

Gibberellin Biosynthesis: Genetic Studies in *Gibberella fujikuroi*

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Abstract

Biochemical genetic studies on the production of gibberellins by the fungus *Gibberella fujikuroi* have identified two genes which control different steps in the biosynthetic pathway. One gene (g_1) controls the production of all of the gibberellins; the second gene (g_2) controls the production of GA₁ and GA₃ only.

Ascospores are not ordered in the ascus of this fungus. This apparent spore slip-page precludes mapping of these genes to their respective centromeres.

Introduction

The fungus *Gibberella fujikuroi* (Saw.) Wr., the first organism shown to produce gibberellins, is unique in that it accumulates relatively large amounts as well as large numbers of chemically distinct gibberellins. As a result of these characteristics, *G. fujikuroi* has been used for chemical and physiological studies on gibberellin biosynthesis. However, there is very little information of the genetic control of gibberellin production in this fungus.

Using isotopic feeding studies, mevalonate, geranylgeranyl pyrophosphate and kaurene have been shown to be metabolic precursors to the gibberellins (Birch *et al.* 1958, Birch *et al.* 1959, Cross *et al.* 1964 a, Graebe *et al.* 1965). Also, it has been shown that GA₉ is a precursor of GA₁₀ (Cross *et al.* 1964 b), GA₁₂ is a precursor to both GA₃ and GA₁₃ (Cross and Norton 1965) and GA₄ and GA₇ are precursors to GA₁ and GA₃ (Geissman *et al.* 1966).

We will demonstrate in this report that physiological-genetic studies on gibberellin production can be made with *Gibberella fujikuroi*. We will also present evidence for the presence of two genes that control different steps in the gibberellin biosynthetic pathway.

Materials and Methods

The two strains of *Gibberella fujikuroi* used in this study, 2794 a and 2900 A, originated from collections made in the field (Spector and Phinney 1966, Phinney and Spector 1967). The cultures used came from single spore isolates of the two original strains.

Media and culture methods. — Potato dextrose agar was used as a medium for the maintenance of all stock cultures. This medium was prepared by autoclaving 200 g of diced russett potatoes in 1 liter of water. This solution was filtered through cheese cloth, and 20 g of dextrose and 15 g of agar were added to the filtrate. This medium was dispensed into test tubes; the test tubes were capped with cotton plugs and autoclaved. Cultures on potato dextrose agar were grown at room temperature.

Potato dextrose liquid was used as a growth medium for the production of gibberellins by the fungus. This medium was prepared in the same manner as potato dextrose agar, except that agar was omitted. Fifty ml aliquots of potato dextrose liquid medium were dispensed into 125 ml Erlenmeyer flasks. These flasks were capped with stainless steel closures and autoclaved. Each potato dextrose liquid culture was grown for 7 days in the light on a rotary shaker (2.5 cm displacement at 180 rpm) at 23°C.

Citrus stem was used as a medium for the production of the perfect stage of the fungus (Phinney and Spector 1967). This medium was prepared by autoclaving two 5 cm pieces of air-dried shoots of *Citrus medica* L. in a 125 ml Erlenmeyer flask containing 10 ml of distilled water. Each flask was stoppered with a cork covered with aluminum foil and then autoclaved. Cultures were maintained at 17–21°C with intermittent illumination from fluorescent lights.

Ascus dissection. — Perithecia were removed from the Citrus stem medium and ruptured in a drop of water. The asci were then transferred with a micropipette to a 2 % agar block, and the ascospores from each ascus were separated using a De Fonbrune micromanipulator. Approximately 8 hours later, the germinated ascospores were transferred individually to potato dextrose agar slants.

Gibberellin extraction. — Each potato dextrose liquid culture was centrifuged to separate the mycelia from the culture medium. The supernatant medium was adjusted to pH 2.5 and partitioned 3 times with $1/2$ volumes of ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness at room temperature. The resultant extract residue was subsequently redissolved and assayed for the amounts and kinds of gibberellins produced.

Separation of the gibberellins. — The individual gibberellins present in the extract were separated from each other by a variety of thin layer chromatographic systems (MacMillan and Suter 1963). These systems were used to identify gibberellins GA₁, GA₃, GA₄, GA₇ and GA₉. Other gibberellins were not identified in this study.

Three absorbent-solvent combinations were used, CSI, CSII and CSIII. CSI consisted of silica-gel plates developed in di-isopropyl ether/glacial acetic acid (95:5). This system separated the 5 gibberellins into the 3 groups, GA₁-GA₃, GA₄-GA₇ and GA₉. CSII consisted of silica-gel plates developed in the upper phase of benzene/glacial acetic acid/water (8:3:5). This system was used primarily to separate GA₄ from GA₇. CSIII consisted of Kieselguhr plates developed in the upper phase of benzene/glacial acetic acid/water (8:3:5). This system was used primarily to separate GA₁ from GA₃. In the second and third chromatographic systems the plates

were first equilibrated for 15 hours over the aqueous (lower) phase of the solvent and then developed in the benzene phase.

Gibberellin assays. — Two types of assay were used to locate the gibberellins on the chromatograms. The first employed the ultra-violet fluorescence of certain gibberellin decomposition products; the second used the growth stimulatory effects of gibberellins on *d-1* and *d-5* mutants of *Zea mays* L. and on *Cucumis sativus* L. (cucumber) seedlings.

In the fluorescence assay each plate was sprayed with sulfuric acid/water (7:3) and viewed under ultra-violet light. Under these conditions, there was a green fluorescence in positions corresponding to GA₃ and GA₇. The chromatogram was then heated in an oven at 120°C for 10 minutes and again viewed under ultra-violet light. After heating there was green fluorescence in the positions corresponding to GA₁, GA₃, and GA₇, a blue spot corresponding in position to GA₄ and a powder blue spot corresponding in position to GA₉. The location of each gibberellin was determined by its characteristic UV fluorescence and by its chromatographic identity to authentic samples.

To locate the gibberellins on chromatograms by bioassay, the extract residue from one fungal culture was spotted and developed in CSI. A control solution of GA₃, GA₄ and GA₉ was added as a single spot at the origin to the right of the extract residue. Following development, the adsorbent (excluding the region containing the controls) was scraped from the plate as 1 cm wide zones parallel to the front. The location of the control gibberellins was then determined by fluorescence directly from the plates. The adsorbent from each of the 15 zones was transferred individually to 15 ml centrifuge tubes, suspended in ethyl alcohol and centrifuged. This elution was carried out three times with 3 ml aliquots of ethyl alcohol. The eluates from one zone were combined in a 10 ml beaker and allowed to air dry. Thus, 15 samples were obtained from one chromatogram. Each sample was assayed on *d-1*, *d-5* and cucumber seedlings.

The three bioassays were used because of the differential biological activity of some of the gibberellins in these assay systems (Brian *et al.* 1962). GA₁ and GA₃ are highly active on *d-1* and *d-5* but show only small activity on the cucumber seedling assay. GA₄ and GA₇ are active on all three bioassay systems. GA₉ is active on the cucumber seedling assay and the *d-5* assay but shows only slight activity on the *d-1* assay.

The *d-1* and *d-5* *Zea mays* assays were patterned after the methods of Phinney and West (1961) and Katsumi (1964). The extract to be assayed was dissolved in 50 % acetone, and 0.1 ml aliquots of this solution were added to the first unfolding leaf of 5 eight-day-old seedlings. Ten days after treatment the lengths of the first and second leaf sheaths were measured and the sum of these lengths determined for each seedling. The mean and standard error of these sums were used as a measure of the response in the assay.

The cucumber assay was patterned after the methods of Katsumi (1964). Seedlings, approximately 6 days old, were marked with india ink 2 cm below the cotyledonary node. The apical bud was then removed from each seedling with fine pointed forceps and 5 seedlings were treated with 0.01 ml aliquots of the ethanolic solution to be assayed. Three days after treatment the distance between the cotyledonary node and the ink spot was measured and the mean and standard error determined for each treatment. The difference between the initial length (2 cm) and the final mean length of the hypocotyls was used as a measure of the response in the assay.

Results

The sexual stage

One of the early problems in this study was to find a medium which would support the development of the sexual stage of the fungus. A number of natural and synthetic media were tested and found to be ineffective in stimulating perithecial production. However, when fungal strains of opposite mating type were grown on stems of *Citrus medica* L. (Figure 1 A), perithecia were regularly produced in from 3 to 6 weeks (Spector 1964, Spector and Phinney 1966, Phinney and Spector 1967).

The perithecia were found to be an intense blue-purple when seen through transmitted light. They were pyriform with an inconspicuous ostiol at the upper (narrow) end (Figure 1 B). At maturity the perithecia ranged in size from 0.2 to 0.4 mm in width and from 0.3 to 0.5 mm in length.

The rupture of a perithecium in water released one to several fan shaped

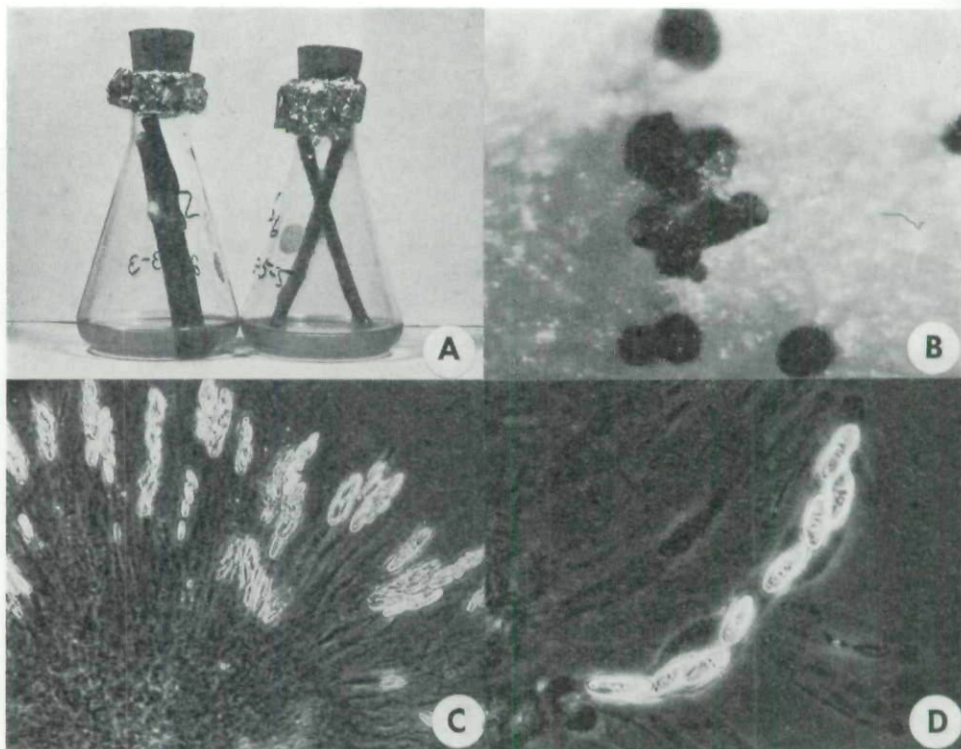


Figure 1 A. Culture flasks containing *Gibberella fujikuroi* growing on *Citrus* stems.
Figure 1 B. Perithecia of *Gibberella fujikuroi* produced on stems of *Citrus* medium. Ascospores are exuding through the ostiol of mature perithecia.
Figure 1 C. Asci with overlapping ascospores.
Figure 1 D. An ascus with eight ascospores.

masses of ascogenous hyphae (Figure 1 C). While the number of ascospores present in an ascus varied, a maximum number of 8 spores was frequently observed (Figure 1 D). Occasionally the ascospores were arranged linearly within the ascus; more commonly the ascospores overlapped with one another to a varying degree. A representative length for a mature ascus was 90 microns. The ascospores had a characteristic shape when mature; their ends were round with a prominent constriction at their septa. Most ascospores were two-celled; occasionally 3 or 4 celled ascospores were observed. When mature, ascospores germinated regularly within 8 hours at room temperature. Ascospores ranged in size from 17 to 19 microns in length and 5 to 7 microns in width.

Ordered spores

A test was made to determine whether spores derived from the same meiotic product (*i.e.*, the mitotic pair) occupied adjoining positions within the ascus.

Sixty-one asci with linearly arranged spores were selected. The ascospores were removed in order and transferred to potato dextrose agar slants. The two spores originating from each meiotic product were identified by the color similarities of the cultures derived from the spores (Figure 2). Sixteen of the 61 asci were found to have eight ascospores arranged in four pairs. Each of the remaining 45 asci had at least one mitotic pair of spores that

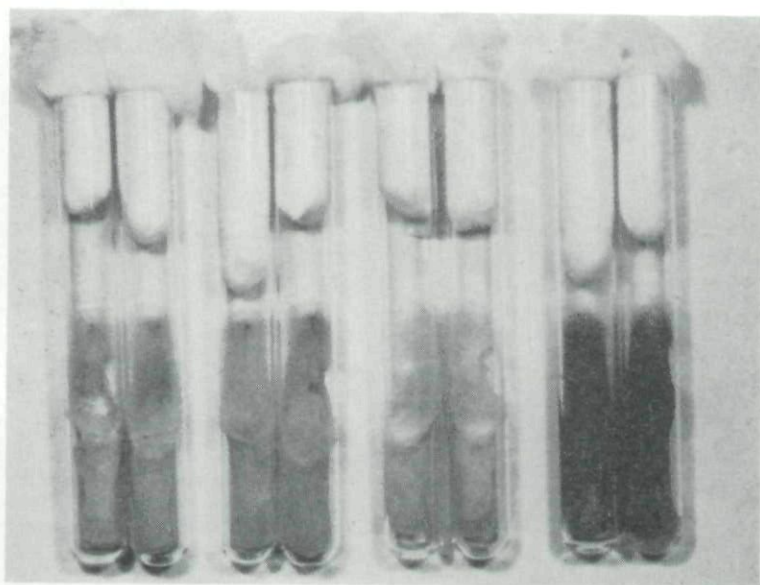


Figure 2. The eight cultures derived from the ascospores of one ascus. These cultures are grouped into four pairs based on visible phenotypic differences. The four groups represent cultures derived from the four meiotic products.

did not occupy adjacent positions within the ascus. These results demonstrate the lack of a consistent spore order within the asci.

Genetic control of gibberellin production

Preliminary experiment. — As indicated in a preliminary report (Spector and Phinney 1966), a mating between a high and low gibberellin producing strain gave tetrads that segregated 2:2 in terms of the total amount of gibberellin produced by the progeny. One parent, 2794 a, and half of the progeny, produced relatively high amounts of GA₁, GA₃, GA₄, GA₇ and GA₉ in addition to a number of other unidentified gibberellin-like materials. The other parent, 2900 A, accumulated trace amounts of GA₄ and GA₇ only; the remaining half of the progeny accumulated either trace amounts of GA₄ and GA₇ or no detectable gibberellins.

One tetrad from this mating was of special interest, since one of the four strains (strain 3, Table 1) produced high amounts of GA₄, GA₇ and GA₉ but no GA₁ and GA₃.

The *d*-5 biological activity from the chromatogrammed (CSI) extracts of 2794 a, and strain 3, are shown in Figure 3. For 2794 a, zones of high activity were observed in positions on the chromatogram corresponding to GA₁-GA₃ (Zone 2), GA₄-GA₇ (Zones 6 and 7) and GA₉ (Zone 13). For strain 3 zones of high activity were observed in positions on the chromatogram corresponding to GA₄-GA₇ and GA₉ but only a small amount of activity was present in

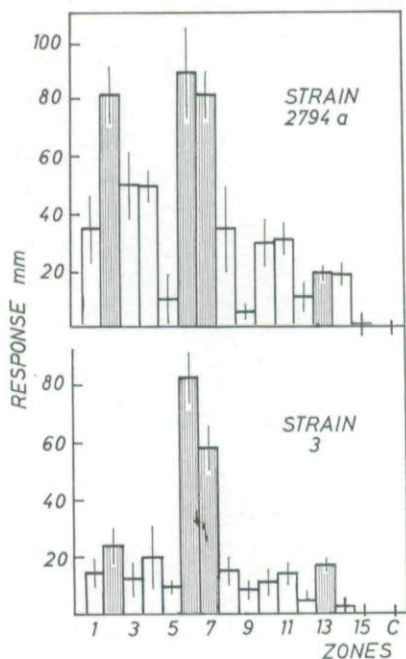


Figure 3. Bioassay response data from the chromatogrammed extract from strain 2794 a and strain 3. Equal quantities of the eluate from each of the 15 zones of a chromatogram were assayed on *d*-5 *Zea mays* seedlings. The zones of the chromatogram that correspond in position to GA₁-GA₃ (Zone 2), GA₄-GA₇ (Zone 6, 7), and GA₉ (Zone 13) are indicated by shading.

Table 1. The gibberellin phenotypes, mating types and assumed genotypes of the members from the "unusual" tetrad.

Spore	Gibberellins GA ₁ -GA ₃	Produced GA ₄ -GA ₇ -GA ₉	Mating type	Assumed genotype
1	—	—	A	g ₁ ⁻ g ₂ ⁻
2	—	—	A	g ₁ ⁻ g ₂ ⁺
3	—	+	a	g ₁ ⁺ g ₂ ⁻
4	+	+	a	g ₁ ⁺ g ₂ ⁺

the GA₁-GA₃ zone. For both strains, other zones of activity were also present. Similar results were obtained in the *d-1* and cucumber bioassay systems.

An extract from strain 3 was developed in CSI and the GA₁-GA₃ region (Zone 2) eluted and developed in CSIII. No fluorescence appeared in the regions corresponding to GA₁ or GA₃. However, at least three fluorescent spots appeared in other regions of this chromatogram. It is therefore suggested that the small amount of activity in the GA₁-GA₃ region (zone 2) from strain 3 is due to other gibberellin-like substances and not to GA₁ or GA₃.

The appearance of the 3 kinds of strains in the preliminary study could mean that 2 different genes are controlling gibberellin production. One gene (g₁) would affect a step early in the gibberellin biosynthetic pathway and control the overall production of gibberellin. The second gene (g₂) would affect a step later in the pathway and control only the production of GA₁ and GA₃. On this basis, presumptive genotypes have been assigned to the members of the "unusual" tetrad (Table 1). To determine the validity of these genotypes, three crosses were made among the members of this tetrad.

Experiment 1. — A cross was made between strains two and four (g₁⁻ g₂⁺ × g₁⁺ g₂⁺) and sixteen asci were dissected. From each ascus there was a 2:2 segregation for gibberellin production. The two gibberellin producing strains accumulated all five gibberellins; the two remaining strains produced no detectable gibberellin. Thus, for each of the 16 asci, half of the members of a tetrad were like the non-producing parent and the remaining half of the members were like the producing parent.

Experiment 2. — A cross was made between strains one and three (g₁⁻ g₂⁻ × g₁⁺ g₂⁻) and 16 asci were dissected. Each tetrad again segregated 2:2 for gibberellin production. The two gibberellin producing strains had GA₄, GA₇ and GA₉, but no GA₁ or GA₃; the remaining two strains produced no detectable gibberellin. Thus, again, half of the members of the tetrad were like the non-producing parent and the remaining half of the members were like the producing parent.

Experiment 3. — A cross was made between strains one and four (g₁⁻ g₂⁻ × g₁⁺ g₂⁺) and 91 asci were dissected. Each tetrad segregated 2:2 for gibberellin production. There were, however, three types of tetrads, based on the presence or absence of GA₁ and GA₃ among the gibberellin producing members within each tetrad. The assumed genotypes of these three kinds of tetrads are shown in Table 2.

For the first type of tetrad (parental ditype), two members produced no gibberellins and the remaining two members produced all five gibberellins.

Table 2. *The gibberellin phenotypes and assumed genotypes of the members of the tetrads from Experiment 3.*

Spore	Gibberellins produced		Assumed genotypes
	GA ₁ -GA ₃	GA ₄ -GA ₇ -GA ₉	
A. Type I 12 tetrads			
1	-	-	g ₁ ⁻ g ₂ ⁻
2	-	-	g ₁ ⁻ g ₂ ⁻
3	+	+	g ₁ ⁺ g ₂ ⁺
4	+	+	g ₁ ⁺ g ₂ ⁺
B. Type II 53 tetrads			
1	-	-	g ₁ ⁻ g ₂ ⁻
2	-	-	g ₁ ⁻ g ₂ ⁺
3	-	+	g ₁ ⁺ g ₂ ⁻
4	+	+	g ₁ ⁺ g ₂ ⁺
C. Type III 26 tetrads			
1	-	-	g ₁ ⁻ g ₂ ⁺
2	-	-	g ₁ ⁻ g ₂ ⁺
3	-	+	g ₁ ⁺ g ₂ ⁻
4	-	+	g ₁ ⁺ g ₂ ⁻

For the second type of tetrad (tetratype) two of the members (strain one and two) produced no gibberellins, one of the members (strain 4) produced all of the five gibberellins, and one of the members (strain 3) produced GA₄, GA₇ and GA₉, but no GA₁ or GA₃.

Thus strain 3 was a recombinant type; the expression of the second recombinant type could not be detected because it would have a genotype g₁⁻ g₂⁺ and the g₁⁻ allele would mask the expression of the g₂⁺ allele. This second recombinant type would lack all gibberellins and therefore be either strain 1 or strain 2.

For the third type of tetrad (non-parental ditype), two of the members (strains 3 and 4) produced GA₄, GA₇ and GA₉ but no GA₁ or GA₃. They were thus recombinants. The expression of the remaining two members (strains 1 and 2) were again not detected because of the presence of the g₁⁻ alleles.

The occurrence of these three types of tetrads demonstrates assortment between the two pairs of alleles responsible for gibberellin production.

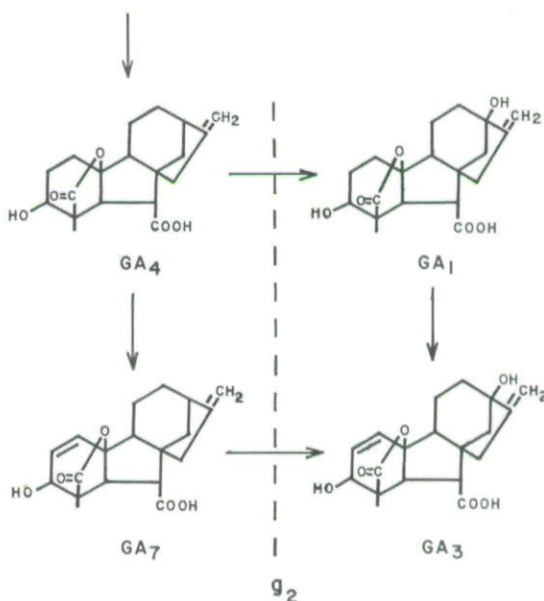
The results from experiments 1, 2 and 3 are all consistent with the assigned genotypes originally given to the strains of the "unusual" tetrad (Table 1).

Discussion

The fungus *Gibberella fujikuroi* is well suited as a research organism, first, because it produces a variety of different gibberellins in a relatively large amount, and second, because matings can be made readily between different strains in a relatively short time.

As indicated in a preliminary report (Spector and Phinney 1966), ascospores from a single ascus have represented among them all four meiotic products enabling tetrad analysis to be made. However, the apparent lack

Figure 4. Metabolic interrelationship of GA₁, GA₃, GA₄ and GA₇ based on the labelling studies of Geissman *et al.* The position of the genetic block is indicated by the dotted line.



of spore order within the ascus suggests that spore slippage is common; therefore, at present, the mapping of genes to their respective centromeres is precluded.

The results presented in this report are consistent with the hypothesis that one pair of alleles, gene *g*₁, is responsible for the control of overall gibberellin production. The segregation pattern from 127 asci are all consistent with this interpretation. The results are also consistent with the hypothesis that a second pair of alleles, gene *g*₂, controls the production of GA₁ and GA₃. These two pair of alleles are non-allelic and probably not linked.

Gene *g*₂ apparently controls a biosynthetic step prior to the synthesis of GA₁ and GA₃. These two gibberellins are therefore related in their biosynthetic pathways. The fact that the presence of other gibberellins was not influenced by gene *g*₂ may indicate that these other gibberellins are either produced prior to GA₁ and GA₃ or are on alternate pathways.

It has been suggested by Geissman *et al.* (1966) that GA₄ and GA₇ are precursors to GA₁ and GA₃. GA₁ differs from GA₄ and GA₃ differs from GA₇, only in the presence of a hydroxyl group at the seven position of the gibbane ring system (Figure 4). Thus it is possible that GA₄ and GA₇ are converted to GA₁ and GA₃ by a single step. We suggest that the *g*₂ locus controls this hydroxylation step as shown in Figure 4.

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