. 4). These unidentified compounds, which are not present in the untransformed strains, may be formed by the unspecific action of endogenous enzymes on *ent*-kaurenoic acid, for which they compete with the relatively low-abundance GA biosynthetic enzymes. The low activity of the GA enzymes in *F. proliferatum* is evident from the much lower amounts of GAs found in the complemented strains compared with those in *F. fujikuroi* wild-type strain m567.

In the complementation experiments, the site of integration of the introduced gene seems to be important not only for its expression but also for functionality of the encoded enzyme. Integration of the entire *P450-4* gene of either *F. fujikuroi* or *F. proliferatum* into the genome of strain D02945 did not always result in detectable expression of the transgene (34). Similar results were obtained in experiments with *Aspergillus parasiticus*, in which β -glucuronidase reporter gene assays with the aflatoxin biosynthetic genes *ver1* and *nor1* clearly demonstrated that only integration into the homologous site resulted in expression of the fused *uidA* gene (7). Studies with *Aspergillus* spp. showed the existence of a global transcriptional regulator, LaeA, for secondary metabolism that is specific for a gene cluster, as transcription of genes upstream and downstream of secondary metabolism gene clusters was unaffected (4). In addition, some transformants of strain D02945 with high expression levels of *P450-4* (C) exhibited low levels of activity of the encoded *ent*-kaurene oxidase, resulting in the formation of low amounts of *ent*-kaurenoic acid. Therefore, the genetic environment seems to play a fundamental role not only for gene expression but also for activity and functionality of the encoded protein, although the exact reasons for this are still not identified.

Complementation of the *F. fujikuroi* desaturase deletion strain Δdes (C) with the corresponding desaturase gene from *F. proliferatum* strain D02945 resulted in the restoration of GA₃ and GA₇ production (Table 2), demonstrating the functionality of *des* in strain D02945. In contrast, strain D00502 produces only [¹⁴C]GA₁ and [¹⁴C]GA₄ (as observed for the *des* [C] deletion strain) when incubated with precursors such as *ent*-[¹⁴C]kaurenoic acid or [¹⁴C]GA₁₂-aldehyde, indicating that the *des* gene in this strain does not encode a functional enzyme and/or is poorly expressed (34). Thus, genetic diversity between GA biosynthesis genes occurs not only between isolates of different species but even between sexually compatible strains of one species.

In summary, it is noteworthy that several mutations in coding and 5' noncoding regions of different genes of the GA cluster led to the total loss of GA production in *F. proliferatum*. Major blocks were determined at the first three enzymes of the GA biosynthesis pathway: GGS2, CPS/KS, and *ent*-kaurene oxidase (P450-4). It was clearly shown by expression studies that the regulatory mechanisms are present in *F. proliferatum* and were not affected during evolution. Thus, the *F. fujikuroi* *ggs2*, *cps/ks*, and *P450-4* genes were highly expressed in the *F.*

proliferatum background, allowing the restoration of GA production.

The results presented here raise questions about the origin and evolution of fungal GA gene clusters. After completion of the cloning and characterization of GA biosynthetic genes in *F. fujikuroi* (reviewed in reference 65), and to a large extent in *Arabidopsis thaliana* and other plants (reviewed in reference 14), we can exclude the possibility of horizontal gene transfer from higher plants to the fungus for several reasons (reviewed in reference 15). Thus, several oxidation steps are catalyzed by dioxygenases in plants but by P450 monooxygenases in the fungus. Furthermore, the fungal GA biosynthetic genes are organized in a gene cluster but are dispersed in the genome of higher plants.

Surprisingly, only the species in the *G. fujikuroi* species complex contain the entire gene cluster or parts of it, whereas other species, such as *F. graminearum*, *F. oxysporum*, *F. sporotrichoides*, and *F. culmorum*, do not contain any of the GA biosynthetic genes in their genomes (33). On the other hand, members of at least two other fungal genera, *Sphaceloma* spp. and *Phaeosphaeria* spp., neither of which are closely related to *Fusarium*, and also bacteria such as *Rhizobium phaseoli*, *Azospirillum lipoferum*, and *Azospirillum brasilense* were also found to produce GAs (reviewed in reference 31). So far, only one gene, *cps/ks*, has been characterized from *Phaeosphaeria* species (56). As in *F. fujikuroi*, the encoded enzyme CPS/KS is bifunctional and catalyzes the first two cyclization steps to produce *ent*-kaurene via *ent*-copalyl diphosphate.

Recently, a new diterpene gene cluster has been identified in the fungus *Phoma betae* which is responsible for the production of aphidicolin, a compound with a GA-like structure (57). This gene cluster contains two P450 monooxygenase genes and a *cps/ks*-like bifunctional diterpene cyclase gene encoding aphidicolan-16 β -ol synthase. Again, the diterpene cyclase gene acts as a functional unit together with a pathway-specific GGDP synthase gene. All these data indicate that different fungal genomes contain basic units of a diterpene biosynthetic gene cluster with a diterpene cyclase-encoding gene linked to a pathway-specific *ggs* gene by a bidirectional promoter region.

The availability of an increasing amount of fungal sequence data makes it possible to study the distribution and evolution of secondary metabolite gene clusters in general. Thus, a sirodesmin gene cluster was identified in *Leptosphaeria maculans* (12), and very similar gene clusters have been found in at least four additional distantly related ascomycetes, such as *Aspergillus fumigatus*, *Chaetomium globosum*, *Magnaporthe grisea*, and *F. graminearum*.

The reasons for the organization of fungal genes involved in secondary metabolism in clusters and the continued retention of such large gene units even if there is no obvious or essential function in the corresponding organism are interesting questions. If such cooperating genes are conditionally dispensable but have adaptive value for colonizing certain ecological niches, the incipient cluster would be maintained by positive selection in those environments (23, 45, 47). The increasing number of sequenced fungal genomes will give us the opportunity to study the evolutionary origin and advantages of secondary metabolite gene clusters in the near future.

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