Characterization of the Final Two Genes of the Gibberellin Biosynthesis Gene Cluster of Gibberella fujikuroi

des AND P450-3 ENCODE GA DESATURASE AND THE 13-HYDROXYLASE, RESPECTIVELY*

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Recently, six genes of the gibberellin (GA) biosynthesis gene cluster in Gibberella fujikuroi were cloned and the functions of five of these genes were determined. Here we describe the function of the sixth gene, P450-3, and the cloning and functional analysis of a seventh gene, orf3, located at the left border of the gene cluster. We have thereby defined the complete GA biosynthesis gene cluster in this fungus. The predicted amino acid sequence of orf3 revealed no close homology to known proteins. High performance liquid chromatography and gas chromatography-mass spectrometry analyses of the culture fluid of knock-out mutants identified GA1 and GA2, rather than GA2 and GA3, as the major C19 GA products, suggesting that orf3 encodes the GA1,2-desaturase. This was confirmed by transformation of the SG139 mutant, which lacks the GA biosynthesis gene cluster, with the desaturase gene renamed des. The transformants converted GA2 and GA3, and also metabolized GA9 (3-deoxyGA4) to GA20 (1,2-didehydroGA9), but the 2α-hydroxylated compound GA20, was the major product in this case. We demonstrate also by gene disruption that P450-3, one of the four cytochrome P450 monoxygenase genes in the GA gene cluster, encodes the 13-hydroxylase, which catalyzes the conversion of GA2 to GA3, in the last step of the pathway. This enzyme also catalyzes the 13-hydroxylation of GA2 to GA3. Disruption of the des gene in an UV-induced P450-3 mutant produced a double mutant lacking both desaturase and 13-hydroxylase activities that accumulated high amounts of the commercially important GA3. The des and P450-3 genes differ in their regulation by nitrogen metabolite repression. In common with the other five GA biosynthesis genes, expression of the desaturase gene is repressed by high amounts of nitrogen in the culture medium, whereas P450-3 is the only gene in the cluster not repressed by nitrogen.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AJ417493.

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G. fujikuroi IMI 58289 and the GA-defective mutant strain SG139 (15) were kindly provided by J. Avalos (Sevilla, Spain). SG139 has completely lost the GA-biosynthetic gene cluster as demonstrated by Southern blotting and PCR analysis. Culture conditions for preparing mycelia for DNA or RNA isolation were as described previously (14). For analysis of GA production, the fungus was grown in the complex optimized GA production medium, OFM (14), or in 20% C1 medium (16) for 7–10 days at 28 °C on a rotary shaker (200 rpm).

Bacterial Strains and Plasmids—Escherichia coli strain Top10 (Invitrogen) was used for plasmid propagation. Vector pUC19 was used to clone DNA fragments carrying the G. fujikuroi orf3 gene or parts of it. The plasmid porf3-Sal carries a 3.4-kb fragment with the entire gene. For inactivation of orf3, an internal BamHI/XhoI fragment of plasmid porf3-Sal, instead of the hygromycin resistance cassette from pGPC1 (17). For inactivation of P450-3, three different gene disruption vectors, pP450-3-GD1, pP450-3-GD2, and pP450-3-GD3, were constructed by cloning internal fragments of the gene into the vectors pUCH2–8 (18) or pAN7–1 (19), both of which carry the hygromycin B resistance cassette. The internal fragments of P450-3 were first amplified by PCR, cloned into the PCR cloning vector pCR2.1 (Invitrogen), cut with KpnI/XhoI (pP450-3-GD2 and pP450-3-GD3), and then cloned into pUCH2–8. For pP450-3-GD1, the fragment was cut with HindIII/XhoI and cloned into pAN7–1. For gene expression of orf3 in the GA-deficient mutant SG139, the 3.4-kb SalI fragment from plasmid porf3-Sal was cloned into pUCH2–8, resulting in plasmid porf3-GC. For expression of P450-3 in SG139, a 2.3-kb XhoI fragment with the entire gene was cloned into pAN7–1, resulting in the vector p450–3-GC. In addition, the cosmid clone, cos5 carrying the entire P450–3 gene and 40 kb to the right of P450–3 was used for complementation of SG139. The Uni-Zap XR vector of the orf3 cDNA clones allowed in vivo excision and recircularization of cloned inserts to form a phagemid in pBluescript SK–) carrying the insert. This subcloning step was performed according to the manufacturer’s protocol (Stratagene, La Jolla, CA).

PCR—Conditions were as described previously (14). The primers for amplifying the internal fragments of P450–3 were as follows: pP450–3-GD1 (P450–3–F1, 5′-TGGAGCATGAAGACAAGTTTCAGG-3′; P450–3–R1, 5′-CTTAGCGGATCAGACGGAAGGG-3′; pP450–3–GD2 (P450–3–R2, 5′-CTTAGCGGATCAGACGGAAGGG-3′); and P450–3–GD3 (P450–3–F1, 5′-TGGACGATGAAGACAAGTTTCAGG-3′, and P450–3–R2, 5′-GATGCACTGCTTCTTGCAGG-3′). The mutant allele of P450–3 from strain 6314 was amplified with the forward primer P450–3–MF in combination with the reverse primer P450–3–MR and P450–3–R1 as follows: P450–3–MF (5′-CCTCTTGGCCTGGATCAGCG-3′), P450–3–MR (5′-AGCCCTCAGTATTCTCTTG-3′), and P450–3–R1 (5′-CTACCAAGTGAAGCTTTCAGG-3′). To identify the transformants of SG139 in which orf3 and P450–3 had been integrated correctly, the following PCR primers were used: orf3-F1 (5′-ATCTGTGGTCCTAAACAACTCTCAGC-3′), orf3-R1 (5′-CTTCACCTCTTCCTTCTTCTTCCCC-3′); P450–3–F1 (5′-ACCCCGAGGGGCGTGAGGT-3′), and P450–3–R3 (5′-ATCACCTGATGTCCTCTGACG-3′). All of the PCR primers were synthesized by MWG-Biotech (München, Germany).

Southern and Northern Blot Analysis—DNA and RNA isolation and Southern and Northern analyses were as described previously (14). The G. fujikuroi small subunit of rRNA was used as a control for RNA transfer.

Screening of G. fujikuroi cDNA and Genomic EMBL3 Libraries —The expression library (UniZap™XR vector, Stratagene) was constructed from RNA isolated from mycelium grown under optimal conditions for GA3 formation (4). Approximately 30,000 recombinant phages were plated at ~7,500 plaques/150-mm diameter Petri dishes and transferred to nylon membranes. For screening of the genomic library (20), ~40,000 recombinant phages were plated and transferred to membranes. The hybridization was performed at high stringency (65 °C), and the blots were washed at the same temperature in 2× SSC, 0.1% SDS, followed by 0.1× SSC, 0.1% SDS. Positive recombinant clones were used for a second round of plaque purification.

Sequence Analysis—DNA sequencing of recombinant plasmid clones was accomplished by the dideoxy chain termination method (21) using an automatic sequencer “LI-COR 4000” (MWG-Biotech, München, Germany).

Transformation of G. fujikuroi—Preparation of protoplasts and transformations were carried out as described previously (20). 106 protoplasts (100 μl) of the strain IMI 58289 or the mutant 6314 were transformed with 10 μg of the SalI fragment from the gene replacement vector pORF3-GR or one of the circular disruption vectors pP450–3–GD1, pP450–3–GD2, and pP450–3–GD3. Transformed protoplasts were regenerated at 28 °C in a complete regeneration agar (0.7 m sucrose, 2× YES).
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RESULTS

Cloning and Expression of orf3—A genomic 3.4-kb SalI fragment (Fig. 2A) of the left end of the GA-biosynthesis gene cluster, spanning from the gene P450–4 (12) to the sugar membrane transporter gene smt (25), was subcloned into...
pUC19. Sequence analysis revealed a 1029-bp open reading frame (designated orf3) transcribed in the same orientation as P450–4. The cDNA sequence data indicated that the open reading frame is not interrupted by any introns. The orf3 gene is deposited under the GenBank™ accession number AJ417493. Scrutiny of the GenBank™ data base for similar sequences revealed only a weak homology with ORF8 of the two-component 7a-cephem-methoxylase from Nocardiada lactamurans (26), providing little indication of its possible function in GA biosynthesis. In the 5′ non-coding region of orf3, 10 putative GATA motifs for binding of the major nitrogen regulatory protein, ARE1, were identified, indicating that expression of orf3 may be regulated by nitrogen repression as are most of the other GA-biosynthetic genes (5, 12, 13).

Functional Analysis of orf3—A gene replacement vector pORF3-GR was obtained by replacing the central BamHI/XbaI fragment of plasmid porf3-Sal carrying the 3.4-kb SalI fragment of orf3 (Fig. 2B) with the hygromycin B resistance cassette from the vector pGCP1 (17). The wild-type strain IMI58289 was transformed with the 4.3-kb SalI-fragment from pORF3-GR. TLC and HPLC analysis revealed that 5 of 42 transformants (T18, T21, T23, T55, and T60) were not able to produce GA3, the final product of the GA-biosynthetic pathway in G. fujikuroi, or its immediate precursor, GA20. Southern blot analysis confirmed that the transformants have lost the 6.3-kb PatI wild-type band because of homologous integration of the replacement cassette into the orf3 locus (data not shown). GC-MS analysis of culture filtrates of transformant T23 and wild-type indicated that GA1 rather than GA20 was the major C19-GA in the orf3-deficient line (Fig. 3). Fuxenoic acid and GA20 (Fig. 1) were major metabolites in both lines. Three of the transformants were shown by HPLC analysis to contain GA1 and GA20 in ratios from 3:1 to 5:1 after cultivation in the synthetic 20% ICI medium or in OPM for 10 days (Table I). Since the mutants produced no GA20 or GA20 and accumulated GA1 and GA20, the mutation appears to block the 1,2-desaturation of GA20 to GA1 (Fig. 1), indicating that orf3 encodes GA20 desaturase.

To confirm the function of the orf3 expression product, we transformed the deletion mutant SG139, which has lost the entire GA gene cluster, with the gene complementation vector porf3-GC carrying the wild-type orf3 gene. From 20 analyzed transformants, 12 were shown by PCR to contain a 2.1-kb fragment expected after correct integration of the gene into the SG139 genome. Analysis of five transformants by Northern hybridization confirmed that the gene was expressed in strain SG139 despite the loss of the entire GA gene cluster (data not shown). Four strains SG139-T1, SG139-T2, SG139-T4, and -T5, which expressed orf3 at levels comparable with the wild-type, were incubated with the radiolabeled substrates [14C]GA3 (3β-hydroxylated) or [14C]GA20 (non-hydroxylated) in medium buffered at pH 3.0. GC-MS analysis of the HPLC-purified product from incubation of [14C]GA3 with transformants T1, T2, T4, and T5 identified [14C]GA3 as sole product (shown for T1 in Table II), whereas SG139 did not metabolize this substrate (data not shown). Thus, the desaturase gene is expressed as an active enzyme in SG139. In addition, the orf3 transformants efficiently utilized the non-hydroxylated substrate [14C]GA20, producing two more polar fractions of radioactivity on HPLC in 95% yield. The more polar of these (65%) was found by GC-MS to contain [14C]GA40 (2α-hydroxyGA20), whereas the less polar fraction (28%) contained GA120 (1,2-didehydroGA20) (Table II and Fig. 4) and three isomers of a diene dicarboxylic acid derivative assumed to be formed from GA120 by rearrangement during work-up and derivatization. The enzyme will thus accept both 3β-hydroxylated and non-hydroxylated substrates, although in the latter case, there is an apparent loss of regiospecificity.

Functional Analysis of P450–3—To determine the function of the remaining gene of the cluster, P450–3, the wild-type strain IMI58289 was transformed with the gene disruption vector pP450–3-GD1, which contained the hygromycin-resistant gene, hph (Fig. 2C). After 100 hygromycin-resistant transformants by TLC, all were found to produce high amounts of GAs after 7 days of cultivation in OPM but three lines, T52, T59, and T61, did not produce GA20, although its precursors, GA20 and GA20, were present. To be sure that this change in GA profile was the result of P450–3 gene inactivation, we constructed two additional disruption vectors pP450–3-GD2 and pP450–3-GD3. In pP450–3-GD3, a mutation in the heme-binding domain was introduced via PCR (Fig. 2C, primer P450–3-R3). We analyzed 100 hygromycin-resistant transformants for both vectors by TLC. One of the transformants from pP450–3-GD2 (T92) and three of the transformants from pP450–3-GD3 (T11, T49, and T55) produced GA20 as final product instead of GA20, which was confirmed by HPLC. GC-MS analysis of the culture fluid from transformant pP450–3-T55 confirmed that GA20 was the major product and that no GA120 was produced (Fig. 3). Southern blot analysis (data not shown) revealed that transformants with all of the three vectors had produced two more polar fractions of radioactivity on HPLC in 95% yield. The more polar of these (65%) was found by GC-MS to contain [14C]GA40 (2α-hydroxyGA20), whereas the less polar fraction (28%) contained GA120 (1,2-didehydroGA20) (Table II and Fig. 4) and three isomers of a diene dicarboxylic acid derivative assumed to be formed from GA120 by rearrangement during work-up and derivatization. The enzyme will thus accept both 3β-hydroxylated and non-hydroxylated substrates, although in the latter case, there is an apparent loss of regiospecificity.
Characterization of des and P450-3

Table I

Yields of GAs (mg/liter⁻¹) produced by the wild-type (IMI58289) and mutant 6314 strains and in des-deletion mutants (Δdes) obtained from both strains after 10 days of cultivation in 20% ICI medium or OPM.

The GA concentration was determined by HPLC and is the mean of three measurements. N.D., non-detectable by HPLC.

| Strain          | GA₄ | GA₇ | GA₁ | GA₃ |
|-----------------|-----|-----|-----|-----|
| 20% ICI medium  |     |     |     |     |
| IMI58289        | 22  | 62  | N.D.| 268 |
| IMIΔorfST23     | 37  | N.D.| 198 | N.D.|
| IMIΔorfST33     | 55  | N.D.| 119 | N.D.|
| IMIΔorfST55     | 62  | N.D.| 162 | N.D.|
| 6314            | 34  | 414 | N.D.| 18  |
| 6314ΔorfST1     | 165 | 4   | N.D.| 4   |
| 6314ΔorfST2     | 267 | N.D.| 22  | N.D.|
| 6314ΔorfST8     | 381 | N.D.| 8   | N.D.|
| OPM             |     |     |     |     |
| IMI58289        | 79  | 97  | N.D.| 761 |
| IMIΔorfST23     | 92  | N.D.| 432 | N.D.|
| IMIΔorfST33     | 83  | N.D.| 389 | N.D.|
| IMIΔorfST55     | 89  | N.D.| 412 | N.D.|
| 6314            | 34  | 790 | N.D.| 35  |
| 6314ΔorfST1     | 762 | N.D.| N.D.| N.D.|
| 6314ΔorfST2     | 657 | N.D.| N.D.| N.D.|
| 6314ΔorfST8     | 654 | N.D.| N.D.| N.D.|

Table II

GC-MS identification of products from incubation of [14C]GA₄ and [14C]GA₇ with SG139 des transformants.

| Substrate Product a and authentic samples | m/z                      | % relative intensity |
|-----------------------------------------|--------------------------|----------------------|
| [14C]₁GA₄ [14C]₂GA₄ (100%)              | M⁺ 418(2), 386(7), 358(12), 300(12), 284(14), 225(51), 224(100), 223(30), 222(26), 195(29), 181(33), 179(28), 155(40) |
| GA₇ b                                   | M⁺ 416(9), 384(14), 356(22), 298(20), 282(35), 223(73), 222(100), 193(43), 181(31), 179(42), 155(48) |
| [14C]₂GA₅ [14C]₃GA₁₆ (65%)             | M⁺ 426(3), 395(4), 379(93), 371(46), 349(100), 343(46), 321(15), 305(84), 299(37), 292(20), 284(10), 275(10), 249(13), 231(38), 229(56), 225(18), 223(25) |
| GA₁₄                                   | M⁺ 418(8), 387(4), 371(100), 343(83), 315(7), 299(79), 284(78), 269(7), 243(8), 225(57), 223(29) |
| [14C]₃GA₂₁₀ (28%)                     | M⁺ 336(8), 318(5), 304(43), 290(26), 276(33), 274(54), 268(38), 259(25), 249(100), 233(44), 219(15), 199(21), 187(35) |
| GA₂₁₀ b                                 | M⁺ 328(21), 310(5), 296(40), 284(21), 282(15), 268(45), 224(38), 223(100), 211(18), 195(21), 181(35) |

* Percent conversion in parenthesis
* Data taken from library spectra

Transformants produced no GA₁, another 13-hydroxylated GA, which is produced in small amounts (~1.3% total GAs) in the wild-type. Thus, P450-3 is responsible for 13-hydroxylation of both GA₇ and GA₄ (Fig. 4).

To confirm the function of P450-3, complementation of SG139 with the complementation vector pP450-3-GC and cosmid clone cos5, both carrying the entire gene P450-3, was performed. However, Northern analysis indicated that none of the resulting transformants expressed the gene despite the correct integration of the gene into the SG139 genome (data not shown). Furthermore, none of the transformants from both complementation vectors were able to metabolize [14C]GA₇ or [14C]GA₄ to radiolabeled GA₁ and GA₃, respectively. Several intermediates of the GA biosynthetic pathway were tested as possible inducers of P450-3 expression by adding them to cultures of the P450-3 transformants at 350–500 μM. We found that ent-kaurenoic acid, GA₁₄, GA₄, or GA₇ were ineffective in inducing GA 13-hydroxylase activity in the SG139-P450-3 transformants, which remained unable to convert [14C]GA₄ or [14C]GA₇ under these conditions. The lack of P450-3 expression in the SG139 + P450-3 transformants is in contrast with the efficient expression of P450-1 (13), P450-2 (14), P450-4 (12), and des in SG139. Interestingly, complementation of SG139 with a cosmid carrying the entire gene cluster (cos1) fully restored 13-hydroxylase activity.

Characterization of the 13-Hydroxylase Mutant 6314—The identification of P450-3 as the 13-hydroxylase that catalyzes the conversion of GA₇ to GA₃ allowed us to classify the GA₇-overproducing mutant 6314 as a putative P450-3 mutant. This strain was isolated after mutagenesis of the highly GA₃-overproducing wild-type strain m567 and screening for GA-overproducing mutants.² To identify the nature of the mutation, the coding region of the P450-3 gene from the mutant was amplified by PCR using the primer pairs P450-3-MF and P450-3-MR as well as P450-3-MF and P450-3-31. Nine independent clones were sequenced from both strands. A point mutation was found at position 844, resulting in an amino acid substitution from arginine to tryptophan at position 221 (Fig. 5). This substitution results in a dramatic change in the GA product spectrum, indicating greatly reduced 13-hydroxylation activity, whereas the wild-type strain m567 produced ~84% GA₃, ~2% GA₇ and ~14% GA₄. As determined by HPLC, the mutant 6314 produced 3% GA₃, 3% GA₄, and 94% GA₇ under the same conditions.

² B. Tudzynski, unpublished data.
The substrates GA4 and GA7 are formed from the 3β-hydroxylation and non-3β-hydroxylation pathways, respectively. The pathway to GA3 is dehydrated GA3 synthesis as demonstrated by GC-MS analysis of total RNA revealed a single band of ~1.0 kb in 10% ICI medium, the transcript level increasing in abundance with culture time in a similar manner to P450–4 (Fig. 7A), cepsks and ggs2 (data not shown). No transcript was observed for des or P450–4 in mycelia grown in 100% ICI medium. In contrast to the other six genes of the cluster, P450–3 appears to be expressed more highly in medium with high N than with low N (Fig. 7A, C). After 15 h in culture in 10% ICI medium, when some nitrogen still remains, only very low levels of expression of des, P450–4 (Fig. 7A) and the other four genes (data not shown) can be detected, whereas P450–3 is highly expressed. With depletion of nitrogen, transcript abundance for des and P450–4 increases, whereas P450–3 transcript levels decrease (Fig. 7A). In addition, transcript abundance for P450–3 is not reduced in areA mutants confirming that AREA does not control expression of P450–3, whereas des expression (Fig. 7B) and that of the other genes (data not shown) require the active nitrogen regulator, AREA. The pH of the culture medium in the range 3.5 to 8.0 does not affect the expression of des, P450–3 (Fig. 7C) or any of the other GA-biosynthetic genes (data not shown).

To confirm that the differential regulation of des and P450–3 expression can be observed at the biochemical level, we investigated metabolism of [14C]GA4 or [14C]GA7 in P450–2 disruption mutants. These mutants are blocked for the oxidative removal of C-20 from GA14 and are therefore not able to produce GA4, GA7, GA1, or GA3 (Fig. 1) (14). The experiments with the gene-disruption mutants P450–2-T35 and T39, demonstrated clear differential inhibition of the desaturase and 13-hydroxylase activities by N. Under high N conditions (100% ICI), [14C]GA4 was mainly converted to [14C]GA1, together with a small amount of [14C]GA3 (Table III), while incubations in ICI medium with no N gave almost complete conversion of [14C]GA4 to [14C]GA3 (Table III). On the other hand, [14C]GA7 was converted efficiently to [14C]GA3 in both high and low N conditions, thus confirming that the 13-hydroxylase shows no significant inhibition by high N concentration. In contrast, the above results demonstrate that the desaturase is strongly inhibited by N, in agreement with the expression analysis on the des and P450–3 genes described above (Fig. 7A). The product distribution obtained from [14C]GA4 under high N conditions,
is consistent with the spectrum of GAs found in des disruption mutants, which accumulate mainly GA₁ (Table I).

**DISCUSSION**

We describe the cloning of the seventh and final gene of the GA biosynthesis gene cluster in G. fujikuroi and demonstrate that it encodes GA₄ desaturase (DES). The gene is located between the recently identified membrane transporter gene smt (25) and the ent-kaurene oxidase gene P450–4 (12) at the left side of the cluster. The gene is not related to any other GA-biosynthetic gene from G. fujikuroi from higher plants, but it has low homology with ORF8 in the cephamycin C cluster of N. lactamdurans (26). This protein forms part of the two-protein 7a-cephem-methoxylase, which has both hydroxylase and methyltransferase activities. However, ORF8 has no apparent activity in isolation and did not assist us in determining the function of DES, which was demonstrated by a combination of gene replacement and biochemical analysis. Transformants of the SG139 mutant containing the wild-type des gene in the absence of the other GA biosynthesis genes converted [¹⁴C]GA₄ to [¹⁴C]GA₃ and also metabolized the non-hydroxylated substrate [¹⁴C]GA₉ to [¹⁴C]GA₁₂₀ (1,2-didehydroGA₉). However, GA₄₀ (2α-hydroxyGA₉) was the major product from GA₉, showing that 2α-hydroxylation is an additional activity of the desaturase. This enzyme would thus account for the presence of 2α-hydroxylated GAs in G. fujikuroi cultures (28). No 2α-hydroxylated products were detected in incubations of the des transformants with the 3β-hydroxylated substrate GA₅, suggesting that the presence of the 3β-hydroxyl group directs 1,2-desaturation in favor of 2α-hydroxylation. However, the occurrence of 2α,3β-dihydroxylated GAs in fungal cultures (28) indicates that 2α-hydroxylation of GA₅ may occur if at a low rate. Although we have no information on the mechanism of the desaturation, the formation of a hydroxylated product implies a requirement for oxygen.

In plants, oxidation of ring A of the GAs is catalyzed by 2-oxoglutarate-dependent dioxygenases, many of which are
TABLE III

| % N<sup>b</sup> | Substrate | Products | Relative amount<sup>a</sup> |
|--------------|-----------|----------|--------------------------|
| 100          | [14C]GA<sub>4</sub> | [14C]GA<sub>1</sub>, [14C]GA<sub>2</sub> | 2                        |
| 0            | [14C]GA<sub>4</sub> | [14C]GA<sub>1</sub> | 1                        |
| 100          | [14C]GA<sub>7</sub> | [14C]GA<sub>3</sub>, [14C]GA<sub>1</sub> | 1                        |
| 0            | [14C]GA<sub>7</sub> | [14C]GA<sub>3</sub> | 1                        |

<sup>a</sup> Ammonium nitrate content in the ICI culture medium (16). 100% = 4.75 liter<sup>-1</sup>.
<sup>b</sup> Determined by GC-MS from the intensities of the ions at m/e 508 ([<sup>14</sup>C]GA<sub>1</sub>) and 506 ([<sup>14</sup>C]GA<sub>3</sub>.

multifunctional, catalyzing desaturation, and hydroxylation reactions (29). For example, a GA 3-oxidase from Phaseolus vulgaris catalyzes dehydration of GA<sub>3</sub> at C-2,3 and 2β-hydroxylation in addition to its major 3β-hydroxylase activity (30). Furthermore, the same multifunctional dioxygenase is probably responsible for GA<sub>3</sub> synthesis from the 2,3-didehydro intermediate (GA<sub>2</sub>) by rearrangement of the double bond from C-2,3 to C-1,2 followed by 3β-hydroxylation (31). Thus, in this case, desaturation at C-1,2, which results in the loss of the 1β,2β-H atoms (32), is a side reaction of the main pathway to GA<sub>1</sub>. In contrast, in G. fujikuroi direct 1,2-desaturation by loss of the 1α,2α-H atoms (33) is the major reaction and is accompanied by 2α-hydroxylation. It is of interest that immature seeds of Prunus persica contain a number of 3-deoxy 1,2-didehydro GAs including GA<sub>120</sub> that are probably formed by direct 1,2-desaturation (34). The seeds also contain 1α-hydroxy GAs, which could be formed as byproducts of the desaturation. Nothing is known regarding the nature of the putative desaturase/1α-hydroxylase in this case.

We have also shown by gene disruption that P<sub>450</sub>-3, the fourth cytochrome P<sub>450</sub> monoxygenase gene in the GA gene cluster, encodes the 13-hydroxylase, which catalyzes the final reaction in the biosynthesis of GA<sub>3</sub>. The same enzyme catalyzes also the 13-hydroxylation of GA<sub>2</sub> to form GA<sub>1</sub>. However, little GA<sub>1</sub> (2–3% total GA content) is produced in these two wild-type strains of G. fujikuroi, suggesting either that the fungal cultures contain much more GA<sub>3</sub> desaturase than 13-hydroxylase activity or that GA<sub>3</sub> has a higher affinity for the desaturase than for the 13-hydroxylase. Removal of desaturase activity by disruption of the des gene resulted in approximately a 100-fold accumulation of GA<sub>3</sub> but only a 2-fold accumulation of GA<sub>1</sub>. The mutant strain could thus provide the means to produce large quantities of GA<sub>1</sub> for agricultural or experimental purposes. Similarly, the double mutant lacking both desaturase and 13-hydroxylase activities could be used for the production of the commercially important GA<sub>1</sub>.

There was an unexpected difference in the regulation of the des and P<sub>450</sub>-3 genes. Expression of des is high under conditions of low N that promote GA production but very low in the growth phase when the N content is high. These expression patterns correspond to those of ggs2, cpsiks, P<sub>450</sub>-1, P<sub>450</sub>-2, and P<sub>450</sub>-4, which are all under control of the major nitrogen regulator, AREA (35, 36). In contrast, the expression pattern of P<sub>450</sub>-3 differs from that of the other six GA genes. Northern analysis with mycelia grown in medium with a high or low N content showed that P<sub>450</sub>-3 is expressed under both conditions, and in fact, more transcript was present under high N conditions. Thus, P<sub>450</sub>-3 is the only gene in the GA cluster for which transcript is detectable in 100% ICI medium, which contains 4.8 gliter<sup>-1</sup> NH<sub>4</sub>N<sub>3</sub>O<sub>4</sub>. These results are in agreement with those from incubations of <sup>13</sup>C-labeled GA<sub>4</sub> and GA<sub>2</sub> with P<sub>450</sub>-2 knock-out mutants, which do not contain these GAs and later metabolites because the conversion of GA<sub>4</sub> to GA<sub>2</sub> is blocked (14). In cultures growing in low N, both desaturase and 13-hydroxylase activities were present so that GA<sub>3</sub> was the major product formed from GA<sub>4</sub>, whereas in high N, more GA<sub>1</sub> was produced than GA<sub>4</sub> due to low desaturase activity (Table III). Furthermore, GA<sub>3</sub> was converted to GA<sub>15</sub> regardless of the N content of the medium. The lack of repression of P<sub>450</sub>-3 expression by N is consistent with the absence of the double GATA sequence elements in its promoter. These elements, which bind the AREA transcriptional regulator (37), are present in the promoters of the other six GA-biosynthesis genes. In addition to its different regulation by N, P<sub>450</sub>-3 is the only gene in the cluster that cannot be expressed in the deletion mutant SG139, which lacks the gene cluster. In contrast, it was effectively expressed in 6314, which has a point mutation in P<sub>450</sub>-3, as well as in SG139 complemented with cosmid 1 containing the entire GA gene cluster. We have not yet investigated how many of the other genes need to be present and active for P<sub>450</sub>-3 to be expressed but have addressed the possibility that its expression requires induction by a GA-biosynthetic intermediate by applying precursors to SG139-P<sub>450</sub>-3 transformants. However, the presence of ent-kaurenoic acid, GA<sub>15</sub>, GA<sub>4</sub>, or GA<sub>3</sub>, which are the end products of P<sub>450</sub>-4, P<sub>450</sub>-1, P<sub>450</sub>-2, and DES, respectively, did not improve expression. The fusion of the P<sub>450</sub>-3 gene to a strong and/or N-repressible promoter will determine whether or not the low expression of the gene under N-limiting conditions is responsible for the failure to complement P<sub>450</sub>-3 in SG139 or whether other elements of the gene cluster are needed for gene expression and enzyme activity.

The functional characterization of des and P<sub>450</sub>-3 completes the analysis of the GA gene cluster in G. fujikuroi. Disruption of smt (25) and an alcohol dehydrogenase gene<sup>2</sup> at the left border of the cluster and of orf<sub>1</sub> and orf<sub>2</sub> at the right of P<sub>450</sub>-3<sup>2</sup> did not affect the production of GA<sub>3</sub> or its regulation. Thus, the biosynthesis of the final product, GA<sub>3</sub>, in 13 steps beginning with the formation of GGPP by the pathway-specific gera

lygeranyl-diphosphate synthase GGS2 requires only seven enzymes, many of which are multifunctional. In contrast to many other fungal secondary metabolite gene clusters, e.g. the aflatoxin gene cluster in Aspergillus parasiticus (38) and the trichothecene gene cluster in Fusarium sporotrichoides (39), the GA gene cluster does not contain a pathogenesis-specific regulatory gene. We have evidence that, in addition to the general regulator AREA, a regulatory gene that controls expression of all of the seven GA-biosynthesis genes exists and must be located elsewhere in the genome.

We are now able to compare GA biosynthesis in G. fujikuroi with that in higher plants at the chemical, biochemical, and gene levels. Although the fungus and plants produce structurally identical GAs and the early enzymatic steps up to the formation of GA<sub>12</sub>-aldehyde are similar, the pathways thereafter differ fundamentally as do the character of enzymes involved and the regulation of their genes. An important difference is that the early 3β-hydroxylation and 20-oxidation steps in the fungus are catalyzed by cytochrome P<sub>450</sub> monoxygenases, whereas soluble dioxygenases are responsible for these reactions in higher plants. Another major difference is that 13-hydroxylation occurs early (at the stage of GA<sub>12</sub>) in plants, whereas it is the last step in the fungus. Even in the first part of the pathways in which cytochrome P<sub>450</sub>s participate in the fungus and plants, the equivalent enzymes in G. fujikuroi and plants have low levels of amino acid identity, e.g. only 10% for ent-kaurene oxidase of the fungus (P<sub>450</sub>-4) and A. thaliana (40).

In higher plants, GAs play a key role in development and...
their concentration is maintained at a very low level by a number of endogenous and environmental factors including feedback regulation (7, 41). In contrast, wild-type strains of *G. fujikuroi* produce approximately 1 liter⁻¹ GA₃ under laboratory conditions. The absence of feedback regulation in the fungus (14) suggests that GAs do not have an important role in N repression. However, the molecular mechanism of the AREA-mediated regulation including N sensing, signal transduction, and modulation of activity of the transcription factor AREA is still to be investigated.

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