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# GIBBERELLIN METABOLISM IN SUSPENSION CULTURED CELLS OF *RAPHANUS SATIVUS*

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**Key Word Index**—*Raphanus sativus*; Cruciferae; Japanese radish; [$^3$H]gibberellins; [$^2$H]gibberellins; suspension cultured cells; metabolism.

**Abstract**—Gibberellin $A_1$ ($GA_1$), $GA_4$, $GA_9$, $GA_{19}$ and $GA_{20}$, which are known to be native to Japanese radish (*Raphanus sativus*), were applied as [$^3$H]GAs and [$^2$H]GAs to cell suspension cultures of *R. sativus*. As the metabolites in [$^2$H]GA-feeds, [$^2$H]$GA_8$ from [$^2$H]$GA_1$, [$^2$H]$GA_1$ and [$^2$H]$GA_2$ from [$^2$H]$GA_4$, [$^2$H]$GA_1$, [$^2$H]$GA_4$ and [$^2$H]$GA_{20}$ from [$^2$H]$GA_9$, [$^2$H]$GA_{20}$ from [$^2$H]$GA_{19}$, and [$^2$H]$GA_1$ and [$^2$H]$GA_{20}$-15-ene from [$^2$H]$GA_{20}$ were identified by GC-SIM. The distribution of [$^3$H]GA metabolites after HPLC corresponded closely with that of the [$^2$H]GA metabolites, except in the case of the [$^2$H]$GA_{20}$-feeds. Based on the metabolic patterns of applied GAs, it is supposed that 13-hydroxylation from $GA_4$ is much more dominant than 3$\beta$-hydroxylation from $GA_{20}$ in pathways leading to $GA_1$ in suspension cultured cells of *R. sativus*.

## INTRODUCTION

Japanese radish (*Raphanus sativus* L.) is a cold-requiring long-day plant, in which both seeds and green plants are vernalizable. We are investigating the relationship between endogenous gibberellins (GAs) and stem elongation and flowering of *R. sativus*. As the endogenous GAs of *R. sativus*, 13-hydroxy-GAs, $GA_1$, 3-epi-$GA_1$, $GA_{17}$, $GA_{19}$, $GA_{20}$, $GA_{20}$-15-ene, $GA_{44}$ and $GA_{77}$, and 13-deoxy-GAs, $GA_4$, 3-epi-$GA_4$, $GA_9$, $GA_{15}$, $GA_{24}$, $GA_{24}$-15-ene, $GA_{25}$, $GA_{25}$-15-ene, $GA_{34}$ and $GA_{51}$, have been identified by GC-MS from vegetative and reproductive tissues [Nishijima, T., personal communication], as well as 13-hydroxy-GAs, $GA_1$, 3-epi-$GA_1$, $GA_8$, $GA_{17}$, $GA_{19}$, $GA_{20}$ and $GA_{77}$, and 13-deoxy-GAs, $GA_9$, $GA_{24}$, 12$\beta$-hydroxy-$GA_{24}$ and $GA_{25}$ in mature seeds [1]. In support of the proposed conclusion that endogenous GAs play a regulatory role in stem elongation in cold-requiring long-day plants [2–4], endogenous levels of $GA_1$ and $GA_4$ were found to increase sharply in bolting stems of *R. sativus* under flowering conditions [5]. However, the biosynthetic pathway and movement of these GAs in intact plants of *R. sativus* are still unknown. Metabolic pathways of GAs in cell suspension cultures have been shown to be very similar to those in intact plants of *Pharbitis nil* [6, 7], *Prunus* spp. [8, 9] and *Oryza sativa* [10]. Hence, preparatory to an examination of the GA metabolic sequence and movement in intact *R. sativus* plants, we have analysed the metabolism of $GA_1$ and $GA_4$, and their precursors, $GA_9$, $GA_{19}$ and $GA_{20}$, in cell suspension cultures of *R. sativus*

## RESULTS AND DISCUSSION

### *Distribution of radioactivity*

After applications of [$^3$H]GAs to the cell suspension cultures for 48 hr, only 1.2% of the applied radioactivity was recovered in the free acidic GA fraction of the cell extracts in the [$^3$H]$GA_1$-feeds, with 2.0% in the [$^3$H]$GA_4$-feeds, 2.4% in the [$^3$H]$GA_9$-feeds and 1.7% in the [$^3$H]$GA_{20}$-feeds. In the highly water soluble GA fraction of the cell extracts in the [$^3$H]$GA_1$-feeds, 1.0% of the applied radioactivity was found as well as 1.6% in the [$^3$H]$GA_4$-feeds, 10.5% in the [$^3$H]$GA_9$-feeds and 1.2% in the [$^3$H]$GA_{20}$-feeds. Most of the radioactivity in these fractions may well consist of glucosyl ethers and glucosyl esters of [$^3$H]GAs, as found previously when GAs were applied to cell cultures [7, 9, 10, 11–13]. In the cell residues, 1.7% of the applied radioactivity remained in the [$^3$H]$GA_1$-feeds, as well as 2.6% in the [$^3$H]$GA_4$-feeds, 4.7% in the [$^3$H]$GA_9$-feeds and 0.8% in the [$^3$H]$GA_{20}$-feeds. The total uptakes of [$^3$H]GAs by cultured cells were 5.1% of the applied radioactivity in the [$^3$H]$GA_1$-feeds, 8.2% in the [$^3$H]$GA_4$-feeds, 25.6% in the [$^3$H]$GA_9$-feeds, and 5.7% in the [$^3$H]$GA_{20}$-feeds, respectively. Thus, the uptakes of [$^3$H]$GA_1$, [$^3$H]$GA_4$ and [$^3$H]$GA_{20}$ by cultured cells were rather low in comparison with that of [$^3$H]$GA_9$, although the application of [$^3$H]GAs to the cell cultures was taken at the same time and under almost the same condition. The differences between the amounts of radioactivity taken up in the [$^3$H]GA-feeds seemed to be due to the activity in

highly water soluble GA. However, it is not clear whether the differences between uptakes of GAs by the cells were caused by the different polarities of GAs (i.e. membrane permiabilities of GAs) or different rates of metabolism of GAs to highly water soluble GAs.

*Identification of [$^3$H]GA metabolites in suspension cultured cells*

Because of the high specific radioactivity of the [$^3$H]GAs, tentative identification of [$^3$H]GA metabolites was based only on chromatographic analyses with direct comparison of the chromatographic behaviour with those of authentic standards. However, the results from the [$^2$H]GAs-feeds provide more definitive identification of GA metabolites. As shown in Table 1, [$^3$H]$GA_1$- and [$^3$H]$GA_8$-like peaks were found from the free acidic GA fraction of the [$^3$H]$GA_1$-feeds, with [$^3$H]$GA_1$-, [$^3$H]$GA_4$- and [$^3$H]$GA_8$-like peaks from the [$^3$H]$GA_4$-feeds, [$^3$H]$GA_1$-, [$^3$H]$GA_4$-, [$^3$H]$GA_8$-, [$^3$H]$GA_9$- and [$^3$H]$GA_{20}$-like peaks from the [$^3$H]$GA_9$-feeds, and [$^3$H]$GA_1$-, [$^3$H]$GA_8$- and [$^3$H]$GA_{20}$-like peaks from the [$^3$H]$GA_{20}$-feeds, respectively.

*Identification of [$^2$H]GA metabolites in suspension cultured cells*

The analyses for the [$^2$H]GAs feeds were done as noted above for the [$^3$H]GAs feeds, because each [$^2$H]GA and its corresponding [$^3$H]GA were applied to the same cultures at the same time, except in the case of the [$^2$H]$GA_{19}$-feeds. The biological activity of [$^2$H]GA metabolites in radioactive fractions after HPLC was assayed using dwarf rice seedlings prior to GC-SIM. For the SIM analysis, characteristic ions of speculated [$^2$H]GA metabolites were selected, as well as those of the corresponding protiated GAs. Several characteristic ion peaks of expected deuterated GAs were observed, although no characteristic ion peaks of the corresponding protiated GAs were found. Therefore, we concluded that the characteristic ion peaks found in the samples were not originating from the isotope ions of the corresponding protiated GAs, but from the characteristic ions of [$^2$H]GA metabolites. [$^2$H]GA metabolites identified in the corresponding [$^3$H]GA fractions from the [$^2$H]/[$^3$H]GAs-feeds based on ion intensities and Kovats retention indices (KRI) [14] are shown in Table 2. [$^2$H]$GA_1$ and its 2$\beta$-hydroxylated metabolite, [$^2$H]$GA_8$, were found from the $GA_1$-feeds. [$^2$H]$GA_2$ was identified, as well as [$^2$H]$GA_4$ and its 13-hydroxylated metabolite, [$^2$H]$GA_1$, from the $GA_4$-feeds. However, [$^2$H]$GA_2$ may be an artefact resulting from the addition of water to the exo-double bond of [$^2$H]$GA_4$ rather than a genuine metabolite of [$^2$H]$GA_4$, because $GA_2$ was not found as an endogenous GA in *R. sativus*. From the $GA_9$-feeds, [$^2$H]$GA_4$, the 3$\beta$-hydroxylated metabolite of $GA_9$, and [$^2$H]$GA_{20}$, the 13-hydroxylated metabolite of $GA_9$, were identified, as well as [$^2$H]$GA_1$ and [$^2$H]$GA_9$. The presence of $GA_{34}$ and $GA_{51}$, which are the endogenous 2$\beta$-hydroxylated metabolites of $GA_4$ and $GA_9$, respectively, was not confirmed in any radioactive/biologically active fractions in both the $GA_4$-feeds and the $GA_9$-feeds. [$^2$H]$GA_{19}$ and its metabolite, [$^2$H]$GA_{20}$, were found from the $GA_{19}$-feeds. From the $GA_{20}$-feeds, [$^2$H]$GA_{20}$ and its 13-hydroxylated metabolite, [$^2$H]$GA_1$, were identified. [$^2$H]$GA_{20}$-15-ene was also tentatively identified from the $GA_{20}$-feeds based on the published mass spectrum and KRI [15]. [$^2$H]$GA_{20}$-15-ene might be an artefact resulting from rearrangement of the exo-double bond under the experimental conditions. However, we confirmed that [$^2$H]$GA_{20}$-15-ene was a metabolite of [$^2$H]$GA_{20}$, since the protiated $GA_{20}$ was not converted into $GA_{20}$-15-ene under the experimental conditions, and also $GA_{20}$-15-ene was identified as an endogenous component in *R. sativus* by GC-SIM [Nishijima, T., personal communication]. No [$^2$H]GA metabolite was found in

Table 1. Radioactivity (as a percentage of extracted radioactivity) in HPLC fractions from extracts of cells in suspension cultures of *R. sativus* incubated with [$^3$H]GAs for 48 hr

| Radioactive region | HPLC fraction | Applied GA | | | |
|---|---|---|---|---|---|
| | | [$^3$H]$GA_1$ | [$^3$H]$GA_4$ | [$^3$H]$GA_9$ | [$^3$H]$GA_{20}$ |
| [$^3$H]$GA_8$ fraction | 14–16 | 9.2 | 4.3 | 2.6 | 5.4 |
| [$^3$H]$GA_1$ fraction | 18–20 | 87.1 | 64.3 | 17.8 | 60.1 |
| [$^3$H]$GA_{20}$ fraction | 21–23 | — | — | 33.3 | 32.2 |
| [$^3$H]$GA_4$ fraction | 24–25 | — | 14.5 | 31.5 | — |
| [$^3$H]$GA_9$ fraction | 26–27 | — | — | 12.1 | — |
| Others | | 3.7 | 16.9 | 2.7 | 2.3 |
| Authentic GA | | | | | |
| [$^3$H]$GA_1$ | 18–19 | | | | |
| [$^3$H]$GA_4$ | 24–25 | | | | |
| [$^3$H]$GA_8$ | 15–16 | | | | |
| [$^3$H]$GA_9$ | 26–27 | | | | |
| [$^3$H]$GA_{19}$ | 23–24 | | | | |
| [$^3$H]$GA_{20}$ | 21–22 | | | | |

Table 2. Identification of GA metabolites by GC-MS for Me or MeTMSi derivatives for radio-/biologically active fractions from HPLC of extracts of cells in suspension cultures of *R. sativus* incubated with [$^2$H]GAs for 48 hr

| HPLC fraction | Applied GA | | | | | Ions monitored for identification ($m/z$) |
|---|---|---|---|---|---|---|
| | [17,17-$^2H_2$]$GA_1$ (GA,* KRI,† RI‡) | [17,17-$^2H_2$]$GA_4$ (GA,* KRI,† RI‡) | [17,17-$^2H_2$]$GA_9$ (GA,* KRI,† RI‡) | [17,17-$^2H_2$]$GA_{19}$ (GA,* KRI,† RI‡) | [17,17-$^2H_2$]$GA_{20}$ (GA,* KRI,† RI‡) | |
| 14–16 | $GA_8$ 2813 0.03 | | | | | 596[M]$^+$,§ 594, 581, 579 |
| 18–20 | $GA_1$ 2664 1.00 | $GA_1$ 2664 0.38 | $GA_1$ 2664 1.05 | | $GA_1$ 2664 trace | 508[M]$^+$,§ 506, 493, 491, 449, 447 |
| 18–20 | | $GA_2$ 2745 0.01 | | | | 510[M]$^+$,§ 508, 495, 493, 477, 475 |
| 21–23 | | | $GA_{20}$ 2477 0.02 | $GA_{20}$ 2477 0.04 | $GA_{20}$ 2476 1.00 | 420[M]$^+$,§ 418, 405, 403, 377, 375 |
| 21–23 | | | | | $GA_{20}$ 2432 0.09 – 15-ene¶ | 420[M]$^+$,§ 418, 391, 389, 361, 359 |
| 24 | | | | $GA_{19}$ 2578 1.00 | | 464[M]$^+$, 462, 436,§ 434, 376, 374 |
| 24–25 | | $GA_4$ 2499 1.00 | $GA_4$ 2500 0.03 | | | 420[M]$^+$, 418, 330, 328, 286,§ 284 |
| 26–27 | | | $GA_9$ 2300 1.00 | | | 322[M]$^+$, 330, 300,§ 298, 288, 286 |

* GAs identified as [17,17-$^2H_2$]GA metabolites.
† Kovats retention indices.
‡ Relative intensities compared with the parent GA.
§ Ions used for comparison of relative intensities.
¶ Tentatively identified based on the mass spectrum and KRI data.

each $GA_8$-like fraction after HPLC, except in the case of the $GA_1$-feeds, when [$^2$H]$HA_8$ was identified.

*Metabolism of GAs*

The distribution of radioactivity in the [$^3$H]GA metabolites after HPLC corresponded in most cases with that of the [$^2$H]GA metabolites, based on mass spectral relative intensities, except in the case of the $GA_{20}$-feeds, when a trace amount of [$^2$H]$GA_1$ (*ca* 0.1% intensity of [$^2$H]$GA_{20}$) was identified, although more than 60% of the extractable radioactivity was found in the [$^3$H]$GA_1$ fraction. In the fraction, only a slight biological activity was observed and other possible GA metabolites, e.g. [$^2$H]$GA_6$ and [$^2$H]$GA_{29}$, were not detected. From the metabolic patterns of applied GAs, it is clear that $GA_1$ is subject to 2$\beta$-hydroxylation, $GA_4$ is easily subject to 13-hydroxylation, and $GA_9$ is subject to 3$\beta$-hydroxylation, as well as 13-hydroxylation. However, it is still unclear whether $GA_{20}$ undergoes 3$\beta$-hydroxylation in the cells. Previously [1 and Nishijima, T., personal communication], two major biosynthetic pathways, the early-13-hydroxylation pathway ($\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}\rightarrow GA_1\rightarrow GA_8$) which leads to $GA_1$, which is recognized as the physiologically active GA in regulating shoot elongation in maize, rice and pea [16], and the non-13-hydroxylation pathway ($\rightarrow GA_{15}\rightarrow GA_{24}\rightarrow GA_9\rightarrow GA_4\rightarrow$), were proposed for *R. sativus* on the basis of the identification and quantification of endogenous GAs. However, the present findings show that 13-hydroxylation of $GA_4$ is much more dominant than 3$\beta$-hydroxylation from $GA_{20}$ in pathways leading to $GA_1$ in suspension cultured cells of *R. sativus*. The hypothetical metabolic pathways of GAs in suspension cultured cells are shown in Fig. 1. As mentioned before, metabolic pathways of GAs in cell suspension cultures of some plants have been shown to be very similar to those in the corresponding intact plants. Thus, the pattern of GA metabolism in suspension cultured cells of *R. sativus* will be expected to be similar to that existing in intact plants, although quantitative differences will occur between these tissues in their metabolism of GA.

## EXPERIMENTAL

*Culture origin and maintenance.* Cell cultures of Japanese radish (*Raphanus sativus* L. cv Taibyo-sobutori) were derived from hypocotyl tissues of germinated seeds. The cultures were maintained by bi-weekly subculturing into MS liquid medium containing $2\times10^{-6}$ M of 2,4-D. The subcultures were grown at 27° in continuous light (100–120 $\mu$mol m$^{-2}$ s$^{-1}$) on a shaker (80 rpm).

*Application of GAs.* Seven-day-old cultures were incubated for 48 hr with [$^3$H]GAs (212 KBq of $GA_1$, 197 KBq of $GA_4$, 168 KBq of $GA_9$ and 163 KBq of $GA_{20}$, each 40 ~ 100 GBq × mmol$^{-1}$) and [17,17-$^2H_2$]GAs ($GA_1$, $GA_4$, $GA_9$, $GA_{19}$ and $GA_{20}$). 100 $\mu$g of each [$^2$H]GA were dissolved in 200 $\mu$l 95% EtOH with the corresponding [$^3$H]GA, except for the [$^2$H]$GA_{19}$-feeds, diluted to 5 ml with the medium and then added to the

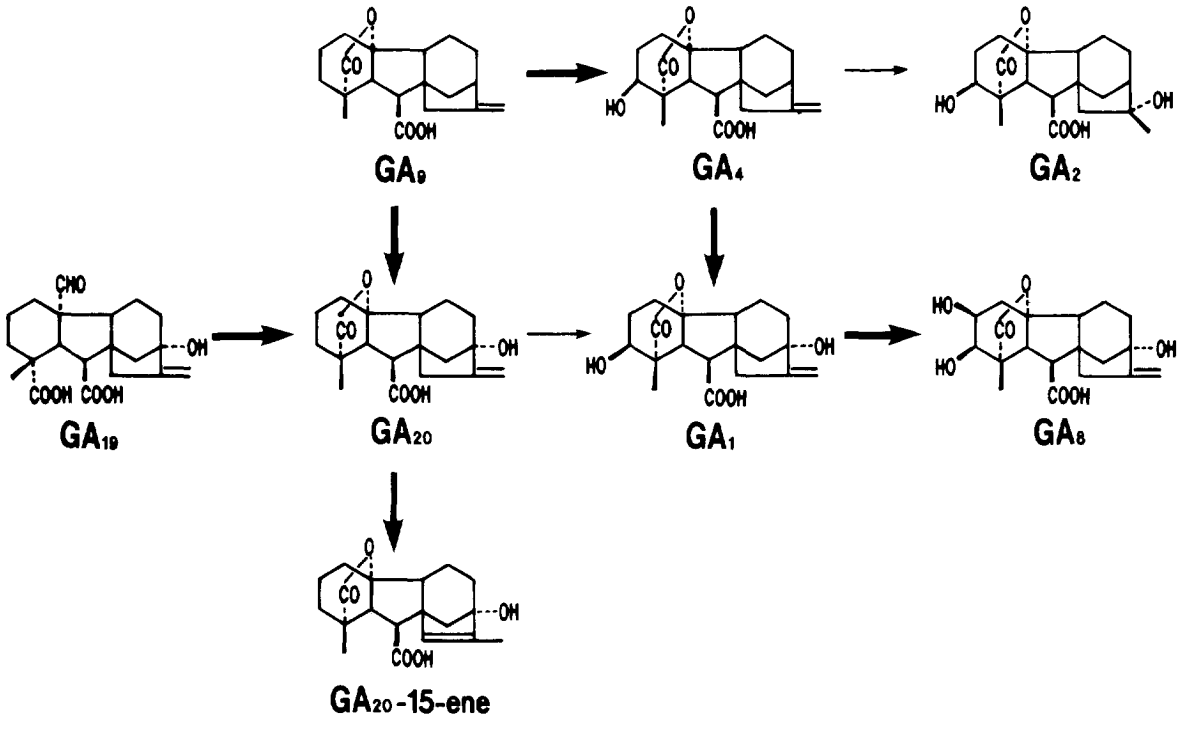


Fig. 1. Hypothetical metabolic pathways of GAs in cell suspension cultures of *R. sativus*. → Major metabolic pathway.

suspension cultures. After 48 hr incubation, the cells were harvested by filtration, washed with 20 ml distilled $H_2O$ ( × 3), frozen in liquid $N_2$, then freeze-dried. The fr. wt and dry wt of each batch of cultured cells were 10.23 g and 1.66 g in the $GA_1$-feeds, 10.32 g and 1.60 g in the $GA_4$-feeds, 10.11 g and 1.64 g in the $GA_1$-feeds, 9.79 g and 1.67 g in the $GA_{19}$-feeds, and 9.68 g and 1.58 g in the $GA_{20}$-feeds, respectively.

*Extraction and purification of GA metabolites.* Freeze-dried cells were extracted with aq. 80% MeOH ( × 3), concd *in vacuo* and solvent-fractionated to obtain an acidic EtOAc-soluble (acidic GA) fr. The acidic GA fr. was dried and then dissolved in 2 ml MeOH. The soln was loaded on to a column of Bondesil (diethylaminopropyl, DEA; Varian; 5 g, packed with MeOH). After sample loading the column was washed with MeOH (100 ml), and the GAs were eluted with MeOH containing 0.5% HOAc (100 ml). The effluent was then reduced to dryness *in vacuo*. The residue was dissolved in 1 ml 45% MeOH in 0.1% aq. HOAc. The soln was purified by gradient-eluted Develosil ODS HPLC (10 × 150 mm) with the following gradient: 10% MeOH in 0.1% aq. HOAC, isocratic (10 min); linear gradient from 10% MeOH in 0.1% aq. HOAc to 73% MeOH in 0.1% aq. HOAc (10–40 min); 73% MeOH in aq. HOAc, isocratic (40–50 min); 100% MeOH, isocratic (50–70 min). The total elution time was 70 min, with a flow rate of 2 ml $min^{-1}$, and 2-min frs were collected. The frs were assayed by liquid scintillation spectrometry and by a Tan-ginbozu dwarf rice micro-drop bioassay [17]. The radioactivity in cell residues was assayed by means of a sample oxidizer and liquid scintillation spectrometry.

*GC-SIM analysis of GA metabolites.* The frs showing biological activity and/or containing radioactivity were converted to methyl esters (Me) with ethereal $CH_2N_2$, and then to TMSi ethers with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide. The derivatized samples were analysed by GC-SIM [18], with splitless injection into a WCOT DB-1-15N column (film thickness 0.25 μm, 0.25 mm × 15 m) installed in a Hewlett Packard 5890 GC connected to a JEOL Automass 20 mass spectrometer. For the identification of possible [$^2$H]GA metabolites, characteristic ions of the corresponding deuteriated and protiated GAs, their relative intensities and KRIs, were used.

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