Light-Stimulated Gibberellin Biosynthesis in Gibberella fujikuroi

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ABSTRACT

Gibberellins (GAs) are a group of plant growth hormones that were first isolated from the fungus Gibberella fujikuroi. The biosynthesis of GA in liquid cultures of the fungus has been examined using high-performance liquid chromatography and combined gas chromatography-mass spectrometry. GA, was the predominant GA in well-aerated cultures. GA and GA, intermediates in GA biosynthesis, accumulated in cultures with low levels of dissolved oxygen, but were not detectable in more highly aerated cultures. Light stimulated the production of GA in G. fujikuroi cultures grown from young stock cultures. Cell-free enzyme studies revealed a significant stimulation in the levels of kaurenic acid oxidation in cultures grown in the light in comparison with those grown in the dark. However, measurements of the relative rates of [14C]mevalonic acid incorporation into kaurene showed no effect of light on this early part of the pathway. Preliminary experiments indicated that blue light is most effective in enhancing kaurenic acid oxidation.

GAs were first isolated from the Ascomycete Gibberella fujikuroi (Sawanda) Wollenweber (Fusarium moniliforme Sheld, imperfect stage). They have since been found to occur widely in higher plants. GAs have also been found in the lower vascular plants Psilotum nudum (28) and Lygodium japonicum (31); in two other Ascomycetes; Sphaceloma manihitica (25) and Neurospora crassa (17); and in the bacteria Rhizobium phaseoli (1) and Azospirillum lipoflorum (4).

GAs are synthesized from MVA via the isoprenoid pathway. Four molecules of MVA are incorporated into ent-kaurene, a tetracyclic compound (9) that is converted through a series of oxidative reactions to GA,aldehyde (2). GA,aldehyde is a branch point from which either 3-3-hydroxylated or non-3-3-hydroxylated GAs may be formed (13).

There have been reports of light acting as a regulator of GA biosynthesis in G. fujikuroi. Zweig and DeVay (32) found that "cultures which were kept in darkness usually produced less GA than cultures which were kept in the light." Mertz and Henson (21, 22) also observed light stimulation of GA biosynthesis in the fungus. Light increased levels of GA-like materials in fungal cultures by 112%, as measured by the Zea mays dwarf-1 bioassay. Incorporation of [14C]acetate into compounds that co-chromatographed with GAs was found to be higher in light-grown cultures. This light-stimulated increase in acetate incorporation appeared to be prevented by AMO-1618, a GA biosynthesis inhibitor. [14C]Leucine incorporated into compounds that co-chromatographed with GAs was also enhanced in light-grown cultures (20).

Isoprenoid biosynthesis in other ascomycetes is also stimulated by light. Blue-light irradiation rapidly enhanced phytoene production in N. crassa (18, 27). In cell-free Neurospora extracts, at least one of the photoregulated steps occurs after the formation of isopentenyl pyrophosphate (27). Carotenoid synthesis in Fusarium aquaeductuum is also stimulated by blue light (26).

The present studies were undertaken to confirm and extend the earlier reports of effects of light on the GA biosynthetic pathway in G. fujikuroi. Light stimulation of GA biosynthesis in G. fujikuroi was analyzed by HPLC and GC-MS. We also used isotope feeding studies and cell-free enzyme extracts to examine the possible enzymatic site(s) of light regulation in the GA biosynthetic pathway of G. fujikuroi.

MATERIALS AND METHODS

Culture of Fungi

Gibberella fujikuroi strain gf-1a was obtained from B. O. Pinney and cultured on potato dextrose agar. Liquid cultures were grown in media described previously (8). Glucose and minerals were autoclaved separately. Inocula were originally prepared by transferring a small amount of mycelia from the agar slant to 250-mL Erlenmeyer flasks containing 50 mL of sterile media. These flasks were incubated on an orbital shaker (250 rpm) at room temperature under 2.4 × 105 ergs cm-2 sec-1 of light for 7 d. At this time, 1-mL aliquots of mycelial suspension were used to inoculate new 50-mL cultures, which were grown under the same conditions. Liquid culture stock was maintained by periodically inoculating media with 1-mL transfers from actively growing (3-6-d-old) cultures. Purity and viability of the cultures were checked by plating small amounts on Petri plates containing potato dextrose agar. Oxygen concentrations were measured with a polarographic oxygen sensor (Yellow Springs Instruments).
Light Treatments

To examine the effects of darkness, cultures were covered with boxes (37 × 26 × 22 cm) that had been painted with black followed by silver enamel paint. The area of contact between the boxes and the top was sealed with light-proof tape. Each box was fitted with two pieces of rubber tubing (4-mm i.d.) to facilitate a flow of air (1000 cu ft/min). The air ensured a constant supply of oxygen and temperatures varied no more than 0.5°C between any two treatments.

To test the effects of white light, a box topped with Saran Wrap plastic wrap was used. Light (2.4 × 10^5 ergs cm^{-2} s^{-1}) was provided by a 52-W incandescent bulb (Sylvania) and overhead fluorescent lighting.

Isolation and Quantification of Gibberellins

Cultures were filtered through Whatman No. 1 paper rinsed with de-ionized water. The filtrates were refiltered through Whatman No. 5 paper and, if not immediately extracted, stored at -20°C. Mycelial mats were saved for dry weight determination or enzyme isolation.

The filtrates were adjusted to pH 3.0 and extracted three times with one-third-volume of EtOAc. The organic fractions were combined, dried with anhydrous NaSO_{4}, and evaporated to dryness with a rotary evaporator at 30° to 40°C. The residues were redissolved in MeOH and passed through Sep-Pak C-18 cartridges (Baker). Each cartridge was rinsed with 2 mL of MeOH, and the combined methanolic fractions were evaporated under reduced pressure using an Evapomix. The dried samples were taken up in 0.01 M H_{3}PO_{4},MeOH (60:40, v/v; pH 3.0) for HPLC analysis.

Samples were chromatographed on a 5 μm ODS C-18 HPLC column (10 mm × 250 mm), using a MeOH:0.01 M H_{3}PO_{4} (pH 3.0) gradient and a flow rate of 2.5 mL/min. Gibberellins were eluted with a step gradient starting with 40% MeOH, then 75%, 80%, and finally 100% MeOH over a total period of 35 min. GAS were detected at 205 using a variable wavelength detector. GA_{3} was quantified by comparison with a standard curve for authentic GA_{3} (Abbott).

Combined GC-MS was used primarily to confirm the presence and identity of GAS in HPLC fractions. GC-MS was also used as an additional means of quantifying GAS by comparison with standard curves.

The MeOH in collected HPLC fractions was evaporated under reduced pressure. The remaining aqueous portions were adjusted to pH 3.0, and extracted three times with EtOAc. The organic fractions were combined, evaporated to dryness, and reacted with ethereal diazomethane to form methyl esters. The samples were then derivatized with bis-(trimethylsilyl)trifluoroacetamide plus 1% v/v trimethylchlorosilane to form the trimethylsilyl ethers of the methylated GAS. HPLC grade pyridine (Fisher), dried with KOH, was added to each sample for a final bis-(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane:pyridine ratio of 2:3.

Derivatized samples were injected into a Hewlett-Packard 5890 gas chromatograph coupled with a HP 5970 mass selective detector. The chromatography column was a J & W Scientific DB-1 W.C.O.T.-fused silica capillary column (30 m long, 0.25-mm i.d.). Helium was the carrier gas at a linear flow rate of 25 cm/s. The injector and detector temperatures were both set at 280°C. During the course of a run, column temperature was increased at a rate of 20°/min from 50° to 280°C over the first 11.5 min; the column temperature was then maintained at 280°C until the end of the run (23.5 min). The identities of presumptive GAS were confirmed by comparing the spectra of their MeTMS derivatives to those of authentic standards. Standard curves for MeTMS-GA_{3} and MeTMS-GA_{13} were prepared using derivatized authentic standards.

Determination of Percent Recovery

To determine percent recovery, 6 × 10^{5} Bq [^{14}C]GA_{3} (7.3 × 10^{5} Bq/μmol) were added to mature *G. fujikuroi* liquid media cultures. The media were extracted and analyzed as described above. GA_{3}-containing HPLC fractions were collected and the recovered radioactivity measurements (liquid scintillation spectrometry), were used to estimate percent recovery through HPLC.

Incorporation of [^{14}C]Mevalonic Acid into [^{14}C]GA_{3} by Cultures of *G. fujikuroi*

Flasks containing 50 mL of liquid media were inoculated with 1 mL of cell suspension from a 2-old dark grown culture. After 72 h, incubation under either dark or light conditions, 6.6 × 10^{8} Bq of 2-[^{14}C]MVA (specific activity, 10 μCi/μmol) was added to each flask. The cultures were incubated an additional 48 h and harvested and extracted as described previously. Radiolabeled GA_{3} was detected with an on-line solid scintillation monitor (Romona D). GA_{3}-containing fractions were collected and quantified by liquid scintillation spectrometry.

Biosynthesis of ent-Kaurene in Cell-Free Extracts from *G. fujikuroi*

The mycelia from 4-old light- and dark-grown *G. fujikuroi* liquid cultures were separated from the media by filtration through Whatman No. 1 filters. All of the cultures grown under one set of experimental conditions (three to six cultures) were combined. A portion of the filtrate was saved for measurement of GA content. The mycelial mats were rinsed once with de-ionized water and twice with 50 mm Tes buffer, pH 7.1. Mats were frozen in liquid N_{2} and stored either in liquid N_{2} or in a −20°C freezer.

The frozen mycelial mats were crushed in a chilled (−20°C) Sager press at 13,000 psi and homogenized in 50 or 100 mm Tes buffer (pH 7.1) containing 10 mm β-mercaptoethanol using a Teflon to glass homogenizer. One milliliter of buffer was used for each g of mycelial fresh weight. The resulting homogenate was filtered through Miracloth and was centrifuged at 10,000 g for 15 min. After the lipid layer was removed, the supernatant fraction (S_{10}) was recentrifuged for 1.5 h at 150,000 g. The supernatant from the high-speed centrifugation (S_{10}) was used as the enzyme source. Enzyme preparations were stored in liquid N_{2} until used.

Reaction mixtures contained 50 nmol of [^{14}C]MVA (Amer- sham) diluted with unlabeled R,S-MVA (Sigma) to a final
specific activity of \(1.32 \times 10^8 \text{ Bq}/\mu\text{mol}\), 20 to 250 \(\mu\text{L}\) enzyme extract, 2 mM \(\text{MgCl}_2\), 2 mM \(\text{MnCl}_2\), 3 mM ATP, and 50 mM Tris buffer (pH 7.1) in a total volume of 1 mL. Reactions were incubated 30 min at 30°C in a shaking water bath (100 rpm) and stopped with 1 mL of acetone. Mixtures were extracted three times with 1 mL of benzene:acetone (3:1, v/v). The organic extracts were dried under reduced pressure and chromatographed on silica gel G TLC plates (EM Reagent, 2 × 20 cm) in hexanes. To quantify the kaurene produced, the silica in the region 10 to 15 cm from the origin was scraped from the plates and counted by liquid scintillation.

**Biosynthesis of Oxidized Derivatives of ent-Kaurenoic Acid in Cell-Free Extracts of G. fujikuroi**

To isolate oxidative enzymes, the above steps for obtaining soluble enzymes were followed, except that 100 mM Tricine (pH 8.0) and 100 mM Tricine (pH 8.0) containing 0.29 mM sucrose and 10 mM \(\beta\)-mercaptoethanol were used for rinsing and homogenizing, respectively (23). The pelleted fraction from an initial high-speed centrifugation that contained microsomal enzymes (P150) was resuspended in the Tricine-sucrose-\(\beta\)-mercaptoethanol buffer and stored in liquid N\(_2\) until used.

Reaction mixtures contained 1.6 nmol of 17-[\(^{14}\)C]kaurenoic acid (specific activity: \(1.17 \times 10^9 \text{ Bq}/\mu\text{mol}\)), 50 or 100 \(\mu\text{L}\) of P150 enzyme preparation, 0.5 \(\mu\text{M}\) flavin-adenine dinucleotide, 50 \(\mu\text{M}\) NADPH, and buffer (50 mM Tricine, pH 8.0, with 10 \(\mu\text{M}\) \(\beta\)-mercaptoethanol) in a total volume of 1 mL. Preparations from light- and dark-grown cultures contained equal levels of total protein. Reactions were incubated for 30 or 60 min under aerobic conditions in a 30°C shaking water bath (150 rpm). Reactions were stopped with 1 mL of acetone, acidified to pH 3.0, and extracted three times with 1 mL of EtOAc. The combined organic fractions were evaporated to dryness under reduced pressure and chromatographed on silica gel G TLC plates, developed in benzene: ethanol: \(\text{NH}_4\text{OH} \) (65:35:0.5, v/v/v). The GAs were quantified by removing the silica 2 cm up from the origin and counting the radioactivity by liquid scintillation.

Total protein in enzyme extracts was measured using the method of Bradford (5).

**RESULTS**

**Time Course for Growth and Gibberellin Production**

Growth of fungal cultures, as measured by dry weight, was linear for the first 72 h, slowed between d 3 and 4, and then leveled off from the fourth to the sixth day after resuspension (Fig. 1). Measurable amounts of GA\(_3\) were first detected in the 2-d-old samples. GA\(_3\) accumulation followed a pattern similar to that of growth, but the linear phase lagged slightly behind that for growth. GA\(_{13}\) was not detectable until the third day of growth. The levels of this GA in the cultures increased slightly over the following 72 h.

**Effect of Oxygen Concentration on Gibberellin Accumulation in G. fujikuroi Liquid Media Cultures**

In the studies of growth and GA accumulation, the cultures contained 50 mL of liquid media and were grown at 250 rpm on an orbital shaker. These conditions resulted in high levels of GA\(_3\) and GA\(_{13}\) (Fig. 2A). However, the immediate precursors to GA\(_3\), GA\(_4\), and GA\(_5\) were not detected. Growing the
cultures at a slower shaker speed (160 rpm) resulted in lower levels of GA₃, but again GA₄ and GA₇ were not detected (Fig. 2B). When cultures were grown in 100 mL at 160 rpm, however, UV absorbance peaks corresponding to the retention times of GA₄ and GA₇ were observed in the HPLC traces of the acidic ethyl acetate extracts (Fig. 2C). GC-MS analysis of these fractions confirmed the presence of GA₄ and GA₇.

Measurements of the pH and O₂ content of cultures grown under the conditions given above revealed no significant difference in pH among the treatments (Table I). There were, however, marked differences in dissolved O₂. Fifty-milliliter cultures incubated at 250 or at 160 rpm, and 100 mL cultures incubated at 160 rpm, had average O₂ concentrations of 5.1, 4.6, and 1.43 ppm, respectively.

Gibberellin Production by Light- and Dark-Grown G. fujikuroi Cultures

The ability of light to stimulate GA biosynthesis in G. fujikuroi was examined in cultures inoculated with stock grown under a variety of conditions. In one set of experiments, cultures were inoculated with 6-d-old light-grown stock and grown for 3 d, which is the end of the linear phase of growth. Analysis of the GA₃ showed only a slightly greater amount of GA₃ in the light-grown cultures. When cultures inoculated with mature light-grown stock were grown for 6 d before harvesting, light still did not significantly affect GA biosynthesis. When light-grown 2-d-old stock was used as inoculum, there was a 35% increase in GA₃ production in the light-grown cultures. In cultures grown from 2-d-old dark-grown stock, white-light irradiation resulted in an average 43% increase in GA₃ production (Table II).

![Figure 3. ent-Kaurene biosynthesis in cell-free extracts from light- and dark-grown G. fujikuroi cultures. Reactions contained S₅₀ enzyme extract, 50 nmol of [¹⁴C]-MVA, 2 mM MgCl₂, 2 mM MnCl₂, 3 mM ATP, and 50 mM Tes buffer (pH 7.1) in a total volume of 1 mL.](image)

**Incorporation of [¹⁴C]MVA into [¹⁴C]GA by Cultures of G. fujikuroi**

The time-course experiments for GA production showed that the middle of the linear phase of GA₃ accumulation occurs on the third day of growth. This is also the time when GA₁₃ accumulation begins (Fig. 1). This stage was chosen for feeding experiments with [¹⁴C]MVA. HPLC analysis of the acidic ethyl acetate extracts of culture filtrates revealed three major peaks of radioactivity (data not shown). The first peak corresponded with the solvent front and may have contained unmetabolized [¹⁴C]MVA. The second peak co-chromatographed with GA₃. The third major peak had the same retention time as GA₁₃ (21.2 min).

More [¹⁴C]MVA was incorporated into [¹⁴C]GA₃ in G. fujikuroi cultures grown in the light than in cultures maintained under dark conditions. Average yields of [¹⁴C]GA₃ in these experiments were 15.27 nmol/g fresh weight and 20.50 nmol/g fresh weight in dark- and light-grown cultures, respectively (Table II). This represents a 34% increase in incorporation in the light-grown cultures.

**Cell-Free Incorporation of [¹⁴C]MVA into Kaurene**

In an attempt to locate the site(s) of the light-stimulated step(s) in the GA biosynthetic pathway, cell-free extracts were used to incorporate [¹⁴C]MVA into kaurene. Incubations with soluble (S₁₅₀) enzymes were carried out for 30 min, which was in the linear range for enzyme activity with regard to time. Virtually no difference was found between the activities of the preparations isolated from light-grown cultures and those from dark-grown samples over the range of enzyme concentrations tested (Fig. 3).

**Cell-Free Oxidation of [¹⁴C]Kaurenoic Acid**

Cell-free experiments were also carried out using microsomal extracts from light- and dark-grown fungal cultures. In these studies, the metabolism of kaurenoic acid was examined.
The products isolated for quantification were highly polar and did not move significantly from the origin in the TLC developing system used. In subsequent experiments in our laboratory, we have identified GA_{14} by GC-MS as one of the primary polar products (Jennings and Coolbaugh, unpublished results). The ability of the extracts from light-grown cultures to oxidize kaurenoic acid was significantly greater (40–60%) than that of the dark-grown preparations (Fig. 4).

**DISCUSSION**

HPLC analysis allowed for the consistent recovery of over 70% of the total GA found in filtrates of _G. fujikuroi_ cultures. In this study, GC-MS was used to confirm the presence of GA_{1}, GA_{4}, GA_{7}, and GA_{13} in fractions collected during HPLC analysis of _G. fujikuroi_ liquid culture extracts.

The time course for growth and GA production in _G. fujikuroi_ liquid media cultures was examined by Bu'Lock *et al.* (6). Growth as well as GA content increased through the 5 d of the study, with the linear phase of growth preceding the period of rapid accumulation. Significant amounts of GAs were found in the cultures after 3 d. These patterns for growth and GA production are similar to those observed in our 6-d time-course study in which GA levels sharply increased following the onset of linear growth and then accumulated at a lower rate after growth had plateaued.

Growing the cultures at rapid shaker speeds and in an amount of media that allows for a high surface-to-volume ratio resulted in substantial amounts of GA production. GA_{1} was the most prevalent GA produced by these fungal cultures, typically 62% of the total. This is in accord with earlier reports (3, 11) on GA metabolism in _G. fujikuroi_. In well-aerated cultures the only other GA to accumulate in detectable amounts was GA_{13} (38% of total).

In _G. fujikuroi_, the immediate precursors to GA_{1} are GA_{4} and GA_{7} (3, 29). These GAs were found to accumulate in _G. fujikuroi_ cultures only when cultures were grown in high (100 mL) volumes of media and at slow (160 rpm) shaker speeds, conditions that resulted in low (1.43 ppm) oxygen concentrations (Table I). GA_{4} and GA_{7} were not observed when dissolved oxygen levels were raised by increasing the shaker speed, or decreasing the volume of media and increasing the shaker speed.

In higher plants, at least some GA interconversion reactions are oxygen dependent (16, 24). It has been suggested that they are oxoglutarate-dependent dioxygenases (12). The data from this study suggest that the enzymes involved in the late stages of GA biosynthesis in _G. fujikuroi_ also require oxygen. However, the exact nature of the enzymes involved in the conversion of GA_{4} to GA_{7} to GA_{1} is unknown.

The first suggestion that light stimulates GA biosynthesis in _G. fujikuroi_ was that of Zweig and DeVay (32). Using bioassays, Mertz and Henson (21, 22) found higher levels of GA-like activity in light-grown cultures than in dark grown-cultures. They also observed light-induced incorporation of [14C]acetate into GA-like compounds. The present studies, in which GA production was analyzed using HPLC followed by GC-MS, confirmed these earlier reports. GA biosynthesis in _G. fujikuroi_ is not totally light dependent; rather, light leads to higher levels of accumulation. This situation is similar to carotenoid biosynthesis in Phycomyces, in which a significant amount of carotenoids is synthesized in the dark but their levels increase in the light (14).

In the current study, it was found that light stimulated GA synthesis in light-grown cultures grown from young (2-d-old) inocula. These data are consistent with those of Mertz (20), who reported over 60% greater incorporation of [14C]leucine into GA when the cultures were irradiated than when they were maintained in the dark. The difference between the levels of GA production in the light- and dark-grown daughter cultures was greater if the stock cultures had been maintained in darkness. Only slight differences in GA content were noted in light- and dark-grown cultures inoculated with older (6-d-old) stock. Older mycelia appear to lose their capacity for light stimulation of GA synthesis.

Mertz (20) proposed that light affects GA biosynthesis by adjusting the partitioning of acetate units. The results in the present study showing increased levels of incorporation of MVA into GA_{1} suggest that there is at least one additional light-stimulated step(s) later in the GA biosynthetic pathway. Working with peas, Ecklund and Moore (10) found evidence for light-stimulated activity between MVA and kaurene. From the results reported here, this early part of the GA biosynthetic pathway does not seem to be light-regulated in _G. fujikuroi_. The rates of incorporation of MVA into kaurene for cell-free systems prepared from light- and dark-grown cultures were virtually identical (Fig. 3). Kaurenoic acid oxidation, however, does appear to be a light-stimulated. Rates of oxidation to products having GA-like properties were increased by up to 60% in enzyme preparations from light-grown cultures (Fig. 4). The oxidative reactions observed involve the conversion of kaurenoic acid to ent-7-a-hydroxykaurenoic acid and the further oxidation to GA_{3}-aldehyde and GA_{7}. These reactions require oxygen and NADPH. They are also inhibited by carbon monoxide. West (30) suggested that this combination of properties may indicate the involvement of a mixed-func-

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**Figure 4.** Oxidation of kaurenoic acid in microsomal cell-free extracts from light- and dark-grown _G. fujikuroi_ cultures. Reaction mixtures contained P_{150} enzyme extract, 1.6 nmol of 17-[14C]kaurenoic acid, 0.5 mM flavin adenine dinucleotide, 50 mM NADPH, and buffer (50 mM Tricine, pH 8.0, 10 mM β-mercaptoethanol) in a total volume of 1 mL.
tion oxidase. In order to determine exactly which step(s) is photostimulated, further cell-free experiments using ent-7-α-hydroxykaurenoic acid and GA12-aldehyde as substrates need to be undertaken. The results presented do not exclude the possibility of light-stimulation of the biosynthetic steps between kaurene and kaurenolic acid.

Blue light has been found to stimulate isoprenoid biosynthesis in several of the Ascomycetes. Carotenogenesis in *Fusarium aquaeductuum* is markedly increased by exposure to blue light (26). In *Botrytis cinerea*, abscisic acid synthesis is reportedly increased more than 300-fold by blue-light irradiation (19). The action spectrum of light-stimulated kaurene biosynthesis in extracts of pea seedlings also has a peak in the blue region of the spectrum (7). We have preliminary results that suggest that light-stimulated GA biosynthesis in *G. fujikuroi* also involves blue-light reception (15). Efforts are currently underway to confirm and extend this observation.

**LITERATURE CITED**