スギ花粉に含まれる内生ジベレリン

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論　文

Endogenous gibberellins in the pollen of *Cryptomeria japonica* D. DON

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HONMA, T., YAMAGUCHI, I., NAKAJIMA, M., MUROFUSHI, N. and MIGITA, K.: **Endogenous gibberellins in the pollen of *Cryptomeria japonica* D. DON. J. Jpn. For. Soc. 77: 358～365, 1995** Shoot elongation and flower bud formation of sugi (*Cryptomeria japonica* D. DON) are promoted by exogenous gibberellins (GAs). This suggests that these physiological events are regulated by endogenous GAs, too. Little is known about the endogenous GAs of conifers. GAs are characterized from only four species of Pinaceae. Although the occurrence of GA-like substances in *C. japonica* was shown by bioassay using dwarf rices, their identification has not been reported. As the first step to study the relationship between endogenous GAs and the physiological and morphological changes in the growth of *C. japonica*, we analyzed endogenous GAs in the pollen of this plant. The occurrence of GA_1 and/or GA_3, GA_4, GA_9, GA_{12}, and GA_{15} was suggested by combination of bioassays using dwarf rice (*Oryza sativa* L. *cv.* Tan-ginbozu) and enzyme-linked immunosorbent assay (ELISA) after purification by high performance liguid chromatography (HPLC). Eventually, GA_9, GA_{12}, and GA_{15} were conclusively identified by gas chromatography-mass spectrometry (GC/MS). However, the identification of GA_1 and/or GA_3 and GA_4 by either GC/MS or gas chromatography-selected ion monitoring (GC/SIM) was unsuccessful, because these amounts were too small for the detection. This result shows that GAs in the early-non-hydroxylation biosynthetic pathway are predominant in the pollen of *C. japonica*.

本間　環・山口五十麿・中嶋正敏・室伏　旭・右田一雄：**スギ花粉に含まれる内生ジベレリン　日林誌 77: 358～365, 1995**　スギ（*Cryptomeria japonica* D. DON）の伸長成長および花芽形成は，他の針葉樹と同様にジベレリン（GAs）の投与によって促進される。このことは，これらの生理現象が内生GAsによって調節されている可能性をも示している。しかし，針葉樹の内生GAsに関する知見は少ない。これまでに，針葉樹の内生GAsの報告はマツ科の4種に限られている。一方，スギのジベレリン様物質は矮性イネ「短銀坊主」によるバイオアッセイにより確認されているものの，その同定は未だ報告されていない。本研究では，内生GAsとスギの生理学的および形態学的変化との関連を解明する研究の第一段階として，花粉に含まれる内生GAsの分析を行った。その結果，高速液体クロマトグラフィーにより精製した試料を「短銀坊主」によるバイオアッセイおよびエンザイムイムノアッセイ（ELISAs）を用いて分析し，GA_1 および/または GA_3，GA_4，GA_9，GA_{12}，GA_{15} の存在を推測した。それらのうち GA_9，GA_{12} および GA_{15} をガスクロマトグラフィー質量分析（GC/MS）により同定した。しかしながら，ELISAによりその存在が示唆された GA_1 および/または GA_3 と GA_4 については，含有量が少ないためにGC/MSあるいはGC/SIMのいずれにおいても同定することはできなかった。これらの結果から，スギの花粉にはearly-non-hydroxylation pathwayが主要な生合成経路として機能している可能性が示された。

I. Introduction

The physiological effects of exogenously applied gibberellins (GAs) on conifers have been studied by many research groups (PHARIS and KING, 1985). Exogenously applied GAs promote shoot elongation and flower bud formation in many species of conifers. KATO *et al.* (1958) reported that exogenously applied GAs

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(gibberellin powder) promoted flower bud formation of sugi (*Cryptomeria japonica* D. DON), a species of Taxodiaceae, and HASHIZUME (1959) reported the promotion of shoot elongation of the same species by exogenously applied GAs. These observations suggest that endogenous GAs are involved in these physiological events in nature. The occurrence of GA-like substances in 31 conifer species were reported by DUNBERG and ODÉN (1983). However, endogenous GAs were identified from only four species of Pinaceae, and little is known about the presence of endogenous GAs of other species. We are interested in the relationship between these physiological events and the dynamics of endogenous GAs of *C. japonica*. The occurrence of GA-like substances in this plant was reported by HASHIZUME (1961) and OGIYAMA and PHARIS (1980) based on the biological activities in dwarf rice bioassays, but none of them was identified. Thus, we tried to identify endogenous GAs of this species as many as possible for the future study on the relationship between its physiological events and the dynamics of endogenous GAs. The pollen was selected as the first material for the survey of GAs, because the concentration of GAs was expected to be high in the pollen of *C. japonica* as in *Pinus attenuata* LEMM. (KAMIENSKA *et al.*, 1976a). In this paper, the GAs in the pollen of *C. japonica* were carefully surveyed by combination of dwarf rice bioassay, enzyme-linked immunosorbent assay (ELISA) and, gas chromatography-mass spectrometry (GC/MS).

II. Materials and Methods

1. Plant materials

Branches with male flowers collected just before blooming at the Chiba Experimental Forest of the University of Tokyo in February 1993, were kept in water pails at 18°C for 14 days until full bloom. The pollen was collected from the fully open anthers of male flowers, and stored at −85°C until processed for analysis.

2. Processing of the pollen for analysis

The purification procedure of the pollen is shown in Fig. 1.

1) Extraction and solvent fractionation—The pollen (700 g fresh weight, fr wt) was soaked and homogenized in 80% acetone (v/v) and extracted with the same solvent. After, filtration, the extracts were concentrated *in vacuo*. The aqueous concentrate was subjected to a solvent fractionation, and an acidic ethyl acetate (AE) fraction was obtained (YAMAGUCHI *et al.*, 1990).

2) High performance liquid chromatography (HPLC)—The dried AE fration was dissolved in a small volume of methanol (MeOH) and loaded on a Bond Elut DEA cartridge (18 g 40 μm, Analytichem International Co., Ltd., USA.), which was eluted with MeOH (20 ml) and 0.5% acetic acid (AcOH) in MeOH (30 ml), successively. The eluates of the acidic MeOH were concentrated and subjected to HPLC (L-6000 pump and L-6200 intelligent pump, Hitachi Ltd., Tokyo) of an Octadecylsilan (ODS) column (15 cm×6 mm inside diameter, SCC-ODS-H2151, Senshu Scientific Co., Tokyo) using the following gradient elution between solvents A (acetonitorile (MeCN) : H_2O, 2 : 8, containing 0.5% AcOH) and solvent B (MeCN : H_2O, 8 : 2, containing 0.5% AcOH) ; 0～5 min : isocractic solvent A, 5～35 min : linear gradient from 100% solvent A to 100% solvent B, 35～45 min : isocractic solvent B. The flow rate was 1.0 ml/min, and the eluates were collected in 1 min fractions.

3) Dwarf rice bioassay—Biological activity in each fraction of ODS-HPLC was assayed by the microdrop method (MURAKAMI, 1968) using the dwarf rice (*Oryza sativa* L. *cv.* Tan-ginbozu). The concentrate of each fraction was dissolved in 50% aqueous acetone (v/v), and an aliquot (20 g fr wt equivalent (equiv.)) was applied to each plant.

4) Enzyme-linked immunosorbent assay (ELISA)—ELISA was conducted in the same manner as reported by YAMAGUCHI *et al.* (1990). An anti-GA_1-Me- or an anti-GA_{20}-Me-antiserum and GA-labeled alkaline phosphatase as a tracer (YAMAGUCHI *et al.*, 1987, 1990) were used for each assay. The cross-reactivities of each GA-Me against the anti-GA_1-Me- and the anti-GA_{20}-Me-antiserum are summarized in Fig. 2. A portion of each fraction from ODS-HPLC was concentrated, methylated with diazomethane (CH_2N_2), dried, and re-dissolved in 5% MeOH in 50 mM tris buffer (pH 7.6). An aliquot (3 g fr wt equiv.) of each methylated fraction was used for the assays. The absorbance at 410 nm of nitrophenol was measured by a microplate

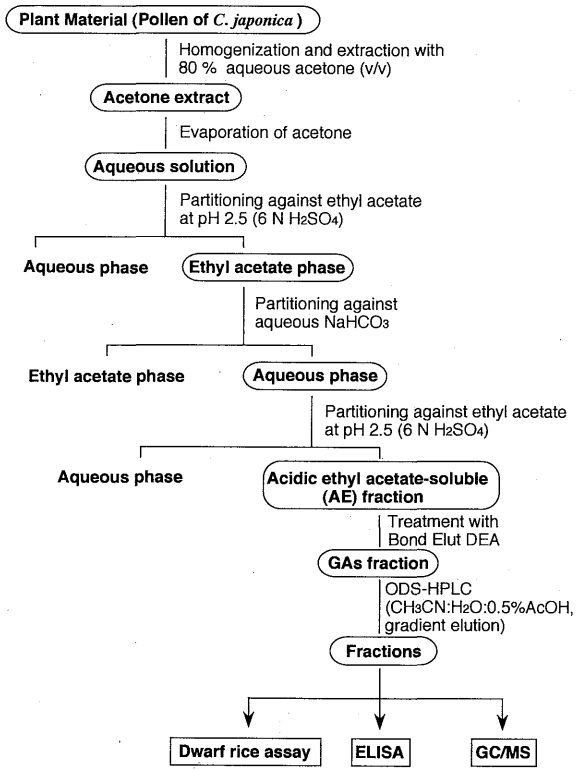

Fig. 1. Procedure for the extraction, purification and identification of GAs in the pollen of *C. japonica*

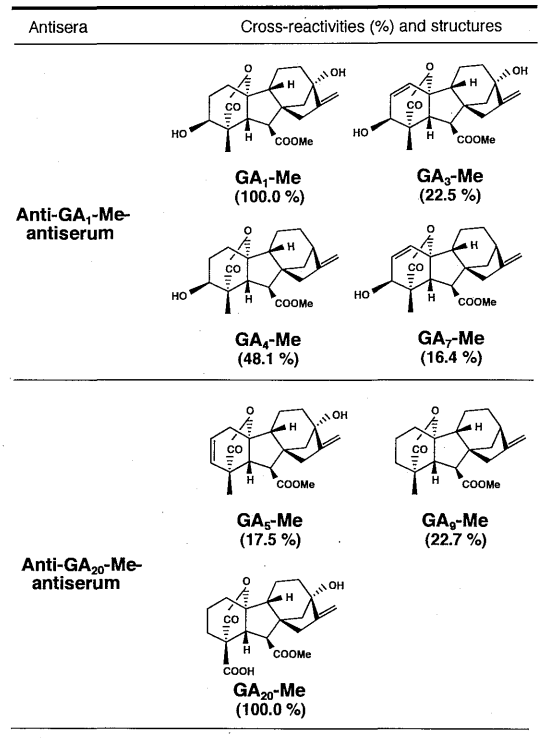

Fig. 2. Structures of GA methyl esters showing high cross-reactivity against an anti-GA_1-Me- and an anti-GA_{20}-Me-antiserum

reader (MR 5000, Dynatech, USA.).

5) Gas chromatography-mass spectrometry (GC/MS)—A Hitachi M-80B mass spectrometer equipped with a Hewlett Packard 5980 Agas chromatograph and a capillary column Ultra # 1 (25 m×0.2 mm *i. d.*, Hewlett Packard, USA.) was used under the following conditions; injection temperature: 250°C, column temperature: 60°C (1.5 min), linear gradient up to 200°C at 60°C/min, to 280°C at 5°C/min, and kept at 280°C (10 min). Ionization voltage was 70 eV. The remainder of each fraction from ODS-HPLC were subjected to GC/MS and/or GC/SIM after methylation with CH_2N_2 and trimethylsilylation with *N*-methyl-*N*-trimethylsilytrifluoroacetamide. In GC/SIM, characteristic ions, *m/z*, (mass to charge ratio peak) 506 (M^+, molecular ion peak), 491, and 448 for GA_1, *m/z* 504(M^+), 489, and 447 for GA_3, and *m/z* 418 (M^+), 390, and 386 for GA_4, were monitored. Hydrocarbons C_{18} to C_{30} with even numbers of carbon atoms, were used for the calculation of the modified Kovats retention indices for the identification of GAs.

III. Results

Figure 3 shows a histogram of biological activities on Tan-ginbozu of the fractions from ODS-HPLC. Three peaks were observed in the fractions of, retention times (t_R) 25～26 min, 29～30 min, and 31～33 min, GA_5, GA_{20}, and GA_{44} were put up for the candidates of the biologically active substances at t_R 25～26 min, GA_9 and GA_{15} to those at t_R 29～30 min and GA_{12} to that at t_R 31～33 min.

A histogram of immunoreactivities against the anti-GA_1-Me-antiserum of the fractions from ODS-HPLC is shown in Fig. 4. Two clear immunoreactive peaks were observed at t_R 4～5 and 10～11 min with some small peaks scattered over the fractions of t_R 15～19, 23～27, and 32～35 min, Judging from the t_R and the specificity of the antiserum, GA_1 and GA_3 could be put up as candidates of the active principles at t_R 10～11

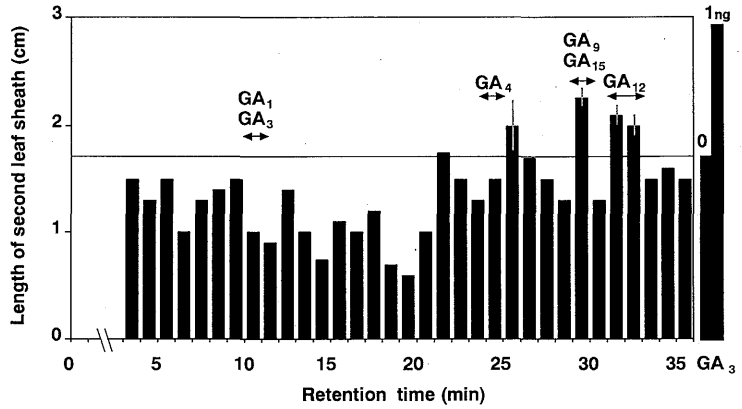

Fig. 3. Histogram of growth promoting activities on dwarf rice (Tan-ginbozu) of the fractions from ODS-HPLC of the AE fraction of the pollen (*C. japonica*)

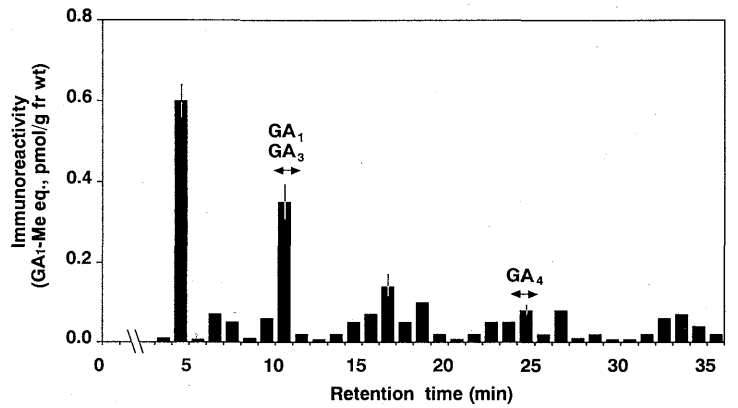

Fig. 4. Histogram of immunoreactivities against anti-GA_1-Me- antiserum of the fractions from ODS-HPLC of the AE fraction of the pollen (*C. japonica*)

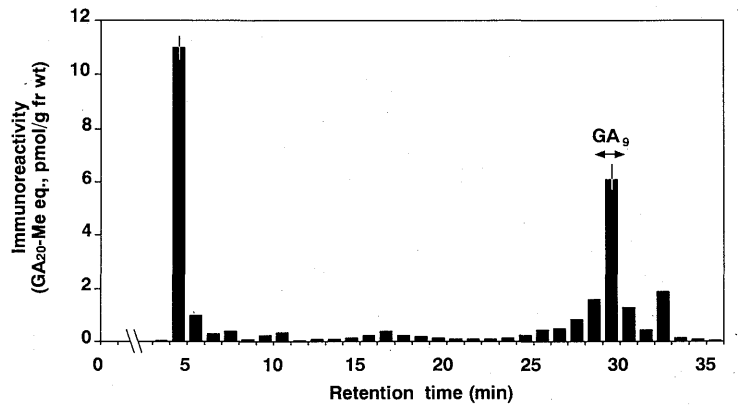

Fig. 5. Histogram of immunoreactivities against anti-GA_{20}-Me-antiserum of the fractions from ODS-HPLC of the AE fraction of the pollen (*C. japonica*)

Table 1. Retention times (ODS-HPLC and GC), modified Kovats retention indices (KRI), and characteristic ions of GAs identified from the pollen of *C. japonica*

GAs		Retention time (min)		KRI	Characteristic ions, *m/z* (relative intensity)
		HPLC	GC		
GA_9	Sample	29~30	10.37	2349	300[M^+]. (8), 298(69), 270(100)
	Authentic		10.45	2353	330[M^+]. (10), 298(83), 270(100), 243(60)
GA_{15}	Sample	29~30	13.95	2665	344[M^+]. (18), 312(23), 284(68), 239(100)
	Authentic		13.98	2667	344[M^+]. (19), 312(23), 284(68), 239(100)
GA_{12}	Sample	32~33	10.65	2404	360[M^+]. (0), 328(20), 300(100), 285(25), 240(42)
	Authentic		10.70	2410	360[M^+]. (0), 328(20), 300(100), 285(25), 240(39)

min, but none for that at t_R 4~5 min. At the elution zone of GA_4 (t_R 23~25 min), only a small immunoreactivity was observed. The other histogram of immunoreactivities against anti-GA_{20}-Me-antiserum is shown in Fig. 5. Prominent immunoreactive peaks were observed at t_R 4~5 min and 29~30 min with a medium one at t_R 32~33 min. Judging from the t_R and the specificity of the anti-GA_{20}-Me-antiserum, GA_9 was put up as the candidate for the immunoreactive substance at t_R 29~30 min. There were no candidates for the activity at t_R 4~5 min, because the t_R is too small for known GAs with high cross-reactivity to anti-GA_{20}-Me-antiserum. The possibility of GA_5 and GA_{20} as the candidates of biological activity on dwarf rice at t_R 25~26 min was excluded because no immunoreactivity was detected by ELISA using anti-GA_{20}-Me-antiserum.

To identify GAs definitely, the fractions which showed either biological activity in dwarf rice assay or immunoreactivity against anti-GA_1-Me- or anti-GA_{20}-Me-antiserum were subjected to full scan GC/MS or GC/SIM. GA_9, GA_{12}, and GA_{15} were identified based on the modified KOVATS' retention index and full scan mass spectra. The details are summarized in Table 1. The fractions of t_R 10~11 min and t_R 24~25 min, which corresponded to the elution zones of GA_1 and/or GA_3 and GA_4 and showed immunoreactivities against anti-GA_1-Me-antiserum, were examined by GC/SIM. However, none of the expected GAs was identified because of their small quantities.

IV. Discussion

The first identification of endogenous GAs from conifers by GC/MS was reported by KAMIENSKA *et al.* (1976a). They identified GA_3, GA_4, and GA_7 from the pollen of *Pinus attenuata*. GA_9 was identified from shoots of *Picea abies* L. KARST. (ODÉN *et al.*, 1982) and GA_1, GA_3, and GA_9 were identified from shoots of the same species (ODÉN *et al.*, 1987). GA_1, GA_4, and GA_9 were identified from shoots of *Picea sitchensis* BONG. CARR. during the period of cone-bud differentiation (MORIZ *et al.*, 1989a). GA_1, GA_3, GA_4, and GA_9 were identified from shoots of *Picea sitchensis* during the period of flower-bud differentiation and shoot elongation (MORIZ *et al.*, 1990a, 1990b). GA_1, GA_3, GA_4, GA_7 and GA_9 were identified from shoots of *Pseudotsuga menziesii* MIRB. FRANCO. (DOUMAS *et al.*, 1992). Glucosyl esters of GA_9 and *iso*-GA_9 were identified from needle of *Picea sitchensis* (LORENZI *et al.*, 1976, 1977). A conjugate of GA_9 was identified from shoots of *Picea sitchensis* (MORIZ *et al.*, 1990a, 1990b). Glucosyl ester of GA_9 was identified from shoots of *Pseudotsuga menziesii* (DOUMAS *et al.*, 1992). These reports show that both the early-13-hydroxylation pathway and the early-non-hydroxylation pathway of GA-biosynthesis may operate in these conifers. However, no evidence of the organ specific occurrence of endogenous GAs has not been reported in conifers. In the survey of endogenous GAs in the pollen of *C. japonica*, we identified GA_9, GA_{12}, and GA_{15} all of which are members in the early-non-hydroxylation biosynthetic pathway (Fig. 6). The occurrence of GA_1 and/or GA_3 was also suggested by ELISA (Fig. 4) after ODS-HPLC, but neither of these GAs were detected by GC/MS or GC/SIM. However, judging from the earlier reports describing the distribution of GA_1 and/or GA_3 in all conifers so far examined, the possibility of the occurrence of GA_1 and/or GA_3 at low levels in the pollen of *C. japonica* remains. Judging from the results of ELISA (Fig. 4), the content of GA_4, which is expected to be an active GA in the early-non-hydroxylation pathway must be less than a half of GA_1 and/or GA_3, if

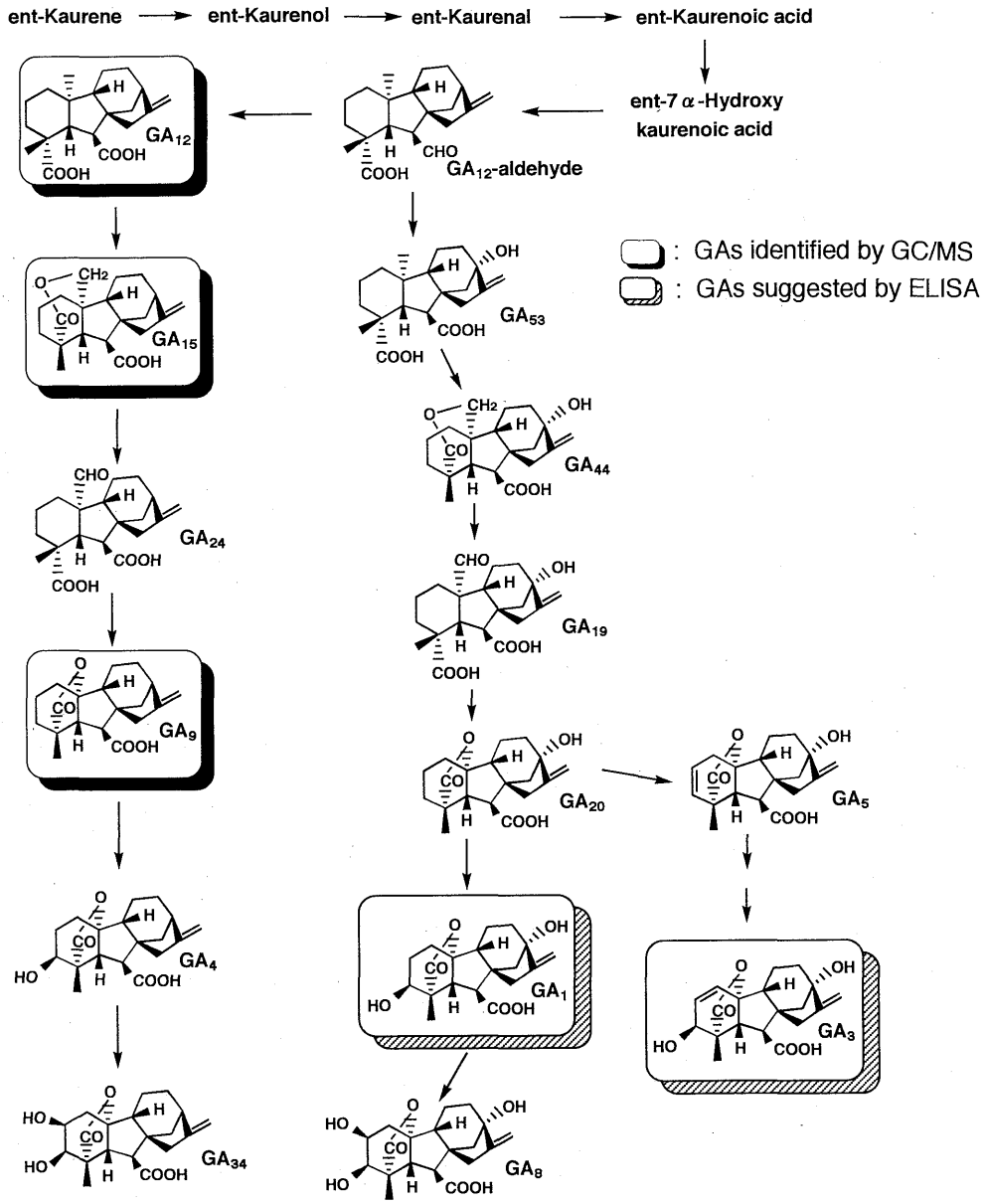

Fig. 6. Early-13-hydroxylation and early non-hydroxylation pathways of GA-biosynthesis

any. Nevertheless, GAs in the early-non-hydroxylation biosynthetic pathway are predominant in the pollen. This suggests that either the early-non-hydroxylation pathway is the major biosynthetic pathway in *C. japonica*, as in cucumber (GRAEBE, 1987) or that the GAs in this pathway distribute organ specificity in the pollen as in anthers of rice (KOBAYASHI *et al.*, 1984).

An immunoreactivity was detected in the fraction of t_R 4～5 min from ODS-HPLC by both ELISAs using anti-GA_1-Me-antibody and anti-GA_{20}-Me-antibody. Although it is impossible to deduce the immunoreactive principle in this fraction from available data, unknown GA conjugates, which show cross-reactivites to both antibodies, may be contained in the fraction as in the case of anthers of rice (HASEGAWA *et al.*, 1994).

In the present study, we could not identify 3β-hydroxylated C_{19}-GAs, which are expected to be active GAs, from the pollen of *C. japonica*, although the occurrence of GA_1 and/or GA_3 was strongly suggested by ELISA. So far, GA_{20} which is a direct precursor of GA_1 in the early-13-hydroxylation biosynthetic pathway has not been identified in many conifers, inspite of GA_1 and/or GA_3 having been identified from several species. This may suggest that a biosynthetic pathway of late 13-hydroxylation operates as a characteristic of conifers. Exogenously applied GA_4 was metabolized into GA_1 and GA_{34} in germinating pollen of *Piuns attenuata* (KAMIENSKA *et al.*, 1976b). Exogenous applied GA_9 was metabolized into GA_4 and GA_1, and GA_4 into GA_1 in *Picea sitchensis* (MORIZ *et al.*, 1989b). These observations suggested the possibility that GA_1 is formed by the late 13-hydroxylation of C_{19}-GAs in Pinaceas. The same pathway may operate in *C. japonica*, too. As it is important to clarify the distribution of GAs in organs and their biosynthesis and translocation for the study of the role of GAs in the expression of physiological phenomena of *C. japonica*, we now are extensively surveying 3β-hydroxylated GAs and 13-hydroxylated GAs in this plant.

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