# Loss of Gibberellin Production in *Fusarium verticillioides* (*Gibberella fujikuroi* MP-A) Is Due to a Deletion in the Gibberellic Acid Gene Cluster[▽]

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***Fusarium verticillioides* (*Gibberella fujikuroi* mating population A [MP-A]) is a widespread pathogen on maize and is well-known for producing fumonisins, mycotoxins that cause severe disease in animals and humans. The species is a member of the *Gibberella fujikuroi* species complex, which consists of at least 11 different biological species, termed MP-A to -K. All members of this species complex are known to produce a variety of secondary metabolites. The production of gibberellins (GAs), a group of diterpenoid plant hormones, is mainly restricted to *Fusarium fujikuroi* (*G. fujikuroi* MP-C) and *Fusarium konzum* (MP-I), although most members of the *G. fujikuroi* species complex contain the GA biosynthesis gene cluster or parts of it. In this work, we show that the inability to produce GAs in *F. verticillioides* (MP-A) is due to the loss of a majority of the GA gene cluster as found in *F. fujikuroi*. The remaining part of the cluster consists of the full-length *F. verticillioides des* gene (Fv*des*), encoding the $GA_4$ desaturase, and the coding region of Fv*P450-4*, encoding the *ent*-kaurene oxidase. Both genes share a high degree of sequence identity with the corresponding genes of *F. fujikuroi*. The GA production capacity of *F. verticillioides* was restored by transforming a cosmid with the entire GA gene cluster from *F. fujikuroi*, indicating the existence of an active regulation system in *F. verticillioides*. Furthermore, the $GA_4$ desaturase gene *des* from *F. verticillioides* encodes an active enzyme which was able to restore the GA production in a corresponding *des* deletion mutant of *F. fujikuroi*.**

*Fusarium verticillioides* (Sacc.) Nirenberg (synonym *Fusarium moniliforme*; teleomorph *Gibberella fujikuroi* MP-A or *Gibberella moniliformis*) is a widespread pathogen of maize plants causing ear and stalk rot disease (4, 10) and belongs to the *Gibberella fujikuroi* (Sawada) species complex. This complex includes important fungal pathogens of a variety of crops, such as maize, rice, barley, sugarcane, pine, mango, pineapple, and sorghum (26). It comprises 11 sexually fertile biological species, also known as mating populations (MPs; MP-A to -K), and more than 30 anamorphic species (genus *Fusarium*, sections *Liseola*, *Elegans*, and *Dlaminia*) (21, 24, 44, 45, 65). Members of the species complex produce a broad variety of mycotoxins and other secondary metabolites, such as moniliformin (25), fusaric acid (1), beauvericin (30), gibberellins (GAs) (58), bikaverin (29), and fumonisins (3, 4, 39, 43).

It is noteworthy that some secondary metabolites are produced by more than one species in the complex, whereas other metabolites are produced only by one species. Thus, fumonisins are produced by *F. proliferatum* and *F. fujikuroi*, as well as by *F. verticillioides* (12, 37, 49, 51), moniliformin by *F. verticillioides*, *F. fujikuroi* and *F. thapsinum* (25, 38), and beauvericin by isolates of *F. fujikuroi*, *F. proliferatum*, and *F. subglutinans* (12, 55). In contrast, the production of GAs, a group of plant hormones, is so far mainly restricted to *F. fujikuroi* (MP-C) and *F. konzum* (MP-I), although several species of the *G. fujikuroi* species complex contain the entire GA-biosynthetic gene cluster (32, 33, 34).

The genetics and biochemistry of GA production in the rice pathogen *F. fujikuroi* have been well characterized in recent years (53, 61, 62, 63). The GA biosynthesis genes in *F. fujikuroi* are organized in a gene cluster (28, 58), as is often the case for secondary-metabolite biosynthesis pathways in fungi, e.g., the genes for the biosynthesis of fumonisins in *F. verticillioides* (48), aurofusarin in *Fusarium graminearum* (35), and aflatoxin and sterigmatocystin in *Aspergillus* species (20).

Beside genes encoding a pathway-specific geranylgeranyl diphosphate synthase (*ggs2*) and the bifunctional *ent*-copalyl diphosphate/*ent*-kaurene synthase (*cps*/*ks*), the GA gene cluster includes four cytochrome P450 monooxygenase genes (*P450-1* to *P450-4*) and the $GA_4$ desaturase gene (*des*) (53, 58, 61, 62, 63). Thirteen enzymatic steps are involved in the biosynthesis of gibberellic acid ($GA_3$) from geranylgeranyl diphosphate, indicating that most of the cluster genes encode multifunctional enzymes (Fig. 1). Recently, we demonstrated that the expression of six of the seven genes is under positive control by the general transcription factor AreA (40, 60) and that high nitrogen levels, which suppress AreA activity, virtually eliminate the expression of these genes and GA production. We have also characterized the cytochrome P450 oxidoreductase gene, *cpr*, and showed that CPR is essential for the functionality of the GA-specific cytochrome P450 monooxygenases in this fungus (31).

Here we show that the genome of *F. verticillioides* contains a rudimentary GA gene cluster due to the complete absence of five of the seven biosynthetic genes. We found that the only complete remaining gene, Fv*des* (*F. verticillioides des*), encoding the $GA_4$ desaturase, is active and is able to complement the

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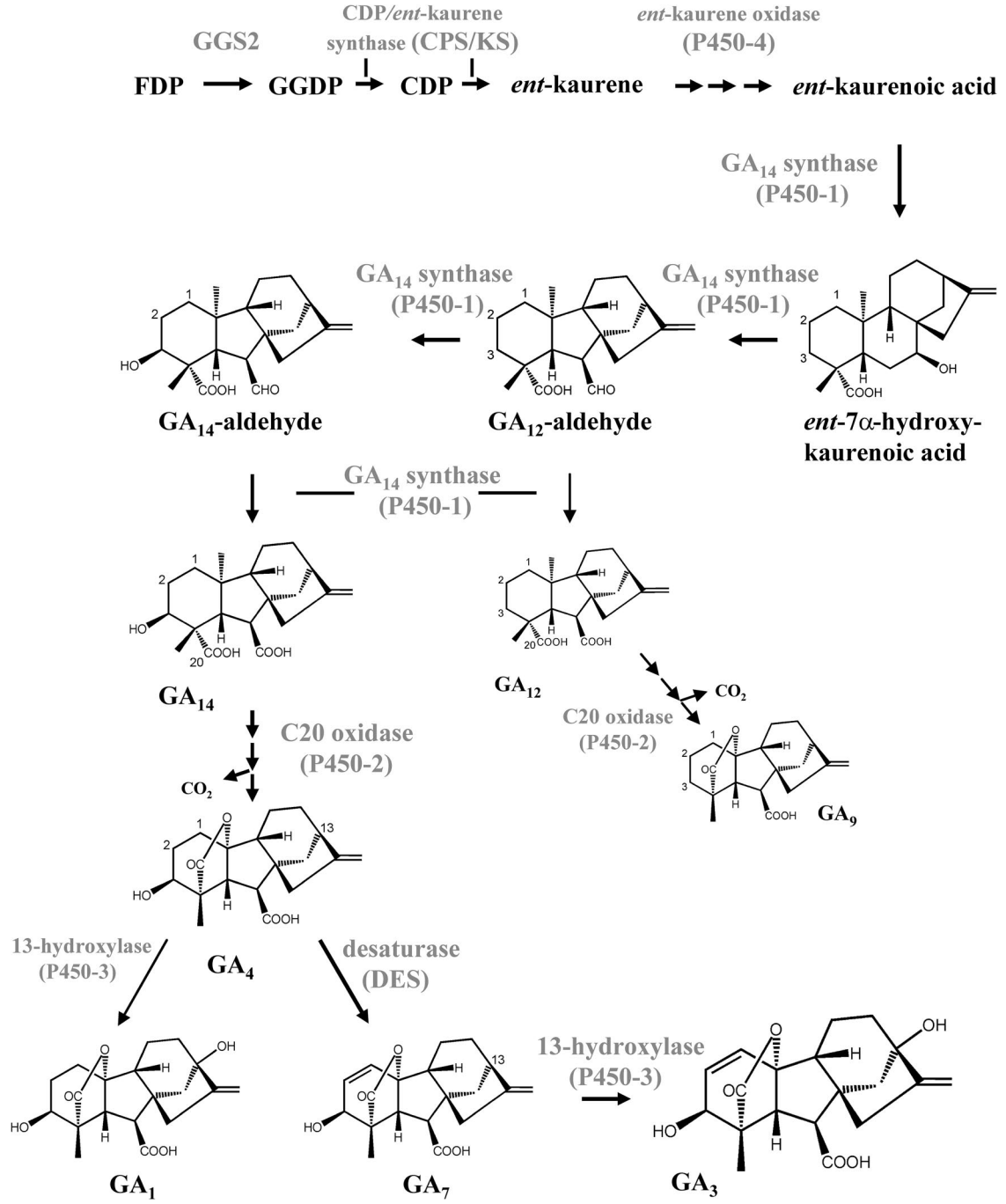


FIG. 1. GA-biosynthetic pathway in *F. fujikuroi*. The major pathway is indicated by bold arrows.

block in $GA_3$ biosynthesis in the *F. fujikuroi des* deletion (Ff$\Delta$*des*) mutant. Furthermore, we show that *F. verticillioides* produces a full spectrum of GAs after transformation with the entire GA gene cluster from *F. fujikuroi*, indicating an active regulation system in *F. verticillioides*.

## MATERIALS AND METHODS

**Fungal strains.** *Fusarium fujikuroi* (MP-C) strains IMI58289 (Commonwealth Mycological Institute, Kew, United Kingdom) and m567 (Fungal Culture Collection, Weimar, Germany) are GA-producing wild-type strains. *F. verticillioides* (MP-A) strains A00149 (FGSC7415), A00999 (FGSC7603), A02949, A03823 (FRC M1212), A03824 (JFK), A04516 (FGSC7606), A04362 (NRRL22052), A04367 (JFL), A04796 (JFL), and A04801 (MRC4315) are isolates from maize kernels at different geographic locations and were kindly provided by J. F. Leslie (Kansas State University, KA). Strain Ff-$\Delta$orf3-T33 (Ff$\Delta$*des*) is a *des* deletion mutant of *F. fujikuroi* strain IMI58289, lacking $GA_3$ and $GA_7$ production (63). The GA-deficient strain SG139 is a UV-induced *F. fujikuroi* mutant that lacks the whole GA gene cluster (14, 61). It was kindly provided by E. Cerdá-Olmedo and J. Avalos (University of Seville, Seville, Spain). Mutant B1-41a, obtained by UV irradiation of *F. fujikuroi* strain GF-1a (2), was donated by J. MacMillan (University of Bristol, Bristol, United Kingdom). This strain was shown to contain a point mutation in the *P450-4* locus and is GA deficient.

**Bacterial strains and plasmids.** *Escherichia coli* strain Top10F′ (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Vectors pUCBM20 (Boehringer, Mannheim, Germany) and pUC19 (Fermentas, St. Leon-Rot, Germany) were used to clone DNA fragments carrying the *Fusarium* cluster genes and gene fragments from *F. fujikuroi* and *F. verticillioides*. Cosmid pCos1, derived from a cosmid library based on strain m567, contains the entire GA gene cluster, including the noncoding 5′ and 3′ regions (about 40 kb) and, additionally, the hygromycin resistance cassette for selection (P. Linnemannstöns and B. Tudzynski, unpublished data). Plasmid pDesA was constructed by PCR using primer pair des-Prom and P450-4-GD1 and contains the full-length Fv*des* gene from strain A00149, encoding the $GA_4$ desaturase, and the rudimentary *P450-4* gene, encoding *ent*-kaurene oxidase. For functional studies with Fv*P450-4*, vectors pFv-P450-4 (genomic gene copy with natural promoter region) and $pFfP4_{prom}$::FvP4 (genomic gene copy under the control of the *F. fujikuroi P450-4* promoter) were generated. To obtain expression vector pFv-P450-4, the genomic copy of Fv*P450-4* with the putative promoter was amplified by using primer pair Fv-P450-4-Prom-R2 and Fv-P450-4-F1 and cloned into pCR2.1TOPO. For expression of the Fv*P450-4* gene under the control of the corresponding *F. fujikuroi* promoter, the gene was amplified with an introduced NcoI site at the translation start codon (primer combination Fv-P450-4-Prom-NcoI-R1 and Fv-P450-4-F1), cloned into pCR2.1TOPO, excised with EcoRI, and ligated into pUC19. The *F. fujikuroi* promoter was generated by PCR using primers Ff-P450-4-1-Prom-NcoI-F1 and Ff-P450-4-1-Prom-NcoI-R1, introducing NcoI sites at the 5′ and 3′ ends. It was ligated into the NcoI-digested pUC19 containing Fv*P450-4*, generating plasmid $pFfP4_{prom}$::FvP4. In cotransformation experiments, pNR1 (nourseothricin resistance) (23, 31) or pAN7-1 (hygromycin resistance) (50), respectively, were used for selection of transformants.

**Media and culture conditions.** For DNA isolation, *Fusarium* strains were grown for 3 days at 28°C on cellophane sheets (Alba Gewürze, Bielefeld, Germany) placed on CM agar (46). The harvested mycelium was frozen in liquid nitrogen, lyophilized for 24 h, and ground to a fine powder with a mortar and pestle. *F. verticillioides* strains were cultivated on V8 juice agar (200 ml/liter V8 juice, 0.3% $CaCO_3$, and 2% agar) for sporulation. For RNA isolation, fungal strains were grown in 100%, 20%, or 0% ICI medium (16), containing 8% glucose, 0.5% $MgSO_4$, 0.1% $KH_2PO_4$, and 0.5, 0.1, or 0% $NH_4NO_3$, respectively.

For analysis of gene expression, *Fusarium* strains were cultivated for 7 to 10 days in 20% ICI medium on a rotary shaker at 28°C. To elucidate nitrogen regulation, strains were harvested after 4 days of cultivation in 10% ICI medium, the mycelium was washed, and 1.5-g (wet weight) amounts were transferred to 100 ml of 0% or 100% ICI medium for 3 h. For GA production, the strains were grown for 10 days on a rotary shaker (190 rpm) at 28°C in 300-ml Erlenmeyer flasks containing 100 ml of 20% ICI medium.

**DNA and RNA isolation.** Genomic DNA was isolated from lyophilized mycelium as described by Doyle and Doyle (13). Plasmid DNA was extracted by using Qiagen columns following the manufacturer's protocol (Qiagen, Hilden, Germany). RNA was isolated by using an RNAgents total RNA isolation kit (Promega, Mannheim, Germany).

**PCR.** The PCR mixture contained 25 ng DNA, 10 ng of each primer (Operon Biotechnologies GmbH, Köln, Germany), 0.2 mM deoxynucleoside triphosphates, and 1 U DNA polymerase (BioTherm; GeneCraft GmbH, Lüdinghausen, Germany) in a total volume of 50 μl. PCR was carried out at 94°C for 4 min, followed by 36 cycles of 94°C for 1 min, 50 to 60°C for 1 min, 70°C for 1 to 4.5 min, and a final 10 min at 70°C. Annealing temperature and elongation time were applied differently, depending on the annealing temperature of each primer and the length of the amplified fragment. For analysis of the GA gene cluster and cloning of the remaining GA genes in *F. verticillioides* (MP-A) strain A00149, the following primers (Table 1) were synthesized on the basis of sequence data from *F. fujikuroi* genes and the published *F. verticillioides* genome sequence (*Fusarium verticillioides* Sequencing Project, Broad Institute of Harvard and MIT [http://www.broad.mit.edu]).

For reverse transcription-PCR (RT-PCR), 1 μg of total RNA of nitrogen-starved wild-type mycelium served as template to create cDNA by using a One-Step quantitative RT-PCR kit (Invitrogen, Groningen). For RT-PCR analysis of the *des* and *P450-4* genes, primer pairs des-1 (5′-GCC AGT GCG CAA GAG TGT CAC TGC-3′) and des-2 (5′-TCT CAC TTC CTC CTT GTC AGT TCC-3′) and Fv-P450-4-RT-1 (5′-GCA TCG CGT GAG AGG GGA CGG C-3′) and Fv-P450-4-RT-2 (5′-GCA GAA CCG TGT GAT TCA GC-3′), respectively, were used.

**Southern and Northern blot analysis.** After digestion with restriction endonucleases and electrophoresis, genomic DNA was transferred onto Hybond $N^+$ filters (Amersham Pharmacia, Freiburg, Germany). $^{32}$P-labeled probes were prepared by using the random oligomer-primer method (36). Filters were hybridized at 65°C in 5× Denhardt's solution containing 5% dextran sulfate (36). Filters were washed at the same temperature used for hybridization in 1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7]), 0.1% sodium dodecyl sulfate.

Northern blot hybridizations were accomplished by the method of Church and Gilbert (8). The *F. fujikuroi* rRNA gene was used as a control hybridization probe to confirm RNA transfer.

**Sequencing.** DNA sequencing of recombinant plasmid clones was accomplished with the automatic sequencer LI-COR 4200 (MWG, München, Germany). The two strands of overlapping subclones obtained from the genomic DNA clones were sequenced by using the universal and the reverse primers. DNA and protein sequences were aligned by using DNAStar (Madison, WI).

**Transformation of *Fusarium* strains.** The preparation of protoplasts and the transformation procedure were carried out as previously described (59), with the following modifications for strain A00149: strain A00149 was precultivated for 7 to 10 days on V8 agar. About $5 \times 10^8$ spores were inoculated into 100 ml CM medium. For complementation experiments, $10^7$ protoplasts (in 50 μl) of strain A00149, Ff-Δorf3-T33 (FfΔ*des*), SG139, or B1-41a were transformed with up to 15 μg of the cosmid pCos1, carrying the entire GA gene cluster from *F. fujikuroi*, or one of the circular complementation vectors pDesA, pFv-P450-4, and $pFfP4_{prom}$::FvP4. Plasmids were cotransformed with pNR1 (nourseothricin resistance marker) (31) or pAN7-1 (hygromycin resistance) (50).

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, Bad Soden, Germany) or 120 μg/ml nourseothricin (Werner BioAgents, Jena, Germany) for 6 to 7 days.

**GA assays.** The complete GA spectrum produced by the different strains and transformants was determined by gas chromatography-mass spectrometry (GC-MS) in ethyl acetate extracts of culture filtrates as described previously (63). Gibberellic acid ($GA_3$) was quantified in the culture fluid of B1-41a and B1-41a transformants by GC-MS after 5 days of incubation in 0% ICI medium (16). For incubation with [$^{14}$C]$GA_4$, cultures grown in 40% ICI were transferred to 0% ICI (10 ml) buffered at pH 3.0 and 300,000 dpm [$^{14}$C]$GA_4$ was added as a methanol solution. The mixture was further incubated for 3 days at 28°C, the culture fluid

TABLE 1. Primers used in this study

| Primer | Sequence (5′→3′)[a] |
|---|---|
| des-Fus1 | TGC TGC CGC TGC TTG ATT T |
| P450-4-Fus2 | CCC AGG GCG GTT CTA TGC |
| P4-MPA-R1 | GAG ACA TCA TTC GCT CCC AGC AAG GTA AT |
| des-Prom | CTA CCA GAA TGC AAT GAA |
| P450-4-GD1 | GGT CCA GAG CAC TGC CGC |
| Ff-P450-4-1-Prom-NcoI-F1 | TGG ACT TAC cCA Tgg TGT TAT TG |
| Ff-P450-4-1-Prom-NcoI-R1 | GGT TCG CCA Tgg TCT GAC AAC |
| Fv-P450-4-seq1 | GGT CTC CCC TTT CCA TCA GG |
| Fv-P450-4-seq2 | GGT AGA TGC GAT TGA ATG TTA CTG G |
| Fv-P450-4-seq3 | GGA TAA AAC CAG TCA TGA AGA GG |
| Fv-P450-4-seq4 | GCA TTT ATG AAC CCA GCT CAA GC |
| Fv-P450-4-seq5 | GCT GAA TCA CAC GGT TCT GC |
| Fv-P450-4-seq6 | GGG ATG AAA GGA GGT GGA TG |
| Fv-P450-4-seq7 | GGA GCA GCG TTT CAT TAA TGG |
| Fv-P450-4-seq8 | GGT AGA CTC TCC TCA TAA GCG G |
| Fv-P450-4-seq9 | GGG AAC TTC TTG TGT GCG AG |
| Fv-P450-4-seq10 | GTG GCT GAG GCG CAA ACG G |
| Fv-des-P4 | GGA TCA TGA CCT TCT TGG |
| Fv-P450-4-F1 | GCC CGA AGC AGG AGC TGA ATA TG |
| Fv-P450-4-Prom-NcoI-R1 | CAA AAC ACc ATG gAT AAG TTC AAC |
| Fv-P450-4-Prom-R2 | GGC TCC AGC CTT CTA ATG GG |

[a] Introduced restriction sites are underlined. Lowercase letters show nucleotides changed to introduce the corresponding restriction site.

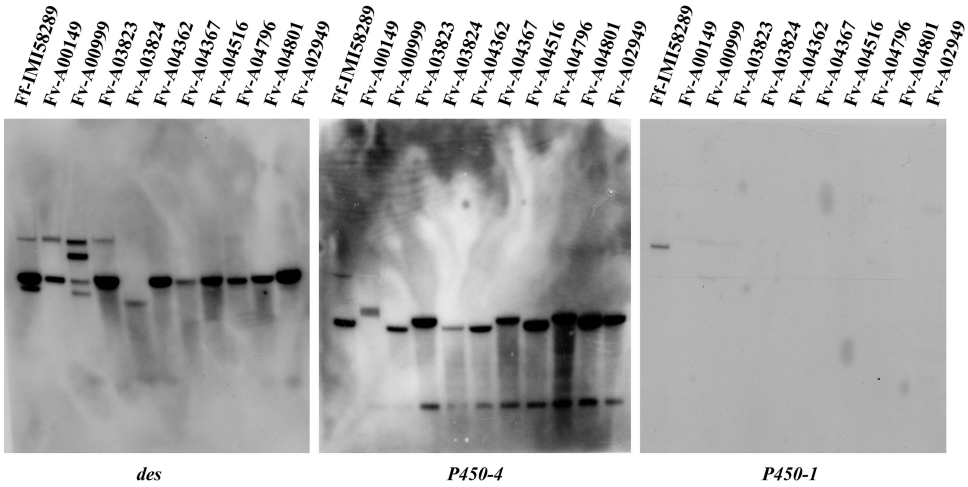


FIG. 2. Southern blot analysis of *F. fujikuroi* IMI58289 and several *F. verticillioides* isolates. The genomic DNA of all strains was digested with HindIII and hybridized with a 1:2 mixture of the gene probes from *F. fujikuroi* and *F. verticillioides* (for *des* and *P450-4*) or with the *F. fujikuroi* gene (*P450-1*). Ff, *F. fujikuroi*; Fv, *F. verticillioides*.

was separated by filtration, and GAs were extracted as described previously and analyzed by GC-MS (63).

**Nucleotide sequence accession numbers.** The gene sequences for Fv*des* and Fv*P450-4* of *F. verticillioides* A00149 have been deposited in the GenBank database under accession numbers AM946176 and AM946177, respectively.

## RESULTS

**Presence of a remnant GA gene cluster in *F. verticillioides* (MP-A) strains.** Previously, we have shown that *F. verticillioides* strains A00999 and A00149 contain only two of the seven GA-biosynthetic genes in the genome; these encode the $GA_4$ desaturase (DES) and the *ent*-kaurene oxidase (P450-4) (33). To confirm that this finding is a general feature in *F. verticillioides*, we analyzed eight additional *F. verticillioides* strains, all isolated from maize kernels in different regions of the United States (J. F. Leslie, personal communication), by Southern blot analysis using the seven *F. fujikuroi* GA-biosynthetic genes as probes. All *F. verticillioides* strains revealed hybridizing bands for *des* and *P450-4* but no hybridization signals for the other GA cluster genes (Fig. 2, results shown for *des*, *P450-4*, and *P450-1*). Therefore, *F. verticillioides* contains only the left border of the GA gene cluster found in *F. fujikuroi* and in most of the other species in the *G. fujikuroi* species complex (33).

BLAST comparison of the genes flanking the *F. fujikuroi* GA gene cluster to the corresponding region (Broad Institute; http://www.broad.mit.edu) flanking the remnant putative *F. verticillioides* GA genes identified some highly homologous regions, as well as some striking differences (Fig. 3). Thus, the composition of the genomic region upstream of *des* is almost identical to that in *F. fujikuroi*. Genes encoding a sugar membrane transporter (*smt*) (64) and an ankyrin domain-containing protein (*ank*), as well as an alcohol and an aldehyde dehydrogenase (*alc-dh* and *ald-dh*) (reviewed in reference 57), are located upstream of *des* in both species (Fig. 3). However, *smt* and *ank*, which are immediately adjacent to *des* and share a bidirectional promoter region, are inverted and orientated in the opposite direction in *Fusarium verticillioides* compared with their orientation in *F. fujikuroi.* Interestingly, about 50 kb downstream of Fv*P450-4* is a region that shares 90% identity to the genomic region downstream of the *F. fujikuroi* GA gene cluster (*orf1*, *orf2*, and *mfs*), though in the opposite direction, suggesting an inversion of this genomic fragment (Fig. 3).

**Expression of the two putative GA biosynthesis genes.** To analyze the expression of the two remaining GA-biosynthetic genes, Northern blot analyses were carried out with RNA isolated from different strains of *F. verticillioides* and compared

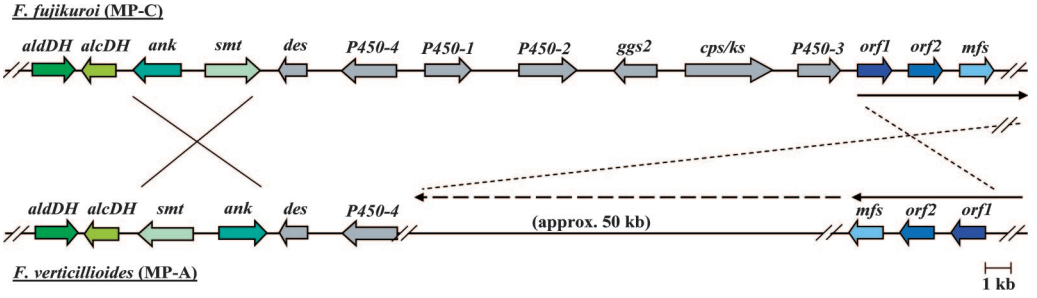


FIG. 3. Comparison of the GA gene clusters in *F. fujikuroi* and *F. verticillioides.* Crosses indicate gene inversions, dashed lines demonstrate putative inversions, and arrows show the orientation of transcription. GA genes are shown in gray, and genes not belonging to the cluster are colored.

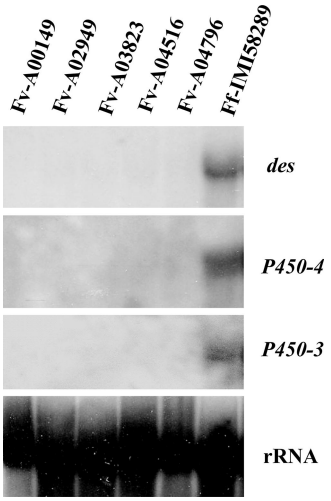


FIG. 4. Northern blot analysis of *F. verticillioides* isolates and the *F. fujikuroi* wild-type strain IMI58289. Total RNA was hybridized with the probes as indicated. *P450-3* is not present in the genome of *F. verticillioides* and was used as a negative control. Fv, *F. verticillioides*; Ff, *F. fujikuroi*.

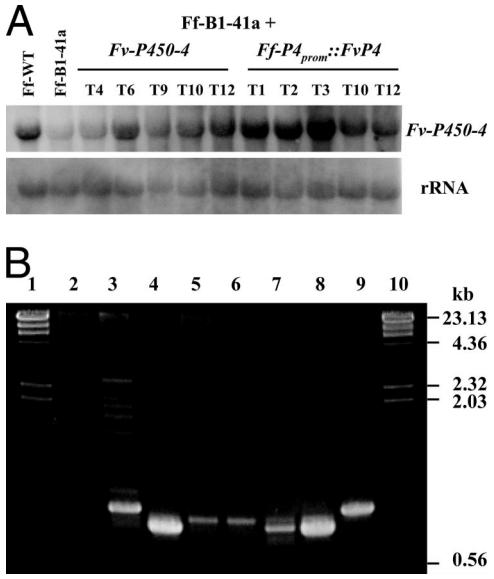


FIG. 5. Analysis of *ent*-kaurene oxidase *P450-4* expression. (A) Northern blot analysis of *F. fujikuroi* wild-type strain IMI58289, the *F. fujikuroi* mutant B1-41a carrying a point mutation in the gene *P450-4*, and transformants of B1-41a transformed (+) with the *F. verticillioides ent*-kaurene oxidase gene Fv*P450-4*, driven either by its own promoter or the promoter of the *F. fujikuroi P450-4* gene (Ff$P4_{prom}$::Fv*P4*). (B) RT-PCR analysis of the *P450-4* gene in different fusaria and mutant strains. Lanes: 1 and 10, markers; 2, *F. verticillioides* cDNA; 3, *F. verticillioides* genomic DNA; 4, *F. fujikuroi* cDNA; 5, B1-41a cDNA; 6, B1-41a Fv*P450-4* T6 cDNA; 7, B1-41a Ff$P4_{prom}$::Fv*P4* T3 cDNA; 8, *F. fujikuroi* cDNA; 9, *F. fujikuroi* genomic DNA. Ff, *F. fujikuroi*; Fv, *F. verticillioides*; WT, wild type.

to that from *F. fujikuroi* by using *des* and *P450-4* as probes (Fig. 4). In addition, the filter was probed with the *P450-3* gene, which is located at the right border of the cluster in *F. fujikuroi* and is missing in the remnant *F. verticillioides* cluster (Fig. 3). In *F. fujikuroi*, all three genes were expressed under conditions of nitrogen starvation, but no signal was obtained for *des* and *P450-4* in *F. verticillioides*. Although we expected Fv*P450-4* not to be expressed or to be expressed only at low levels due to the missing homologous promoter region, there is no obvious reason for the lack of *des* expression. To scrutinize the expression of these genes, we performed RT-PCR analyses with primers des-1 and des-2 (*des*) and Fv-P450-4-RT-1 and Fv-P450-4-RT-2 (*P450-4*), respectively, with RNA isolated from nitrogen-starved mycelium of *F. verticillioides* strain A00149. By this more sensitive method, a transcript was detected for the desaturase gene, and its identity was confirmed by sequencing the RT-PCR fragment (data not shown). However, no transcript was present for *P450-4*. Thus, only one of the two remaining GA cluster genes is expressed in *F. verticillioides*, which might encode a functional $GA_4$ desaturase.

**Functional analysis of the *P450-4* and *des* genes from *F. verticillioides*.** P450-4 catalyzes the first oxidation steps in GA biosynthesis, converting *ent*-kaurene in three steps to *ent*-kaurenoic acid (61). To determine if the corresponding gene from *F. verticillioides* encodes a functional protein despite it not being expressed, we fused the promoter of Ff*P450-4* with the Fv*P450-4* coding sequence (Ff$P4_{prom}$::Fv*P4*). We complemented the *F. fujikuroi* mutant B1-41a (2) with the fusion construct and with the Fv*P450-4* gene driven by the native promoter. Strain B1-41a produces significantly less $GA_3$ than the wild type due to a point mutation in the Ff*P450-4* gene that interferes with the splicing of intron 2 and thus translation but not transcription (61). The mutant was cotransformed with vector pNR1 carrying the nourseothricin resistance gene. The complete integration of the *P450-4* constructs was confirmed by PCR using primer combinations Fv-P450-4-F1 and Fv-P450-4-Prom-R2 (original *F. verticillioides* gene) and Ff-P450-4-1-Prom-NcoI-R1 combined with Fv-P450-4-F1 (*F. verticillioides* coding sequence fused with the *F. fujikuroi* promoter). Northern experiments with five verified transformants each revealed a higher expression level than that of B1-41a, particularly for those which were transformed with the construct Ff$P4_{prom}$::Fv*P4* (Fig. 5A). Since the two types of *P450-4* transcripts that accumulate in the transformed B1-41a, the mis-spliced native Ff*P450-4* gene and the introduced Fv*P450-4* genes, could not be distinguished by Northern analysis, we performed RT-PCR to differentiate between the longer mis-spliced Ff*P450-4* transcripts and the shorter, spliced Fv*P450-4* transcripts. By this means, we could show that the fusion of the *F. verticillioides* coding sequence to the *F. fujikuroi* promoter enabled the expression of *FvP450-4* in the B1-41a background (Fig. 5B, lane 7). None of the transformants exhibited a restored GA production, as evidenced by similar $GA_3$ levels found in the cultures of B1-41a (0.71 μg/10 ml) and of the B1-41a Fv*P450-4* (1.04 μg/10 ml) and B1-41a Ff$P4_{prom}$::Fv*P4* transformants (1.46 μg/10 ml). The B1-41a mutant produces only 2.5% of the amount of $GA_3$ produced by the wild-type GF-1a strain (2); thus, the above results demonstrate that Fv*P450-4* does not encode a functional protein.

In contrast to Fv*P450-4*, Fv*des* is expressed but at a low level in *F. verticillioides*. To determine if it encodes a functional $GA_4$ desaturase, Fv*des* was cloned from strain *F. verticillioides* A00149 by PCR with primers des-Prom and P450-4-GD1, and three independent clones were sequenced in both directions.

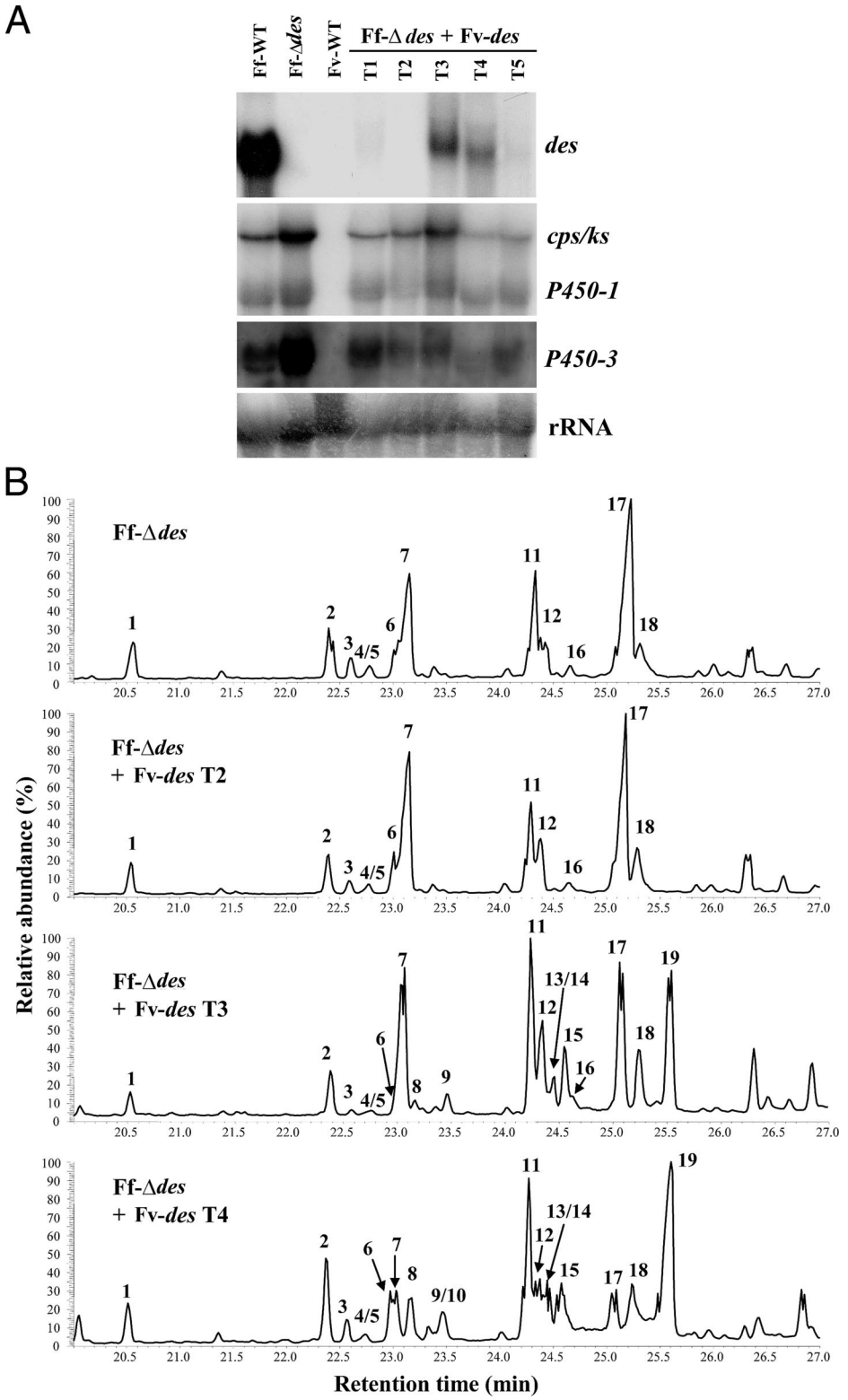


FIG. 6. (A) Northern blot analysis of strains *F. fujikuroi* IMI58289, the *F. fujikuroi* Ff$\Delta$*des* mutant, and transformants of the Ff$\Delta$*des* mutant complemented (+) with Fv*des*. Total RNA was hybridized with the probes as indicated. (B) GC-MS analysis of culture filtrates of Ff$\Delta$*des* and of transformants of Ff$\Delta$*des* complemented (+) with Fv*des* (T2, T3, and T4). Total ion currents are shown for ethyl acetate extracts as methyl esters trimethylsilyl ethers. Components were identified by comparison of their mass spectra and GC retention times with published data (15). Peaks: 1, $GA_9$; 2, $GA_{25}$; 3, $GA_{14}$; 4, $GA_{20}$; 5, $GA_{24}$; 6, 7$\beta$-hydroxykaurenolide; 7, $GA_4$; 8, gibberellenic acid; 9, $GA_7$; 10, $GA_{40}$; 11, $GA_{13}$; 12, fujenoic acid; 13, $GA_{47}$; 14, $GA_{16}$; 15, iso-$GA_3$; 16, $GA_{36}$; 17, $GA_1$; 18, 7$\beta$,18-dihydroxykaurenolide; 19, $GA_3$. Ff, *F. fujikuroi*; Fv, *F. verticillioides*; WT, wild type.

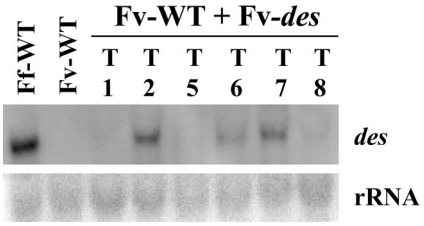


FIG. 7. Northern blot analysis of *F. fujikuroi* wild-type strain IMI58289, *F. verticillioides* wild-type strain A00149, and A00149 transformed (+) with additional copies of the Fv*des* gene from strain A00149. Total RNA was hybridized with Fv*des*. Ff, *F. fujikuroi*; Fv, *F. verticillioides*; WT, wild type.

The gene revealed 92% and 94% identity at the nucleotide and amino acid levels, respectively, to Ff*des* (63) (AJ417493, CAD10289), as well as 89% and 95% identity, respectively, to *des* from *F. proliferatum* (Fp*des*) (34) (AJ628021, CAF31351) (32). The Fv*des* gene was transformed into a *des* deletion strain of *F. fujikuroi* (FfΔ*des*). This mutant was shown to accumulate $GA_1$ and $GA_4$ instead of $GA_7$ and $GA_3$ (63) (Fig. 1). The results of Southern blot analysis of five transformants clearly showed that some contained multiple copies of the transformed DNA (data not shown), and the results of Northern analysis indicated that Fv*des* was highly expressed in some transformants (Fig. 6A). To test the enzymatic activity of FvDES, five of the transformants were cultivated for 10 days in the synthetic ICI medium (1.0 g/liter $NH_4NO_3$ as nitrogen source), and the secreted GAs were analyzed by GC-MS. Transformants T3 and T4, which were shown to have high levels of Fv*des* expression, were able to produce $GA_3$ in addition to $GA_1$ (Fig. 6B). Therefore, Fv*des* of strain A00149 encodes a functional protein that fully complemented the biosynthetic block in the FfΔ*des* mutant. As shown in Fig. 6A, the expression level of Fv*des* is much higher in the genetic background of *F. fujikuroi* than in that of *F. verticillioides*. One of the transformants with a high expression level, T3, contains at least three gene copies, while for T4 only one copy was present (data not shown), suggesting that the integration site in the genome plays an important role in the expression level.

To investigate if the genomic environment may be responsible for the low expression level of Fv*des* in *F. verticillioides*, we transformed Fv*des* back into its original source strain. Transformants were shown to have integrated the *des* gene in copy numbers from 1 to 3 at different loci (data not shown). Some of these transformants (T2 with one copy, T6 with three copies, and T7 with one copy) indeed have a higher expression level than the recipient strain, *F. verticillioides* A00149 (Fig. 7). Therefore, the genomic region where the GA biosynthesis genes are located seems to play an important role in their expression level.

**The GA regulatory network is functional in *F. verticillioides*.** The results of Northern blot analyses indicated that the expression of *P450-4* and *des* in *F. verticillioides* remained absent or low regardless of the strain or culture medium (Fig. 4, 6A, 7, and 8B). However, the much higher expression level of Fv*des* in *F. fujikuroi* than in *F. verticillioides* suggests that nucleotide differences in the Fv*des* promoter region in comparison with the Ff*des* promoter cannot be the only reason for the different expression levels in these genetic backgrounds. To investigate

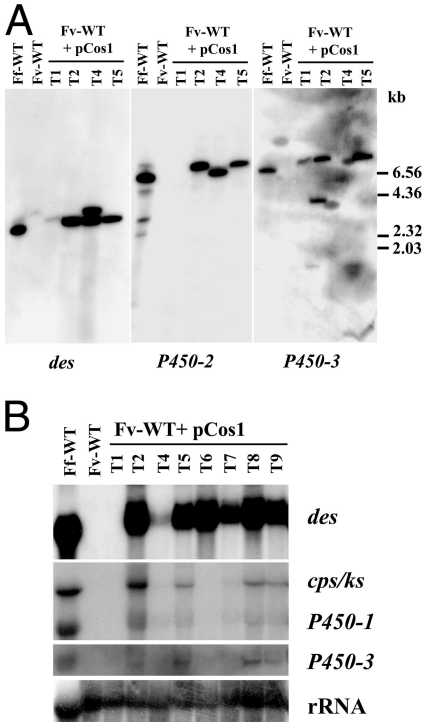


FIG. 8. Transformation (+) of *F. verticillioides* A00149 with the cosmid (pCos1) carrying the entire GA gene cluster from *F. fujikuroi*. (A) Southern blot analysis of *F. fujikuroi* IMI58289, *F. verticillioides* A00149, and transformants of A00149 carrying pCos1. The genomic DNA was restricted with HindIII. The filter was probed with GA-biosynthetic genes from *F. fujikuroi* as indicated. (B) Northern blot analysis of *F. fujikuroi* IMI58289, *F. verticillioides* A00149, and transformants of strain A00149 with pCos1. The filter was probed with GA-biosynthetic genes from *F. fujikuroi* as indicated. Ff, *F. fujikuroi*; Fv, *F. verticillioides*; WT, wild type.

if the transcription factors that regulate the expression of GA biosynthesis genes are active in *F. verticillioides*, we transformed the cosmid pCos1, carrying the entire GA gene cluster from the GA-producing strain *F. fujikuroi* IMI58289, into *F. verticillioides* strain A00149. The results of Southern blot analysis revealed the integration of the entire GA gene cluster of *F. fujikuroi* (including the 5′ and 3′ neighborhood of the GA cluster) into pCos1 transformants T2, T5, T7, T8, and T9 of *F. verticillioides*, whereas transformant T4 did not contain *P450-3*, the right border of the GA gene cluster, and T1 had integrated none of the cluster genes (Fig. 8A, results for T1, T2, T4, and T5).

In contrast to the recipient strain *F. verticillioides* A00149, most of the transformants express the GA pathway genes at about the same level as *F. fujikuroi* IMI58289 (results shown for *des*, *P450-3*, *cps/ks*, and *P450-1* in Fig. 8B). Six of the seven GA genes (except for *P450-3*) are found to be regulated by nitrogen: large amounts of glutamine or ammonium almost totally repressed gene expression, as for *F. fujikuroi* (40; data not shown). Surprisingly, transformant T4 does not express the GA genes, although six of the seven genes are integrated into the genome. However, it is not likely that the lack of *P450-3* is the reason for the inhibition of gene expression since *F. fujiku-*

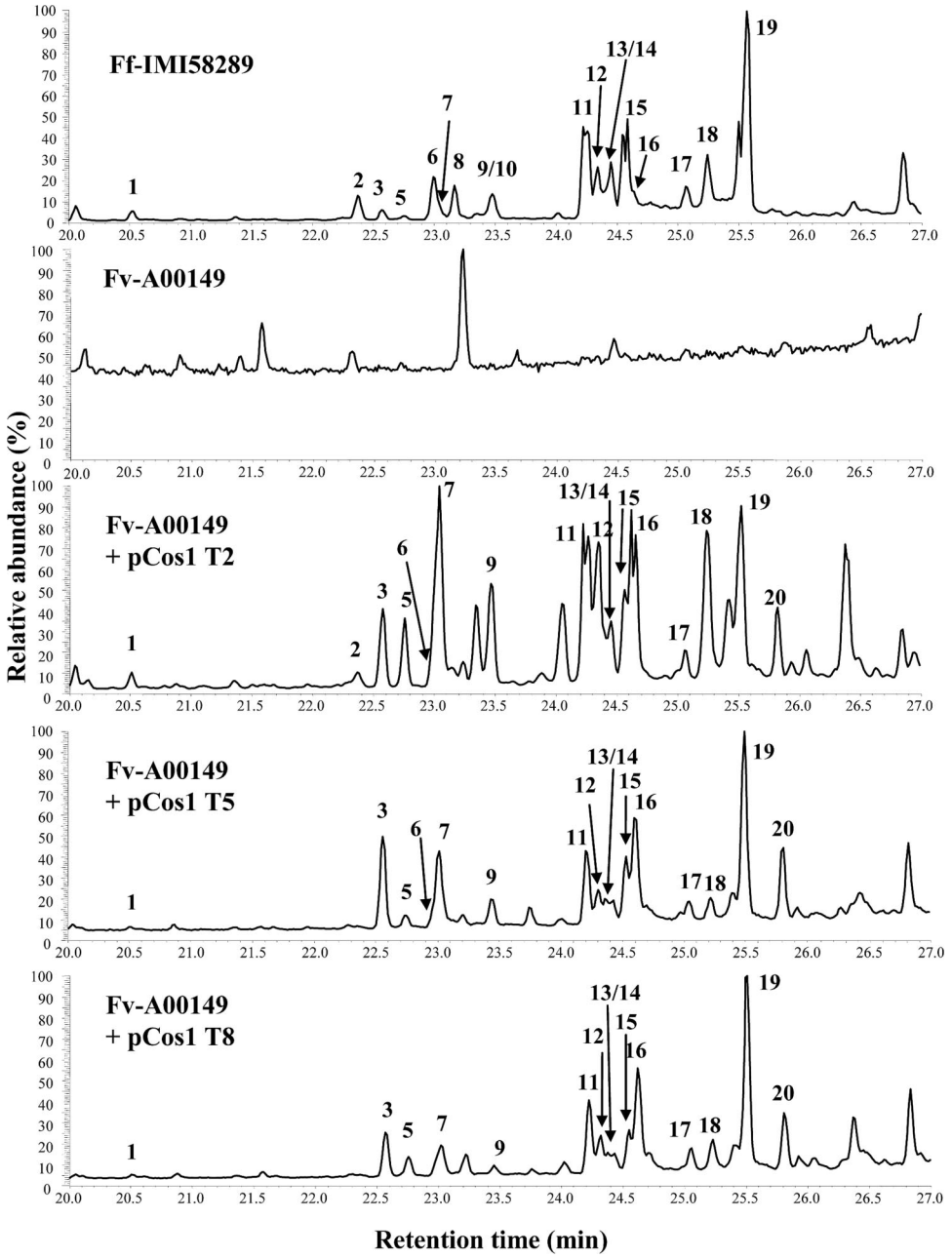


FIG. 9. GC-MS analysis of culture filtrates of wild-type strains *F. fujikuroi* IMI58289 and *F. verticillioides* A00149 and strains of A00149 transformed (+) with pCos1 carrying the entire *F. fujikuroi* GA gene cluster. Total ion current for ethyl acetate extracts after derivatization to methyl esters trimethylsilyl ethers is shown. Peak identities from comparison with published mass spectra (15) are as listed for Fig. 6B, with, additionally, $GA_{42}$ (peak 20). Ff, *F. fujikuroi*; Fv, *F. verticillioides*.

*roi* Δ*P450-3* mutants express the other GA cluster genes in a wild-type-like manner and produce large amounts of $GA_7$ (63).

In order to compare GA production, we performed GC-MS analysis of the culture extracts after incubating *F. verticillioides* A00149, three pCos1 transformants of A00149 (T2, T5, and T8) carrying the entire gene cluster, and the *F. fujikuroi* wild-type strain IMI58289 in synthetic ICI medium for 10 days (Fig. 9). The transformants were able to produce the full spectrum of GAs as in the wild-type *F. fujikuroi* strain, demonstrating that all essential regulators needed for GA gene expression are present in the genome of *F. verticillioides*.

We directly compared the enzyme activities of FvDES and FfDES in the *F. fujikuroi* background by transforming Fv*des* into the *F. fujikuroi* mutant SG139, which lacks the entire GA gene cluster (61). Transformants were screened for integration of the Fv*des* gene by diagnostic PCR using the primers des-Prom and des-2. The results of Southern blot analysis revealed that the gene was integrated with one to three

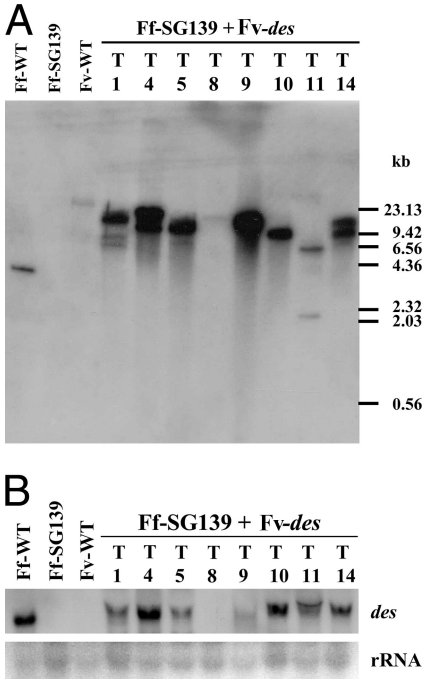


FIG. 10. Heterologous expression of Fv*des* in *F. fujikuroi* mutant SG139. The entire GA gene cluster is missing in strain SG139. (A) Southern blot analysis of *F. fujikuroi* IMI58289 (wild type), *F. fujikuroi* SG139, and transformants (+) of SG139 with the gene Fv*des.* Genomic DNA was digested with HindIII and probed with the genomic fragment of gene Fv*des.* (B) Northern blot analysis of strains *F. fujikuroi* IMI58289 (wild type) and *F. fujikuroi* SG139 and transformants of SG139 which were transformed (+) with the gene Fv*des.* The filter was probed with the genomic fragment of gene Fv*des.* Ff, *F. fujikuroi*; Fv, *F. verticillioides*; WT, wild type.

copies (Fig. 10A). The results of Northern blot analyses showed a high level of expression of Fv*des* in *F. fujikuroi* SG139, in contrast to its low level of expression in the *F. verticillioides* wild-type strain (Fig. 10B). Cultures of two SG139 transformants with Fv*des* (T4 and T10) and two previously described transformants with Ff*des* (T1 and T2) (63) were incubated with [$^{14}$C]$GA_4$ for 3 days in nitrogen-free medium, and its conversion to [$^{14}$C]$GA_7$ was investigated by GC-MS. The DES enzymes of both species revealed similar enzymatic activities: SG139 transformants with Fv*des* gave 12.7% conversion of [$^{14}$C]$GA_4$ to [$^{14}$C]$GA_7$, while SG139 with Ff*des* gave 19% conversion. Thus, Fv*des* encodes a functional GA desaturase with an activity similar to that of the desaturase from *F. fujikuroi*, consistent with the rescue of the *F. fujikuroi* Ff$\Delta$*des* mutant by Fv*des* described above.

## DISCUSSION

The *G. fujikuroi* species complex is known for the substantial production of a broad variety of secondary metabolites (reviewed in reference 9). Differences in metabolic chemotypes were part of recent investigations to characterize different *Fusarium* strains of this species complex (27, 33, 47, 52). *F. verticillioides* (*G. fujikuroi* MP-A) isolates from various areas and host plants produce large amounts of the mycotoxin fumonisin (7, 11, 12, 25). Fumonisin production is not restricted to *F. verticillioides*, as several isolates of *F. proliferatum* (MP-D), *Fusarium nygamai* (MP-G), and *F. fujikuroi* (MP-C) were also reported to contain the fumonisin gene cluster and to produce significant levels of this group of mycotoxins (9, 49). Recently, a correlation between the production of fumonisins and the host specificity has been shown for *F. verticillioides* isolates. While members of a distinct population of *F. verticillioides* are pathogenic on bananas and nonpathogenic on maize and are not able to produce fumonisins, another *F. verticillioides* subgroup of isolates infect maize and produce fumonisins (41, 42). The banana strains were shown to have a deletion of the fumonisin biosynthesis gene cluster, with only parts of the two terminal *FUM* genes remaining, suggesting that the ability to produce fumonisins is not essential for banana infection. Transformation-mediated complementation of banana strains with the complete *FUM* gene cluster restored fumonisin production, as well as pathogenicity on maize seedlings (17). Thus, there seems to be a correlation between fumonisin production and host specificity. The ubiquitous presence of the *FUM* cluster in maize isolates on one hand and the apparent deletion of almost the entire *FUM* gene cluster in banana isolates on the other hand suggests that the discontinuous distribution of gene clusters in highly related strains and species is due to specialization to different host plants and infection strategies during evolution.

We found a similar situation for the presence of the GA gene cluster and the ability to produce GAs in the *G. fujikuroi* species complex. While we did not find any GA-nonproducing *F. fujikuroi* strain isolated from rice, almost all the other *Fusarium* species of the *G. fujikuroi* species complex with different host plants have lost the ability to produce GAs due to multiple mutations in some GA cluster genes (33). Other genes in these clusters still encode functional enzymes, as they were able to complement the corresponding mutants of *F. fujikuroi* and restore the GA biosynthesis capability (34).

For *F. proliferatum*, which is one of the closest relatives to *F. fujikuroi*, we have studied the reasons for the loss of GA production in more detail. Despite the overall high degree of sequence identity of GA biosynthesis genes in these species, several mutations accumulated in the 5′ noncoding (e.g., Fp*P450-1* and Fp*P450-4*) or coding regions (e.g., Fp*ggs2* and Fp*cps*/*ks*) of GA genes in *F. proliferatum* strain D02945. Complementation of this nonproducing strain with the *ggs2* and *cps*/*ks* genes from *F. fujikuroi* restored the ability to produce GAs, demonstrating that the mutations in these genes were the main reason for the loss of GA production (32, 34). In contrast to these results, we recently isolated a GA-producing strain, *F. proliferatum* ET-1, from the roots of tropical orchids (56). Since *F. fujikuroi* and *F. proliferatum* are closely related species, we verified the identity of the latter strain by phylogenetic analysis with five genetic markers. These results clearly demonstrate that the capacity to synthesize GAs and the spectrum of GAs present may differ even between strains of one species.

A similar situation was found for the aflatoxin gene cluster in different species of the *Aspergillus* section *Flavi*, which includes species such as *A. parasiticus*, *A. flavus*, *A. sojae*, and *A. oryzae*. Some of the species produce aflatoxin, while others have lost this ability for different reasons. Recently, it was shown that several nonproducing strains have retained the entire gene

cluster but have acquired mutations in the regulatory gene *aflR* or in the noncoding or coding regions of three other open reading frames encoding biosynthesis genes. Other strains harbored small or large (more than 1 to 2 kb) deletions in the gene cluster. In several cases, the lack of expression of pathway genes can be explained by mutations in the AreA, FacB, and AflR binding motifs in their promoters (54). The authors suggest that losses of aflatoxin biosynthesis in *A. oryzae* and *A. sojae* are the result of a long history of use in industrial brewing processes where this pathway is apparently not needed by the fungus.

In this paper, we show that *F. verticillioides*, a member of the *G. fujikuroi* species complex, has lost its ability to produce GAs due to a substantial deletion of the GA gene cluster (Fig. 3). With Fv*des*, encoding $GA_4$ desaturase, and Fv*P450-4*, encoding a nonfunctional *ent*-kaurene oxidase, there are only two genes left in the genomes of the 10 *F. verticillioides* strains analyzed, but only Fv*des* seems to encode a functional enzyme, albeit with slightly reduced activity. Interestingly, downstream of the two remaining genes there is a genomic region of about 50 kb that is not found in *F. fujikuroi*, followed by a region with about 90% sequence identity to the *F. fujikuroi* region immediately adjacent to the GA gene cluster, though with the opposite orientation (Fig. 3). These data demonstrate that not only deletions but also genome rearrangements took place during the evolution of the *G. fujikuroi* species complex. A similar phenomenon of intercalary inversions, as well as other chromosome rearrangements, has been described from interlineage crosses of *Gibberella zeae* (18) and from interspecies crosses between isolates of *Gibberella fujikuroi* and *Gibberella intermedia* (27).

Recently, similar events of gene duplications, multiple losses, and chromosome inversions were shown for the *Magnaporthe grisea ACEI* cluster (22). A complete *ACEI* cluster was found in the genomes of only 4 of the 23 sequenced Pezizomycotina genomes. The authors showed that the main part of the cluster has been distributed from an ancestor of *M. grisea* to other species by horizontal gene transfer but that evolutionary constraints acted to maintain this cluster in only a few species, probably because of an important role of the yet-unknown product for their pathogenic life style (22). Similarly, highly conserved GA gene clusters might have been distributed by horizontal gene transfer from an ancestor species to other ascomycetes, but only some distantly related species, such as *Sphaceloma manihoticola* (6) and *Phaeosphaeria* sp. (19) maintained these clusters and produce GAs. Even among the closely related species of the *G. fujikuroi* species complex, only some are still able to produce GAs, while the others have lost this ability due to mutations in the GA gene cluster and/or to losses of one or more genes (32, 33, 34). *F. verticillioides* is an example for both GA gene losses and mutations. The remaining two genes are very poorly expressed. There might be different reasons for this. First, the loss of five GA cluster genes could result in low levels of expression of the remaining genes. However, this is not very likely as no gene(s) coding for pathway-specific transcription factors are located in the GA gene cluster in *F. fujikuroi* or any other species. In this case we would have expected a coordinated expression of all cluster genes, but in fact Fv*des* is highly expressed in the *F. fujikuroi* mutant SG139, which has lost the entire gene cluster by UV mutation. Second, the accumulation of point mutations, e.g., in some of the GATA sequence elements, could be one reason for the reduced expression level of Fv*des*, as has been shown for the GA genes Fp*P450-1* and Fp*P450-4* in *F. proliferatum* (32). However, Fv*des*, with its slightly altered promoter region with respect to that of Ff*des*, is expressed in the genetic background of *F. fujikuroi* much more strongly than in *F. verticillioides* and was able to complement the GA production defect of the *F. fujikuroi* Δ*des* mutant. These results suggest that additional factors, such as the genomic environment, seem to be responsible for the low level of expression of Fv*des*. Indeed, by transforming Fv*des* back into its original source strain, we revealed several transformants with different numbers of gene copies that have a higher expression level than the recipient strain *F. verticillioides* A00149.

To check if *F. verticillioides* has the regulatory network for the synthesis of GAs, we transformed a cosmid with the entire GA gene cluster from *F. fujikuroi* strain IMI58289 into *F. verticillioides* A00149. Surprisingly, the seven GA-biosynthetic genes were expressed in some transformants at almost the same high level as in *F. fujikuroi*. This is only possible if all the necessary transcriptional regulators, which might act in addition to AreA, exist and are active in *F. verticillioides.* These results clearly corroborate our previous findings in *F. proliferatum*, where the general transcription regulator AreA and other yet-unknown GA-specific regulator(s) were found to be functional (34).

Recent data from work with *Aspergillus nidulans* have demonstrated that the chromosomal position of gene clusters appears to affect transcription (5). We could show that the integration of Fv*des* into different loci in the *F. verticillioides* genome can significantly increase the expression level, confirming that the differing histone structure caused by the deletion of the GA gene cluster in *F. verticillioides* might indeed be very important.

To summarize, we showed that the genomic region containing the remnant GA cluster in *F. verticillioides* has undergone two rearrangements, a sequence deletion and an inversion of a 50-kb fragment relative to that in the GA cluster of *F. fujikuroi*. Of the two remaining genes, only one, Fv*des*, is still expressed (although at a low level) and encodes a functional enzyme, which has slightly lower activity than the corresponding *F. fujikuroi* enzyme. The activity was high enough to fully restore $GA_3$ production in the *F. fujikuroi* Δ*des* mutant. We suggest that the presence of an active GA gene cluster in all *F. fujikuroi* strains studied so far and in some *F. konzum* (33) and *F. proliferatum* (56) isolates on one hand and the loss of GA production capacity in other species of the *G. fujikuroi* species complex on the other hand might be correlated with the specialization of the latter to different host plants and/or to pathogenic versus endophytic life styles.

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