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Influence of Growth Temperature on the Amounts of Tocopherols, Tocotrienols, and γ-Oryzanol in Brown Rice

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Brown rice is a valuable source of lipid-soluble antioxidants including ferulated phytosterols (i.e., γ-oryzanol), tocopherols, and tocotrienols. To evaluate the impact of temperature on the accumulation of these compounds, seeds from six different rice lines grown to maturity in replicate greenhouses in Gainesville, FL, were analyzed. The lines represented *Oryza sativa indica*, *O. sativa japonica*, and *Oryza glaberrima* of different origins. Temperatures were maintained near ambient at one end of each greenhouse and at approximately 4.5 °C above ambient at the other end. γ-Oryzanols, tocopherols, and tocotrienols were extracted from whole seed (i.e., brown rice) and analyzed by HPLC. Tocotrienols and tocopherols varied widely between lines but changed only slightly with respect to temperature. In general, the proportions of α-tocotrienol and/or α-tocopherol increased at elevated temperature, whereas γ-tocopherol and γ-tocotrienol decreased. Six γ-oryzanol peaks, identified on the basis of absorbance maxima at 330 nm and HPLC−mass spectrometry, were quantified. The most abundant component was 24-methylenecycloartanyl ferulate, present at 40−62% of total. Its levels increased 35−57% at elevated temperature in five of six lines, accounting for most of the change in total γ-oryzanol. The results suggest that the physiological action of individual ferulated phytosterols should be investigated because their relative proportions in γ-oryzanol can change.

KEYWORDS: Antioxidant; climate change; temperature; tocopherols; tocotrienols; oryzanol; *Oryza sativa*; *Oryza glaberrima*

INTRODUCTION

Rice bran constitutes about 10% of the dry matter of a seed and consists of the outer layers (pericarp, seed coat, nucellus, and aleurone) and the embryo or germ (*1*). Bran is about 15−20% oil, of which a relatively large proportion (ca. 4%) compared to other vegetable oils is unsaponifiable material, primarily free and esterified phytosterols as well as tocotrienols and tocopherols (collectively referred to tocols). The esterified sterols include 10 or more different compounds linked to ferulic acid (*2*) and are known collectively as γ-oryzanol.

Similar to oat bran, rice bran and rice bran oil have significant hypocholesterolemic effects on humans and other species. Unlike oat bran, rice bran lipids and unsaponifiable components appear to be responsible (*3*−*6*). Isolated tocols, tocotrienols in particular (*7*), and γ-oryzanols (*8*) have both been implicated as active components in cholesterol reduction.

Environment (i.e., planting location or planting year) as well as genetics has been reported to affect the contents and/or composition of tocols and/or γ-oryzanols in rice seed (*9*, *10*). In addition to affecting product quality, such variation may have a significant effect on nutrition study outcomes if different sources of bran or oil are compared. Conversely, environmental manipulation in conjunction with genetic selection could be used to produce bran or oil varying in the content and composition of tocols and/or γ-oryzanols. The impact of environmental variables (e.g., temperature, drought, light, etc.) on rice phytochemical composition has not been determined.

In soybean seeds, however, elevated temperatures (28 compared to 23 °C) or severe drought during seed development was shown to alter tocopherol metabolism, increasing the proportion of α-tocopherol (*11*). In other studies, seed development at 30 versus 15 °C resulted in 95% increases in total soybean phytosterols averaged across nine lines with a relative increase in campesterol compared to stigmasterol and β-sitosterol (*12*). Large decreases in total tocopherols were also reported in

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Table 1. Characteristics of Rice Lines (*Oryza* spp.)

line	species/ecotype/habit	origin	grain size	temperature response[a]
Arborio	*sativa*/*japonica*/semidwarf	Italy	medium	intermediate
Kaybonnet	*sativa*/*japonica*/tall	Texas	long	intermediate
Jodon	*sativa*/*indica*/semidwarf	Texas	long	intermediate
Tellahamsa	*sativa*/*indica*/tall	India	medium	tolerant
CG-17	*glaberrima*/tall	Cotonou, Benin[b]	long	intermediate
Italica[c]	*sativa*/*japonica*/semidwarf	Italy	medium	sensitive

[a] The relative sensitivity of spikelet fertility to elevated greenhouse temperature in 2003 (P. V. V. Prasad, personal communication). [b] West African Rice Development Association (Africa Rice Center). [c] Italica Livorna.

companion measurements on the same material (*13*). Because sterols and tocopherols both depend on isoprenoid metabolism, the possibility for interactions should be considered (*14*). However, phytosterols are not necessarily affected by environment or location (*15*, *16*), even under conditions when temperature differences were sufficient to affect fatty acid saturation (*16*).

Rice is currently grown near its temperature optimum in much of the world and, similar to other grain crops, further increases in temperature can reduce yield (*17*, *18*). Temperature gradient greenhouses have proven to be useful to investigate the influence of temperature on crops (*17*) and were used here to grow rice reproducibly at ambient or elevated temperature (ca. +4.5 °C). Seeds were analyzed from six lines representing *Oryza sativa indica*, *O. sativa japonica*, and *Oryza glaberrima* originating from different parts of the globe (**Table 1**). On the basis of spikelet fertility, Tellahamsa, a tall *indica* line of *O. sativa* from India, is relatively heat tolerant, whereas Italica Livorna, a semidwarf *japonica* line from Italy, is relatively sensitive (**Table 1**). *O. glaberrima*, a species still cultivated in Africa, is of interest because of its potential for increased tolerance to stress (*19*). However, CG-17 was intermediate in its response to elevated temperature (**Table 1**). Like other *O. glaberrima* lines, CG-17 seeds are red, indicating the presence of anthocyanins (*20*).

The purpose of this study is to assess whether season-long differences in growth temperature affect the concentration and/or composition of tocols and γ-oryzanol in brown rice seeds. In general, there is relatively little information on how environmental stress during growth and development affects phytochemicals in seeds, and specific effects of temperature on tocols and γ-oryzanol have not been described previously. In addition, the possible significance of such changes on either the nutritional value of brown rice or the ability of rice seed to tolerate heat stress will be considered. Both considerations are important in view of predicted increases in global temperature.

MATERIALS AND METHODS

Cultivation. Rice seeds were sown on May 28, 2003, in soil (natural Millhopper fine sand). Plots were fertilized with 60 kg ha^{-1} each of N, P, and K at sowing and with additional N at panicle initiation and seed filling (60 kg ha^{-1} each time). Plots were located in three replicate temperature-gradient greenhouses in Gainesville, FL (29° 68′ N, 82° 27′ W), and were irrigated daily to maintain soil moisture near saturation. Details of the temperature-gradient greenhouse layout and operation are described elsewhere (*17*, *18*). In brief, the long axis of each greenhouse was 27.4 m and oriented north-to-south. The houses were 4.4 m wide and 2.2 m high in the middle and covered with polyethylene that transmitted ca. 90% of photosynthetically active solar radiation (400−700 nm). The lengths of the greenhouses were separated into four zones 5 m in length and 4.4 m wide. The first zone was maintained at near-ambient temperatures (30/23 °C day/night maximum/minimum growing-season-long average), whereas the fourth zone was maintained at ca. +4.5 °C (i.e., 35/27 °C day/night). The middle (second and third) zones were not used in this study. Temperatures were 1−2 °C warmer on average during 2003 as compared to 2001 and 2002 (*17*).

Table 2. Rice Samples for Analysis

	GH 1[a]		GH 2[b]		GH 3[c]	
line	E[d]	W[e]	E	W	E	W
Arborio	A[f]	A	A	A	A	A
Kaybonnet	A	A	A	NA[g]	A	NA
Jodon	A	A	NA	A	NA	NA
Tellahamsa	NA	A	NA	NA	A	A
CG-17	NA	NA	NA	A	A	A
Italica	NA	NA	NA	NA	A	A

[a] Greenhouse 1. [b] Greenhouse 2. [c] Greenhouse 3. [d] East. [e] West. [f] Available (includes both ambient and elevated temperature sections). [g] Not available (i.e., not planted or insufficient seed).

Sampling. Intact panicles from multiple plants were collected from east and/or west sides of each greenhouse zone as available (see **Table 2**) and air-dried. In practice, Arborio was the only line for which east and west harvests were available for each greenhouse zone, whereas Arborio and Kaybonnet were the only lines represented in all three greenhouses. Each sample (east or west) was subdivided to create two duplicates for analysis. All results from a given greenhouse zone were averaged on the basis of these two or four measurements depending on availability of east and west seed samples to calculate a greenhouse zone mean. SigmaStat (ver. 2.03, SPSS Science, Chicago, IL) was used to determine significant treatment effects ($p < 0.05$) on rice phytochemicals by two-way ANOVA (rice line × temperature) after validation of normal distribution and equal variance. Significance of differences ($p < 0.05$) between rice lines at ambient temperature and significance of effects of temperature on each line were determined by Tukey pairwise multiple-comparison tests.

Whole seeds (i.e., brown rice including bran and embryo) were analyzed because it is difficult to get reproducible bran samples for very small seed samples such as were available for this study. Moreover, milling duration can easily and differentially affect the amounts of tocols and γ-oryzanols present (*1*, *21*). For example, the first 10 s of milling released most of the γ-oryzanol, indicating this compound was concentrated in the outermost pericarp, seed coat, and nucellus layers of the bran (*1*). In contrast, a further 10 s of milling yielded a bran fraction with about the same amount of tocopherols and tocotrienols as the first 10 s, indicating tocols were well represented in the deeper aleurone layer, which contains large numbers of lipid bodies. In addition, significant amounts of phytochemicals were detected in rice seed even after almost 10% of seed mass was removed by milling (*21*).

Analysis of Tocols and γ-Oryzanols. Seeds were carefully dehusked by gentle abrasion using nylon screens and then freeze-dried and ground to a fine flour in a cyclone mill. Samples (100 mg) were weighed into conical centrifuge tubes and extracted three times in absolute ethanol (5 mL total volume) with 0.1% butylated hydroxytoluene (BHT) at 60 °C for 5 min each time. Samples were vortexed prior to and following heating and then centrifuged for 5 min at high speed (18000*g*) in a benchtop centrifuge. Supernatants (5 mL total volume) were collected, combined, and stored on ice. Following these steps, water (1.5 mL) was added to each sample, after which the extracts were washed three

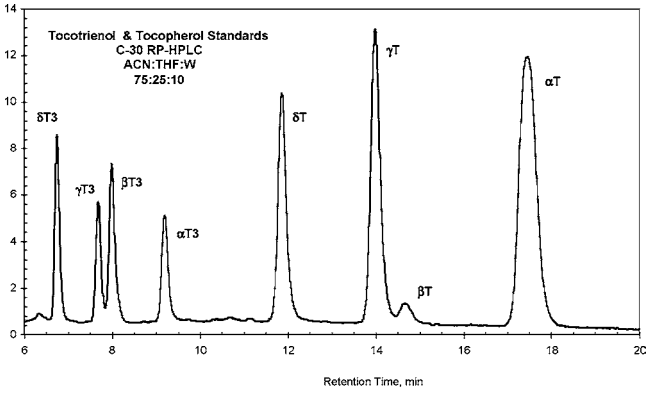

Figure 1. HPLC chromatogram of tocotrienol and tocopherol standards monitored at 292 nm: δ-tocotrienol, δT3; γ-tocotrienol, γT3; β-tocotrienol, βT3; α-tocotrienol, αT3; δ-tocopherol, δT; γ-tocopherol, γT; β-tocopherol, βT; and α-tocopherol, αT.

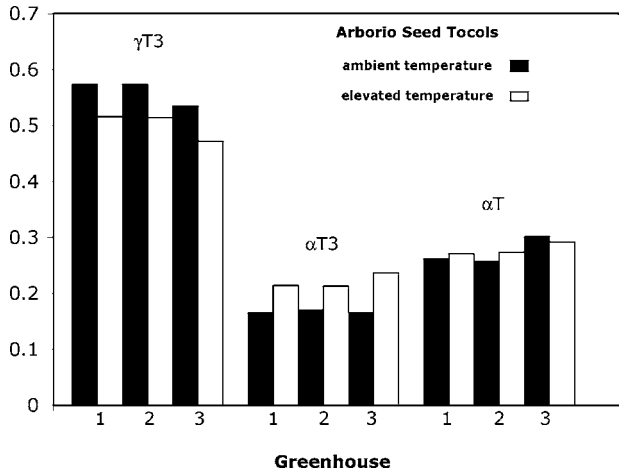

Figure 2. Relative tocol concentrations in Arborio seeds produced at ambient or elevated temperature in three replicate greenhouses.

times with hexane (3.5 mL total volume) to remove lipids. Hexane supernatants were kept on ice, combined, and then dried under N_2. Residues were dissolved in 0.5 mL of absolute ethanol with 0.1% BHT and transferred in an amber vial immediately to an HPLC (Agilent model 1100), where they were stored in a refrigerated sampling unit for up to 12 h before analysis. In some experiments, samples of different mass (e.g., 50, 100, or 150 mg) were extracted to ascertain that measured tocols and γ-oryzanols were proportional to sample mass.

Extracts (20 μL) were injected on a YMC C-30 reverse-phase (RP) "carotenoid" column (4.6 $\times$ 250 mm, 3 μm; Waters Corp., Milford MA) run at 25 °C and 1.0 mL min^{-1} with 100% solvent B (acetonitrile/tetrahydrofuran/water 70:25:10, v/v/v) between 0 and 25 min followed by a linear gradient to 100% solvent A (acetonitrile/tetrahydrofuran 50:50, v/v) from 25 to 35 min, 100% solvent A from 35 to 45 min, and ending with 100% solvent B from 45 to 60 min. Sample absorbance was monitored with a diode array detector. Tocotrienol and tocopherol standards (Calbiochem, San Diego, CA) monitored at 292 nm were separated between 6.5 and 19 min (**Figure 1**), whereas a γ-oryzanol standard (Tokyo Kasei Kogyu, Portland, OR) monitored at 330 nm eluted between 37 and 41 min (**Figure 3**, inset). HPLC−mass spectrometry was conducted with an Agilent 1100 MSD equipped with an atmospheric pressure chemical ionization interface operated in the positive mode (drying gas at 6.0 L min^{-1}, nebulizer pressure at 60 psi, drying gas temperature at 350 °C, vaporizer gas temperature at 325 °C, capillary voltage at 4000 V, corona current at 4.0 μA, and fragmentor at 80 V). All solvents were of HPLC grade, and all chemicals were of reagent grade.

Use of a C-30 column for the simultaneous separation of tocols, γ-oryzanol, and carotenoids in rice bran oil has previously been reported (*22*). This column is sufficient to resolve all eight standard tocols [α-tocotrienol (αT3), β-tocotrienol (βT3), γ-tocotrienol (γT3), and δ-tocotrienol (δT3) and α-tocopherol (αT), β-tocopherol (βT), γ-tocopherol (γT), and δ-tocopherol (δT)]. The structural isomers γT3 and βT3 as well as γT and βT are usually not resolved by standard C-18 RP-HPLC. In practice, this was not an issue because βT3 and βT were not detected in any rice samples. The identity of tocols in rice samples was confirmed by cochromatography with co-injected individual standards. A fluorescence detector was also used in some separations to confirm that all putative tocol peaks exhibited characteristic fluorescence.

The γ-oryzanol standard revealed five main peaks that coeluted with peaks in rice samples. These peaks and an additional minor component found primarily in Arborio samples were quantified in rice extracts assuming the extinction coefficients for ferulic acid were identical for all peaks. Therefore, the mass of each peak was calculated on the basis of its contribution to total A_{330} [i.e., mass (peak i) = $\sum A_{330}$ (peak i)/$\sum A_{330}$ (peaks 1, ..., n) $\times$ mass of γ-oryzanol standard injected, where there are n peaks with A_{330} above baseline]. For the purposes of this calculation, several minor peaks with detectable A_{330} were included in total A_{330}. The total absorbance of the six peaks in the standard accounted for 90−95% of the total A_{300}. Rice samples generally had more detectable peaks than the standard; the six quantified peaks typically accounted for 80−85% of total A_{330}.

RESULTS

Tocols. Brown rice seeds contained about 100 nmol g^{-1} of dry matter of total tocols (**Table 3**), except for Italica Livorna, which had about double this amount. The major individual tocol was γT3, except again for Italica Livorna, which had very high levels of αT. Although Italica Livorna samples were available from only one greenhouse (**Table 2**), eight samples representing duplicates from both east and west sides as well as ambient and elevated temperature zones were consistent with respect to total tocols and αT. Variable amounts and proportions of αT, γT, and αT3 were found in all of the remaining lines with the exception of Arborio, which lacked detectable γT. Detectable levels of δT3 (5% or less of total) were found only in Kaybonnet and CG-17. Because rice tocols are concentrated in the 10% of seed mass formed by the bran and germ, estimated values for total tocols per unit of bran mass are comparable to other oil seeds and other reports of rice bran.

Elevated temperature caused small but significant increases in αT (five of six lines), αT3 (four of six lines), and γT3 (one of six lines). There were also small but significant decreases in γT (three of six lines) and δT3 (one of six lines). The changes are comparable to those reported in the literature for rice tocols from different locations or years (*9*). Although ANOVA determined there was an overall significant effect of temperature on total tocols ($p = 0.016$), the Tukey test identified a significant increase in total tocols for only Kaybonnet. However, rice seeds decrease about 10% in mass at elevated temperature (data not shown), so increases in tocols on a mass basis could be an artifact if the tocol content per seed remains constant.

In fact, real changes in tocol metabolism probably occur because the proportion of individual tocols with respect to total changed significantly at elevated temperature. Thus, γT3 decreased 6.5%, whereas αT3 increased 5.6% on average, at elevated temperature for Arborio. Although small, these changes were highly reproducible (**Figure 2**). Although absolute values for αT increased significantly (**Table 3**), αT as a proportion of total tocols did not change. The pattern of change depended on rice line. In Kaybonnet, for example, αT3 increased significantly, similar to Arborio, but γT3 did not change (data not shown). In contrast, δT3 and γT, neither of which is detected in Arborio, did decrease significantly. Italica Livorna was the only line without significant changes in the proportion of individual tocols.

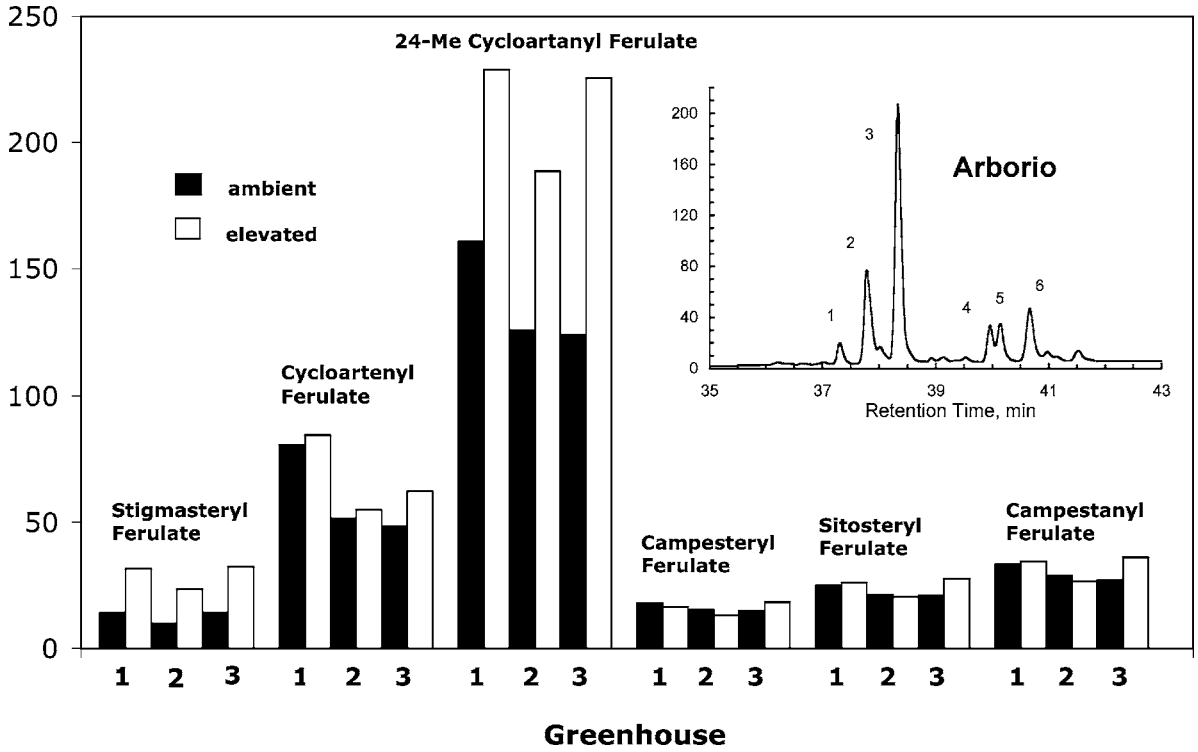

Figure 3. Concentration of stigmasteryl ferulate (peak 1), cycloartenyl ferulate (peak 2), 24-methylenecycloartanyl ferulate (peak 3), campesteryl ferulate (peak 4), sitosteryl ferulate (peak 5), and campestanyl ferulate (peak 6) in Arborio seeds produced at ambient or elevated temperature in three replicate greenhouses. (Inset) HPLC chromatogram of Arborio γ-oryzanol monitored at 330 nm; peaks tentatively identified by HPLC-MS-APCI (**Table 4**).

Table 3. Comparison of Tocols in Rice Lines Grown at Ambient or Elevated Temperature

line	total tocols[a]	δT3[b]	γT3[c]	αT3[d]	γT[e]	αT[f]
	Concentration at Ambient Temperature (Nanomoles per Gram of Dry Matter)[g,h]					
Arborio	91 c[i]		52 c	15 ab		25 b
Kaybonnet	140 b	5 a	95 a	13 bc	8 d	19 c
Jodon	135 b		93 ab	14 abc	7 d	21 c
Tellahamsa	99 c		72 bc	2 d	12 c	13 d
CG-17	97 c	4 a	63 c	4 d	18 b	7 e
Italica	216 a		57 c	18 a	31 a	109 a
	Concentration Increase (Decrease) at Elevated Temperature (Nanomoles per Gram of Dry Matter)					
Arborio	9		(1)	7 z		3 z
Kaybonnet	18 z[j]	(5) z	11	8 z	(2)	6 z
Jodon	0		1	4 z	(7) z	2
Tellahamsa	5		6 z	1	(7) z	5 z
CG-17	11	0	5	2 z	(2)	5 z
Italica	20		2	3	4 z	11 z

[a] Sum of δT3, γT3, αT3, γT, and αT. [b] δ-Tocotrienol. [c] γ-Tocotrienol. [d] α-tocotrienol. [e] γ-Tocopherol. [f] α-Tocopherol. [g] Seed dry matter. [h] Mean of 1 (Italica), 2 (Jodon, Tellahamsa, CG-17), or 3 (Arborio, Kaybonnet) greenhouse zones. [i] Values in a column followed by different letters are significantly different ($p < 0.05$). [j] Increases (decreases) followed by "z" are significantly different ($p < 0.05$) from the value at ambient temperature.

Table 4. Tentative Identification of γ-Oryzanol Peaks via HPLC-MS-APCI

peak	retention time (min)	major sterol fragment ion = $[M - 18 + 1]^+$	proposed chemical structure
1	37.3	395	stigmasteryl ferulate
2	37.8	409	cycloartenyl ferulate
3	38.4	423	24-methylenecycloartanyl ferulate
4	40.0	383	campesteryl ferulate
5	40.2	397	sitosteryl ferulate
6	40.8	385	campestanyl ferulate

γ-Oryzanol. Six peaks of phytosteryl ferulates (γ-oryzanol) separated via HPLC were detected via UV absorption at 330 nm (**Figure 3**, inset), and the chemical structures of these esters were identified using HPLC-MS-APCI in the positive ion mode (**Table 4**). As observed previously, the major fragment ions of each of the phytosteryl esters were the $[M - \text{water}]^+$ of the phytosteryl moieties (*23*). All lines had similar amounts of γ-oryzanol under ambient temperature conditions (**Table 5**). The concentrations are comparable to other reports for brown rice (*10*, *21*) or bran (*9*) after correction for the proportion of bran mass to seed. The distribution of individual γ-oryzanols was similar for all six lines. About 40−60% of the total was present in peak 3, which was tentatively identified as 24-methylenecycloartanyl ferulate on the basis of LC-MS APCI data. Approximately 20−35% of total γ-oryzanol was found in peak 2, which was identified as cycloartenyl ferulate. These chemical structures are consistent with other studies using C-18 RP-HPLC (*2*) and C-30 RP-HPLC (*22*) and indicate the majority of γ-oryzanol consists of 4,4′-dimethylsteryl ferulates derived from early steps in phytosterol metabolism (*24*−*26*).

Peaks 4, 5, and 6 were tentatively identified, in order, as campesteryl ferulate, sitosteryl ferulate, and campestanyl ferulate. A seventh peak, not quantified in these studies, was assigned to sitostanyl ferulate. Peaks 4−6 together constituted about 17−26% of total γ-oryzanol. Peak 1, a minor component ranging from <1% of the total to 4% in Arborio, appeared to be composed of stigmasteryl (or possibly stigmastenyl) ferulate, consistent with an earlier study (*2*).

Arborio, Kaybonnet, Tellahamsa, and CG-17 had large increases (>30%) in total γ-oryzanol at elevated temperature. Most of the increase in all four lines (60−80%) resulted from increases in 24-methylenecycloartanyl ferulate (**Figure 3**;

Table 5. Comparison of γ-Oryzanols in Rice Lines Grown at Ambient or Elevated Temperature

line	total γ-oryzanol[a]	peak 1[b]	peak 2[c]	peak 3[d]	peaks 4 + 5 + 6[e]
	Concentration at Ambient Temperature (Microgram per Gram of Dry Matter)[f]				
Arborio	277 a[g]	12 a	60 a	137 a	68 a
Kaybonnet	252 a	4 bc	87 a	101 a	60 ab
Jodon	259 a	4 bc	84 a	104 a	67 ab
Tellahamsa	228 a	1 c	42 a	130 a	55 ab
GC-17	180 a	1 c	47 a	199 a	34 b
Italica	220 a	10 ab	49 a	108 a	53 ab
	Concentration Increase (Decrease) at Elevated Temperature (Micrograms per Gram of Dry Matter)				
Arborio	106 z[h]	17 z	7	78 z	4
Kaybonnet	99 z	3	17	55 z	24 z
Jodon	17	0	(12)	36 z	(7)
Tellahamsa	98 z	2	16	69 z	11
CG-17	56 z	2	6	45 z	2
Italica	6	0	7	(1)	1

[a] Sum of peaks 1, 2, 3, 4, 5, and 6 (see **Figure 3** for HPLC; **Table 4** for tentative identification). [b] Stigmasteryl ferulate. [c] Cycloartenyl ferulate. [d] 24-Methylenecycloartanyl ferulate. [e] Sum of campesteryl ferulate, sitosteryl ferulate, and campestanyl ferulate. [f] Mean of 1 (Italica), 2 (Jodon, Tellahamsa, CG-17), or 3 (Arborio, Kaybonnet) greenhouse zones. [h] Values in a column followed by different letters are significantly different ($p < 0.05$). [h] Increases (decreases) followed by "z" are significantly different ($p < 0.05$) from the value at ambient temperature.

Table 5). The remainder of the increase in γ-oryzanol came from different compounds in different lines. For example, stigmastenyl ferulate (peak 1) contributed 17% to the total increase in Arborio but was a minor component in Kaybonnet, Tellahamsa, and CG-17. Peaks 4−6 together contributed 24% of the total γ-oryzanol increase in Kaybonnet, but were minor components in Arborio, Tellahamsa, and CG-17. There were also relatively large increases in cycloartenyl ferulate (peak 2) in Kaybonnet and Tellahamsa. Miller and Engel (*10*) emphasized the importance of comparing 4,4′-dimethylsteryl ferulates (i.e., cycloartenyl ferulate and 24-methylenecycloartanyl ferulate) with 4-desmethylsterols (i.e., all of the rest). Taken together, the dimethylsteryl ferulates accounted for 75−90+% of the increase in total γ-oryzanol in Arborio, Kaybonnet, Tellahamsa, and CG-17.

Changes in total γ-oryzanols were not significant in the two remaining lines, Jodon and Italica Livorna, although Jodon had relatively large increases in peak 3 that were partially negated by decreases in peaks 2 and 4−6.

Greenhouse Effects. Greenhouse 1 was distinguished from the other two houses on the basis of higher levels of γT3, αT3, αT, cycloartenyl ferulate, and 24-methylenecycloartanyl ferulate in Arborio seeds grown under ambient temperature conditions (**Figure 3** and data not shown). Similar results were noted for Kaybonnet with respect to δT3, γT3, γT, cycloartenyl ferulate, sitosteryl ferulate, and campestanyl ferulate (data not shown). On the other hand, αT3, αT, 24-methylenecycloartanyl ferulate, and campesteryl ferulate in Kaybonnet were similar in all houses. Differences between greenhouses did not appear to interfere with the effect of temperature, which was superimposed on higher ambient baseline levels (**Figure 3**).

DISCUSSION

The main result is that a 4.5 °C increase in season-long growth temperature resulted in large increases in one fraction of γ-oryzanol, 24-methylenecycloartanyl ferulate, in five of six lines representing a relatively wide distribution of rice ecotypes. In four of these five lines there were also small increases in cycloartenyl ferulate such that between 75 and 90% of the total increase in γ-oryzanol can be ascribed to 4,4′-dimethylsteryl ferulates. Because these compounds may be less effective in blocking cholesterol uptake and absorption in animal models than the 4-desmethylsterols (*27*), it is possible that elevated temperature results in diminished bioactivity of γ-oryzanol. However, there may be potential for genetic improvements in specific 4-desmethyl phytosterols. For example, elevated temperature caused 140% increases in a minor peak in Arborio that may represent stigmasteryl or stigmastenyl ferulate and approximately 50% increases in two peaks in Kaybonnet that may represent sitosteryl ferulate and campestanyl ferulate.

On the other hand, 24-methylenecycloartanyl ferulate has been shown to be a more effective inhibitor of free radical initiated cholesterol oxidation in vitro than either cycloartenyl ferulate or campesteryl ferulate (*28*). The two dimethylsterols, cycloartenyl and 24-methylenecycloartanyl, were more effective than various 4,4′-desmethyl phytosterols with respect to anti-inflammatory action induced by phorbol esters (*29*). Although esterification of 4-desmethyl phytosterols with *trans*-ferulic acid increased their activity in this test, it did not make these compounds more effective than the 4,4′-dimethylsterols. More research is required to evaluate the bioactivity of individual components of γ-oryzanol and to evaluate the relative importance of the free phytosterol versus esterified ferulic acid. It is possible that the benefit from γ-oryzanols derives from ferulic acid and that the major impact of the steryl component is to affect bioavailability (*30*).

The implications of these findings for sterol metabolism are unclear because the biosynthesis of sterols is complicated and it is not understand how ferulation is regulated. In fact, few studies have examined both γ-oryzanol and bulk sterols. In one comparison, γ-oryzanol and phytosterols in rice seeds were present in approximately equal amounts (*21*). Bulk sterols consisted, as expected, mainly of 4-desmethylsterols (chiefly β-sitosterol). Although it appears therefore that sterols are not simply ferulated in proportion to their abundance in the seed, the situation is more complicated because γ-oryzanols were concentrated in the outermost layers of the bran, whereas bulk sterols, similar to tocols and lipids, were distributed more evenly in deeper layers.

Phytosterols are important components of plant membranes; changes in temperature affect the relative composition of sterols in ways likely to stabilize membranes (*25*). The function of γ-oryzanols in plants, however, is unknown. That moderate increases in temperature induce increases in these compounds suggests they are involved in plant stress responses. For example, γ-oryzanols did not increase at elevated temperature in seeds of Italica Livorna, which were relatively sensitive to high temperature (**Table 1**) and which had typical levels of total and individual γ-oryzanols at ambient temperature (**Table 5**). On the other hand, seeds of Tellahamsa, which was relatively tolerant to elevated temperature (**Table 1**), were comparable to other lines with intermediate temperature sensitivity with respect to γ-oryzanols and the changes induced by elevated temperature (**Table 5**). Additional research over a wider range of genotypes and growing conditions is needed and may lead to improvements in the ability of rice to adapt to more stressful conditions. In particular, it will be useful to investigate the impact of elevated atmospheric CO_2. In soybeans seeds, by comparison, elevated CO_2 appeared to ameliorate the impact of elevated temperature

and reduced changes in tocopherols and isoflavones induced by elevated temperature (*31*).

Changes in tocopherols and tocotrienols appear to be less important than changes in γ-oryzanols for the adaptation of rice to elevated temperature. Thus, seeds of the sensitive Italica Livorna had very high levels of αT, whereas seeds of the tolerant Tellahamsa contained relatively low levels of total tocols. Nonetheless, the extreme variability in tocopherol and tocotrienol composition noted here for different lines of rice may be useful to evaluate the significance of these compounds with respect to serum cholesterol level reduction by rice bran or oil (*32*). Comparing seeds from 26 lines of rice, Horvath et al. (*33*) also noted wide variability in total tocals, in the relative amounts of tocopherols and tocotrienols, and in the ratios αT/γT and αT3/γT3. Moreover, the ratios αT/γT and αT3/γT3 were often different in the same line.

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LITERATURE CITED

(1) Rohrer, C. A.; Siebenmorgen, T. J. Nutraceutical concentrations within the bran of various rice kernel thickness fractions. *Biosyst. Eng*. **2004**, *88*, 453−460.

(2) Xu, Z.; Godber, J. S. Purification and identification of components of γ-oryzanol in rice bran oil. *J. Agric. Food Chem*. **1999**, *47*, 2724−2728.

(3) Wilson, T. A.; Idreis, H. M.; Taylor, C. M.; Nicolosi, R. J. Whole fat rice bran reduces the development of early aortic atherosclerosis in hypercholesterolemic hamsters compared with wheat bran. *Nutr. Res*. **2002**, *22*, 1319−1332.

(4) Berger, A.; Rein, D.; Schaefer, A.; Monnard, I.; Gremaud, G.; Lambelet, P.; Bertoli, C. Similar cholesterol-lowering properties of rice bran oil, with varied γ-oryzanol, in mildly hypercholesterolemic men. *Eur. J. Nutr*. **2005**, *44*, 163−173.

(5) Most, M. M.; Tulley, R.; Morales, S.; Lefevre, M. Rice bran oil, not fiber, lowers cholesterol in humans. *Am. J. Clin. Nutr*. **2005**, *81*, 64−68.

(6) Chen, C.-W.; Cheng, H.-H. A rice bran oil diet increases LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity in rats with streptozotocin/nicotinamide-induced type 2 diabetes. *J. Nutr*. **2006**, *136*, 1472−1476.

(7) Qureshi, A.; Sami, S. A.; Salser, W. A.; Khan, F. A. Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF_{25}) of rice bran in hypercholesterolemic humans. *Atheroschlerosis* **2002**, *161*, 199−207.

(8) Rong, N.; Ausman, L. M.; Nicolosi, R. J. Oryzanol decreases cholesterol absorption and aortic fatty streaks in hamsters. *Lipids* **1997**, *32*, 303−309.

(9) Bergman, C. J.; Xu, Z. Genotype and environment effects on tocopherol, tocotrienol, and γ-oryzanol contents of southern U.S. rice. *Cereal Chem.* **2003**, *80*, 446−449.

(10) Miller, A.; Engel, K.-H. Content of γ-oryzanol and composition of steryl ferulates in brown rice (*Oryza sativa* L.) of European origin. *J. Agric. Food Chem.* **2006**, *54*, 8127−8133.

(11) Britz, S. J.; Kremer, D. Warm temperatures or drought during seed maturation increase free α-tocopherol in seeds of soybean (*Glycine max* [L.] Merr.). *J. Agric. Food Chem.* **2002**, *50*, 6058−6063.

(12) Vlahakis, C.; Hazebroek, J. Phytosterol accumulation in canola, sunflower, and soybean oils: effects of genetics, planting location, and temperature. *J. Am. Oil Chem. Soc*. **2000**, *77*, 49−53.

(13) Dolde, D.; Vlahakis, C.; Hazebroek, J. Tocopherols in breeding lines and effects of planting location, fatty acid composition, and temperature during development. *J. Am. Oil Chem. Soc.* **1999**, *76*, 349−355.

(14) Shewmaker, C. K.; Sheehy, J. A.; Daley, M.; Colburn, S.; Ke, D. Y. Seed-specific overexpression of phytoene synthase: increase in carotenoids and metabolic effects. *Plant J*. **1999**, *20*, 401−412.

(15) Maatta, K.; Lampi, A.-M.; Petterson, J.; Fogelfors, B. M.; Piironen, V.; Kamal-Eldin, A. Phytosterol content in seven oat cultivars grown at three locations in Sweden. *J. Sci. Food Agric*. **1999**, *79*, 1021−1027.

(16) Hamama, A. A.; Bhardwaj, H. L.; Starner, D. E. Genotype and growing location effects on phytosterols in canola oil. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1121−1126.

(17) Prasad, P. V. V.; Boote, K. J.; Allen, L. H., Jr.; Sheehy, J. E.; Thomas, J. M. G. Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field Crops Res*. **2006**, *95*, 398−411.

(18) Allen, L. H.; Albrecht, S. A.; Boote, K. J.; Thomas, J. M. G.; Newman, Y. C.; Skirvin, K. W. Soil organic carbon and nitrogen accumulation in plots of rhizome perennial peanut and bahiagrass grown in elevated carbon dioxide and temperature. *J. Environ. Qual*. **2006**, *35*, 1405−1412.

(19) Linares, O. F. African rice (*Oryza glaberrima*): history and future potential. *Proc. Natl. Acad. Sci. U.S.A*. **2002**, *99*, 16360−16365.

(20) Abdel-Aal, E.-S. M.; Young, J. C.; Rabalski, I. Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J. Agric. Food Chem.* **2006**, *54*, 4696−4704.

(21) Ha, T.-Y.; Ko, S.-N.; Lee, S.-M.; Kim, H.-R.; Chung, S.-H.; Kim, S.-R.; Yoon, H.-H.; Kim, I. H. Changes in nutraceutical lipid components of rice at different degrees of milling. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 175−181.

(22) Stöggl, W.; Huck, C.; Wongyai, S.; Scherz, H.; Bonn, G. Simultaneous determination of carotenoids, tocopherols, and γ-oryzanol in crude rice bran oil by liquid chromatography coupled to diode array and mass spectrometric detection employing silica C30 stationary phases. *J. Sep. Sci*. **2005**, *28*, 1712−1718.

(23) Moreau, R. A.; Powell, M. J.; Hicks, K. B. Evaluation of a commercial enzyme-based serum cholesterol test kit for the analysis of phytosterol and phytostanol products. *J. Agric. Food Chem.* **2003**, *51*, 6663−6667.

(24) Piironen, V.; Lindsay, D. G.; Miettinen, T. A.; Toivo, J.; Lampi, A.-M. Plant sterols: biosynthesis, biological function and their importance to human nutrition. *J. Sci. Food Agric.* **2000**, *80*, 939−966.

(25) Moreau, R. A.; Whitaker, B. D.; Hicks, K. B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid Res.* **2002**, *41*, 457−500.

(26) Benveniste, P. Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* **2004**, *55*, 429−457.

(27) Meijer, G. W.; Bressers, M. A. J. J.; De Groot, W. A.; Rudrum, M. Effect of structure and form on the ability of plant sterols to inhibit cholesterol absorption in hamsters. *Lipids* **2003**, *38*, 713−721.

(28) Xu, Z.; Hua, N.; Godber, J. S. Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2′-azobis(2-methylpriopionadmidine) dihydrochloride. *J. Agric. Food Chem*. **2001**, *49*, 2077−2081.

(29) Akihisa, T.; Yasukawa, K.; Yamaura, M.; Ukiya, M.; Kimura, Y.; Shimizu. N.; Arai, K. Triterpene alcohols and sterol ferulates from rice bran and their anti-inflammatory effects. *J. Agric. Food Chem.* **2000**, *48*, 2313−2319.

(30) Ou, S.; Kwok, K.-C. Ferulic acid: pharmaceutical functions, preparation and application in foods. *J. Sci. Food Agric*. **2004**, *84*, 1261−1269.

(31) Caldwell, C. R.; Britz, S. J.; Mirecki, R. M. Effect of temperature, elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [*Glycine max* (L.) Merrill] grown in controlled environments. *J. Agric. Food Chem.* **2005**, *53*, 1125−1129.
(32) Schaffer, S.; Mueller, W. E.; Eckert, G. P. Tocotrienols: constitutional effects in aging and disease. *J. Nutr*. **2005**, *135*, 151−154.
(33) Horvath, G.; Wessjohann, L.; Bigirimana, J.; Jansen, M.; Guisez, Y.; Caubergs, R.; Horemans, N. Differential distribution of tocopherols and tocotrienols in photosynthetic and non-photosynthetic tissues. *Phytochemistry* **2006**, *67*, 1185−1195.

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