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MINI-REVIEW

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Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology

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Abstract Gibberellins (GAs) constitute a large family of tetracyclic diterpenoid carboxylic acids, some members of which function as growth hormones in higher plants. As well as being phytohormones, GAs are also present in some fungi and bacteria. In recent years, GA biosynthetic genes from *Fusarium fujikuroi* and *Arabidopsis thaliana* have been cloned and well characterised. Although higher plants and the fungus both produce structurally identical GAs, there are important differences indicating that GA biosynthetic pathways have evolved independently in higher plants and fungi. The fact that horizontal gene transfer of GA genes from the plant to the fungus can be excluded, and that GA genes are obviously missing in closely related *Fusarium* species, raises the question of the origin of fungal GA biosynthetic genes. Besides characterisation of *F. fujikuroi* GA pathway genes, much progress has been made in the molecular analysis of regulatory mechanisms, especially the nitrogen metabolite repression controlling fungal GA biosynthesis. Basic research in this field has been shown to have an impact on biotechnology. Cloning of genes, construction of knock-out mutants, gene amplification, and regulation studies at the molecular level are powerful tools for improvement of production strains. Besides increased yields of the final product, GA_3, it is now possible to produce intermediates of the GA biosynthetic pathway, such as *ent*-kaurene, *ent*-kaurenoic acid, and GA_{14}, in high amounts using different knock-out mutants. This review concentrates mainly on the fungal biosynthetic pathway, the genes and enzymes involved, the regulation network, the biotechnological relevance of recent studies, and on evolutionary aspects of GA biosynthetic genes.

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Introduction

The fungus *Fusarium fujikuroi* has played a crucial role in gibberellin (GA) research for more than 100 years. GAs were first identified as secondary products of the rice pathogenic fungus causing overgrowth symptoms ("bakanae" disease) in rice seedlings (see Phinney 1983 for review). The compound GA_3 was first isolated by Japanese scientists (Cross et al. 1959). The ability of GA_3 to restore normal growth of plant dwarf mutants (Lang 1956), and the occurrence of GA_3-like substances in higher plants (Radley 1956) prompted the suggestion that GAs are natural plant hormones regulating growth and development in higher plants.

The biosynthesis of GAs has been investigated for many years—as soon as the structure of GA_3 was known—both in the fungus and in higher plants. Birch and coworkers determined incorporation patterns from ^{14}C-labelled acetate and mevalonate into GA_3 in fungal cultures and concluded that GAs were diterpenoid compounds (Birch et al. 1958, 1959). At that time, several GA-deficient mutants of *F. fujikuroi* were available in which GA biosynthesis is blocked at different points of the pathway. Such mutants played an important role in the discovery of major steps of GA biosynthesis. Potential intermediates were isolated from cultures of these mutants and used to demonstrate their incorporation into GA_3 without the requirement for isotopically labelled substrates. The most famous mutant obtained by UV mutagenesis, B1-41a, has a genetic block in *ent*-kaurene oxidation (Bearder et al. 1974). Other mutants, such as R9 (Bearder 1983) and SG123 and SG133 (Avalos et al. 1997, 1999), have a defect in C-13 hydroxylation and secrete mainly GA_7 instead of GA_3. These feeding experiments allowed determination of the basic biosynthetic pathway to GA_3 in the 1960s and early 1970s. The development of combined gas chromatography-mass spectrometry (GC-MS) for GA analysis at about this time was an essential pre-requisite for identification of intermediates and products of fungal and plant GA biosynthesis (Binks et al. 1969; MacMillan et al. 1967).

GA research in higher plants profited from successful experiments with cultures of *F. fujikuroi*, but advanced more slowly. The plant biosynthetic pathway was established mostly by using cell-free systems from developing seeds, particularly those from *Marah macrocarpus* (Graebe et al. 1965), *Cucurbita maxima* (pumpkin) (Graebe et al. 1974), and *Pisum sativum* (garden pea) (Kamiya and Graebe 1983). This work enabled the determination of GA pathways in these organisms, characterisation of the enzymes involved and, finally, cloning of the corresponding genes in the 1990s (reviewed in MacMillan 1997). Almost all GA biosynthetic genes in *Arabidopsis thaliana* have now been identified following the sequencing of the *Arabidopsis* genome. However, despite the initial identification of GAs, and determination of the main biosynthetic steps first in the fungus *F. fujikuroi*, there had been little advance in the fungal system for almost 20 years. The breakthrough came with the discovery that the genes for the GA biosynthetic pathway were clustered on the *F. fujikuroi* genome (Tudzynski and Hölter 1998). Since then, all pathway genes have been cloned, and the character of the corresponding enzymes has been identified. On the basis of recent data from the fungus and from higher plants, it is now possible to compare GA biosynthetic genes and genome organisation, the character of the encoded enzymes, pathway steps, and regulation of gene expression in both systems. The profound differences between *F. fujikuroi* and higher plants on the one hand, and the widespread occurrence of GAs in plants, different fungi, and some bacteria on the other, raise questions regarding the evolutionary origin of GA biosynthetic genes.

This article updates a previous review (Tudzynski 1999), and concentrates on recent progress in characterisation of the fungal GA pathway, regulation of gene expression, and evolutionary aspects of GA biosynthetic genes.

The *G. fujikuroi* species complex and GA production capability

Fusarium fujikuroi belongs to the *Gibberella fujikuroi* (Sawada) species complex, which contains species from the *Fusarium* sections *Liseola* and *Elegans*. This species complex is composed of at least nine reproductively isolated biological species (mating populations) denoted by the letters A through I, and 32 additional asexual species (Britz et al. 1999; Kerényi et al. 1999; Leslie 1995; Marasas 2001; Nirenberg and O'Donnell 1998; Nirenberg et al. 1998; O'Donnell et al. 1998; Zeller et al. 2003). The elevation of the mating populations to distinct species was supported by DNA sequence analysis of multiple unlinked loci (Nirenber and O'Donnell 1998; Nirenberg et al. 1998; O'Donnell et al. 1998; Steenkamp et al. 1999; Yun et al. 2000).

All these *Fusarium* species are important fungal pathogens of various crops such as maize, rice, barley, sugarcane, pine, mango, pineapple, sorghum, and many more (Leslie and Plattner 1991; Leslie 1995, 1996; Leslie et al. 2004). Moreover, these species differ in their ability to produce secondary metabolites such as fumonisins (Desjardins et al. 1995, 2000; Kedera et al. 1999; Leslie et al. 1992; Proctor et al. 1999), fusaric acid (Bacon et al. 1996); beauvericin (Logrieco et al. 1998; Torres et al. 2001), moniliformin (Leslie et al. 1996; Desjardins et al. 2000), fusarins (Wiebe and Bjeldanes 1981; Song et al. 2004), the pigments neurosporaxanthin and bikaverin (Linnemannstöns et al. 2002a,b), and GAs (El-Bahrawi 1977; Tudzynski and Hölter 1998; Desjardins et al. 2000).

So far, the ability to produce GAs and to cause "bakanae" disease has been confirmed only for rice isolates belonging to the species *F. fujikuroi* (sexual stage: *G. fujikuroi* MP-C). However, some strains of MP-A (anamorph *F. verticillioides*) and MP-D (anamorph *F. proliferatum*) have also been isolated from rice in different geographic regions (Desjardins et al. 1997). It is not yet clear if these *Fusarium* species are also associated with the symptoms of "bakanae" disease, or are present only as saprophytes. Only now, since the genes in the GA biosynthetic pathway have been cloned and characterized from *F. fujikuroi* (*G. fujikuroi* MP-C), can we determine if members of the other MPs of the *G. fujikuroi* species complex also contain these genes, and if they are organised in a similar gene cluster. Recently, it was found that all MPs of the *G. fujikuroi* species complex, except for *F. verticillioides* (MP-A) and *F. circinatum* (MP-H), contain the entire GA biosynthetic gene cluster but are not able to produce GAs due to several mutations in the coding and non-coding regions of the GA biosynthetic genes (S. Malonek and B. Tudzynski, unpublished). Experiments aimed at restoring GA production capability in *F. verticillioides* and *F. proliferatum* by complementing the mutated or missing pathway genes with the corresponding genes from *F. fujikuroi* are underway.

The GA biosynthetic pathway in *F. fujikuroi*

The GA biosynthetic pathways in plants and *F. fujikuroi* have been summarised in many reviews, most recently by MacMillan (1997), Tudzynski (1999), and Hedden et al. (2001).

GAs, like other diterpenoids, are produced from hydroxymethylglutaryl (HMG) coenzyme A via mevalonic acid, isopentenyl diphosphate, geranyldiphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP), which is a precursor not only for GAs, but also for the carotenoid neurosporaxanthin (Domenech et al. 1996; Linnemannstöns et al. 2002a) and ubiquinones. *Ent*-kaurene, the first GA-specific intermediate, is produced in two cyclisation steps from GGDP via *ent*-copalyl diphosphate (CPP). Sequential oxidation of *ent*-kaurene at C-19 via *ent*-kaurenol and *ent*-kaurenal yields *ent*-kaurenoic acid, which is further oxidised to *ent*-7α-hydroxykaurenoic acid. A final oxidation at C-6β, resulting in contraction of ring B, leads to formation of GA_{12}-aldehyde (Fig. 1). These first steps of the pathway

are identical in the fungus and in higher plants. After GA_{12}-aldehyde, the pathways in higher plants and *F. fujikuroi* differ. In *F. fujikuroi*, GA_{12}-aldehyde is first 3β-hydroxylated to GA_{14}-aldehyde, which is then oxidized at C-7 to form GA_{14} (Hedden et al. 1974; Urrutia et al. 2001). GA_{14} is then converted to the 19-carbon gibberellin GA_4 by 20-oxidation. GA_4, the first biologically active GA, is desaturated to GA_7, which is then converted to GA_3 by late 13-hydroxylation. GA_1 is formed in a minor side reaction by 13-hydroxylation of GA_4 (Fig. 1).

In plants, GA_{12}-aldehyde is converted to GA_{12}, which is either oxidised at C-20 to form the 19-carbon gibberellin, GA_9, or is first 13-hydroxylated to GA_{53}, which is then oxidized at C-20 to yield GA_{20} (Fig. 2). GA_9 and GA_{20} are formed in parallel pathways, both involving oxidation of C-20 to alcohol and aldehyde, and the final formation of biological active 19-carbon GAs by loss of C-20. In plants, the oxidation and removal of C-20 is catalysed by a multifunctional GA 20-oxidase (Lange et al. 1994; Phillips et al. 1995). At the end of the pathway, both GA_9 and GA_{20} are converted to GA_4 and GA_1, respectively, by introduction of a 3β-hydroxyl group.

Thus, a major difference between the GA pathways in *F. fujikuroi* and plants is the stage at which the hydroxyl groups are introduced. In the fungus, GA_{12}-aldehyde is 3β-hydroxylated to GA_{14}-aldehyde, whereas in plants GA_{12}-aldehyde is converted to GA_{12}, which is then 13-hydroxylated to GA_{53} (Fig. 2). In fungi, 13-hydroxylation takes place only in the final step to form GA_3 from GA_7, whereas in plants the final step is the 3β-hydroxylation of GA_9 and GA_{20} to GA_4 and GA_1, respectively (Fig. 2).

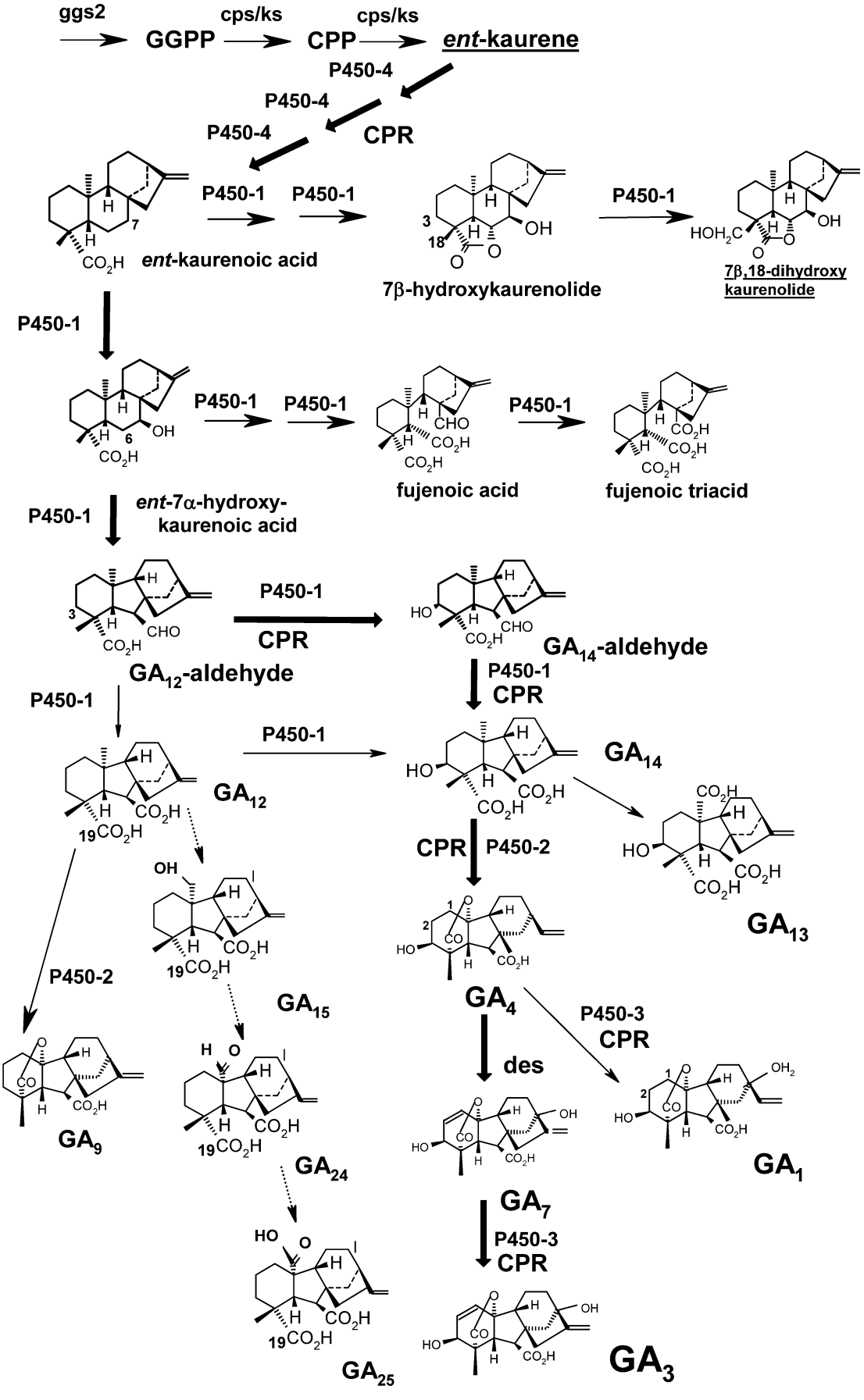
GA biosynthetic genes in *F. fujikuroi*

The main isoprenoid genes and enzymes involved in GA biosynthesis are highly similar in plants and the fungus. In both systems mevalonic acid is converted to GGDP via HMG, GDP and FDP. The corresponding genes are highly conserved in plants, animals and fungi, and were cloned easily from *F. fujikuroi* (Table 1). All three genes, encoding HMG-CoA reductase (*hmg*; Woitek et al. 1997), FDP synthase (*fpps*; Homann et al. 1996), and GGDP synthase (*ggs1*; Mende et al. 1997), are constitutively expressed: light, pH, and the concentration of nitrogen or carbon sources do not affect their transcription level (Mende et al. 1997, Woitek et al. 1997, Homann et al. 1996).

In contrast to the central isoprenoid pathway genes, the genes encoding enzymes that catalyse later steps in the pathway have only a weak similarity to those in plants. Therefore, only one gene encoding the *ent*-copalyldiphosphate/*ent*-kaurene synthase CPS/KS has been cloned by PCR on the basis of sequence data from plant genes (Tudzynski et al. 1998). The homologous *cps*/*ks* gene had already been cloned previously from another GA-producing fungus, *Phaeosphaeria* sp. L487 (Kawaide et al. 1997).

Table 1 Genes of the gibberellin (GA) biosynthetic gene cluster and adjacent genes: function and regulation. *HMG-CoA* Hydroxymethylglutaryl coenzyme A, *GDPP* geranylgeranyl diphosphate

Gene	Enzyme function	Regulation of gene expression	Reference
General isoprenoid pathway			
hmgr	HMG-CoA reductase	Constitutive expression	Woitek et al. (1997)
fpps	Farnesyl diphosphate synthase	Constitutive expression	Homann et al. (1996)
ggs (*ggs1*)	General GGDP-synthase, primary metabolism	Constitutive expression	Mende et al. (1997)
GA biosynthetic gene cluster			
ggs2	GA-specific GGDP-synthase	AREA-control	Tudzynski and Hölter (1998)
cps/ks	Bifunctional *ent*-copalyl-*ent*-kaurene synthase	AREA- control	Tudzynski et al. (1998)
P450-4	*ent*-kaurene oxidase (P450 monooxygenase)	AREA- control	Tudzynski et al. (1999)
P450-1	Multifunctional cytochrome P450 monooxygenase, GA_{14}-synthase	AREA- control	Rojas et al. (2001)
P450-2	GA 20-oxidase, oxidative elimination of the carbon 20 (P450 monooxygenase)	AREA- control	Tudzynski et al. (2002)
des	GA_4 1.2-desaturase, conversion of GA_4 to GA_7	AREA- control	Tudzynski et al. (2003)
P450-3	13-hydroxylase, conversion GA_7 to GA_3	No N-metabolite repression	Tudzynski et al. (2003)
smt	Sugar membrane transporter upstream of the gene cluster	Weak nitrogen regulation	Voß et al. (2001)
orf1, *orf2 mfs*	Genes of unknown function (downstream of the gene cluster)	Constitutive expression	B. Tudzynski, unpublished


ggs2
cps/ks
cps/ks
GGPP
CPP
ent-kaurene
P450-4
P450-4
P450-4
CPR
P450-1
P450-1
P450-1
ent-kaurenoic acid
7β-hydroxykaurenolide
7β,18-dihydroxy kaurenolide
P450-1
P450-1
P450-1
P450-1
fujenoic acid
fujenoic triacid
P450-1
ent-7α-hydroxy-kaurenoic acid
P450-1
CPR
GA12-aldehyde
GA14-aldehyde
P450-1
CPR
P450-1
P450-1
GA12
GA14
CPR
P450-2
GA13
P450-2
GA15
GA4
P450-3
CPR
des
GA9
GA24
GA1
GA7
P450-3
CPR
GA25
GA3

◀ **Fig. 1** Gibberellin (GA)-biosynthetic pathways in *Fusarium fujikuroi*, indicating reactions of the main (3β-hydroxylated) and the minor (non-hydroxylated) pathways as well as side reactions catalysed by the enzyme P450-1. All reactions affected in the *cpr-Gf* mutant and dependent on cytochrome P450 oxidoreductase (*CPR*) are marked. *Dotted arrows* Proposed sequence of reactions. *GGPP* Geranylgeranyl diphosphate, *CPPent*-copalyl diphosphate

The next pathway-specific gene of *F. fujikuroi*, *P450-1*, was cloned via a differential cDNA screening approach under conditions of nitrogen starvation/nitrogen excess. A second P450 monooxygenase gene, *P450-2*, has been identified on the same genomic fragment (Tudzynski and Hölter 1998), suggesting the possibility of clustering of some or all GA pathway genes. It is well known that genes involved in biosynthesis of secondary metabolites, such as aflatoxins and penicillin, are often organised in gene clusters in filamentous fungi (Keller and Hohn 1997). In order to identify more potential GA biosynthetic genes upstream and downstream of *P450-1* and *P450-2*, a chromosome walking approach was used. Altogether, a genomic region of about 40 kb was cloned and sequenced in *F. fujikuroi* m567 (Tudzynski and Hölter 1998; Voss et al. 2001; Tudzynski et al. 2003; B. Tudzynski, unpublished results). An overview of all genes cloned is given in Table 1 and Fig. 3.

The next gene downstream of *P450-2* is a gene with 60% sequence identity to the *F. fujikuroi* GGDP synthase-encoding gene *ggs1*, which is involved in the central isoprenoid pathway (Mende et al. 1997). It has been suggested that *ggs2* in *F. fujikuroi* might encode a GGDP synthase specifically responsible for supplying GGDP for GA production. The gene *ggs2* is transcribed from a bidirectional promoter in the opposite direction from the already identified *cps*/*ks* gene encoding the key enzyme of the GA pathway.

The conversion of GGDP to *ent*-kaurene requires two cyclisation reactions. In contrast to higher plants, in which these two steps are catalysed by two enzymes, CPS and KS, the two-step cyclisation of GGDP is catalysed by only one bifunctional enzyme, the so called "fungal-type" CPS enzyme, in both *Phaeosphaeria* and *F. fujikuroi* (Kawaide et al. 1997; Tudzynski et al. 1998).

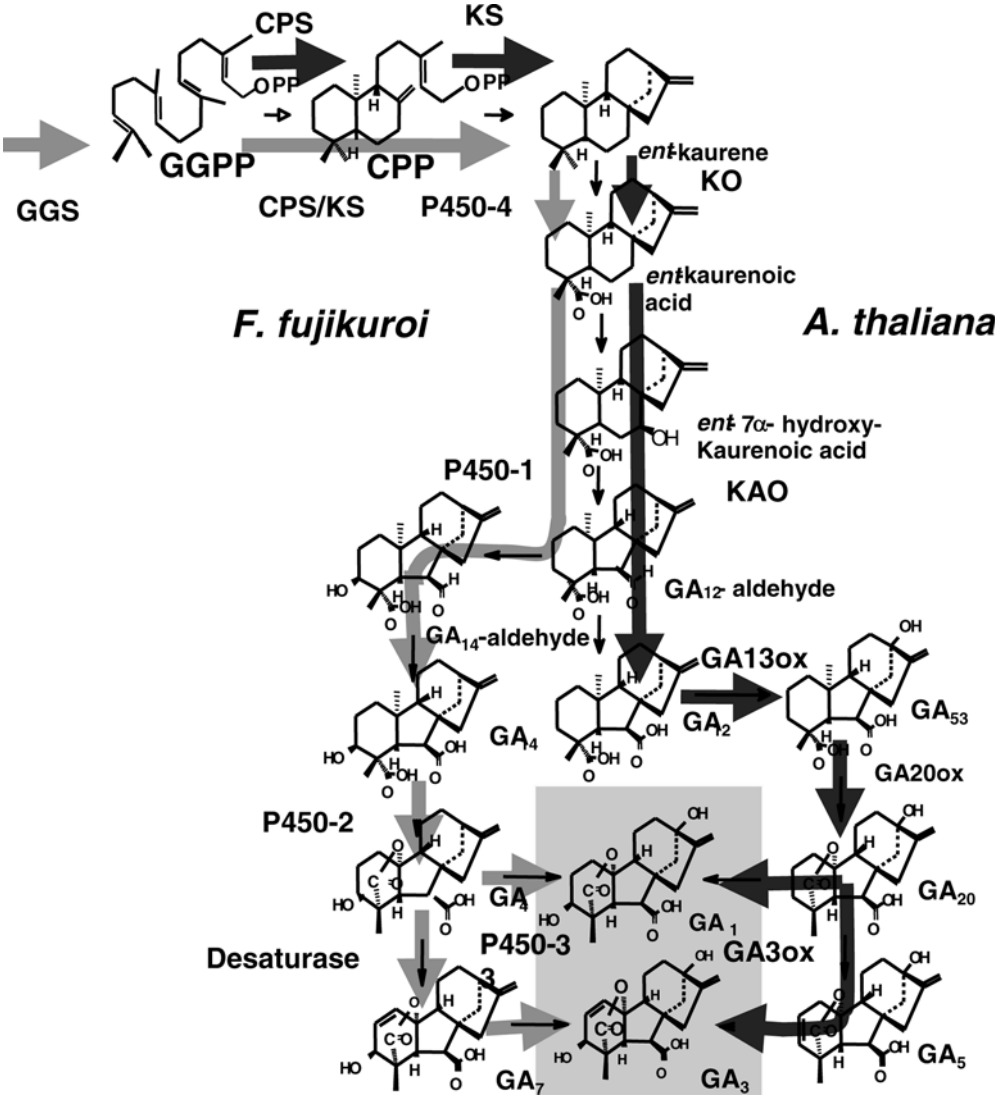


Fig. 2 Biosynthetic pathways to GA_1 and GA_3 in higher plants and the fungus *F. fujikuroi*. Higher plant enzymes and reactions are shown on the right, fungal enzymes on the left (modified from Hedden et al. 2001)

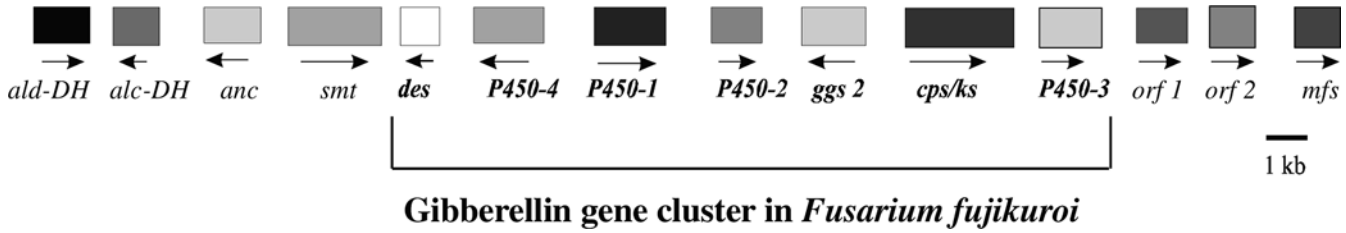


Fig. 3 The GA biosynthetic gene cluster in *F. fujikuroi*. The *arrows* indicate the direction of transcription

A third P450 monooxygenase gene, *P450-3*, two open reading frames without sequence similarity to known genes (*orf1* and *orf2*), and a gene encoding a membrane transporter (*mfs*) are located downstream of the *cps/ks* gene (Hölter and Tudzynski 1998; B. Tudzynski, unpublished results) (Table 1, Fig. 3).

Upstream of *P450-1*, a fourth P450 monooxygenase gene (*P450-4*), a gene without any similarity to other fungal genes (*orf3*, later named *des*), and genes encoding a sugar membrane transporter (*smt*, Voß et al. 2001), an ankyrine domain-containing enzyme (*ank*, T. Voss and B. Tudzynski, unpublished) as well as an aldehyde (*ald-DH*) and an alcohol dehydrogenase (*alc-DH*) genes (T. Voss and B. Tudzynski, unpublished) have been found.

Functional characterisation of genes involved in GA biosynthesis

Disruption of the *F. fujikuroi cps/ks* gene results in total loss of GA production, confirming the prediction that this gene encodes the key enzyme of the GA biosynthetic pathway, the bifunctional CPS/KS (Tudzynski et al. 1998). In order to find out if the genes upstream and downstream of *cps/ks* also encode GA biosynthetic enzymes, all genes in the cluster have been disrupted and the intermediates that accumulate in the resulting mutants have been identified.

The *P450-1* and *P450-4* gene complex

Two of the four P450 monooxygenase genes, *P450-1* and *P450-4*, are closely linked in the gene cluster, sharing the same promoter and being transcribed in opposite directions (Fig. 3). The corresponding deduced proteins have only 33% amino acid identity (Rojas et al. 2001; Tudzynski et al. 2001). Disruption of *P450-1* and *P450-4* resulted in strains producing no GAs. ΔP450-4 mutants accumulate *ent*-kaurene as the only intermediate in the GA biosynthetic pathway. After feeding ^{14}C-labelled *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenal, and *ent*-kaurenoic acid to cultures of ΔP450-4 mutants, only *ent*-kaurenoic acid was efficiently converted to GAs (Tudzynski et al. 2001). On the other hand, ΔP450-1 mutants accumulate *ent*-kaurenoic acid, but no later intermediates. To define precisely the metabolic steps catalysed by *P450-4* and *P450-1*, wild-type gene copies were transformed into the GA-deficient mutant strain SG139. This mutant was first described as a regulatory mutant as none of the expected intermediates were detected (Barrero et al. 1999). However, it was later shown by Southern blot and PCR-analysis, that this mutant strain completely lacks the entire GA gene cluster (B. Tudzynski, unpublished), making this strain an excellent tool for functional analysis of single GA genes. Both the *P450-1* and *P450-4* genes were highly expressed in SG139 under nitrogen starvation conditions. SG139, which failed to metabolise *ent*-[^{14}C]kaurene and *ent*-[^{14}C]kaurenoic acid, converted these substrates after transformation with the *P450-4* gene (Tudzynski et al. 2001), and metabolised labelled *ent*-kaurenoic acid, *ent*-7α-hydroxykaurenoic acid, GA_{12}-aldehyde and GA_{12} to the major product [^{14}C]GA_{14} after transformation of *P450-1* (Rojas et al. 2001). These results clearly demonstrate that both *P450-1* and *P450-4* are multifunctional monooxygenases catalysing several steps in the GA pathway. *P450-4* encodes *ent*-kaurene oxidase, catalysing the oxidation of *ent*-kaurene to *ent*-kaurenoic acid via *ent*-kaurenol and *ent*-kaurenal (see Fig. 1).

P450-1 was shown not only to catalyse the 7β-hydroxylation of *ent*-kaurenoic acid, but also to convert *ent*-kaurenoic acid to GA_{14}, thus catalysing four steps in the GA pathway: hydroxylation at C-7, oxidation at C-6 resulting in contraction of ring B, 3β-hydroxylation, and finally oxidation at C-7 (see Fig. 1). Intermediates in this sequence, *ent*-7β-hydroxykaurenoic acid and GA_{12}-aldehyde, are converted by this enzyme predominantly to GA_{14}. Furthermore, it has been shown that P450-1, as well as catalysing multiple reactions in the GA biosynthetic pathway, may be involved in two branch pathways—from *ent*-kaurenoic acid to the kaurenolides and from *ent*-7β-hydroxykaurenoic acid to seco-ring B products (Rojas et al. 2001, 2004) (see Fig. 1).

Sequential oxidations at a single carbon atom as, for example, the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid by *P450-4* (Tudzynski et al. 2001), or the loss of C-14 in steroid biosynthesis (Lamb et al. 1990), have been described. However, the reactions catalysed by *P450-1* involve oxidations at four different carbon atoms: C-6, C-7, C-3, and C-18, most of which are nonadjacent. Prior to the description of P450-1, the only previously described examples of a single P450 monooxygenase catalysing oxidations of multiple carbons were a mammalian corticoid oxidase (Sun et al. 1995) and a cortisol hydroxylase of the fungus *Curvularia lunata* (Suzuki et al. 1993). Interestingly, in plants the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, and the conversion of *ent*-kaurenoic acid to GA_{12}, are also catalysed by P450 monooxygenases

(Helliwell et al. 1998, 2001). However, 3β-hydroxylation, which is one of the activities of P450-1 in *F. fujikuroi*, is catalysed by 2-oxoglutarate-dependent dioxygenases in plants and occurs late in the pathway (Hedden et al. 2001).

The *ggs2-cps/ks* complex

cps/ks was the first gene shown to be involved in the GA pathway in *F. fujikuroi* (Tudzynski et al. 1998). Adjacent to *cps/ks*, a second *F. fujikuroi* GGDP synthase-encoding gene, *ggs2*, has been found (Fig. 3) (Tudzynski and Hölter 1998). At that time, the two-step cyclisation of GGDP to *ent*-kaurene had been defined as the first GA pathway-specific step. However, deletion of *ggs2* revealed a total block of GA biosynthesis, demonstrating that GGS2 catalyses formation of GGDP specifically for GA biosynthesis (B. Tudzynski, unpublished). The close linkage of *ggs2* and *cps/ks*—they share the same promoter—hints at strict co-regulation of both genes catalysing early steps in the GA pathway leading to *ent*-kaurene. Interestingly, the recently identified carotenoid biosynthesis gene cluster in *F. fujikuroi* does not contain a pathway-specific *ggs* gene (Linnemannstöns et al. 2002a), whereas the paxillin gene cluster in *Penicillium paxilli* contains a second copy of GGDP synthase (*paxG*) besides the weakly expressed gene *ggs1* outside the gene cluster (Young et al. 2001). Deletion of the pathway-specific *ggs* gene, *paxG*, resulted in total loss of paxillin formation. These results demonstrate that the *ggs1* homologues in *P. paxilli* and *F. fujikuroi* are unable to compensate for loss of function of *paxG* and *ggs2*, respectively. The data provide genetic support for the suggestion of cellular partitioning and different metabolic functions of both GGS1 enzymes (primary metabolism) and PaxG and GGS2 (secondary metabolism) in the two fungi. The concept of metabolic channels had already been proposed for isoprenoid biosynthesis in plants (Chappel 1995).

P450-2

P450-2 is located in the GA biosynthetic gene cluster between *P450-1* and *ggs2* (Fig. 3). Disruption of *P450-2* results in mutants that produce only GA_{14} according to TLC, HPLC, and GC-MS analysis (Tudzynski et al. 2002). In contrast, wild-type *F. fujikuroi* produces mainly GA_4, GA_7, GA_{13}, and GA_3. This result indicates that P450-2 activity is required for oxidation of GA_{14} at C-20, and the final removal of C-20, through which 20-carbon GAs are converted to biologically active 19-carbon-GAs. Interestingly, in all higher plants analysed so far, the removal of C-20 by progressive oxidation of the C-20 methyl group is catalysed by a 2-oxoglutarate-dependent dioxygenase. To confirm this unexpected result, i.e. that in *F. fujikuroi* this reaction is indeed catalysed by a P450 monooxygenase, a copy of the *P450-2* gene was introduced into the cluster-deletion mutant SG139. In most of the transformants, *P450-2* was highly expressed under optimum conditions (nitrogen starvation). The labelled GA precursor [^{14}C]GA_{14} was efficiently metabolised to [^{14}C]GA_4, whereas the non-3β-hydroxylated [^{14}C]GA_{12} was converted to [^{14}C]GA_9 and smaller amounts of [^{14}C]GA_{25}, the tricarboxylic acid product (Tudzynski et al. 2002). These results confirm the suggestion that P450-2 is responsible for oxidation of GA_{14} at C-20 and the final removal of the 20-carbon in *F. fujikuroi*.

In contrast, incubation of both labelled substrates with the mutant SG139 gave no conversion, whereas the wild-type converted these substrates mainly to [^{14}C]GA_3 and, in

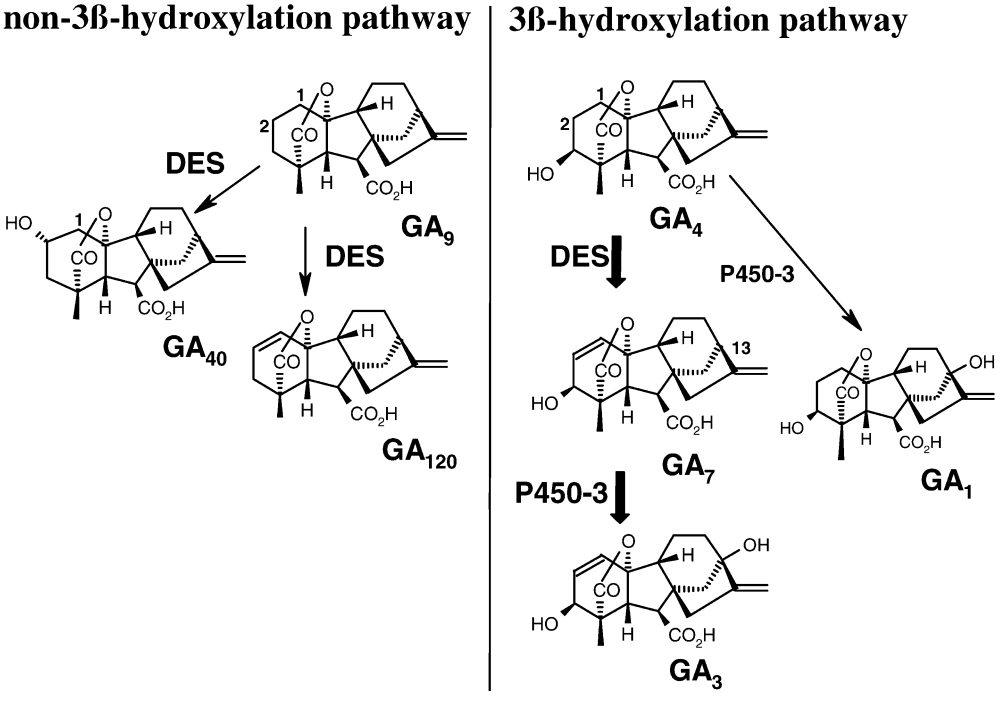


Fig. 4 Reactions catalysed by the enzymes P450-3 (13-hydroxylase) and DES (GA4 1.2 desaturase) in the GA biosynthetic pathway of *F. fujikuroi* (modified from Tudzynski et al. 2003)

the minor pathway, to [^{14}C]GA_9 and [^{14}C]GA_{25} (non-3β-hydroxylated GAs).

Thus, the P450-2 protein has a function analogous to that of the soluble GA 20-oxidases (dioxygenases) in plants (Lange et al. 1994; Hedden and Phillips 2000a). However, in contrast to the activities of P450-4 and P450-1 (Urrutia et al. 2001), it has so far not been possible to demonstrate in vitro activity of the fungal GA_{20} oxidase, P450-2.

The two final genes of the GA pathway

Only recently, a gene upstream of *P450-4*, called *orf 3*, was cloned and functionally characterised (Fig. 3). Sequence analysis revealed a 1,029 bp open reading frame uninterrupted by introns (Tudzynski et al. 2003). The amino acid sequence has only a weak similarity with a 7α-cephem-methoxylase from *Nocardia lactamdurans* (Coque et al. 1995), providing no indication of its possible function in GA biosynthesis. Knock-out mutants are unable to produce GA_7 and GA_3, the final products of the GA-biosynthetic pathway in *F. fujikuroi*. GC-MS analysis of culture filtrates of wild-type and mutants indicated that GA_1 and GA_4 rather than GA_3 were the major C19-GAs (Fig. 4). The ratio of GA_1 and GA_4 was 3:1 to 5:1 depending on the culture medium. Therefore, the mutation appears to block the 1.2 desaturation of GA_4 to GA_7 (Fig. 4), indicating that the new gene (*des*) encodes GA_4 desaturase (Tudzynski et al. 2003).

To confirm the function of the desaturase, the *des* gene was expressed in the deletion mutant SG139. Transformants carrying *des* as the only GA-biosynthetic gene were incubated with the radiolabelled substrates [^{14}C]GA_4 (3β-hydroxylated) or [^{14}C]GA_9 (non-hydroxylated). GC-MS analysis of the HPLC-purified product from incubation with labelled GA_4 identified [^{14}C]GA_7 as the sole product, whereas labelled GA_9 was converted to [^{14}C]GA_{40} (2α-hydroxy GA_9) and [^{14}C]GA_{120} (1.2-didehydro GA_9). Therefore, the desaturase accepts both 3β-hydroxylated and non-hydroxylated substrates (Tudzynski et al. 2003).

The remaining gene downstream of *cps/ks* is *P450-3*, one of the four cytochrome P450 monooxygenase genes of the cluster (Fig. 3). Gene disruption resulted in loss of the ability to produce GA_3, suggesting that *P450-3* encodes the missing 13-hydroxylase catalysing conversion of GA_7 to GA_3. Furthermore, ΔP450-3 mutants produced not GA_1, but another 13-hydroxylated GA, which is produced in small amounts (1-2% total GAs) in the wild-type, but is the main product in Δdes mutants (Tudzynski et al. 2003) (Fig. 4).

In contrast to the other three P450 monooxygenase genes and *des*, *P450-3* was not expressed in the cluster deletion mutant SG139. None of the transformants were able to metabolise [^{14}C]GA_7 or [^{14}C]GA_4 to labelled GA_3 or GA_1, respectively. Feeding the transformants with several precursors did not induce *P450-3* gene expression. On the other hand, the 13-hydroxylase UV mutant 6314, which was previously isolated as a GA_7-overproducing mutant (B. Tudzynski, unpublished), could be successfully complemented with a copy of the *P430-3* gene, restoring the ability to produce GA_3. Sequence analysis of the mutant *P450-3* gene copy revealed a point mutation at nucleotide position 844 resulting in a single amino acid substitution from arginine to tryptophan at position 221 (Tudzynski et al. 2003). These results suggest that *P450-3*, in contrast to the other genes, requires the existence of the GA gene cluster for its expression.

The functional characterisation of *des* and *P450-3* completed analysis of the GA gene cluster in *F. fujikuroi*. Disruption of the border genes *smt* upstream of *des* (Voß et al. 2001) and of *orf1* and *orf2* downstream of *P450-3* (see Fig. 3) (B. Tudzynski, unpublished) did not affect production of GAs or its regulation. Therefore, the biosynthesis of the final product, GA_3, in 15 steps beginning with the formation of GGDP by the pathway-specific GGDP synthase GGS2 requires only seven enzymes, many of which are multifunctional.

Cytochrome P450 oxidoreductase affects GA biosynthesis in *F. fujikuroi*

Eukaryotic non-mitochondrial cytochrome P450 monooxygenases are membrane proteins that require association with a NADPH-cytochrome P450 oxidoreductase (CPR) for activity. CPRs facilitate the transfer of electrons from NADPH via FAD and FMN to the prosthetic heme group of the P450 monooxygenases. Only one CPR-encoding gene, but about 40 different P450 monooxygenases, have been identified in the sequenced genomes of filamentous fungi such as *Fusarium graminearum*, *Magnaporthe grisea*, *Aspergillus nidulans* and *Neurospora crassa*.

The *F. fujikuroi* CPR would be expected to have a strong influence on GA biosynthesis since four P450 monooxygenases (*P450-1– P450-4*) are involved in this pathway. Thus, loss of CPR activity should affect the rates of several GA biosynthetic steps. A phenotype similar to that expected for *cpr* mutants has been described for strain SG138. This UV-induced mutant has lost most oxidation steps catalysed by P450 monooxygenases and does not produce any bioactive GAs (Barrero et al. 2001).

The *cpr* gene has been cloned from *F. fujikuroi*, and its role in GA biosynthesis and primary metabolism studied (Malonek et al. 2004). Knock-out mutants, as well as UV mutant SG138, showed a reduction in growth rate on agar plates—more significantly on minimal medium. The mutants did not produce GA_4, GA_7, or GA_3, the last three products of the pathway, whereas complementation of the Δcpr mutant with the wild-type gene resulted in full restoration of GA production (Fig. 5). However, small amounts of non-hydroxylated GAs, such as GA_{15} and GA_{24}, and the high level of *ent*-kaurene found in Δcpr mutants indicate low residual activities of *ent*-kaurene oxidase (*P450-4*), and GA_7 oxidase (one of the P450-1 activities), in the absence of the reductase, suggesting the participation of a second electron transport protein, e.g. cytochrome *b5*, in GA biosynthesis (Malonek et al. 2004).

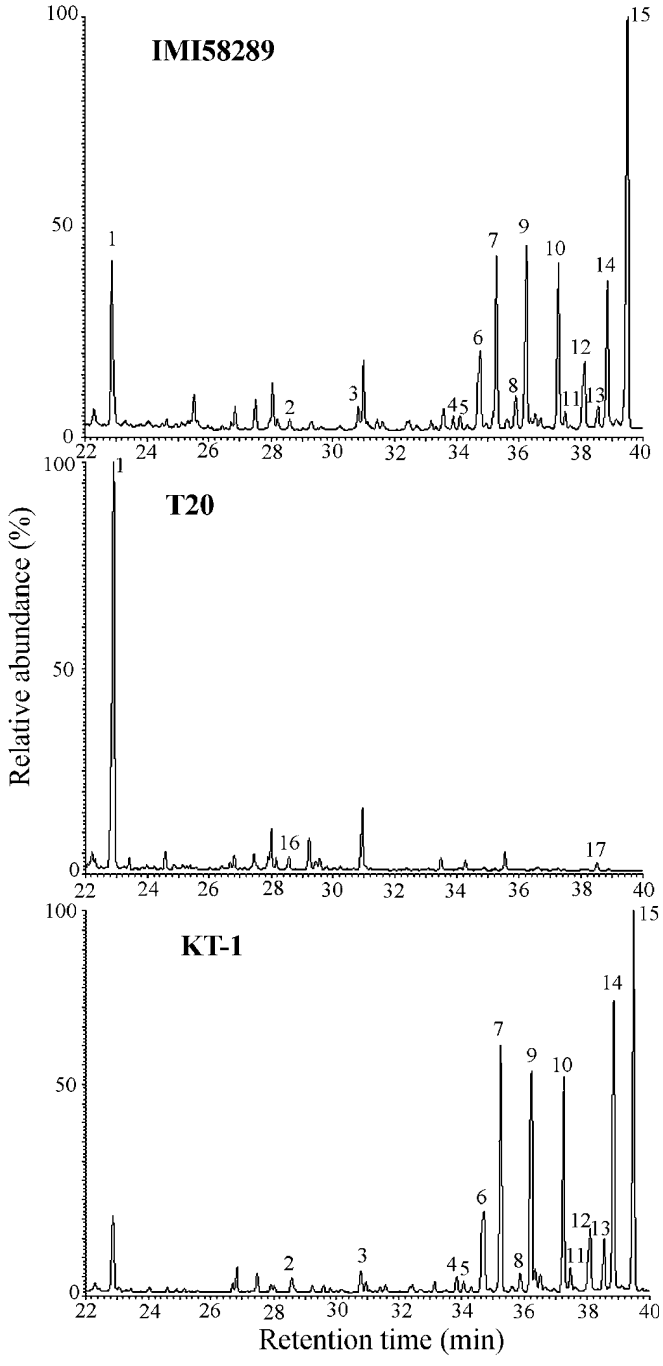


Fig. 5 Gas chromatography-mass spectrometry (GC-MS) analysis of culture filtrates of the wild-type (IMI58289), *cpr*-disruption mutant (ΔCPR-T20) and transformant KT-1, in which T20 has been complemented with the *F. fujikuroi cpr* gene. Total ion currents are shown for extracts as methyl esters trimethylsilyl ethers. Peaks: *1ent*-kaurene, *2ent*-kaurenoic acid, *3* GA_9, *4* GA_{25}, *5* GA_{24}, *6* GA_{14} and 7β-hydroxykaurenolide, *7* GA_4, *8* GA_7, *9* fujenoic acid, *10* GA_{13}, *11* GA_{36}, *12* GA_3 isolactone, *13* 7β, 18-dihydroxykaurenolide, *14* GA_1, *15* GA_3, *16ent*-kaurenol, *17* GA_{15}. Unlabeled peaks represent compounds unrelated to GA biosynthesis. The peak at the same retention time as *ent*-kaurene in the KT-1 extract contains no *ent*-kaurene. Modified from Malonek et al. 2004

On the other hand, the 3β-hydroxylase activity of the multifunctional enzyme P450-1 and production of biologically active 19-carbon GAs by GA 20-oxidase (P450-2) appears to be completely blocked (see Fig. 1).

Sequence comparison between the wild-type *cpr* gene and the *cpr* copy of the mutant strain SG138 confirmed the suggestion that loss of almost all oxidative enzymatic activities of the GA-specific P450 monooxygenases in this mutant is due to a point mutation in the *cpr* gene in SG138 (Malonek et al. 2004). Interestingly, GA production capability could be restored not only by transformation with a wild-type copy of *F. fujikuroi cpr*, but also by the *Aspergillus niger* gene *cprA* (van den Brink et al. 1995). This result indicates that CPRs act non-specifically as general electron donors for P450 monooxygenases from different pathways, e.g. in detoxification of benzoate by the P450 enzyme benzoate-*p*-hydroxylase (Malonek et al. 2004).

Regulation of GA biosynthesis in *F. fujikuroi*

The conditions for optimal production of GAs by *F. fujikuroi* are of biotechnological interest and have been studied intensively over the last 50 years (Borrow et al. 1955, 1964; Brückner and Blechschmidt 1991; Candau et al. 1992; Darken et al. 1959; Geissman et al. 1966; Avalos et al. 1997, 1999; Tudzynski 1999). The most obvious regulatory principle is the strong repression of GA production by high amounts of nitrogen (e.g. ammonium, glutamine, glutamate, asparagine, nitrate) in the culture medium (Muñoz and Agosin 1993; Brückner and Blechschmidt 1991).

The study of the molecular mechanism of nitrogen regulation began after the GA biosynthetic genes were cloned. Although GAs do not contain any nitrogen in their structure, it has been found that AREA (NIT2), the major nitrogen regulatory protein in filamentous fungi (Caddick 1994; Feng et al. 1995), controls expression of GA biosynthetic genes. Replacement of the *F. fujikuroi* homologue of *areA* and *nit-2*, *areA-Gf*, resulted in a significant reduction in GA production yields (Tudzynski et al. 1999). Complementation of ΔareA-Gf mutants with the wild-type gene led to full recovery of GA production.

Recent studies have shown that the expression level of six of the seven GA biosynthetic genes is drastically reduced in mutants lacking *areA-Gf* (Mihlan et al. 2003). Only the expression of *P450-3*, catalysing the final 13-hydroxylation of GA_7 to form GA_3, is not repressed by high amounts of nitrogen (Fig. 6). Electrophoretic gel mobility shift assays (EMSA) with promoter fragments of GA biosynthetic genes revealed binding of the zinc finger peptide of *F. fujikuroi* AREA-Gf to all GATA sequence elements in vitro. However, mutation and deletion of those elements in the bidirectional promoter of *P450-1*/*P450-4* and its fusion to the *Escherichia coli* β-glucuronidase (*uidA*) gene clearly demonstrated that only two double GATAs out of the nine GATA elements play an important

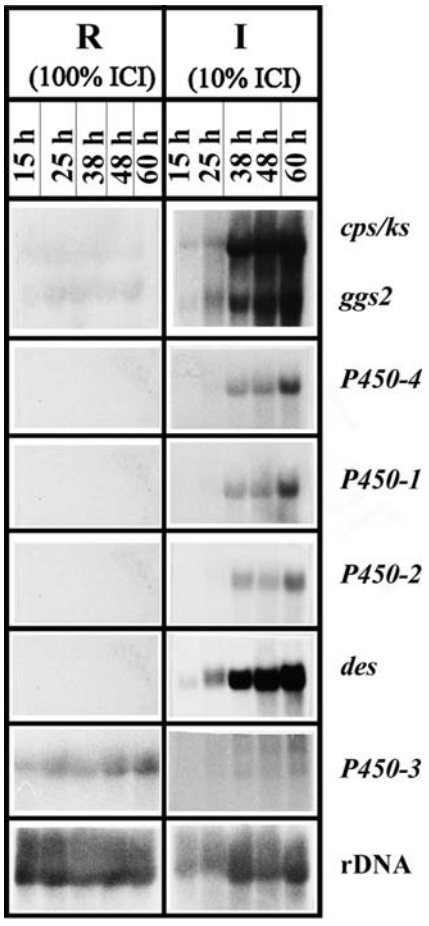


Fig. 6 Time-course of expression of GA biosynthetic genes in 100% ICI medium (high in nitrogen) and 10% ICI (low in nitrogen). *R* Repressing conditions, *I* inducing conditions

role in transcription regulation in vivo (Mihlan et al. 2003).

In *A. nidulans* and *N. crassa*, the activity of AREA (NIT2) is regulated post-translationally by direct binding of a second regulatory protein, NMR (Jarai and Marzluf 1990; Andrianopoulos et al. 1998). However, deletion of the homologous gene *nmr-Gf* in *F. fujikuroi* did not result in the expected repression of GA biosynthetic genes. Overexpression of *nmr-Gf* led to slightly more repression of AREA-regulated genes (Mihlan et al. 2003). However, the limited role of NMR-Gf in *F. fujikuroi* suggests that other regulatory proteins must exist in this fungus that are involved in nitrogen metabolite repression, e.g. by interaction with AREA.

Interestingly, besides *areA-Gf*, the glutamine synthetase (GS) of *F. fujikuroi* seems to be involved in nitrogen regulation in general, and unexpectedly also in the control of N-regulated secondary metabolite pathways. Deletion of the GS-encoding gene *glnA-Gf* resulted in total loss of GA- and bikaverin (red pigment)-biosynthesis even under nitrogen starvation conditions (Teichert et al. 2004).

Some secondary metabolite gene clusters in fungi, e.g. the aflatoxin gene cluster in *Aspergillus parasiticus*, contain pathway-specific regulatory genes (Keller and Hohn 1997). The GA biosynthetic gene cluster in *F. fujikuroi* does not include a gene encoding a pathway-specific transcription factor. Point mutations in the *P450-4* gene promoter revealed a 30 bp region important for a high transcription level of *P450-4*. This sequence element could be the binding motif for a putative GA pathway-specific transcription factor that should be cloned by a one-hybrid approach in the near future.

Biotechnological importance of cloning and functional analysis of GA biosynthetic genes

Gibberellins play important roles in plant development, controlling such processes as seed germination, internode elongation, flower development, and fruit set in higher plants (Crozier 1983). Therefore, cloning and characterisation of GA biosynthetic genes in higher plants and *F. fujikuroi* is not only of scientific, but also of biotechnological interest.

The spectacular increases in wheat and rice yields during the 'Green Revolution', were enabled by the introduction of dwarfing traits into the plants (Hedden 2003). Now, identification of the genes responsible for these traits shows that they interfere with the action or production of gibberellins. Mutation and overexpression of plant genes encoding GA-biosynthetic enzymes have been shown to affect plant growth and developmental processes (Hedden and Phillips 2000b; Hedden 2003). Thus, GA 20-oxidase is a regulatory enzyme controlled by developmental and environmental stimuli (Phillips et al. 1995) and, as such, is a prime target for genetic manipulation. In *Arabidopsis*, overexpression of GA 20-oxidase genes produced a GA-overproduction phenotype with elongated hypocotyls and stem and early flowering (Huang et al. 1998). Among the GA 20-oxidases known to date, only that from developing pumpkin seeds has been shown to produce biologically inactive GAs as the major products; the pumpkin enzyme converts the aldehyde intermediates GA_{24} and GA_{19} to the tricarboxylic acids, GA_{25} and GA_{17}, respectively (Lange 1998). Overexpression of this GA 20-oxidase gene in transgenic plants could, therefore, result in a reduction of biologically active GAs by diverting the pathway to tricarboxylic acids. Thus, the GA 20-oxidase gene is a potentially useful tool for controlling plant growth (Niki et al. 2001).

In addition to the modification of GA biosynthesis in crop plants to improve agriculturally important traits, modification of GAs in *F. fujikuroi* for commercial production is also of increasing importance. The cloning and characterisation of genes encoding the final two enzymes in the GA biosynthetic pathway in *F. fujikuroi* is of special biotechnological interest. Certain agricultural and horticultural applications specifically require GA_4 (Hedden et al. 1993; Almqvist 2003; Tudzynski and Sharon 2002). Therefore, strains producing GA_4 in the absence of GA_3 and GA_7 would have considerable utility. Double mutants lacking both desaturase and 3β-hydroxylase activities were constructed and shown to produce

almost exclusively GA_4 in high amounts. Deletion of *P450-3* resulted in accumulation of GA_7, whereas GA_4 desaturase (*des*) mutants produce a mixture of GA_1 and GA_4 (Fig. 7) (B. Tudzynski, unpublished results).

Intermediates of GA biosynthesis, such as *ent*-kaurene, *ent*-kaurenoic acid, GA_{12}-aldehyde, and GA_{14}, are not yet commercially available and are synthesised in small amounts from pumpkin endosperms for scientific experiments. By using *F. fujikuroi* mutants with defined genetic blocks at specific steps of the pathway, large scale biotechnological production of these intermediates is now possible. Thus, ΔP450-4, ΔP450-1, and ΔP450-2 mutants accumulate *ent*-kaurene, *ent*-kaurenoic acid, and GA_{14}, respectively, in high amounts.

In the last 10 years, several attempts have been made to improve secondary metabolism in microorganisms by genetic engineering (Newbert et al. 1997; Perez-Llarena et al. 1997; Kosalkova et al. 2001). Amplification of entire gene clusters, genes encoding enzymes that catalyse rate-limiting steps, or of genes encoding pathway-specific transcription factors, are possible strategies for strain improvement. A prerequisite for successful application of recombinant DNA technology to improve strain performance is identification of the optimal genetic change. Thus, in *Penicillium chrysogenum*, the elevated copy number of the entire penicillin gene cluster and increased gene transcription levels largely explains the high penicillin titres of production strains resulting from classical mutagenesis and selection-based strain development programs over the last 50 years (Newbert et al. 1997). Using a single copy low-producing strain, the effect of overexpressing the penicillin biosynthetic genes has been confirmed by molecular approaches. Transformants in which the penicillin gene cluster was amplified showed the largest increase in penicillin production—up to 176% (Theilgaard et al. 2001).

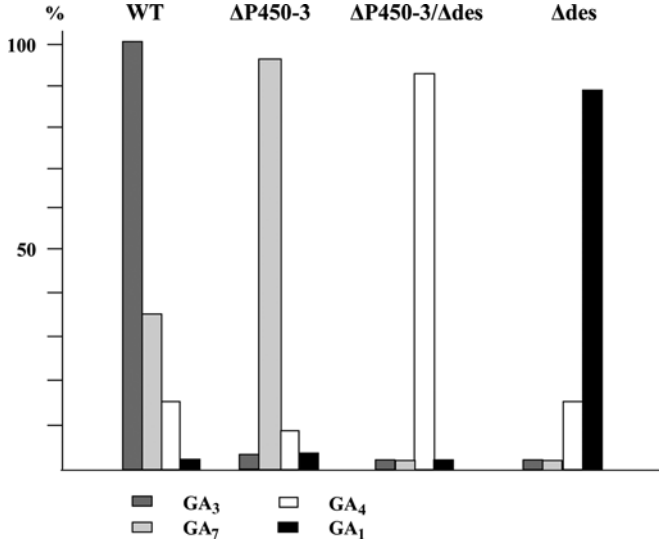


Fig. 7 GA production profiles in the wild-type strain IMI 58289, the ΔP450-3 mutant, the ΔP450-3/Δdes double mutant and the Δdes mutant (P. Linnemannstöns and B. Tudzynski, unpublished results)

In *F. fujikuroi*, amplification of a genomic fragment carrying the *ggs2* and *cps/ks* genes encoding the first pathway-specific enzymes, did not result in significant increase of GA production. However, amplification of all GA biosynthetic genes by transforming a cosmid carrying the entire GA gene cluster resulted in a significant increase (up to 300%) of GA production yields (P. Linnemannstöns and B. Tudzynski, unpublished).

Another promising approach for development of GA-overproducing strains is the blocking of competing pathways, such as the bikaverin and carotenoid biosynthetic pathways. The genes encoding the key enzymes of both pathways have been deleted in *F. fujikuroi*, but without any significant effect on GA production capability (Linnemannstöns et al. 2002a,b). However, although disruption of *pks4* (encoding the bikaverin-specific polyketide synthase PKS4) did not improve GA production, the mutants should be useful in large scale production of GAs since the absence of the red pigment will simplify purification of GAs from the fermentation medium (Linnemannstöns et al. 2002b).

Another important approach to strain improvement will be modulation of gene expression after detailed study of regulation principles. Knowledge of the transcription factors involved, and their specific binding motifs in the promoters of GA biosynthetic genes, will allow manipulations such as overexpression of pathway-specific transcription factors, amplification of sequence elements in the promoters, and/or fusion of strong promoters to GA biosynthetic genes.

Furthermore, it has been shown in many studies that complex culture media containing plant components, such as plant oils, plant meals, or corn steep liquor, are much better as GA production media than synthetic media with glucose and defined nitrogen sources (Darken et al. 1959; Fuska et al. 1961; Muromtsev and Agnistova 1984). The identification of the inducing substances in these complex plant materials and confirmation of their positive effect on gene expression would help to further optimise the composition of production media.

Evolution of GA biosynthetic genes

The identity of GAs produced by higher plants and *F. fujikuroi*, as well as the identity of the first pathway steps as far as GA_{12}-aldehyde in both systems, led to the suggestion that fungi acquired the GA pathway genes from plants by horizontal gene transfer (Chapman and Regan 1980). However, after cloning of all pathway genes in plants and *F. fujikuroi* it became clear that there are dramatic differences in the character of enzymes involved, the organisation of GA biosynthetic genes in the two genomes, and the pathway stages at which hydroxyl groups are introduced. As shown above, the final stages of GA biosynthesis in plants, comprising the removal of C-20 by GA 20-oxidases (GA20ox) as well as the introduction of the 3β-hydroxyl group by GA 3-oxidases (GA3ox), are carried out by soluble 2-oxoglutarate-

dependent dioxygenases that are assumed to be localised in the cytosol (Hedden and Phillips 2000a; Hedden et al. 2001). In *F. fujikuroi*, the same activities are the responsibility of cytochrome P450 monooxygenases (*P450-1*, *P450-2*), demonstrating that GA biosynthesis has evolved independently in plants and *F. fujikuroi*. If this is the case, the question of the origin of the GA biosynthesis gene cluster in *F. fujikuroi* arises. Our recent data show that seven of the nine sexually fertile species of the *Gibberella fujikuroi* species complex contain all seven (e.g. *F. proliferatum*, MP-D; *F. subglutinans*, MP-E), and two species (*F. verticilloides*, MP-A; *F. circinatum*, MP-H) contain only one or two GA cluster genes. However, none of these species except *F. fujikuroi* (MP-C) are able to produce GAs (S. Malonek and B. Tudzynski, unpublished).

Recently, the entire genome of *F. graminearum* has been sequenced. Despite the high degree of sequence identity between primary metabolism genes of *F. graminearum* and *F. fujikuroi*, there are dramatic differences in secondary metabolite production by the two species. Most surprisingly, *F. graminearum* does not contain any obvious GA biosynthetic genes. On the other hand, a number of other fungal species not related to *Fusarium* produce GAs at high concentrations (milligram quantities per litre of culture fluid; Rademacher 1994). Several species of the ascomycete *Sphaceloma* produce GA_4, but not GA_3, and therefore seem to lack 1.2 desaturase and 13-hydroxylase activities. A species of *Phaeosphaeria* produces GA_1 via a pathway in which 3β-hydroxylation occurs after C19-GA formation, as is the case in higher plants (Kawaide et al. 1995). However, similar to *F. fujikuroi* but unlike in plants, 13-hydroxylation occurs late in the pathway. Thus, the pathway to GA_1 includes GA_9 as an intermediate, which is 3β-hydroxylated to GA_4 and then 13-hydroxylated to GA_1 (Kawaide et al. 1995).

Genomic libraries of two *Sphaceloma* species were recently constructed, and analysis of the GA biosynthesis genes is underway C. Bömke and B. Tudzynski, unpublished). The characterisation of these genes and their organisation in the genomes of both fungi will facilitate the study of the evolutionary origin of GA biosynthetic genes in fungi.

In addition to plants and fungi, some bacteria (e.g. *Acetobacter diazotropicus*, *Azospirillum lipoferum*; *Rhizobium phaseoli*; and *Bacillus pumilus*) produce GAs (reviewed in MacMillan 2001). However, the relevant biosynthetic genes have not yet been cloned. Thus, *Azospirillum* spp., inoculated on rice GA-deficient mutants, metabolised GA_{20} to GA_1 and reversed dwarfism, showing an in vivo capacity to perform 3β-hydroxylation. The bacterial 3β-hydroxylase may be a 2-oxoglutarate-dependent dioxygenase similar to those of plants (Cassan et al. 2001).

The evolution of the ability to synthesise specialised metabolites is likely to have been the key for survival and diversification of different plant, fungal and bacterial species. For further study of the phylogenetic origin of the GA gene cluster in the *F. fujikuroi* species complex, it will be interesting to compare the GA genes of *F. fujikuroi* with those in other MPs, but also with those of *Sphaceloma* and *Phaeosphaeria* in order to find out if there is a general pattern of inheritance of this secondary gene cluster during evolution. It is still not clear if the GA gene cluster evolved from one ancestral species, with divergence during speciation resulting in the inability of most of ascomycete species to produce GAs. The general question is why organisms retain such large gene units, like gene clusters, without their having obvious functionality in the organism. If such cooperating genes are conditionally dispensable, but have an adaptive value in colonising certain ecological niches, the incipient cluster could be maintained by positive selection in those environments (Lawrence 1997). However, to answer this question, it will be necessary to investigate the role of GA production for the fungus itself, e.g. in its effects on host plants, in more detail.

Summary and conclusion

In parallel with progress in higher plants and bacteria, advances have been made in understanding GAs in the fungus *F. fujikuroi*. The clustering of GA biosynthetic genes has facilitated their rapid isolation, sequencing, and functional characterisation following identification of the first gene. Knock-out mutants of all pathway genes are now available and allow the production of high amounts of GA intermediates and precursors of GA_3, such as *ent*-kaurene, *ent*-kaurenoic acid, GA_{14}, GA_4, GA_7 and GA_1. The profound differences in GA biosynthesis in plants and *F. fujikuroi* identified in these studies strongly suggest that GA biosynthesis pathways evolved independently in these two groups of organisms. These data raise questions regarding the evolutionary origin of GA biosynthetic genes in *Fusarium*, since *Fusarium* species outside the *G. fujikuroi* species complex, such as *F. oxysporum*, or *F. graminearum*, do not have GA biosynthetic genes. On the other hand, fungi from other taxonomic groups, such as *Sphaceloma manihoticola* and *Phaeosphaeria* sp. have been shown to produce GAs. However, the sequence, character and organisation of GA genes in the genomes of these fungi, as well as regulation of gene expression compared with *F. fujikuroi*, remain to be studied.

The availability of all pathway genes in *F. fujikuroi* now allows a detailed analysis of the regulation of gene expression on a molecular level. One aspect of future research will be a thorough study of nitrogen metabolite repression, its mechanism, and components involved in addition to the general regulator AREA. On the other hand, in addition to nitrogen regulation, it seems likely that GA biosynthesis is also under the control of pathway-specific transcription factors that have yet to be identified. A detailed knowledge of the mechanisms of gene regulation and identification of the transcription factors involved are important prerequisites for further strain improvement programs by metabolic engineering.

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