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Gibberellin biosynthesis: metabolic evidence for three steps in the early 13-hydroxylation pathway of rice

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Abstract

[$^{14}C_4$]GA_{53}, [$^{14}C_4$]GA_{44}, and [$^{2}H_2$ /$^{14}C_4$]GA_{19} were injected separately into seedlings of rice (*Oryza sativa)* using a dwarf mutant (d_{35}) that has low levels of endogenous gibberellins (GAs). After 8 h incubation, the shoots were extracted and the labeled metabolites were identified by full-scan gas chromatography–mass spectrometry (GC–MS) and Kovats retention indices (KRIs). Our results document the metabolic sequence, $GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}$ and the presence of endogenous GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_1. Previous metabolic studies have shown the presence of the step, $GA_{20}\rightarrow GA_1$ in rice. Taken together, the data establish in vegetative shoots of rice the presence of the early 13-hydroxylation pathway, a pathway that originates from GA_{12} and leads to bio-active GA_1. © 2000 Published by Elsevier Science Ltd.

Keywords: Oryza sativa; Graminaceae; Rice; *Dwarf-35* (d_{35}, *dx*; cultivar Tanginbozu); Gibberellins; Metabolism; Plant growth regulators; Early 13-hydroxylation pathway; GAs; $GA_{1\text{-}n}$.

1. Introduction

Gibberellins (GAs) are diterpene acids that are of widespread occurrence in higher plants (angiosperms, gymnosperms and ferns) (MacMillan, 1999), and to a limited extent in fungi (MacMillan, 2000a) and bacteria (MacMillan, 2000b). While gibberellins control a wide variety of plant responses, only a limited number of the 121 identified GAs are bio-active per se in the control of shoot elongation (reviewed in MacMillan and Phinney, 1987). The other GAs are either bio-active as precursors to the active GAs, or bio-inactive as terminal branch products of the main pathway(s) (MacMillan, 1987). Interestingly, the gibberellins have yet to be shown to have a biological function in fungi and bacteria. GAs originate from GA_{12} by branch pathways, the early 13-hydroxylation pathway, the early 3-hydroxylation pathway, the early 3,13-hydroxylation pathway and/or the non-early 3,13-hydroxylation pathway. The number of branches vary depending on the species and organ, the branches differing from each other in the timing and pattern of hydroxylation. For flowering plants (angiosperms), the biosynthesis of GA_1 is generally assumed to occur via the early 13-hydroxylation pathway, which originates from GA_{12} and leads to bio-active GA_1. However, there is limited in vivo metabolic evidence for this generalization, where most of the information is based solely on the endogenous presence of GAs in any one species (reviewed in MacMillan, 1997). The most complete information on the early 13-hydroxylation pathway in flowering plants is from vegetative shoots of maize where all members of the pathway have been shown to be endogenous (Fujioka et al., 1988); in this comprehensive series of in vivo studies, each step has been established by feeds of labeled substrates followed by the GC–MS identification of the metabolites from each experiment (Kobayashi et al., 1994, l996, Spray et al., 1996). While we have previously shown GA_{20} to be metabolized to bio-active GA_1 in rice (Kobayashi et al., 1994), we now present additional evidence for the

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presence of the early 13-hydroxylation pathway by studying the metabolism of the three gibberellins, GA_{53}, GA_{44} and GA_{19}. In addition, GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_1 are identified as naturally occurring in rice. The mutant d_{35} was used in the studies since this semi-dwarf has been reported to have reduced levels of endogenous GAs due to a genetic block for GA-biosynthesis at a step before GA_{12} (Ogawa et al., 1996).

2. Results and discussion

2.1. Metabolism of [$^{14}C_4$]GA_{53}

The metabolites, [$^{14}C_4$]GA_{44} and [$^{14}C_4$]GA_{19}, and non-metabolized [$^{14}C_4$]GA_{53} were identified by the KRI and full-scan GC–MS data shown in Table 1. These identifications were confirmed in a duplicate experiment (data not shown). The isotopic content in the substrate (115 Ci mol^{-1}) was diluted in the non-metabolized [$^{14}C_4$]GA_{53} (102.2 Ci mol^{-1}) and in the metabolites [$^{14}C_4$]GA_{44} (90.1, 92.6 and 95.8 Ci mol^{-1}) and [$^{14}C_4$]GA_{19} (84.4, 94.6 and 95.5 Ci mol^{-1}). We conclude that GA_{53} was metabolized to GA_{44} and GA_{19}, and that GA_{53}, GA_{44} and GA_{19} are endogenous in rice.

2.2. Metabolism of [$^{14}C_4$]GA_{44}

The metabolite, [$^{14}C_4$]GA_{19}, and non-metabolized [$^{14}C_4$]GA_{44} were identified by the KRI and full-scan GC–MS data shown in Table 2. These identifications were confirmed by a second experiment (data not shown). We conclude that GA_{44} was metabolized to GA_{19}. Further evidence for the presence of endogenous GA_{44} and GA_{19} was provided by the dilution of label in the substrate (160 Ci mol^{-1}) for the non-metabolized [$^{14}C_4$]GA_{44} (95.8 Ci mol^{-1}), and in the metabolite, [$^{14}C_4$]GA_{19} (94.6 Ci mol^{-1}).

2.3. Metabolism of [$^{2}H_2$/$^{14}C_4$]GA_{19}

[$^{2}H_2$]GA_{20} and [$^{2}H_2$]GA_1 were identified by the KRI and full scan GC–MS data (Table 3) using [$^{14}C_4$]GA_{19} as a radioactive marker. 3-epi[$^{2}H_2$]GA_1 was also identified but is probably an artefact formed from [$^{2}H_2$]GA_1 (Gaskin et al., 1995). [$^{2}H_2$]GA_{17} was tentatively identified by GC–SIM at the correct retention time. The identification of [$^{2}H_2$]GA_{20} as a metabolite of [$^{2}H_2$]GA_{19} was confirmed by a second experiment using [$^{3}H_2$]GA_{20} as a radioactive marker (data not shown). The isotope content in the substrate [$^{2}H_2$]GA_{19} (86 $^{2}H_2$ mol^{-1}) was diluted in the metabolites, [$^{2}H_2$]GA_{20} (81 $^{2}H_2$ mol^{-1}) and [$^{2}H_2$]GA_1 (79 $^{2}H_2$ mol^{-1}). We conclude that GA_{19} is metabolized to GA_{20} and that GA_{20} and GA_1 are endogenous in rice.

The data on dilution of label documents the endogenous presence in rice of the five gibberellins, GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_1. The present results also provide metabolic evidence in rice for the three steps, $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$. We had previously shown that GA_{20} is metabolized to GA_1 in shoots of rice (Kobayashi et al., 1994). Thus we have now established four steps of the early 13-hydroxylation branch pathway (Fig. 1), a pathway generally presumed to occur in rice shoots.

Table 1
GAs identified by GC–MS as MeTMSi derivatives[a]

ODS HPLC fraction	$N(CH_3)_2$HPLC fraction	Radioactivity (Bq)	Identified GA	Isotopic content (Ci mol^{-1})	Identification data KRI: significant ions, m/z (rel. int)[b]	
19–21	10–11	134	GA_{44}	90.1, 92.6, 95.8[c]	2791:	440 (12), 432 (29), 425 (4), 417 (8), 373 (6), 238 (11), 209 (70), 207 (100)
19–21	14–15	101	GA_{19}	84.4, 94.6, 95.5[d]	2596:	462 (7), 447 (6), 442 (21), 434 (100), 402 (7), 374 (72), 345 (31)
25	5–6	184	GA_{53}	102.2[c]	2505:	456 (14), 448 (26), 424 (9), 416 (10), 389 (32), 209 (100), 207 (85)

[a] Seedlings were harvested and processed 8 h after injection of [$^{14}C_4$]GA_{53} (467 Bq per plant) to 59 seedlings of d_{35} rice at the 4–5 leaf stage. An additional feed (419 Bq per plant) to 40 seedlings provided replicate isotopic content values.
[b] Determined by full scan GC–MS.
[c] GC–SIM on M^+ ion cluster at correct GC retention time.
[d] GC–SIM on M^+-28 ion cluster at correct GC retention time.

Table 2
GAs identified by GC–MS as MeTMSi derivatives[a]

ODS HPLC fraction	$N(CH_3)_2$HPLC fraction	Radioactivity (Bq)	Identified GA	Isotopic ratio $^2H_0$:$^2H_1$:$^2H_2$	Identification data KRI: significant ions, m/z (rel. int)[b]	
11–13	19–20	595	ND[c]			
19–21	12	696	GA_{44}	95.8[d]	2803:	440 (26), 438 (11), 432 (16), 379 (10), 373 (4), 240 (34), 238 (17), 209 (100), 207 (51)
19–21	17–18	181	GA_{19}	94.6[e]	2601:	442 (48), 434 (100), 410 (10), 402 (28), 380 (56), 378 (35), 374 (72)

[a] Seedlings were harvested and processed 8 h after injection of [$^{14}C_4$]GA_{44} (823 Bq per plant) to 50 seedlings of d_{35} rice at the 4–5 leaf stage.
[b] Determined by full scan GC–MS.
[c] No GAs detected.
[d] GC–SIM on M^+ ion cluster at correct GC retention time.
[e] GC–SIM on M^+-28 ion cluster at correct GC retention time.

Table 3
GAs identified by GC–MS as MeTMSi derivatives[a]

ODS HPLC fraction	$N(CH_3)_2$fraction fraction	Radioactivity (Bq)	Identified GA	Isotopic ratio $^2H_0$:$^2H_1$:$^2H_2$	Identification data KRI: significant ions, m/z (rel. int)[b]	
9–10	11	10	GA_1	19.0:2.0:79.0	2672:	508 (100), 506 (18), 493 (8), 450 (26), 378 (21), 315 (18), 237 (12), 209 (54), 207 (21)
			3-epiGA_1	20.0:4.5:74.5	2793:	508 (100), 506 (3), 493 (8), 450 (21)
16–18	14–15	235	GA_{20}	15.8:2.9:81.3	2488:	420(100), 418 (13), 405 (16), 403 (2), 377 (59), 361 (13), 303 (23), 301 (4)
20–22	7	7	GA_{17}	16.4:0.0: 83.6		[c]

[a] Seedlings were harvested and processed 8 h after injection of a mixture of [$^2H_2$]GA_{19} (50 ng per plant) and [$^{14}C_4$]GA_{19} (370 Bq per plant) into 30 seedlings of d_{35} rice at the 4-5 leaf stage.
[b] Determined by full scan GC–MS.
[c] GC–SIM on M^+ ion cluster at the correct GC retention time.

Our metabolic evidence that the three sequential steps, $GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}$, occur in rice extends the information obtained from the cloning of a GA 20-oxidase from rice (Toyomasu et al., 1997). The recombinant protein expressed by a cDNA clone from rice seedlings catalyzed the conversion of GA_{53} to a mixture of GA_{44}, GA_{19} and GA_{20}. However, the individual steps were not investigated. Of particular interest would have been information on the metabolism of GA_{44} by the recombinant protein since our present paper shows that the closed lactone of GA_{44} is metabolized in vivo to GA_{19}, as is the case for vegetative shoots of maize (Kobayashi et al., 1996).

3. Experimental

3.1. Plant material

Seedlings of the *dwarf-35* (d_{35}) mutant of rice (*Oryza sativa*) were used for the feeding studies. This mutant was originally isolated as the rice cultivar, Tanginbozu, subsequently called *dx*, and currently named d_{35} (Mitsunaga et al., 1994; Futsuhara and Kikuchi, 1995). The seed were a gift from Dr. Hitoshi Saka (National Institute of Agrobiological Resources, Tsukuba-shi, Japan). Seedlings were grown in the UCLA greenhouse and used at the 4-leaf stage (3 weeks after germination).

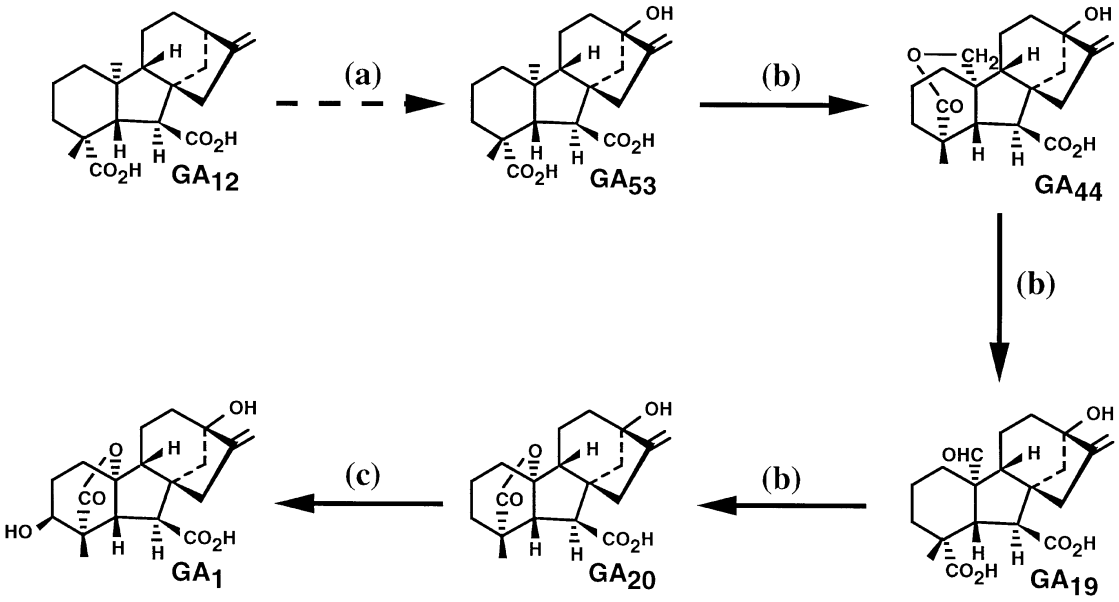

Fig. 1. Metabolic steps in the early 13-hydroxylation pathway for vegetative shoots of rice: (a) putative; (b) established in this paper; (c) established by Kobayashi et al. (1994).

3.2. Labeled substrates

$[^{14}C_4]GA_{53}$ (4.29 GBq mmol^{-1}) (115 Ci mol^{-1}), $[^{14}C_4]GA_{44}$ (5.92 GBq mmol^{-1}) (160 Ci mol^{-1}), and $[^{14}C_4]GA_{19}$ (3.36 GBq mmol^{-1}) (90.26 Ci mol^{-1}) were prepared as described by Kamiya and Graebe (1983). $[^2H_2]GA_{19}$ (7:7:86; $^2H_0$:$^2H_1$:$^2H_2$) was obtained from Professor L. N. Mander (Australian National University, Canberra, Australia). The substrates were at least 95% chemically pure based on GC–MS.

3.3. Feeds, extraction and purification

Each substrate was dissolved in ethanol:water (1:1) and injected with a microsyringe into sets of seedlings, the numbers in each set varying depending on the experiment. The amounts and combinations of substrates applied to each plant are given in Tables 1–3.

Sets of shoots were harvested 8 h after treatment and homogenized in dry ice. Each homogenate was extracted and purified to give an EtOAc-soluble acid fraction (AE), which was further purified by column chromatography, the details of which are given in Spray et al. (1996).

3.4. GC–MS

Radioactive fractions from the Nucleosil $5N(CH_3)_2$ columns were methylated and trimethysilylated, then analyzed by full-scan GC–MS and/or GC–SIM as described in Gaskin and MacMillan (1991).

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