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Concentrations of *Ent*-Kaurene and Squalene in Vegetative Rice Shoots

Thomas C. Moore,[1] Hisakazu Yamane, Noboru Murofushi, and Nobutaka Takahashi

Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Abstract. *Ent*-kaurene (*ent*-kaur-16-ene) and squalene were analyzed in extracts of the shoots of three cultivars of rice (*Oryza sativa* L.) of 14 and 28 days of age by gas chromatography–mass spectrometry (GS-MS) and GC–selected ion monitoring (GS-SIM). *Ent*-kaurene occurred at approximate concentrations of <1 to 13 ng/g f.w. in 14-day-old plants and 26 to 147 ng/g f.w. in 28-day-old plants. Shoots of the dwarf cultivar Waito-C contained much less *ent*-kaurene than the other two cultivars at both developmental stages. The level of *ent*-kaurene in the dwarf cultivar Tan-ginbozu was similar to that in the normal cultivar Nihonbare at 14 days but was lower than in Nihonbare at 28 days. Trace amounts of *ent*-isokaurene (*ent*-kaur-15-ene) were also found in the extracts of all three cultivars of shoots at 28 days. Squalene occurred in approximate concentrations from as low as 19 ng/g f.w. in 28-day-old Waito-C shoots to as much as 626 ng/g f.w. in 14-day-old Nihonbare shoots. Both Tan-ginbozu and Waito-C shoots contained less squalene than Nihonbare shoots at both developmental stages.

It has been reported that the rate of biosynthesis of *ent*-kaurene (*ent*-kaur-16-ene) may be rate-limiting for gibberellin (GA) biosynthesis during the development of seeds (Coolbaugh and Moore 1969) and shoots (Ecklund and Moore 1974) of pea (*Pisum sativum* L.). However, the concentrations of endogenous *ent*-kaurene have apparently not been determined for the tissues of any higher plants. The purpose of this paper is to report data on the approximate pool sizes of *ent*-kaurene and the triterpenoid precursor squalene as well in extracts

[1] Permanent address: Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA.

of shoots of three cultivars of rice (*Oryza sativa* L.) at two immature stages of their development.

Materials and Methods

Plant Material

Nihonbare (normal), Tan-ginbozu (dwarf), and Waito-C (dwarf) cultivars of rice were grown for 14 and 28 days in a greenhouse under simulated paddy conditions—i.e., in flooded, fertilized soil. Entire shoots were excised, weighed, and stored submerged in methanol in a cold room (4°C) before analysis. Shoot samples of the 14-day-old plants were: Nihonbare, 34.1 g; Tan-ginbozu, 34.8 g; and Waito-C, 34.2 g. For the 28-day-old plants, the shoot samples weighed: Nihonbare, 65.6 g; Tan-ginbozu, 85.7 g; and Waito-C, 82.9 g. Average shoot lengths of 14-day-old plants were: Nihonbare, 15.1 cm; Tan-ginbozu, 9.0 cm; and Waito-C, 7.5 cm. Fourteen-day-old plants were at the four-leaf stage, and the month-old plants were at the six-leaf stage (Kurogochi et al. 1979).

Extraction and Fractionation

Extracts were prepared and subjected to solvent fractionation essentially as described by Kurogochi et al. (1979) and as outlined in the first part of the flow diagram (Fig. 1), except that in the case of the month-old plants, the tissue was not extracted with n-hexane following the initial extraction with methanol.

Silica Gel Column Chromatography

Each hexane fraction was applied to a column consisting of 5–7.5 g of silica gel slurried in n-hexane and approximately 9 cm in length and 1.5 cm in diameter. The column was eluted with 50–100 ml of n-hexane under a slight head pressure. This step was repeated when necessary to rid the eluate of most of the chlorophyll.

High-Performance Liquid Chromatography (HPLC)

Each hexane fraction of a silica gel column was evaporated and stored in a Teflon-stoppered microtube in a cold room (4°C) until it was prepared for HPLC. Then each hexane fraction residue was dissolved in 200 μl of 5% n-hexane in methanol. A 100-μl sample was subjected to HPLC under the following conditions: column, Develosil 5-ODS, 8 mm diameter and 15 cm length; column temperature, 30°C; flow rate, 3 ml/min; pressure 50 kg/cm^2; wavelength on detector, 210 nm; and solvent, methanol. Eluate samples were collected at 1-min intervals. Fractions encompassing the retention times for samples of authentic *ent*-kaurene and *ent*-isokaurene and of squalene were saved for further analysis. The retention times for both *ent*-kaurene and *ent*-

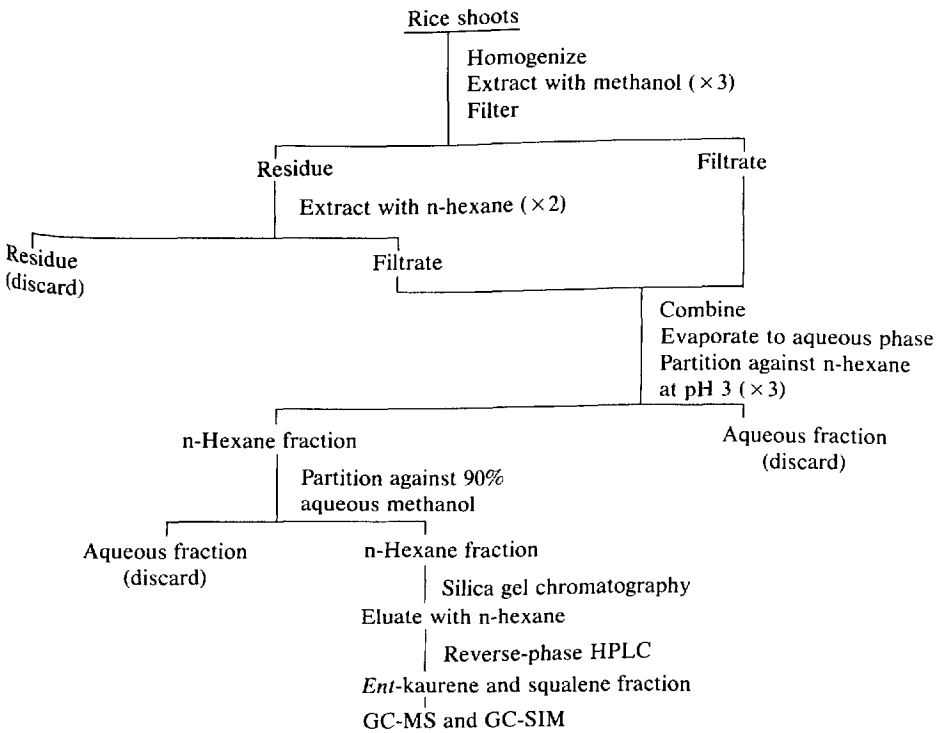

Fig. 1. Steps in the analysis of *ent*-kaurene, *ent*-isokaurene, and squalene in extracts of rice shoots.

isokaurene were 9.8 min; that for squalene, 12.4 min (Table 1). Whereas *ent*-kaurene and *ent*-isokaurene had identical retention times on HPLC, they were separated on the mass chromatograms during GC-MS.

Gas Chromatography–Mass Spectrometry (GS-MS)

GC-MS was carried out with a JEOL DX 303 (ionization voltage, 70 eV). Samples were dissolved in n-hexane and injected onto a fused silica capillary column DB-1 (J&W Scientific Inc., CA, 0.258 mm diameter and 15 m length, 0.25-μm-thick stationary phase) at 120°C in the splitless mode. After a 2-min isothermal hold, the column temperature was programmed at 16°C/min to 280°C with a 10-min isothermal hold at the end of the program. The pressure of the He carrier gas was 0.65 kg/cm^2. The column was led directly into the ion source.

GC–Selected Ion Monitoring (GC-SIM)

GC-SIM was performed on a Hewlett-Packard 5890A gas chromatograph coupled to a Hewlett-Packard 5970 series mass selective detector. The critical

Table 1. *Ent*-kaurene, *ent*-isokaurene, and squalene identified by GC-MS analyses in 14-day-old and 28-day-old rice plants of Nihonbare, Tan-ginbozu, and Waito-C.

Sample	Rt (min) on HPLC	Rt (min) on GC-MS	Compound	Principal ions and relative abundance (% base peak)
Authentic				
ent-isokaurene	9.8	7.53		272 (M^+, 100), 257 (70), 244 (22), 229 (55), 213 (24), 201 (19), 187 (37)
Authentic				
ent-kaurene	9.8	7.78		272 (M^+, 87), 257 (100), 229 (75), 213 (38), 201 (15), 187 (20), 175 (25)
Authentic				
squalene	12.4	11.98		410 (M^+, 10), 367 (6), 341 (9), 273 (6), 231 (9), 217 (8), 137 (100), 121 (70)
Nihonbare				
14-day-old	8–11	7.82	*ent*-kaurene	272 (M^+, 58), 257 (100), 229 (56)
	11–15	11.97	squalene	410 (M^+, 10), 367 (6), 341 (11), 273 (6), 137 (100)
28-day-old	8–11	7.53	*ent*-isokaurene	272 (M^+, 100), 257 (77), 244 (33), 229 (50), 213 (18)
	8–11	7.82	*ent*-kaurene	272 (M^+, 83), 257 (100), 229 (50), 213 (27), 201 (23)
	11–15	11.98	squalene	410 (M^+, 10), 367 (6), 341 (9), 273 (6), 137 (100)
Tan-ginbozu				
14-day-old	8–11	7.80	*ent*-kaurene	272 (M^+, 65), 257 (100), 229 (63), 213 (22), 201 (20)
	11–15	11.59	squalene	410 (M^+, 10), 367 (6), 341 (12), 273 (6), 137 (100)
28-day-old	8–11	7.57	*ent*-isokaurene	272 (M^+, 100), 257 (60), 229 (28)
	8–11	7.80	*ent*-kaurene	272 (M^+, 60), 257 (100), 229 (42), 213 (22), 201 (20)
	11–15	11.95	squalene	410 (M^+, 10), 367 (6), 341 (8), 273 (6), 137 (100)
Waito-C				
14-day-old	8–11	7.82	*ent*-kaurene	272 (M^+, 75), 257 (100), 229 (38), 213 (25)
	11–15	11.98	squalene	410 (M^+, 10), 367 (7), 341 (11), 273 (5), 137 (100)
28-day-old	8–11	7.55	*ent*-isokaurene	272 (M^+, 100), 257 (84), 229 (42)
	8–11	7.83	*ent*-kaurene	272 (M^+, 80), 257 (100), 229 (70), 213 (36), 201 (20)
	11–15	11.95	squalene	410 (M^+, 10), 367 (6), 341 (5), 273 (6), 137 (100)

operating conditions were: column composition, a capillary column HP-1 (0.2 mm diameter and 12.5 m length, 0.33-μm-thick stationary phase); He carrier gas, 0.5 kg cm^{-2}; 160°C (for 2 min) to 280°C at 16°C/min; ionization voltage, 70 eV; and selected ions, m/z 272, 257, and 229 (for *ent*-isokaurene and *ent*-kaurene) and m/z 410, 367, and 341 (for squalene). For quantitation, the ions at m/z 272 (for *ent*-kaurene and *ent*-isokaurene) and at m/z 410 (for squalene) were used. Standard calibration curves were produced using known amounts of authentic compounds. Retention times of *ent*-isokaurene, *ent*-kaurene, and squalene in these analytical conditions were 6.34, 6.62, and 11.10 min, respectively.

Results and Discussion

Ent-kaurene and squalene were identified by retention times and full mass spectra from GC-MS analyses of purified 14-day-old and 28-day-old extracts of all three cultivars (Table 1). *Ent*-isokaurene was similarly identified in the 28-day-old plant extracts of all three cultivars, but not any 14-day-old plant extracts. Since no information on recovery ratios of such very lipophilic compounds was available, the approximate recovery ratio of *ent*-kaurene was investigated. Ten micrograms of authentic *ent*-kaurene was subjected to the same procedures as in the case of purification of the plant extracts, and its recovery ratio at each purification step was determined by GC-SIM. The percentages of recovery of *ent*-kaurene after extraction with n-hexane against an aqueous layer, extraction with n-hexane against 90% aqueous methanol, silica gel chromatography, and HPLC were approximately 100, 92, 87, and 38, respectively. Recovery ratios were not determined for *ent*-isokaurene and squalene. No correction was made for the losses of compounds that occurred during the purification procedures. Since the analyses were performed only once, the data should be regarded as conclusive qualitatively but as only closely approximate quantitatively.

The concentrations of *ent*-kaurene were as low as <1 ng/g f.w. in extracts of 14-day-old Waito-C (dwarf) shoots to as much as approximately 13 ng/g f.w. in extracts of 14-day-old Tan-ginbozu (dwarf) shoot extracts (Table 2). Concentrations were approximately an order of magnitude higher in extracts of shoots of 28-day-old plants and ranged from approximately 26 ng/g f.w. for Waito-C to approximately 147 ng/g f.w. for Nihonbare. It is relevant that Ecklund and Moore (1974) observed an approximately exponential increase from nearly zero in the rate of synthesis of *ent*-kaurene in cell-free enzyme extracts prepared from pea shoots from the third to the ninth day after planting. Also, Kurogochi et al. (1979) found an increase of more than fivefold in endogenous GA_{19} in Nihonbare rice plants from the sixth-leaf stage to the panicle-initiation stage. Thus large changes in endogenous *ent*-kaurene and GA levels evidently do occur.

The concentrations of *ent*-kaurene in the shoots of Waito-C plants were considerably lower than those in Tan-ginbozu and Nihonbare. Murakami (1972) postulated that Waito-C plants have a genetic block in the conversion of GA_{20} to GA_1, the latter being considered to be the GA active in the growth of rice

Table 2. Concentrations of *ent*-kaurene (K) and *ent*-isokaurene (IK) in extracts of rice shoots.

Cultivar	14-day-old plants (ng/g f.w.)		28-day-old plants (ng/g f.w.)	
	K	IK	K	IK
Nihonbare	10	—[a]	147	2.8
Tan-ginbozu	13	—	102	6.1
Waito-C	<1	—	26	Trace

[a] Not detected by GC-MS.

Table 3. Concentrations of squalene in extracts of rice shoots.

Cultivar	14-day-old plants (ng/g f.w.)	28-day-old plants (ng/g f.w.)
Nihonbare	626	499
Tan-ginbozu	441	232
Waito-C	485	19

(Kurogochi et al. 1979, Suzuki et al. 1981). Synthesis of a considerably large amount of *ent*-kaurene and its relatively rapid conversion to GAs in Waito-C seems unlikely, since Kobayashi (1987) did not find a large accumulation of GA_{20} in shoots of month-old Waito-C plants. He found a few ng/g f.w. of GA_{20} in Waito-C shoots compared to <1 ng/g f.w. in Nihonbare.

Dwarfism in the Tan-ginbozu cultivar appears not to be correlated with a deficient production of *ent*-kaurene in 14-day-old plants, although it might be in 28-day-old plants. The cause of dwarfism in Tan-ginbozu has previously been attributed to deficiency in the biosynthesis of *ent*-kaurene (Murakami 1972), whereas the results presented in this paper do not support that conclusion. Further investigation on the cause of dwarfism in Tan-ginbozu will be required.

Ent-isokaurene is a "dead-end" isomer of *ent*-kaurene which is not converted to GA (Hedden et al. 1977). Whether the trace amounts of *ent*-isokaurene found in these investigations were formed enzymically from copalyl pyrophosphate, as postulated for the dwarf-5 mutant of *Zea mays* (Hedden and Phinney 1979), or isomerized nonenzymically from *ent*-kaurene, as described by Appleton et al. (1966), during extraction is not known. Whichever the case, the amounts of *ent*-isokaurene found in the present investigations appear to be too small to be important in dwarfism even if formed enzymically.

Substantial concentrations of squalene were also found in the same extracts containing *ent*-kaurene from all three cultivars of rice at both ages examined (Table 3). Concentrations were lower in the dwarf cultivars Waito-C and Tan-ginbozu than in the normal Nihonbare cultivar. That finding may be important to understanding the regulation of triterpenoid biosyntheses, including biosynthesis of the brassinosteroids.

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