Influence of electron transport proteins on the reactions catalyzed by *Fusarium fujikuroi* gibberellin monooxygenases

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Abstract

The multifunctional cytochrome P450 monooxygenases P450-1 and P450-2 from *Fusarium fujikuroi* catalyze the formation of GA_{14} and GA_4, respectively, in the gibberellin (GA)-biosynthetic pathway. However, the activity of these enzymes is qualitatively and quantitatively different in mutants lacking the NADPH:cytochrome P450 oxidoreductase (CPR) compared to CPR-containing strains. 3β-Hydroxylation, a major P450-1 activity in wild-type strains, was strongly decreased in the mutants relative to oxidation at C-6 and C-7, while synthesis of C_{19}-GAs as a result of oxidative cleavage of C-20 by P450-2 was almost absent whereas the C-20 alcohol, aldehyde and carboxylic acid derivatives accumulated. Interaction of the monooxygenases with alternative electron transport proteins could account for these different product distributions. In the absence of CPR, P450-1 activities were NADH-dependent, and stimulated by cytochrome *b*5 or by added FAD. These properties as well as the decreased efficiency of P450-1 and P450-2 in the mutants are consistent with the participation of cytochrome *b*5:NADH cytochrome *b*5 reductase as redox partner of the gibberellin monooxygenases in the absence of CPR. We provide evidence, from either incubations of GA_{12} (C-20 methyl) with cultures of the mutant suspended in $[^{18}O]H_2O$ or maintained under an atmosphere of $[^{18}O]O_2$:N_2 (20:80), that GA_{15} (C-20 alcohol) and GA_{24} (C-20 aldehyde) are formed directly from dioxygen and not from hydrolysis of covalently enzyme-bound intermediates. Thus these partially oxidized GAs correspond to intermediates of the sequential oxidation of C-20 catalyzed by P450-2.

Keywords: *Fusarium fujikuroi*; Gibberellin biosynthesis; GA_{14} synthase; Gibberellin 20-oxidase; P450 monooxygenases; Electron transport proteins

1. Introduction

The rice pathogen *Gibberella fujikuroi* mating population C, renamed *Fusarium fujikuroi* (O'Donnell et al., 1998), synthesizes high amounts of gibberellins (GAs), mainly gibberellic acid (GA_3) (**14**) plus GA_4 (**11**) and GA_7 (**13**) at lower levels (Rademacher, 1994) (see Fig. 1). GAs have a hormone function in plants modulating growth and development (Graebe, 1987) and thus the fungal system is currently used for commercial production of these diterpenes, which are applied as growth modulators in agricultural processes (Tudzynski and Sharon, 2002). The structural requirements for growth promoting activity include a 19,10-γ-lactone function, a 3β-hydroxyl group, and a carboxylate function at position 7 (Graebe, 1987). We have been interested in the fungal reactions that generate these structural features as well as in the corresponding enzymes, the genes for which have been recently isolated, allowing the enzymes to be identified as cytochrome P450 monooxygenases (Rojas et al., 2001; Tudzynski et al., 2002; Hedden et al., 2002). In *F. fujikuroi*, the GA-biosynthetic genes are arranged in a cluster as found for genes for other fungal secondary metabolites (Keller and Hohn, 1997; Tudzynski and Hölter, 1998; Yu

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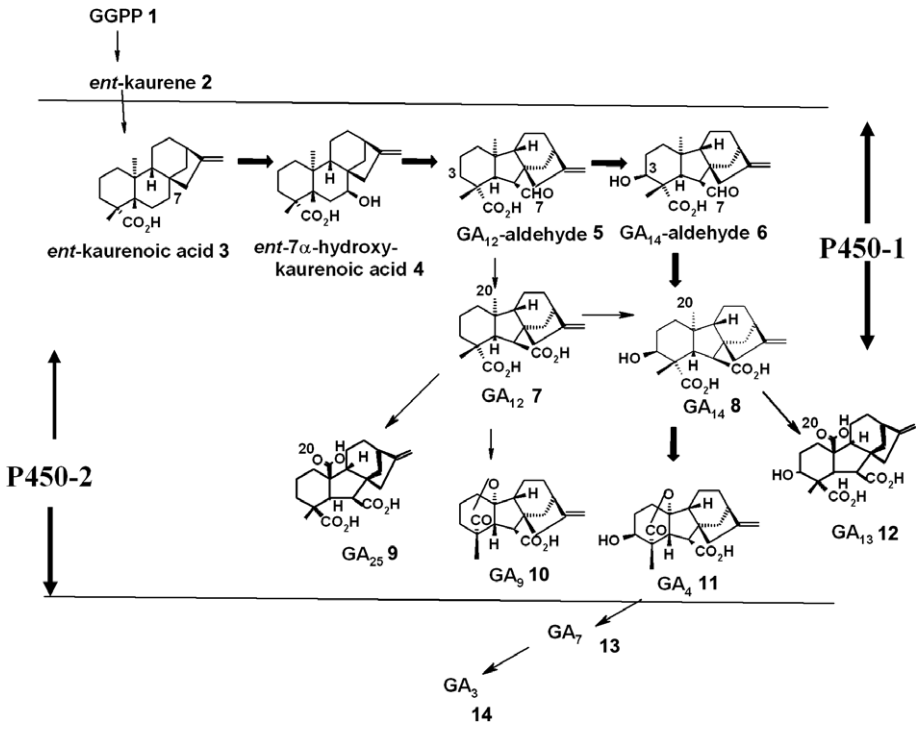
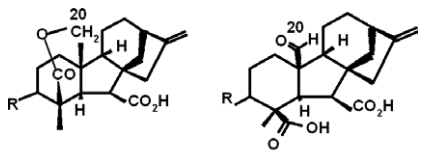


Fig. 1. Reactions catalyzed by P450-1 and P450-2 GA monooxygenases in CPR-containing *Fusarium fujikuroi* strains. Thick arrows denote the major GA biosynthesis pathway.

et al., 2004). They include four cytochrome P450 monooxygenase genes (*P450-1*, *P450-2*, *P450-3* and *P450-4*) encoding multifunctional enzymes that catalyze 10 of the 15 reactions of the pathway to GA_3, **14** (Rojas et al., 2001; Tudzynski et al., 2001, 2002); these include 7-oxidation, 3β-hydroxylation and the formation of the 19,10-γ-lactone (Fig. 1). P450-1 (GA_{14} synthase) catalyzes four sequential oxidation steps from *ent*-kaurenoic acid (**3**):7β-hydroxylation, ring B contraction initiated by oxidation at C-6, 3β-hydroxylation and finally oxidation of C-7 from an aldehyde to a carboxylic acid function (Rojas et al., 2001). P450-2 (GA 20-oxidase) catalyzes oxidation of the C-20 methyl group resulting in its elimination as CO_2 to give C_{19}-GAs or alternatively C-20 carboxylic acid products (Fig. 1) (Tudzynski et al., 2002).

Cytochrome P450 monooxygenases are extremely versatile catalysts that can mediate multiple oxidative reactions at single active sites. For activity, they require interaction with electron transport proteins that supply electrons for reduction of O_2 in the formation of the iron–oxygen species that serves as the oxidant (Malonek et al., 2004). Multifunctional P450s have been described in glucocorticoid and sex steroid synthesis (Pandey and Miller, 2005), in the inactivation pathway of vitamin D (Prosser and Jones, 2004) and in cyanogenic glucoside biosynthesis (Sibbesen et al., 1995). These reactions include oxygen insertion into unactivated C—H bonds as well as C—C bond cleavage. The fungal P450-1 shows remarkable multifunctionality catalyzing oxidation at non-adjacent carbon centers during the synthesis of 3β-hydroxylated GAs, plus several side reactions involved in kaurenolide and fujenoic acid synthesis (Rojas et al., 2001, 2004). In Arabidopsis and barley, as well as in pea, the corresponding function is carried out by *ent*-kaurenoic acid oxidase, a monooxygenase that lacks 3β-hydroxylase activity in contrast to its fungal counterpart (Davidson et al., 2003; Helliwell et al., 2001). Interestingly, the two major activities of P450-1, 3β-hydroxylase and 7-oxidase, can be dissociated by changing the electron source (Urrutia et al., 2001). Both activities are detected with NADPH, while only 7-oxidase is found in the presence of NADH, suggesting that interaction of P450-1 with different electron transport proteins changes its catalytic function (Rojas et al., 2004). The NADPH–cytochrome P450 oxidoreductase (CPR) encoded by *cpr* is the main electron transport protein associated with the GA monooxygenases in *F. fujikuroi* (Malonek et al., 2004). Targeted disruption of *cpr* decreased GA production to about 3% of the levels found in wild-type strains (Malonek et al., 2004). Gibberellin synthesis was however not totally abolished indicating the existence of a second fungal electron transport system.

The mechanism for the sequential oxidation of C-20 by P450-2, including the identification of intermediates, has not yet been established. It has been demonstrated that C-20 is lost as CO_2 (Dockerill and Hanson, 1978) and that both oxygen atoms of the 19,10-γ-lactone in the C_{19}-GA product come from the C-19 carboxylate group (Bearder et al., 1976). C-20 alcohol (**15**/**16**) and C-20 aldehyde (**17**/**18**) GAs (Fig. 2) are not formed at significant levels or converted into C_{19}-GAs when applied (Bearder et al., 1975; Tudzynski et al., 2002), suggesting that they may not be



GA_{15} R =H , 15 GA_{24} R =H , 17
GA_{37} R =OH , 16 GA_{36} R =OH , 18

Fig. 2. Structures of fungal GAs partially oxidized at C-20.

intermediates in the reaction sequence catalyzed by P450-2. However, GA_{15} (C-20 alcohol), **15**, and GA_{24} (C-20 aldehyde), **17**, were found as main products of the pathway in CPR-deficient Δ*cpr* and SG138 strains (Malonek et al., 2004), and may result from hydrolysis of covalently enzyme-bound intermediates that accumulate under low oxidation conditions. In contrast to P450-2, plant GA 20-oxidases, which are 2-oxoglutarate-dependent dioxygenases, accumulate alcohol and aldehyde intermediates in the sequential C-20 oxidation; these intermediates serve as substrates for the enzyme (Lange et al., 1994). The loss of CO_2 occurs from the C-20 aldehyde possibly giving rise to a C-10 radical intermediate (Ward et al., 2002). Two oxidation cycles are required to give CO_2, a sequence for which an enzyme-bound C-20 thioester has been proposed (Hedden, 1997; Ward et al., 2002).

The finding of non-hydroxylated C-20 GAs as main products of the pathway in CPR-deficient strains suggests that P450-1 and P450-2 monooxygenases could catalyze different oxidation reactions in the mutants compared to CPR-containing strains. However, this has to be directly demonstrated, since the altered GA pattern could result from reduced available substrate due to low *ent*-kaurene oxidase activity in the mutants as evidenced by *ent*-kaurene accumulation in Δ*cpr* cultures (Malonek et al., 2004). In this study, we investigate if indeed some catalytic activities of P450-1 and P450-2 GA monooxygenases are selectively reduced or suppressed in CPR-deficient mutants due to interaction with an alternative electron transport sytem. We directly demonstrated the reactions catalyzed by P450-1 and P450-2 in cultures of Δ*cpr* and SG138 and show that the properties of the associated electron transport proteins that support P450-1 activity in these mutants are consistent with cytochrome *b*5:NADH-cytochrome *b*5 oxidoreductase (cyt *b*5:cyt *b*5 reductase). Finally, production of GA_{15} (C-20 alcohol), **15**, and GA_{24} (C-20 aldehyde), **17**, by Δ*cpr* gave us the opportunity to investigate the participation of these GAs in C-20 oxidation by P450-2.

2. Results and discussion

2.1. Products formed by P450-1 and P450-2 monooxygenases in CPR-deficient mutants

Reactions catalyzed by P450-1 and P450-2 were investigated in Δ*cpr* and SG138 mutants by incubating with ^{14}C-labelled substrates under GA-producing conditions (Table 1). The Δ*cpr* disruption mutant lacks CPR, while SG138 contains a truncated CPR that lacks part of the NADPH binding site (Malonek et al., 2004). After 3 days of incubation, *ent*-[$^{14}C_4$]kaurenoic acid (**3**), *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**4**) and [$^{14}C_4$]GA_{12}-aldehyde (**5**) were converted into a main product identified by GC–MS as the non-3β-hydroxylated C-20 alcohol [$^{14}C_4$]GA_{15}, **15** (Table 1; Fig. 2). This requires oxidation at C-7 and C-6 by P450-1, as well as partial oxidation of the C-20 methyl by P450-2. The same labelled product was obtained from the C-20 methyl precursor [$^{14}C_4$]GA_{12} (**7**). In some incubations with [$^{14}C_4$]GA_{12} (**7**) or [$^{14}C_4$]GA_{12}-aldehyde (**5**), [$^{14}C_4$]GA_{24} (C-20 aldehyde), **17**, was found in addition to [$^{14}C_4$]GA_{15} (**15**), although at lower levels (Table 1; Fig. 2). No labelled 3β-hydroxylated or C_{19}-GAs, the main products of the pathway in wild-type strains (Bearder et al., 1975; Hedden et al., 1974), were detected, in agreement with the major endogenous GAs present in Δ*cpr* or SG138, which contain only trace levels of GA_4 (**11**) and GA_7, **13** (Malonek et al., 2004).

Thus, results shown in Table 1 confirm that the reactions catalyzed by P450-1 and P450-2 in CPR-deficient mutants differ from those catalyzed by these GA monooxygenases in wild-type strains, suggesting interaction with alternative electron transport proteins. The differences found do not depend on substrate concentration since GA_{15} (**15**) and GA_{24} (**17**) were the main oxidation products in incubations of Δ*cpr* with *ent*-kaurenoic acid (**3**), in the range of 0.08–50 μg or with GA_{12} (**7**) in the range of 0.08–20 μg, conditions under which CPR-containing strains synthesize mainly 3β-hydroxylated C_{19}-GAs. The 3β-hydroxylase activity of P450-1 is strongly reduced but not completely absent in the mutants since low levels of unlabelled 3β-hydroxylated intermediates, GA_{37} (**16**) and/or GA_{36} (**18**), were detected together with unlabelled GA_{15} (**15**), GA_{24} (**17**) and/or GA_{25} (**9**), besides the labelled products in some incubations. The presence of *ent*-6α,7α-dihydroxy[$^{14}C_4$]kaurenoic acid (Table 1), an intermediate formed by P450-1 on a branch pathway to fujenoic acid and which does not accumulate in wild-type strains (Rojas et al., 2001, 2004), suggests that this monooxygenase has a low activity in Δ*cpr*. The C-20 oxidation products formed by Δ*cpr* or SG138 mutants differ from those synthesized by CPR-containing strains in which the methyl group of GA_{12} (**7**) or GA_{14} (**8**) is removed as CO_2 in the formation of C_{19}-GAs (Tudzynski et al., 2002). Synthesis of GAs partially oxidized at C-20 by the mutants suggests that P450-2 has a reduced catalytic efficiency in the absence of CPR. GA_{15} (**15**) and GA_{24} (**17**) may correspond to intermediates that either dissociate from the enzyme under inefficient catalytic conditions, or alternatively may result from hydrolysis of enzyme-bound intermediates covalently linked through C-20. If the oxidation sequence is slow, bound intermediates would hydrolyze to give the C-20 alcohol and aldehyde GAs at a higher rate than they are oxidized.

Table 1
Products identified by GC–MS by comparison with published spectra (Gaskin and MacMillan, 1992) from incubations of ^{14}C-GA precursors with CPR-deficient *F. fujikuroi* mutants

Mutant	Substrate	Products	Mass spectrum *m/z* (% relative abundance)[a]
Δ*cpr*	*ent*-[$^{14}C_4$]Kaurenoic acid, **3**	[$^{14}C_4$]GA_{15}, **15** (66%)[b]	352(7), 350(8), 320(5), 318(9), 290(31), 288(23), 284(7), 245(100), 243(93), 239(38), 201(40), 199(39)
		7β-Hydroxy [$^{14}C_4$]kaurenolide (12%)	353(8), 351(6), 345(5), 306(45), 304(62),298(25), 278(11), 276(14), 141(100), 139(85), 137(60)
		ent-6α, 7α-diOH-[$^{14}C_4$]kaurenoic acid (10%)	485(76), 483(90), 477(27), 410(12), 408(17), 402(5), 273(100), 271(66), 269(36)
	ent-7α-Hydroxy [$^{14}C_4$]kaurenoic acid, **4**	[$^{14}C_4$]GA_{15}, **15** (40%)	290(22), 288(19), 245(100), 243(92), 239(23), 201(41), 199(36), 195(13)
		ent-6α, 7α-diOH-[$^{14}C_4$]kaurenoic acid (20%)	485(51), 483(84), 477(29), 410(11), 408(9), 273(100), 271(60), 269(41)
	[$^{14}C_4$]GA_{12}-aldehyde, **5**	[$^{14}C_4$]GA_{15}, **15** (80%)	352(8), 344(4), 320(9), 312(4), 290(30), 288(5), 284(21), 245(100), 243(29), 239(58), 201(34), 199(10), 195(20)
	[$^{14}C_4$]GA_{12}, **7**	[$^{14}C_4$]GA_{15}, **15** (78%)	352(10), 350(2), 344(7), 320(12), 318(2), 312(9), 290(33), 288(7), 284(26), 245(100), 243(32), 239(72), 201(40), 199(14), 195(30)
		[$^{14}C_4$]GA_{24}, **17** (14%)	350(24), 342(14), 322(67), 314(68), 310(25), 293(59), 292(40), 290(47), 286(61), 285(42), 260(24), 254(30), 232(90), 231(100), 227(71), 226(85), 225(83)
SG138	[$^{14}C_4$]GA_{12}-aldehyde, **5**	[$^{14}C_4$]GA_{15}, **15** (63%)	352(8), 350(3), 344(3), 320(12), 318(4), 312(3), 290(32), 288(8), 284(11), 245(100), 243(35), 239(33), 201(44), 199(16), 195(13)
		[$^{14}C_4$]GA_{24}, **17** (9%)	350(13), 322(83), 314(54), 310(10), 293(60), 292(60), 290(44), 286(55), 285(24), 260(33), 254(22), 232(77), 231(100), 227(48), 226(66), 225(24)
	[$^{14}C_4$]GA_{12}, **7**	[$^{14}C_4$]GA_{15}, **15** (70%)	352(8), 350(2), 344(4), 320(11), 318(4), 312(6), 290(35), 288(7), 284(19), 245(100), 243(29), 239(66), 201(47), 199(16), 195(24)

[a] For diagnostically important ions.
[b] Percent substrate conversion into each product is shown in parenthesis.

2.2. Rates of the reactions catalyzed by P450-1 and P450-2 in Δcpr and SG138

In order to establish if the catalytic efficiencies of P450-1 and P450-2 monooxygenases are decreased in the mutants the oxidation rates of the respective substrates ([$^{14}C_4$]GA_{12}-aldehyde (**5**) or [$^{14}C_4$]GA_{12} (**7**)) were determined in CPR-containing and CPR-deficient strains.

The time course of [$^{14}C_4$]GA_{12}-aldehyde (**5**) conversion by Δ*cpr* and SG138 was compared to that by SG139-P450-1, a mutant that contains CPR and P450-1, but none of the other GA-biosynthetic enzymes (Rojas et al., 2001) (Fig. 3a). This last strain allowed the reactions catalyzed by P450-1 to be examined without interference by activities of the other enzymes of GA biosynthesis or by the presence of endogenous GAs. Δ*cpr* and SG138 oxidized [$^{14}C_4$]GA_{12}-aldehyde (**5**) at rates of 0.42 pmol/min and 0.38 pmol/min, respectively, while SG139-P450-1 gave a value of 1.4 pmol/min, 3.3 and 3.7 times higher. The activity of P450-1 is thus decreased in CPR-deficient mutants but still retained at a significant level. A similar dependence on the P450 reductase has been described for the GA P450-3 monooxygenase (13-hydroxylase) that retains about 25% of its activity in Δ*cpr* compared to the wild-type strain IMI 58289 (Malonek et al., 2004). The time course of [$^{14}C_4$]GA_{12} (**7**) oxidation by P450-2 was compared in CPR mutants with that in the CPR-containing strain SG139-P450-2 (Fig. 3b). Rates of 0.15 pmol/min and 0.2 pmol/min were found for Δ*cpr* and SG138, respectively, about one-tenth of the value found for SG139-P450-2 (2.0 pmol/min). Thus P450-2 has a stronger dependence on CPR than does P450-1 and

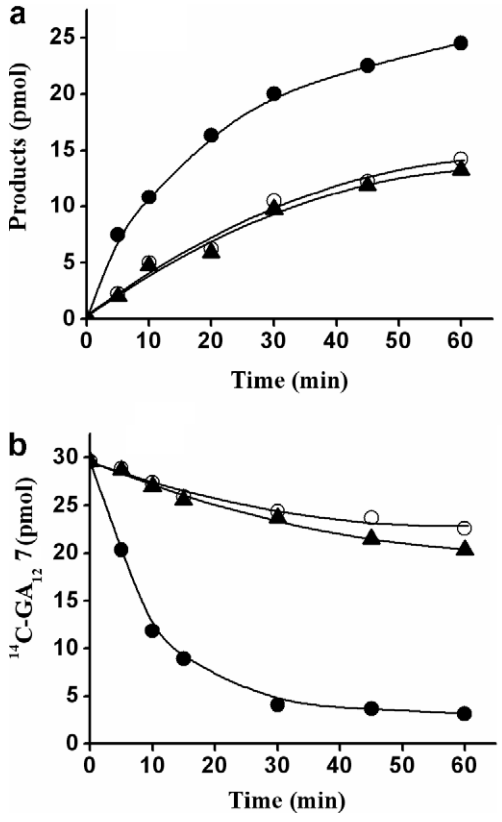


Fig. 3. Time course of enzymatic conversion of P450-1 and P450-2 substrates. (a) Conversion of [^{14}C]GA_{12}-aldehyde (**5**) by cultures of the CPR-deficient mutants Δ*cpr* (○) or SG138 (▲) and of the CPR-containing strain SG139-P450-1 (●). (b) [^{14}C]GA_{12} (**7**) oxidation by cultures of Δ*cpr* (○), SG138 (▲) or SG139-P450-2 (●).

together with P450-4 (*ent*-kaurene oxidase), the activity of P450-2 would have a major influence on the levels of GAs formed by the mutants. In particular, reduced P450-4 activity in Δ*cpr* and SG138 (Malonek et al., 2004) would result in low substrate availability for P450-1 and P450-2, decreasing further their catalytic efficiencies. Altogether this would account for highly inefficient GA synthesis by the CPR-deficient strains.

2.3. Electron source for the reactions catalyzed by P450-1 in CPR mutants

The cofactor requirement of reactions catalyzed by P450-1 was investigated in mycelial microsomal fractions from Δ*cpr* or SG138 in order to characterize the alternative electron source for the fungal GA monooxygenases (Table 2). The activity of this monooxygenase is easily detectable *in vitro* in contrast to P450-2 and P450-3, which are inactive in cell-free extracts (Rojas et al., 2004; Urrutia et al., 2001). Incubations of *ent*-[$^{14}C_4$]kaurenoic acid (**3**), *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**4**) or [$^{14}C_4$]GA_{12}-aldehyde (**5**) with the microsomal fractions in the presence of NADPH gave no enzymatic conversion of these substrates (Table 2). In contrast, when NADH was added as reductant *ent*-[$^{14}C_4$]kaurenoic acid (**3**) was oxidized at C-7 and C-6 to give *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid, **4** (49–60%) plus lower amounts of [$^{14}C_4$]GA_{12} (**7**) as final product (8–18%). No [$^{14}C_4$]GA_{14} (**8**), the main product of P450-1 in CPR-containing strains, was detected from this substrate or from *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**4**). When [$^{14}C_4$]GA_{12}-aldehyde (**5**) was used as substrate, [$^{14}C_4$]GA_{12} (**7**) was the main product (79–83%) and very low levels of [$^{14}C_4$]GA_{14} (**8**) were formed (6–10%) confirming residual 3β-hydroxylase activity in Δ*cpr* and SG138 mutants. In contrast to [$^{14}C_4$]GA_{12}-aldehyde (**5**), *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**4**) was only partially converted, consistent with its accumulation from incubations with *ent*-[$^{14}C_4$]kaurenoic acid, **3** (Table 2). Thus, in CPR-deficient mutants P450-1 catalyzes three sequential oxidation reactions from *ent*-kaurenoic acid (**3**) with NADH as exclusive electron source. These reactions include 7β-hydroxylation, contraction of ring B initiated by oxidation at C-6 and further oxidation at C-7, while hydroxylation at C-3β is severely reduced. Ring B contraction is slow compared to 7-oxidation since *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid (**4**) accumulates in contrast to [$^{14}C_4$]GA_{12}-aldehyde (**5**) that was not detected in any incubation. The same oxidation reactions were demonstrated with microsomes of Δ*cpr* or SG138 which confirms that both mutants are functionally equivalent. Thus, in contrast to CPR that supplies electrons from NADPH to the GA mooxygenases (Malonek et al., 2004), the second electron transport system present in *F. fujikuroi* utilizes exclusively NADH as reductant.

Restoration of NADPH-dependent reactions was demonstrated in microsomes from Δ*cpr* or SG138 complemented with the *cpr* gene from *F. fujikuroi* (Δ*cpr*-*cprFf*, SG138-*cprFf*) or from *Aspergillus niger* (SG138-*cprAn*) (Table 3). [$^{14}C_4$]GA_{12}-aldehyde (**5**) was converted mainly into [$^{14}C_4$]GA_{14} (**8**) when incubated with NADPH, while [$^{14}C_4$]GA_{12} (**7**) was the only product formed in the presence of NADH. Addition of NADH, in the range of 0.5–5 mM, to assays containing NADPH and [$^{14}C_4$]GA_{12}-aldehyde (**5**) did not alter the rate of [^{14}C]GA_{14} (**8**) synthesis.

The exclusive requirement for NADH of P450-1-catalyzed reactions in CPR-deficient mutants, as well as reduced reaction rates found for P450-1 and P450-2 in the mutants, are consistent with involvement of cyt *b5*:cyt *b5* reductase as a redox partner of the GA monooxygenases. Cytochrome *b5* reductase, a flavoprotein that has an NADH binding site, can provide electrons to P450 monooxygenases through cytochrome *b5* (Bewley et al., 2001). It has been described that the cyt *b5*:cyt *b5* reductase system is less efficient than CPR in electron donation to P450s (Lamb et al., 1999; Mokashi et al., 2003;

Table 2
Pyridine nucleotide specificity for reactions catalyzed by P450-1 in CPR-deficient mutants

Mutant	Substrate	Cofactors[a]	Products
Δ*cpr*	*ent*-[$^{14}C_4$]Kaurenoic acid, **3**	NADPH/FAD	–[b]
		NADH/FAD	*ent*-7α-Hydroxy [$^{14}C_4$]kaurenoic acid, **4** (51%)[c], [$^{14}C_4$]GA_{12}, **7** (14%)
	ent-7α-Hydroxy [$^{14}C_4$]kaurenoic acid, **4**	NADPH/FAD	–[b]
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (24%)
	[$^{14}C_4$]GA_{12}-aldehyde, **5**	NADPH/FAD	–[b]
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (72%) [$^{14}C_4$]GA_{14}, **8** (7%)
SG138	*ent*-[$^{14}C_4$]Kaurenoic acid, **3**	NADPH/FAD	–[b]
		NADH/FAD	*ent*-7α-Hydroxy [$^{14}C_4$]kaurenoic acid, **4** (40%), [$^{14}C_4$]GA_{12}, **7** (4%)
	ent-7α-Hydroxy [$^{14}C_4$]kaurenoic acid, **4**	NADPH/FAD	–[b]
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (12%)
	[$^{14}C_4$]GA_{12}-aldehyde, **5**	NADPH/FAD	–[b]
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (76%) [$^{14}C_4$]GA_{14}, **8** (4%)

[a] 1 mM NADPH plus 5 μM FAD or 1 mM NADH plus 250 μM FAD.
[b] The substrate was recovered unconverted and no products were detected in assays with NADPH as reductant.
[c] Percent substrate conversion into each product. Based on recovered radiactivity after HPLC separation.

Table 3
Pyridine nucleotide specificity for P450-1-catalyzed reactions in mutants complemented with *cpr* from *F. fujikuroi* or from *A. niger*

Complemented mutant	Substrate	Cofactors[a]	Products
Δ*cpr-cprFf*	[$^{14}C_4$]GA_{12}-aldehyde, **5**	NADPH/FAD	[$^{14}C_4$]GA_{14}, **8** (61%)[b] [$^{14}C_4$]GA_{12}, **7** (30%)
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (80%)
SG138-*cprFf*	[$^{14}C_4$]GA_{12}-aldehyde, **5**	NADPH/FAD	[$^{14}C_4$]GA_{14}, **8** (50%) [$^{14}C_4$]GA_{12}, **7** (33%)
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (66%)
SG138-*cprAn*	[$^{14}C_4$]GA_{12}-aldehyde, **5**	NADPH/FAD	[$^{14}C_4$]GA_{14}, **8** (67%) [$^{14}C_4$]GA_{12}, **7** (19%)
		NADH/FAD	[$^{14}C_4$]GA_{12}, **7** (25%)

[a] 1 mM NADPH plus 5 μM FAD or 1 mM NADH plus 250 μM FAD.
[b] Percent substrate conversion. Based on recovered radiactivity after HPLC separation.

Venkateswarlu et al., 1998) giving lower oxidation rates (Miller, 2005). For many P450s, the cyt *b5* electron transport system can either assist CPR by supplying the second, but not the first, electron of the catalytic cycle (Guengerich and Johnson, 1997) or by acting as an allosteric activator (Porter, 2002). In sterol and glucocorticoid synthesis, the two CPR-dependent activities of CYP17 (17,20 lyase and 17α-hydroxylase) are modulated differently by cyt *b5* (Pandey and Miller, 2005). Interestingly, we found for the fungal GA monooxygenases that the electron transport system present in CPR mutants can support entirely the respective catalytic cycles substituting for the P450 reductase in several oxidation reactions although with a lower efficiency.

2.4. Additional properties of the electron transport system associated with P450-1 in Δcpr

The electron transport system present in Δ*cpr* is membrane-bound, since all P450-1 activities were found in the 100,000*g* pellet with no activity or enhancement of activity associated with the 100,000*g* supernatant. The inclusion of 0.57 μM cyt *b5* in the reaction media containing [$^{14}C_4$]GA_{12}-aldehyde (**5**) and NADH increased by twofold the rate of [$^{14}C_4$]GA_{12} (**7**) synthesis (Fig. 4). The same reaction assayed with microsomes of the wild-type strain IMI 58289 and NADH was increased by a factor of 1.6 by added cyt *b5*, while no effect was found for NADPH-dependent [$^{14}C_4$]GA_{12}-aldehyde (**5**) oxidation (Fig. 4). [$^{14}C_4$]GA_{12} (**7**) synthesis from [$^{14}C_4$]GA_{12}-aldehyde (**5**) by Δ*cpr* microsomes was activated fivefold upon addition of 300 μM FAD, indicating that a flavoprotein is involved in this reaction and consistent with participation of cyt *b5* reductase which has an FAD prosthetic group (Lamb et al., 1999; Bewley et al., 2001). The activating effect showed a hyperbolic dependence on FAD concentration with a half-saturating concentration of 25 μM, 50 times higher than the half-saturating concentration (0.5 μM) described for activation by FAD of CPR-mediated [$^{14}C_4$]GA_{14} (**8**) synthesis by wild-type microsomes (Urrutia et al., 2001). Added cyt *b5* would enhance coupling of cyt *b5* reductase to the P450-1 monooxygenase facilitating electron transport (Schenkman and Jansson, 2003), while added FAD would restore bound flavin that may have been lost from the cyt *b5* reductase during microsome preparation. Finally we found that [$^{14}C_4$]GA_{12} (**7**) synthesis from [$^{14}C_4$]GA_{12}-aldehyde (**5**) in Δ*cpr* microsomes was inhibited by cytochrome *c* or potassium ferricyanide, two electron acceptors that can interact with the cytochrome *b5* electron transport system (concentration for half maximal activity, I_{50}, 2 μM and 2 mM, respectively). Cyt *c* forms stable complexes with cyt *b5* (Shao et al., 2003), while ferricyanide is an artificial substrate for flavin reductases such as cyt *b5* reductase (Bewley et al., 2001). The I_{50} value for cyt *c* is 150 times lower than the value found for inhibition of NADPH-dependent [^{14}C]GA_{14} (**8**) synthesis mediated by CPR (0.3 mM), which supports participation of different electron transport proteins in GA biosynthesis by the mutants and wild-type strains. Thus, all the properties found for the reactions catalyzed by P450-1 in the mutants are consistent with involvement of the cyt *b5* electron transport system (Porter, 2002).

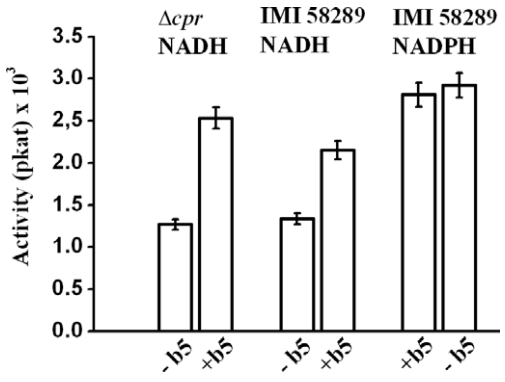


Fig. 4. Effect of added cyt *b5* (0.57 μM) on [^{14}C]GA_{12}-aldehyde (**5**) oxidation by Δ*cpr* or wild-type (IMI 58289) microsomes in the presence of 1 mM NADH or 1 mM NADPH. Results are average of three independent determinations. Error bars represent standard deviation.

Interaction of P450-1 with either CPR or alternatively with cyt *b5*:cyt *b5* reductase would account for the different catalytic activities found for this monooxygenase in the mutants and wild-type strains. In the wild-type, 3β-hydroxylation of GA_{12}-aldehyde (**5**) is favored relative to 7-oxida-

tion (Rojas et al., 2004; Urrutia et al., 2001) in contrast to CPR-deficient mutants in which 7-oxidation is the main reaction catalyzed. Substrate orientation relative to the heme center determines the carbon positions oxidized by P450 monooxygenases (White et al., 1984). Thus, association of P450-1 with CPR or with cyt *b*5:cyt *b*5 reductase would change substrate orientation and accessibility of the different carbon centers to the iron oxidizing species, giving different products and/or oxidation rates. It is possible that in CPR-containing strains C-3 of GA_{12}-aldehyde (**5**) is more accessible to the iron center than C-7 and thus is oxidized at a higher rate while in CPR mutants C-7 would be the more accessible atom resulting in the product pattern found. The truncated CPR present in SG138 is unable to bind NADPH due to lack of part of the NADPH binding domain (Malonek et al., 2004) and thus would not interact with P450-1. Therefore the reactions catalyzed by this monooxygenase are the same in Δ*cpr* and SG138 mutants. Orientation of GA_{12}-aldehyde (**5**) at the active site of P450-1 in CPR mutants may be similar to that in plant *ent*-kaurenoic acid oxidases which produce GA_{12} as exclusive final product (Davidson et al., 2003; Helliwell et al., 2001).

2.5. Gibberellin C-20 oxidation in CPR-deficient mutants

To obtain additional information about the oxidation sequence in the synthesis of C_{19}-GAs by *F. fujikuroi*, C-20 oxidation was further examined in cultures of Δ*cpr* in which the catalytic efficiency of P450-2 is decreased by 10-fold (Fig. 3b). [$^{14}C_4$]GA_{12} (**7**) and [$^{14}C_4$]GA_{14} (**8**) as well as isotopically labelled C-20 alcohol or aldehyde GAs ([17-^{14}C]GA_{15} (**15**), [17-^{14}C]GA_{24} (**17**), [17-^{2}H]GA_{37} (**16**) or [17-^{2}H]GA_{36} (**18**)) were incubated with Δ*cpr* cultures to establish if they are metabolized by P450-2 and to confirm the final C-20 oxidation products (Table 4; Fig. 2).

Conversion of [$^{14}C_4$]GA_{14} (**8**) into the C-20 aldehyde [$^{14}C_4$]GA_{36} (**18**) and the tricarboxylic acid [$^{14}C_4$]GA_{13} (**12**) indicates that the final C-20 oxidation product is the carboxylic acid. [$^{14}C_4$]GA_{36} (**18**) as well as [^{14}C]GA_{15} (**15**) and [^{14}C]GA_{24} (**17**), the alcohol and aldehyde GAs formed from [^{14}C]GA_{12} (**7**), may correspond to intermediates in the oxidation sequence as indicated by their conversion into the same final products. The C-20 alcohol precursors [^{14}C]GA_{15} (**15**) and [^{2}H]GA_{37} (**16**), added as the 19,20 lactones, were converted into the corresponding tricarboxylic acids [^{14}C]GA_{25} (**9**) and

Table 4
Identification by GC–MS of products from incubations of isotopically labelled GA_{12} (**7**), GA_{14} (**8**), C-20 alcohol (**15/16**) and C-20 aldehyde (**17/18**) GAs with Δ*cpr* cultures

Substrate	Products	Retention time (min)	Mass spectrum *m/z* (% relative abundance)[a]
[$^{14}C_4$]GA_{14} **8**	[$^{14}C_4$]GA_{36}, **18**	23.57	462(7), 447(5), 438(17), 436(14), 430(30), 408(6), 402(14), 348(34), 346(21), 340(45), 318(16), 316(18), 312(48), 290(62), 288(27), 284(100)
	[$^{14}C_4$]GA_{13}, **12**	23.46	477(11), 436(33), 406(50), 400(40), 372(14), 342(20), 318(78), 310(100)
[^{2}H]GA_{37}, **16**	[^{2}H]GA_{36}, **18**	23.59	464(5), 449(6), 432(68), 414(11), 404(22), 386(14), 376(30), 342(27), 314(36), 286(100), 239(20), 227(26)
	[^{2}H]GA_{13}, **12**	23.45	479(15), 477(15), 462(17), 460(28), 438(19), 436(26), 402(100), 400(97), 374(34), 372(49), 351(41), 349(47), 344(25), 342(35), 312(68), 310(77), 284(52), 282(46)
[^{2}H]GA_{36}, **18**	[^{2}H]GA_{13}, **12**	23.41	494(4), 479(11), 462(15), 460(11), 438(28), 402(100), 400(53), 374(33), 372(17), 351(47), 344(24), 312(79), 310(20), 284(40), 282(21)
[$^{14}C_4$]GA_{12}, **7**	[$^{14}C_4$]GA_{15}, **15** (heavily diluted)[b]	24.28	344(7), 312(17), 290(9), 288(7), 284(67), 245(16), 243(18), 239(100), 201(12), 199(13), 195(64)
	[$^{14}C_4$]GA_{24}, **17** (heavily diluted)[b]	21.29	342(8), 322(6), 314(49), 310(49), 293(4), 292(5), 290(6), 286(54), 285(22), 254(62), 231(15), 227(51), 226(100), 225(66)
[^{14}C]GA_{15}, **15**	[^{14}C]GA_{24}, **17** (diluted)[b]	21.21	344(13), 342(17), 316(23), 314(60), 312(19), 310(69), 288(20), 287(18), 286(68), 285(26), 282(47), 256(21), 254(47), 229(17), 228(39), 227(64), 226(100), 225(68)
	[^{14}C]GA_{25}, **9** (diluted)[b]	21.14	374(14), 372(19), 314(20), 312(66), 286(42), 284(100), 227(21), 225(51)
[^{14}C]GA_{24}, **17**	[^{14}C]GA_{25}, **9**	21.13	406(4), 374(22), 372(16), 314(71), 312(61), 286(100), 284(84), 227(57), 225(45)
Standards	GA_{15}, **15**	24.22	344(7), 312(21), 298(22), 284(88), 239(100), 195(73)
	GA_{13}, **12**	23.40	447(4), 460(6), 436(18), 400(84), 372(32), 349(41), 342(27), 310(100), 283(45), 282(63), 223(57), 129(20)
	GA_{24}, **17**	21.28	342(9), 314(57), 310(59), 286(46), 284(31), 282(32), 254(53), 226(100), 225(74)
	GA_{25}, **9**	21.13	372(5), 312(73), 284(100), 225(45), 224(36)
	GA_{36}, **18**	23.53	462(2), 447(3), 430(38), 402(17), 374(21), 340(22), 312(38), 284(100), 283(21), 225(43), 224(34), 171(27)

Mass spectra and retention times were compared with those of standards.
[a] Shown for diagnostically important ions.
[b] Dilution with endogenous GAs was found for some of the labelled products.

[^{2}H]GA_{13} (**12**) as well as into C-20 aldehyde products [^{14}C]GA_{24}, **17** and [^{2}H]GA_{36}, **18** (Table 4). Enzymatic conversion of [^{2}H]GA_{37} (**16**) was higher at pH 3.0 than at pH values of 4.5 or 6.4 which supports oxidation of the intact 19,20 lactonic form of the substrate by P450-2. This is different from most plant 20-oxidases that utilize only the free 20-alcohol forms (open lactones) of GA_{15} or GA_{44} (MacMillan, 1997). Added [^{14}C]GA_{24}, **17** or [^{2}H]GA_{36}, **18** (C-20 aldehyde) were also converted by Δ*cpr* into the tricarboxylic acid products [^{14}C]GA_{25} (**9**) and [^{2}H]GA_{13} (**12**), respectively (Table 4). No labelled C_{19}-GAs were formed although traces of unlabelled GA_7 were detected in some incubations together with unlabelled GA_{15} (**15**), GA_{24} (**17**), GA_{25} (**9**) and GA_{36} (**18**), indicating that oxidation of C-20 to CO_2, a major P450-2 activity in CPR-containing strains, although severely reduced, is not completely absent in Δ*cpr*.

In order to establish if the C-20 alcohol and aldehyde products are formed directly or are generated by hydrolysis of intermediates covalently linked to P450-2 through C-20, the origin of the oxygen atom at C-20 in GA_{15} (**15**) and GA_{24} (**17**) was investigated (Table 5). Unlabelled GA_{12}, **7** (20 μg) was either added to Δ*cpr* cultures suspended in [^{18}O]H_2O or alternatively maintained under an atmosphere of [^{18}O]O_2:N_2 (20:80) in the presence of AMO 1618 to inhibit formation of endogenous GAs (Rademacher, 1992). Control incubations were carried out in either [^{16}O]H_2O or under an atmosphere of [^{16}O]O_2:N_2 (20:80). Hydrolysis of enzyme-bound intermediates would result in oxygen incorporation from [^{18}O]H_2O in contrast to sequential oxidation through free intermediates in which the oxygen atom at C-20 would originate solely from [^{18}O]O_2. In all incubations, GA_{12}, (**7**) was converted by Δ*cpr* into GA_{15}, **15** (75–80%) and GA_{24}, **17** (13–15%), together with trace levels of 3β-hydroxylated GAs (GA_{36}, **18**; GA_{37}, **16**). Traces of the tricarboxylic acid products GA_{25} (**9**) and GA_{13} (**12**) were also detected. Comparison of the mass spectra of GA_{15} (**15**) or GA_{24} (**17**) formed in the presence of [^{18}O]H_2O or [^{16}O]H_2O (Table 5) demonstrated that no ^{18}O label was incorporated in these products as determined from the relative abundance of the ions in the molecular ion cluster for GA_{15} (**15**) or for the fragment at *m*/*z* 342 (M^+-32) for GA_{24} (**17**). The molecular ion for GA_{24} (**17**) is too weak for accurate determination of the isotopic abundance while the fragment at *m*/*z* 342 has retained the C-20 aldehyde residue. In contrast, both GA_{15} (**15**) and GA_{24} (**17**) formed under an atmosphere of [^{18}O]O_2:N_2 (20:80) contained one ^{18}O at high isotopic abundance (Table 5) determined by comparison of the mass spectra with those of GA_{15} (**15**) and GA_{24} (**17**) formed in the presence of [^{16}O]O_2:N_2 (20:80). GA_{25} (**9**) and GA_{13} (**12**), the tricarboxylic acid products, were formed at too low levels to determine their ^{18}O content accurately. Control incubations with *ent*-kaurenoic acid (**3**) in the presence of [^{18}O]O_2 or [^{16}O]O_2 were carried out to test oxygen incorporation into *ent*-7α-hydroxykaurenoic acid (**4**), a reaction that is known to occur from O_2 (Rojas et al., 2001). As expected, *ent*-7α-hydroxykaurenoic acid (**4**) formed in the presence of [^{18}O]O_2 contained one ^{18}O at C-7 (data not shown). Several other products also contained ^{18}O; these included non-hydroxylated GAs (GA_{12}, **7**; GA_{15} **15**; GA_{24}, **17**) and low levels of 3β-hydroxylated products (GA_{36}, **18** and GA_{13}, **12**), but not C_{19}-GAs, confirming that the end products of the GA pathway in CPR-deficient mutants are the tricarboxylic acids.

The C-20 alcohol and C-20 aldehyde GAs correspond to intermediates in the fungal C-20 oxidation sequence since the oxygen atom at C-20 of GA_{15} (**15**) and GA_{24} (**17**) comes entirely from O_2 and not from H_2O as would be the case if these compounds were formed by hydrolysis of enzyme-bound intermediates. However, the failure of exogenous C_{20}-GAs apart from GA_{12} (**7**) and GA_{14} (**8**) to be efficiently metabolized by CPR-containing strains (Bearder et al., 1975; Tudzynski et al., 2002) suggests that there is channeling of these intermediates, which would remain non-covalently bound at the catalytic site of P450-2. The

Table 5
Origin of the oxygen atom at C-20 of alcohol and aldehyde GAs. GC–MS analysis of GA_{15} (**15**) and GA_{24} (**17**) formed by Δ*cpr* in the presence of [^{18}O]H_2O or [^{18}O]O_2

Substrate	Incubation conditions	Products[a]	*m*/*z* (% relative abundance)	% ^{18}O incorporation at C-20
GA_{12}, **7**	[^{18}O]H_2O	GA_{15}, **15**	344(100)[b], 345(40), 346(11), 347(12), 348(8)	0
		GA_{24}, **17**	342(100)[c], 343(36), 344(15), 345(4), 346(29)	0
GA_{12}, **7**	[^{16}O]H_2O	GA_{15}, **15**	344(100)[b], 345(29), 346(22), 347(13), 348(6)	n.d.[d]
		GA_{24}, **17**	342(100)[c], 343(28), 344(12), 345(13), 346(36)	n.d.[d]
GA_{12}, **7**	[^{18}O]O_2:N_2 (20:80)	GA_{15}, **15**	344(100)[b], 345(128), 346(672), 347(243), 348(66)	85
		GA_{24}, **17**	342(100)[c], 343(137), 344(457), 345(206), 346(234)	76
GA_{12}, **7**	[^{16}O]O_2:N_2 (20:80)	GA_{15}, **15**	344(100)[b], 345(25), 346(10), 347(16), 348(3)	n.d.[d]
		GA_{24}, **17**	342(100)[c], 343(28), 344(4), 345(3), 346(1)	n.d.[d]

[a] GAs present in EtOAc extracts of culture filtrates were analyzed as methyl esters by GC–MS.
[b] Relative abundance of molecular ion cluster (M^+ at *m*/*z* 344 set to 100%).
[c] Relative abundance of ion cluster for the fragment ion at *m*/*z* 342 (M^+-32); ion at *m*/*z* 342 set to 100%.
[d] Not determined.

high efficiency of electron transfer from the P450 reductase to P450-2 would strongly favor further oxidation and thus would avoid dissociation of intermediates from the enzyme in successive catalytic cycles. In contrast, in CPR-deficient strains that contain an inefficient electron transfer system these intermediates would dissociate due to their relatively low affinity for the enzyme. Dissociated intermediates, which apparently re-associate with the enzyme slowly, would be oxidized at low rates and thus accumulate in the reaction media.

The formation of C-20 carboxylic acid products by P450-2 in Δ*cpr*, in contrast to almost undetectable levels of C_{19}-GAs, indicates that the synthesis of these products may have different catalytic requirements. Both the tricarboxylic acid and the C_{19} γ-lactone products are formed from the aldehyde intermediate, but synthesis of the tricarboxylic acid involves a single hydroxylation step while γ-lactone synthesis has been shown to involve loss of C-20 as CO_2 (Dockerill and Hanson, 1978), requiring two oxidation cycles. In this case, an additional intermediate is required between the aldehyde and the lactone, although such an intermediate has not been detected either in the fungus or in higher plants. Participation of an active site residue in C-20 oxidation to CO_2, that would not be properly oriented upon association with the cyt *b*5 system, could explain the very low levels of C_{19}-GAs formed by CPR mutants. Similarly, structural differences involving a catalytic residue required for the synthesis of C_{19}-GAs would account for the altered catalytic properties of a 20-oxidase from *Cucurbita maxima* that gives mainly C_{20}-GAs (Lange et al., 1994). Differences in the C-terminal region of the *C. maxima* enzyme compared to other plant 20-oxidases were implicated in its inability to synthesize C_{19}-GAs (Lange et al., 1997).

2.6. Concluding remarks

Our studies with *F. fujikuroi* mutants that lack CPR provide support for the participation of cyt *b*5:cyt *b*5 reductase in GA biosynthesis. Interaction of GA monooxygenases with this electron transport system would explain the different oxidation reactions catalyzed by P450-1 (GA_{14} synthase) and P450-2 (GA 20-oxidase) in Δ*cpr* and SG138 mutants compared to CPR-containing strains. These include differences in regiospecificity and in reaction rates. Whereas cyt *b*5:cyt *b*5 reductase supplies electrons to the GA monooxygenases in the absence of CPR, we found no evidence for its participation in CPR-containing strains. The GA pattern found in wild-type strains indicates that CPR would be the main electron transport protein associated with the GA monooxygenases and that the cyt *b*5 system would participate only if CPR is not available. Disruption of *cyt b5* and *cyt b5 reductase* fungal genes together with characterization of the respective mutants will give more insight into the physiological relevance of the cyt *b*5 electron transport system in GA biosynthesis by *F. fujikuroi*.

3. Experimental

3.1. General experimental procedures

[^{18}O]H_2O (min 97 at.%) and [^{18}O]O_2 (min 97 at.%) were purchased from Aldrich (USA). Cytochrome *b*5 (human, recombinant, *Escherichia coli*) was obtained from Calbiochem (USA).

3.2. Fungal strains

Δ*cpr* mutant was obtained from the wild-type *F. fujikuroi* strain IMI 58289 (Commonwealth Mycological Institute, Kew, UK) by disruption of the *cpr* gene as previously described (Malonek et al., 2004). The SG138 UV mutant was kindly provided by Dr. J. Avalos, Universidad de Sevilla, Spain. SG138 contains a point mutation in *cpr* that results in a truncated CPR that lacks part of the NADPH binding domain (Malonek et al., 2004). SG139-P450-1 and SG139-P450-2 transformants were prepared as described (Rojas et al., 2001; Tudzynski et al., 2002) from SG139 *F. fujikuroi* deletion mutant that lacks the entire GA gene cluster (Linnemanstöns et al., 1999) by complementation with *P450-1* or *P450-2* genes. The complementation mutants of Δ*cpr* or SG138 with the *cpr* genes from *F. fujikuroi* or from *A. niger* (Δ*cpr-cprFf*, SG138-*cprFf* and SG138-*cprAn*) were obtained as described (Malonek et al., 2004).

3.3. Culture conditions

Fungal cultures were maintained in potato dextrose agar and used for inoculation into 40% ICI (Imperial Chemical Industries) liquid medium containing 8% glucose, 0.5% $MgSO_4$, 0.1% KH_2PO_4 and 2 g/L ammonium nitrate (Geissmann et al., 1966). After incubation at 28 °C with shaking for 3–4 days, the culture was transferred into 0% ICI medium, with the same composition but containing no ammonium nitrate (Geissmann et al., 1966), and which was supplemented with 200 μM AMO 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride) to inhibit GA biosynthesis (Rademacher, 1992).

3.4. Labelled substrates

ent-[$^{14}C_4$]kaurenoic acid, **3** (7.3×10^3 Bq/nmol), *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid, **4** (7.7×10^3 Bq/nmol), [$^{14}C_4$]GA_{12}-aldehyde, **5** (6.6×10^3 Bq/nmol), and [$^{14}C_4$]GA_{12}, **7** (4.5×10^3 Bq/nmol) were synthesized from *R*-[2-^{14}C]mevalonic acid (Amersham) by incubation with an endosperm preparation from *C. maxima* in the presence of ATP, $MgCl_2$ and NADPH (Urrutia et al., 2001). Unlabelled GA_{12} (**7**) and *ent*-kaurenoic acid (**3**) were gifts from Professor M. Beale (Rothamsted Research, UK). [17-^{2}H]GA_{37}, **16**, [17-^{2}H]GA_{36}, **18**, [17-^{14}C]GA_{15}, **15** (1.6×10^3 Bq/nmol) and [17-^{14}C]GA_{24}, **17** (1.6×10^3

Bq/nmol) were obtained from Professor L. Mander (Australian National University, Canberra, Australia). [$^{14}C_4$]GA_{14}, **8** (6.6×10^3 Bq/nmol) was prepared from [$^{14}C_4$]GA_{12} aldehyde (**5**) by incubation with cultures of the SG139-P450-1 *F. fujikuroi* transformant (Rojas et al., 2001).

3.5. Isolation of microsomes

The mycelia grown for 7 days in 0% ICI (Geissmann et al., 1966) containing 200 μM AMO 1618 were harvested and washed alternatively with water and 50 mM Tris–HCl pH 7.5. About 700 mg of mycelia were suspended in 10 mL of the extraction solution (50 mM Tris–HCl pH 8.0; 5 mM EDTA; 4 mM DTT; 2.5 M sucrose and 10 mg/mL bovine serum albumin) frozen at −20 °C and disrupted under 28,000 psi in a chilled X-Press (Edebo, 1983). The thawed homogenate was centrifuged at 10,000*g* for 20 min and the supernatant was further centrifuged at 100,000*g* for 1 h. The 100,000*g* pellet was suspended in 2 mL of a solution containing 50 mM Tris–HCl pH 7.5, 4 mM DTT, 10 mg/mL bovine serum albumin and 2 M sucrose. P450-1 is stable for at least one year in this fraction, stored at −80 °C.

3.6. Incubation of fungal cultures with GA precursors

The mycelia grown in 40% ICI for 3–4 days were washed and resuspended in 0% ICI medium containing 200 μM AMO1618 (Rademacher, 1992). Five milliliter of fresh 0% ICI medium in 25 mL flasks were inoculated with 1 mL of the resuspended mycelia and labelled substrates were added as MeOH solutions (10–150 μL). When ^{14}C-labelled substrates were used, 0.5–8.3 kBq (0.065–1.85 nmol) per flask were added while for deuterated substrates 1–5 μg were utilized. The cultures were incubated for 3 days at 28 °C with shaking and the products were extracted from the culture filtrate and purified by partition in EtOAc, solid phase extraction and HPLC as already described (Rojas et al., 2001). For time course experiments, mycelia (5 mg dry wt/mL for incubations with [$^{14}C_4$]GA_{12}-aldehyde, **5** or 10 mg dry wt/mL for incubations with [$^{14}C_4$]GA_{12}, **7**) were incubated with labelled substrates at 28 °C in a total volume of 5 mL 0%ICI medium. Aliquots were taken at different times and analyzed as above.

3.7. Enzyme assays

P450-1 activity was determined in the microsomal fractions by incubating the microsomal suspension (10–80 μL) with labelled substrates, 0.5 kBq: *ent*-[$^{14}C_4$]kaurenoic acid, **3** (0.68 μM), *ent*-7α-hydroxy[$^{14}C_4$]kaurenoic acid, **4** (0.65 μM) or [$^{14}C_4$]GA_{12}-aldehyde, **5** (0.75 μM), cofactors (1 mM NADH plus 250 μM FAD or 1 mM NADPH plus 5 μM FAD), 100 mM NaCl and 50 mM Tris–HCl pH 7.5 in a final volume of 100 μL. After incubation for 1 h with shaking at 30 °C, the reaction was stopped by addition of 10 μL of HOAc and 1 mL H_2O. Products and unreacted substrate were purified on C18 Bakerbond cartridges (J.T. Baker) and further analyzed by HPLC.

3.8. Incubations with [^{18}O]H_2O or [^{18}O]O_2

Mycelia of the Δ*cpr* mutant grown in 40% ICI were washed with 0% ICI medium containing 200 μM AMO1618 and resuspended in 10 mL of the same solution. The mycelial suspension was transferred into a closed 50 mL Erlenmeyer flask from which air was eliminated through several vacuum and nitrogen purge cycles. The substrate (20 μg GA_{12}, **7** or 50 μg *ent*-kaurenoic acid, **3**) was added to the solution and two additional nitrogen purge cycles were carried out. Finally the artificial air mixture, [^{18}O]O_2:N_2 (20:80) or [^{16}O]O_2:N_2 (20:80), was added (23 mL) to the flask and the solution incubated with shaking at 28 °C for 2 days. Fresh artificial air was replaced twice during the incubation. For [^{18}O]H_2O incubations the mycelia was washed with 0% ICI containing 200 μM AMO1618, filtered and suspended in 1.5 mL [^{18}O]H_2O. The suspension was incubated for 3 days with shaking at 28 °C. Products were isolated by HPLC and analyzed by GC–MS analysis as described in Section 3.10. The incorporation of ^{18}O at C-20 was determined from the molecular ion cluster for GA_{15} (**15**) and from the M-32 cluster for GA_{24} (**17**) using the relative abundance of the ions at m and $m+2$ after subtracting the contribution to the $m+2$ ions from natural heavy isotopes, determined empirically.

3.9. HPLC conditions

Labelled products were separated on a C18 Symmetry column (5 μm; 250 × 4; Waters) in a Waters 600 HPLC instrument. A linear gradient from 60% to 100% MeOH in H_2O, pH 3.0, over 30 min was used for elution. The flow rate was 1 mL/min. Fractions were collected and the radioactivity measured by liquid scintillation counting.

3.10. Product identification

^{14}C-Labelled products eluted from HPLC were derivatized as methyl esters (ethereal diazomethane), TMSi ethers (*N*-methyl-*N*-trimethylsilyltrifluoracetamide at 90 °C for 30 min) and analyzed by GC–MS, using either a Trio2A mass spectrometer (Micromass, Manchester, UK) connected to an Agilent 5890 gas chromatograph as described in Tudzynski et al. (2003) (for data in Table 1), or a GCQ GC–MS system (ThermoFinnigan) (for the data in Tables 4 and 5). In the latter case, samples diluted in EtOAc were injected (1 μL) splitless into a TR-1 WCOT column (30 m × 0.25 mm × 0.25 μL) (ThermoFisher Scientific) at an oven temperature of 50 °C. The split (50:1) was opened after 2 min and the oven temperature was increased at 20 °C min^{-1} to 200 °C and then at 4 °C min^{-1} to 300 °C. The He flow was maintained at a constant linear velocity of 40 cm s^{-1}, and the injector, transfer line and MS source

temperatures were 220 °C, 270 °C and 210 °C, respectively. Full scans were obtained from 50 to 650 amu at 1 scan s^{-1}. Compounds were identified by comparison of their mass spectra with those of authentic samples and/or with published spectra (Gaskin and MacMillan, 1992). For incubations with deuterated precursors the EtOAc extract was derivatized directly for GC–MS analysis.

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