THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 24, Issue of June 14, pp. 21246–21253, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

The Gibberellin 20-Oxidase of *Gibberella fujikuroi* Is a Multifunctional Monooxygenase*

Received for publication, February 18, 2002, and in revised form, March 28, 2002
Published, JBC Papers in Press, April 9, 2002, DOI 10.1074/jbc.M201651200

Bettina Tudzynski‡, María Cecilia Rojas§, Paul Gaskin¶, and Peter Hedden¶‖

From the ‡Westfälische Wilhelms-Universität Münster, Institut für Botanik, Schloβgarten 3, D-48149 Münster, Germany, §Laboratorio de Bioorgánica, Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile, and the ¶IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, United Kingdom

The genes for gibberellin (GA) biosynthesis are clustered in the fungus *Gibberella fujikuroi*. In addition to genes encoding a GA-specific geranylgeranyl diphosphate synthase and a bifunctional *ent*-copalyl diphosphate/*ent*-kaurene synthase, the cluster contains four cytochrome P450 monooxygenase genes (*P450-1*, *-2*, *-3*, *-4*). Recently it was shown that *P450-4* and *P450-1* encode multifunctional enzymes catalyzing the three oxidation steps from *ent*-kaurene to *ent*-kaurenoic acid and the four oxidation steps from *ent*-kaurenoic acid to GA_{14}, respectively. Here we describe the functional analysis of the *P450-2* gene by gene disruption and by expressing the gene in a mutant that lacks the entire GA biosynthesis gene cluster. Mutants in which *P450-2* is inactivated by the insertion of a large piece of DNA accumulated GA_{14} and lacked biosynthetically more advanced metabolites, indicating that the gene encodes a 20-oxidase. This was confirmed by incubating lines containing *P450-2* in the absence of the other GA biosynthesis genes with isotopically labeled substrates. The *P450-2* gene product oxidized the 3β-hydroxylated intermediate, GA_{14}, and its non-hydroxylated analogue GA_{12} to GA_4 and GA_9, respectively. Expression of *P450-2* is repressed by high amounts of nitrogen in the culture medium but is not affected by the presence of biosynthetically advanced GAs, *i.e.* there is no evidence for feedback regulation. The fact that the GA 20-oxidase is a cytochrome P450 monooxygenase in *G. fujikuroi* and not a 2-oxoglutarate-dependent dioxygenase as in plants, together with the significant differences in regulation of gene expression, are further evidence for independent evolution of the GA biosynthetic pathways in plants and fungi.

Considerable progress has been made in isolating and characterizing the genes encoding enzymes of gibberellin (GA)[1] biosynthesis in plants (1, 2) and in the fungus *Gibberella fujikuroi* in which, in contrast to plants, all the GA biosynthesis genes are organized in a cluster (3–6). Comparison of the products of the plant and fungal genes indicates important biochemical differences in GA biosynthesis between these kingdoms. For example, formation of the early hydrocarbon intermediate *ent*-kaurene from geranylgeranyl diphosphate requires two enzymes in plants but is catalyzed by a single bifunctional terpene cyclase in *G. fujikuroi* (7) and a *Phaeosphaeria* species (8). Although *ent*-kaurene oxidase, which converts *ent*-kaurene to *ent*-kaurenoic acid, is a cytochrome P450 monooxygenase in plants and *G. fujikuroi*, the enzymes encoded by *GA3* in *Arabidopsis thaliana* and *P450-4* in *G. fujikuroi* are highly diverged and are placed in different P450 families (6). Recently, we showed that in *G. fujikuroi* the four steps by which *ent*-kaurenoic acid is converted to GA_{14} are catalyzed by a single multifunctional cytochrome P450, encoded by *P450-1* (4). This enzyme is also responsible for the formation of GA_{12} and for the kaurenolides and fujenoic acids, which are products of branches from the main pathway (4).

Although the biosynthetic pathway from *trans*-geranylgeranyl diphosphate to GA_{12}-aldehyde is the same in the fungus and in all higher plants studied so far, thereafter it differs between *G. fujikuroi* and plants. In *G. fujikuroi*, GA_{12}-aldehyde is 3β-hydroxylated to GA_{14}-aldehyde and then oxidized on C-7 to GA_{14} (main pathway) or oxidized directly on C-7 to GA_{12} (minor pathway) by the same multifunctional enzyme, P450-1 (Fig. 1 and Refs. 4 and 9). The products of P450-1 are then converted by oxidative removal of C-20 to GA_4 and GA_9, respectively. In most higher plant species, GA_{12}, formed from *ent*-kaurenoic acid by *ent*-kaurenoic acid oxidase (10), is either 13-hydroxylated to GA_{53}, which is converted to GA_{20} by GA 20-oxidase (13-hydroxylation pathway) or oxidized directly by 20-oxidase to GA_9 (non-13-hydroxylation pathway).

In plants, the removal of C-20, through which C_{20}-GAs are converted to C_{19}-GAs, requires the progressive oxidation of the C-20 methyl through a hydroxymethyl to an aldehyde, from which C-20 is lost as CO_2 in the formation of a γ-lactone (11). This series of reactions is catalyzed by multifunctional 2-oxoglutarate-dependent dioxygenases, for which the alcohol and aldehyde intermediates are both products and substrates. For all known plant GA 20-oxidases, the main final products are C_{19}-GAs (12), with the exception of an enzyme from immature pumpkin seeds, which produces mainly tricarboxylic acid products (13, 14). In contrast to plant systems, the oxidation sequence at C-20 to give the C_{19}-GAs has not been clearly established in *G. fujikuroi* (Fig. 1). Although fungal cultures convert GA_{14} to GA_4 (major pathway) and GA_{12} to GA_9 (minor pathway), it has not been possible to demonstrate the involvement of intermediates in these conversions. Potential intermediates, such as GA_{37}, GA_{36}, and GA_{13}, are present in cultures, but these are not formed from GA_{14} when they are applied to the

* This work was supported by the Deutsche Forschungsgemeinschaft (Tu101-7), the Deutscher Akademischer Austauschdienst/Consejo Nacional de Ciencia y Tecnología Cooperation Program, and FONDECYT Grant 1020140. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel.: 44-1275-549263; Fax: 44-1275-394281; E-mail: peter.hedden@bbsrc.ac.uk.

[1] The abbreviations used are: GA, gibberellin; GC-MS, combined gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; OPM, optimized GA_3 production medium.

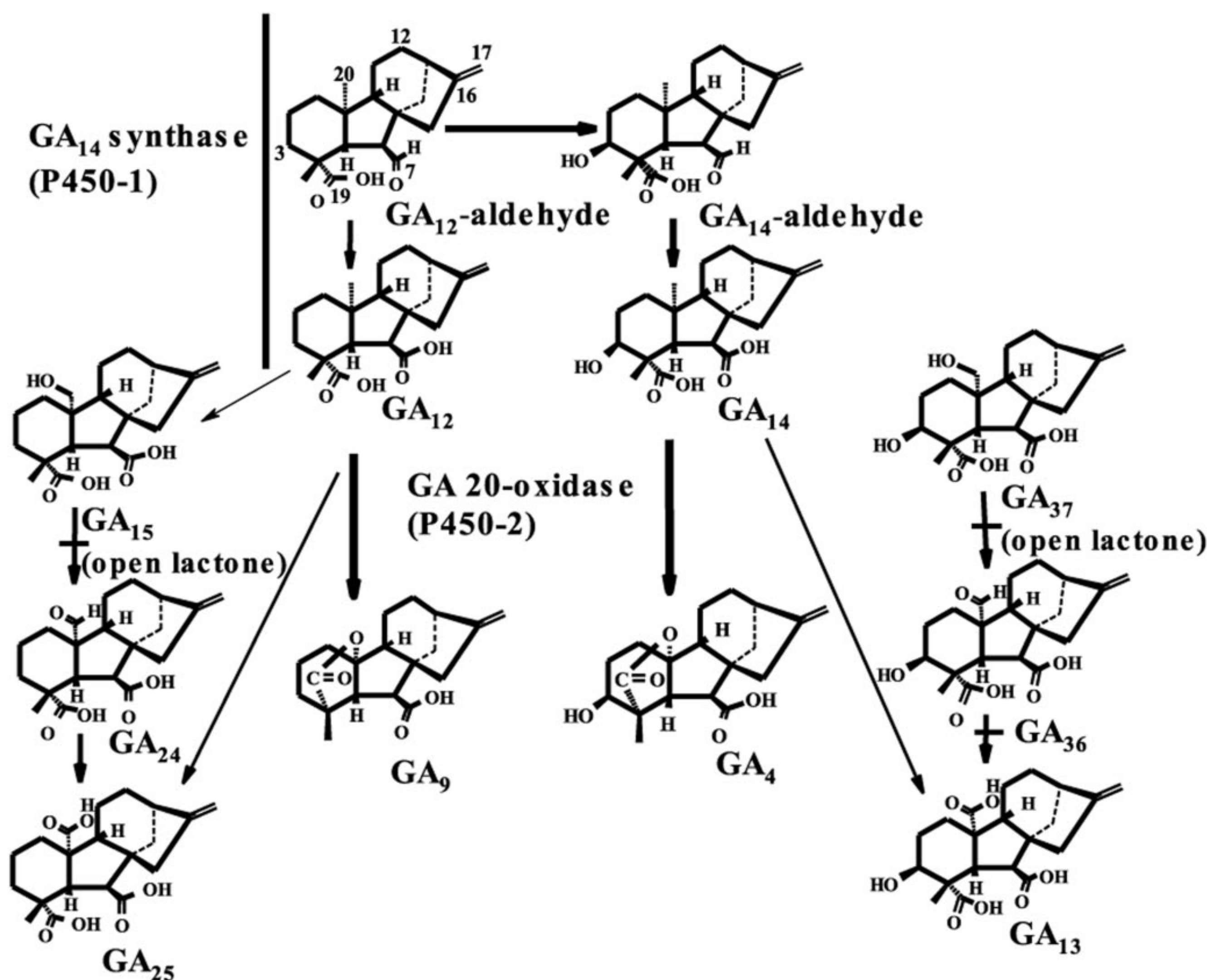

FIG. 1. **Metabolic relationship of GAs discussed in the text.** GA_{12} and GA_{14}, which are formed from GA_{12}-aldehyde by GA_{14} synthase, are converted to the C_{19}-GAs GA_9 and GA_4, respectively, by GA 20-oxidase. GAs with C-20 at higher oxidation levels are not metabolized to C_{19}-GAs. The numbers of relevant C atoms are shown for GA_{12}-aldehyde.

fungal mutant strain B1-41a, nor are they metabolized to GA_4 (15). Similar results are found for GA_{15}, GA_{24}, and GA_{25}, the non-hydroxylated analogs of these possible intermediates.

Although all GA biosynthetic steps after GA_{53} are catalyzed by dioxygenases in plants (1, 2), the *G. fujikuroi* GA gene cluster contains no dioxygenase genes. Instead, four cytochrome P450 monooxygenase genes are closely linked in the cluster, indicating that, in contrast to higher plants, the 20-oxidation might be catalyzed by a monooxygenase in the fungus. Here we report the functional analysis of one of the four P450 monooxygenase genes, *P450-2*, demonstrating that this gene fulfills the function of the plant 20-oxidases. The enzyme catalyzes the removal of C-20 from GA_{14} and GA_{12} to produce the C_{19}-GAs, GA_4 and GA_9, respectively. In addition to the differences in enzyme character, expression of the plant and fungal GA 20-oxidase genes is regulated differently.

EXPERIMENTAL PROCEDURES

Fungal Strains and Culture Conditions—G. fujikuroi m567, a wild-type strain from rice, was provided by the Fungal Culture Collection (Weimar, Germany). The wild-type strain IMI58289 and the GA-defective mutant strain SG139 (16) were kindly provided by E. Cerda-Olmedo and J. Avalos (University of Sevilla, Sevilla, Spain). SG139 is missing the entire GA gene cluster as demonstrated by Southern blot and PCR analysis.

Bacterial Strains and Plasmids—Escherichia coli strain Top10 (Invitrogen) was used for plasmid propagation. The gene disruption vector, pP450-2-GD, constructed by cloning a 0.9-kb internal *Hin*dIII/*Sal*I fragment of *P450-2* from plasmid pP450-S containing a 6.7-kb *Sal*I fragment spanning from P450-4 to the heme binding domain of P450-2 (5), was cloned into the vector pGPC1 (17) carrying the hygromycin B resistance cassette. For complementation of strain SG139 with the wild-type *P450-2* gene, a 4.0-kb *Bam*HI fragment carrying the entire *P450-2* gene was cloned into pGPC1 (17).

Media and Culture Conditions—For DNA isolation the fungal strains were grown in 100 ml of complete liquid medium optimized for *Fusarium* spp. (18) for 3 days at 28 °C on a rotary shaker set at 200 rpm. The mycelium was harvested by filtration through a sterile glass filter (G2, Schott Jena, Germany), washed with sterile distilled water, frozen in liquid nitrogen, and lyophilized for 24 h. The lyophilized mycelial tissue was ground to a fine powder with a mortar and pestle. For RNA isolation, fungal strains were grown in an optimized GA_3 production medium (OPM), containing 6% sunflower oil, 0.05% $(NH_4)_2SO_4$, 1.5% corn-steep solids (Sigma), and 0.1% KH_2PO_4. Mycelium was harvested after 15 h (growth phase) and after 3–6 days of cultivation (production phase). For study of feedback control by GAs the mycelium was cultivated in 10% ICI medium (19) for 4 days and then resuspended in 0% ICI with or without the addition of 1 g/liter of GA_3. For analysis of GAs, fungal strains were grown in the GA production medium for 7–10 days at 28 °C on a rotary shaker (200 rpm). Cultures for metabolism studies were established in 100 ml of 100% ICI medium for 4 days at 25 °C on a rotary shaker, then subcultured into 100 ml of 40% ICI medium, and,

after 5 days, into 100 ml of 10% ICI medium containing 1 mM AMO-1618 (Calbiochem).

DNA and RNA Isolation—Genomic DNA was isolated from lyophilized mycelium according to Doyle and Doyle (20). Lambda DNA from positive lambda clones was prepared according to Ref. 21. Plasmid DNA was extracted using Genomed columns following the manufacturer's protocol (Genomed, Bad Oeynhausen, Germany). RNA for Northern blot analysis was isolated by using the RNAgents total RNA isolation kit (Promega, Mannheim, Germany).

Southern and Northern Blot Analysis—After incubation with restriction endonucleases and electrophoresis, genomic or lambda DNA was transferred to Hybond N^+ filters (Amersham Biosciences) (22). ^{32}P-labeled probes were prepared using the random oligomer primer method. Filters were hybridized at 65 °C in 5× Denhardt's solution containing 5% dextran sulfate (21). Filters were washed at the hybridization temperature in 2× SSPE, 0.1% SDS and then 1× SSPE, 0.1% SDS. Northern blot hybridization was accomplished by the method of Church and Gilbert (23). Probing with *G. fujikuroi* rRNA was used as a control for RNA transfer.

Transformation of G. fujikuroi—Preparation of protoplasts and the transformation procedure were carried out as previously described (24). For gene disruption, 10^7 protoplasts (100 μl) of strain IMI58289 were transformed with 10 μg of the gene disruption vector pP450-2-GD. For complementation of the mutant strain, SG139, with the intact *P450-2* gene, protoplasts were transformed with 10 μg of the circular complementation vector pP450-2-GC.

Transformed protoplasts were regenerated at 28 °C in a complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$ containing 120 μg/ml of hygromycin B (Calbiochem)) for 6–7 days. Single conidial cultures were established from hygromycin B-resistant transformants and used for DNA isolation and Southern blot analysis.

Gibberellin Analysis—For analysis of GA formation, the wild-type strain and *P450-2*-disrupted mutants were cultivated in 100 ml Erlenmeyer flasks containing 20 ml of OPM medium. Cultures were incubated for 7 days on a rotary shaker (200 rpm) at 28 °C. GA_3 was analyzed by high performance liquid chromatography (HPLC) according to Barendse *et al.* (25) using a Merck HPLC system with a UV detector and a Lichrospher 100 RP18 column (5 μm; 250 × 4; Merck). GA_3 GA_4 and GA_7 also were analyzed by thin layer chromatography eluted with ethyl acetate/chloroform/acetic acid (60:40:5). Extracts of culture filtrates and mycelia were analyzed by gas chromatography-mass spectroscopy (GC-MS) as described previously (3).

PCR—Genomic DNA of strains SG139 and complemented transformants were used as templates for amplification of the entire *P450-2* gene. The specific primers, which were synthesized by MWG-Biotech AG, had the following sequences: P450-2-F, 5′-ATGATCACCAGCTATGCGGGTGCC-3′ and P450-2-R, 5′-ATCCAACTCAACTGACTCCCGACGC-3′. DNA amplification was performed in 50 μl mixtures using 2 units of *Taq*DNA polymerase (Red-*Taq*, Sigma), 50 ng/μl genomic DNA, 50 μM each dNTP, 200 nM each primer and 1× *Taq* buffer (Sigma). PCR was carried out for 36 cycles, each comprising 1 min of denaturation at 94 °C, 0.5 min of annealing at 62 °C, and 1.5 min of extension at 72 °C. The PCR product was purified using the gel extraction kit (Genomed), precipitated with 0.3 M sodium acetate and 2 volumes of ethanol and cloned using the pCR2.1 vector system (Invitrogen).

Incubations with Isotopically Labeled Substrates—[1,7,12,18-$^{14}C_4$]GA_{12} (4.40 TBq mol^{-1}) and [1,7,12,18-$^{14}C_4$]GA_{12}-aldehyde (6.81 TBq mol^{-1}) were prepared from *R*-[2-^{14}C]mevalonic acid (Amersham Biosciences) using a cell-free system from pumpkin endosperm, as previously described (26). [^{14}C]GA_{14} (6.81 TBq mol^{-1}) was prepared from [^{14}C]GA_{12}-aldehyde by incubation with cultures of the *P450-1* transformant SG139-T7 of *G. fujikuroi* (4). [18-$^2H_1$]GA_{12} was a gift from Dr. M. H. Beale (IACR-Long Ashton Research Station) and was used to prepare [$^2H_1$]GA_{14} by incubation with cultures of the *P450-1* transformant SG139-T7 (4). [17-^{14}C]GA_{24} (1.64 TBq mol^{-1}), [17-^{14}C]GA_{15} (1.64 TBq mol^{-1}), [17-^{2}H]GA_{36}, and [17-^{2}H]GA_{37} were obtained from Professor L. Mander (Australian National University, Canberra, Australia). The open lactones of [^{14}C]GA_{15} and [^{2}H]GA_{37} were prepared by heating the respective lactones at 100 °C in 0.1 N KOH for 2 h. For incubations with radiolabelled substrates, fungal cultures of SG139 transformants were grown in 40% ICI medium for 2 days at 28 °C then harvested and the mycelia washed with 0% medium (19). Mycelia were resuspended in 0% ICI, and 1-ml aliquots were transferred to 25 ml sterile flasks containing 5 ml of the same medium. The radiolabelled substrates (500 Bq/flask) were added to the cultures in methanol (10–40 μl). After incubation on an orbital shaker at 28 °C for 2 days, cultures were filtered and GAs extracted and purified as described in Ref. 4. HPLC analysis was made on a C_{18} column (Symmetry, Waters) in a Waters 600 instrument using a linear gradient from 60 to 100% methanol/H_2O, pH 3.0, over 30 min. The flow rate was 1 ml/min. Fractions (1 ml) were collected, and the radioactivity was measured by liquid scintillation counting. Products were identified by combined GC-MS as described previously (3).

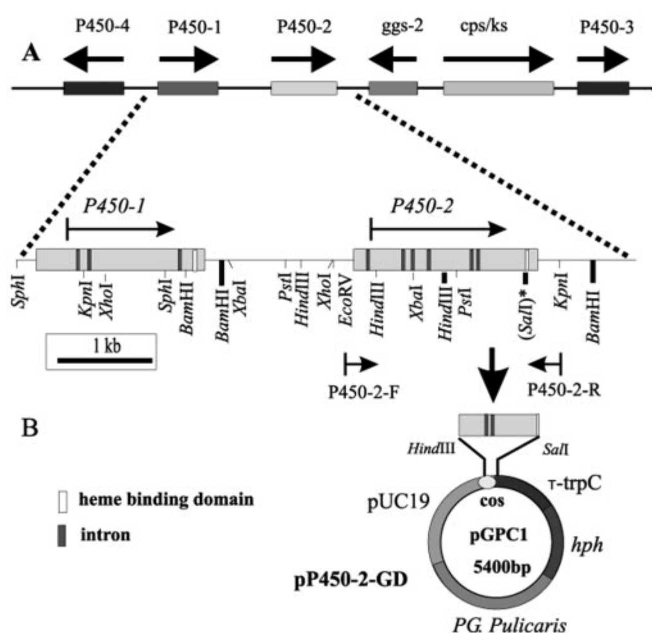

FIG. 2. **The *P450-2* gene of *G. fujikuroi*.** *A*, physical map of *P450-2* in the GA biosynthesis gene cluster showing the position of the *Bam*HI sites used to excise the *P450-2* gene for the complementation experiment. The DNA fragment for the construction of the disruption vector pP450-2-GD (shown in *B*) was excised from a lambda clone by digestion with *Hin*dIII and *Sal*I. The *Sal*I site marked with an *asterisk* is not present in the genomic DNA. The position of the primers used in PCR to determine the presence of *P450-2* in the SG139 transformants are also marked.

RESULTS

Disruption of the P450-2 Gene—*P450-2* is part of the *G. fujikuroi* GA biosynthesis gene cluster and was mapped to the right of *P450-1* and to the left of *ggs2* (5) (Fig. 2*A*). The gene (GenBank™ accession no. Y15278) contains 6 introns and encodes a cytochrome P450-like protein of 1,568 bp.

To characterize the biosynthetic steps in which the gene might be involved we performed gene disruption experiments. An internal 0.9-kb *Hin*dIII/*Sal*I fragment ending in the middle of the heme binding domain of the enzyme, was cloned into the vector pGPC1 carrying the hygromycin resistance gene. The resulting disruption vector pP450-2-GD (Fig. 2*B*) was transformed into the wild-type strain IMI58289. Among the 24 transformants generated, two showed no GA production. The gene disruption event was confirmed by Southern blot analysis (Fig. 3). Both transformants, ΔP450-2-T35 and ΔP450-2-T39, had lost the 4.0-kb *Bam*HI wild-type band and instead produced hybridizing bands consistent with the integration of the vector into the *P450-2* locus by a single crossover. Additional hybridizing bands in the transformants are due to ectopic vector integrations.

Analysis of the Intermediates Produced in the Mutants—Neither transformant produced GA_4, GA_7, or GA_3, as shown by TLC and HPLC. To characterize the step in the pathway that was blocked by disruption of *P450-2*, the wild-type strain IMI58289 and the transformant T35 were cultivated in 20% ICI production medium for 5 days. Extracts of the culture fluids and the mycelia were analyzed by GC-MS. The total ion current traces for the culture fluid extracts are shown in Fig. 4. The wild-type produced mainly GA_3, GA_{13}, GA_7, and 7β,18-dihy-

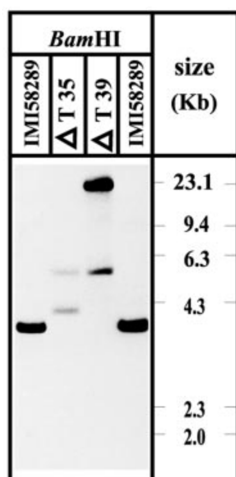

FIG. 3. **Southern blot analysis of the *P450-2* disruption mutants.** The analysis was performed on genomic DNA from the wild type (IMI58289) and disruption mutant strains ΔP450-2-T35 and -T39. The DNA was digested with *Bam*HI and probed with full-length *P450-2* cDNA.

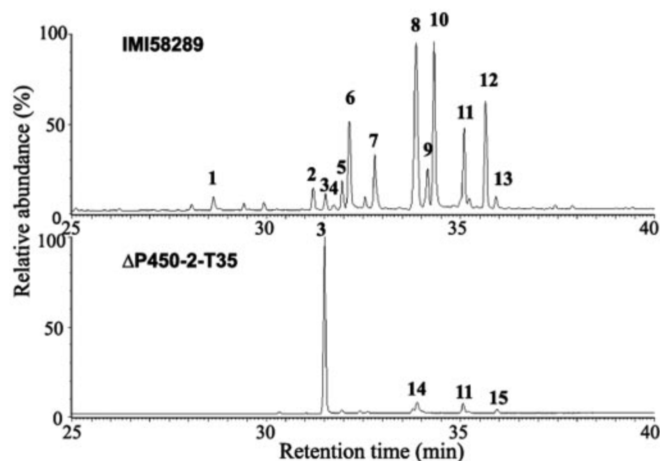

FIG. 4. **GC-MS analysis of culture filtrates of the wild-type (IMI58289) and disruption mutant (ΔP450-2-T35) strains.** Total ion current traces are shown for ethyl acetate extracts after derivatization to methyl esters trimethylsilyl ethers. Components identified by comparison of their mass spectra and GC retention times with published data (39) are as follows: *peak 1*, GA_9; *peak 2*, GA_{25}; *peak 3*, GA_{14}; *peak 4*, GA_{24}; *peak 5*, 7β-hydroxykaurenolide; *peak 6*, isoGA_7; *peak 7*, GA_7; *peak 8*, GA_{13}; *peak 9*, GA_{16}; *peak 10*, isoGA_3; *peak 11*, 7β,18-dihydroxykaurenolide; *peak 12*, GA_3; *peak 13*, GA_{78}; *peak 14*, fujenoic acid; *peak 15*, GA_{42}. isoGA_3 and isoGA_7 (the 19,2α-lactone, 1,10-enes) are formed from the respective GAs either during extraction or GC-MS analysis.

droxykaurenolide, whereas the mutant ΔP450-2-T35 accumulated GA_{14} but later intermediates of the pathway were not detected. This result indicated that P450-2 activity was required for oxidation of GA_{14} at C-20, a reaction that in plants is catalyzed by dioxygenases.

Expression of P450-2 in the Deletion Mutant SG139—To confirm that P450-2 was a 20-oxidase and to define precisely the reactions catalyzed by this enzyme we transformed the GA-deficient mutant strain SG139 with the gene complementation vector pP450-2-GC containing an intact copy of *P450-2* and the hygromycin resistance cassette as selection marker. Strain SG139 has lost the entire GA gene cluster and was used previously to demonstrate the functions of P450-4 (6) and P450-1 (4). Ten of the resulting hygromycin-resistant transformants were analyzed by PCR for correct integration of the complete *P450-2* gene using primers P450-2-F and P450-2-R

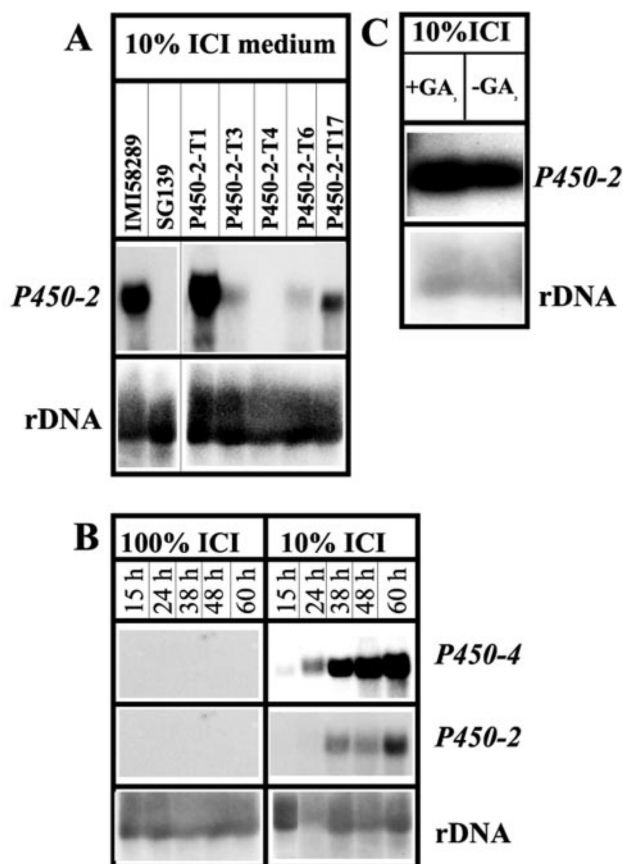

FIG. 5. **Northern blot analysis of *P450-2* gene expression.** *A*, expression of *P450-2* in the transformed SG139 mutant was confirmed by determining transcript levels in selected transformants P540-2-T1, -T3, -T6, and -T17 that were shown by PCR to contain *P450-2*. For comparison expression was also examined in the wild-type (IMI58289), SG139 deletion mutant, and transformant P450-2-T4, which was shown by PCR to contain the gene but gave no expression. *B*, the effect of growing the wild-type strain IMI58289 for different times in media with high N_2 (100% ICI) or low N content (10% ICI) on expression of *P450-2* and, for comparison, *P450-4*, was determined. *C*, the effect of GA_3 content on *P450-2* expression was determined in the wild-type strain IMI58289 with or without supplementing the culture medium with GA_3. In each experiment total RNA was probed with the *P450-2* cDNA. As controls for RNA loading, the blots were hybridized with ribosomal RNA from *G. fujikuroi*.

(see Fig. 2). Six of the transformants amplified the expected 2,500-bp PCR fragment corresponding to the size of the wild-type gene (data not shown). To confirm that the gene is expressed in the complemented transformants, Northern blot analysis was performed on some of the transformants as well as on the wild-type and mutant strain SG139 (Fig. 5*A*). *P450–2* was expressed in the wild type as well as in transformants T1, T3, T6, and T17 but was not expressed in the recipient strain SG139 or in T4, even though this transformant produced a PCR product of the correct size. Transformants T1, T6, and T17 were used for further expression and metabolism studies.

Regulation of Gene Expression—We showed previously that the genes encoding CPS/KS (*ent*-kaurene synthase), P450-1 (GA_{14} synthase), and P450-4 (*ent*-kaurene oxidase) are expressed only in GA production conditions (low nitrogen) (4–6). To determine whether *P450-2* is co-regulated with these genes, the wild type was grown for 15, 24, 38, 48, and 60 h in 10 and 100% ICI medium containing 0.5 and 5.0 g/liter NH_4NO_3, respectively. Northern blot analysis of total RNA revealed a single band of ~1.8 kb only in 10% ICI medium clearly demonstrating strong nitrogen repression of gene expression. The *P450-2* transcript was just detectable at 24 h and increased in

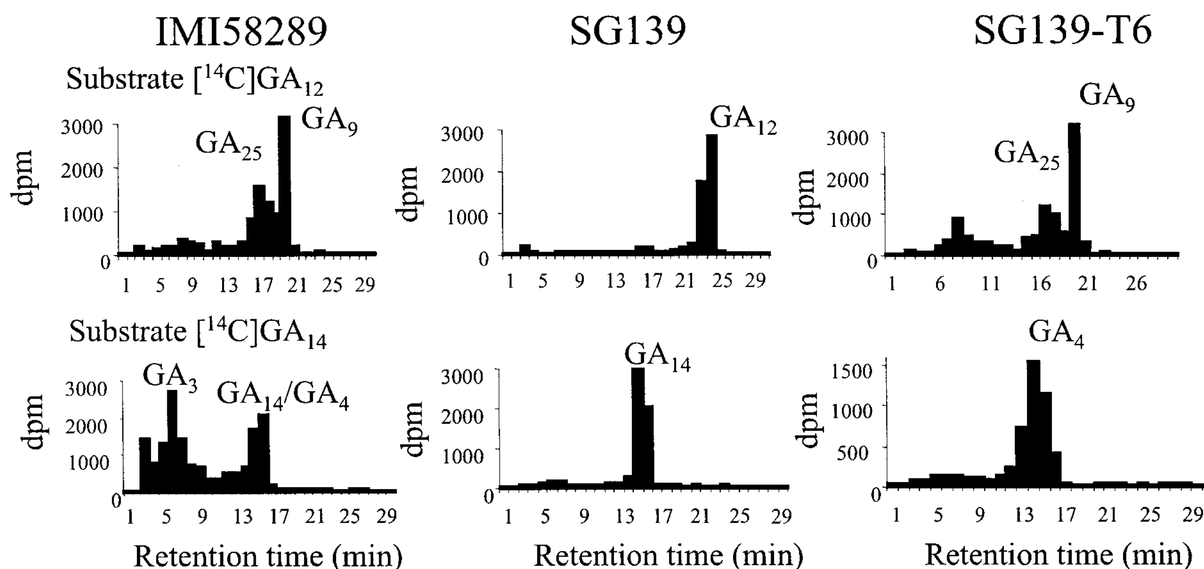

FIG. 6. **Metabolism of GA_{12} and GA_{14} in fungal cultures.** ^{14}C-labeled substrates were incubated with cultures of the wild-type strain IMI58289, deletion mutant SG139, and the *P450-2* transformant T6. Total extracts were separated by HPLC and the radioactivity profiles produced by liquid scintillation counting of aliquots from each fraction. The identity of products was confirmed by GC-MS. Retention times are: GA_{12}, 24 min; GA_{14}, 16 min; GA_3, 6 min; GA_4, 15 min; GA_9, 20 min; GA_{25}, 17 min; 16,17-dihydrodihydroxy-GA_9, 8 min.

abundance with culture time (Fig. 5*B*). Its expression pattern corresponded closely to that of *P450-4* (Fig. 5*B*) and four other genes in the GA cluster (4, 5).

In higher plants GA 20-oxidases are key regulatory enzymes for GA biosynthesis, and their expression is under negative feedback control by bioactive GAs. However, addition of 1 g/liter GA_3, the final product of GA biosynthesis in *G. fujikuroi*, to cultures of the wild-type strain did not reduce *P450-2* transcript abundance (Fig. 5*C*), suggesting its expression is not under feedback control.

Incubation of Labeled Gibberellin Precursors with P450-2 Transformants—$[^{14}C]GA_{14}$ and $[^{14}C]GA_{12}$ were both metabolized efficiently by the three P450-2 transformants, SG139-T1, -T6, and -T17, grown under GA-producing conditions. Complete conversion of $[^{14}C]GA_{14}$ to $[^{14}C]GA_4$ was demonstrated by HPLC and GC-MS, whereas $[^{14}C]GA_{12}$ was converted to $[^{14}C]GA_9$ and smaller amounts of $[^{14}C]GA_{25}$, the tricarboxylic acid product (Fig. 6 and Table I). In some incubations with $[^{14}C]GA_{12}$, $[^{14}C]GA_9$ 16,17-dihydrodiol was also detected (Table I). The dihydrodiol was apparently a product of the P450-2 monooxygenase because $[^{14}C]GA_9$ was not transformed when incubated with the recipient strain SG139. Incubations of $[^{14}C]GA_{12}$ or $[^{14}C]GA_{14}$ with SG139 also gave no conversion of these precursors (Fig. 6), whereas the wild-type strain IMI58289 converted these substrates to mainly $[^{14}C]GA_9$ plus $[^{14}C]GA_{25}$ (Fig. 6, *retention time 20* and *17 min*, respectively), and $[^{14}C]GA_3$ (Fig. 6, *retention time*, *7 min*), respectively.

Although $[^{14}C]GA_9$ and $[^{14}C]GA_{25}$ were produced in a 2:1 ratio from $[^{14}C]GA_{12}$ by the transformants, only $[^{14}C]GA_4$ and no $[^{14}C]GA_{13}$ (the 3β-hydroxylated tricarboxylic acid analog of GA_{25}) was detected when 0.24 μg of $[^{14}C]GA_{14}$ was incubated with these strains. Thus, the C_{19} lactones are the main products of the fungal GA 20-oxidase. When a higher amount of substrate (83 μg of $[^2H]GA_{14}$) was incubated with the T17 transformant, $[^2H]GA_{13}$ was detected in addition to $[^2H]GA_4$ at a ratio of 5:1 GA_4/GA_{13} (Table I). A small amount of $[^2H]GA_{15}$ (1.8% total products) was formed when 100 μg of $[^2H]GA_{12}$ was incubated with T17. GA_{15} contains C-20 at the alcohol oxidation level and, as the free alcohol, has been demonstrated as an intermediate in C-20 oxidation by plant dioxygenases (12, 13). In contrast, GA_{24}, GA_{36}, or GA_{37}, which have been proposed as intermediates of C-20 oxidation in *G. fujikuroi*, were not detected under our incubation conditions, although a trace of labeled 12-hydroxy-GA_{24} (stereochemistry at C-12 not determined) was present when $[^2H]GA_{12}$ was incubated at high concentration (Table I). Furthermore, small amounts of 12α-hydroxy-GA_9 (GA_{70}), 12α-hydroxy-GA_{12} (GA_{111}), and 12β-hydroxy-GA_{12} (GA_{112}), as well as the 16,17-dihydrodiols of GA_{12} and GA_9, were obtained from GA_{12} under these conditions. The tricarboxylic acid GA_{25} is a final product and not an intermediate in C-20 oxidation because it remained unchanged when incubated with cultures of the *P450-2* transformants (data not shown).

To investigate further the possible participation of alcohol and aldehyde intermediates in fungal GA C-20 oxidation, $[^{14}C]GA_{15}$, and $[^{14}C]GA_{24}$ as well as the corresponding 3β-hydroxylated compounds, $[^2H]GA_{37}$ and $[^2H]GA_{36}$, were incubated with cultures of the *P450-2* transformants (Table I). $[^{14}C]GA_{15}$ and $[^2H]GA_{37}$ were incubated as lactones or as opened lactones prepared by hydrolysis of the lactones with KOH because many plant GA 20-oxidases oxidize the C-20 alcohol only in the free form (27). GA_{15} is otherwise stable to strong base, but there is likely to be some epimerization of the 3β-hydroxyl group of GA_{37} under these conditions. The lactone form of $[^{14}C]GA_{15}$ was incubated with the transformant in culture medium at pH 3.0 or 4.5, after which it remained unchanged as did the free alcohol form in cultures buffered at pH values from 5.0 to 7.0. The higher pH values would retard relactonization in the medium. The same results were found for $[^2H]GA_{37}$ and its free alcohol form. The non-hydroxylated $[^{14}C]GA_{24}$, containing C-20 as the aldehyde, was about 50% metabolized by the T1, T6, or T17 transformants to $[^{14}C]GA_{25}$ as sole product under conditions of complete conversion of $[^{14}C]GA_{12}$. No $[^{14}C]GA_9$ was detected from these incubations. The mutant strain SG139 failed to metabolize $[^{14}C]GA_{24}$ (data not shown), confirming that oxidation to GA_{25} was due to the activity of P450-2. Unexpectedly, the 3β-hydroxylated analog, $[^2H]GA_{36}$, was not transformed by the *P450-2* transformants to any detectable products.

Oxidation of $[^{14}C]GA_{12}$ and $[^{14}C]GA_{14}$ by the *P450-2* transformants was highly dependent on the pH level of the culture medium. Efficient transformation was found at low pH levels,

TABLE I
GC-MS identification of products from incubations of isotopically labelled GA_{12}, GA_{14} and C-20 alcohol and aldehyde GAs with SG139 P450–2 transformants

HPLC fractions containing radioactivity, in the case of ^{14}C-labeled substrates, or total extracts of culture filtrates, in the case of ^{2}H-labeled substrates, were analyzed as methyl esters trimethylsilyl ethers.

Transformant	Substrate	Products (% relative amount)[a]	Mass spectrum
			m/z (% relative abundance)
T1, T6[b]	$[^{14}C_4]GA_{12}$ (0.5 μg)	$[^{14}C_4]GA_9$ (57)	306(63), 304(21), 298(53), 276(1000), 274(39), 270(98), 251(76), 249(29), 243(50), 232(65), 230(71), 226(79), 189(35), 183(42)
		$[^{14}C_4]GA_{25}$ (29)	380(12), 372(19), 320(72), 312(75), 292(87), 290(48), 284(100), 231(85), 230(48), 225(48), 224(36)
		$[^{14}C]GA_9$ 16,17-dihydrodiol (14)	413(100), 411(28), 405(82), 351(14), 345(9), 307(17), 301(16), 263(18), 255(24)
T17	$[^{2}H]GA_{12}$ (100 μg)	$[^{2}H]GA_9$ (50)	331(2), 299(50), 287(11), 271(100), 244(47), 228(26), 227(54), 226(33), 225(34)
		$[^{2}H]GA_{25}$ (32)	373(9), 313(68), 312(20), 285(100), 284(35), 226(47), 225(42)
		$[^{2}H]GA_{15}$ (2)	345(10), 313(24), 299(24), 285(88), 284(28), 240(100), 239(44), 226(17), 196(53)
		$[^{2}H]GA_{70}$ (12α-OHGA$_9$) (trace)	419(12), 372(22), 359(17), 329(14), 312(25), 297(100), 284(24), 283(53), 269(94), 224(96)
		$[^{2}H]GA_{111}$ (12α-OHGA$_{12}$) (1)	417(22), 389(46), 358(23), 327(12), 299(100), 240(43), 239(40)
		$[^{2}H]GA_{112}$ (12β-OHGA$_{12}$) (trace)	449(11), 417(62), 389(100), 374(21), 317(18), 299(81), 284(30), 272(20), 240(80), 239(58)
		$[^{2}H]$12-OHGA$_{24}$ (trace)	313(69), 287(58), 285(91), 259(31), 228(45), 227(100), 226(82), 199(20)
		$[^{2}H]GA_{12}$ 16,17-dihydrodiol (2)	436(100), 406(18), 376(86), 316(16), 299(14), 286(30), 256(12)
		$[^{2}H]GA_9$ 16,17-dihydrodiol (9)	494(2), 406(100), 374(3), 359(4), 346(8), 316(4), 302(18), 293(11), 270(7), 256(15), 228(12)
T1,[b] T6	$[^{14}C_4]GA_{14}$	$[^{14}C_4]GA_4$	426(12), 408(11), 398(26), 336(25), 308(25), 304(37), 292(96), 290(48), 284(30), 233(45), 231(82), 230(100), 229(54), 224(35), 205(31)
T17	$[^{2}H]GA_{14}$ (83 μg)	$[^{2}H]GA_4$ (83)	419(10), 400(9), 391(26), 387(18), 359(11), 329(23), 301(27), 297(33), 290(24), 285(100), 226(60), 225(93), 202(32)
		$[^{2}H]GA_{13}$ (17)	478(16), 461(19), 437(22), 433(22), 400(71), 373(33), 349(51), 343(30), 311(100), 310(40), 284(47), 283(72), 224(45), 223(38)
T17, T1, T6[b]	$[^{14}C]GA_{24}$	$[^{14}C]GA_{25}$	374(16), 314(74), 286(100), 271(3), 254(7), 232(11), 227(43), 226(28)
T1, T6	$[^{14}C]GA_{15}$[c]	no conversion	
	$[^{2}H_2]GA_{37}$[c]	no conversion	
	$[^{2}H_2]GA_{36}$	no conversion	

[a] In the case of ^{14}C-labeled substrates, the % relative abundance of each product was based on the recovered radioactivity after HPLC separation. For ^{2}H-labeled substrates, relative abundance was estimated from mass spectral total ion currents.
[b] Data presented from an incubation with this transformant.
[c] Assayed as lactones (at pH 3 and 4.5) or as free alcohols (at pH 7.0; 6.0 and 5.0). Conditions described under "Experimental Procedures."

whereas conversion of these substrates was markedly reduced when the culture medium was buffered at pH 7.0 (shown for $[^{14}C]GA_{12}$ in Table II). In contrast, $[^{14}C]GA_{12}$-aldehyde was completely transformed by the wild-type strain IMI58289 at pH values from 3.0 to 7.0 to give GA_3 and GA_9 in similar relative amounts regardless of the pH of the culture medium (Table II). Moreover, expression of *P450-2* in terms of transcript abundance was also independent of pH level (data not shown).

DISCUSSION

Gibberellin biosynthesis in *G. fujikuroi* is a complex process involving at least 16 enzymatic steps. Among the seven GA biosynthesis genes that were found to be organized in a gene cluster on chromosome IV, four cytochrome P450 monooxygenase, but no dioxygenase genes were identified (3–6). Two of the P450 genes, *P450-4* and *P450-1*, have been shown to code for the multifunctional *ent*-kaurene oxidase and GA_{14}-aldehyde synthase, respectively (4, 6). In this study, we have characterized the *P450-2* gene at both the molecular and biochemical levels.

The *P450-2* disruption mutants accumulated the C_{20}-GA precursor GA_{14}, suggesting a role for P450-2 in the oxidation of C-20, which results in the loss of this carbon atom and the formation of the C_{19} product GA_4. The function of P450-2 was confirmed by expressing the gene in the mutant SG139, which lacks the GA biosynthesis gene cluster. The transformants converted GA_{12} and GA_{14} to GA_9 and GA_4, respectively, whereas SG139 did not metabolize these substrates. Thus, the P450-2 protein has an analogous function to the soluble GA 20-oxidases in plants (2, 12, 13, 28). It had not previously been possible to determine the nature of the fungal GA 20-oxidase because this enzyme was not active in cell-free preparations. In fact, although all of the P450-catalyzed reactions required for the formation of GA_{14} could be demonstrated in such preparations (9, 29–31), none of the activities for later steps were present. The reason for this is still not clear despite the enzymatic nature of the 20-oxidase being known. The derived amino acid sequence of P450-2 shows 12 putative membrane-binding motifs, indicating that, in common with the P450s responsible for the earlier steps, the P450-2 protein should be associated with membranes.

There was no indication that the fungal 20-oxidase utilized the 3β-hydroxylated substrate GA_{14} more efficiently than the non-hydroxylated analog GA_{12}. Thus, the relative fluxes through the minor non-hydroxylation pathway to GA_9 and the

TABLE II
Influence of the pH of the culture medium on metabolism of [^{14}C]GA$_{12}$ and [^{14}C]GA$_{12}$-aldehyde by SG139-T1 and IMI58289 strains, respectively

Products were separated by HPLC. In incubations with [^{14}C]GA$_{12}$-aldehyde, besides [^{14}C]GA$_3$ and [^{14}C]GA$_9$, the remainder of the radioactivity eluted with the retention time of [^{14}C]GA$_{14}$. In incubations with [^{14}C]GA$_{12}$, the remainder of the radioactivity corresponded to [^{14}C]GA$_{25}$.

Substrate	pH of culture medium	% conversion	C_{19}-GA products	
			[^{14}C]GA$_9$	[^{14}C]GA$_3$
			dpm (% total products)	
[^{14}C]GA$_{12}$[a]	3.0	98.0	13200 ± 530 (66%)	–
[^{14}C]GA$_{12}$[a]	7.0	8.0	1000 ± 48 (5%)	–
[^{14}C]GA$_{12}$-aldehyde[b]	3.0	98.5	1200 ± 65 (6%)	5900 ± 354 (29.5%)
[^{14}C]GA$_{12}$-aldehyde[b]	7.0	99	2100 ± 120 (10.5%)	3900 ± 190 (19.5%)

[a] 20,000 dpm were incubated with the GA 20-oxidase transformant SG139-T1.
[b] 20,000 dpm were incubated with the wild type, IMI58289.

main 3β-hydroxylation pathway leading to GA$_3$ is probably determined by the availability of GA$_{12}$ and GA$_{14}$ rather than by the efficiency with which they are converted by the 20-oxidase. As we reported recently (4), GA$_{12}$ and GA$_{14}$ are both formed from *ent*-kaurenoic acid by the P450-1 monooxygenase (GA$_{14}$ synthase), which channels the GA intermediates preferentially into the 3β-hydroxylation pathway.

Although the C_{19} lactones, GA$_9$ or GA$_4$, were the major products of P450-2, the C_{20} tricarboxylic acid, GA$_{25}$, was formed from GA$_{12}$ in about 30% yield. However, none of the 3β-hydroxytricarboxylic acid GA$_{13}$ was detected from incubations with [^{14}C]GA$_{14}$, although GA$_{13}$ accounted for about 17% of the product when higher concentrations of substrate were used. These tricarboxylic acids are present in cultures of the wild-type strains of *G. fujikuroi* (see Fig. 4), and this probably reflects the high levels of intermediates present in such cultures. *G. fujikuroi* cultures also contain low amounts of GAs with C-20 at the alcohol and aldehyde oxidation levels, although, with the exception of the small amount of GA$_{15}$ (2% total products) and a trace of 12-hydroxy-GA$_{24}$ that accumulated when high levels of GA$_{12}$ were incubated, these GAs were not detected from incubations of GA$_{12}$ or GA$_{14}$ with the *P450-2* transformants. Furthermore, the 20-alcohols (as lactone or free alcohol) or aldehydes were not converted to C_{19}-GAs. The alcohol GAs, GA$_{15}$ and GA$_{37}$ and the 3β-hydroxyaldehyde, GA$_{36}$, were not metabolized by the transformant, whereas the 3-deoxyaldehyde GA$_{24}$ was converted to GA$_{25}$, the carboxylic acid, but not to GA$_9$. The same results were obtained previously from incubations with the mutant strain B1-41a (15). Because *G. fujikuroi* is capable of metabolizing a large range of GA substrates with different polarities, it seems unlikely that these results can be explained by failure to transport the compounds into the cells. It seems more likely that C-20 oxidation to form the C_{19}-GAs in *G. fujikuroi* does not go through the free alcohols or aldehydes and that the true intermediates remain bound to the enzyme. The small amounts of C-20 alcohols and aldehydes as well as the tricarboxylic acid products found in fungal cultures could be explained by the release from the enzyme of the covalently bound intermediates, probably as the result of hydrolysis. In contrast, C-20 alcohols and aldehydes are true intermediates in the GA 20-oxidase reaction in plants because they are produced and metabolized by these enzymes (12, 13, 32). The plant enzymes are 2-oxoglutarate-dependent dioxygenases, which may require full dissociation of products from the active site after each catalytic cycle, whereas in sequential reactions catalyzed by cytochrome P450 monooxygenases intermediates often do not accumulate and may not need to be released from the enzyme. It is of interest that intermediates also do not accumulate in reactions catalyzed by the other multifunctional P450 monooxygenases of the fungal GA biosynthetic pathway, *ent*-kaurene oxidase (6) and GA$_{14}$ synthase (4), indicating that the intermediates may not be released from the enzyme active site. However, in contrast to the GA 20-oxidase, these enzymes metabolize the intermediates efficiently so that they are unlikely to be covalently bound to the enzymes.

In incubations with GA$_{12}$, 12α-hydroxy, 12β-hydroxy, and 16,17-dhydrodihydroxy (probably formed by epoxidation of the 16,17-double bond and subsequent hydration) products were obtained in addition to GA$_9$ and GA$_{25}$. These appear to be true byproducts of P450-2 activity because they were not formed in incubations with the SG139 parent strain. Thus the enzyme is not strictly regiospecific with this substrate, whereas these side reactions were not apparent when GA$_{14}$ was the substrate, perhaps indicating that it is more rigidly bound at the enzyme active site.

In plants, GA 20-oxidase is an important regulatory enzyme for GA biosynthesis, its expression being negatively feedback-regulated by biologically active GAs to maintain GA homeostasis (2, 12, 33). In addition, *GA20ox* expression is promoted by long days in rosette plants as part of the photoperiod-dependent bolting response (34) and is suppressed by KNOTTED-type homeobox transcription factors, which give positional control by excluding GA biosynthesis from meristems (35). There was no evidence for feedback regulation of the corresponding fungal gene, *P450-2*. However, expression of *P450-2* is strongly suppressed by nitrogen, in common with five of the other six genes of the GA biosynthesis gene cluster in *G. fujikuroi* (4–6). Thus, most of the genes of the cluster are coordinately regulated to ensure that GA production occurs only at low nitrogen levels. The effector for this regulation is thought to be glutamine (36). Interestingly, *P450-2* transcript abundance is independent of the pH of the culture medium, whereas conversion of its substrates GA$_{12}$ and GA$_{14}$ by the SG139 transformants was much more effective under acid conditions (pH 3–4) than at neutral pH. However, the earlier intermediate GA$_{12}$-aldehyde was converted as efficiently at pH 7.0 as at pH 3.0 into GA$_3$ and GA$_9$ by the wild-type strain IMI58289, indicating that the GA 20-oxidase is active at neutral pH and suggesting that the difference in the utilization of substrates at acidic and neutral pH is due to preferential transport into the cells at low pH when the carboxylic groups are fully protonated (15). GA$_{12}$-aldehyde contains only the C-19 carboxyl group, which is very weakly acidic and likely to be protonated at pH 7 (37).

In summary, oxidation of GAs at C-20 in *G. fujikuroi* and plants differs markedly in the type of enzymes involved, their localization, the nature of the intermediates, and in the regulation of expression of the genes. The participation of a multifunctional membrane-bound GA 20-oxidase in the fungus in the removal of C-20, apparently without the release of intermediates from the enzyme, enables efficient formation of C_{19}-GAs, consistent with the very high yields of GA production in the fungal cultures. It is clear from the results reported here and in earlier findings that GA biosynthesis in the fungus and in higher plants developed independently and is not a result of horizontal gene transfer, as has been suggested (38).

Acknowledgments—We thank Jessica Schulte (Westfälische Wilhelms-Universität, Münster, Germany) for technical assistance and Dr. Michael Beale for the gift of [18-^{2}H]GA_{12}.

REFERENCES

1. Hedden, P., and Kamiya, Y. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 431–460
2. Hedden, P., and Phillips, A. L. (2000) *Trends Plant Sci.* **5,** 523–530
3. Linnemannstöns, P., Voss, T., Hedden, P., Gaskin, P., and Tudzynski, B. (1999) *Appl. Environ. Microbiol.* **65,** 2558–2564
4. Rojas, M. C., Hedden, P., Gaskin, P., and Tudzynski, B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5838–5843
5. Tudzynski, B., and Hölter, K. (1998) *Fungal Genet. Biol.* **25,** 157–170
6. Tudzynski, B., Hedden, P., Carrera, E., and Gaskin, P. (2001) *Appl. Environ. Microbiol.* **67,** 3514–3522
7. Tudzynski, B., Kawaide, H., and Kamiya, Y. (1998) *Curr. Genet.* **34,** 234–240
8. Kawaide, H., Imai, R., Sassa, T., and Kamiya, Y. (1997) *J. Biol. Chem.* **272,** 21706–21712
9. Urrutia, O., Hedden, P., and Rojas, M. C. (2001) *Phytochemistry* **56,** 505–511
10. Helliwell, C. A., Chandler, P. M., Poole, A., Dennis, E. S., and Peacock, W. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2065–2070
11. Kamiya, Y., Takahashi, N., and Graebe, J. E. (1986) *Planta* **169,** 524–528
12. Phillips, A. L., Ward, D. A., Uknes, S., Appleford, N. E. J., Lange, T., Huttly, A. K., Gaskin, P., Graebe, J. E., and Hedden, P. (1995) *Plant Physiol.* **108,** 1049–1057
13. Lange, T., Hedden, P., and Graebe, J. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8552–8556
14. Lange, T. (1994) *Planta* **195,** 108–115
15. Bearder, J. R., MacMillan, J., and Phinney, B. O. (1975) *J. Chem. Soc. Perkin Trans. I*, 721–726
16. Barrero, A. F., Oltra, J. R., Cabrera, E., Reyes, F., and Álvarez, M. (1999) *Phytochemistry* **50,** 1133–1140
17. Desjardins, A. E., Gardner, H. W., and Weltring, K. M. (1992) *J. Ind. Microbiol.* **9,** 201–211
18. Pontecorvo, G. V., Roper, J. A., Hemmonns, L. M., MacDonald, K. D., and Buften, A. W. J. (1952) *Adv. Genet.* **5,** 141–238
19. Geissman, T. A., Verbiscar, A. J., Phinney, B. O., and Cragg, G. (1966) *Phytochemistry* **5,** 933–947
20. Doyle, J. J., and Doyle, J. L. (1990) *Focus* **12,** 13–15
21. Maniatis, T., Sambrook, J., and Fritsch, E. F. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
22. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517
23. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1991–1995
24. Tudzynski, B., Mende, K., Weltring, K.-M., Unkles, S. E., and Kinghorn, J. R. (1996) *Microbiol.* **142,** 533–539
25. Barendse, G. W. M., van de Werken, P. H., and Takahashi, N. (1980) *J. Chromatogr.* **198,** 449–455
26. Graebe, J. E., Hedden, P., Gaskin, P., and MacMillan, J. (1974) *Phytochemistry* **13,** 1433–1440
27. Ward, J. L., Jackson, G. J., Beale, M. H., Gaskin, P., Hedden, P., Mander, L. N., Phillips, A. L., Seto, H., Talon, M., Willis, C. L., Wilson, T. M., and Zeevaart, J. A. D. (1997) *Chem. Commun.*, 13–14
28. Lange, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6553–6558
29. Ashman, P. J., Mackenzie, A., and Bramley, P. M. (1990) *Biochim. Biophys. Acta* **1036,** 151–157
30. Jennings, J. C., Coolbaugh, R. C., Nakata, D. A., and West, C. A. (1993) *Plant Physiol.* **101,** 925–930
31. West, C. A. (1973) in *Biosynthesis and its Control in Plants*, (Milborrow, B. V., ed) pp. 143–169, Academic Press, London
32. Lange, T., Kegler, C., Hedden, P., Phillips, A. L., and Graebe, J. E. (1997) *Physiol. Plant.* **100,** 543–549
33. Xu, Y. L., Li, L., Wu, K. Q., Peeters, A. J. M., Gage, D. A., and Zeevaart, J. A. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6640–6644
34. Wu, K. Q., Li, L., Gage, D. A., and Zeevaart, J. A. D. (1996) *Plant Physiol.* **110,** 547–554
35. Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M. (2001) *Genes Dev.* **15,** 581–590
36. Munoz, G. A., and Agosin, E. (1993) *Appl. Environ. Microbiol.* **59,** 4317–4322
37. Hendrick, C. A., and Jefferies, P. R. (1964) *Aust. J. Chem.* **17,** 915–933
38. Chapman, D. J., and Ragan, M. A. (1980) *Annu. Rev. Plant Physiol.* **31,** 639–678
39. Gaskin, P., and MacMillan, J. (1992) *GC-MS of the Gibberellins and Related Compounds: Methodology and a Library of Spectra*, Cantock's Enterprises, Bristol, UK