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Original Paper

Generation of transgenic tobacco plants with enhanced tocotrienol levels through the ectopic expression of rice homogentisate geranylgeranyl transferase

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Abstract Tocotrienols with three double bonds in the hydrocarbon tail are the major form of vitamin E in the seeds of most monocots and certain dicots. They have recently been attracting increasing attention for their various biological properties for human health. Homogentisate geranylgeranyl transferase (HGGT) catalyzes the committed step of tocotrienol biosynthesis. HGGT, except for enzyme from barley, has been not analyzed in detail, although cDNAs encoding HGGT have been isolated from barley, wheat, and rice. Since tocotrienol levels are higher in rice than in barley, rice HGGT (OsHGGT) may have superior enzymatic characteristics to those of barley HGGT. In the present study, we generated transgenic tobacco plants introducing *OsHGGT* cDNA into a nuclear genome and measured their tocopherol and tocotrienol levels. We demonstrated that the ectopic expression of *OsHGGT* enhanced tocotrienol levels without decreasing tocopherol levels in tobacco plants. The results of the present study may lead to a better understanding of the manipulation of vitamin E biosynthesis in leafy vegetables.

Key words: Homogentisate geranylgeranyl transferase, tocotrienol, transgenic tobacco, vitamin E.

Vitamin E comprises chemically distinct compounds that are separated into tocopherols and tocotrienols based on the saturation of the hydrophobic tails (Falk and Munné-Bosch 2010). Tocopherols have a fully saturated tail, whereas tocotrienols have an unsaturated tail that contains three double bonds (DellaPenna 2005). Similar to tocopherols, tocotrienols have been distributed into α-, β-, γ-, and δ-forms (Falk and Munné-Bosch 2010; Mène-Saffrané and DellaPenna 2010). Tocotrienols have recently been attracting increasing of due to its superior biological properties over tocopherols, such as antioxidative, antihypercholesterolemic, anticancer, and neuroprotective activities (Ghosh et al. 2009; Goh et al. 1994; Khanna et al. 2005; Qureshi et al. 2002; Theriault et al. 1999; Wada et al. 2005). Tocopherols occur widely in plants and its α-form is especially abundant in the leaves of all plants (Szymanska and Kruk 2008). On the other hand, the accumulation of tocotrienols is limited to the seeds of monocots such as cereal grains and some dicots (Falk and Munné-Bosch 2010; Horvath et al. 2006; Yang et al. 2011). Therefore, it is desirable to accumulate high levels of tocotrienols in leafy vegetables using gene transfer technology with biosynthesis-related genes.

The biosynthetic pathways of tocopherols and tocotrienols in plastids have been extensively examined (Mène-Saffrané and DellaPenna 2010). Tocopherols arise from the condensation reaction of homogentisic acid (HGA) with phytyl diphosphate (PDP), which is catalyzed by plastidic homogentisate phytyltransferase (HPT), whereas tocotrienols arise from the condensation reaction of HGA with plastidic geranylgeranyl diphosphate (GGDP), which is catalyzed by homogentisate geranylgeranyl transferase (HGGT) (Figure 1). Subsequent reactions are catalyzed by common enzymes, 2-methyl-6-phytylbenzoquinone methyltransferase (MPBQMT) and tocopherol cyclase

Abbreviations: DMGGBQ, 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; GGDP, geranylgeranyl diphosphate; HGA, homogentisic acid; HGGT, homogentisate geranylgeranyl transferase; HPT, homogentisate phytyltransferase; HPP, *p*-hydroxyphenylpyruvate; HPPD, *p*-hydroxyphenylpyruvate dioxygenase; MEP, methylerythritol phosphate; MGGBQ, 2-methyl-6-geranylgeranylbenzoquinone; MPBQ, 2-methyl-6-phytylbenzoquinone; MPBQMT, 2-methyl-6-phytylbenzoquinone methyltransferase; PDP, phytyl diphosphate; TC, tocopherol cyclase; γ-TMT, γ-tocopherol methyltransferase.

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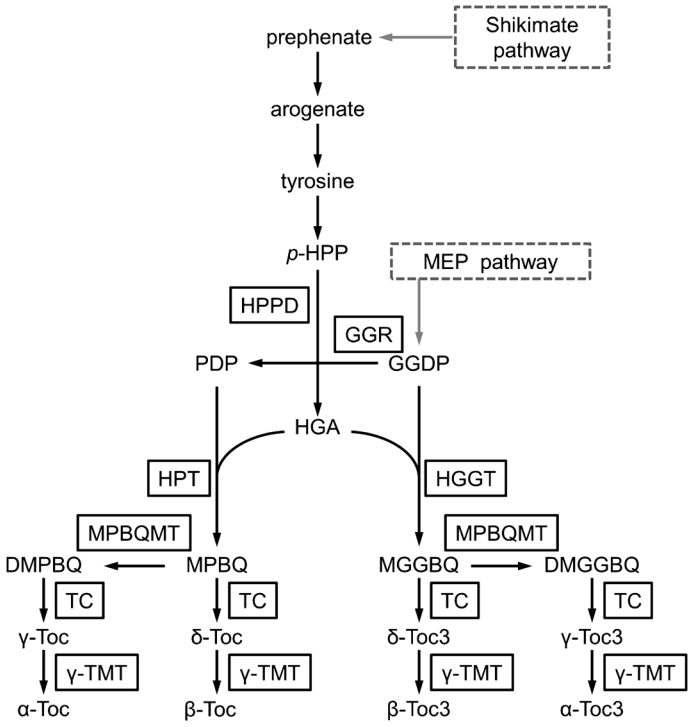

Figure 1. Biosynthetic pathway for tocopherols and tocotrienols in plants. HPPD, *p*-hydroxyphenylpyruvate dioxygenase; GGR, geranylgeranyl diphosphate reductase; HPT, homogentisate phytyltransferase; MPBQMT, 2-methyl-6-phytylbenzoquinone methyltransferase; TC, tocopherol cyclase; *γ*-TMT, *γ*-tocopherol methyltransferase; MEP, 2-C-methylerythritol 4-phosphate; PDP, phytyl diphosphate; GGDP, geranylgeranyl diphosphate; *p*-HPP, *p*-hydroxyphenylpyruvate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytylbenzoquinone; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; MGGBQ, 2-methyl-6-geranylgeranylbenzoquinone; DMGGBQ, 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone; Toc, tocopherol; Toc3, tocotrienol.

(TC), for tocopherol and tocotrienol biosynthesis (Cahoon et al. 2003; Cheng et al. 2003; Porfirova et al. 2002).

HGGT homologue genes have been identified on the genomes of a wide range of monocot plants, but not on those of dicot plants (Cahoon et al. 2003), suggesting that HGGT is a key enzyme for tocotrienol biosynthesis. In fact, Cahoon et al. (2003) have reported that the transgenic tobacco calli overexpressing barley, wheat or rice HGGT accumulated tocotrienol and that the overexpression of barley *HGGT* (*HvHGGT*) caused the highest accumulation of tocotrienol. Furthermore, tocotrienols were found to accumulate in Arabidopsis leaves when *HvHGGT* was overexpressed (Cahoon et al. 2003). Although the overexpression of rice *HGGT* (*OsHGGT*) produces less amount of tocotrienol than *HvHGGT* in tobacco calli, there has been no report of tocotrienols in the leaves of transgenic plants which were transformed *OsHGGT*.

Rice is one of the most important food crops consumed worldwide and is a good source of both tocopherols and tocotrienols. Tocotrienols generally account for at least 60% of total vitamin E (tocopherols and tocotrienols) in rice seeds (Heinemann et al. 2008). Furthermore, brown rice was previously shown to contain higher tocotrienol levels than husked barley (Shammugasamy et al. 2013), suggesting that the OsHGGT may have better enzymatic characteristics than that of HvHGGT.

In the present study, we generated transgenic tobacco plants introducing *OsHGGT* cDNA into a nuclear genome, in which tocotrienol levels were significantly increased and tocopherol levels remained unchanged. These results indicated that the introduction of *OsHGGT* conferred tocotrienol-biosynthetic ability to tobacco plants with increased levels of tocotrienols and without reducing the content of tocopherols.

Materials and methods

Chemicals

α-, *β*-, *γ*-, and *δ*-tocopherols were purchased from Eisai Food & Chemical (Tokyo, Japan). *α*-, *β*-, *γ*-, and *δ*-tocotrienols were obtained from Dr. Koichi Abe (Eisai Food & Chemical Co., Ltd.). All other chemicals were of analytical grade and were used without further purification.

Plant materials and growth conditions

Wild-type tobacco plants (*Nicotiana tabacum* cv. Xanthi) and transgenic plants expressing the *OsHGGT* gene were grown on Murashige-Skoog medium in a growth chamber under a 12-h light (25°C) and 12-h dark (20°C) cycle with moderate light intensity ($100\,\mu mol\,m^{-2}s^{-1}$).

Construction and transformation of the OsHGGT gene

The transformation vector pRI101/*OsHGGT* was constructed as follows. *OsHGGT* cDNA was amplified by reverse transcription-PCR (RT-PCR) using the following primer sets: OsHGGT-F (*Sph*I); 5′-**GCATGC**AAG CCT CAT CGG-3′ (*Sph*I site in bold), OsHGGT-R (*Sal*I); 5′-**GTCGAC**TCA CTG CAA AAT GGT ATA AGG-3′ (*Sal*I site in bold). The DNA fragments generated by PCR were ligated into the vector pT7 Blue (Novagen, San Diego, CA, USA). DNA sequences were confirmed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). From the plasmids digested with *Sph*I and *Sal*I, a 1.3-kbp DNA fragment encoding *OsHGGT* and *Nde*I–*Sph*I linker (5′-TAC ATG-3′) were integrated into pRI101 digested with *Nde* I and *Sal*I (Figure 2A). This construct or empty pRI101 vector was then introduced into leaf disks derived from tobacco using *Agrobacterium*-mediated transformation. Transgenic plants were regenerated from kanamycin-resistant calli and planted in soil as described previously (Shikanai et al. 1998).

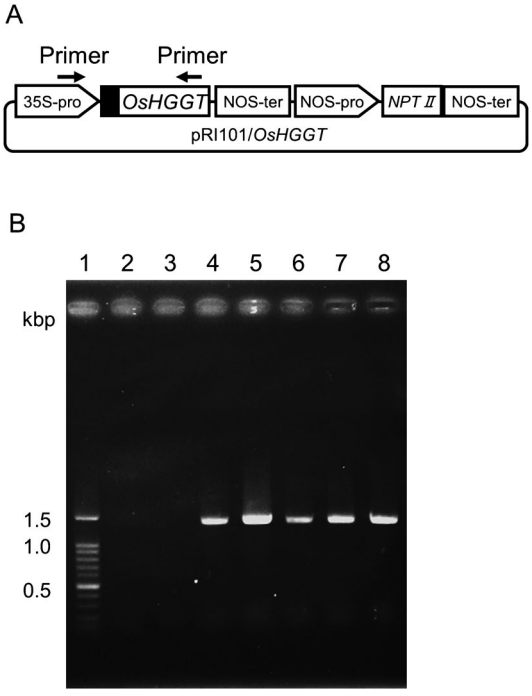

Figure 2. Creation of OsHGGT transgenic tobacco plants. (A) Construction of a transformation vector. The drawing is not to scale. Arrows indicate the locations of the PCR primers. 35S-pro, cauliflower mosaic virus 35S promoter; *NPTII*, neomycin phosphotransferase II; NOS-pro, nopalin synthase gene promoter; NOS-ter, nopalin synthase gene terminator. Black box indicates the plastid transit sequence of *OsHGGT*. (B) Analysis of transgenic plants carrying *OsHGGT* cDNA introduced into the nuclear genome. Aliquots (10 μl) of PCR mixtures were loaded onto the gel slots. Lane 1, DNA marker; lane 2, template DNA from the wild type; lane 3, control (transformed with the empty pRI101 vector); lane 4, OsHGGT-2; lane 5, OsHGGT-3; lane 6, OsHGGT-8; lane 7, OsHGGT-9; lane 8, OsHGGT-15. Detailed procedures are described in the 'Materials and methods' section.

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA (50 μg) was isolated using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan) from the third leaves from the top of tobacco plants, and then treated with DNase I (Takara, Kyoto, Japan) to eliminate any DNA contamination, and converted into first-strand cDNA using ReverTra Ace (Toyobo, Osaka, Japan) and a random hexamer. A semi-quantitative RT-PCR analysis was performed using the following primer sets: OsHGGT-F, 5′-AGC GTA CGT TTG GGT CCA GAA AGA-3′ and OsHGGT-R, 5′-CTC TCT GCC AAA GTG CAA AGG CAA-3′; NtActin-F, 5′-CTA TTC TCC GCT TTG GAC TTG GCA-3′ and NtActin-R, 5′-ACC TGC TGG AAG GTG CTG AGG GAA-3′. The semi-quantitative RT-PCR experiments were repeated at least three times with cDNA prepared from three batches of plant leaves.

Measurement of vitamin E levels

The extraction and determination by HPLC of each tocopherol and tocotrienol from wild-type and transgenic tobacco leaves were carried out with some modifications as described previously (Yabuta et al. 2013). We analyzed an aliquot (20 μl) of the filtrate using a Shimadzu HPLC apparatus (LC-6A pumps, RF-530 fluorescence detector, 20-μl sample loop, and C-R6A chromatopac integrator). The sample was injected in an HPLC column (COSMOSIL 5SL-II column, ϕ4.6×250 mm, Nacalai Tesque). The flow rate was set at 1.0 ml min^{-1}. The eluate was monitored fluorometrically at 295 nm excitation and 340 nm emission. The relationship between the peak area and quantity of each tocopherol and tocotrienol injected was linear from 0.1 to 10 μg.

Data Analysis

The significance of differences between data sets was evaluated with a *t*-test. Calculations were carried out with Microsoft Excel software.

Results and discussion

Rice, a monocot, is a good source of both tocopherols and tocotrienols. Rice seeds contain a large amount of tocotrienols, at least 60% of total vitamin E (tocopherols and tocotrienols). A data-base analysis (TargetP: http://www.cbs.dtu.dk/services/TargetP/) predicts that the OsHGGT protein is distributed in plastids. It has been reported that the green fluorescent protein fused to the N-terminal 49 amino acids of HvHGGT localized in the plastid of Arabidopsis plants (Yang et al. 2011). Furthermore, the N-terminal sequence of OsHGGT shares significant homology with the HvHGGT transit peptide (Yang et al. 2011). So, we generated transgenic tobacco plants (*N. tabacum* cv. Xanthi) by the infection of *Agrobacterium* transformed with a transgene containing *OsHGGT* cDNA including the plastid transit sequence from rice under the control of the cauliflower

mosaic virus 35S promoter (Figure 2A). Ten kanamycin-resistant transgenic plants (T_0) were generated. The T_0 transformants were transferred to soil, grown, and self-pollinated twice to produce T_2 progeny. As shown in Figure 2B, a genomic PCR analysis revealed that the five transgenic lines carried the exogenous gene in the nuclear genome. Thus, the five OsHGGT transgenic lines were used in further analyses. These OsHGGT transgenic lines grew normally and showed no visually detectable phenotypic differences from the control (transformed with the empty pRI101 vector) and non-transgenic wild-type plants (data not shown). Semi-quantitative RT-PCR revealed that the transcripts of *OsHGGT* were expressed in all OsHGGT transgenic plants grown for 5 weeks under normal conditions (Figure 3). The transcript levels of *OsHGGT* in the leaves of OsHGGT-2, -3, -9, and -15 were similar, while those in OsHGGT-8 were the lowest among the five transgenic plants. The transcripts of *OsHGGT* were not detected in the leaves of wild-type and control plants. The transcript levels of *actin*, a housekeeping gene, were similar among the wild-type, control, and OsHGGT transgenic plants.

In order to measure the composition and levels of vitamin E, extracts of leaves from 5-week-old wild-type, control, and OsHGGT transgenic plants were subjected to HPLC. β- and δ-Tocopherols were not detected in the leaves of wild-type, control, or any OsHGGT plants (Table 1). Previous studies reported that β- and δ-tocopherols were the minor forms of vitamin E in many plant species (Chun et al. 2010; DellaPenna 2005). The contents of α-tocopherol in the leaves of wild-type, control, and OsHGGT-2, -3, -8, -9, and -15 plants were 17 ± 3, 15 ± 1, 20 ± 2, 20 ± 5, 15 ± 6, 23 ± 5, and $24\pm2\,\mu g\,g^{-1}$ FW, respectively, while the contents of γ-tocopherol were 1.3 ± 0.3, 1.5 ± 0.5, 2.0 ± 0.7, 1.5 ± 0.7, 1.2 ± 0.3, 1.4 ± 0.2, and $1.5\pm0.2\,\mu g\,g^{-1}$ FW, respectively (Table 1 and Figure 4). No significant differences were observed in the contents of α- and γ-tocopherols between the wild-type, control, and OsHGGT transgenic plants.

Although the leaves of wild-type and control plants had no tocotrienol isoforms, those of the OsHGGT plants contained α- and γ-tocotrienols (Table 1 and Figure 4). The levels of α-tocotrienol in the leaves of OsHGGT-2, -3, -8, -9, and -15 plants were 35 ± 5, 39 ± 5, 0.1 ± 0.2, 49 ± 6, and $58\pm5\,\mu g\,g^{-1}$ FW, respectively, while the levels of γ-tocotrienol were 10 ± 3, 3.0 ± 0.7, 0.3 ± 0.2, 5.0 ± 0.4, and $4.0\pm0.4\,\mu g\,g^{-1}$ FW, respectively. These results suggested that OsHGGT protein was located in the plastids and catalyzed the condensation of HGA with GGDP in transgenic tobacco plants. Furthermore, these results also suggested that the expression levels of *OsHGGT* reflected the accumulation of α- and γ-tocotrienols in the OsHGGT transgenic plants. Neither β- nor δ-tocotrienol was detected in the leaves of wild-type, control, or any OsHGGT plants. β- and δ-Tocopherols were not detected in the leaves of transgenic Arabidopsis plants expressing *HvHGGT*, while β- and δ-tocotrienols had slightly accumulated (Cahoon et al. 2003). The precursors of β- and δ-tocopherols (2-methyl-6-phytylbenzoquinone; MPBQ) and β- and δ-tocotrienols (2-methyl-6-geranylgeranylbenzoquinone; MGGBQ) are converted to 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) or 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone (DMGGBQ), respectively, by MPBQMT (Cheng et al. 2003; Figure 1). These findings suggest that plant leaves have sufficient MPBQMT activity to completely convert MPBQ and MGGBQ to DMPBQ and DMGGBQ, respectively. On the other hand, β- and δ-tocopherols were detected in the leaves of lettuce plants (Yabuta et al. 2013), suggesting that MPBQMT activity in these leaves was lower than that in tobacco and Arabidopsis plants.

Accompanying the increase in tocotrienol levels, the levels of total vitamin E in the leaves of OsHGGT plants, with the exception of OsHGGT-8, were higher than those in wild-type and control plants (Table 1 and Figure 4). Total vitamin E levels in the leaves of OsHGGT-2, -3, -9, and -15 plants were 3.70-, 3.43-, 4.31-, and 4.78-fold, respectively, higher than those in the leaves of wild-type plants. These results indicated that the ectopic expression of *OsHGGT* as a key enzyme for tocotrienol biosynthesis was useful for the accumulation of tocotrienols in dicots. Similar findings have been reported for Arabidopsis leaves and corn seeds expressing *HvHGGT* (Cahoon et al. 2003).

α-Tocotrienol was the major form of vitamin E in the leaves of OsHGGT plants, while those of *HvHGGT* expressing Arabidopsis contained γ-tocotrienol as the predominant form. This difference may have been caused by a difference in γ-TMT activity between the tobacco and Arabidopsis plants or in substrate specificity of γ-TMT, but not by the difference of transgene.

A previous study reported that recombinant HvHGGT exhibited activity toward PDP and also that PDP was used approximately only 17% as effectively as GGDP

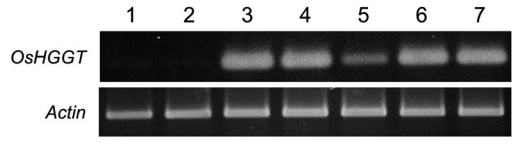

Figure 3. Expression of *OsHGGT* in transgenic plants. Semi-quantitative RT-PCR was performed using specific primers for the genes encoding *OsHGGT* and tobacco *actin* with total RNA from the third and fourth leaves from the top of 5-week-old wild-type, control, and OsHGGT transgenic plants. Lane 1, wild type; lane 2, control; lane 3, OsHGGT-2; lane 4, OsHGGT-3; lane 5, OsHGGT-8; lane 6, OsHGGT-9; lane 7 OsHGGT-15. The PCR profile was 20 or 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Aliquots of the products were analyzed on a 2% agarose gel. Detailed procedures are described in the 'Materials and methods' section.

Table 1. Tocopherols and tocotrienols levels (μg/g Fresh Weight) in the leaves of wild-type, control and transgenic plants.

	α-Toc	β-Toc	γ-Toc	δ-Toc	total Toc	α-Toc3	β-Toc3	γ-Toc3	δ-Toc3	total Toc3	Toc+Toc3
Wild type	17±3	n.d.	1.3±0.3	n.d.	18±3	n.d.	n.d.	n.d.	n.d.	n.d.	18±3
Control	15±1	n.d.	1.5±0.5	n.d.	17±1	n.d.	n.d.	n.d.	n.d.	n.d.	17±1
OsHGGT-2	20±2	n.d.	2.0±0.7	n.d.	22±2	35±5	n.d.	10±3	n.d.	45±8	68±9
OsHGGT-3	20±5	n.d.	1.5±0.7	n.d.	21±5	39±5	n.d.	3.0±0.7	n.d.	41±5	63±8
OsHGGT-8	15±6	n.d.	1.2±0.3	n.d.	16±6	0.1±0.2	n.d.	0.3±0.2	n.d.	0.4±0.3	17±6
OsHGGT-9	23±5	n.d.	1.4±0.2	n.d.	24±5	49±6	n.d.	5.0±0.4	n.d.	54±6	79±9
OsHGGT-15	24±2	n.d.	1.5±0.2	n.d.	25±2	58±5	n.d.	4.0±0.4	n.d.	62±4	87±3

Data represent the means of three replicates±SD; n.d., not detectable

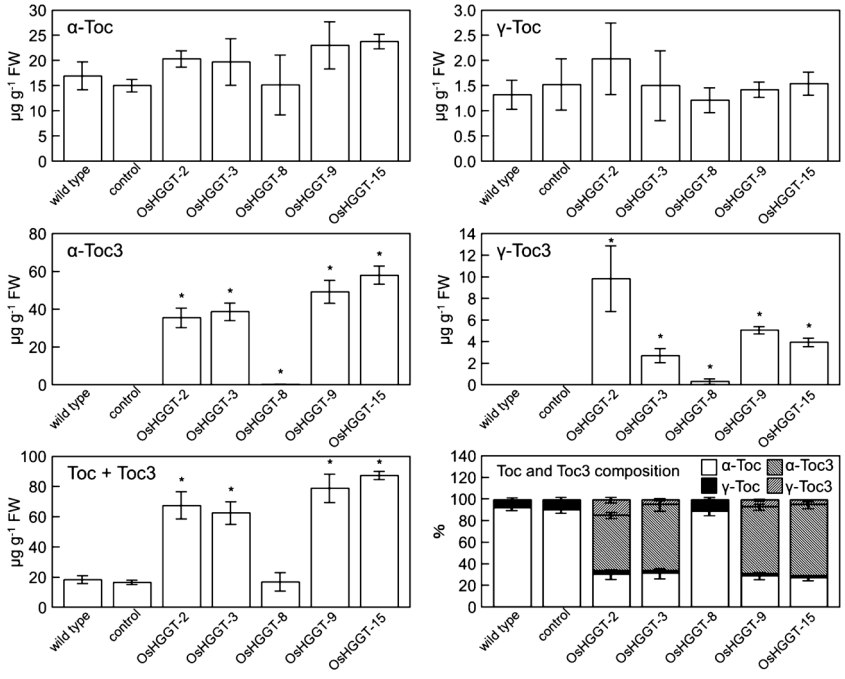

Figure 4. Effects of the ectopic expression of *OsHGGT* on vitamin E levels in wild-type, control, and transgenic plants. Detailed procedures are described in the 'Materials and methods' section. Tocopherols and tocotrienols composition indicate the relative content (%) of the tocopherol and tocotrienol molecular species in total vitamin E content (tocopherol and tocotrienol). Data are mean values±SD for three individual experiments (n=3). Asterisks indicate that mean values are significantly different from those in wild-type plants ($p<0.05$).

by the barley enzyme (Yang et al. 2011). However, an increase in tocopherol levels was not observed in either transgenic plants (Arabidopsis plants expressing *HvHGGT* or tobacco plants expressing OsHGGT). These findings suggest that the exogenous HGGTs in dicots catalyze the condensation of HGA with only GGDP, but not with PDP. Perhaps the different pool size between GGDP and PDP or the difference between in vitro and in vivo function of HGGT are concerned with such things. Furthermore, our results also suggest that the contribution of exogenous HGGTs to the condensation of HGA with PDP may be negligibly small in dicots.

Thus, the overexpression of exogenous *HGGTs* in dicots did not decrease the content of tocopherols. Furthermore, the growth of these transgenic Arabidopsis and tobacco plants was not negatively affected. These results suggest that transgenic Arabidopsis and tobacco plants contain a sufficient pool of HGA and GGDP to synthesize tocotrienols in their cells. However, GGDP is an important precursor not only to tocotrienols, but also to carotenoids, chlorophylls, and gibberellins (Cordoba et al. 2009). The constitutive overexpression of phytoene synthase, which catalyzes the conversion of GGDP to phytoene in tomato plants, caused chlorosis and dwarfism (Fray et al. 1995), suggesting that the GGDP pool was insufficient to synthesize carotenoids, chlorophylls, and gibberellins. Previous studies reported that the co-expression of yeast prephenate dehydrogenase (PDH) and Arabidopsis *p*-hydroxyphenylpyruvate dioxygenase (HPPD) in tobacco plants markedly enhanced the accumulation of tocotrienols (Matringe et al. 2008; Rippert et al. 2004). Similar findings have been reported for transgenic soybean and Arabidopsis plants co-expressing bacterial PDH and Arabidopsis HPPD (Karunanandaa et al. 2005). Their seeds accumulated large amounts of tocotrienols and also HGA, suggesting

that the accumulation of HGA enhanced tocotrienol production (Karunanandaa et al. 2005). A combination of the overexpression of *HGGT* and the enhancements in the HGA and GGDP pool size may be more effective for producing transgenic plants with enhanced tocotrienol levels.

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