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High Expression of Tocochromanol Biosynthesis Genes Increases the Vitamin E Level in a New Line of Giant Embryo Rice

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Supporting Information

ABSTRACT: 'Shangshida No. 5' is a new variety of giant embryo rice derived from a single-point mutation of the *giant embryo* gene (*ge*) in 'Chao2-10' rice. This study quantified the levels of eight vitamin E homologues (*α*-, *β*-, *γ*-, and *δ*-tocopherol and *α*-, *β*-, *γ*-, and *δ*-tocotrienol) in brown rice, embryos, endosperm, and developing seeds of giant embryo and normal embryo rice using a normal phase high-performance liquid chromatographic method. Quantitative RT-PCR analysis was conducted to reveal the different expression patterns of the *ge* gene and tocochromanol biosynthesis genes in developing giant and normal embryo seeds. The total vitamin E content in 'Shangshida No. 5' brown rice was 52.54 mg *α*-tocopherol equivalent (*α*-TE)/kg, of which *α*-tocopherol constituted 49.14 mg/kg, which was approximately 2.2-fold greater than that in 'Chao2-10' brown rice. In giant embryo seeds, the expression level of the *ge* gene was higher than that in normal embryo seeds during early developmental stages. These results are the first to indicate that coup-regulated expression of the *OsHPPD*, *OsHPT*, and *OsMPBQ MT2* genes might be the primary reason for the large accumulation of *α*-tocopherol in giant embryo rice seeds. The different transcription pattern of the tocochromanol biosynthesis genes in 'Shangshida No. 5' rice seeds compared with 'Chao2-10' rice seeds is attributable to the *ge* mutation and the different expression level of the *ge* gene in giant embryo seeds.

KEYWORDS: *giant embryo rice, giant embryo gene (ge), vitamin E, tocopherols, tocotrienols, biosynthetic pathway, normal phase high-performance liquid chromatography, quantitative RT-PCR*

INTRODUCTION

Vitamin E, specifically tocochromanols, comprises a group of structurally related amphiphilic lipid-soluble antioxidants that have diverse biological activities and benefits in humans and animals. Natural tocochromanols mainly consist of eight homologues: *α*-, *β*-, *γ*- and *δ*-tocopherol and *α*-, *β*-, *γ*- and *δ*-tocotrienol. In general, tocopherols and tocotrienols have similar molecular structures that are characterized by a hydrophobic C_{16} side chain attached to the 2-position of an aromatic headgroup. In nature, *α*-tocopherol is found in a single *RRR* configuration.[1] It is generally thought that *RRR*-*α*-tocopherol possesses the highest antioxidative activity of all tocochromanols in humans and animals, and this activity is largely dependent on the preferential capture and distribution of *RRR*-*α*-tocopherol mediated by hepatic *α*-tocopherol transfer protein (*α*-TTP).[2,3] A mixture of tocopherols, containing *α*-, *γ*-, and *δ*-tocopherol in defined volume proportions, has been demonstrated to display better antioxidative and anti-inflammatory activities than *α*-tocopherol alone in in vitro antioxidation reactions.[4,5] Tocotrienols possess many functions that are not shared by tocopherols. Tocotrienols can inhibit cholesterol biosynthesis through a post-transcriptional down-regulation of 3-hydroxy-3-methylglutarylCoA reductase (HMGR), the key enzyme in the cholesterol biosynthesis pathway.[6] Tocotrienols have also been reported to be more effective than tocopherols at preventing cardiovascular diseases, cancer, and some chronic diseases,[7−11] which might be related to the involved signal transduction pathways and the regulation of gene expression profiles. In plants, tocochromanols are well-known as efficient antioxidants that play important roles in protecting photosystem II and membrane lipids from oxidation and peroxidation.[12,13] It has been demonstrated that tocopherols can limit nonenzymatic lipid oxidation during seed storage, germination, and early seedling development.[14] Tocopherols have been shown to be involved in non-antioxidative functions such as the regulation of carbohydrate metabolism in higher plants,[15] which has been proposed to be associated with the involvement of tocopherols in signaling pathway and gene expression.

Vitamin E is mainly synthesized in plant chloroplasts and other photosynthetic organisms. This biosynthesis pathway utilizes cytosolic homogentisic acid (HGA) for headgroup synthesis and plastid phytyl-PP (PDP) for tocopherol tail synthesis and geranylgeranyl diphosphate (GGDP) for tocotrienol tail synthesis.[16] During the past 20 years, several key enzyme genes required for tocochromanol biosynthesis in photosynthetic tissues have been identified and cloned, along with the application of molecular biological approaches and bioinformatics analysis in the model organisms *Synechocystis* sp. PCC6803 and *Arabidopsis thaliana*.[16] Mutant and transgenic studies in plants have provided insight into the function, regulation, and interaction of these key enzymes[17−27] and have provided an important theoretical basis for molecular breeding to modify the content and type of tocochromanols in crops.[20,27−29] Chaudhary and Khurana[30] identified the

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homologous tocochromanol biosynthesis genes (*OsHPPD*, *OsHPT*, *OsHGGT*, *OsMPBQ MT*, *OsTC*, and *OsγTMT*) in rice (*Oryza sativa* L.). Most of these genes have been observed to have higher expression levels in light-grown or photosynthetic tissues, which indicates their possible regulation by light.[30] In addition, almost all of these genes are highly expressed in developing and mature seeds, and *OsHGGT* is specifically expressed only in rice seeds.[30] Furthermore, rice has a duplicated copy of the *OsMPBQ MT* gene, and *OsMPBQ MT2* has a high expression level in all tissues and developmental stages, but *OsMPBQ MT1* has a negligible expression in the same tissues and developmental stages.[30]

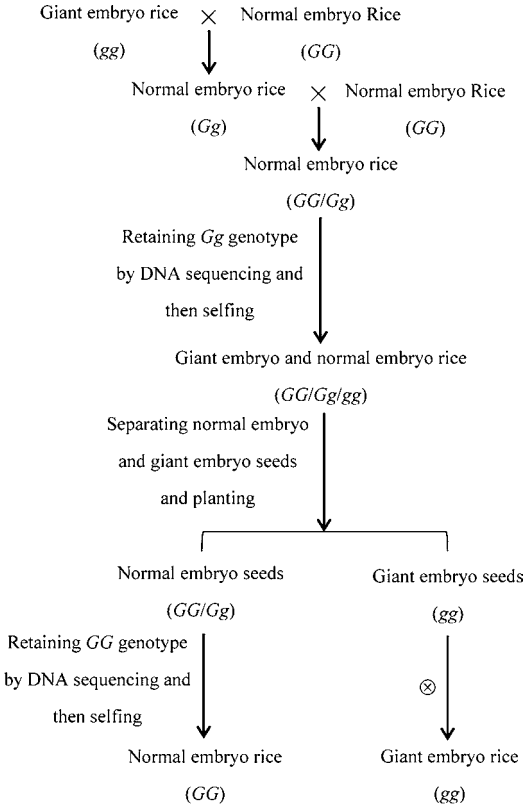
The human daily intake of vitamin E is primarily derived from plant-originated foods or vitamin supplements. Brown rice, which is known as a very good source of tocopherols and tocotrienols, has received considerable research attention.[31−35] Giant embryo rice is characterized by an enlarged embryo that is controlled by a single recessive gene.[36] Mutation of the *giant embryo* gene (*ge*) has been shown to result in the enlargement of the rice embryo.[37,38] The *ge* gene is located on chromosome 7 and encodes a protein member of the cytochrome P450 enzyme family.[37] A significant increase in vitamin E has been reported in brown rice of giant embryo mutants.[38−40] 'Shangshida No. 5' is a new variety of giant embryo rice obtained from tissue culture and regeneration of *japonica* cv. rice 'Chao2-10' mature embryos[41] as a result of a single-point mutation in the *ge* gene coding region. Our study focused on the accumulation process of vitamin E in giant embryo rice. This is the first study to suggest a detailed and dynamic cumulative process of eight vitamin E homologues (*α*-, *β*-, *γ*-, and *δ*-tocopherol/tocotrienol) in giant and normal embryo rice. The molecular mechanism underlying vitamin E enrichment in giant embryo rice was also explored via rice vitamin E biosynthesis genes and *ge* gene expression analyses. The knowledge obtained from these studies should be helpful for identifying the potential value of 'Shangshida No. 5' giant embryo rice and for revealing the process by which tocochromanols accumulate during rice seed development, representing an initial exploration of the relationships between *ge* gene mutation and extensive accumulation of vitamin E in giant embryo rice.

MATERIALS AND METHODS

Chemicals. Tocopherols (*α*-, *β*-, *γ*-, and *δ*-T) and tocotrienols (*α*-, *β*-, *γ*-, and *δ*-T3) were obtained from ChromaDex Inc. (Irvine, CA, USA). The compounds 2,2,5,7,8-pentamethyl-6-chromanol (PMC, internal standard) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hexane and 2-propanol of high-performance liquid chromatography (HPLC) grade were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and Merck (Darmstadt, Germany), respectively. Ethyl acetate and acetic acid were obtained from Dikma Technologies Inc. (Lake Forest, CA, USA). All other chemicals used in this study were of analytical grade.

Rice Samples. The rice cultivars used in this study, homozygous giant embryo rice (*O. sativa* L. *japonica* cv. Shangshida No. 5) and homozygous normal embryo rice (*O. sativa* L. *japonica* cv. Chao2-10), were obtained again from the program described in Scheme 1. According to this program, the 'Shangshida No. 5' rice (used as giant embryo rice, female parent) and 'Chao2-10' rice (used as normal embryo rice, pollen parent) were planted and selected to have a comparable genetic composition overall between the two tested rice cultivars, except for a single-point mutation in the *ge* gene in giant embryo rice. DNA sequencing was used for *ge* gene identification, with the forward primer 5′-ATGACCCTTTGATTACGCT-3′ and the reverse primer 5′-GACGAACACCTCCACCTT-3′. Giant embryo and normal embryo rice were both planted in the test fields of Shanghai Normal University at the same time of year. According to the normal process of rice production, the mature seeds were dried in the sun and then stored at −20 °C.

Scheme 1. Cultivation Program of the Tested Rice Used in the Study[a]



[a]*G*, the wild-type *giant embryo* gene (*GE*); *g*, the mutant *giant embryo* gene (*ge*); *GG* (*GG* genotype), rice or rice seeds with a pair of homozygous wild-type *GE* alleles; *Gg* (*Gg* genotype), rice or rice seeds with a wild-type *GE* gene and a mutant *ge* gene on homologous chromosomes; *gg* (*gg* genotype), rice or rice seeds with a pair of homozygous mutant *ge* alleles.

Standard Stock Solution Preparation. Each stock solution of tocopherols and PMC was prepared in hexane at a concentration of 2 mg/mL, and each tocotrienol stock solution was prepared in hexane at a concentration of 1 mg/mL. All of the stock solutions were stored at −4 °C, tightly closed and protected from light. The working solutions of the standard mixture were prepared from these stock solutions and diluted with hexane to the necessary concentrations.

Rice Sample Extraction. The mature and developing rice seeds (obtained at 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 days after flowering) of the 'Shangshida No. 5' giant embryo rice and 'Chao2-10' normal embryo rice cultivars were separated from rice husk and then ground into powder at a low temperature. Mature embryos of the two tested rice cultivars were removed from the endosperm and ground into powder individually at a low temperature. The extraction procedures were previously described by Jang and Xu.[42] Briefly, 0.5 g of each sample was transferred into a 15 mL test tube (17 × 120 mm), and then 3 mL of hexane and 50 μL of BHT (10 mg/mL) were added. The test tubes were capped, and the mixture was vortexed for 1 min and then placed in a 60 °C water bath for 25 min with intermittent shaking. The supernatant hexane layer was separated and collected by centrifugation at 2000*g* for 15 min, and the residues were further treated twice with the same procedures. The supernatants were collected together, diluted, and filtered to a volume of 10 mL. Then, 20 μL of filtrate was taken for HPLC analysis.

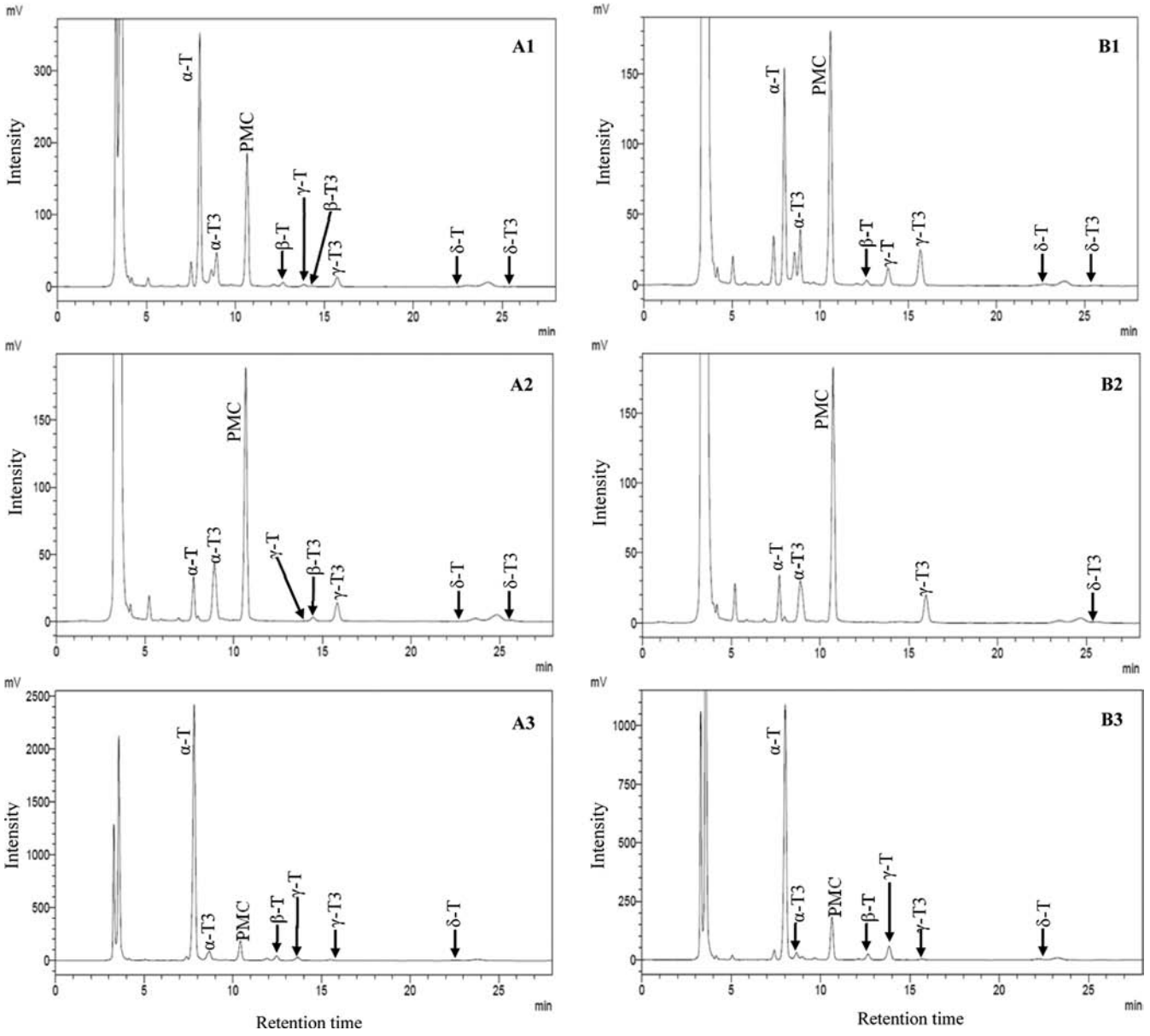


Figure 1. Chromatograms of the vitamin E homologues in brown rice, endosperm, and embryos of the two tested rice cultivars: (A1−A3) 'Shangshida No. 5' rice; (B1−B3) 'Chao2-10' rice; (A1, B1) brown rice; (A2, B2) endosperm; (A3, B3) embryo. *α*-T, *α*-tocopherol; *β*-T, *β*-tocopherol; *γ*-T, *γ*-tocopherol; *δ*-T, *δ*-tocopherol; *α*-T3, *α*-tocotrienol; *β*-T3, *β*-tocotrienol; *γ*-T3, *γ*-tocotrienol; *δ*-T3, *δ*-tocotrienol; PMC, 2,2,5,7,8-pentamethyl-6-chromanol (internal standard).

Quantification of Tocopherols and Tocotrienols in Rice Samples. The HPLC analysis used a method described by Huang and Ng[34] with a change in the mobile phase and a different chromatographic system. Briefly, the Shimadzu HPLC system (Japan), consisting of an LC-20AT pump and an RF-20Axs fluorescence detector set at 290 mm of excitation wavelength and 330 mm of emission wavelength, was used. Chromatographic separation was performed using a normal phase Inertsil SIL-100A column (particle size = 5 μm; column size = 4.6 mm i.d. × 250 mm length, Shimadzu GL Sciences Inc., Japan) coupled with an EasyGuard silica (5 μm; 4.0 mm × 10 mm) guard column (Dikma Technologies Inc.). The mobile phase was composed of hexane/2-propanol/ethyl acetate/acetic acid (99.1:0.3:0.3:0.3, v/v/v/v). The flow rate was 1 mL/min, and the analysis was completed at room temperature. A typical chromatogram of *α*-, *β*-, *γ*-, and *δ*-tocopherol (T) and *α*-, *β*-, *γ*-, and *δ*-tocotrienol (T3) in standard mixture is shown in the Supporting Information (Figure S1). The linear relationship of each single vitamin E homologue was determined at six (for *α*-T) or five (for other homologues) concentration points ranging from 0.1 to 30 μg/mL for *α*-T and from 0.1 to 10 μg/mL for the other homologues. All eight vitamin E homologues produced a high correlation coefficient (r^2 > 0.99) in the concentration ranges tested (Supporting Information Figure S2). The tocopherols and tocotrienols in each rice sample were identified via chromatographic comparisons of retention time with their respective standards and quantified using a calibration curve of internal standards. The reliability and repeatability of this NP-HPLC method was confirmed by a recovery test of tocopherols and tocotrienols as described by Huang and Ng[34] in brown rice samples in triplicate, and the recovery rate of the eight homologues varied from 92.40 to 110.10% (Supporting Information Table S1).

RNA Isolation from Developing Rice Seeds and cDNA Synthesis. Total RNA from the developing seeds (6, 10, 14, 20, 26, and 30 days after flowering, without husk) was extracted using TRIzol reagent (Life Technologies Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the high-quality RNA samples were used to synthesize the first-strand cDNA sequences

Table 1. Content of Tocopherols and Tocotrienols in Brown Rice, Embryos, and Endosperm of the Two Tested Rice Cultivars[a]

	tocopherols (mg/kg, DW)				tocotrienols (mg/kg, DW)			
cv.	α	β	γ	δ	α	β	γ	δ
Brown Rice								
Chao2-10	21.85 ± 0.82	0.94 ± 0.03	3.13 ± 0.09	0.25 ± 0.02	5.20 ± 0.18	ND[b]	6.98 ± 0.23	0.14 ± 0.01
Shangshida No. 5	49.14 ± 0.37**	1.51 ± 0.03**	0.96 ± 0.05**	0.49 ± 0.00**	8.28 ± 0.32**	0.14 ± 0.02	3.95 ± 0.04**	0.08 ± 0.01
Embryo								
Chao2-10	153.31 ± 3.30	5.89 ± 0.33	18.39 ± 0.30	2.36 ± 0.10	2.55 ± 0.46	ND	1.12 ± 0.01	ND
Shangshida No. 5	375.92 ± 13.77**	12.40 ± 0.51*	9.36 ± 0.27*	1.61 ± 0.03*	2.73 ± 0.06	ND	2.82 ± 0.12*	ND
Endosperm								
Chao2-10	0.33 ± 0.02	ND	ND	ND	7.39 ± 0.23	ND	5.32 ± 0.12	0.07 ± 0.00
Shangshida No. 5	0.17 ± 0.00**	ND	0.04 ± 0.00	ND	8.45 ± 0.25*	0.86 ± 0.04	3.36 ± 0.04**	0.11 ± 0.00

[a]Values are the mean ± SD of three analyses ($n = 3$). **, significant difference ($P < 0.01$); *, significant difference ($P < 0.05$); values without an asterisk, no significant difference ($P > 0.05$). [b]ND, not detected. Between two rice cultivars.

according to the manufacturer's instructions of the PrimeScript RT Reagent kit (TaKaRa Bio Inc., Otsu, Shiga, Japan).

Quantitative RT-PCR Analysis of Gene Expression. The transcription levels of known tocochromanol biosynthesis enzyme genes and the *giant embryo* gene (*ge*) in developing rice seeds (without husk) were quantified by quantitative RT-PCR analysis using a C1000 Thermal Cycler and a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers for the tocochromanol biosynthesis genes used in this study were described by Chaudhary and Khurana,[30] and the primers of the *ge* gene and *OsActin* gene were designed using Primer Premier 5 software (Primer Biosoft, Palo Alto, CA, USA) (Supporting Information Table S2). The cDNA samples (100 ng) were mixed with 10 μM of each primer and IQ SYBR Green Supermix (Bio-Rad Laboratories Inc.). Amplification reactions were conducted according to the following parameters: 2 min at 95 °C, 40 cycles of 20 s at 95 °C and 1 min at 60 °C in 96-well white plates (Bio-Rad Laboratories Inc.). The specificity of these reactions was certified by melting curve analysis. The relative transcription levels of each gene in different RNA samples were normalized with respect to the internal standard *OsActin* gene. Each cDNA sample was subjected to quantitative RT-PCR analysis in triplicate. The values in the graphs were calculated using the Livak method ($2^{-\Delta\Delta CT}$).[43]

RESULTS

Tocopherol and Tocotrienol Content in Brown Rice, Embryos, and Endosperm. The chromatograms of eight vitamin E homologues in brown rice and the embryos and endosperm of 'Shangshida No. 5' giant embryo rice and 'Chao2-10' rice are shown in Figure 1. The content of tocopherols and tocotrienols in brown rice and the embryos and endosperm of the two tested rice cultivars ('Shangshida No. 5' rice and 'Chao2-10' rice) are presented in Table 1. The results indicated that the major types of vitamin E present in the two tested brown rice were α-T, α-T3, γ-T, and γ-T3, and the order of abundance of those vitamin E homologues (from highest to lowest) was α-T ≫ α-T3 > γ-T3 > γ-T in 'Shangshida No. 5' brown rice and α-T ≫ γ-T3 > α-T3 > γ-T in 'Chao2-10' brown rice. The distribution profiles of T and T3 in giant embryo brown rice were basically in accordance with that in normal embryo brown rice. Tocotrienols accumulated mainly in the endosperm, but a large portion of tocopherols accumulated in the embryo (Table 1). The α-T and α-T3 contents in 'Chao2-10' brown rice were 21.85 and 5.20 mg/kg, respectively. 'Shangshida No. 5' brown rice presented higher levels of α-T (49.14 mg/kg) and α-T3 (8.28 mg/kg) but, interestingly, lower levels of γ-T (0.96 mg/kg) and γ-T3 (3.95 mg/kg) than 'Chao2-10' brown rice (3.13 and 6.98 mg/kg for γ-T and γ-T3, respectively). The two tested brown rices each had minute amounts of δ-T and δ-T3, and β-T3 was detected only in 'Shangshida No. 5' brown rice (Table 1).

Contents of Tocopherols and Tocotrienols in Developing Rice Seeds. To assess the accumulation process of each type of tocochromanol in giant embryo seeds and normal embryo seeds, 6−30 days after flowering (DAF) developing rice seeds of 'Shangshida No. 5' rice and 'Chao2-10' rice were used for quantitative tests. The accumulation profiles of the tocopherols and tocotrienols in 'Shangshida No. 5' giant embryo seeds and 'Chao2-10' seeds (as a control) are shown in Figure 2, and the content levels are presented in Tables S3 and S4 of the Supporting Information. The accumulation of tocochromanols in developing rice seeds exhibited fluctuations over time. There was only a small amount of vitamin E in 6−16 DAF developing seeds. An obvious increase of α-T in 'Shangshida No. 5' giant embryo seeds was presented after 16 DAF (Figure 2). From that point, the content of α-T in giant embryo seeds continuously increased, and the α-T content in 30 DAF giant embryo seeds was 51.50 mg/kg (Supporting Information Table S3). Large portions of β-T, γ-T, α-T3, and γ-T3 were accumulated since 18 DAF in the two tested rice cultivars, once or twice falling back to low levels. δ-T and δ-T3 were not detected in 28 and 30 DAF 'Chao2-10' rice seeds. The increase of δ-T content peaked at 14, 20, and 26 DAF, with a high concentration point of 1.0 mg/kg during the development of normal embryo seeds (Figure 2). In giant embryo seeds, δ-T was detected at only 22, 26, and 30 DAF, with a final content of 0.16 mg/kg at 30 DAF (Supporting Information Table S3). Similar results were obtained in the δ-T3 quantitative analysis. It is interesting that β-T/T3 but not δ-T/T3 displayed preferential accumulation in both the giant and normal embryo seeds. In general, the α-, β-, and δ-T/T3 levels in 30 DAF giant and normal embryo rice seeds were quite similar to the levels in mature giant and normal embryo brown rice grains. It is rather surprising that the levels of γ-T (6.69 mg/kg) and γ-T3 (6.29 mg/kg) in 30 DAF giant embryo seeds were far higher than the levels in giant embryo brown rice (0.96 and 3.95 mg/kg, respectively). These levels in mature and 30 DAF 'Chao2-10' seeds were quite similar, and the values were 3.13 and 3.73 mg/kg for γ-T and 6.98 and 6.59 mg/kg for γ-T3, respectively (Figure 3).

Expression Profiles of Tocochromanol Biosynthesis Genes and the *ge* Gene. To study the expression pattern of the tocochromanol biosynthesis genes and the *ge* gene in 'Shangshida No. 5' giant embryo rice and 'Chao2-10' normal embryo rice seeds, quantitative RT-PCR analysis was performed on the developing seeds of these two tested rice

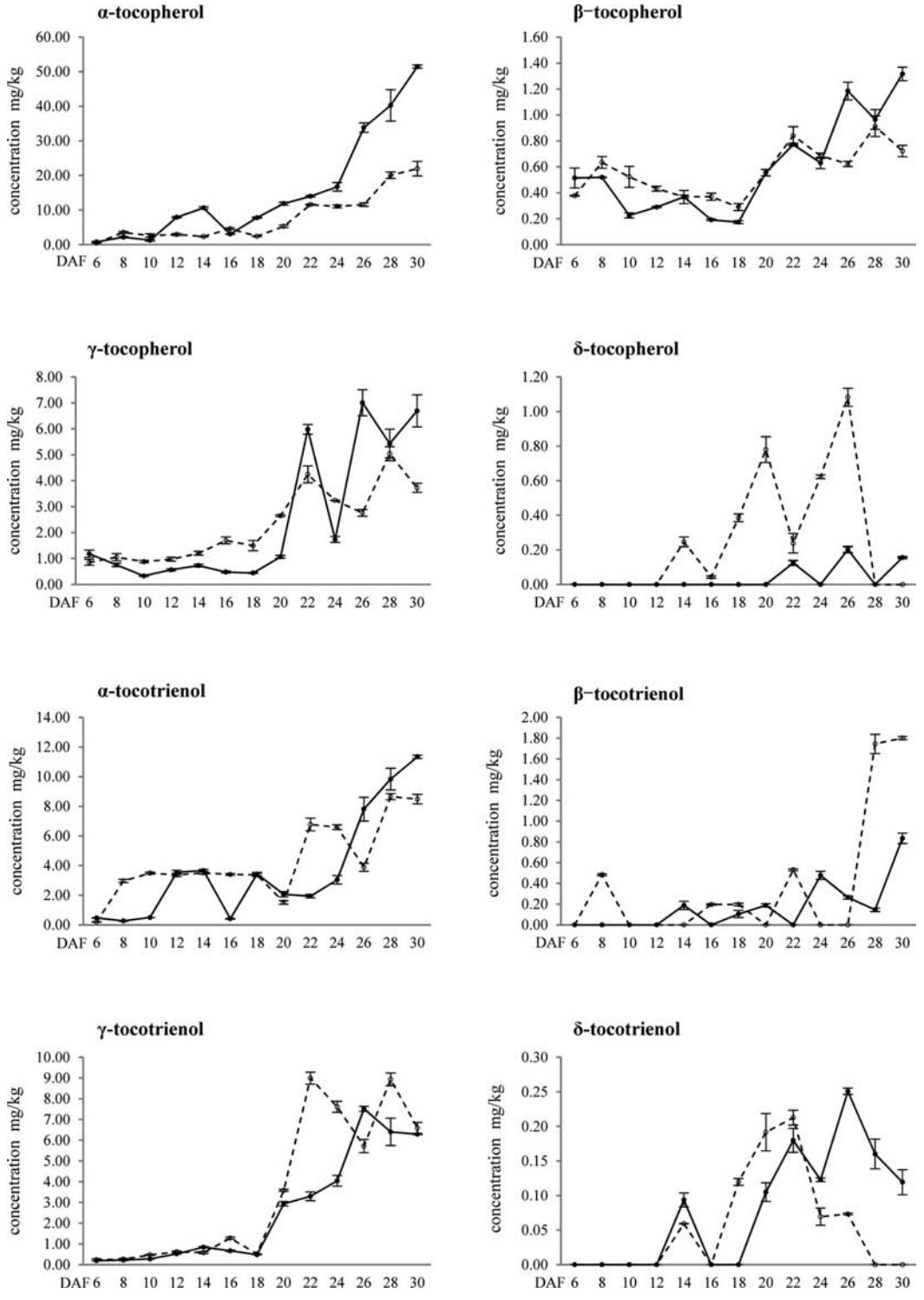


Figure 2. Accumulation profiles of eight vitamin E homologues in the developing seeds of the two tested rice cultivars: (solid lines) 'Shangshida No. 5' giant embryo rice; (dashed lines) 'Chao2-10' rice; (error bars) standard deviation (SD).

cultivars. Total RNA samples were prepared from seeds (without husk) of 'Shangshida No. 5' rice and 'Chao2-10' rice harvested at 6, 10, 14, 20, 26 and 30 DAF. The *OsActin* gene was used as a control. *OsHPPD* is the key enzyme gene in the homogentisic acid biosynthesis, which is a committed step in vitamin E headgroup synthesis.[16] In 'Chao2-10' rice seeds, *OsHPPD* was highly expressed at the last stage of development and peaked at 26 DAF. In 'Shangshida No. 5' giant embryo seeds, the expression of *OsHPPD* increased gradually with seed development and only dramatically increased at 14 DAF (Figure 4). Homogentisate phytyl transferase (HPT) and homogentisic acid geranylgeranyl transferase (HGGT) catalyze the condensation of PDP and HGA in tocopherol synthesis and the condensation of GGDP and HGA in tocotrienol synthesis, respectively.[16] The expression patterns of *OsHPT* were obviously different in the two types of tested rice seeds. In 'Chao2-10' rice seeds, a very low expression level was maintained until 30 DAF. However, in 'Shangshida No. 5'

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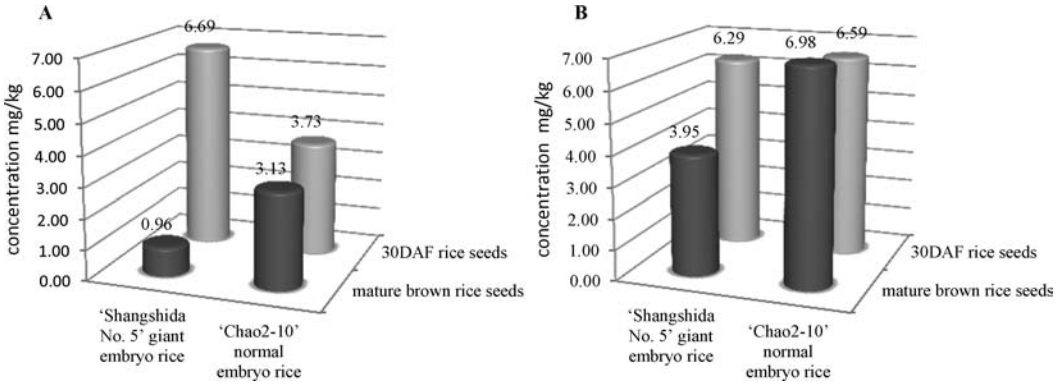

Figure 3. Contents of γ-tocopherol and γ-tocotrienol in mature brown rice seeds and 30 DAF rice seeds of the two tested rice cultivars: (A) γ-tocopherol; (B) γ-tocotrienol.

rice seeds, the *OsHPT* gene was obviously highly expressed at 10 and 26 DAF (Figure 4). The *OsHGGT* gene presented a similar transcription pattern in the two tested rice cultivars, except for the different expression peak at 10 DAF in 'Chao2-10' normal embryo seeds and at 14 DAF in 'Shangshida No. 5' giant embryo seeds (Figure 4). In addition, 2-methyl-6-phytylbenzoquinol methyltransferase (MPBQ MT) and γ-tocopherol methyltransferase (γ-TMT) are key methylation enzymes that determine the type of vitamin E accumulated in rice seeds.[16] In the tested giant embryo and normal embryo seeds, the *OsMPBQ MT2*, *OsγTMT*, and *OsTC* genes did not display a great difference in expression pattern. Similar to the *OsHGGT* gene, the transcription of these genes (*OsMPBQ MT2*, *OsγTMT*, and *OsTC*) peaked at 10 DAF in normal embryo seeds and at 14 DAF in giant embryo seeds (Figure 4). In addition, the transcription level of the *OsMPBQ MT2* gene was evidently increased in 'Shangshida No. 5' giant embryo seeds. The single-point mutation of the *ge* gene led to the enlargement of rice embryos in 'Shangshida No. 5' rice seeds. In the development of normal embryo rice seeds, the *ge* gene was moderately expressed and only peaked at 10 DAF. In contrast, the mutant *ge* gene was highly expressed at 6 and 10 DAF but peaked at 14 DAF in giant embryo seeds (Figure 4).

DISCUSSION

Rice (*O. sativa* L.) is an important crop and staple food in many Asian countries. Brown rice contains abundant phytochemicals such as tocopherols (T) and tocotrienols (T3), which are thought to have essential roles in the oxidation resistance of mammalian cell membranes and the prevention of cardiovascular and cerebrovascular diseases, cancer, and nervous system diseases.[44−46] The average total vitamin E content in brown rice of *japonica* rice (24.2 mg/kg) was shown to be noticeably higher than that in brown rice of *indica* rice (17.1 mg/kg) in a Brazilian study of 32 rice cultivars.[33] In *japonica* brown rice, α-T, α-T3, and γ-T3 were the three most abundant homologues, whereas in *indica* brown rice this order was γ-T3 > α-T > α-T3.[33] Zhang et al.[35] reported on the content of α-T, α-T3, γ-T, and γ-T3 in the brown rice of 34 rice cultivars from mainland China. Consistent with the results of Heinemann et al.,[33] γ-T3, α-T, α-T3, and γ-T (in descending concentration order) were the major vitamin E types in 18 *indica* cultivars.[35] However, in 16 *japonica* cultivars, the concentration of α-T was higher than that of γ-T3, and the concentration order was α-T > γ-T3 > α-T3 > γ-T.[35] In a study by Huang and Ng,[34] 12 commercial rice cultivars from Taiwan were used in a quantitative analysis of 8 vitamin E homologues. The authors found that γ-T3 was the most abundant vitamin E type in *japonica* and *indica* brown rice.[34] In six *japonica* cultivar brown rices, the level of α-T was 4.07−10.06 mg/kg, the level of α-T3 was 2.57−6.81 mg/kg, and the level of γ-T3 was 8.60−16.31 mg/kg.[34] In the present study, *japonica* rice 'Chao2-10' (used as a control) exhibited a high level of total vitamin E (24.27 mg α-TE/kg) compared with the average total vitamin E content (8.86 mg α-TE/kg) in brown rice of six *japonica* cultivars from Taiwan,[34] according to the formula of total vitamin E in the daily diet, expressed as α-tocopherol equivalent (mg α-TE/kg) = 1.0α-T (mg/kg) + 0.5β-T (mg/kg) + 0.1γ-T (mg/kg) + 0.03δ-T (mg/kg) + 0.3α-T3 (mg/kg) + 0.05β-T3 (mg/kg) + 0.01γ-T3 (mg/kg).[47] In addition, *japonica* 'Shangshida No. 5' brown rice, characterized by an enlarged embryo, was demonstrated to have a higher total vitamin E content (52.54 mg α-TE/kg) than 'Chao2-10' brown rice, and α-T was found at the highest level among the vitamin E homologues (49.14 mg/kg), which was approximately 2.2-fold higher than that value (21.85 mg/kg) in the 'Chao2-10' brown rice. Similarly, a study by Jeng et al.[48] suggested an increase in the α-T level in giant embryo mutant TNG17-GE brown rice, but the level in that study was approximately 1.8-fold higher than the value in normal embryo TNG17 brown rice. Surprisingly, 'Shangshida No. 5' giant embryo brown rice displayed a lower level of γ-T and γ-T3 than normal embryo brown rice (Table 1), but this difference was not observed in 30 DAF giant embryo seeds. It has been suggested that γ-T and γ-T3 might be more easily lost than other vitamin E homologues during the sunshine- or hot-air-based drying of the mature giant embryo seeds. In daily rice production, cryopreservation without drying might be a recommended method to maintain the stability of tocochromanols, especially γ-T and γ-T3 in giant embryo mature seeds. Our study verified that tocopherols primarily accumulated in the rice embryo, and the concentration of α-T increased considerably in giant embryos (Table 1). These results suggest a relationship between abundant accumulation of α-T and the mutation of the *ge* gene in 'Shangshida No. 5' rice seeds, but it is interesting that 'Shangshida No. 5' giant embryo seeds had smaller endosperm than 'Chao2-10' seeds but a similar concentration of tocotrienols. Therefore, the embryo or endosperm size changes were not the ultimate reason for the variation in the tocochromanols content.

In general, grain filling of rice seeds occurs form 6 to 20 DAF.[49] The endosperm cellularization and embryo differentiation are almost completed at 6 DAF, and then the size of

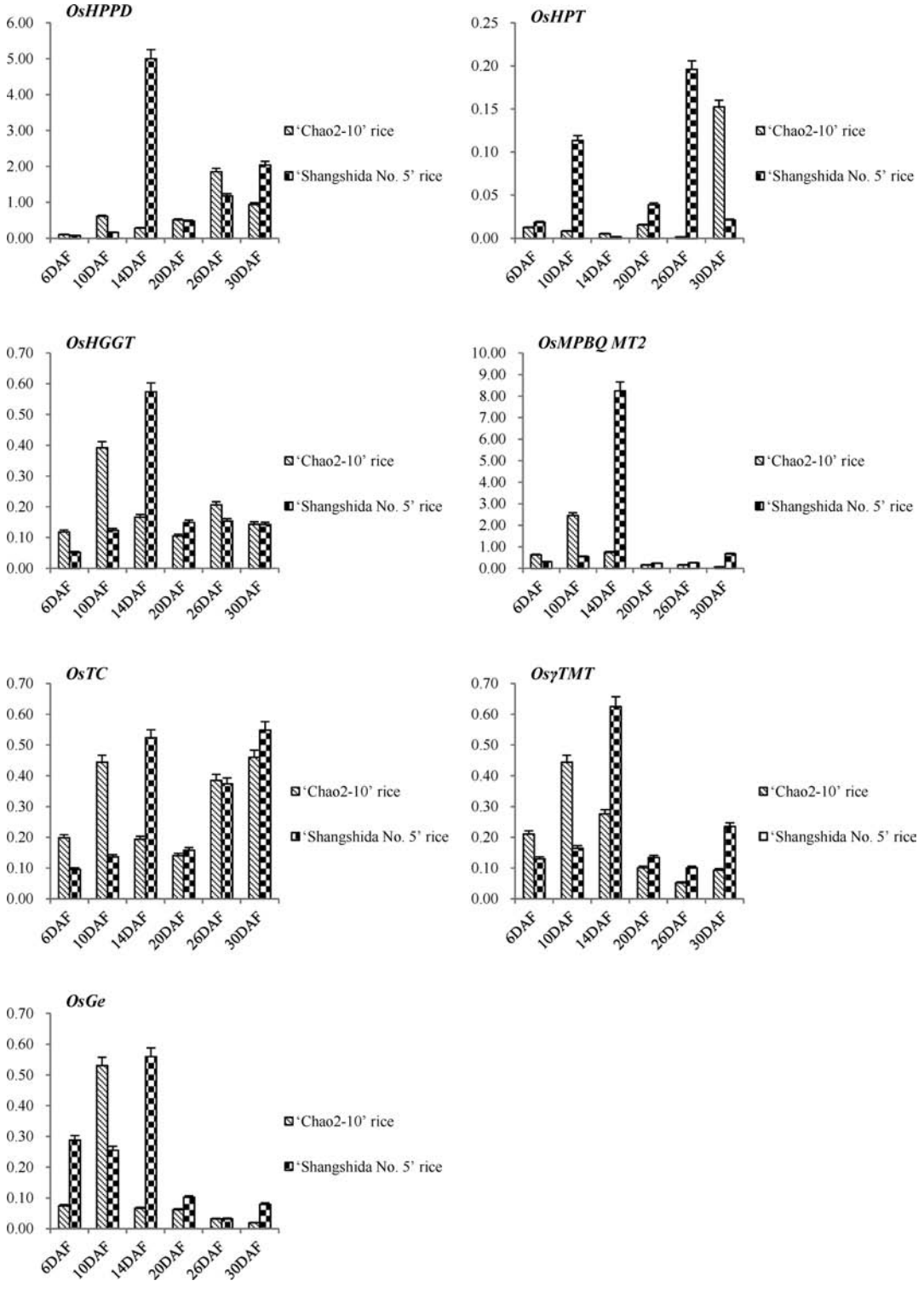

Figure 4. Expression analyses of tocochromanol biosynthesis genes and the *ge* gene in developing seeds of the two tested rice cultivars ('Shangshida No. 5' and 'Chao2-10'). *Y*-axis: relative gene expression using the value of $2^{\Delta CT}$.

developing seeds enlarges without interruption to the size of mature seeds at approximately 12 DAF.[49] Our study revealed a fluctuating accumulation process of eight vitamin E homologues during the development of rice seeds (Figure 2). In addition, the transcription levels of six tocochromanol biosynthesis genes in the developing seeds of the two tested rice cultivars ('Shangshida No. 5' and 'Chao2-10') were quantified to establish their relationship to the tocochromanol accumulation process. During 6−16 DAF, only a small amount of vitamin E was observed in developing rice seeds, and an obvious increase appeared after 16 DAF (Figure 2). For the *OsHGGT*, *OsMPBQ MT2*, *OsTC*, and *OsγTMT* genes, similar expression patterns were displayed in both giant embryo and normal embryo rice seeds, but the expression levels peaked at different development times in the 'Chao2-10' (10 DAF) and 'Shangshida No. 5' seeds (14 DAF) (Figure 4). These results

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can be attributed to the different development progress of embryos or whole seeds in giant and normal embryo rice. The maturity of 'Shangshida No. 5' rice seeds seemingly required a longer time than 'Chao2-10' normal embryo seeds. *OsHPPD* and *OsHPT* genes presented different expression patterns in the two tested rice cultivars. The expression of *OsHPT* was increased, particularly in developing giant embryo seeds, which could lead to a high accumulation of MPBQ, the key intermediate product in tocopherol biosynthesis. Moreover, the increased expression of the *OsMPBQ MT2* gene promoted the accumulation of DMPBQ and DMGGBQ, which determined the stronger preference for α-T/T3 and γ-T/T3 synthesized in 'Shangshida No. 5' giant embryo seeds. In addition, the increase in total vitamin E content was most likely based on the increased transcription level of *OsHPPD*, especially in 14 DAF giant embryo seeds, which might provide enough HGA for headgroup biosynthesis of tocochromanols. It has been suggested that the coup-regulated expression of *OsHPPD*, *OsHPT*, and *OsMPBQ MT2* genes might be the immediate reason for the abundant α-T accumulation in 'Shangshida No. 5' giant embryo seeds. Furthermore, the great accumulation of α-T practically had no effect on the biosynthesis and accumulation process of the other seven vitamin E homologues during the development of giant embryo rice seeds. The *giant embryo* gene (*ge*) mutation led to the enlargement of the rice embryo and, therefore, the transcription level of the mutant *ge* gene was also a salient point in our study. The rice *ge* gene encodes a protein member of the cytochrome P450 family.[37] Plant cytochrome P450 enzymes are NADPH-dependent monooxygenases that might play important roles in the oxidative metabolism of a variety compounds in plants.[37] The *ge* gene is mainly expressed in the early stage of rice seed development, before 10 DAF in normal embryo seeds and before 14 DAF in giant embryo seeds (Figure 4), and plays an important role in rice embryo development. Compared with normal embryo seeds, the transcription level of the mutant *ge* gene was up-regulated during the early stage of giant embryo seeds development (6−14 DAF), but the relationship between this change and the *ge* gene mutation is still not understood. Owing to the comparable genetic composition of the two tested rice cultivars, except for the mutant *ge* gene in 'Shangshida No. 5' giant embryo rice, the changes in the transcription pattern of tocochromanol biosynthesis genes are most likely attributable to the mutation and the different expression level of the *ge* gene in giant embryo rice seeds.

In conclusion, we determined the tocopherol and tocotrienol levels in mature and developing 'Shangshida No. 5' giant embryo seeds. The concentrations of most types of tocochromanols, except γ-tocopherol and γ-tocotrienol, were generally increased in giant embryo seeds, and α-tocopherol was greatly increased in 'Shangshida No. 5' brown rice (49.14 mg/kg), with the level being approximately 2.2-fold higher than the concentration in 'Chao2-10' brown rice. 'Shangshida No. 5' giant embryo brown rice is an excellent source of vitamin E (especially natural α-tocopherol) and is quite valuable as a daily food and for vitamin E supplement research and development. In addition, gene expression analyses of key vitamin E biosynthesis enzymes in developing 'Shangshida No. 5' rice seeds were very helpful for understanding the enrichment of vitamin E in giant embryo rice seeds. The relationship between the *ge* gene mutation and the expression level changes of vitamin E biosynthetic genes remains unclear, and additional studies are needed. A better understanding of the *ge* gene and GE protein functions will be helpful in more clearly describing the process of embryo enlargement and the abundant accumulation of vitamin E (especially α-tocopherol) in giant embryo rice seeds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Lodge, J. K. Vitamin E bioavailability in humans. *J. Plant Physiol.* **2005**, *162* (7), 790−796.

(2) Hosomi, A.; Arita, M.; Sato, Y.; Kiyose, C.; Ueda, T.; Igarashi, O.; Arai, H.; Inoue, K. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett.* **1997**, *409* (1), 105−108.

(3) Traber, M. G.; Arai, H. Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr.* **1999**, *19*, 343−355.

(4) Chen, H.; Li, D.; Saldeen, T.; Romeo, F.; Mehta, J. L. Mixed tocopherol preparation is superior to α-tocopherol alone against hypoxia-reoxygenation injury. *Biochem. Biophys. Res. Commun.* **2002**, *291* (2), 349−353.

(5) Liu, M.; Wallin, R.; Wallmon, A.; Saldeen, T. Mixed tocopherols have a stronger inhibitory effect on lipid peroxidation than α-tocopherol alone. *J. Cardiovasc. Pharmacol.* **2002**, *39* (5), 714−721.

(6) McAnally, J. A.; Gupta, J.; Sodhani, S.; Bravo, L.; Mo, H. Tocotrienols potentiate lovastatin-mediated growth suppression *in vitro* and *in vivo*. *Exp. Biol. Med.* **2007**, *232* (4), 523−531.

(7) Vasanthi, H. R.; Parameswari, R. P.; Das, D. K. Tocotrienols and its role in cardiovascular health − a lead for drug design. *Curr. Pharm. Des.* **2011**, *17* (21), 2170−2175.

(8) Kunnumakkara, A. B.; Sung, B.; Ravindran, J.; Diagaradjane, P.; Deorukhkar, A.; Dey, S.; Koca, C.; Yadav, V. R.; Tong, Z.; Gelovani, J. G.; Guha, S.; Krishnan, S.; Aggarwal, B. B. γ-Tocotrienol inhibits pancreatic tumors and sensitizes them to gemcitabine treatment by modulating the inflammatory microenvironment. *Cancer Res.* **2010**, *70* (21), 8695−8705.

(9) Rickmann, M.; Vaquero, E. C.; Malagelada, J. R.; Molero, X. Tocotrienols induce apoptosis and autophagy in rat pancreatic stellate cells through the mitochondrial death pathway. *Gastroenterology* **2007**, *132* (7), 2518−2532.

(10) Kannappan, R.; Ravindran, J.; Prasad, S.; Sung, B.; Yadav, V. R.; Reuter, S.; Chaturvedi, M. M.; Aggarwal, B. B. γ-Tocotrienol promotes TRAIL-induced apoptosis through reactive oxygen species/extracellular signal-regulated kinase/p53-mediated upregulation of death receptors. *Mol. Cancer Ther.* **2010**, *9* (8), 2196−2207.
(11) Kannappan, R.; Yadav, V. R.; Aggarwal, B. B. γ-Tocotrienol but not γ-tocopherol blocks STAT3 cell signaling pathway through induction of protein-tyrosine phosphatase SHP-1 and sensitizes tumor cells to chemotherapeutic agents. *J. Biol. Chem.* **2010**, *285* (43), 33520−33528.
(12) Trebst, A.; Depka, B.; Holländer-Czytko, H. A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in *Chlamydomonas reinhardtii*. *FEBS Lett.* **2002**, *516* (1−3), 156−160.
(13) Havaux, M.; Eymery, F.; Porfirova, S.; Rey, P.; Dörmann, P. Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. *Plant Cell* **2005**, *17* (12), 3451−3469.
(14) Sattler, S. E.; Gilliland, L. U.; Magallanes-Lundback, M.; Pollard, M.; Dellapenna, D. Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell.* **2004**, *16* (6), 1419−1432.
(15) Hofius, D.; Hajirezaei, M.; Geiger, M.; Tschiersch, H.; Melzer, M.; Sonnewald, U. RNAi-mediated tocopherol deficiency impairs photoassimilate export in transgenic potato plants. *Plant Physiol.* **2004**, *135* (3), 1256−1268.
(16) Dellapenna, D.; Pogson, B. J. Vitamin synthesis in plants: tocopherols and carotenoids. *Annu. Rev. Plant Biol.* **2006**, *57*, 711−738.
(17) Norris, S. R.; Shen, X.; DellaPenna, D. Complementation of the *Arabidopsis pds1* mutation with the gene encoding *p*-hydroxyphenylpyruvate dioxygenase. *Plant Physiol.* **1998**, *117* (4), 1317−1323.
(18) Shintani, D.; Dellapenna, D. Elevating the vitamin E content of plants through metabolic engineering. *Science.* **1998**, *282* (5396), 2098−2100.
(19) Tsegaye, Y.; Shintani, D. K.; DellaPenna, D. Overexpression of the enzyme *p*-hydroxyphenolpyruvate dioxygenase in *Arabidopsis* and its relation to tocopherol biosynthesis. *Plant Physiol. Biochem.* **2002**, *40* (11), 913−920.
(20) Cahoon, E. B.; Hall, S. E.; Ripp, K. G.; Ganzke, T. S.; Hitz, W. D.; Coughlan, S. J. Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. *Nat. Biotechnol.* **2003**, *21* (9), 1082−1087.
(21) Collakova, E.; DellaPenna, D. Homogentisate phytyltransferase activity is limiting for tocopherol biosynthesis in *Arabidopsis*. *Plant Physiol.* **2003**, *131* (2), 632−642.
(22) Collakova, E.; DellaPenna, D. The role of homogentisate phytyltransferase and other tocopherol pathway enzymes in the regulation of tocopherol synthesis during abiotic stress. *Plant Physiol.* **2003**, *133* (2), 930−940.
(23) Cheng, Z.; Sattler, S.; Maeda, H.; Sakuragi, Y.; Bryant, D. A.; DellaPenna, D. Highly divergent methyltransferases catalyze a conserved reaction in tocopherol and plastoquinone synthesis in cyanobacteria and photosynthetic eukaryotes. *Plant Cell* **2003**, *15* (10), 2343−2356.
(24) Rippert, P.; Scimemi, C.; Dubald, M.; Matringe, M. Engineering plant shikimate pathway for production of tocotrienol and improving herbicide resistance. *Plant Physiol.* **2004**, *134* (1), 92−100.
(25) Falk, J.; Brosch, M.; Schafer, A.; Braun, S.; Krupinska, K. Characterization of transplastomic tobacco plants with a plastid localized barley 4-hydroxyphenylpyruvate dioxygenase. *J. Plant Physiol.* **2005**, *162* (7), 738−742.
(26) Kanwischer, M.; Porfirova, S.; Bergmuller, E.; Dormann, P. Alterations in tocopherol cyclase activity in transgenic and mutant plants of *Arabidopsis* affect tocopherol content, tocopherol composition, and oxidative stress. *Plant Physiol.* **2005**, *137* (2), 713−723.
(27) Karunanandaa, B.; Qi, Q.; Hao, M.; Baszis, S. R.; Jensen, P. K.; Wong, Y. H.; Jiang, J.; Venkatramesh, M.; Gruys, K. J.; Moshiri, F.; Post-Beittenmiller, D.; Weiss, J. D.; Valentin, H. E. Metabolically engineered oilseed crops with enhanced seed tocopherol. *Metab. Eng.* **2005**, *7* (5−6), 384−400.
(28) Van Eenennaam, A. L.; Lincoln, K.; Durrett, T. P.; Valentin, H. E.; Shewmaker, C. K.; Thorme, G. M.; Jiang, J.; Baszis, S. R.; Levering, C. K.; Aasen, E. D.; Hao, M.; Stein, J. C.; Norris, S. R.; Last, R. L. Engineering vitamin E content: from *Arabidopsis* mutant to soy oil. *Plant Cell* **2003**, *15* (12), 3007−3019.
(29) Kim, Y. H.; Lee, Y. Y.; Kim, Y. H.; Choi, M. S.; Jeong, K. H.; Lee, S. K.; Seo, M. J.; Yun, H. T.; Lee, C. K.; Kim, W. H.; Lee, S. C.; Park, S. K.; Park, H. M. Antioxidant activity and inhibition of lipid peroxidation in germinating seeds of transgenic soybean expressing *OsHGGT*. *J. Agric. Food Chem.* **2011**, *59* (2), 584−591.
(30) Chaudhary, N.; Khurana, P. Vitamin E biosynthesis genes in rice: molecular characterization, expression profiling and comparative phylogenetic analysis. *Plant Sci.* **2009**, *177* (5), 479−491.
(31) Bergman, C. J.; Xu, Z. Genotype and environment effects on tocopherols, tocotrienols and γ-oryzanol contents of southern U.S. rice. *Cereal Chem.* **2003**, *80* (4), 446−449.
(32) Britz, S. J.; Prasad, P. V. V.; Moreau, R. A.; Allen, L. H.; Kremer, D. F.; Boote, K. J. Influence of growth temperature on the amounts of tocopherols, tocotrienols, and γ-oryzanol in brown rice. *J. Agric. Food Chem.* **2007**, *55* (18), 7559−7565.
(33) Heinemann, R. J. B.; Xu, Z.; Godber, J. S.; Lanfer-Marquez, U. M. Tocopherols, tocotrienols and γ-oryzanol contents in *Japonica* and *Indica* subspecies of rice (*Oryza sativa* L.) cultivated in Brazil. *Cereal Chem.* **2008**, *85* (2), 243−247.
(34) Huang, S.; Ng, L. Quantification of tocopherols, tocotrienols and γ-oryzanol contents and their distribution in some commercial rice varieties in Taiwan. *J. Agric. Food Chem.* **2011**, *59* (20), 11150−11159.
(35) Zhang, G.; Liu, R.; Zhang, P.; Xu, Y.; Zhu, J.; Gu, M.; Liang, G.; Liu, Q. Variation and distribution of vitamin E and composition in seeds among rice varieties. *Acta Agron. Sin.* **2012**, *38* (1), 55−61.
(36) Satoh, H.; Omura, T. New endosperm mutations induced by chemical mutagens in rice, *Oryza sativa* L. *Jpn. J. Breed.* **1981**, *31* (3), 316−326.
(37) Cahoon, R. E.; Elmer, P. H.; Nobuhiro, N.; Hajime, S. Alteration of embryo/endosperm size during seed development. No. US2003/0126645 A1, 2003.
(38) Park, D. S.; Park, S. K.; Lee, B. C.; Song, S. Y.; Jun, N. S.; Manigbas, N. L.; Cho, J. H.; Nam, M. H.; Jeon, J. S.; Han, C. D.; Choi, K. J.; Kim, D. H.; Woo, Y.; Koh, H. J.; Kang, H. W.; Yi, G. Molecular characterization and physico-chemical analysis of a new giant embryo mutant allele (ge^t) in rice (*Oryza sativa* L.). *Genes Genomics* **2009**, *31* (4), 277−282.
(39) Zhang, L. L.; Shu, X. L.; Wang, X. Y.; Lu, H. J.; Shu, Q. Y.; Wu, D. X. Characterization of Indica-type giant embryo mutant rice enriched with nutritional components. *Cereal Res. Commun.* **2007**, *35* (3), 1459−1468.
(40) Seo, W. D.; Kim, J. Y.; Park, D.; Han, S.; Jang, K. C.; Choi, K.; Kim, S.; Oh, S.; Ra, J.; Yi, G.; Park, S.; Hwang, W.; Song, Y.; Park, B.; Kang, H. Comparative analysis of physicochemicals and antioxidative properties of new giant embryo mutant, YR23517Acp79, in rice (*Oryza sativa* L.). *J. Korean Soc. Appl. Biol. Chem.* **2011**, *54* (5), 700−709.
(41) Ren, Y.; Zhang, J.; Zhang, H.; Bai, J.; Liu, D.; Li, J. A new mutants for rice giant-embryo induced thought in vitro culture of mature embryos and analyses of its characters and grain qualities. *J. Shanghai Normal Univ. (Nat. Sci. Ed.).* **2011**, *40* (3), 289−294.
(42) Jang, S.; Xu, Z. Lipophilic and hydrophilic antioxidants and their antioxidant activities in purple rice bran. *J. Agric. Food Chem.* **2009**, *57* (3), 858−862.
(43) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25* (4), 402−408.
(44) Brigelius-Flohé, R.; Traber, M. G. Vitamin E: function and metabolism. *FASEB J.* **1999**, *13* (10), 1145−1155.
(45) Azzi, A.; Stocker, A. Vitamin E: non-antioxidant roles. *Prog. Lipid Res.* **2000**, *39* (3), 231−255.
(46) Sen, C. K.; Khanna, S.; Roy, S. Tocotrienols in health and disease: the other half of the natural vitamin E family. *Mol. Aspects Med.* **2007**, *28* (5−6), 692−728.

(47) Eitenmiller, R.; Lee, J. Nutrition and health implications of vitamin E. In *Vitamin E: Food Chemistry, Composition, and Analysis*; Eitenmiller, R., Lee, J., Eds.; Dekker: New York, 2004; pp 39−88.
(48) Jeng, T. L.; Shih, Y. J.; Ho, P. T.; Lai, C. C.; Lin, Y. W.; Wang, C. S.; Sung, J. M. γ-Oryzanol, tocol and mineral compositions in different grain fractions of giant embryo rice mutants. *J. Sci. Food Agric.* **2012**, *92* (7), 1468−1474.
(49) Xu, S. B.; Li, T.; Deng, Z. Y.; Chong, K.; Xue, Y.; Wang, T. Dynamic proteomic analysis reveals a switch between central carbon metabolism and alcoholic fermentation in rice filling grains. *Plant Physiol.* **2008**, *148* (2), 908−925.