International Food Research Journal 20(6): 3395-3401 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Expression of a key gene involved in the biosynthetic pathway of vitamin E in red pericarp and white rice grains

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Article history

Received: 24 January 2013
Received in revised form: 26 July 2013
Accepted: 5 August 2013

Keywords

Homogentisate geranylgeranyl transferase
Semi quantitative RT-PCR
Tocochromanol content

Abstract

Tocochromanols (tocopherols and tocotrienols) unitedly known as vitamin E, are the necessary antioxidant components of both human and animal diets. There is a considerable interest in plants with increased or customized vitamin E content, due to their potential health benefits. To quantify the tocochromanol content and determine the expression of a key tocotrienol biosynthesis gene among a set of contrasting red pericarp and light brown rice genotypes of advanced breeding lines together with their parents; expression pattern of homogentisate geranylgeranyl transferase (HGGT), the key gene was studied by semi-quantitative RT-PCR in milky and matured grain stages. Vitamin E analysis was carried out by high performance liquid chromatography (HPLC). The chloroform-methanolic extracts prepared from red pericarp and light brown rice advanced breeding lines showed significant differences for vitamin E content. Averaged across all samples, the content of γ-tocotrienol > α-tocopherol > α-tocotrienol > γ-tocopherol > δ-tocotrienol, and total E vitamin content ranged from 10.30 to 31.65 µg/g. Genotype G37 (red pericarp) was found to have higher expression than G7 (light brown) and G33 (red pericarp) at both grain development stages but lower than both parents whereas their transcript levels were comparatively lower in mature grain, which indicates their possible regulation by plant growth stage. HPLC results of γ-tocotrienol content supported gene expression results with the exception of the recurrent parent MR219.

Introduction

Rice (*Oryza sativa* L.) is one of the leading food crops of the world, the staple food of over half the world's population (Juliano, 1985). At least 90% of the world's rice farmers and consumers are in Asia, where rice provides up to 75% of the dietary energy and protein for people (Juliano, 1990). Good nutritional quality of rice is of primary interest to consumers all over the world. Although milled rice is superior to brown rice in palatability and digestibility, the milling process results in fewer nutrients than in brown rice (Misaki and Yasumatsu, 1985). Whole grains contain certain phytochemicals that complete those in fruits and vegetables when consumed together (Liu, 2007). The lipophilic phytochemicals such as vitamin E (refers to any of the 8 naturally occurring forms, α, β, γ, and δ species of both tocotrienols and tocopherols) and γ-oryzanols (ferulic acid ester of phytosterols) are strong antioxidants and have demonstrated various health-valuable effects, including the deterrence of cancer and cardiovascular disease, as well as reduction of oxidative damage in food (Bramley *et al*., 2000; Cicero and Gaddi, 2001; Packer, 1995; Yasukawa *et al*., 1998; Qureshi *et al*., 2002). The vitamin E in rice bran, as in seeds of most monocots, is made up of high percentage of tocotrienols than tocopherols. Studies have shown that tocotrienols have more potent antioxidant capacity than tocopherols and have specific neuroprotective effects, independent of their antioxidant activity (Packer, 1995). The biosynthesis of tocotrienols is hypothesized to take place in plastids and include reactions similar to those correlated with tocopherol biosynthesis (Soll *et al*., 1980; Yang *et al*., 2011). Homogentisate geranylgeranyl transferase (HGGT) has been known as a key enzyme in tocotrienol biosynthesis and was found to be located on chromosome 6 of rice (Chaudhary and Khurana, 2009; Horvath *et al*., 2006). The isolation of HGGT gene from rice, wheat, and barley present a biochemical and genetic explanation for the incidence of tocotrienols in monocot seeds (Cahoon *et al*., 2003). Rice HGGT is highly expressed in caryopsis, as shown by microarray analysis (Chaudhary and Khurana, 2009).

Although most of rice varieties grown around the

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world have light-brown bran, there are some special varieties of rice that have other colour pigments, such as red rice which are obvious after eliminating the hull that includes the caryopsis. From the time of its appearance, red rice has gained much attention among rice growers and scientists. Red rice contains great levels of vitamin E and γ-oryzanol in its bran layer (Aguilar-Garcia *et al.*, 2007).

The present study aimed to evaluate the selected transgressive variants for tocochromanol content especially γ-tocotrienol and the expression of HGGT gene involved in tocochromanol biosynthesis in two different growth stages of selected light brown and red pericarp transgressive variants of rice (Fasahat *et al*., 2012a; Fasahat *et al*., 2012b). Brown rice was chosen rather than milled rice since the milled rice composition would be influenced by the degree of milling, which is difficult to control.

Materials and Methods

Plant materials and growth conditions

The seeds of *O. rufipogon* IRGC105491, *O. sativa* ssp. indica cv. MR219 (Fasahat *et al.*, 2012c) and BC2F7 genotypes were provided from a National Rice Breeding Program which involved wild relative, *O. rufipogon* as a female parent crossed with Malaysian rice variety, MR219 a popular high yielding Malaysian rice cultivar as a recurrent parent (Fasahat *et al*., 2012b). All materials were collected from the Seed Gene Bank of the Malaysian Agricultural Research and Development Institute (MARDI) in Seberang Perai, Pinang, Malaysia. BC2F7 line G7 was identified as a relatively high yielding light brown grain progeny line; whereas, BC2F7 lines G33 and G37 were identified as a relatively high yielding red pericarp progeny lines among 266 backcrossed progenies (Bhuiyan *et al*., 2011) under this program (Table 1). Designation of variants in Table 1 refer to pedigree record. According to IBPGR-IRRI Rice Advisory Committee (1980) classification for bran colour, genotypes G33, G37 and *O. rufipogon* are classified as red pericarp while G7 and MR219 are light brown grain (IBPGR, 1980).

Oryza rufipogon, *Oryza sativa* ssp. indica cv. MR219, BC2F7 genotypes G7, G33 and G37 were planted in cycles. All seeds were germinated in the laboratory and grown in the greenhouse at the Plant Biotechnology Laboratory of the National University of Malaysia, following natural daylight. About 15 seeds of each genotype were placed in each Petri dish to germinate. After that, they were transferred to a plastic tray with loam soil for 20 to 21 days for further growth. The plastic tray was flooded with water. After 23-24 days, each rice seedling was transferred to a single pot containing half loam soil. Two seedlings were transferred per pot and then thinned to one seedling per pot. After 4 weeks, 1.5 g of nitrogen: phosphorus: potassium (15:15:15) fertilizer pellets were added to each plot every 2 weeks until the milky grain stage. Seed samples were collected from each plant at milky and fully matured grain stages. All the genotypes showed an average of 108 days to milky grain stage similar to parents. The cultivar, MR219 followed by genotypes G33, G37 and G7 showed shorter days to maturity (<138 days) whereas *O. rufipogon* showed longer growth duration (178 days). The seed samples collected at each stage were frozen in liquid nitrogen and used immediately for RNA extraction.

Table 1. List of rice samples used in this study

Sample Number	Designation of genotypes	Genotype code of BC_2F_7 generation	Grain type*	Tissue
1	R14-3-66-4-B-B	G33	Long	Grain
2	R19-2-93-3-B-B	G37	Long	Grain
3	R6-2-31-2-B-B	G7	Long	Grain
4	MR219	MR219	Long	Grain
5	*O. rufipogon*	*O. rufipogon*	Medium	Grain

*Grain type was identified according to scale of RTWG (1997).

Extraction and determination of tocochromanol contents

The extraction and determination of tocopherols and tocotrienols were performed, according to the method of Fasahat *et al*. (2012a).

RNA isolation and cDNA synthesis

Total RNA was extracted during grain development using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The quality of total RNA extracted was monitored by electrophoresis on 1.2% agarose gel. The isolated RNA was quantified with NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, USA) and then used for cDNA synthesis using Oligo (dT)18 primer and High-Capacity cDNA reverse transcription kit (Fermentase Company, USA). Four µl of 5X RT Buffer, 2 µl of 10 mM dNTP Mix, 1 µl of Oligo (dT)18 primer, 1 µl of RevertAidTM M-MuLV Reverse Transcriptase (200 u/µl) and 1 µl of RibolockTM RNase Inhibitor (20 u/µl) were added with dH2O to 12 µl into a 0.5 ml microcentrifuge tube. A total volume of 8 µl of total RNA (5 µg μl^{-1}) was added to the mixture. The PCR profile for cDNA synthesis was 37°C for 30 min, 65°C for 10 min, 42°C for 60 min and 70°C for 5 min.

Primer design

The Primer Premier V.5.0 software (Premier Biosoft International, Palo Alto, CA) was used to

design all primers that were used in this study. Primers were always chosen according to the following parameters: length between 18 and 25 bases, optimal 20-22 bases; Tm comprised between 57 and 65°C, optimal Tm 60-62°C; length of amplification product between 200 and 500 bp. Primers of putative OsHGGT were designed according to the gene sequence of *Oryza sativa* ssp. *japonica* (AK063383). Primers were designed from 3'UTRs region of the gene. The following primers were used: VitE-a F, 5'-TTTCAAATCACCCACCGTCAG-3'; VitE-a R, 5'-TAGGAGCATACAGTTTAGAGCA-3'. Eukaryotic elongation factor 1-alpha (*eEF-1α*), (Accession No. AK061464) was used as a housekeeping gene: *eEF-1α* F, 5'-TTTCACTCTTGGTGTGAAGCAGAT-3; *eEF-1α* R, 5'-GACTTCCTTCACGATTTCATCGTAA-3' (Jain *et al*., 2006).

Semi quantitative PCR expression analysis

The semi-quantitative PCR can be processed with conventional thermocycler that makes this assay a more economic method for the evaluation of expression levels. PCR was performed in a volume of 12.5 µl containing 3 µl of cDNA template, 0.1 mM each of dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each forward and reverse VitE-a primer, 1X PCR buffer and 0.5 U/µl *Taq* DNA Polymerase (5 U/µl). The cycle parameters in the PCR program were as follows: 94°C for 5 min, 35 cycles of: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 45 seconds followed by a final extension at 72°C for 7 min. Housekeeping gene *eEF-1α* was also amplified under the same conditions using primers eEF-1αF and eEF-1αR as the control. About 4 µl of each PCR product was analyzed by agarose gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide. To ensure reproducibility the experiment was replicated; both experiments produced identical results.

Statistical analysis

Tocochromanol content assay was carried out three times from the same extract in order to determine their reproducibility. Analysis of the variance was used to determine any difference in antioxidant activities resulting from these methods. Duncan's new multiple-range test was used to determine significant differences. Statistical significance was declared at $p < 0.05$ using SAS version 9.1.3 (SAS Institute Inc., 2003).

Results

Yields of methanolic extracts

Efficiency of extraction is an essential factor for comparability of antioxidant compounds. Because of the different solubility of active compounds, antioxidants in grains are difficult to be extracted (Miller *et al*., 2000). Methanol-chloroform was selected as an extraction solvent in this study. The yield of methanol-chloroform extracts obtained from grains is presented in Table 2 and the grains gave a yield of 2.4–3.4 %.

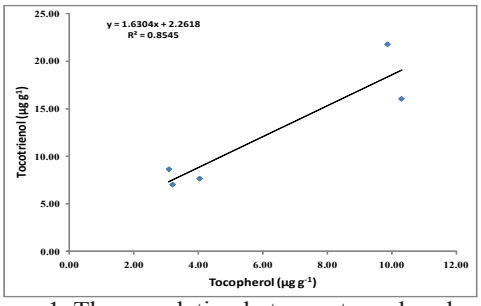

Figure 1. The correlation between tocopherols and tocotrienols content of grain extracts

The contents of tocopherols and tocotrienols

Concentration of tocopherol (α-T and γ-T) and tocotrienol (α-T3, γ-T3 and δ-T3) homologues in brown rice powder of different colours are listed in Table 2. Brown rice is composed of a starchy endosperm and embryo covered by the layer of bran. The concentration of total vitamin E (the sum of tocopherols and tocotrienols) in the whole rice grain samples ranged from 10.30 to 31.65 µg/g rice (dry basis). *Oryza rufipogon* (red pericarp) contained the highest concentration of total vitamin E, followed by the G37 (red pericarp), G33 (red pericarp) and MR219 (light brown) being equal; while G7 (light brown) had the lowest concentration, demonstrating that the colour of rice bran does not necessarily contribute to the total vitamin E concentration. The levels of γ-tocotrienol, the most abundant tocol found in the samples, ranged between 5.10 and 12.45 µg/g and averaged at 8.14 µg/g, which corresponds to 45% of total tocols content (91.65 µg/g). α-tocopherol, α-trocotrienol, γ-tocopherol and δ-trocotrienol were present at lower concentrations representing 21%, 15%, 12%, and 7% of total tocols, respectively and no other vitamin E homologues were detected. Pearson's correlation analysis demonstrated that α-tocotrienol was positively correlated to γ-tocotrienol and δ-tocotrienol ($r = 0.86$ and 0.87, respectively, $p < 0.01$); as α-tocotrienol was to α-tocopherol ($r = 0.95$, $p < 0.01$). The correlation between α-tocopherol and γ-tocopherol was not significant. All 5 tocochromanol compounds were well separated in a total run time of 30 min, with good peak resolution, sharpness and symmetry.

Figure 1 shows the correlation between tocopherols and tocotrienols content of grain extracts. Results showed a positive correlation coefficient

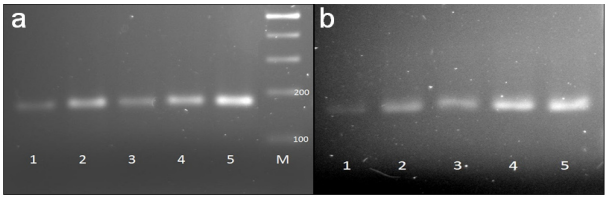

Figure 2. Expression pattern of HGGT gene at two different rice grain maturity stages: milky grain stage (a) and matured stage (b), 1) G33, 2) G37, 3) G7, 4) MR219, 5) *O. rufipogon*

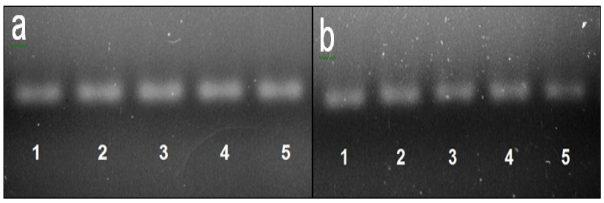

Figure 3. *eEF-1α* amplification product at milky grain stage (a) and matured stage (b), 1) G33, 2) G37, 3) G7, 4) MR219, 5) *O. rufipogon*

between tocopherols and tocotrienols content of plants extracts (r = 0.92) which was highly significant ($p < 0.01$). Meanwhile, coefficient of determination (R^2) was measured on how well the regression line represents the data which shows the association between total tocopherols and total tocotrienols content ($R^2 = 0.85$) in Figure 1.

Expression of rice tocochromanol biosynthesis gene (HGGT)

Partial HGGT gene was successfully amplified from cDNA of all samples. Due to the weak expression of HGGT gene for all samples at maturity stage, the PCR cycles were increased to 60 cycles. Semi qPCR analysis revealed that G37 had higher expression compared to G33 and G7. Expression results of HGGT gene for all samples on agarose gel at different developmental stages are shown in Figure 2. MR219 showed higher expression than the two red pericarp transgressive variants which was contrary to γ-tocotrienol HPLC results. Putative HGGT transcript level at mature grain stage was lower compared to milky grain stage. Expression of putative HGGT gene in O. rufipogon was highly up-regulated at both stages compared to MR219 and the BC2F7 genotypes G7, G33 and G37. In this study *eEF-1α* was selected as the reference gene to examine the transcribed RNA quantities across all samples where amplification intensities were similar (Figure 3).

Discussion

Tocochromanol content of light brown and red pericarp genotypes

The physiological role of tocopherols and tocotrienols is thought to be the protection of proteins, pigments, and polyunsaturated fatty acids (PUFA) from lipid oxidation by scavenging free radicals in cell membranes and other lipophilic environments (Kamal-Eldin and Appelqvist, 1996; Semchuk *et al*., 2009). Of the vitamin E components, the tocopherols and tocotrienols were 33.3 and 66.6% of total vitamin E content, respectively. Similar to this study, tocopherol was detected in lower content (18.07% of total average), than tocotrienol (81.52%), in three brown rice cultivars grown in three different locations in Brazil which are consistent with other studies (Chen and Bergman, 2005; Heinemann *et al.*, 2008; Auilar-Garcia *et al.*, 2008; Pascual *et al*., 2011).

The concentration of total tocotrienols was more than 2-fold higher than that of total tocopherols in all rice samples used in this study. The lack of association between the levels of α- and γ-homologues indicates that the biosynthesis of these tocols happens through different metabolic pathways, which may describe why cultivars have specific homologues as major components (Heinemann *et al*., 2008; Bergman, 2003). Previous research showed the levels of α-tocopherol in rice bran (26.4–35.0 μg/g) and brown rice (7.83–8.51 μg/g) for three varieties of Venezuelan rice (Aguilar-Garcia *et al*., 2007). In this study, γ-tocopherol (γ-T) was higher than α-tocopherol in some samples including MR219 and G33 and similar to brown rice samples used by Aguilar-Garcia *et al.* (2007), γ-tocopherol was found to be a more active antioxidant at higher concentrations than α-tocopherol in fats and oils, meaning less α-tocopherol is needed for maximum antioxidant protection and α-tocopherol commonly showed better antioxidant activity than γ-tocopherol (Seppanen *et al*., 2010). Hence, genotypes G7 and G37 with high α-tocopherol are predicted to have high antioxidant activity. Previous study (Khatoon and Gopalakrishna, 2004) reported slightly higher levels of α-tocopherol contents than were found in the present study for Basmati brown rice (12.1 μg/g) and Jaya brown rice (9.9 μg/g). These differences in tocopherols concentration may be due to the differences between rice genotypes, different methods of extraction used in the studies and may also be due to growth conditions. The expression of genes responsible for the synthesis of tocopherols in plants can be activated by oxidative stress (Sandorf and Hollander-Czytko, 2002). A half-century ago tocotrienols were discovered, but most of their biology has been uncovered only in the last decade. The levels of α-tocotrienol in whole grain were in the range of 0.30-6.35 μg/g which were slightly lower than that reported by previous study which was 8.70 μg/g (Ha *et al*., 2006). The order of γ-tocotrienol content was

Table 2. The contents of tocopherols (T) and tocotrienols (T3) (in whole grain) of different genotypes (µg/g)*

		Tocopherols			Tocotrienols				Total vitamin E
	Yield (%)	α-tocopherol	γ-tocopherol	Total	α-tocotrienol	γ-tocotrienol	δ-tocotrienol	Total	
G7	2.4	2.30 ± 0.01^{c}	0.90 ± 0.0^{e}	3.20 ± 0.02^{d}	1.30 ± 0.01^{c}	5.10 ± 0.02^{e}	0.60 ± 0.0^{e}	7.00 ± 0.02^{e}	10.30 ± 0.04^{d}
G33	2.6	1.00 ± 0.0^{d}	2.09 ± 0.0^{c}	3.09 ± 0.0^{d}	0.30 ± 0.01^{e}	7.61 ± 0.01^{c}	0.70 ± 0.0^{d}	8.61 ± 0.02^{c}	11.70 ± 0.02^{c}
G37	3.0	8.81 ± 0.02^{a}	1.50 ± 0.0^{d}	10.31 ± 0.03^{a}	5.33 ± 0.0^{b}	9.30 ± 0.02^{b}	1.39 ± 0.0^{b}	16.03 ± 0.03^{b}	26.34 ± 0.05^{b}
MR219	3.4	0.62 ± 0.0^{e}	3.42 ± 0.0^{a}	4.04 ± 0.0^{c}	0.58 ± 0.0^{d}	6.23 ± 0.0^{d}	0.82 ± 0.0^{c}	7.63 ± 0.0^{d}	11.67 ± 0.0^{c}
O. rufipogon	2.7	6.88 ± 0.02^{b}	3.00 ± 0.0^{b}	9.87 ± 0.02^{b}	6.35 ± 0.01^{a}	12.45 ± 0.01^{a}	2.97 ± 0.01^{a}	21.77 ± 0.0^{a}	31.65 ± 0.02^{a}

*Data expressed as means ± standard deviation. Within each column for whole grain, means with the same letter are not significantly different ($p < 0.05$).

as follows: *O. rufipogon* > G37 > G33 > MR219 > G7 with 12.45, 9.30, 7.61, 6.23 and 5.10 µg/g, respectively. γ-tocotrienol is better than α-tocotrienol as an antioxidant (Schroeder *et al.*, 2006). Recent studies have reported that tocotrienols are more effective antioxidants than tocopherols and have hypocholesterolemic, antitumor, and antiangiogenic, and neuroprotective effects while α-tocopherol was believed to be the most active form of vitamin E homologue in biological systems (Packer, 1995; Packer *et al*., 2001; Qureshi *et al.*, 2002; Inokuchi *et al*., 2003; Wada *et al.*, 2005). γ-tocotrienol was colinear with total tocotrienols.

Differential expression of rice tocochromanol biosynthesis gene, HGGT

Tocotrienol accumulation is produced from the condensation of homogentisate (HGA) and geranylgeranyl diphosphate (GGDP) (rather than phytyl) into 2-methyl- 6-geranylgeranyl-benzoquinol (MGGBQ) that is catalyzed by homogentisic acid geranylgeranyl transferase (HGGT) (Mène-Saffrané and DellaPenna, 2010). In a previous research the expression pattern of tocochromanol biosynthesis genes was done in different rice tissues and revealed that all tocochromanol biosynthesis genes were found to be highly expressed in developing and mature seed, although at different levels and had higher expression in seed, than shoot and root (Chaudhary and Khurana, 2009). Recently, in a study expressing barley HGGT, tocotrienols accounted for ~60% of tocochromanols in mature seeds (Yang *et al*., 2011) Semi-quantitative PCR analysis results of HGGT gene, which encodes enzymes that play a key role in tocotrienol synthesis, were compared with those obtained by HPLC analysis. Although there were some cases for which the levels of HGGT and the γ-tocotrienol were parallel, a tight correlation between these two parameters was not always found. Genotype G37 was found to have higher expression than G7 and G33 in both stages of grain development but lower than MR219 whereas their transcript levels were comparatively lower in mature seed, which indicates their possible regulation by plant growth stage. These results corroborate those obtained by HPLC for γ-tocotrienol content except for MR219 which may be due to translational repression (Figure 2). The expression patterns of HGGT were consistent in rice endosperm at different growing stages (Figure 2).

A lack of correlation between relative gene expression and γ-tocotrienol production for some samples has been observed previously. For example, in a recent study, seven barley cultivars with different vitamin E levels were grown under controlled conditions and activity of HGGT was measured at four, eight and twelve days after pollination. The results showed no relationship between the activity of HGGT gene and the final tocotrienol content in barley grain (Kosař *et al.*, 2010). Gene expression does not always correlate with the final product. Even though a gene may seemingly be expressed more highly, we do not know how much enzyme accumulates or if regulatory mechanisms exist for post-translational modifications (e.g., phosphorylation) that may activate or inactivate the enzymes (Cahoon EB, pers. comm.). In another study, expression of only a small number of aflatoxin biosynthetic pathway genes were directly coupled with aflatoxin biosynthesis (Scherm *et al*., 2005). The basic presumption when using housekeeping genes to assess gene expression results is that they are expressed at continuous levels across the samples and that their expression does not vary in response to the experimental manipulation. Since the expression of the target gene is measured comparative to the housekeeping gene, it is essential that these assumptions hold (Abruzzo *et al*., 2005). The candidate reference genes were amplified in cDNA samples from the same 2 grain development stages. In this study *eEF-1α* was found to be highly expressed for all rice samples (Figure 3). Ten housekeeping genes were validated in a previous

study and UBQ5 and *eEF-1α* were identified as the most stably expressed control genes in a given set of rice tissue samples (Jain *et al.*, 2006). Using housekeeping gene confirms the fact that all the steps needed to achieve the final PCR measurement are at optimal conditions (Huggett *et al*., 2005)

In conclusion, the experimental results presented above demonstrated that whole kernel rice transgressive variants used in this study are a good source of tocols. Consumption of whole rice grain, which has been shown to be a rich source of these compounds, could then be recommended in ordinary diet to enhance the daily intake of nutrients.

Acknowledgements

The authors are grateful to the Ministry of Science, Technology and Innovation (MOSTI) for funding of this study under Project No.UKM-ABI-NBD0001-2007. We thank Dr. Abdul Gapor Mat Top from the Malaysian Palm Oil Board, for facilitating the vitamin E analysis and Ms. Mahani for her technical assistance. Cooperation provided by Melissa M.L. Wong and UPM-BERNAS Research Laboratory staff are gratefully acknowledged.

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