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# Quantitation of Tocotrienol and Tocopherol in Various Rice Brans

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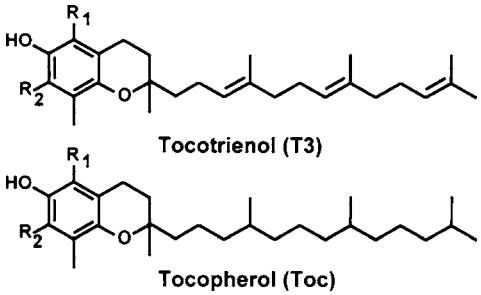
Rice bran is abundant in bioactive compounds including tocotrienol (T3, unsaturated vitamin E). T3 has been reported about its potential functionalities (i.e., antiangiogenic effect), so much attention has been paid on usability of rice bran T3. Hence, we developed a rapid screening method for T3-rich rice bran by one-step equilibrium direct solvent extraction followed by normal phase high-performace liquid chromatography (HPLC). The method gave high-extraction rate of rice bran T3 and tocopherol (above 90%), and the determination of vitamin E by HPLC was completed within 15 min. Using the method, an average of total T3 content in 109 kinds of rice bran samples was 830 $\mu$g/g dry wt. Kouchi-Akamai, Joushuu, and Wataribune were found as the T3-rich rice bran varieties (1350−1430 $\mu$g T3/g dry wt). According to T3 ratio against total vitamin E (wt %), the average ratio was 61%. Hirayama, Moritawase, and Kaneko were found as the varieties having the highest T3 ratio (80−86%). Since T3 content in Koshihikari rice bran (the leading variety in Japan) was a little above the average, we cross-fertilized Koshihikari with T3-rich varieties and found that T3 content or ratio in F1 was improved compared with Koshihikari. The varieties found rich in T3 could be used for nutraceutical purpose.

**KEYWORDS: Rice bran; tocotrienol; tocopherol; FLD-HPLC; screening**

## INTRODUCTION

Rice bran has been known to be abundant in some functional compounds such as tocotrienol (T3, unsaturated vitamin E, **Figure 1**) (*1*,*2*). Recent studies reported that T3 had potential physiological functionalities including cholesterol lowering and antithrombotic effects (*3*−*6*). We found that T3 acted as an effective antiangiogenic compound (*7*−*9*) useful for preventing angiogenic-related disorders (i.e., diabetic retinopathy, rheumatoid arthritis, and cancer). These reports (*3*−*9*) suggest that rice bran T3 has potential use as functional foods for prevention or treatment of cardiovascular disease and angiogenic disorders. For this reason, how to make use of rice bran T3 is now in focus.

Since about 60 million metric tons of rice bran is annually produced as byproduct, rice bran is thought as the best T3 source in term of availability. Rice harvested from different areas vary in shape, texture, and flavor (*10*), suggesting a possibility for different levels of T3 in rice bran (T3-rich rice bran may be available). However, until now there has been no evidence concerning T3 contents in many rice bran varieties. This has been partly due to the lack of a convenient method for determining rice bran T3.




| | $R_1$ | $R_2$ |
|---|---|---|
| α- | $CH_3$ | $CH_3$ |
| β- | $CH_3$ | H |
| γ- | H | $CH_3$ |
| δ- | H | H |

**Figure 1.** Chemical structure of tocotrienol (T3). T3 has an unsaturated isoprenoid tail, which differs from tocopherol (Toc) bearing a saturated phytyl side chain.

In this study, we developed a screening method for rice bran vitamin E, with particular emphasis on T3, by a one-step equilibrium direct solvent extraction coupled with fluorescence detection (FLD)−normal-phase high-performance liquid chro-

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matography (HPLC). Using the developed method, for screening T3-rich rice bran, T3 and tocopherol (Toc) contents in 109 of different kinds of rice bran were determined. On the basis of the result, some varieties high in T3 profile were selected and cross-fertilized with Koshihikari, and F1 rich in T3 will be used as functional foods.

## MATERIALS AND METHODS

**Chemicals.** Four isomers of tocotrienol (α-, *β*-, *γ*-, and *δ*-T3), four isomers of tocopherol (α-, *β*-, *γ*-, and *δ*-Toc), 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), acetonitrile, methanol, hexane, 1,4-dioxane, and 2-propanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). All reagents used were of analytical grade.

**Rice Bran Samples.** The seeds of 109 varieties including rice diversity research set of germplasm (RDRS) provided from National Institute of Agrobiological Sciences (NIAS) Genebank were sown on March 31, 2004 (*11*). Fourteen seedlings of each variety were individually transplanted by 25 × 25 cm spacing at the experiment farm of Toyama Agricultural Research Center on April 26, 2004. Rice grains were harvested at maturity from eight plants in the middle of the plot. The whole rice crops were dehulled, and rice bran samples were prepared by using Pearlest grain polish machine (Kett Electric Laboratory, Tokyo, Japan). The weight of each sample was equal to $^1/_{10}$ of that of the whole grain. The rice bran samples were stored at −30 °C with controlled humidity.

**Optimization of T3 and Toc Analysis by HPLC.** Focusing on T3 separation and analytical time, the HPLC operation condition for determining rice bran vitamin E was optimized as follows. The HPLC system consisted of a JASCO PU-980 pump (Japan Spectroscopic Co., Tokyo, Japan), a JASCO CO-860 column oven, and a Reodyne 7125 injector (Cotati, CA). Inersil SIL 100A-5 (4.6 × 250 mm; GL Science, Tokyo, Japan) was used as the HPLC column. The mixture of hexane/1,4-dioxane/2-propanol (100:40:5, v/v/v) was used as the mobile phase. The flow rate was adjusted to 1.0 mL/min and the temperature was maintained at 35 °C. T3 and Toc were detected by a RF-10AXL FLD detector (excitation 294 nm, emission 326 nm; Shimadzu, Kyoto, Japan). All peak areas were registered using a SIC Chromatocorder 21J integrator (System Instruments, Tokyo, Japan). By using this condition, all vitamin E homologues were successfully separated without peak overlapping, and the analytical time was about 15 min (**Figure 2A**). The peaks were sorted as α-Toc (7.1 min), α-T3 (8.1 min), *β*-Toc (9.5 min), *γ*-Toc (10.2 min), *β*-T3 (11.0 min), *γ*-T3 (11.8 min), *δ*-Toc (13.1 min), and *δ*-T3 (13.3 min). PMC (8.8 min; an internal standard) appeared between peaks of α-T3 and *β*-Toc. The standard curves of T3 and Toc were linear in a concentration range of 2−750 pmol. Analytical sensitivity of vitamin E was low as 2 pmol (*S*/*N* 3).

**Extraction and Determination of Rice Bran T3 and Toc.** Focusing on rapidity to be applied in a large number of samples, the one-step equilibrium direct solvent extraction was selected. One-step equilibrium direct solvent extraction was conducted by the method of Chen and Bergman (*12*) with some modification. Each 50 mg of rice bran sample was extracted by using a vortex mixer for 1 min with 3 mL of 2-propanol containing 30 nmol of PMC (internal standard) and 0.75 mg butylated hydroxyl toluene (as antioxidant). Then, the mixture was sent to centrifugation at 825*g* for 10 min. A 1.0 mL of the supernatant was collected and diluted with hexane to make a final volume of 5.0 mL. The prepared solution was filtered with Minisart RC15 (0.45-*μ*m pore size; Hannover, Germany), and then a portion (40 *μ*L) of the final solution was subjected to the FLD-normal phase HPLC. T3 and Toc concentrations in rice bran samples were calculated using equations of calibration curves of standard T3 and Toc and then were corrected using the peak area ratios of the vitamin E isoforms to internal standard (PMC). The determination was made three times in each sample. The time needed for the extraction was about 15 min.

In recovery rate test, rice bran of Koshihikari was used as a representative for the 109 samples. The added amount of each T3 or Toc was about the premeasured levels of vitamin E in the sample. Recovery rate was calculated by the equation $R\% = [(C_s - C_p)/C_a] \times 100$. Where $R$ (%) is the percent recovery of added vitamin E, $C_s$ is the vitamin E content in spiked sample, $C_p$ is the vitamin E content in sample,; and $C_a$ is the vitamin E added.

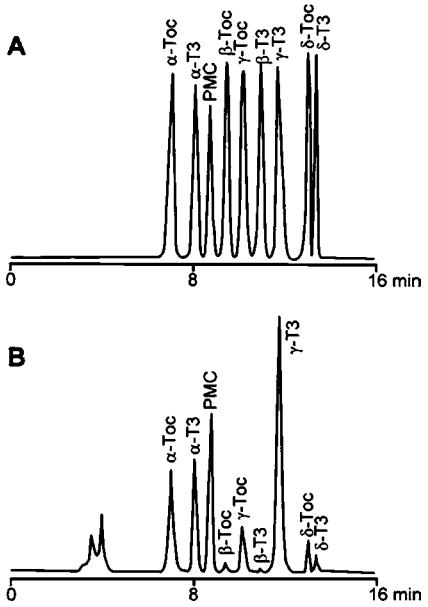


**Figure 2.** Typical HPLC chromatograms of standard vitamin E (**A**) and a rice bran extract (**B**). An Inersil SIL 100A-5 (4.6 × 250 mm) column was used with a mobile phase of hexane/1,4-dioxane/2-propanol (100:40:5, v/v/v) at a flow rate of 1.0 mL/min. The column temperature was maintained at 35 °C. The fluorescence detection was set as 294 nm for excitation and 326 nm for emission wavelengths.

**Screening of T3-Rich Rice Bran and Improvement of Koshihikari T3 by Cross-Fertilization.** After optimization of both vitamin E extraction and quantitative HPLC analysis, the method was applied for screening T3-rich rice bran from 109 rice bran samples. On the basis of the result, some varieties high in T3 profile were selected and cross-fertilized with Koshihikari. In the cross-fertilization, emasculation was applied by soaking panicles of seed parents (Koshihikari) in the hot water of 42 °C for 7 min just before flowering. After pollination, each panicle was covered with a sack of vitriol paper to prevent contamination of other pollens. Hybridization was done in a very humid condition because pollens could be easily damaged by drying (*13*). T3 and Toc contents in F1 generation rice bran were determined and compared with Koshihikari.

**Statistical Analysis.** The data were expressed as mean ± SD ($n$ = 3). Statistical comparisons were made with Student's *t*-test.

## RESULTS

**Extraction of Rice Bran T3 and Toc.** The chromatogram of Koshihikari is shown in **Figure 2B**. The peak area of each T3 and Toc of Koshihikari was found in a range of standard curves. The recovery of T3 and Toc (as well as PMC) was high (above 90%) (**Table 1**). With high-extraction rates and clear chromatogram of the rice bran, the extraction method was rapid and effective enough to apply for screening T3-rich rice brans.

**T3 and Vitamin E Contents in 109 Rice Bran Samples.** By using the developed method, vitamin E in 109 rice bran samples was extracted and determined. The contents of T3 and Toc in 109 rice bran samples are individually presented in alphabetical order in **SI Table 1**. Considering T3 and total vitamin E contents, the data of some varieties (Koshihikari, first 10 and last 10 quantitatively ordered varieties) are reported in **Figure 3**. As a result, a large variation of vitamin E contents was observed in 109 rice bran samples. Among vitamin E compounds, *γ*-T3 and *β*-T3 were the predominant and the smallest constituents, respectively.

**Table 1.** Recovery Rates of 2,2,5,7,8-Pentamethyl-6-hydroxychromane (PMC), Tocotrienol (T3), and Tocopherol (Toc)[a]

| | concentration ($\mu$g/g dry wt) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PMC | $\alpha$-T3 | $\beta$-T3 | $\gamma$-T3 | $\delta$-T3 | $\alpha$-Toc | $\beta$-Toc | $\gamma$-Toc | $\delta$-Toc |
| $C_p$ | | 296.4 | 4.49 | 567 | 31.2 | 455.5 | 17.0 | 85.4 | 39.7 |
| $C_a$ | 265.8 ± 2.5 | 297.2 ± 0.8 | 4.50 ± 0.09 | 567 ± 1.1 | 31.4 ± 0.7 | 454.5 ± 4.6 | 17.0 ± 0.01 | 85.9 ± 0.5 | 39.7 ± 0.1 |
| $C_s$ | 263.2 ± 0.6 | 587.5 ± 3.8 | 8.80 ± 0.17 | 1121 ± 1.9 | 62.6 ± 0.4 | 900.7 ± 10 | 33.8 ± 0.3 | 169.3 ± 0.5 | 79.1 ± 0.2 |
| $C_s - C_p$ | 263.3 ± 0.6 | 291.1 ± 2.8 | 4.30 ± 0.01 | 554 ± 0.1 | 31.4 ± 0.1 | 445.3 ± 0.8 | 16.8 ± 0.1 | 83.9 ± 0.4 | 39.4 ± 0.2 |
| $R$% | 99.0 ± 0.7 | 98.0 ± 1.6 | 97.3 ± 1.60 | 97.7 ± 1.7 | 100.1 ± 0.7 | 98.0 ± 1.0 | 98.8 ± 1.5 | 97.7 ± 1.5 | 99.4 ± 1.4 |

[a] Values are expressed as mean ± SD, $n = 3$. Recovery rate was calculated by the equation $R\% = [(C_s - C_p)/C_a] \times 100$ where $R$ (%) is the percent recovery of added vitamin E; $C_s$ is the vitamin E content in spiked sample; $C_p$ is the vitamin E content in sample; and $C_a$ is the vitamin E added.

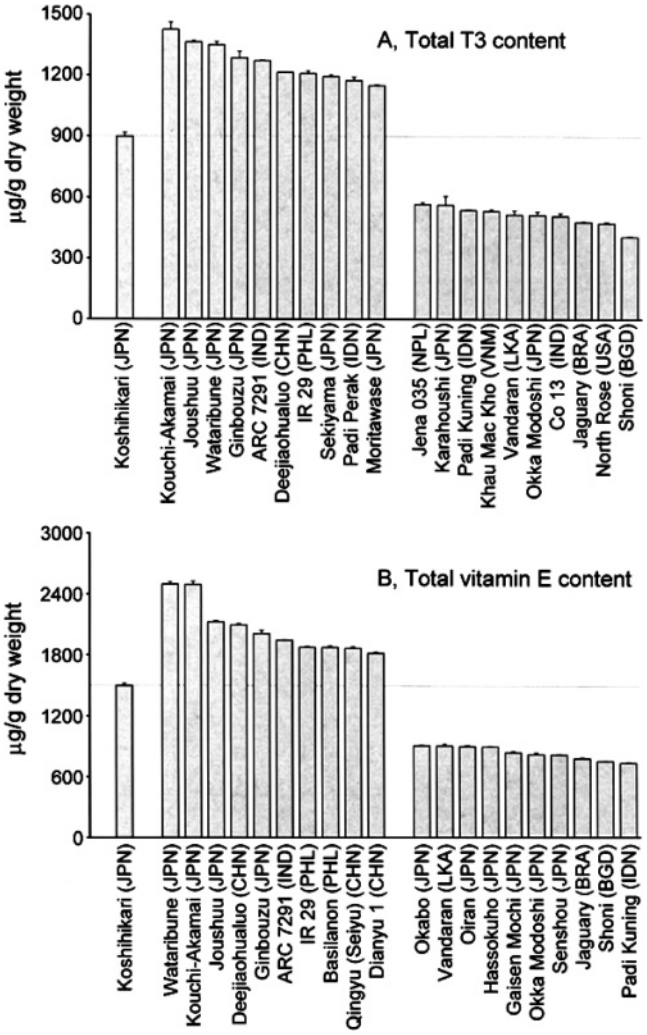


**Figure 3.** Tocotrienol (T3) and vitamin E contents in Koshihikari, first 10 and last 10 quantitatively ordered varieties. (**A**) Total T3 content (sum of T3 isomers); (**B**) total vitamin E content (sum of T3 and tocopherol). Mean ± SD ($n = 3$). BGD, Bangladesh; BRA, Brazil; CHN, China; IDN, Indonesia; IND, India; JPN, Japan; LKA, Sri Lanka; NPL, Nepal; PHL, Philippines; VNM, Vietnam; USA, United States of America.

Considering total T3 isomers, an average T3 content was 830 $\mu$g/g dry wt. Kouchi-Akamai (1430 $\mu$g/g dry wt), Joushuu (1365 $\mu$g/g dry wt), and Wataribune (1351 $\mu$g/g dry wt) were the varieties having highest values of total T3 contents, whereas the lowest level was found in Shoni (410 $\mu$g/g dry wt). According to vitamin E levels (sum of T3 and Toc), the average value was 1360 $\mu$g/g dry wt. The highest vitamin E content was 2495 $\mu$g/g dry wt (Wataribune) which was 3.5 times higher than the lowest content (Padi Kuning, 740 $\mu$g/g dry wt).

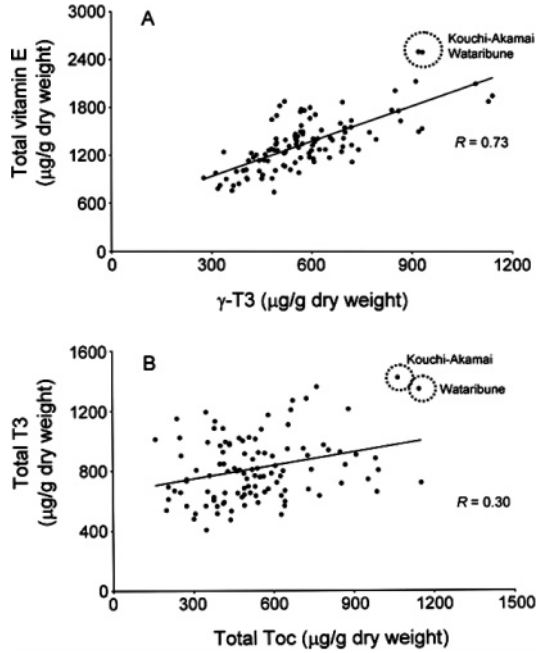


**Figure 4.** Correlation plots of the vitamin E contents in 109 rice bran samples: (**A**) between $\gamma$-tocotrienol (T3) and total vitamin E content and (**B**) between total tocopherol (Toc) and T3.

Regarding the ratio of T3 against vitamin E (wt %), the average ratio was 61%. The varieties containing the highest T3 ratio were Hirayama (86%), Moritawase (83%), and Kaneko (80%), whereas Basilanon (38%) had the smallest ratio. On the other hand, the correlations between $\gamma$-T3 and total vitamin E content and between total T3 and Toc are shown in **Figure 4**.

**Improvement of Rice Bran T3 Content by Cross-Fertilization.** To improve rice bran T3 content, cross-fertilization was conducted. Koshihikari was used as a seed parent because of its popularity and availability. With limitation in farming, we selected nine kinds of Japanese varieties as pollen parents including varieties rich in T3 content (Kouchi-Akamai, Joushuu, and Wataribune), varieties high in T3 ratio (Hirayama and Moritawase), and other varieties having higher T3 than Koshihikari (Kameji, Aikoku, Hiyadachitou, and Nipponbare). The T3 contents in F1 are shown in **Figure 5**. Compared with Koshihikari (905 $\mu$g T3/g dry wt), there was an improvement in T3 contents in all F1 rice bran, except in Koshihikari/Hirayama and Koshihikari/Moritawase. Nevertheless, T3 ratios in the F1 Koshihikari/Hirayama and Koshihikari/Moritawase were heightened to about 70% compared with Koshihikari (60%) (data not shown). These results showed that when Koshihikari was crossbred with T3-rich varieties, the T3 profile of F1 generation was found improved from that of parent Koshihikari.

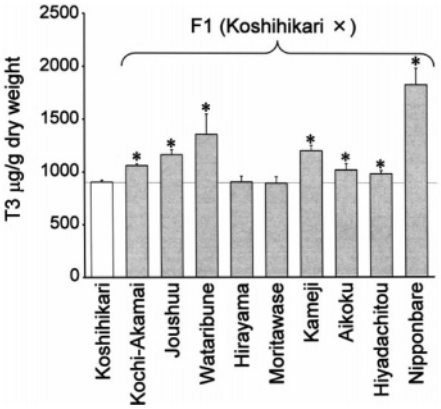


**Figure 5.** Tocotrienol (T3) content of Koshihikari (F1 generation) when Koshihikari were cross-fertilized with T3-rich varieties (Kouchi-Akamai, Joushuu, Wataribune, Hirayama, Moritawase, Kameji, Aikoku, Hiyadachitou, and Nipponbare). Mean ± SD ($n = 3$). *, significant difference compared to Koshihikari ($P < 0.05$).

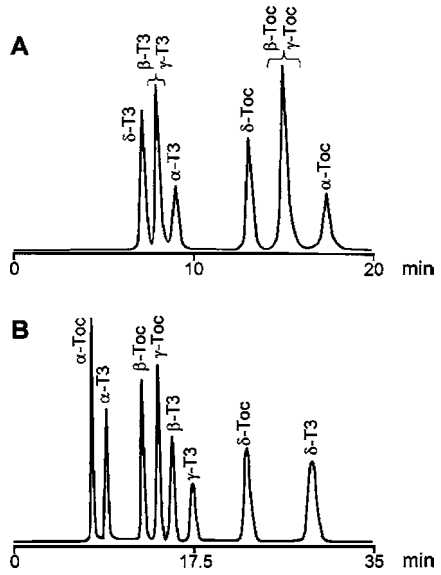


**Figure 6.** Different HPLC methods for standard T3 and Toc. (**A**) Reverse-phase HPLC (column, TSK-Gel ODS-80Ts, 4.6 × 250 mm; mobile phase, methanol/acetonitrile (1/5, v/v)). (**B**) Normal-phase HPLC (column, ZORBAX RX-SIL, 4.6 × 250 mm; mobile phase, hexane/ethanol (98/2, v/v)). The flow rates were 1.0 mL/min. The column temperatures were maintained at 35 °C. T3 and Toc were detected by FLD (excitation 294 nm, emission 326 nm).

## DISCUSSION

The eight vitamin E compounds might have different biological functionalities (*14*), so it is necessary to be able to have quantitative data on the biological level of each vitamin E separately. Chromatographically, normal-phase HPLC shows clear separation of all eight tocols, while there is a peak overlapping between $\beta$- and $\gamma$-isoforms in reverse-phase HPLC (**Figure 6**). This indicates the normal-phase HPLC to be the suitable quantitative tool for determination of rice bran T3 and Toc. Generally, determination methods of vitamin E require 20−25 min or more in a replication (*15*, *16*). In contrast, our analytical time of vitamin E was reduced to within 15 min, which is considered as the rapid analysis, by using optimized condition (mobile phase, *n*-hexane/1,4-dioxane/2-propanol (100:40:5, v/v/v); flow rate, 1.0 mL/min; column temperature, 35 °C) (**Figure 2A**).

Among vitamin E extraction methods including liquid−liquid-phase extraction, one-step equilibrium direct solvent extraction, solid-phase extraction, and supercritical fluid extraction (*12*, *17*−*19*), we chose the one-step equilibrium direct solvent extraction method. According to the study of Chen and Bergman (*12*) using one-step equilibrium direct solvent extraction, methanol was used as an extraction solvent for rice bran phytochemicals (T3, Toc, and $\gamma$-oryzanol). In our study, the polarity of methanol was a concern, because our method to determine rice bran vitamin E was normal-phase HPLC. We therefore selected 2-propanol as the extraction solvent, because the polarity of 2-propanol was not too high to be submitted to normal-phase HPLC after dilution with hexane but still high enough to extract rice bran T3 and Toc (**Table 1**). The 2-propanol extract of rice bran was directly submitted into our developed normal-phase HPLC after dilution with hexane, and the chromatogram showed clear peak separation in a short period of time (**Figure 2B**).

Taking together both extraction and HPLC determination of vitamin E, the rapid screening method for T3-rich rice bran was developed. The characteristics and advantages of our developed screening method can be explained as follows. (1) By using one-step equilibrium direct solvent extraction method, no special instrumentation or complicated procedure is needed. (2) The operation time was reduced and the screening method was simple and convenient enough to be applied to a large number of samples. (3) The method was safe because no seriously toxic chemicals or high temperature was included in the process. (4) Besides its simplicity and convenience, the method was satisfactorily selective and effective for quantification of T3 and Toc present in rice bran. (5) The method is cost-effective compared to the other analytical methods of vitamin E.

Rice cultivars have a wide variation range in their physical and physiobiological aspects. It was reported that many rice cultivars contained the difference in their kernel composition (i.e., amylase, mineral matter, crude fat, and crude protein) (*20*). Because of our screening results, a large variation of T3 and vitamin E contents was found in 109 rice bran samples (**SI Table 1** and **Figure 3**). Kouchi-Akamai, Joushuu, and Wataribune had the highest values of T3 content, while Hirayama, Moritawase, and Kaneko had the highest T3 ratio. These varieties would be considered as attractive sources of T3 or vitamin E products. By the way, the different levels of T3 and Toc contents may be due to the subtle difference at a molecular or genetic level of each cultivar. Explanatorily, homogentistic acid geranyl geranyl transferase (HGGT, T3 synthetase) and homogentistic acid phytyl transferase (HPT, Toc synthetase) are quite identical and can be altered to the other class with a subtle change in amino acid sequences (*21*). Cahoon et al. (*22*) reported that an increase in HGGT activity in transgenic plants (i.e., *Arabidopsis thaliana* and tobacco callus) caused the production of a considerable amount of T3. Therefore, the T3-rich rice varieties may have the genetic characteristics responsible for HGGT or even enzymes related to biosynthesis of vitamin E precursors (i.e., acetyl CoA, geranylgeranyl diphosphate, and homogentistic acid). Using our data as the reference, the genetic characteristic of the varieties high in T3 profile could be better clarified.

By the way, as shown in **Figure 4**, the positive correlation between $\gamma$-T3 and total vitamin E content was found, and Kouchi-Akamai and Wataribune (varieties high in $\gamma$-T3 and Toc) were separated around the top right of the chart, suggesting their unique characteristics in vitamin E composition. In contrast,

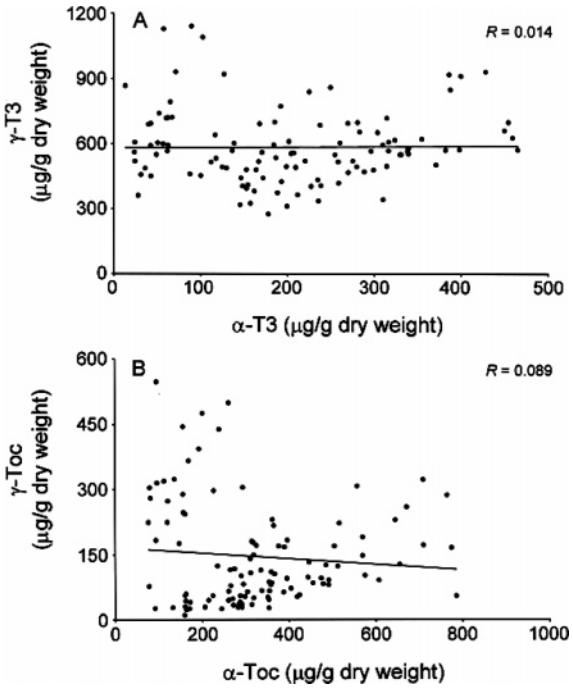


**Figure 7.** Correlation plots between α- and γ-forms of both tocotrienol (T3) and tocopherol (Toc): (**A**) between α-T3 and γ-T3 and (**B**) between α-Toc and γ-Toc.

there was a loose correlation found between total Toc and total T3, indicating that the relationship between T3 and Toc is not proportional and that T3 and Toc biosynthesis might not be directly related. In plants, γ-T3 and γ-Toc were believed to be separately synthesized and, then, partially converted to α-form by the enzymatic activity of methyltrasferase. However, there was no correlation found between α- and γ-forms of both T3 and Toc (**Figure 7**), suggesting the possibility that the α-form of vitamin E may not be directly related to the conversion of the γ-form by the methyltransferase.

Crossbreeding has been used as a tool for development of some interesting characteristics in plants or animals. In rice farming, crossbreeding has been used to improve some characteristics of the rice trees (i.e., weed competitiveness in aerobic rice) (*23*). In our study, we found that T3 in the cross-fertilized F1 rice bran generation was improved compared with the parent Koshihikari (**Figure 5**). Since some T3-rich rice varieties (e.g., Wataribune and Kouchi-Akamai) have an inferior flavor and taste, the study on the cross-fertilization needs more replications, and the organoleptic examination (a test for appearance, odor, flavor, and texture) is needed to confirm the best variety (a variety having preferable sensory characteristics and being high in nutritional profiles including T3).

As there is interest now in T3 because of its physiological functionalities (i.e., antithrombotic, antitumor, and antiangiogenic effects), how to make use of natural T3 is in focus. However, there are some concerns about not only the limited T3 sources but also its coexistence with Toc. According to most T3 studies, T3-rich fractions, partially containing Toc, prepared from rice bran oil or palm oil have been used (*24*, *25*). As one of our goals is to prepare a large amount of high-purity T3 for its therapeutic benefits, we are studying many related fields such as farming T3-rich rice paddy, the technology for a large scale separation between T3 and Toc, as well as production of T3 functional foods. After a lot of pure T3 is obtained, many studies concerning therapeutic and nutraceutical properties of T3 will be employed.

In conclusion, we developed the rapid screening method for T3-rich rice bran, and by using the method, Kouchi-Akamai, Joushuu, and Wataribune were found as the T3-rich rice varieties. Accordingly, some varieties high in T3 profile were cross-fertilized with Koshihikari, and it was an improvement in T3 content or the ratio in the F1 rice bran compared with the parent Koshihikari, suggesting the cross-fertilization process as an effective tool for improving T3 level in rice cultivars.

## ABBREVIATIONS USED

FLD, fluorescence detection; HGGT, homogentistic acid geranyl geranyl transferase; HPLC, high-performance liquid chromatography; HPT, homogentistic acid phytyl transferase; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; Toc, tocopherol; T3, tocotrienol.

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**Supporting Information Available:** Tocotrienol (T3) and tocopherol (Toc) contents in 109 rice bran varieties. This material is available free of charge via the Internet at http://pubs.acs.org.

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