Gibberellin Biosynthesis in gib Mutants of Gibberella fujikuroi*

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The Ascomycete Gibberella fujikuroi synthesizes gibberellins, fujenal, carotenoids, and other terpenoids. Twelve gib mutants, isolated through the modified gibberellin fluorescence of their culture media, were subjected to chemical and biochemical analyses. Two mutants were specifically defective in the hydroxylation of carbon 13; their total gibberellin production was normal, but their main gibberellin was GA₃ instead of GA₄. Four mutants were blocked in the early reactions between geranylgeranyl pyrophosphate and 7-hydroxykaurenonic acid; two of them could not synthesize kaurene and another one was blocked in several oxidative steps. Six mutants had partial defects in early reactions, leading to the production of one-fifth to one-third of the wild type amounts of gibberellins and fujenals. Two of these produced considerable amounts of kaurenilones due to a defect in the conversion of kaurenic acid to 7-hydroxykaurenonic acid. Another one produced no carotenoids, but attempts to isolate mutants of reactions shared by the carotenoid and gibberellin pathways failed. The gib mutations did not modify the ability of the fungus to live as a saprophyte.

The gibberellins are terpenoid hormones that regulate plant growth and development (1, 2) and have found many applications in agriculture and brewing (3–5). They are present at low concentrations in plant tissues and are obtained industrially from the culture media of the fungus Gibberella fujikuroi ssp. fujikuroi (6, 7).

Gibberella synthesizes about 20 different gibberellins (8, 9), of which the most abundant is gibberellic acid (GA₃) as well as other related metabolites, such as fujenal and the kaurenoides. The biosynthetic pathway (Fig. 1) has been deduced from the determination of chemical structures, the use of labeled precursors, and the analysis of biotransformations (10, 11). A major role in this work was played by a mutant called B1–41a, which is unable to synthesize kaurenic acid, but can convert it to gibberellins. This mutant was isolated after testing 50,000 survivors of ultraviolet exposure in a bioassay with barley seeds.

Ease of growth in the laboratory, availability of active cell-free systems for biochemical analysis, and suitability for the isolation of recessive mutations make Gibberella an attractive organism for the investigation of gibberellin biosynthesis (12, 13). A simplified method to detect changes in gibberellin production led to the isolation of 14 gib mutants from about 4,000 colonies tested (14). We have now characterized these mutants to gain information on the biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Strains—The wild type IMI58298 of G. fujikuroi, ssp. fujikuroi came from the Commonwealth Mycological Institute, Kew, United Kingdom. The 12 gib mutants defective in gibberellin biosynthesis (14) listed in Table 1 and the carotenoid-supercproducing mutant S225 (15) were derived from IMI58298 after exposure of its spores to N-methyl-N'-nitro-N-nitrosoguanidine (12). The same mutagenesis procedure was used to obtain white mutants of S225. For chemical and biochemical analyses, low-nitrogen (0.48 g/liter NH₄NO₃) minimal liquid medium (16) was inoculated with spores grown on sporulation agar (13) and incubated in the dark at 30 °C in an orbital shaker (150 rpm).

Analysis of Gibberellins and Related Compounds—Aliquots (21 ml) of culture media from 200-ml cultures, grown in 500-ml Erlenmeyer flasks, were separated from the mycelia by filtration and brought to pH 8 with NaOH. They were extracted three times with ethyl acetate, brought to pH 2 with HCl, and extracted three more times with ethyl acetate. The dried extracts were methylated with diazomethane, mixed with 40 μl of tetrahydrofuran and 40 μl of N-O-bis(trimethylsilyl)-trifluoroacetamide and heated at 60 °C for 30 min to form methyltrimethylsilyl derivatives for gas chromatography (Hewlett-Packard 5890A; capillary column HP-1 of cross-linked methylsilicone gum, 25 m × 0.2 mm × 0.33-μm film thickness; programmed temperature increase from 120 to 220 °C at 5 °C/min and from 220 to 280 °C at 3 °C/min; injector temperature 260 °C; flame ionization detector at 290 °C; carrier gas N₂ at 25 ml/min). The gas chromatogram was coupled to a Hewlett-Packard 5988A mass spectrometer operating at 70 eV.

Compounds were identified by comparison of mass spectra with those previously described (17) and by using our own standards (18). The amounts of the different gibberellins were calculated by reference to known amounts of GA₃ subjected to the same procedures. The amounts of GA₄ and GA₅ include those of their isomers marked with * and iso (Fig. 2), formed during the extraction procedure. The gibberellins and fujenal (isolated in its diacid form) were identified as follows: GA₃ (retention time 29.57 min), 330 (M*+, 14), 298 (71), 270 (69), 243 (57), 227 (54), 226 (60); GA₄* (Rt 31.40): 430 (M*+, 6), 370 (16), 311 (51), 281 (71), 221 (100); GA₅ (Rt 32.69): 404 (M*+, 0, 372 (17), 312 (60), 284 (100), 253 (9), 225 (80); GA₆ (Rt 32.80): 574 (M*+, 3), 314 (84), 386 (62), 285 (40), 226 (100), 225 (95); iso-GA₄ (Rt 33.84): 416 (M*+, 11), 394 (27), 356 (65), 223 (72), 222 (100); GA₉ (Rt 34.02): 418 (M*+, 15), 283 (35), 284 (100), 225 (91), 224 (81); GA₁₀ (Rt 34.54): 416 (M*+, 10), 384 (50), 356 (70), 223 (63), 222 (100); Fujenal diacid (Rt 34.89): 376 (M*+, 0.4), 343 (3), 344 (2), 316 (5), 227 (37), 195 (86), 167 (64), 107 (100); GA₉* (Rt 35.01): 518 (M*+, 3), 418 (15), 399 (9), 399 (19), 309 (11), 257 (22), 227 (27); GA₁₀ (Rt 36.06): 492 (M*+, 2), 477 (5), 436 (9), 400 (12), 310 (33), 282 (25); iso-GA₁₀ (Rt 36.92): 504 (M*+, 100), 489 (12), 370 (23), 347 (18), 208 (27); GA₁₂ (Rt 37.30): 506 (M*+, 19), 416 (19), 390 (100), 357 (37), 360 (31), 340 (26), 300 (39); GA₉ (Rt 37.54): 506 (M*+, 100), 491 (10), 448 (18), 377 (22), 313 (17); GA₁₀ (Rt 38.65): 504 (M*+, 100), 489 (9), 370 (11), 347 (2), 208 (57).

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§ The abbreviations used are: kaurenolic acid, ent-kauren-16-en-19-oid acid; 7-hydroxykaurenolic acid, ent-7-hydroxykauren-16-en-19-oid acid; kaurene, ent-kauren-16-ene.

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The high peaks at m/e 73 in the spectra of GA_{1}, GA_{12}, GA_{13}, GA_{36}, iso-GA_{12}, GA_{20}, iso-GA_{20}, and GA_{25} were not included when calculating ion abundances.

The alkaline extracts were dissolved in a mixture of pyridine and N,O-bis-(trimethylsilyl)-trifluoroacetamide (10 μl each per mg of extract), heated at 100 °C for 30 min in a closed vial, and analyzed by gas chromatography and mass spectrometry. The quantitative results were deduced from the peak area given by the detector and the total weight of the extracts.

Mycelia were washed, dried, ground in a mortar, and extracted with ethyl acetate for 24 h. The extracts (containing abundant triglycerides) were saponified with 2 mol KOH in methanol (10 μl per mg of extract) for 12 h, diluted with water, extracted with tert-butylmethyl ether and analyzed as the alkaline extracts above.

Mycelial pigments were extracted with acetone; the dried extracts were partitioned three times between methanol/water (30:1) and petroleum ether (b.p. 40–60 °C). The bikaverins were retained in the hypophase. The dried petroleum ether extract was redissolved in n-hexane for spectrophotometry. The total amounts of colored carotenoids (predominantly neurosporaxanthin) were estimated using an absorbance coefficient (1-cm optical path) of 2000 for a 1% (w/v) solution. The absence of phytoene in strain SG127 was confirmed by chromatography (19).

*In Vitro Terpenoid Biosynthesis*—Washed mycelia from 500-ml cultures grown for 8 days in 1-liter flasks were lyophilized, extracted, and incubated as described (20, 21), except that the buffer included 20% (w/v) glycerol and the cofactor mixture did not contain NADH or NADPH. Incubation mixtures (0.5 ml) contained 1.2-2.0 mg of protein and 18,500 Bq of sodium 3R-[2-14C]mevalonate (2.0 TBq/mol), prepared from the lactone purchased from Amersham (Bucks, United Kingdom). After stopping the reaction with methanol, the mixture was extracted three times with petroleum ether. The radioactivity in aliquots of the extract was used to estimate total terpenoid biosynthesis. The rest of the extract was reduced to about 0.1 ml for chromatography on Silica Gel G thin layers (20 × 5 cm) developed with 15% toluene in petroleum ether. The kaurene band (R_{f} 0.7) was scraped off and radioassayed (21). The results are expressed as geometrical means and their standard errors in two independent experiments, with two determinations in each (four independent experiments in the case of the wild type).

**RESULTS**

The mutants used in this study showed the growth pattern of the wild type and had no special growth requirements. The slower growth of three mutants in comparison with the wild type (Table I) may be due, not to the *gib* mutations, but to other mutations introduced into the same genome by the high dose of mutagen.

The *Gibberellin Pathway*—The wild type and the mutants differed in the production of gibberellins, fujenal, kaurenoles, and their precursors (Fig. 3, Tables I and II). The acid extracts of the wild type contained considerable amounts of gibberellic acid (GA_{3}) and fujenal, and smaller amounts of various compounds, among which GA_{2}, GA_{16}, and GA_{12} were prominent. The alkaline extracts contained kaurenoles and kaurene, but
**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>SG 123</th>
<th>SG 133</th>
<th>SG 139</th>
<th>SG 121</th>
<th>SG 138</th>
<th>SG 136</th>
<th>SG 128</th>
<th>SG 129</th>
<th>SG 124</th>
<th>SG 127</th>
<th>SG 122</th>
<th>SG 135</th>
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<td>8.2</td>
<td>8.5</td>
<td>5.5</td>
<td>7.6</td>
<td>7.1</td>
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<td>7.7</td>
<td>7.4</td>
<td>5.9</td>
<td>7.4</td>
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<td><strong>Carotenoids</strong></td>
<td>0.58</td>
<td>0.53</td>
<td>0.39</td>
<td>0.68</td>
<td>0.19</td>
<td>0.98</td>
<td>0.64</td>
<td>0.22</td>
<td>0.41</td>
<td>0.43</td>
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<td>1.28</td>
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<td>23</td>
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<td>1</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>7</td>
<td>9</td>
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<td><strong>Gibberellins</strong></td>
<td>93</td>
<td>64</td>
<td>143</td>
<td>0</td>
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<td>2</td>
<td>0</td>
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<td>34</td>
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<td>19</td>
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<td><strong>GA_{15}</strong></td>
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<td>0</td>
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<td>0</td>
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<td>2</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>2</td>
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<td>3</td>
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<tr>
<td><strong>GA_{16}</strong></td>
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<td>66</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
<td>7</td>
<td>17</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>GA_{3}</strong></td>
<td>58</td>
<td>8</td>
<td>34</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>GA/GA_{7}</strong></td>
<td>2.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1.3</td>
<td>0.5</td>
<td>1.0</td>
<td>2.2</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Fig. 2.** GA<sub>7</sub> and GA<sub>4</sub>, isomers formed during the extraction procedure.

Negligible quantities of gibberellins.

Strain SG123 accumulated GA<sub>7</sub>, at the expense of GA<sub>9</sub>, and showed a drastic decrease in GA<sub>15</sub>. The ratio of GA<sub>7</sub> to GA<sub>9</sub> in 14-day-old cultures was 17 times larger in this mutant than in the wild type, although both strains were similar in their overall production of gibberellins and in their content of other terpenoids. Therefore, the effect of the mutation in strain SG123 was an impairment of the ability to hydroxylate the carbon at position 13 (Fig. 1). The same phenotype, quantitatively less marked, was found in strain SG133.

Four mutants (SG139, SG121, SG138, and SG136) produced no gibberellins, fujenal, or kaurenoioids, or only very small amounts, indicating a virtually complete block in a step before kaurenoic acid, the last common precursor of the absent isoprenoids. As expected, therefore, negligible kaurenoic acid (less than 0.05 mg/liter) was detected in these mutants, while wild type cultures produced 1.3 mg/liter.

Strains SG139 and SG121 produced very little kaurene (less than 1 mg/liter in 14-day cultures), in contrast to the wild type level of 22 mg/liter, of which 70% was in the mycelium. The defect was confirmed by the low level of kaurene biosynthesis from labeled mevalonate in vitro (Fig. 4). SG139 was the tightest of the gib mutants, as it contained no detectable gibberellins, fujenal, or kaurenoioids.

Strain SG138 made kaurene in vitro and in vivo (13 mg/liter, of which 55% was in the mycelium), indicating a defect in the oxidation of kaurene to kaurenoic acid. This strain was slightly leaky and produced small amounts of GA<sub>15</sub> and GA<sub>9</sub>. The gibberellins hydroxylated at carbon 3 and those with 19 carbons, which predominate in the wild type, were completely absent. A similar strain, SG136, synthesized kaurene in vitro (Fig. 4).

The partial blocks found in six strains (SG128, SG129, SG124, SG127, SG122, and SG135) led to gibberellin and fujenal contents one-fifth to one-third of those of the wild type, each compound being reduced to about the same extent. An excep-
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Fig. 3. Analysis of acid extracts of *G. fujikuroi* culture media. Gas chromatograms of the wild type and two gib mutants grown for 8 days in low-nitrogen minimal medium.

tion to this was the GA₃ content of strains SG129, SG128, and SG124, which was closer to that of the wild type, thus leading to higher GA₂/GA₃ ratios than in the latter.

The main defect in strains SG128 and SG129 was probably the conversion of kaurenoic acid to 7-hydroxykaurenoic acid, since they produced more kaurenoles than would be expected from the overall leakiness of their mutations (Table II). Strains SG124 and SG127 contained early blocks in the pathway, since they did not produce kaurene.

Other Metabolites—With the exception of strain SG127, the carotenoid contents of the mutants were very similar to that of the wild type. The variations (about double in SG122, about half in SG121, and smaller variations in others) are possibly indirect effects.

Strain SG127 produced no carotenoids, whether in the light or dark, in liquid media or on agar. Other strains deficient in both gibberellins and carotenoids could not be obtained. A total of 29 new white mutants were derived from strain SG22, but they produced approximately normal amounts of gibberellins, as shown by the fluorescence assay (14).

The gibberelin-containing culture media were red in color, due to the presence of bikaverin, as judged from the solubility of the pigment and its absorption spectrum (22, 23). The red color was absent in strain SG121 and less pronounced in strain SG127 than in the wild type.

**DISCUSSION**

This report represents the most exhaustive description of gibberelin mutants of *Gibberella* to date. The gibberellins produced by 12 mutants have been analyzed and compared with those produced by the wild type. The results confirm the usefulness of the fluorescence method (14) for the isolation of mutants with quantitative and qualitative changes in gibberelin production. Gibberellins do not seem to carry out any important function in the life of *Gibberella* as a saprophyte, in contrast to higher plants, where the characteristic phenotypic changes associated with decreased gibberelin contents (e.g., dwarfism) reflect the physiological role of these hormones (24).

One pair of mutants (SG123 and SG135), defective in the hydroxylation of carbon 13 of the gibberelin molecule, are similar to two natural *Gibberella* strains (25, 26). The phenotype of these mutants suggests that the same 13-hydroxylase acts on different substrates, such as GA₅ and GA₇.

Other mutants (SG139 and SG121) are defective in kaurene synthesis. Since they accumulate normal carotenoid concentrations, they must produce geranylgeranyl pyrophosphate, and are therefore presumably defective in kaurene synthetase, which catalyzes in two steps the four cyclizations needed to convert geranylgeranyl pyrophosphate into kaurene (27). Alternatively, they could be regulatory mutants, unable to carry out all the reactions in the pathway.

The oxidations of kaurene to kaurenolic acid (28) and the subsequent hydroxylation to 7-hydroxykaurenolic acid (29) are catalyzed by monooxygenases dependent on cytochrome P-450. The mutant B1–41a (10) made gibberellic acid and other compounds when incubated with kaurenolic acid, but not when incubated with earlier intermediates (11). Strain SG138 is also deficient in the oxidation of kaurene, but different from B1–41a. The structure of the minor gibberellins in SG138 implies additional defects in the hydroxylation at carbon 3 and in the loss of carbon 20. This suggests that a common gene product participates in three seemingly different biochemical modifications. Under this interpretation, strain SG138 would not be expected to produce gibberellic acid from kaurenolic acid.

Strain SG136 is a clean mutant which produces very small amounts of gibberellins very late in the growth cycle. There are several possible explanations for the contradiction between the absence of kaurene in this strain *in vivo* and its ability to produce it *in vitro* (Fig. 4). For example, the mutant may be unable to target its kaurene synthetase to the correct subcellular compartment or the enzyme may be subject to an inhibition *in vivo* that is lost *in vitro*.

The six strains with partial early blocks in the gibberellin pathway do not lend themselves to detailed biochemical analyses. Two of them, SG128 and SG129, seem to be defective in the hydroxylation of kaurenolic acid at carbon 7. Their existence confirms that such a hydroxylation is not required for kaurenomide biosynthesis, which begins with the introduction of a double bond between carbons 6 and 7 (30, 31).

The white, carotenoid-less phenotype of SG127 could be due to a partial loss of a specific prenyl transferase that converts farnesyl pyrophosphate to geranylgeranyl pyrophosphate, as it occurs in the *al-3* mutants of *Neurospora crassa* (32). We failed to isolate such mutants by screening white *Gibberella* mutants for gibberellin production. The alternative hypothesis, that SG127 is a rare double mutant, was not confirmed because of the lack of suitable strains to cross with our *Gibberella* strain.

Mutants exhibiting very small decreases in gibberellin production are easily obtained (33–35) and many are probably unspecific. The competition for a common substrate (gera-
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Table II

<table>
<thead>
<tr>
<th>Kaurenoide production in the wild type and the mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kde</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>11.4</td>
</tr>
<tr>
<td>18-OH-Kde</td>
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<td>3a-OH-Kde</td>
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</tbody>
</table>

Acknowledgment—R. F. M. thanks Prof. John R. Bower (Department of Biochemistry, Royal Holloway and Bedford New College) for his hospitality.

REFERENCES


Fig. 4. In vitro kaurene biosynthesis. Incorporation of 3R-[2-14C]mevalonic acid into terpenoids (radioactivity in petroleum ether extracts) and kaurene (relative to incorporation into terpenoids) in crude extracts of the wild type and several gibr mutants.

nylgeranyl pyrophosphate) explains why mutants with increased or decreased carotenoid content exhibit a small variation of opposite sign in gibberellin content (14). In addition, the reduced biosynthesis of gibberellins in mutants isolated for resistance to peflurazone (36), an inhibitor of sterol biosynthesis, suggests that some gene products may act on both the gibberellin and the sterol pathways.

Most of the chemical modifications needed to produce gibberellins are not specifically blocked in any of the gibr mutants, and none is blocked in more than two mutants. This indicates that the present collection is incomplete and that repeated application of the fluorescence test should yield new mutant types. Some steps may be refractory to mutational analysis for various reasons, including a similar fluorescence of the accumulated gibberellins in the mutants and the wild type.

The results in this paper support the existence of “gene-saving devices” in the development and diversification of secondary metabolism (37). Foremost is the versatility of enzymes to carry out similar reactions on different substrates, so that the same enzyme may be used repeatedly for successive reactions and different enzymes may act in any order along the pathway. Some reactions, particularly those yielding minor side products, may occur spontaneously during the week-long incubations.