Distribution of gibberellin biosynthetic genes and gibberellin production in the *Gibberella fujikuroi* species complex

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Abstract

Gibberella fujikuroi is a species-rich monophyletic complex of at least nine sexually fertile biological species (mating populations, MP-A to MP-I) and more than 30 anamorphs in the genus *Fusarium*. They produce a variety of secondary metabolites, such as fumonisins, fusaproliferin, moniliformin, beauvericin, fusaric acid, and gibberellins (GAs), a group of plant hormones. In this study, we examined for the first time all nine sexually fertile species (MPs) and additional anamorphs within and outside the *G. fujikuroi* species complex for the presence of GA biosynthetic genes. So far, the ability to produce GAs was described only for *Fusarium fujikuroi* (*G. fujikuroi* MP-C), which contains seven clustered genes in the genome all participating in GA biosynthesis. We show that six other MPs (MPs B, D, E, F, G, and I) and most of the anamorphs within the species complex also contain the entire gene cluster, except for *F. verticillioides* (MP-A), and *F. circinatum* (MP-H), containing only parts of it. Despite the presence of the entire gene cluster in most of the species within the *G. fujikuroi* species complex, expression of GA biosynthetic genes and GA production were detected only in *F. fujikuroi* (MP-C) and one isolate of *F. konzum* (MP-I). We used two new molecular marker genes, *P450-4* from the GA gene cluster, and *cpr*, encoding the highly conserved NADPH cytochrome P450 reductase to study phylogenetic relationships within the *G. fujikuroi* species complex. The molecular phylogenetic studies for both genes have revealed good agreement with phylogenetic trees inferred from other genes. Furthermore, we discuss the role and evolutionary origin of the GA biosynthetic gene cluster.

Keywords: *Gibberella fujikuroi*; Gibberellin biosynthesis; Species complex; Phylogenetic tree; Gene cluster; P450 reductase

1. Introduction

The *Gibberella fujikuroi* species complex includes important fungal pathogens of various crops, such as maize, rice, barley, sugarcane, pine, mango, pineapple, sorghum, and many more (Leslie, 1995, 1999). Anamorphs within this complex belong to the genus *Fusarium* section *Liseola*. This species complex may be divided into at least nine sexually fertile biological species (also known as mating populations, MP-A to -I) and so far 32 additional asexual species (Britz et al., 1999; Kerényi et al., 1999; Leslie, 1995; Marasas et al., 2001; Nirenberg and O'Donnell, 1998; Nirenberg et al., 1998; O'Donnell et al., 1998, 2000; Steenkamp et al., 1999; Zeller et al., 2003). In recent years species concepts in this complex have been intensively studied using morphology (Nirenberg and O'Donnell, 1998), mating experiments (Leslie, 1995; Leslie et al., 2004),

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and phylogenetic analysis based on DNA sequence data from unlinked loci, such as mitochondrial small subunit (mtSSU) rDNA, nuclear 28S rDNA, β-tubulin, calmodulin, translation elongation factor EF-1α, and histone H3 genes (Waalwijk et al., 1996; O'Donnell and Cigelnik, 1997, 1998, 2000; Kerényi et al., 1999; Steenkamp et al., 1999, 2000; Yun et al., 2000; Jimenez et al., 2000). Other molecular techniques, such as RAPDs (DuTeau and Leslie, 1991; Mitter et al., 2002; Voigt et al., 1995), mitochondrial RFLPs (Correll et al., 1992), AFLPs (Chulze et al., 2000; Marasas et al., 2001; Zeller et al., 2003), and CHEF-gel karyotypes (Xu et al., 1995) have been also used to differentiate between members of the *G. fujikuroi* species complex. Based on the results of these analyses, the *G. fujikuroi* complex has been delineated into three lineages, designated as the African, Asian, and American clades (O'Donnell et al., 1998, 2000).

Moreover, members of these mating populations can be found preferentially on different host plants (Leslie and Plattner, 1991; Zeller et al., 2003) and differ in their ability to produce secondary metabolites, such as fumonisins (Desjardins et al., 1995, 2000; Kedera et al., 1999; Leslie et al., 1992a,b; Proctor et al., 1999, 2004), fusaric acid (Bacon et al., 1996), beauvericin (Logrieco et al., 1998; Torres et al., 2001; Reynoso et al., 2004), fusaproliferin (Reynoso et al., 2004), moniliformin (Leslie et al., 1996; Desjardins et al., 2000), fusarins (Wiebe and Bjeldanes, 1981; Song et al., 2004), and gibberellins (GAs) (El-Bahrawi, 1977; Tudzynski and Hölter, 1998; Desjardins et al., 2000). In some cases, strains from more than one mating population can produce a particular secondary metabolite, while in other cases this ability seems to be species-specific. Thus, fumonisins are produced by members of MP-A, -C, -D, and -G (Proctor et al., 2004), moniliformin by members of MP-A, -C, and -F (Marasas et al., 1986; Leslie et al., 1996), and beauvericin and fusaproliferin by isolates of MP-D and -E (Torres et al., 2001; Desjardins et al., 2000; Reynoso et al., 2004). In contrast, GA production was described so far only for the species *F. fujikuroi* (MP-C), the causative agent of bakanae disease of rice. The genetic and biochemical background of GA biosynthesis by *F. fujikuroi* has been well characterized in the last six years (Tudzynski and Hölter, 1998; Tudzynski et al., 2001, 2002, 2003; Rojas et al., 2001; Tudzynski, 2005). As it is the case for many fungal secondary metabolites, GA biosynthetic genes are organized in a gene cluster (Tudzynski and Hölter, 1998; Linnemanstöns et al., 1999). In addition to genes encoding a pathway-specific geranylgeranyl diphosphate synthase (*ggs2*) and the bifunctional *ent*-copalyl diphosphate/*ent*-kaurene synthase (*cps*/*ks*), the GA gene cluster consists of four cytochrome P450 monooxygenase genes (*P450-1* to *P450-4*) and a GA_4 desaturase gene (*des*) (Rojas et al., 2001; Tudzynski et al., 2001, 2002, 2003). At least 16 enzymatic steps (Fig. 1(a)) are involved in the biosynthesis of gibberellic acid (GA_3), and most of the genes encode multifunctional enzymes. Six of the seven cloned genes are under control of the positively acting general transcription factor AREA (Tudzynski et al., 1999; Mihlan et al., 2003), and high levels of preferred nitrogenous compounds can drastically reduce the expression of GA-biosynthetic genes and GA amounts produced. We also cloned and characterized the cytochrome P450 oxidoreductase gene, *cpr-Gf*, and showed that CPR is responsible for the electron supply to the GA-specific cytochrome P450 monooxygenases and probably all of the other P450s in this fungus. Deletion of *cpr-Gf* resulted in the loss of GA production and a lower growth rate demonstrating that CPR also is essential for primary metabolism (Malonek et al., 2004).

Only now after the cloning of all genes responsible for GA biosynthesis, we are able to elucidate, whether other species within the *G. fujikuroi* complex have also a similar GA biosynthetic gene cluster, and if so, whether they produce GAs. Apart from *F. fujikuroi* (MP-C), some strains of MP-A (*F. verticillioides*) and MP-D (*F. proliferatum*) have been also isolated from infected rice seedlings in different geographic regions (Desjardins et al., 1997). However, these studies did not finally answer the question if these *Fusarium* species are also associated with symptoms of "bakanae" disease, or are present only as saprophytes.

Consequently, the aim of this work is to determine whether all sexual fertile species (MPs) and also some anamorphs of the *G. fujikuroi* species complex contain the GA biosynthetic gene cluster, and if strains carrying the entire gene cluster are additionally able to produce GAs. Therefore, we examined about 50 *Fusarium* species within and outside the *G. fujikuroi* species complex (Table 1) for the presence of the GA biosynthetic genes. Our results indicate that most of the species within the complex have the entire GA biosynthetic gene cluster or at least some of the genes. However, intriguingly none of these species except for *F. fujikuroi* (MP-C) and one strain of *F. konzum* (MP-I) express the GA biosynthetic genes at detectable levels, and none of them except for *F. fujikuroi* and so far one *F. konzum* isolate are able to produce GAs. Contrary to that, *Fusarium* strains not related to the *G. fujikuroi* species complex contain none of the genes.

2. Results

2.1. Identification and expression of GA biosynthetic genes

By Southern blot analyses we examined several strains from the nine sexually fertile and some anamorphic species within the *G. fujikuroi* species complex as

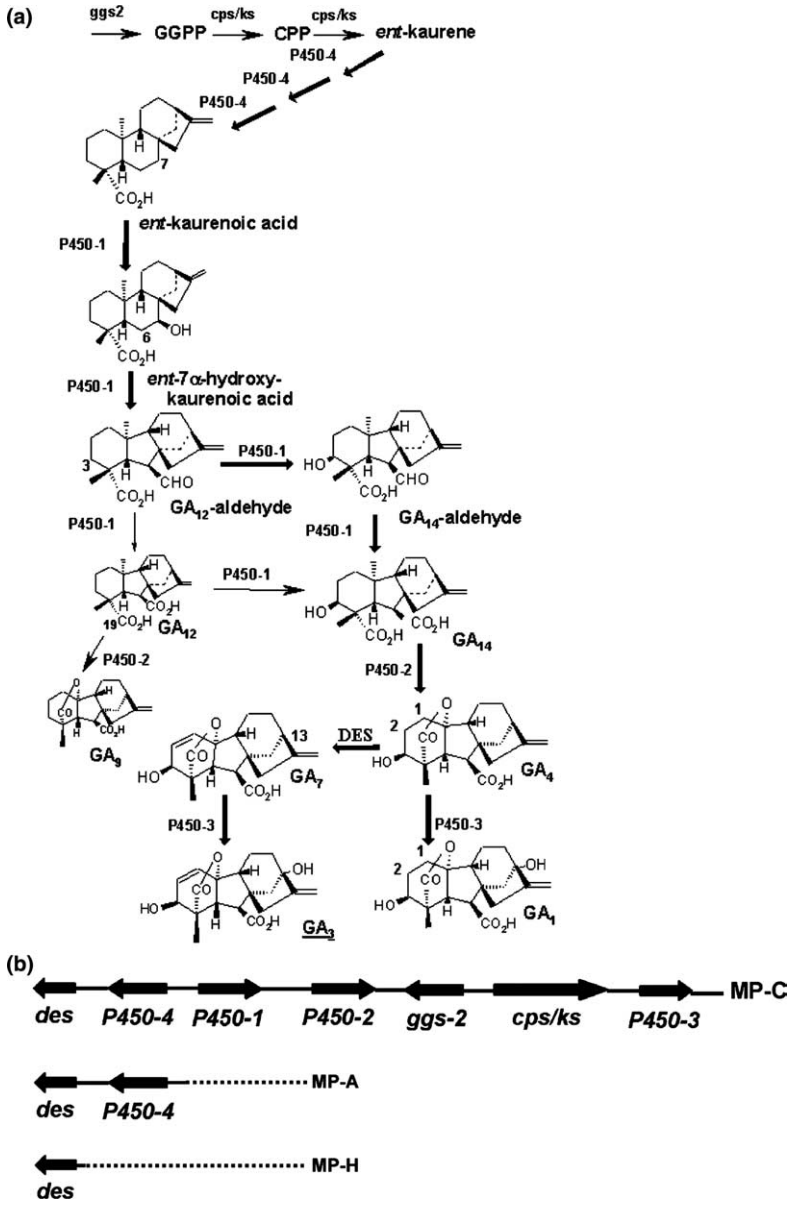

Fig. 1. (a) Gibberellin biosynthesis pathway indicating the genes, enzymes and products. (b) Organization of the gene cluster of the GA biosynthesis pathway, including all seven genes in MP-C, and the rudimental clusters in MP-A (only two genes left) or MP-H (one gene left).

well as some other *Fusarium* species (Table 1) for the presence of the GA biosynthetic genes. cDNA fragments of three cluster genes (*des*, *P450-4*, and *P450-3*) of *F. fujikuroi* (MP-C) were used as probes. The genes are distributed across the entire length of the GA biosynthetic gene cluster (Fig. 1(b)). Chromosomal DNA from all sexually fertile species within the *G. fujikuroi* complex except for *F. verticillioides* (MP-A) and *F. circinatum* (MP-H) hybridized to all probes under stringent hybridization conditions (Fig. 2(a)–(c), exemplarily shown only for some strains). For strains of *F. verticillioides* (MP-A), hybridization signals were detected with only two genes, *des* and *P450-4*, whereas *F. circinatum* (MP-H) strains hybridized only to the *des* gene of the GA cluster (Fig. 1(b), Fig. 2(a)–(c); Table 2). To confirm our suggestion that the GA gene cluster in MP-A and MP-H broke off downstream of *P450-4* and *des*, respectively, we probed the same filters with *P450-1* (see Fig. 1(b)). As expected, for strains of MPs A and H no hybridizing bands were obtained, whereas all the other MPs hybridized to the *P450-1* probe (data not shown) and most likely contain the entire gene cluster.

In some cases, RFLPs were observed for strains of the same species, e.g. for the gene *des* in MP-B and MP-F, or for *P450-4* in MP-A (Fig. 2(a)–(c)). Interestingly, only one of the MP-I strains hybridized to the *P450-3* probe (Fig. 2(c)) although both strains contained the *cps/ks* gene upstream of *P450-3* (data not shown).

Table 1
Strains used in this study, their host plants and source

Strain	Host plant	Source
F. verticillioides (*G. fujikuroi* MP-A)		
A-00501 (A1)	Maize, Kansas	J.F. Leslie
A-00552 (A2)	Maize, Kansas	J.F. Leslie
A-00999 (A3)	Maize, Kansas	J.F. Leslie
A-00149 (A4)	Maize, Kansas	J.F. Leslie
A-00488 (A5)	Maize, South Africa	J.F. Leslie
F. sacchari (*G. fujikuroi* MP-B)		
B-00278 (B1)	Sugarcane, Taiwan	J.F. Leslie
B-00281 (B2)	Sugarcane, Taiwan	J.F. Leslie
B-01722 (B3)	Sorghum, Phillippines	J.F. Leslie
B-01724 (B4)	Sorghum, Phillippines	J.F. Leslie
B-01725 (B5)	Sorghum, Phillippines	J.F. Leslie
F. fujikuroi (*G. fujikuroi* MP-C)		
C-m566 (C1)	Rice, Japan	FCCW*
C-m556 (C2)	Rice, Japan	FCCW
C-m567 (C3)	Rice, Japan	FCCW
C-1993 (C4)	Rice, Taiwan	J.F. Leslie
C-1995 (C5)	Rice, Taiwan	J.F. Leslie
C/D-2968**	Laboratory cross	J.F. Leslie
F. proliferatum (*G. fujikuroi* MP-D)		
D-02877 (D1)	Sorghum, Missouri	J.F. Leslie
D-02887 (D2)	Corn stalk, Indiana	J.F. Leslie
D-02945 (D3)	Sorghum, Missisippi	J.F. Leslie
D-02894 (D4)	Maize, Ohio	J.F. Leslie
D-02930 (D5)	Sorghum, Alabama	J.F. Leslie
D-4854 (D6)	Laboratory cross	J.F. Leslie
F. subglutinans (*G. fujikuroi* MP-E)		
E-00731 (E1)	Sorghum, Kansas	J.F. Leslie
E-00507 (E2)	Maize, Kansas	J.F. Leslie
E-00993 (E3)	Maize, Illinois	J.F. Leslie
E-00990 (E4)	Maize, Illinois	J.F. Leslie
E-00551 (E5)	Maize, Kansas	J.F. Leslie
F. thapsinum (*G. fujikuroi* MP-F)		
F-00728 (F1)	Sorghum, Kansas	J.F. Leslie
F-00965 (F2)	Sorghum, Kansas	J.F. Leslie
F-03869 (F3)	Sorghum, South Africa	J.F. Leslie
F-01054 (F4)	Sorghum, Kansas	J.F. Leslie
F-04084 (F5)	Peanut soil debris, Texas	J.F. Leslie
F. nygamai (*G. fujikuroi* MP-G)		
G-01762 (G1)	Laboratory cross	J.F. Leslie
G-01783 (G2)	Laboratory cross	J.F. Leslie
G-11150 (G3)	Grassland root debris, Australia	J.F. Leslie
G-11155 (G4)	Sorghum, Colorado	J.F. Leslie
G-01796 (G5)	Soil, cropped to sorghumSouth Africa	J.F. Leslie
F. circinatum (*G. fujikuroi* MP-H)		
H-6213 (H1)	Pine, South Africa	M.J. Wingfield
H-7438 (H2)	Pine, USA	M.J. Wingfield
F. konzum (*G. fujikuroi* MP-I)		
I-10653 (I3)	Prairie grass, Kansas	J.F. Leslie
I-10595 (I4)	Prairie grass, Kansas	J.F. Leslie
I-10681 (I5)	Prairie grass, Kansas	J.F. Leslie
I-10638 (I6)	Prairie grass, Kansas	J.F. Leslie
I-10678 (I7)	Prairie grass, Kansas	J.F. Leslie
Anamorphs within the *G. fujikuroi* complex		
F. hostae 71576	Hosta	H. Nirenberg
F. foetens 72034	*Begonia*	H. Nirenberg
F. anthophilum 63270	*Euphorbia pulcherrima*	H. Nirenberg

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Table 1 (*continued*)

Strain	Host plant	Source
F. bactridioides BBA63602	*Cronartium conigunum*	H. Nirenberg
F. begoniae BBA72230	*Elatior begonie*	H. Nirenberg
F. globosum BBA70242	Maize	H. Nirenberg
F. lateritium BBA72233	*Calycanthus*	H. Nirenberg
F. miscanthi BBA71315	*Miscanthus sinensis*	H. Nirenberg
F. napiforme BBA70780	*Pennisetum typhoides*	H. Nirenberg
F. udum BBA70731	*Cajanus cajan*	H. Nirenberg
F. nisikadoi BBA71956	Wheat	H. Nirenberg
F. acutatum BBA71935	*Vicia faba*	H. Nirenberg
F. chlamydosporum BBA62171	Soil	H. Nirenberg
Fusarium species outside the *G. fujikuroi* complex		
F. tricinctum BBA71010	*Leonurus sieboldii*	H. Nirenberg
F. solani BBA72084	Sugar beet	H. Nirenberg
F. oxysporum f. sp. *lycopersici* 4287	Tomato	A. Di Pietro
F. poae BBA65613	*Avena sativa*	H. Nirenberg
F. graminearum BBA64884	*Triticum durum*	H. Nirenberg
Nectria heamatococca *44100*	Pea	H. van Etten
F. avenaceum BBA62155	*Triticum aestivum*	H. Nirenberg

Abbreviations: *FCCW, Fungal Culture Collection Weimar, Germany; **C/D-2968 is an isolate that can interbreed with tester strains of *F. fujikuroi* (MP-C) and *F. proliferatum* (MP-D) (J.F. Leslie, pers. communication).

These results suggest that even strains of one species can differ in the composition of GA biosynthetic genes.

In addition, Southern blot analyses were performed for some anamorphs within the *G. fujikuroi* complex. For *F. globosum*, *F. bactridioides*, *F. udum*, *F. miscanthi*, *F. acutatum*, *F. begoniae*, and *F. foetens*, all three genes of the GA gene cluster were detected suggesting the existence of the entire gene cluster (Fig. 2(d), for example after probing with *P450-4*). For *F. napiforme*, a signal was detected only for *P450-3* (data not shown), whereas the genomic DNA of *F. chlamydosporum*, *F. hostae*, and *F. tricinctum* did not hybridize to any of the probes (Fig. 2(d), shown for *P450-4*). Therefore, at least seven fertile and seven anamorphic species contain the entire GA gene cluster and could potentially produce GAs under appropriate conditions.

To find out if *Fusarium* species not belonging to the *G. fujikuroi* species complex contain the entire GA gene cluster or at least parts of it, we performed Southern blot analyses for several strains (Table 1) under stringent and non-stringent (54 °C) hybridization conditions. For none of these *Fusarium* strains were any signals detected for the GA biosynthetic genes.

To examine if the GA biosynthetic genes are expressed under nitrogen starvation conditions (optimum conditions for expression in the GA-producing species *F. fujikuroi*), we performed Northern blot analyses for two strains of each MP. However, despite the existence of the entire gene cluster in seven of the nine MPs, GA genes were expressed only in strains of *F. fujikuroi* and one of the two analyzed *F. konzum* strains, I-3, at a detectable level (Fig. 3, for three genes and one strain from each MP). For the different *F. fujikuroi* strains significant differences in transcript levels were observed. Thus, expression of *des*, *cps/ks* and *P450-3* in strain C-m567 was at least twice as high as in strain C-IMI58289 and more than five times higher than in C-1995 (data not shown). *F. konzum* strain I-3 is the only strain of a species other than *F. fujikuroi*, expressing the GA-biosynthetic genes at detectable levels (Fig. 3).

2.2. Production of GAs by various strains in the G. fujikuroi species complex

We further analyzed strains of those sexually fertile species and anamorphs within the *G. fujikuroi* species complex that contain the entire gene cluster (Table 2) for their ability to produce GAs. All strains were cultivated for 10 days in the optimized production medium OPM. Despite the presence of all seven GA biosynthetic genes in these species, only strains of the species *F. fujikuroi* (MP-C), and surprisingly, one strain of *F. konzum* (I-3) produced GAs (Figs. 4 and 5; Tables 2 and 3) which was found to be in accordance with the results of Northern blot analyses (Fig. 3). The highest amounts of GA_3 (1.24 g/l) were detected in the culture filtrate of strain C m567, which showed also the highest level of gene expression (Fig. 3 and Table 3). The other strains, such as C IMI58289, C1993, and C m566 accumulated less GA_3 and showed less GA gene transcript levels compared to C-m567. The culture fluid of strain I10653 (I-3) of *F. konzum* was shown by GC–MS to contain large amounts of GAs and related metabolites. These include in decreasing order of abundance GA_1, GA_3, fujenoic acid, isoGA_3, GA_{13}, 7β, 18-dihydroxy-kaurenolide, gibberellenic acid, GA_{20}, GA_{25}, GA_{24}, GA_4, GA_{40}, and GA_9 (Fig. 5).

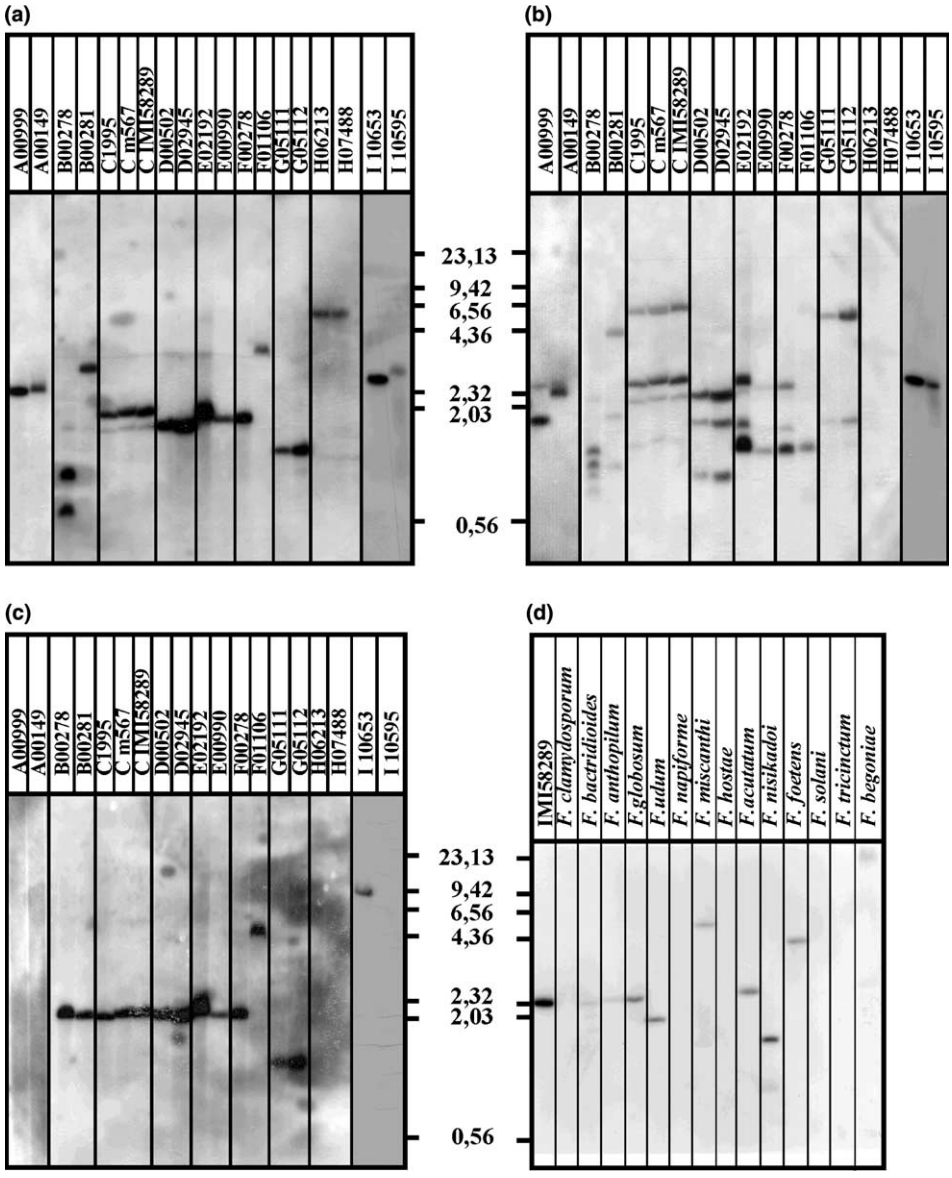

Fig. 2. High stringency Southern blot analysis of all MPs and some anamorphs of the *G. fujikuroi* species complex. The genomic DNA of all strains was restricted by *Hin*dIII and probed with the cDNA-clones of the corresponding genes of *F. fujikuroi* (MP-C). (a) The genomic DNA of two strains of each MP was hybridized to the ^{32}P-labeled *des* gene. (b) The genomic DNA of two strains of each MP was hybridized to the ^{32}P-labeled *P450-4* gene. (c) The genomic DNA of two strains of each MP was hybridized to the ^{32}P-labeled *P450-3* gene. (d) The genomic DNA of the anamorphs was hybridized to the ^{32}P-labeled *P450-4* gene.

Thus, the expression level of GA genes appears to correlate with GA production. For C1995 no GA_3, but significant levels of GA_1 (Fig. 4 and Table 3) and very low amounts of GA_4 and GA_7, were found suggesting a mutation in the desaturase gene, *des*, which catalyzes the formation of GA_7 from GA_4 (Tudzynski et al., 2003).

2.3. Sequence analysis of P450-4 (GA pathway) and cpr

In order to investigate if we could infer identical or similar phylogenetic relationships between members and non-members of the *G. fujikuroi* species complex with the help of two new genetic markers, a dispensable GA biosynthetic gene and a highly conserved gene not directly involved in GA biosynthesis, a comparative sequence analysis was performed.

Nucleotide sequence analysis of fragments of the GA biosynthetic gene *P450-4* encoding the *ent*-kaurene oxidase (Tudzynski et al., 2001) and *cpr* encoding the highly conserved NADPH cytochrome P450 reductase (Malonek et al., 2004) were conducted for five strains each of the sexually fertile and for some anamorphic *Fusarium* species within the *G. fujikuroi* complex. In the case of *cpr*, sequence data from some *Fusarium* species outside the *G. fujikuroi* complex were integrated into this

Table 2
The sexually fertile species (MPs) of the *Gibberella fujikuroi* species complex: host plants, first description, presence of the GA biosynthetic genes and GA production

Anamorph	Teleomorph	Host plant	References	GA cluster genes	GA production
F. verticillioides (*F. moniliforme*)	*G. fujikuroi* MP-A	Maize (rice)	Nelson (1992)	Only *des* and *P450-4*	No
F. sacchari	*G. fujikuroi* MP-B	Sugarcane, sorghum	Leslie (1991)	Entire GA genecluster	No
F. fujikuroi	*G. fujikuroi* MP-C	Rice	Hsieh et al. (1977)	Entire GA gene cluster	Yes
F. proliferatum	*G. fujikuroi* MP-D	Rice, maize, sorghum	Kuhlman (1982)	Entire GA gene cluster	No
F. *subglutinans*	*G. fujikuroi* MP-E	Maize	Nelson (1992)	Entire GA gene cluster	No
F. thapsinum	*G. fujikuroi* MP-F	Sorghum	Klittich et al. (1997)	Entire GA gene cluster	No
F. nygamai	*G. fujikuroi* MP-G	Laboratory crosses	Klaasen and Nelson (1996)	Entire GA gene cluster	No
F. circinatum (*subglutinans* f. sp. *pini*)	*G. fujikuroi* MP-H	*Pinus* spp.	Britz et al. (1999)	Only *des*	No
F. konzum	*G. fujikuroi* MP-I	Prairie grasses	Zeller et al. (2003)	Entire GA gene cluster	Yes (strain I-3) No (other isolates)
F. globosum BBA70242	–	Maize, South Africa	O'Donnell et al. (1998)	Entire GA gene cluster	No
F. bactridioides BBA63602	–	*Pinus* spp. Arizona		Entire GA gene cluster	No
F. udum BBA70731	–	*Cajanus cajan*	O'Donnell et al. (1998)	Entire GA gene cluster	No
F. miscanthi BBA71315	–	*Miscanthus sinensis*	O'Donnell et al. (1998)	Entire GA gene cluster	No
F. acutatum BBA71935	–	*Vicia faba*, Sudan	O'Donnell et al. (1998)	Entire GA gene cluster	No
F. begoniae BBA72230	–	*Elatior begonie*	O'Donnell et al. (1998)	Entire GA gene cluster	No
F. foetens BBA72034	–	*Begonia*	H. Nirenberg, pers. communication	Entire GA gene cluster	No
F. napiforme BBA70780	–	*Pennisetumtyphoides*	O'Donnell et al. (1998)	Only *P450-3*	No
F. nisikadoi BBA71956	–	Wheat, Japan	O'Donnell et al. (1998)	Only *des* and *P450-4*	No
F. anthophilum BBA63270	–	*Euphorbia pulcherrima*	O'Donnell et al. (1998)	Only *des* and *P450-4*	No
F. chlamydosporum BBA62171	–	Soil, Pakistan	O'Donnell et al. (1998)	No GA cluster genes	No
F. hostae BBA71576	–	*Hosta*	O'Donnell et al. (1998)	No GA cluster genes	No
F. tricinctum BBA72019	–	*Leonurus sieboldii*	O'Donnell et al. (1998)	No GA cluster genes	No

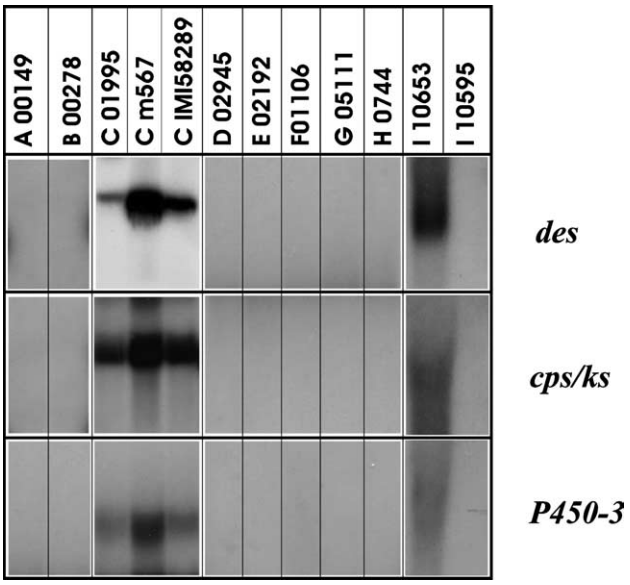

Fig. 3. Northern blot analysis of all MPs (shown for some isolates each), with representative data for *des*, *P450-4* and *P450-3* genes as probes. For RNA analysis, all strains were grown for 5 days in 20% ICI medium.

analysis. The amplified *P450-4*-fragments were between 650 and 654 bp in length and included one intron at a conserved position (see Fig. 6). The *cpr*-fragments were between 820 and 834 bp in length and did not contain any intron. Both amplified products contained highly conserved domains required for the function of the encoded enzymes (Fig. 6) – the heme binding region (P450-4) and FAD binding modules (CPR-GF). *P450-4* fragments have been retrieved with high similarity to the *F. fujikuroi* (84% and 94% on nucleotide level) gene from strains of all MPs except for *F. circinatum* (MP-H). The highest degree of sequence identity (94%) to the *F. fujikuroi P450-4* (MP-C) was omitted for both *F. proliferatum* (MP-D) and *F. sacchari* (MP-B).

We have used the maximum likelyhood method for the phylogenetic analysis of the *P450-4* protein sequences (Fig. 7). The first clade included the isolates of MP-A, -G, and –F belonging to the ''African'' clade according to O'Donnell et al. (1998, 2000). The second or ''American'' clade showed typical strains from MP-E, and -I, as well as *F. begoniae* and *F. bactridioides*, and the third or ''Asian'' clade included isolates of MP-C, -D, and -B, as well as the anamorphic species *F. globosum*. The deep nodes are fairly reliable with a bootstrap value of 94. Five anamorphic species, *F. foetens*, *F. oxysporum*, *F. miscanthi*, *F. nisikadoi*, and *F. hostae*, do not belong to these three clades of species within the *G. fujikuroi* species complex (Fig. 7). As strains of MP-H do not contain *P450-4* homologues in their genome, they could therefore not be included into this analysis.

The percentage of sequence identity for the *cpr*-fragments of all species was even higher (89–99%) than for *P450-4*, and therefore, the phylogenetic tree has been inferred from DNA sequences. Among the *Fusarium* species, the highest similarity to *cpr-Gf* (MP-C) was obtained for *F. proliferatum* (MP-D) (98%). Due to the high degree of sequence conservation for fungal *cpr* genes, we extended the phylogenetic analysis to some *Fusarium* species outside the *G. fujikuroi* species complex (Table 1) and added *cpr* sequences of *F. verticillioides* and *Fusarium graminearum* from the known databases (http://www.tigr.org/tdb/tgi/cw/cwgi2/ and http://www.broad.mit.edu/annotation/fungi/fusarium/). Phylogenetic analysis for the highly conserved *cpr* sequences revealed a tree consistent with those published earlier for other genes (O'Donnell et al., 2000) (Fig. 8). Composition of the clades were similar, but not identical to those obtained for *P450-4*. MPs C, D, and B as well as *F. globosum* remained in one clade (''Asian'' clade), whereas the ''African'' clade contains MP-F, MP-A, and MP-G. MP-I, which was not classified until now, clustered together with MPs E and H and the anamorphs *F. begoniae*, *F. anthophilum*, *F.udum*, *F. acutatum*, and *F. bactridioides* within the third (''American'') clade. As expected, *F. graminearum*, *F. poae*, and *Nectria haematococca* are outgroups of the *G. fujikuroi* species complex. Summarizing the results of phylogenetic analyses, the phylogenetic trees obtained for both, *P450-4* and *cpr* genes are very similar. However, in the *P450-4* tree, one of the MP-D strains, D3, clusters within the MP-C rather than the other MP-D strains.

3. Discussion

In this paper, we present for the first time an overview of the distribution of GA biosynthetic genes, their expression as well as GA production capability of all sexually fertile and several anamorphic species within the *G. fujikuroi* species complex. Interestingly, no strains of any species other than *F. fujikuroi* (MP-C) except for one isolate of *F. konzum* (MP-I; strain I10653) produced GAs, although all seven GA biosynthetic genes were identified in most of the mating populations. According to this, only strains of *F. fujikuroi* and one strain of *F. konzum* expressed the GA biosynthetic genes at a level detectable in Northern blot analysis. Altogether, our group analyzed more than 50 *F. fujikuroi* (MP-C) strains from different origins and culture collections for their ability to produce GAs, and intriguingly all of them produced significant amounts of GAs, although with differences in yield and composition (B. Tudzynski, unpublished).

Recently, we analyzed the GA biosynthetic gene cluster of *F. proliferatum* (MP-D) in more detail (Malonek et al., 2005a,b). This species is very closely related to *F. fujikuroi* according to phylogenetic trees based on the similarity of sequenced loci (Bacon et al., 1996;

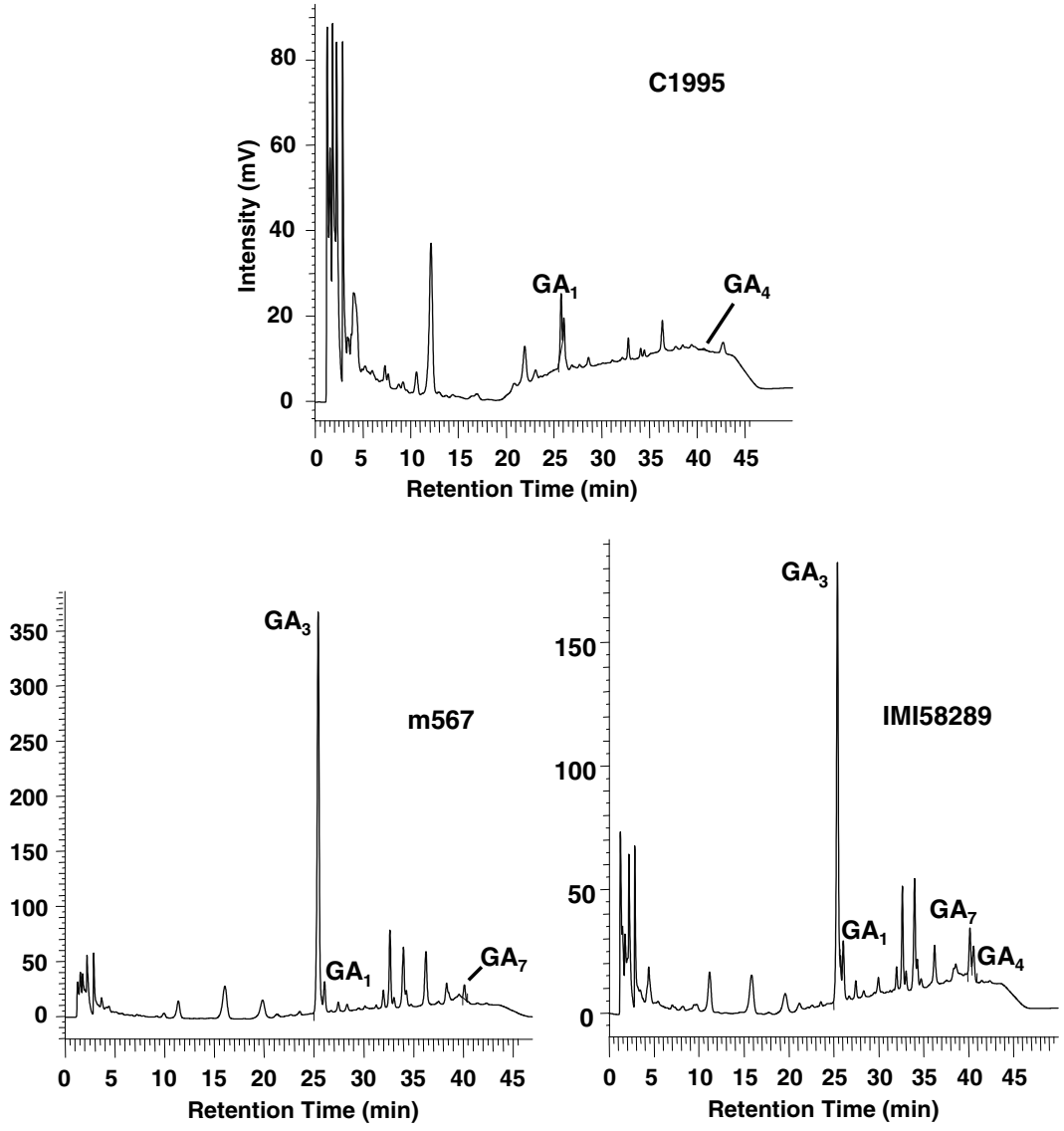

Fig. 4. GA analysis by HPLC in purified culture filtrates from strains of MP-C incubated in OPM for 10 days.

Steenkamp et al., 1999, 2000; O'Donnell et al., 1998, 2000; Proctor et al., 2004; this study). The organization of the GA biosynthetic gene cluster appeared to be identical to that in *F. fujikuroi*, and the overall level of sequence similarity of the GA biosynthetic genes is about 94%. However, the genes are not or only slightly expressed. Results further indicate that one reason for the loss of GA production is the accumulation of several mutations in the coding and 5′-non-coding regions of different GA cluster genes rather than any defect in the GA regulatory system in *F. proliferatum* (Malonek et al., 2005a).

In this study, we demonstrated that beside *F. proliferatum*, most of the other sexually fertile and analyzed anamorphic species of the *G. fujikuroi* species complex also contain the entire GA gene cluster or at least some of the genes. However, as in *F. proliferatum*, the GA biosynthetic genes are neither expressed under nitrogen starvation conditions nor by addition of plant compounds such as plant oil or cornsteep solids, both optimal conditions for GA production in *F. fujikuroi* (Tudzynski, 1999). Thus, we suggest that the non-detectable expression level for the GA biosynthetic genes and the inability to produce any GAs in all these species of the *G. fujikuroi* complex is probably due to several mutations in the 5′-non-translated as well as in the coding regions, as it is the case in *F. proliferatum* although defects in the GA regulation system in those strains cannot be fully excluded (Malonek et al., 2005a,b). However, we found one exclusion: *F. konzum* strain I10653 (MP-I), in contrast to the other strains of the same species and all tested isolates of the other species, expressed the GA-biosynthetic genes under nitrogen-limiting conditions and produced GA_3, GA_1, GA_4, and several other GAs and

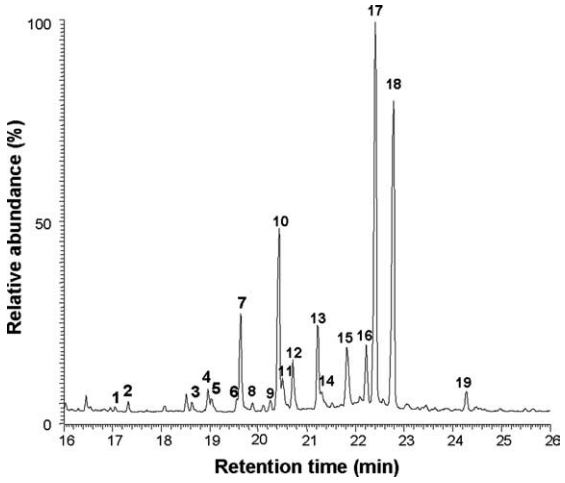

Fig. 5. GC–MS analysis of the culture filtrate of *F. konzum* strain I-3 (*G. fujikuroi* MP-I). The total ion current is shown for an ethyl acetate extract after derivatization to methyl esters trimethylsilyl ethers. Peak identities after comparison of the mass spectra with published data (Gaskin and MacMillan, 1992) are as follows: (**1**) GA_9; (**2**) dehydroallogibberic acid; (**3**) GA_4 diacid; (**4**) GA_{25}; (**5**) GA_{24}; (**6**) GA_{20}; (**7**) dioctylphthalate (present as impurity); (**8**) GA_4; (**9**) GA_{40}; (**10**) fujenoic diacid; (**11**) fujenoic triacid: (**12**) gibberellinic acid; (**13**) GA_{13}; (**14**) GA_{36}; (**15**) *iso*GA_3; (**16**), 7β, 18-dihydroxykaurenolide; (**17**) GA_1; (**18**) GA_3; (**19**) 3-*epi*-GA_1.

intermediates in detectable amounts. Interestingly, this is the only strain of this species containing the gene *P450-3* in its GA gene cluster. All the other MP-I strains analyzed so far have only six of the GA biosynthetic genes in their genome, and they do not express these genes and do not produce any GAs. The reason for these differences between isolates of one species are not clear.

Several other secondary metabolites, such as fumonisins, fusaric acid, fusaproliferin, beauvericin, and moniliformin, are produced by different species within and some of them even outside the *G. fujikuroi* complex (Desjardins et al., 1997; Bacon et al., 1996; Proctor et al., 2004; Reynoso et al., 2004). In contrast, the existence of the GA gene cluster is restricted to species inside the *G. fujikuroi* species complex. Thus, we suggest that the GA gene cluster developed or was acquired by horizontal gene transfer late in the evolution of the genus *Fusarium*.

So far, only for fumonisins (Proctor et al., 2004) and GAs (Malonek et al., 2005a,b; this study) the phylogenetic analysis of secondary metabolite production in the *G. fujikuroi* species complex has been successfully combined with molecular studies in order to show whether non-producing species of *Fusarium* lack the biosynthetic genes or if these genes are non-functional. The fact that from all these species that have the entire gene cluster, only one species, the rice pathogen *F. fujikuroi*, does produce GAs, let us speculate that GAs play an important role in the process of infecting and/or invading rice seedlings. Despite the presence of the GA gene cluster, none of the isolates (except for *F. konzum* strain I10653) of the other species within the complex, preferentially isolated from other host plants, were found to produce GAs. In contrast, all so far analyzed rice isolates of the species *F. fujikuroi* (MP-C) produced significant amounts of biologically active GAs without any exclusion. Up to now, nothing is known about the relevance or necessity of GA production by *F. fujikuroi* for pathogenicity or virulence of the strains. Microarray analysis of GA-regulated gene expression in rice seedlings showed that GAs influence growth and development by coordinately regulating the expression of specific groups of genes (Yang et al., 2004). As for these experiments only low amounts of GAs were applied to the rice seedlings, it would be interesting to look for

Table 3
GA production of some isolates of MP-C in the *Gibberella fujikuroi* species complex in the GA production medium OPM after 10 days

MP/strain	GA_3 (mg/ml)*	GA_7 (mg/ml)*	GA_4 (mg/ml)*	GA_1 (mg/ml)*
C1995	<0.001	<0.001	<0.01	**0.580**
C-m567	**1.24**	0.05	<0.01	<0.01
C-IMI58289	**0.61**	0.08	0.045	<0.01
C1993	**0.28**	0.03	0.025	<0.001
C-m566	**0.45**	0.12	0.023	<0.001

* All data are average amounts from three independent samples.

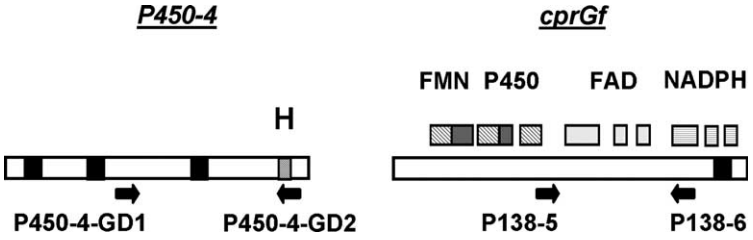

Fig. 6. Schematic genetic map of *P450-4* and *cpr-Gf*. Primer positions are indicated as arrows, important functional domains of *cpr-Gf* (FAD, FMN, P450, NADPH) as well as for *P450-4* (H, heme binding domain) are shown as rectangles.

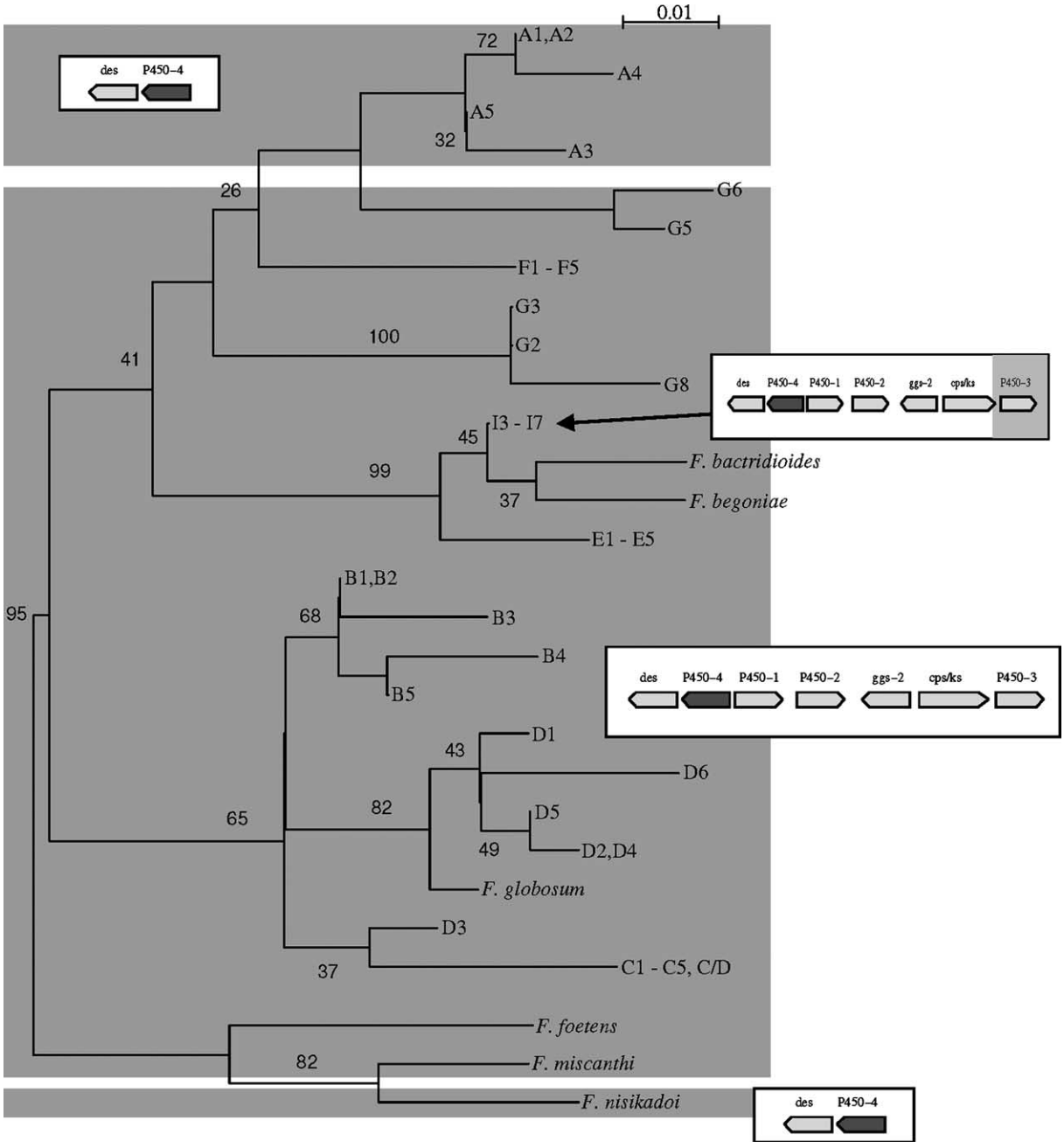

Fig. 7. Phylogeny of the GA biosynthetic gene *P450-4* (maximum likelihood for protein sequences). Numbers at the nodes are bootstrap values (100 replicates). Branch lengths were inferred using consensus bootstrapped tree. All species except for *F. nisikadoi* and MP-A (two genes) consist of the entire GA gene cluster. MP-H contains only one gene, *des*, and could not be involved in this phylogenetic analysis. The abbreviations for the strains are explained in Table 1. The full or partial existence of the GA biosynthetic gene cluster is shown.

gene expression patterns after application of higher GA concentrations comparable to those produced by the fungus on the plant.

Both phylogenetic trees (Figs. 7 and 8) were in good concordance with those based on other conserved sequence markers (O'Donnell et al., 1998, 2000; Steenkamp et al., 1999, 2000), even though the sequence fragments were quite short and highly similar. The recently described sexually fertile species *F. konzum* (*G. fujikuroi* MP-I) (Zeller et al., 2003) was phylogenetically analyzed for the first time and shown to cluster within the American clade together with MPs E and H. Reassuringly, the topologies of the trees for *cpr* (maximum likelihood, DNA) and *P450-4* (maximum likelihood, protein) were almost identical. The GA producers *F. fujikuroi* (MP-C), clusters in the "Asian" clade together with *F. sacchari* (MP-B), and *F. proliferatum* (MP-D), which is consistent with previous reports for other genes,

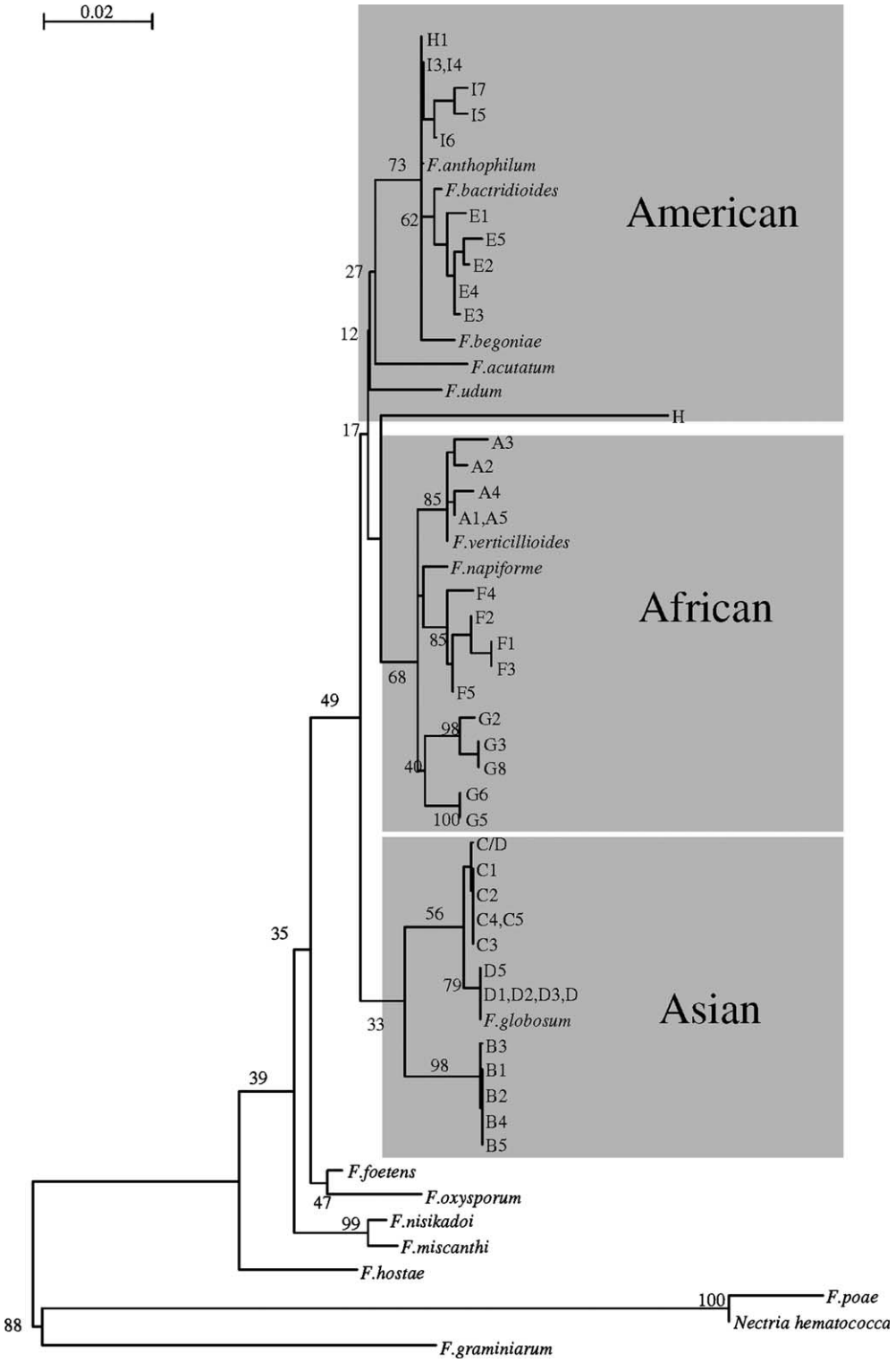

Fig. 8. Phylogeny of the *cpr* genes of *Fusarium* species within and outside the *G. fujikuroi* species complex (maximum likelihood for DNA sequences). Numbers at the nodes are bootstrap values (100 replicates). Branch lengths were inferred using consensus bootstrapped tree. The abbreviations for the strains are explained in Table 1.

e.g. histone H3 and MAT-genes, 28S rDNA, mtSSU, β-tubulin, translation initiation factor EF-1α, and calmodulin (Steenkamp et al., 1999, 2000; O'Donnell et al., 1998, 2000). However, there is also one remarkable difference between the *P450-4* tree and the tree inferred from *cpr*: in the case of the *P450-4* gene, the strain D3 is more closely related to strains of MP-C than to all other MP-D strains (Fig. 7). This grouping has been confirmed by

other phylogeny methods, such as parsimony (data not shown). Recent studies of interfertility between members of MP-C and MP-D showed that these two mating populations are indeed very closely related and may exchange genetic material under field conditions (Leslie et al., 2004). Strain D6 (*F. proliferatum* D4854) and C/D2968 are two examples of those strains that can interbreed with strains of both species, *F. fujikuroi* and *F. proliferatum* (J.F. Leslie, personal communication).

Moreover, the *cpr-Gf* sequence marker was proven to be a useful tool, not only for deciphering members of the *G. fujikuroi* species, but also of other *Fusarium* species, e.g. *F. graminearum*, *F. oxysporum*, *F. poae*, and *F. solani*. On the other hand, the *P450-4* gene and probably also the other GA biosynthetic genes are solely specific markers for identification of species belonging to the *G. fujikuroi* complex, and for distinguishing between these species. The conservation of the *P450-4* sequences is remarkable, considering that the entire GA biosynthetic gene cluster is not present in all species and that the majority of the species lost the ability to produce GAs. The sequenced fragments contain one intron. The mutations accumulate in the intron, which suggests that during evolution there was a selective pressure against mutations within the exons. Consequently, it is very likely that the gene is still functional in some way.

One of the most surprising results of our study is that the GA biosynthetic gene cluster is present in almost all species within the *G. fujikuroi* species complex with only some exceptions: Strains of the species *F. verticillioides* (MP-A), *F. anthophilum*, and *F. nisikadoi* have only two cluster genes, *des* and *P450-4*, whereas strains of *F. circinatum* (MP-H) and *F. napiforme* each have only one gene left, *des* and *P450-3*, respectively. Isolates of the recently identified species *F. konzum* may have all seven GA biosynthetic genes or only six of them by missing the right border gene *P450-3*.

In contrast to the species within the complex, none of the other *Fusarium* species analyzed in this study contained any of the GA biosynthetic genes. In accordance with our Southern blot analysis, no putative homologues of GA-specific genes, such as *cps/ks* encoding the key enzyme of the GA biosynthetic pathway, were found in the recently sequenced genome of *F. graminearum*. The lack of the GA biosynthetic genes in *F. graminearum* and other species outside the *G. fujikuroi* complex raises questions about the origin of the GA gene cluster, especially since Hedden et al. (2002) showed that horizontal transfer of the GA biosynthetic genes from higher plants to the fungus (Chapman and Regan, 1980) was highly unlikely. Our results let us speculate that the GA biosynthetic gene cluster was aquired very late in the evolution of the genus *Fusarium* since only species within the *G. fujikuroi* species complex contain the entire cluster or parts of it. On the other hand, two fungal species, *Sphaceloma manihoticola* and *Phaeosphaeria* sp. (MacMillan, 2002), not closely related to *Fusarium* can produce GAs (Rademacher, 1992; Kawaide and Sassa, 1993). 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 (Toyomasu et al., 2004). This gene cluster contains two P450 monooxygenase genes and a *cps/ks*-like bifunctional diterpene cyclase gene, encoding aphidicolan-16β-ol synthase. Interestingly, the diterpene cyclase gene acts as functional unit together with a pathway-specific GGDP synthase gene as it is the case in the GA biosynthetic pathway. All these data lead us to suggest 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. One or more P450 monooxygenase-encoding genes are additional typical components of these clusters. The conserved intron positions in the known diterpene cyclase genes, as well as the functional unit with a pathway-specific *ggs* gene let us propose that these gene units were distributed to different fungi from one ancestor by horizontal gene transfer.

Sequencing of increasing numbers of fungal genomes revealed the presence of similar secondary metabolite gene clusters in several distantly related ascomycetes suggesting their phylogenetic origin from a putative parental gene cluster (Gardiner et al., 2004). Whether these parental clusters were distributed by horizontal gene transfer only to species of the *G. fujikuroi* complex, or by vertical transmission and subsequent gene loss in species that do not contain homologous genes is unknown. Thus, the recent identification of the GA biosynthetic genes in *F. fujikuroi* provides new prospects to the study of the evolutionary origin of the GA biosynthetic gene cluster in the *G. fujikuroi* species complex and in fungi in general.

4. Experimental

4.1. Fungal strains

The strains used in this study were provided by J.F. Leslie, Kansas State University, USA, H. Nirenberg, Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany, and M.J. Wingfield, University of Pretoria, South Africa, H. van Etten, University of Arizona, and A. DiPietro, University of Cordoba, Spain (Table 1).

4.2. Bacterial strains and plasmids

Escherichia coli strain Top10 (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation.

Vector pCR2.1 (Invitrogen) was used to clone PCR fragments carrying *G. fujikuroi P450-4* and *cpr* genes of all MPs.

4.3. Media and culture conditions

For DNA isolation, fungal strains were grown on cellophane sheets (Alba Gewürze, Bielefeld, Germany) put on CM agar (Pontecorvo et al., 1953) for 3 days at 28 °C. The mycelium was harvested, frozen in liquid nitrogen, and lyophilized for 24 h. The lyophilized mycelia were ground to a fine powder with a mortar and pestle. For RNA isolation, fungal strains were grown in 20% ICI medium (Geissman et al., 1966), containing 8% glucose, 0.5% $MgSO_4$, 0.1% KH_2PO_4, and 5.0, 1.0 or 0 g/l NH_4NO_3. For GA production, the strains were grown for 7–10 days on a rotary shaker (200 rpm) at 28 °C in 300-ml Erlenmeyer flasks containing 100 ml of either 20% ICI or optimized production medium (OPM), containing 6% sunflower oil, 0.05% $(NH_4)_2SO_4$, 1.5% corn-steep solids (Sigma–Aldrich, Taufkirchen, Germany), and 0.1% KH_2PO_4.

4.4. DNA and RNA isolation

Genomic DNA was isolated from lyophilized mycelium as described by Doyle and Doyle (1990). Plasmid DNA was extracted with Genomed columns following the manufacturer's protocol (Genomed, Bad Oeynhausen, Germany). RNA was isolated using the RNAgents total RNA isolation kit (Promega, Mannheim, Germany).

4.5. PCR

PCRs contained 25 ng DNA, 10 ng of each primer, 0.2 mM dNTPs and 2 U *Taq* polymerase (Red Taq, Sigma–Aldrich, Deisenhofen, Germany) in 50 µl. PCR was carried out at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The resulting PCR-fragments of the *P450-4* and *cpr* genes of the *G. fujikuroi* MPs A-H were cloned into pCR2.1 (Invitrogen, Groningen, The Netherlands) and sequenced. The following primers were used:

cpr:	P138-5	5′-AAC CCC TAC ATT GCC CCT ATC-3′
	P138-6	5′-TCG GCA ACC AAA GAA CAA GAG TG-3′
P450-4:	P450-4-GD1	5′-TTT CTC GGT CCA GAG CAC TGC CGC-3′
	P450-4-GD2	5′-CGT GGT CTT CCT TTC CCA TCT GGC-3′

4.6. Southern blot analysis

After digestion with *Hin*dIII and electrophoresis, genomic DNA was transferred to Hybond N^+ filters (Amersham–Pharmacia, Freiburg, Germany). ^{32}P-labelled cDNA fragments of all seven GA biosynthetic genes were used as probes. Filters were hybridized at 65 °C in 5× Denhardt's solution containing 5% dextran sulfate. Filters were washed at 65 °C in 2× SSPE, 0.1% SDS and 1× SSPE 0.1% SDS. Northern blot hybridizations were made as previously described (Church and Gilbert, 1984). The *G. fujikuroi* rDNA gene was used as a control to confirm RNA transfer.

4.7. Sequencing

Recombinant plasmid clones were sequenced with an automatic sequencer "LI-COR 4000" (MWG Biotech, München, Germany). Both strands of overlapping subclones obtained from the genomic DNA were sequenced by using the universal and reverse primers or specific oligonucleotides obtained from MWG Biotech. DNA and protein sequence alignments were made with DNA Star (Madison, WI).

4.8. Phylogenetic analysis

Phylogenetic analysis of the *cpr* and *P450-4* genes was done with the PHYLIP package (Felsenstein, 1989) using the bootstrapped (100 replicates) maximum likelihood method. Analysis was done with bootstrapped (100 replicates) maximum likelihood method. For the *P450-4* genes, protein sequences have been derived from the sequenced PCR products after excising the intron sequences. Redundant (identical) sequences were removed. The *cpr* sequences were, however, highly similar, and therefore the phylogeny has been inferred from DNA sequences. Consensus tree was calculated from the bootstrap replicas, and branch lengths for this tree were obtained using again proml and dnaml programs from the PHYLIP package, respectively. The results have been confirmed using the parsimony method (data not shown, all input sequences and trees can be found in supplementary material on the web page http://www.uni-muenster.de/Biologie.Botanik/ebb/projects/fusarium/).

4.9. Gibberellin assays

GA_3, GA_1, GA_4, and GA_7 in the culture fluids of all strains were analyzed by HPLC (Barendse et al., 1980) with a Merck HPLC system with a UV detector and a Lichrospher 100 RP18 column (5 µm; 250 × 4 mm). The GA_1 standard was provided by Dr. P. Hedden, Rothamsted Research (UK). GA_3, GA_4, and GA_7 reference compounds were provided by Agtrol (Houston, Texas). GC–MS analysis was performed as described in Gaskin and MacMillan, 1992.

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