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Isolation and Identification of Phenolic Compounds Accumulated in Brown Rice Grains Ripened under High Air Temperature

Hiroshi Nakano,*[,†] Hiroshi Ono,[‡] Norio Iwasawa,[†,§] Toshiyuki Takai,[†] Yumiko Arai-Sanoh,[†] and Motohiko Kondo[†]

[†]NARO Institute of Crop Science, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan

[‡]National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

[§]Agricultural Research Institute, Ibaraki Agricultural Center, 3402 Kamikuniicho, Mito, Ibaraki 311-4203, Japan

ABSTRACT: This study aimed to examine the compounds increasing or decreasing in concentration in brown rice grains ripened under high air temperature during ripening using a heat-tolerant cultivar Fusaotome, a heat-intolerant cultivar Hatsuboshi, and an intermediate cultivar Koshihikari. 6-*O*-Feruloylsucrose (**1**), 3′,6-di-*O*-sinapoylsucrose (**2**), 3′-*O*-sinapoyl-6-*O*-feruloylsucrose (**3**), 3′,6-di-*O*-feruloylsucrose (**4**), cycloartenyl ferulate (**5**), and 24-methylenecycloartanyl ferulate (**6**) were isolated from the extracts of brown rice grains. The structures of the isolated compounds (**1**−**6**) were elucidated on the basis of spectroscopic analyses. The mean concentrations of compounds **2**, **3**, and **6** in the grains ripened under high air temperature were markedly higher than those ripened under normal air temperature. In contrast, the mean concentration of compound **5** in the grains ripened under high air temperature was markedly lower than those ripened under normal air temperature. Thus, compounds **2**, **3**, **5**, and **6** constitute potential biomarkers of heat stress in the cultivars used. The mean concentrations of compound **4** in the grains of Fusaotome were the highest in all cultivars. In contrast, the mean concentration of compound **5** in the grains of Fusaotome was the lowest. Therefore, the unique composition of heat-tolerant Fusaotome combines a high concentration of compound **4** with a low concentration of compound **5**.

KEYWORDS: *rice, Oryza sativa, grain, phenolic compound*

INTRODUCTION

Japan had 4.6 million ha of arable land, of which 2.5 million was paddy field in 2012.[1] Most of the rice (*Oryza sativa*) cultivars grown in the field are *japonica* type, and the amount of production of brown rice grains was 8.5 million tons in 2012. Recently, a significant deterioration in the quality of the rice grains, caused by high air temperature during ripening, has been reported in several regions.[2,3] This is considered to be partially caused by global warming.[4] Because rice deterioration decreases farmers' income, the development of breeding programs to select heat-tolerant cultivars has become a priority.

High air temperature during rice plant ripening reduces grain weight and generates several types of chalky grains described as milky-white, white-core, white-back, white-belly, or basal-white.[5] The chalky parts in the endosperm have air spaces between starch granules appearing white areas by light scattering.[6] High air temperature during ripening also increases the thickness of the bran layer,[7] suggesting changes in chemical constituents. However, the rice grain constituents affected by high air temperature during ripening have not been isolated from the bran layer.

Previous studies reported natural variation in heat tolerance during ripening among the different *japonica* cultivars. For example, Fusaotome and Koshijiwase are categorized as heat-tolerant cultivars whereas Hatsuboshi and Chiyonishiki were categorized as heat-intolerant cultivars.[8−10] Quantitative trait locus (QTL) analyses of grain chalkiness have been performed using genetic materials derived from a cross between heat-tolerant and heat-intolerant cultivars such as Hana-echizen × Niigatawase and Koshijiwase × Chiyonishiki. Several QTLs have been associated with the occurrence of white-back and basal-white.[11,12] However, the genes responsible for white-back and basal-white have not been identified. In addition, QTLs specific for the other grain chalkiness (i.e., milky-white, white-core, and white-belly) have not been well examined.

The genetic analysis of heat stress-related chalky grains has been stalled by the lack of precise methodology to evaluate grain chalkiness. Studies evaluating the quality of grains ripened under high air temperature have used equipment or visual examination. Accordingly, quality assessment varies with the types of equipment or experimenter. In contrast, quality assessment relying on the concentrations of specific rice grain compounds sensitive to heat would provide a reliable and reproducible measure of the extent of damage. This approach may lead to the development of biomarkers (i.e., indicators) of heat stress and selection criteria for chalky grains in breeding programs. The objective of the present study was to isolate the compounds increasing or decreasing in concentration in brown rice grains ripened under high air temperature by using heat-tolerant (Fusaotome), heat-intolerant (Hatsuboshi), and intermediate (Koshihikari) cultivars. We then tested the potential of each compound as biomarkers of heat stress and

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compared their concentrations in the three cultivars to determine the compound profile of heat-tolerant Fusaotome.

MATERIALS AND METHODS

General Procedures. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 800 and 500 spectrometers, respectively. High-resolution electron impact mass spectrometry (HRESIMS) spectra were recorded on a Bruker Daltonics Apex II 70e. High-performance liquid chromatography (HPLC) was performed using a Waters Alliance HPLC system.

Plant Materials for Comparison of Extracts and Determination of Compounds. Rice cultivars Fusaotome, Koshihikari, and Hatsuboshi were grown at the NARO Institute of Crop Science (36°03′ N, 140°09′ E) in 2012. Daily mean temperature and solar radiation in 2012 were shown in Table 1. The germinated seeds were sown in nursery boxes in late April. The seedlings were transplanted by hand into 1/5000 a Wagner pots filled with paddy soil at a density of two seedlings per pot in late May. The plants were grown outside and then moved to Espec phytotrons after flowering (early August). In Japan, rice plants flower between July and August that are the hottest months (Table 1). As a result, rice plants are not exposed to high temperature during vegetative stage. Therefore, the temperature treatment was conducted after flowering. The air temperature in the phytotron was 32/26 °C (14/10 h, day/night) for the high air temperature treatment or 26/20 °C (14/10 h, day/night) for the normal air temperature treatment. At maturity (mid-September for Fusaotome and late September for Koshihikari and Hatsuboshi), the panicles were harvested, and then, air-dried panicles were threshed. The rough grains were dehusked, weighed, and then ground using a Retsch MM301 mixer mill. The weights of brown rice grains ripened under high air temperature (22.7, 21.5, and 21.6 mg/seed for Fusaotome, Koshihikari, and Hatsuboshi, respectively) were lower than those ripened under normal air temperature (25.7, 23.4, and 25.4 mg/seed for Fusaotome, Koshihikari, and Hatsuboshi, respectively).

Table 1. Temperature and Solar Radiation at the NARO Institute of Crop Science, Tsukuba, Ibaraki, Japan, in 2012

month	stage of month	mean temp (°C)	max temp (°C)	min temp (°C)	solar radiation (MJ m^{-2} s^{-1})
April	early	10.0	16.9	1.8	20.9
	middle	12.3	17.8	7.1	16.0
	late	15.2	19.7	11.4	13.8
May	early	18.0	23.2	13.6	15.2
	middle	17.5	23.6	11.5	21.6
	late	18.5	24.1	14.2	20.7
June	early	19.8	25.2	15.6	19.5
	middle	19.7	24.0	16.4	16.3
	late	19.6	24.3	16.0	20.6
July	early	23.0	28.1	19.5	17.7
	middle	26.0	30.6	22.9	19.6
	late	26.4	31.5	22.3	20.0
August	early	26.7	32.3	22.6	22.3
	middle	27.7	33.3	23.9	18.6
	late	28.4	35.0	23.3	23.1
September	early	25.9	31.8	22.0	18.0
	middle	26.3	31.8	22.6	16.4
	late	21.2	25.0	17.9	10.5

Comparison of Extracts. Powdered grains (50 seeds) of Fusaotome, Koshihikari, or Hatsuboshi were extracted with aqueous acetone (acetone/H_2O, 1:1 (v/v)) or acetone (20 mL) at 25 °C for 1 day in the dark. To compare HPLC chromatograms of the extracts, the aqueous acetone extracts were subjected to C_{18} HPLC (TSKgel ODS-80Ts, Tosoh, 4.6 mm × 250 mm; eluent, CH_3CN/H_2O/TFA, 5:95:0.1 to 35:65:0.1 (v/v) for 60 min by linear gradient; flow rate, 0.8 mL/min; UV detection at 320 nm), and the acetone extracts were subjected to C_{18} HPLC (Sunfire, Waters, 4.6 mm × 250 mm; eluent, CH_3CN/MeOH, 15:85 (v/v); flow rate, 0.8 mL/min; UV detection at 320 nm).

Plant Materials for Isolation of Compounds. Koshihikari was grown at the NARO Institute of Crop Science (36°03′ N, 140°09′ E) in 2011. The germinated seeds were sown in nursery boxes in late April. The seedlings were transplanted by hand into the paddy field in late May at a density of 22.2 hills m^{-2} with one seedling per hill. After flowering, the plants were covered with plastic sheeting to raise the air temperature. At maturity, the plants were harvested, and then, air-dried plants were threshed. The rough grains were dehusked and then ground in a CMT TI-100 vibrating sample mill.

Isolation of Compounds. Powdered grains (200 g) of Koshihikari were extracted with aqueous MeOH (MeOH/H_2O, 1:1 (v/v)) (1000 mL) at 25 °C for 4 days in the dark. The aqueous MeOH extract was filtered through a sheet of paper and then concentrated to dryness in vacuo at 30 °C. The extract (8.9 g) was subjected to step-gradient Sep-Pak C_{18} cartridge chromatography (Sep-Pak, Waters, 10 g, 55−105 μm) eluted with MeOH/H_2O (step 1, 0:100 (v/v); step 2, 20:80 (v/v); step 3, 40:60 (v/v); step 4, 60:40 (v/v); step 5, 100:0 (v/v)) to afford fractions Sm0 (7251.0 mg), Sm20 (511.4 mg), Sm40 (468.2 mg), Sm60 (392.2 mg), and Sm100 (151.8 mg), respectively. Half of the Sm40 fraction was subjected to C_{18} HPLC (TSKgel ODS-80Ts, Tosoh, 4.6 mm × 250 mm; eluent, MeOH/H_2O, 35:65 (v/v); flow rate, 0.8 mL/min; UV detection at 320 nm) to afford **1** (1.3 mg, 0.0013% yield, t_R = 11.5 min). Half of the Sm60 fraction was subjected to C_{18} HPLC (TSKgel ODS-80Ts, Tosoh, 4.6 mm × 250 mm; eluent, CH_3CN/H_2O, 26:74 (v/v); flow rate, 0.8 mL/min; UV detection at 320 nm) to afford **2** (0.4 mg, 0.0004% yield, t_R = 10.0 min), **3** (0.7 mg, 0.0007% yield, t_R = 11.0 min), and **4** (0.1 mg, 0.0001% yield, t_R = 12.0 min). In addition, powdered grains (50 g) of Koshihikari were extracted with acetone (250 mL) at 25 °C for 2 days in the dark. The acetone extract (1.8 g) was filtered through a sheet of paper and then concentrated to dryness in vacuo at 30 °C. The acetone extract was subjected to step-gradient Sep-Pak C_{18} cartridge chromatography (Sep-Pak, Waters, 10 g, 55−105 μm) eluted with MeOH/H_2O (80:20 (v/v)) and then with MeOH/acetone (80:20 (v/v)) to afford fractions Sw20 (7251.0 mg) and Sa20 (511.4 mg), respectively. Fraction Sa20 was subjected to C_{18} HPLC (Sunfire, Waters, 4.6 mm × 250 mm; eluent, MeOH/CH_3CN, 85:12 (v/v); flow rate, 0.8 mL/min; UV detection at 320 nm) to afford **5** (1.0 mg, 0.0020% yield, t_R = 30.0 min) and **6** (3.2 mg, 0.0064% yield, t_R = 32.5 min).

Determination of Compounds. Powdered grains (50 seeds) of Fusaotome, Koshihikari, or Hatsuboshi were extracted with aqueous acetone (acetone/H_2O, 1:1 (v/v)) or acetone (20 mL) at 25 °C for 1 day in the dark. The aqueous acetone extracts were subjected to C_{18} HPLC (TSKgel ODS-80Ts, Tosoh, 4.6 mm × 250 mm; eluent, CH_3CN/H_2O/TFA, 5:95:0.1 to 35:65:0.1 (v/v) for 60 min by liner gradient; flow rate, 0.8 mL/min; UV detection at 320 nm) to determine **1** (t_R = 27.5 min), **2** (t_R = 43.7 min), **3** (t_R = 44.6 min), and **4** (t_R = 45.3 min). The acetone extracts were subjected to C_{18} HPLC (Sunfire, Waters, 4.6 mm × 250 mm; eluent, CH_3CN/MeOH, 15:85 (v/v); flow rate, 0.8 mL/min; UV detection at 320 nm) to determine **5** (t_R = 29.4 min), **6** (t_R = 31.5 min), and γ-oryzanol (sum of **5**, **6**, **7** (t_R = 33.6 min), and **8** (t_R = 37.4 min)). The identity of peaks **5**−**8** for γ-oryzanol were confirmed with commercially available γ-oryzanol (Wako Chemicals) and by comparison with literature data.[13,14] The amounts of **1**−**6** and γ-oryzanol were calculated using standard curves based on peak areas. The recovery rates of compounds **1**−**6** were considered to be more than 95%. The detection limits of compounds **1**−**6** were estimated to be about 10 nmol per 25.0 μL injection (S/N = 3). This experiment was replicated three times.

Statistical Analysis. Statistical analyses were performed using a general linear model in SPSS (version 17.0, SPSS Inc., Chicago, IL). Treatments included two air temperatures during ripening and three cultivars analyzed by a randomized complete block design with three replicates. Analysis of variance was used to test the effect of air temperature during ripening and cultivar on concentrations of compounds **1**−**6** and γ-oryzanol. Air temperature during ripening

and cultivar was considered as fixed effects, while replication was considered as a random effect. Significant treatment effects ($P < 0.05$) were explored using Fisher's protected least significant difference (LSD).

*6-O-Feruloylsucrose (**1**).* Colorless solid; ^{1}H NMR (800 MHz, CD_3OD) δ 3.32 (1H, dd, J = 9.3, 10.1 Hz, H-4), 3.45 (1H, dd, J = 3.8, 9.3 Hz, H-2), 3.60 (1H, d, J = 12.3 Hz, H-1′a), 3.62 (1H, d, J = 12.3 Hz, H-1′b), 3.72 (1H, t, J = 9.3 Hz, H-3), 3.76 (1H, dd, J = 2.4, 11.8 Hz, H-6′a), 3.79 (1H, ddd, J = 2.4, 6.5, 8.3 Hz, H-5′), 3.86 (1H, dd, J = 6.5, 11.8 Hz, H-6′b), 3.88 (1H, s, H-10″), 4.04 (1H, t, J = 8.3 Hz, H-4′), 4.09 (1H, d, J = 8.3 Hz, H-3′), 4.11 (1H, ddd, J = 2.0, 6.2, 10.1 Hz, H-5), 4.26 (1H, dd, J = 6.2, 11.9 Hz, H-6a), 4.49 (1H, dd, J = 2.0, 11.9 Hz, H-6b), 5.41 (1H, d, J = 3.8, H-1), 6.43 (1H, d, J = 15.9, H-8″), 6.80 (1H, d, J = 8.3 Hz, H-5 ″), 7.09 (1H, dd, J = 1.9, 8.3 Hz, H-3″), 7.22 (1H, d, J = 1.9 Hz, H-2″), 7.63 (1H, d, J = 15.9 Hz, H-7″); ^{13}C NMR (125 MHz, CD_3OD) δ 56.5 (C-10″), 64.2 (C-6′), 64.3 (C-1′), 65.1 (C-6), 71.9 (C-4), 72.1 (C-5), 73.2 (C-2), 74.6 (C-3), 76.1 (C-4′), 79.3 (C-3′), 83.9 (C-5′), 93.3 (C-1), 105.2 (C-2′), 111.7 (C-2″), 115.3 (C-8″), 116.5 (C-5″), 124.3 (C-6″), 127.6 (C-1″), 147.1 (C-7″), 149.5 (C-3″), 150.9 (C-4″), 169.2 (C-9″); HRESIMS m/z 541.1514 $[M + Na]^+$, calcd for $C_{22}H_{30}O_{14}Na$, 541.1528.

*3′,6-Di-O-sinapoylsucrose (**2**).* Colorless solid; ^{1}H NMR (800 MHz, CD_3OD) δ 3.30 (1H, m, H-4), 3.46 (1H, dd, J = 3.4, 9.5 Hz, H-2), 3.58 (1H, d, J = 12.2 Hz, H-1′a), 3.61 (1H, d, J = 12.2 Hz, H-1′b), 3.66 (1H, t, J = 9.5 Hz, H-3), 3.83 (1H, m, H-6′a), 3.89 (3H, s, H-11″), 3.89 (3H, s, H-10″), 3.89 (3H, s, H-11‴), 3.89 (3H, s, H-10‴), 3.89 (1H, m, H-6′b), 3.97 (1H, ddd, J = 2.1, 6.1, 8.1 Hz, H-5′), 4.20 (1H, dd, J = 7.5, 11.7 Hz, H-6a), 4.28 (1H, ddd, J = 1.7, 7.5, 9.7 Hz, H-5), 4.49 (1H, t, J = 8.1 Hz, H-4′), 4.68 (1H, J = 1.7, 11.7 Hz, H-6b), 5.50 (1H, d, J = 8.1 Hz, H-3′), 5.51 (1H, d, J = 3.4 Hz, H-1), 6.44 (1H, d, J = 15.8 Hz, H-8‴), 6.46 (1H, d, J = 15.9 Hz, H-8″), 6.88 (1H, s, H-6″), 6.88 (1H, s, H-2″), 6.93 (1H, s, H-6‴), 6.93 (1H, s, H-2‴), 7.59 (1H, d, J = 15.9 Hz, H-7″), 7.67 (1H, d, J = 15.8 Hz, H-7‴); ^{13}C NMR (125 MHz, CD_3OD) δ 56.8 (C-11″), 56.8 (C-10″), 56.9 (C-11‴), 56.9 (C-10‴), 63.9 (C-6′), 65.7 (C-6), 65.7 (C-1′), 72.0 (C-4), 72.5 (C-5), 73.1 (C-2), 74.2 (C-4′), 75.1 (C-3), 79.3 (C-3′), 84.4 (C-5′), 92.7 (C-1), 104.9 (C-2′), 106.9 (C-6″), 106.9 (C-2″), 107.1 (C-6‴), 107.1 (C-2‴), 115.5 (C-8‴), 115.9 (C-8″), 126.6 (C-1″), 126.6 (C-1‴), 139.6 (C-4″), 139.7 (C-4‴), 147.3 (C-7″), 147.9 (C-7‴), 149.4 (C-5″), 149.4 (C-3″), 149.4 (C-5‴), 149.4 (C-3‴), 168.2 (C-9‴), 169.1 (C-9″); HRESIMS m/z 777.2205 $[M + Na]^+$, calcd for $C_{34}H_{42}O_{19}Na$, 777.2213.

*3′-O-Sinapoyl-6-O-feruloylsucrose (**3**).* Colorless solid; ^{1}H NMR (800 MHz, CD_3OD) δ 3.32 (1H, m, H-4), 3.46 (1H, dd, J = 3.3, 9.3 Hz, H-2), 3.58 (1H, d, J = 12.3 Hz, H-1′a), 3.62 (1H, d, J = 12.3 Hz, H-1′b), 3.66 (1H, t, J = 9.3 Hz, H-3), 3.82 (1H, dd, J = 2.5, 12.0 Hz, H-6′a), 3.86 (3H, s, H-10″), 3.87 (3H, s, H-11‴), 3.87 (3H, s, H-10‴), 3.89 (1H, dd, J = 6.6, 12.0 Hz, H-6′b), 3.96 (1H, ddd, J = 2.5, 6.6, 8.1 Hz, H-5′), 4.22 (1H, dd, J = 7.1, 11.6 Hz, H-6a), 4.26 (1H, ddd, J = 1.5, 7.1, 9.9 Hz, H-5), 4.47 (1H, t, J = 8.1 Hz, H-4′), 4.64 (1H, dd, J = 1.5, 11.6 Hz, H-6b), 5.49 (1H, d, J = 8.1 Hz, H-3′), 5.50 (1H, d, J = 3.3 Hz, H-1), 6.44 (1H, d, J = 15.9 Hz, H-8″), 6.45 (1H, d, J = 15.8 Hz, H-8‴), 6.77 (1H, d, J = 8.2 Hz, H-5″), 6.94 (1H, s, H-6‴), 6.94 (1H, s, H-2‴), 7.03 (1H, dd, J = 1.9, 8.2 Hz, H-6″), 7.19 (1H, d, J = 1.9 Hz, H-2″), 7.60 (1H, d, J = 15.9 Hz, H-7″), 7.68 (1H, d, J = 15.8 Hz, H-7‴); ^{13}C NMR (125 MHz, CD_3OD) δ 56.5 (C-10″), 56.9 (C-11‴), 56.9 (C-10‴), 63.8 (C-6′), 65.5 (C-6), 65.7 (C-1′), 72.0 (C-4), 72.5 (C-5), 73.1 (C-5), 74.2 (C-4′), 75.1(C-3), 79.4 (C-3′), 84.4 (C-5′), 92.7 (C-1), 104.9 (C-2′), 107.1 (C-6‴), 107.1 (C-2‴), 111.6 (C-2″), 115.4 (C-8″), 115.5 (C-8‴), 116.4 (C-5″), 124.3 (C-6″), 126.6 (C-1‴), 127.7 (C-1″), 139.7 (C-4″), 147.1 (C-7″), 147.9 (C-7‴), 149.5 (C-3″), 149.5 (C-5‴), 149.5 (C-3‴), 150.1 (C-4″), 168.2 (C-9‴), 169.2 (C-9″); HRESIMS m/z 747.2083 $[M + Na]^+$, calcd for $C_{33}H_{40}O_{18}Na$, 747.2107.

*3′,6-Di-O-feruloylsucrose (**4**).* Colorless solid; ^{1}H NMR (800 MHz, CD_3OD) δ 3.32 (1H, m, H-4), 3.46 (1H, dd, J = 3.8, 9.3 Hz, H-2), 3.58 (1H, d, J = 12.2 Hz, H-1′a), 3.62 (1H, d, J = 12.2 Hz, H-1′b), 3.66 (1H, t, J = 9.3 Hz, H-3), 3.81 (1H, dd, J = 2.5, 12.1 Hz, H-6′a), 3.86 (3H, s, H-10″), 3.88 (1H, m, H-6′b), 3.89 (3H, s, H-10‴), 3.96 (1H, ddd, J = 2.5, 6.1, 8.1 Hz, H-5′), 4.24 (1H, m, H-6a), 4.24 (1H, m, H-5), 4.46 (1H, t, J = 8.1 Hz, H-4′), 4.63 (1H, m, H-6b), 5.49 (1H, d, J = 8.1, H-3′), 5.49 (1H, d, J = 3.8, H-1), 6.43 (1H, d, J = 15.9 Hz, H-8‴), 6.44 (1H, d, J = 15.9 Hz, H-8″), 6.78 (1H, d, J = 8.2 Hz, H-5″), 6.80 (1H, d, J = 8.2 Hz, H-5‴), 6.78 (1H, d, J = 8.2 Hz, H-5‴), 7.11 (1H, dd, J = 1.9, 8.2 Hz, H-6″), 7.11 (1H, dd, J = 1.8, 8.2 Hz, H-6‴), 7.20 (1H, d, J = 1.9 Hz, H-2″), 7.23 (1H, d, J = 1.8 Hz, H-2‴), 7.61 (1H, d, J = 15.9 Hz, H-7″), 7.69 (1H, d, J = 15.9 Hz, H-7‴); ^{13}C NMR (125 MHz, CD_3OD) δ 56.5 (C-10″), 56.5 (C-10‴), 63.9 (C-6′), 65.5 (C-6), 65.6 (C-1′), 71.9 (C-4), 72.4 (C-5), 73.1 (C-2), 74.2 (C-4′), 75.0 (C-3), 79.4 (C-3′), 84.3 (C-5′), 92.8 (C-1), 104.8 (C-2′), 111.6 (C-2″), 112.1 (C-2‴), 115.1 (C-8‴), 115.4 (C-8″), 116.4 (C-5″), 116.4 (C-5‴),124.2 (C-6‴), 124.4 (C-6″), 127.7 (C-1‴), 127.7 (C-1″), 147.1 (C-7″), 147.7 (C-7‴), 149.4 (C-3‴), 149.4 (C-3″), 150.7 (C-4″), 150.7 (C-4‴), 168.4 (C-9‴), 169.2 (C-9″); HRESIMS m/z 717.1988 $[M + Na]^+$, calcd for $C_{32}H_{38}O_{17}Na$, 717.2001.

*Cycloartenyl Ferulate (**5**).* Colorless solid; ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.37 (1H, d, J = 3.8 Hz, H-19a), 0.60 (1H, d, J = 3.8 Hz, H-19b), 0.82 (1H, m, H-6a), 0.89 (3H, s, H-21), 0.90 (3H, s, H-28), 0.91 (3H, s, H-30), 0.97 (3H, s, H-18), 0.98 (3H, s, H-29), 1.05 (1H, m, H-22a), 1.11 (1H, m, H-7a), 1.14 (1H, m, H-11a), 1.29 (1H, m, H-1a), 1.29 (1H, m, H-15a), 1.29 (1H, m, H-16a), 1.29 (1H, m, H-16b), 1.34 (1H, m, H-7b), 1.39 (1H, m, H-20), 1.44 (1H, m, H-22b), 1.45 (1H, m, H-5), 1.53 (1H, m, H-8), 1.59 (1H, m, H-17), 1.60 (1H, m, H-6b), 1.61 (3H, s, H-27), 1.63 (1H, m, H-12a), 1.63 (1H, m, H-12b), 1.67 (1H, m, H-1b), 1.67 (1H, m, H-2a), 1.69 (3H, s, H-26), 1.84 (1H, m, H-2b), 1.87 (1H, m, H-23a), 1.90 (1H, m, 15b), 2.00 (1H, m, H-11b), 2.05 (1H, m, H-23b), 3.94 (3H, s, H-10′), 4.71 (1H, m, J = 4.4, 11.1 Hz, H-3), 5.10 (1H, m, H-24), 6.30 (1H, d, J = 15.9 Hz, H-2′), 6.92 (1H, d, J = 8.2 Hz, H-8′), 7.04 (1H, d, J = 1.6 Hz, H-5′), 7.08 (1H, dd, J = 1.6, 8.2 Hz, H-9′), 7.60 (1H, d, J = 15.9 Hz, H-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.3 C-29), 17.6 (C-27), 18.0 (C-18), 18.3 (C-21), 19.3 (C-30), 20.2 (C-9), 21.0 (C-6), 24.9 (C-23), 25.5 (C-28), 25.7 (C-26), 25.8 (C-7), 26.0 (C-10), 26.5 (C-11), 27.0 (C-2), 28.1 (C-15), 29.8 (C-19), 31.6 (C-1), 32.9 (C-12), 35.5 (C-16), 35.9 (C-20), 36.3 (C-22), 39.7 (C-4), 45.3 (C-13), 47.2 (C-5), 47.9 (C-8), 48.8 (C-14), 52.3 (C-17), 56.0 (C-10′), 80.5 (C-3), 109.2 (C-5′), 114.6 (C-8′), 116.3 (C-2′), 123.0 (C-9′), 125.3 (C-24), 127.2 (C-4′), 130.9 (C-25), 144.3 (C-3′), 146.7 (C-6′), 147.8 (C-7′), 167.1 (C-1′).

*24-Methylenecycloartanyl Ferulate (**6**).* Colorless solid; ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.37 (1H, d, J = 3.8 Hz, H-19a), 0.61 (1H, d, J = 3.8 Hz, H-19b), 0.82 (1H, m, H-6a), 0.90 (3H, s, H-28), 0.90 (3H, m, H-21), 0.92 (3H, s, H-31), 0.98 (3H, m, H-18), 0.98 (3H, m, H-29), 1.03 (3H, m, H-27), 1.03 (3H, m, H-26), 1.11 (1H, m, H-7a), 1.14 (1H, m, H-11a), 1.15 (1H, m, H-22a), 1.29 (1H, m, H-1), 1.30 (1H, m, H-15a), 1.30 (1H, m, H-16a), 1.30 (1H, m, H-16b), 1.34 (1H, m, H-7b), 1.41 (1H, m, H-20), 1.45 (1H, m, H-5), 1.53 (1H, m, H-8), 1.58 (1H, m, H-22b), 1.60 (1H, m, H-6b), 1.62 (1H, m, H-17), 1.64 (1H, m, H-12a), 1.64 (1H, m, H-12b), 1.67 (1H, m, H-1b), 1.68 (1H, m, H-2a), 1.84 (1H, m, H-2b), 1.89 (1H, m, H-23a), 1.93 (1H, m, H-15b), 2.01 (1H, m, H-11b), 2.13 (1H. m, H-23b), 2.24 (1H, m, H-25), 3.93 (3H, s, H-10′), 4.67 (1H, m, H-30a), 4.72 (1H, m, H-3), 4.72 (1H, m, H-30b), 6.30 (1H, d, J = 15.9 Hz, H-2′), 6.92 (1H, d, J = 8.2 Hz, H-8′), 7.04 (1H, d, J = 1.6 Hz, H-5′), 7.08 (1H, dd, J = 1.6, 8.2 Hz, H-9′), 7.60 (1H, d, J = 15.9 Hz, H-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.3 (C-29), 18.0 (C-18), 18.3 (C-21), 19.3 (C-31), 20.2 (C-9), 21.0 (C-6), 21.9 (C-27), 22.0 (C-26), 25.5 (C-28), 25.8 (C-7), 26.0 (C-10), 26.5 (C-11), 27.0 (C-2), 28.1 (C-15), 29.7 (C-19), 31.3 (C-23), 31.6 (C-1), 32.9 (C-12), 33.8 (C-25), 35.0 (C-22), 35.5 (C-16), 36.1 (C-20), 39.7 (C-4), 45.3 (C-13), 47.2 (C-5), 47.9 (C-8), 48.8 (C-14), 52.2 (C-17), 56.0 (C-10′), 80.5 (C-3), 105.9 (C-30), 109.2 (C-5′), 114.6 (C-8′), 116.3 (C-2′), 123.0 (C-9′), 127.2 (C-4′), 144.3 (C-3′), 146.7 (C-6′), 147.8 (C-7′), 156.9 (C-24), 167.1 (C-1′).

RESULTS AND DISCUSSION

The HPLC chromatograms of the aqueous acetone extracts of brown rice grains ripened under high air temperature and normal air temperature were compared at the level of peak area per seed in the rice cultivars Fusaotome, Koshihikari, and Hatsuboshi (Figures 1 and 2). The extracts of grains ripened

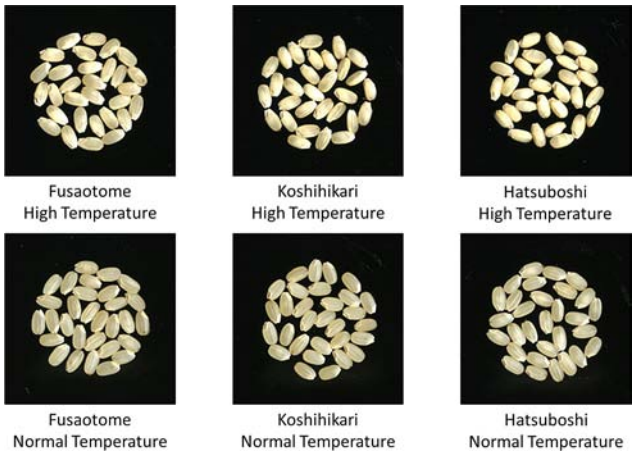


Figure 1. Brown rice grains of the cultivars Fusaotome, Koshihikari, and Hatsuboshi ripened under high or normal temperature.

under high air temperature had two larger peaks **2** and **3**, which were detected at 320 nm, than those ripened under normal air temperature in all cultivars. In contrast, the extracts of grains ripened under high air temperature had a smaller peak **4**, which was detected at 320 nm, than those ripened under normal air temperature in all cultivars. Compounds **2**−**4** corresponding to peaks **2**−**4**, respectively, in the extracts were isolated to identify the constituents affected by ripening air temperature along with the major compound **1** corresponding to peak **1** with a similar UV spectrum to peaks **2**−**4**.

The one- and two-dimensional (1D and 2D) NMR and HRESIMS spectra of compound **1** (Figure 3) revealed the presence of a sucrose moiety and a sinapoyl moiety. The HMBC correlation for H-6 to C-9″ and their chemical shifts indicated that one sinapoyl moiety is attached to C-6 of sucrose. Thus, compound **1** was confirmed to be 6-*O*-feruloylsucrose,[15,16] which was previously isolated from brown rice grains.[16] The 1D and 2D NMR and HRESIMS spectra of compound **2** (Figure 3) revealed the presence of a sucrose moiety and two sinapoyl moieties. The HMBC correlation for H-6 to C-9″ and their chemical shifts indicated that one sinapoyl moiety is attached to C-6 of sucrose. The HMBC correlation for H-3′ to C-9‴ and their chemical shifts indicated that the other sinapoyl moiety is attached to C-3′ of sucrose. Thus, compound **2** was confirmed to be 3′,6-di-*O*-sinapoylsu-

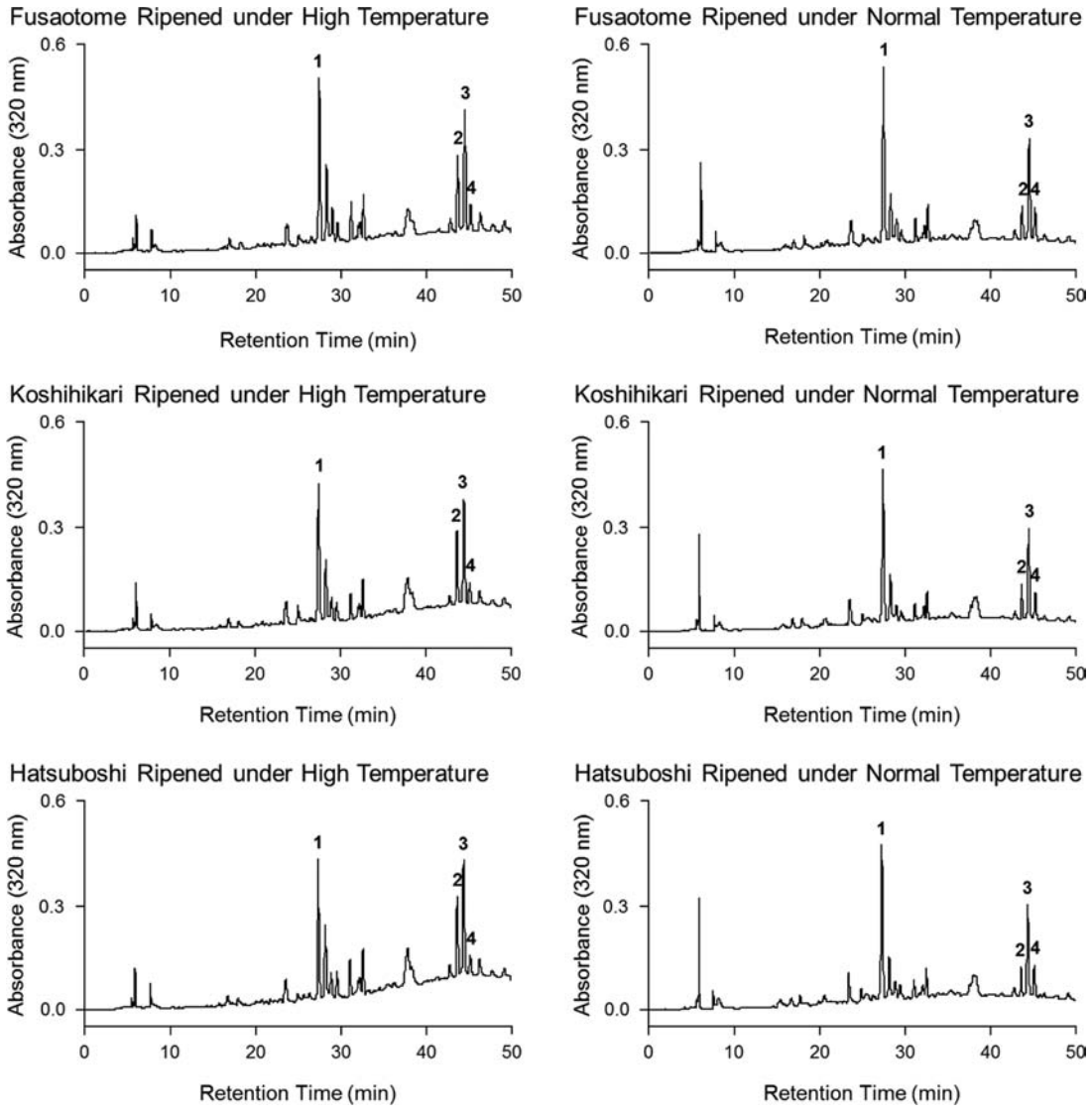


Figure 2. HPLC chromatograms for the aqueous acetone extracts of brown rice grains ripened under high or normal air temperature.

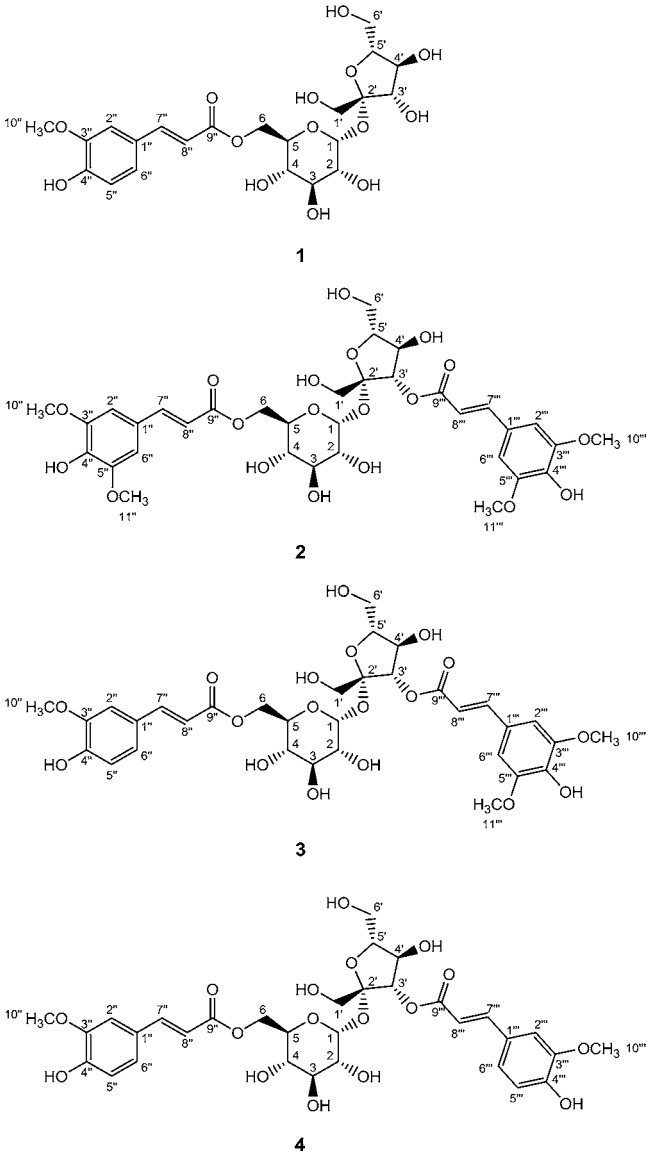


Figure 3. Chemical structures of compounds **1**−**4** isolated from aqueous acetone extracts of brown rice grains.

crose.[15] The 1D and 2D NMR spectra and HRESIMS of compound **3** (Figure 3) revealed the presence of a sucrose moiety, a sinapoyl moiety, and a feruloyl moiety. The HMBC correlation for H-6 to C-9″ and their chemical shifts indicated that a feruloyl moiety is attached to C-6 of sucrose. The HMBC correlation for H-3′ to C-9‴ and their chemical shifts indicated that a sinapoyl moiety is attached to C-3′ of sucrose. Thus, compound **3** was confirmed to be 3′-*O*-sinapoyl-6-*O*-feruloylsucrose.[17] The 1D and 2D NMR and HRESIMS spectra of compound **4** (Figure 3) revealed the presence of a sucrose moiety and two feruloyl moieties. The HMBC correlation for H-6 to C-9″ and their chemical shifts indicated that one feruloyl moiety is attached to C-6 of sucrose. The HMBC correlation for H-3′ to C-9‴ and their chemical shifts indicated that the other feruloyl moiety is attached to C-3′ of sucrose. Thus, compound **4** was confirmed to be 3′,6-di-*O*-feruloylsucrose.[18] This is the first report on the presence of compounds **2**−**4** in Gramineae. Compounds **1**−**4**, which belong to the class of compounds designated as phenylpropanoid sucrose ester and possess some important health care properties, have been isolated from *Polygala* genus, medicinal plants.[19]

The HPLC chromatograms of the acetone extracts of brown rice grains ripened under high air temperature and normal air temperature were compared at the level of peak area per seed in the rice cultivars Fusaotome, Koshihikari, and Hatsuboshi (Figures 1 and 4). The extracts of grains ripened under high air temperature had a larger peak **6**, which was detected at 320 nm, than those ripened under normal air temperature in all cultivars. In contrast, the extracts of grains ripened under high air temperature had a smaller peak **5**, which was detected at 320 nm, than those ripened under normal air temperature in all cultivars. Compounds **5** and **6** corresponding to peaks **5** and **6**, respectively, in the extracts were isolated to identify the constituents affected by ripening air temperature.

The gross structure of compounds **5** and **6** (Figure 4) were elucidated by analyses of ^{1}H and ^{13}C NMR spectra, which were in agreement with literature data of cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, respectively.[20] Compounds **5** and **6** are known as major components of γ-oryzanol, a characteristic constituent in rice bran, which possesses some important health care properties.[21] Peak **7** was considered to be components of γ-oryzanol due to their 1D and 2D NMR and UV spectra and literature data[13] but was not able to be separated and isolated by HPLC. Similarly, peak **8** was also considered to be components of γ-oryzanol due to their UV spectra and literatures,[13] but was not able to be separated and isolated by HPLC.

The concentrations of compounds **1**−**4** were determined in the grains ripened under different air temperatures (Tables 2 and 3). At the level of weight per seed, the mean concentrations of compounds **2** and **3** in the grains ripened under high air temperature were 167 and 54 ng/seed higher than those ripened under normal air temperature, respectively (Table 2). Similarly, at the level of weight per weight, they were 8.0 and 4.1 g/kg higher than those ripened under normal air temperature, respectively (Table 3). Examining the structures of compounds **1**−**4**, in addition to a sucrose moiety, compound **2** has two sinapoyl moieties, compound **3** has a sinapoyl moiety and a feruloyl moiety, compound **1** has a feruloyl moiety, and compound **4** has two feruloyl moieties (Figure 2). Thus, the increased concentration of phenylpropanoid sucrose ester in the grains by high air temperature during ripening increased with increasing the number of sinapoyl moieties or decreasing the number of feruloyl moieties.

γ-Oryzanol consists of a feruloyl moiety and one of the sterol moieties involved in brown rice grains,[21] and sterols do not combine with a sinapic acid in the grains. The concentrations of compounds **5** and **6** and γ-oryzanol were determined in the grains ripened under different air temperatures (Tables 2 and 3). At the level of weight per seed, the mean concentrations of compound **6** and γ-oryzanol in the grains ripened under high air temperature were 1837 and 2216 ng/seed higher than those ripened under normal air temperature, respectively (Table 2). Similarly, at the level of weight per weight, they were 100.2 and 151.8 mg/kg higher than those ripened under normal air temperature, respectively (Table 3). The mean concentration of γ-oryzanol in the grains under normal air temperature was similar to the literature value.[14] In contrast, at the level of weight per seed, the mean concentration of compound **5** in the grains ripened under high air temperature was 1166 ng/seed lower than those ripened under normal air temperature (Table 2). Similarly, at the level of weight per weight, it was 40.7 mg/

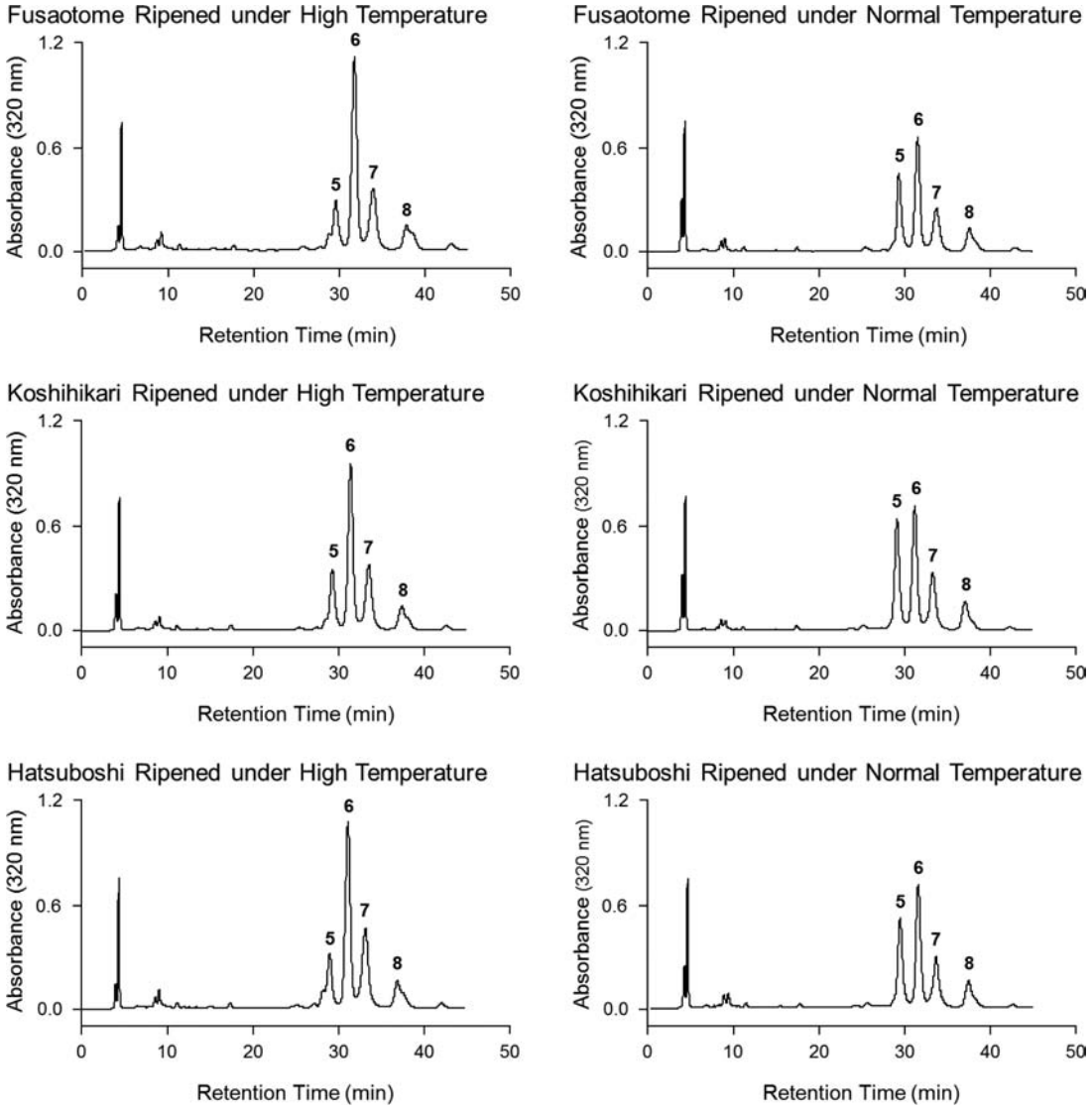


Figure 4. HPLC chromatograms of acetone extracts of brown rice grains ripened under high or normal air temperature.

kg lower than those ripened under normal air temperature (Table 3). High air temperature during ripening increases the thickness of the bran layer,[7] in which most of the γ-oryzanol in brown rice grains is accumulated.[14] Thus, although the γ-oryzanol concentration in the grains increased at high temperature during ripening, its constitution markedly changed.

Useful biomarkers of heat stress during ripening should exhibit a significant change concentration compared with normal temperature. In the present study, high air temperature during ripening markedly increased or decreased the concentrations of compounds **2**, **3**, **5**, and **6** and γ-oryzanol but not compounds **1** and **4**. Therefore, compounds **2**, **3**, **5**, and **6** and γ-oryzanol constitute potential biomarkers of heat stress in cultivars used.

Ferulic acid and sinapic acid are derived from cinnamic acid, which is generated from L-phenylalanine by the catalyzation of L-phenylalanine ammonia-lyase (PAL).[22] PAL is one of the key enzymes in the biosynthesis of phenylpropanoids. It was found that heat stress increased PAL activity and accumulated total phenols in tomato (*Lycopersicon esculentum*) and watermelon (*Citrullus lanatus*) plants.[23] In the present study, compounds with a sinapoyl moiety (or sinapoyl moieties) and a sucrose moiety accumulated in the grains at high air temperature, whereas those with a feruloyl moiety (or feruloyl moieties) and a sucrose moiety did not accumulate (Tables 2 and 3, Figure 3). However, γ-oryzanol, compounds with a feruloyl moiety and a sterol moiety, accumulated at high air temperature (Tables 2 and 3, Figure 5). These results suggest that ferulic acids produced by heat stress may combine more easily with sterol than with sucrose in brown rice grains. In contrast, sinapic acids produced by heat stress may simply combine with sucrose because they cannot combine with sterol.

Studies comparing rice grain quality among different cultivars ripened under high air temperature identified Fusaotome as a heat-tolerant, Hatsuboshi as a heat-intolerant, and Koshihikari as an intermediate cultivar.[8−10] The concentrations of compounds **1**−**4** were determined in the grains of different cultivars (Tables 2 and 3). At the level of weight per seed, the mean concentration of compound **4** of Fusaotome in the grains was the highest in all cultivars (Table 2). Similarly, at the level of weight per weight, the mean concentration of compound **4** of Fusaotome in the grains was the highest in all cultivars (Table 3).

Table 2. Concentrations of Compounds 1−6 and γ-Oryzanol in Brown Rice (ng/seed)

temp	cultivar	1		2		3		4		5		6		γ-oryzanol	
high		582		243	a[a]	356	a	89	b	1341	b	5134	a	12412	a
normal		613		76	b	302	b	104	a	2507	a	3297	b	10197	b
	Fusaotome	653	a	162		353	a	109	a	1651	c	4351		10909	
	Koshihikari	564	b	161		317	b	89	c	2274	a	3984		11397	
	Hatsuboshi	575	b	155		317	b	93	b	1849	b	4312		11608	
high	Fusaotome	639		247		385		102	bA[b]	1147		5486		12392	aAB
	Koshihikari	545		240		343		84	bB	1596		4721		11853	aB
	Hatsuboshi	560		241		339		82	bB	1280		5195		12992	aA
normal	Fusaotome	668		77		320		116	aA	2154		3215		9427	bB
	Koshihikari	582		83		291		94	aC	2951		3248		10941	aA
	Hatsuboshi	590		68		295		103	aB	2417		3429		10223	bAB
ANOVA[c]															
temp (T)		NS		[e]		[e]		[e]		[e]		[e]		[e]	
cultivar (C)		[e]		NS		NS		[e]		[e]		NS		NS	
T × C		NS		NS		NS		[d]		NS		NS		[d]	

[a]Values within a column followed by only the same lowercase letter do not differ significantly ($P < 0.05$). [b]Values within a column followed by only the same lowercase letter do not differ significantly ($P < 0.05$) between temperatures for a given cultivar. Values within a column followed by only the same uppercase letter do not differ significantly ($P < 0.05$) among cultivars for a given temperature. [c]NS, not significant at $P < 0.05$. [d]Significant at $P < 0.05$. [e]Significant at $P < 0.01$.

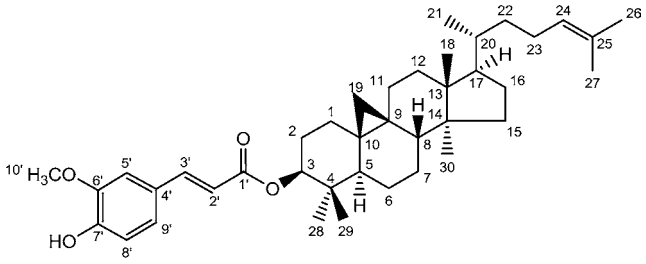
Table 3. Concentrations of Compounds 1−6 and γ-Oryzanol in Brown Rice (mg/kg)

temp	cultivar	1		2		3		4		5		6		γ-oryzanol	
high		26.6	a[a]	11.1	a	16.3	a	4.1		61.3	b	233.6	a	565.3	a
normal		24.6	b	3.1	b	12.1	b	4.2		102.0	a	133.4	b	413.6	b
	Fusaotome	27.0		7.0		14.7		4.5	a	67.2	c	182.9		455.3	b
	Koshihikari	25.0		7.3		14.1		3.9	b	100.4	a	180.0		511.5	a
	Hatsuboshi	24.7		7.0		13.8		4.0	b	77.4	b	187.5		501.6	a
high	Fusaotome	28.2		10.9		17.0		4.5		50.3		240.3		542.6	aB[b]
	Koshihikari	25.3		11.1		15.9		3.9		74.8		221.3		555.6	aAB
	Hatsuboshi	26.3		11.3		15.9		3.9		59.0		239.1		597.8	aA
normal	Fusaotome	25.8		3.0		12.4		4.5		84.1		125.5		368.0	bB
	Koshihikari	24.8		3.5		12.4		4.0		126.1		138.7		467.5	bA
	Hatsuboshi	23.1		2.7		11.6		4.1		95.8		136.0		405.3	bAB
ANOVA[c]															
cultivar (C)		[d]		[e]		[e]		NS		[e]		[e]		[e]	
temp (T)		NS		NS		NS		[e]		[e]		NS		[d]	
C × T		NS		NS		NS		NS		NS		NS		[d]	

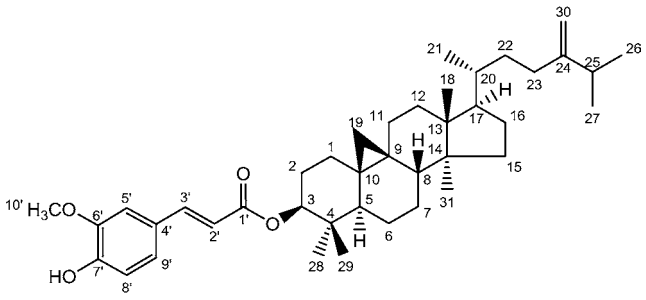
[a]Values within a column followed by only the same lowercase letter do not differ significantly ($P < 0.05$). [b]Values within a column followed by only the same lowercase letter do not differ significantly ($P < 0.05$) between temperatures for a given cultivar. Values within a column followed by only the same uppercase letter do not differ significantly ($P < 0.05$) among cultivars for a given temperature. [c]NS, not significant at $P < 0.05$. [d]Significant at $P < 0.05$. [e]Significant at $P < 0.01$.

The concentrations of compounds **5** and **6** and γ-oryzanol were determined in the grains of different cultivars (Tables 2 and 3). At the level of weight per seed, the mean concentration of compound **5** of Fusaotome was the lowest in all cultivars (Table 2). In normal air temperature, the concentration of γ-oryzanol of Fusaotome was lower than that of Koshihikari and almost the same as that of Hatsuboshi. Similarly, at the level of weight per weight, the concentration of compound **5** of Fusaotome was the lowest in all cultivars (Table 3). In normal air temperature, the concentration of γ-oryzanol of Fusaotome was lower than that of Koshihikari and almost the same as that of Hatsuboshi. These results suggest that ferulic acids may combine more easily with sucrose than with sterol in brown rice grains of Fusaotome. Altogether, these results show that the unique composition of heat-tolerant Fusaotome combines a high concentration of compound **4** with a low concentration of compound **5** and γ-oryzanol. However, the relationship between the concentrations of these compounds and heat-tolerance should be carefully examined by a number of cultivars.

Bran layers in brown rice grains generally have to be removed through the process of polishing. High air temperature during ripening increases the thickness of the bran layer.[7] As a result, the yield of polished rice is reduced by high air temperature.[4] Future studies will establish the relationship between compound **5** and γ-oryzanol concentrations and the thickness of the bran layer of grains ripened under high temperature. Compound **5** and γ-oryzanol could become potential markers to evaluate thickness of the bran layer. This method could improve the efficiency of QTL analyses for grain quality in our changing environment. In addition, future studies will investigate the relationship between the concentrations of compounds **2**, **3**, **5**, and **6** and γ-oryzanol and grain chalkiness.



5



6

Figure 5. Chemical structures of compounds **5** and **6** isolated from acetone extracts of brown rice grains.

In addition to air temperature, solar irradiation affects ripening of rice plants. Both high air temperature and low solar irradiation during rice plant ripening reduces grain weight and generates chalky grains. However, in the present study, the combination effect of high air temperature and low solar irradiation was not examined. The effect of the combination on the constituents of brown rice gain should be examined.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-29-838-8952; fax: +81-29-838-8837; e-mail: nakanohr@affrc.go.jp.

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Notes

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