Gibberellin-like substances in immature berries of seeded and gibberellin-induced seedless Delaware grapes

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Summary

The activity of fractionated gibberellin (GA)-like substances extracted from the seeds and pericarps of seeded and GA-induced seedless berries of Delaware grapes collected at 21 days after full bloom was determined, and the main active components in pericarp tissue were identified by means of selected bioassays and thin-layer chromatography.

In the seeds the highest activity was detected in the basic ethyl acetate fraction with the barley endosperm assay, whereas in the pericarps of seeded and seedless berries the basic and acidic ethyl acetate fractions, especially the acidic ethyl acetate fraction, were predominant in activity among the four fractions of basic ethyl acetate, neutral chloroform, acidic ethyl acetate, and acidic butanol. Moreover, the active components in basic ethyl acetate fractions from seed and pericarp were completely different in Rf on the paper chromatograms.

The higher level of total GA-like substances was found in pericarp of seedless than seeded berries.

The GA-like substances in acidic ethyl acetate fractions from pericarp of seeded and seedless berries exhibited strong activity in dwarf pea, dwarf rice, and barley endosperm assays, but not in a cucumber assay, and they were very similar to GA$_3$ in Rf on the thin-layer chromatograms. These substances therefore seem to be identical with GA$_3$.

Introduction

The discovery that the size of seedless grapes increases markedly with the application of GA$_3$ at flowering or fruit set stage$^{26}$ has provided new approaches to explain the role of endogenous growth regulators in berry growth. The presence of GA-like substances has been demonstrated in grapes$^{4,12,13,27}$ as well as in several other fruits$^{8,10,14,16,17,23,25}$. There is more gibberellin activity in seedless than in seedless Tokey grapes, a mutant of the seeded form$^{27}$ and a peak level of gibberellin activity is detected at the time of fruit set, followed by a more rapid decline in seedless than in seeded berries$^{13}$. These findings suggest that grape seeds are a rich source of GA-like substances, though the direct proof is not yet available.

The chemical form of naturally occurring gibberellins in the tissues of grape berries is still obscure. It is of interest to determine whether the endogenous active component is identical or not with GA$_3$ which is the active exogenous gibberellin in promoting the growth of seedless grape berries. Although Delaware is a seeded variety, dipping its clusters at pre-bloom and again at post bloom in a solution of GA$_3$ has been found to be able to produce seedless berries which are approximately the normal size of the seeded ones$^{3,11,19}$.

The aim of this study is to determine the activity of GA-like substances in the seeds and pericarps of seeded and GA-induced seedless berries of Delaware grapes at the early stage of berry growth, and also to elucidate what sorts of gibberellin are predominant in these tissues.

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Material and Methods

Plant material was obtained from 6-year-old vines of *Vitis labruscana* c.v. Delaware growing in the vineyard of Shimane University, Matsue. In order to induce parthenocarpic berry growth, clusters of selected vines were dipped in GA$_3$ solution at a concentration of 100 ppm 14 days before full bloom and again 10 days after full bloom. This procedure was established by KISHI *et al.* in 1960 and has been adopted widely in commercial vineyards in Japan.

For the extraction of GA-like substances, non-treated (seeded) and GA-treated (seedless) clusters were collected 21 days after full bloom. The pre-bloom application of GA$_3$ accelerated the flowering by 4 or 5 days so that the GA-treated clusters were collected on June 22, 5 days earlier than non-treated ones. Immediately after being sampled, berries were separated from pedicels, washed in 1% hydrochloride solution, soaked in running tap water, and then stored at -20°C. Later the berries for assays were freeze-dried in a lyophilizer. The pericarp tissue of the seeded berries was separated from the seeds after drying, and both portions of tissue were finely ground with a mill. The seedless berries contained aborted seeds which were too small to be separated, so that the whole tissue was regarded as pericarp for convenience and directly ground to a powder.

Generally, 3g dry weight of seed and 50g dry weight of pericarp were used for extraction because the approximate seed-pericarp dry weight ratio at this stage was 3:50. The ground sample was shaken with 150ml (seed) or 800ml (pericarp) of 70% methanol for 1 hr and kept for 24 hrs at 5°C. The liquid was filtered and the residue was extracted two times with the same volumes of 85% and 100% methanol in the order. The combined extracts were evaporated to water phase under reduced pressure at less than 40°C. The pH of the water phase was adjusted to 6.2, and the water phase centrifuged for 10 min. The supernatant was re-adjusted to a pH of 2.5, stirred for 2 hrs after each 5g of activated carbon charcoal (Darco-60) and celite (Hyblo Super-cel) was added. Then it was filtered. The charcoal and celite which remained on the filter papers was washed with distilled water and eluted with acetone. The eluate was evaporated to dryness, and the residue was suspended in phosphate buffer (0.1 M, pH 7.5) and extracted four times with ethyl acetate. The residual water phase was then adjusted to a pH of 6.2 and extracted with chloroform. The water phase was then adjusted to a pH of 2.5 and extracted with ethyl acetate and also n-butanol in the order. These four extracts designated hereafter as the basic ethyl acetate, neutral chloroform, acidic ethyl acetate and acidic butanol fractions were taken up in 2ml of ethanol, respectively. The activity of the extracts was examined with barley endosperm assays.

For the separation of active components of the extracts, chromatography was performed using Toyo No. 51 filter paper strips (2×40 cm) and a mixture of isopropanol, ammonium hydroxide and water (10:1:1, v/v) at 25°C. Fifty µl of a extract were spotted on a paper strip. The developed strips were cut into ten sections according to Rf, and the activity of each section was assessed with a barley endosperm assay.

Further purification of the basic and acidic ethyl acetate fractions was achieved by streaking 0.5 ml of each of the extracts on full sheets (40×40 cm) of Toyo No. 51 paper and developing with the same solvents mixture as mentioned above. The active regions on the paper, which were detected with barley endosperm assays using one-twentieth of the developed
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paper, were eluted with 80% ethanol. The eluates were evaporated to dryness, and the residues were each taken up in 2 ml of ethanol. These ethanol solutions of purified active components were used for selected bioassays with dwarf pea, dwarf rice, cucumber and barley endosperm.

Thin-layer chromatography of the limited eluates was done in order to compare the Rf of unknown active components with that of known gibberellins. Each 50 µl of the purified active components in ethanol and each 5 µg of known gibberellins (GA1, GA3 and GA4+7) in ethanol were spotted on silica-gel G plates (E. Merk F 254, 250 µ). The plates were developed with a mixture of ethyl acetate, chloroform and acetic acid (15 : 5 : 1, v/v) at 25°C. Active components which were separated by the chromatography were viewed under ultraviolet light (360 nm) after the plates were sprayed with 5% sulfuric acid in 95% ethanol and heated for 10 min. at 110°C. The other portions of the active components in the ethanol, GA1 and GA3, were spotted one over the other or separately on the plates. Subsequent chromatographic steps were identical with that mentioned before. Scrapings from 20 sections of the developed plates according to Rf were eluted with ethanol. The activity of each ethanol solution was tested with a barley endosperm assay.

The primary bioassay used in this study was the barley endosperm test described by COOMBE et al. Seeds of naked barley c.v. Akashinriki were used. For the rice seedling assay developed by OGAWA, dwarf rice c.v. Kodake-tamanishiki was used. Seven 2-day-old seedlings were transferred to a test tube (3 x 12 cm) which contained 3 ml of test solution, the pH of which was adjusted to 4.2 with phosphate buffer of 0.001 M. The length of second leaf sheaths of the seedlings was measured after growth for 7 days at 30°C under incandescent light (15,000 lux). For the cucumber bioassay developed by BUKOVAC and WITTWER, seeds of c.v. Suyo were germinated on moist filter paper at 30°C in the dark. After 2 days, seeds were transferred to sterilized sand in flats and grown at the alternating temperature of 25°C during the day and 20°C at night. Just after the cotyledons opened, extracts or known gibberellins dissolved in 50% ethanol containing 0.05% Tween 80 (usually 20 µl per plant) were applied to the growing points. Seven days after treatment, the length of the hypocotyls was measured. In the dwarf pea bioassay described by HAYASHI et al., seeds of c.v. Morse's Progress No. 9 were planted in vermiculite in the dark at 25°C. After 4 days, selected seedlings about 2.5 cm in length were transplanted to vermiculite in flats and grown at 25°C under incandescent light (15,000 lux). One day later, the plants were treated with the solutions containing the extracts or known gibberellins (usually 20 µl per plant). The distance between the cotyledonary node and the highest visible node was measured 7 days after treatment. In the procedure described by HAYASHI et al., red light was originally used as the radiation source. In our preliminary test, however, it was found that pea seedlings grown under incandescent light (15,000 lux) were able to respond to 0.001 µg or less of GA3, though they were more sensitive under red light. Since incubation under incandescent light was easier and more convenient than that under red light, the former was adopted in this study.

Results

The activity of GA-like substances in the basic ethyl acetate, neutral chloroform, acidic ethyl acetate and acidic butanol fractions of seeded and seedless berries of Delaware grapes was
detected with barley endosperm assay. As shown in Fig. 1, each fraction except the neutral chloroform fraction contained active substances. In the seeds the highest activity was found in the basic ethyl acetate fraction, whereas the acidic and basic ethyl acetate fractions, especially the former, were predominant among the four fractions of pericarp of both seeded and seedless berries. The estimated concentration of total active substances in seed, converted to a GA$_3$ equivalent, was rather higher than that in pericarp.

Chromatographic separation of the active substances in each fraction, except the neutral fraction, with a mixture of isopropanol, ammonium hydroxide and water (10 : 1 : 1, v/v) revealed a more interesting pattern. The bioassay results from chromatograms of these fractions are shown in Fig. 2. Although a distinguishably active area was not noted on the chromatograms of acidic butanol fractions probably because of low concentration of active components, good separation were obtained in the chromatography of the basic and acidic ethyl acetate fractions. The main active component in the basic ethyl acetate fraction of seeds did not migrate from the starting line as did that of the pericarp tissue, which migrated to the range between Rf 0.3 and 0.7. As far as the acidic ethyl acetate fractions were concerned, a very similar pattern consisting of an active zone around the starting line and another at the range between Rf 0.3 and 0.6 was found in both seed and pericarp. Greater quantitative responses were obtained from the pericarp of the seedless berries than from that of seeded berries in both the basic and acidic ethyl acetate fractions.

Selected bioassays of the active components in the basic and acidic ethyl acetate fractions were done...
using dwarf pea, dwarf rice and cucumber in addition to barley endosperm assays (Table 1). The eluates from the area around the starting line on the chromatograms of all the fractions tested and also that from the area of Rf 0.3–0.6 of the basic ethyl acetate fractions more or less injured these test plants, so that accurate activity of these eluates could not be measured in the bioassays. However, the components from Rf 0.3–0.6 of the acidic ethyl acetate fractions extracted from pericarp of seeded and seedless berries, especially from that of seedless berries, exhibited strong activity without any phytotoxicity in the dwarf pea and rice seedling assays, as well as in the barley endosperm assay, but not in the cucumber assay. GA1 and GA3, particularly GA3, showed biological responses similar to the responses of these components.

Further investigation was limited to the active components from Rf 0.3–0.6 of the acidic ethyl acetate fractions extracted from the pericarps of seeded and seedless berries. Thin-layer chromatography of the active component of the seedless berries using silica-gel G plate and a mixture of ethyl acetate, chloroform and acetic acid (15:5:1, v/v) showed a distinct spot under ultraviolet light at the area corresponding closely with the Rf of GA3 and being separated

<table>
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<tr>
<th>Seed</th>
<th>Fraction</th>
<th>Rf(b)</th>
<th>Dwarf pea</th>
<th>Dwarf rice</th>
<th>Cucumber</th>
<th>Barley endosperm</th>
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<tr>
<td>Seed</td>
<td>Basic ethyl acetate</td>
<td>0.3–0.6</td>
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<td>85</td>
<td>109</td>
<td>729</td>
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<tr>
<td>Basic ethyl acetate</td>
<td>0.0–0.1</td>
<td>113</td>
<td>93</td>
<td>111</td>
<td>1052</td>
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<td>87</td>
<td>132</td>
<td>45</td>
<td>505</td>
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<td></td>
<td>228</td>
<td>114</td>
<td>111</td>
<td></td>
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<tr>
<td>Seedless berries</td>
<td>Pericarp</td>
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<td>Basic ethyl acetate</td>
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<td>114</td>
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<td>GA1</td>
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<td>105</td>
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<td></td>
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<td>272</td>
<td>108</td>
<td>200</td>
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</table>

a) A mixture of isopropanol, ammonium hydroxide and water (10:1:1, v/v) was used as the developing solvent. Each paper section of indicated Rf was eluted with ethanol, evaporated to dryness, and made up to a volume with ethanol. Fifty μl of each eluate (equivalent to 0.075g of seed or 1.250g of pericarp on dry weight) was applied per vial in the dwarf rice and barley endosperm assays. For the dwarf pea and cucumber assays, one half of each eluate was evaporated to dryness, restored to the former volume with 50% ethanol containing 0.05% of Tween 80 and 20μl of each solution (equivalent to 0.03g of seed or 0.5g of pericarp on dry weight) was applied per plant.

b) Test plants were injured by toxicity.
from GA1 (Fig. 3). In addition, this was the only area where biological activity was detected in a barley endosperm assay even though many other spots appeared on the plate. As to the seeded berries, the area which corresponded with the Rf of GA3 was void of a distinct spot presumably because of lower concentration of the component in the extract. It was difficult to increase the amount to be spotted on a plate, since clear separation could not be obtained with a volume greater than 50 μl. Therefore, the Rf of the component was ascertained in a biological way; the activity of eluates from 20 sections of the developed plates on which the sample, GA1 and GA3 had been spotted one over the other or individually was assessed with a barley endosperm assay. As shown in Fig. 4, the active component migrated to a Rf zone of 4.5 to 6.0 and which was found to be more identical to the Rf zone of GA3 than that of GA1 just like the active component of seedless berries.

Discussion

GA-like activity at the early stage of fruit development was reported to be higher in the seeds than in the pericarps of apricots and peaches15,16~. It was also true in our experiment with seeded Delaware grapes that the level of total GA-like substances, converted to a GA3 equivalent, seemed to be higher in seeds than pericarp, although accurate comparison was difficult because of considerable difference in the doses of extracts used in the bioassays.

There have been reports concerning the GA-like substances occurring in developing grape berries. WEAVER and POOL27~ demonstrated high GA-like activity in the acidic ethyl acetate fractions of seeded and seedless Tokey berries and in the neutral ethyl acetate and acidic butanol fractions of Black Corinth and Thompson Seedless berries. Later IWAHORI et al13~ also noted high activity in the acidic ethyl acetate fractions of both seeded and seedless Tokey berries.
In our experiment, several components were found to contribute to GA-like activity in the seeds and pericarp of Delaware grapes. The most active component was noted in the basic ethyl acetate fraction of seeds and in the acidic ethyl acetate fraction of pericarp. Furthermore, these components were found to be completely different from each other on the basis of relative mobility values in the chromatography. Denne and Nitsch identified GA_4 and GA_7 as predominant components in immature seeds of Golden Delicious apples. On the other hand, Hayashi et al. demonstrated the presence of abundant GA_3 in immature seedless apple fruits, selection New Jersey 12. It is generally accepted that seeds are a rich source of gibberellins, but whether the endogenous gibberellins in pericarp are synthesized in its tissue or transported from seeds or other plant tissues is not yet known. Our data suggest that if pericarp gibberellins are transported from seeds, the conversion of seed gibberellins must occur on the path way. We failed, however, to show the activity of the component in the basic ethyl acetate fraction of seeds in bioassays other than a barley endosperm assay because of phytotoxicity. Therefore, further studies are necessary to elucidate this point which is thought to be an important key in understanding hormonal metabolism in fruit tissue.

It seems significant to note that a higher level of GA-like substances was found in pericarp tissue of GA-induced seedless berries than in seeded berries at the early stage of berry growth. Ito et al. reported that the post bloom application of GA_3 increased the auxin and gibberellin activity to a peak in GA-induced seedless berries of Delaware grapes, but it was markedly lower than that in the seeded berries. They did not separate seeded berries into seed and pericarp tissues for the measurement of the activity as we did in our study. Therefore direct comparison of the results of both experiments seems to be inadequate.

Iwahori et al. found that the GA-like substances in the acidic ethyl acetate fraction of seeded and seedless Frame Tokey berries occurred mainly between Rf 0.3 and 0.6 when the chromatogram was developed with ammonical isopropanol. They also found that these substances were active in dwarf pea, dwarf corn and lettuce hypocotyl assays, but not in the cucumber test. The GA-like substances obtained from acidic ethyl acetate fractions of pericarp of seeded and GA-induced seedless berries of Delaware grapes seem to be very close in chromatographic behavior and biological activity to that demonstrated by Iwahori et al. Moreover, based on the results of selected bioassays and from Rf values on silica-gel plates, it can be said that these substances are most likely GA_3.

Specificity of the gibberellins in inducing parthenocarpy has been revealed in several fruits. Among the gibberellins, GA_4 and GA_7 are most active in apples, although GA_7 is inactive in peaches and plums, less active in grapes, and more active than GA_3 in Japanese persimmons. As indicated in the introduction of this paper, GA_3 has been recognized as the effective agent in inducing parthenocarpy in Delaware and some varieties of grapes and also in stimulating the enlargement of seedless berries. Therefore, it is meaningful to have shown that immature berries of grapes contain several kinds of gibberelin-like substances in seeds and pericarps and that the most active component in pericarps is very similar to GA_3. The roles and properties of the other endogenous gibberelin-like substances remain to be examined further.
LITERATURES CITED


プドウ・デラウェアの有核およびジベレリン処理
無核幼果中のジベレリン様物質

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摘要

満開後21日目に採取したプドウ・デラウェアの有核果およびジベレリン（GA）処理無核果について、種子あるいは果肉中のGA様物質を抽出分別し、それらの生物学的活性を比較するとともに、果肉中の主要GA様物質の同定を行なった。

ハダカムギは乳根の結果、種子または塩基性酸エチル・フラクションで、有核および無核果ともに果肉では塩基性および酸性酸エチル・フラクション、とくに後者で強いGA様活性が認められた。なお種子および果肉の塩基性酸エチル・フラクションの活性物質は、ペーパー・クロマトグラフィーにおいてそれぞれまったく異なるRfを示した。

GA₃に換算した果肉中の総GA様物質の活性は、有核果より無核果において高かった。

有核および無核果の果肉中の酸性酸エチル・フラクションの主要GA様物質は、ハダカムギはい乳、わい性イネ、わい性エンドウ検定で強い活性を示し、キウリ検定では殆ど活性を示さず、GA₃およびGA₄、とくにGA₃と同様の性質であることが認められた。また生物検定あるいは蛍光反応で確認したそれらの物質の薄層クロマトグラム上のRfはGA₃ときわめて類似した。したがってプドウ・デラウェアの幼果中の主要内生GA様物質はGA₃と推定される。