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ARTICLE

Antioxidant Activity and Inhibition of Lipid Peroxidation in Germinating Seeds of Transgenic Soybean Expressing *OsHGGT*

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Tocochromanols are potent lipid-soluble antioxidants and essential nutrients for human health. Genetic engineering techniques were used to develop soybeans with enhanced vitamin E levels, including tocotrienols, which are not found in soybean. The gene encoding rice homogentisate geranylgeranyl transferase (*HGGT*) was overexpressed in soybeans using seed-specific and constitutive promoters. The association between abundance of vitamin E isomers and antioxidant activity was investigated during seed germination. With the exception of β-tocotrienol, all vitamin E isomers were detected in germinating seeds expressing *OsHGGT*. The antioxidant properties of germinating seed extracts were determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals and lipid peroxidation (TBARS). Compared with intact wild-type seeds, transgenic seeds showed increases in radical scavenging of 5.4−17 and 23.2−35.3% in the DPPH and ABTS assays, respectively. Furthermore, the lipid peroxidation levels were 2.0−4.5-fold lower in germinating seeds from transgenic lines than in wild-type seeds. Therefore, it appears that the antioxidant potential of transgenic oil-producing plants such as soybean, sunflower, and corn may be enhanced by overexpressing *OsHGGT* during seed germination.

KEYWORDS: Tocotrienols; tocopherols; seed germination; rice homogentisate geranylgeranyl transferase (*OsHGGT*); antioxidant activity; lipid peroxidation; soybean

INTRODUCTION

Tocochromanols (vitamin E) are the most important lipid-soluble antioxidants found in mammalian cells, and they represent an essential nutrient for human health. These molecules are important for scavenging free radicals and inhibiting lipid peroxidation in biological membranes (*1*). The tocochromanols comprise eight chemically distinct compounds that are separated into tocopherols and tocotrienols, according to saturation of the hydrophobic tails. Tocopherols contain a fully saturated tail, whereas tocotrienols have three unsaturated double bonds (**Figure 1**). The different tocopherol and tocotrienol isomers, that is, alpha (α), beta (β), gamma (γ), and delta (δ), are distinguished by the locations of methyl groups on the chromanol ring.

Tocopherols are abundant in common vegetable oils, whereas tocotrienols are present only in cereal grains such as barley, rice bran, oat, wheat germ, rye, and palm oil (*2*). In recent years, tocotrienols have received much more attention than tocopherols, because they exhibit a different suite of biological effects and because tocotrienols show greater antioxidant activity. For example, α-tocotrienol is more effective at reducing lipid peroxidation than α-tocopherol (*3*). This elevated antioxidant activity is due to higher efficiency recycling from chromanoxyl radicals, more uniform distribution in cell membranes, and better interactions with lipid radicals (*4*, *5*).

Tocotrienols have shown promise in a number of treatment areas, including lowering blood cholesterol and preventing stroke-induced brain damage, as well as exhibiting some anti-inflammatory and antiangiogenesis properties (*2*, *4*−*6*). They show potent anticancer activity in various human cancer cells including prostate, breast, and colon (*7*, *8*), and tocotrienol isomers will suppress up to 50% of the proliferation of breast tumor cells both in vitro and in vivo (*9*). These biological properties have led to the inclusion of tocotrienols in a broad spectrum of dietary supplements, functional foods, and nutraceuticals, as well as their use for cosmeceutical applications. However, tocopherols are the only tocochromanols found in popular oil crops such as soybean, sunflower, corn, and cotton. Thus, it is important to develop plants that can biosynthesize tocotrienols to meet the surging demand for products containing this nutrient.

During the past few decades, there have been significant advances in our understanding of the tocochromanol pathway in plants. Molecular and biochemical studies have revealed that

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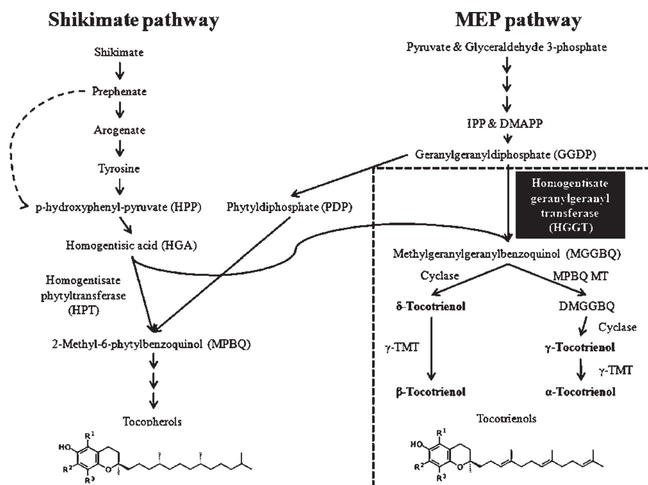

Figure 1. Tocochromanol biosynthesis pathway. Tocotrienols are not found in soybean. In this study, the gene encoding homogentisate geranylgeranyl transferase (*HGGT*) was isolated from rice seeds and introduced into the soybean cultivar Iksannamulkong via *Agrobacterium*-mediated transformation. This generated the tocotrienol biosynthesis pathway (box with dashed line) in transgenic plants.

tocopherols and tocotrienols share the same biosynthetic pathway and that these compounds result from two major biosynthetic pathways. The chromanol headgroup is derived from the shikimate pathway, and the hydrophobic tail is synthesized via the MEP pathway (**Figure 1**). Genes encoding enzymes in the vitamin E pathway have been identified (*10*) and used widely for the development of plants with improved vitamin E content, as well as altered tocopherol or tocotrienol compositions (*11−15*). Different approaches are required to introduce novel tocotrienol biosynthesis pathways into crops that do not normally accumulate tocotrienols. Significant progress was achieved when a novel gene encoding barley homogentisate geranylgeranyl transferase (*HGGT*), a key enzyme that regulates tocotrienol production in cereal crops, was introduced into *Arabidopsis* and corn (*10*). Overexpression of *HGGT* resulted in the accumulation of tocotrienols and a >10-fold increase in total tocochromanols in *Arabidopsis* leaves and corn seeds. In another approach, a significant increase in tocochromanol content, which included tocotrienols, was observed in seeds of transgenic *Arabidopsis*, canola, and soybean plants coexpressing *tyrA*, *At-HPPD*, and *At-VTE2* (*13*). It is likely that the observed increase in tocochromanol levels resulted from an increase in the homogentisate pool and *p*-hydroxyphenyl pyruvate flux through the shikimate branch of the tocochromanol biosynthetic pathway.

This work uses soybean as a model plant to develop methods for biofortification of nutritionally valuable tocotrienols. Because common soybean contains many functional ingredients such as isoflavones and saponins (*16*, *17*), the addition of novel tocotrienols should increase its beneficial health effects. Furthermore, it is possible that the four forms of tocopherols and fortified tocotrienol isomers could function synergistically, providing substantially enhanced inhibition of reactive oxygen species (ROS)-mediated lipid peroxidation. Previous studies have demonstrated that metabolic engineering of biosynthesis pathways can be used to synthesize tocotrienols in soybean plants (*13*). However, little information is available on the antioxidant capacity of these plants in vitro.

In this study, rice *HGGT* (*OsHGGT*) was expressed in soybeans using two different promoters. Tocotrienol accumulation was determined in transgenic soybeans expressing the two different constructs. Antioxidant activities were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays, as well as a lipid peroxidation inhibition test. In transgenic plants, significantly higher antioxidant activities were detected in germinated seeds than in nongerminated seeds. The association between vitamin E isomer content and antioxidant activity was investigated during seed germination to determine which tocotrienol isomers provided the greatest antioxidant activity.

MATERIALS AND METHODS

Chemicals and Instruments. Ethanol, DPPH, ABTS, potassium persulfate, ammonium ferrous sulfate hexahydrate, xylenol orange tetrasodium salt, triphenylphosphine (TPP), hydrogen peroxide solution (H_2O_2, 35.0%), trichloroacetic acid, malondialdehyde tetrabutylammonia, sulfuric acid, and glacial acetic acid were obtained from the Sigma Chemical Co. (St. Louis, MO). Thiobarbituric acid was purchased from MP Biomedicals. Analytical grade methanol, ethyl acetate, *n*-hexane, isopropanol, and water were purchased from J. T. Baker (Phillipsburg, NJ). HPLC was performed on a normal-phase HPLC system comprising a solvent delivery pump (515; Waters, Milford, MA) equipped with a spectrofluorometic detector (2475; Waters) and a Lichrosorb Si60 column (4.6 × 250 mm, 5 μm; Hibar, Darmstadt, Germany).

Vector Construction and Soybean Transformation. Two different vectors for soybean transformation were designed. A full-length cDNA fragment containing the rice *HGGT* gene (GenBank accession no. AY222862) was placed under the control of the seed-specific rice globulin promoter or the constitutive cauliflower mosaic virus (CaMV) 35S promoter (**Figure 2**). These vectors were transferred into *Agrobacterium tumefaciens* strain LBA4404 using the triparental mating method. *Agrobacterium*-mediated transformation of soybean was performed as described previously (*18*). Embryonic axes (*Glycine max* (L.) Merr. cv. Iksannamulkong) were excised from germinating seeds and inoculated in a suspension of *A. tumefaciens* harboring the binary vector *Glb-HGGT* or *35S-HGGT*. After cocultivation for 7 days, embryonic axes were plated onto solid SIM medium [MS salts, 0.4 mg/L BAP, 0.1 mg/L IBA, and 6 mg/L L-phosphinotricin (pH 5.7)] for 2 weeks. The surviving explants were transferred onto shoot elongation medium supplemented with 0.5 mg/L GA3, 0.1 mg/L IAA, 10 mg/L silver nitrate, 1 mg/L zeatin riboside, and 6 mg/L L-phosphinotricin. The elongated shoots (>2 cm long) were transferred to rooting medium [1/2 B5 salt, 2% sucrose, 1 mg/L IBA, and 0.5 g/L activated charcoal (pH 5.6)]. A total of five transgenic plants were selected and transplanted into soil.

Genomic DNA Extraction and Southern Hybridization. Total genomic DNA was extracted from the leaf tissues of T3 progeny plants and nontransformed control plants using the cetyltrimethyl ammonium bromide (CTAB) method (*19*). The remaining RNA was removed by adding 10 μg of RNase A (Sigma-Aldrich Chemie GmbH). Approximately 20 μg of genomic DNA was digested with *Eco*RI and *Dra*I, followed by fractionation through an 0.8% agarose gel in 0.5× TBE buffer. After electrophoresis, Southern blot analysis was conducted with a partial *HGGT* (163 bp) cDNA probe labeled and detected using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Rotkreuz, Switzerland).

Quantitative Real-Time PCR Analysis. Real-time quantitative reverse transcriptase PCR (RT-PCR) was used to analyze *OsHGGT* expression during seed germination of transgenic and wild-type plants. The fluorescent intercalating dye SYBR Green was used to detect amplified fragments. Total RNAs were isolated using the RNeasy Plant Mini Kit, according to the manufacturer's instructions (QIAGEN, Hiden, Germany). The first strands of cDNA were synthesized using the AffinityScript Multiple Temperature Reverse Transcriptase Kit (Stratagene, La Jolla, CA). Quantitative RT-PCR analyses were performed using the gene-specific primer pairs shown in **Table 1**. Soybean 18S rRNA was used as a standard control in RT-PCR reactions. The real-time RT-PCR data show means and standard errors from three independent experiments.

Tocopherol and Tocotrienol Analysis. Soybean seeds were ground in a household grinder. Ground soybeans (5 g) were extracted with 140 mL of *n*-hexane/EtOAc (85:15, v/v, containing 0.01% butylated hydroxytoluene) using a Soxtherm automatic extraction unit (Gerhardt, Germany) for 3 h

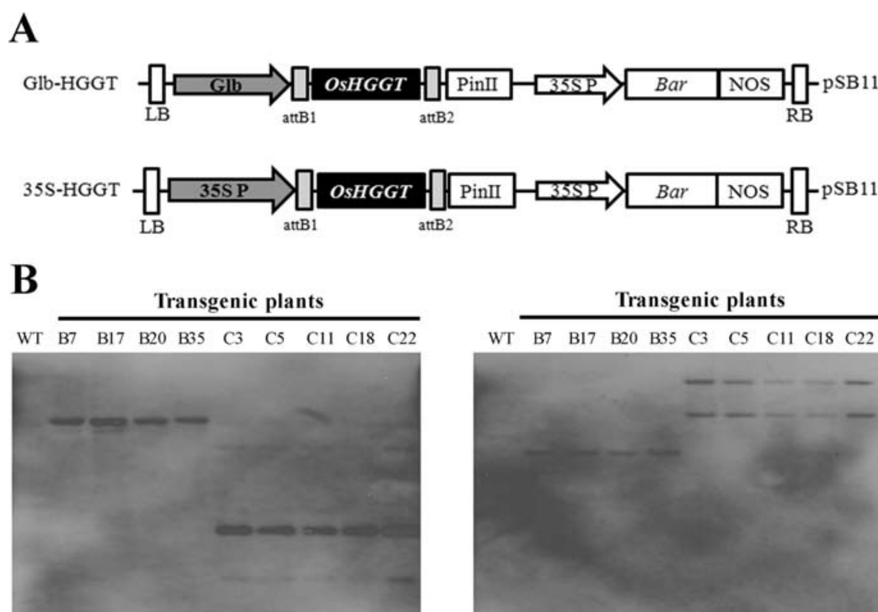

Figure 2. Schematic map of the T-DNA region of plasmids and confirmation of transgenic soybean plants expressing *OsHGGT*. (**A**) Schematic showing the binary vectors *Glb-HGGT* and *35S-HGGT*. Glb, *globulin* promotor; 35S P, cauliflower mosaic virus (*CaMV*) 35S promoter; PinΠ, potato proteinase inhibitor Π (PinΠ) terminator; *Bar*, Bialaphos resistance gene; NOS, *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator. (**B**) Southern blot analysis of a wild-type (WT) plant and nine transgenic lines. Genomic DNA from the leaf tissues of WT and transgenic plants was digested with *Eco*RI (left panel) or *Dra*I (right panel). Hybridization was performed using a 163 bp fragment of *OsHGGT* as a probe.

Table 1. Primers Used in This Study

name	primer sequence[a] (5′ → 3′)	product size (bp)
HGGT-FOR	AAAAAGCAGGCT[a] AGACG**ATG**CAAGCCTCATCGG	1245
HGGT-REV	AGAAAGCTGGGT[b] C**TCA**CTGCACAAATGGTATAAG	
HGGT-RT-F	AGCGTACGTTTGGGTCCAGAAAGA	163
HGGT-RT-R	CTCTCTGCCAAAGTGCAAAGGCAA	
18S rRNA-F	GAATTCTAGTAAGCGCGAGTCATCAG	155
18S rRNA-R	CTACGGAAACCTTGTTACGACTTCTC	

[a] The underlined portions are the attB1 (a) and attB2 (b) sequences for vector construction using the Gateway recombination system. Start and termination codons are shown in bold.

under dim light conditions. Crude lipid extracts were passed through a 0.2 μm filter unit prior to HPLC analysis. For germinating seeds, different extraction methods were applied (*12*). Germinating seeds (1 g) were ground in liquid nitrogen, and the resultant powder was transferred to 3 mL of 1% pyrogallol in ethanol (w/v). The extract was vortexed and centrifuged at 6225*g* for 2 min to remove large chunks of debris. The supernatant was collected in a 10 mL mess flask. Aliquots (1 mL) of supernatant were evaporated with nitrogen gas. Dried samples were re-suspended in *n*-hexane and filtered through a 0.2 μm filter. The tocopherol and tocotrienol contents of soybeans were characterized by normal HPLC. Lipid extract (20 μL) was injected into an analytical Lichrosorb Si60 column, with an isocratic phase of *n*-hexane/isopropanol (99:1, v/v) and a flow rate of 1.5 mL/min. The excitation and emission spectra were 290 and 330 nm, respectively. For quantification and identification purposes, standard stock solutions of four tocopherol isomers (α-, β-, γ-, and δ-tocopherol) and four tocotrienol isomers (α-, β-, γ-, and δ-tocotrienol) were prepared in *n*-hexane (containing 0.01% BHT). Tocopherol and tocotrienol peaks were identified by comparing sample retention times with the standards. Concentrations were calculated from peak areas determined by linear regression (*20*).

DPPH Radical-Scavenging Activity. The antioxidant activity of extracts was determined from the scavenging activity of stable DPPH free radicals using a modification of a method described previously (*21*). In a 96-well microtiter plate, samples containing 100 μL of ethanolic soybean extract (10 mg−50 μg/mL extract in DMSO solution) or standard were added to 150 μL of DPPH in ethanol solution (150 μM). After incubation at 37 °C for 30 min, the absorbance of each solution was determined at 518 nm using a microplate reader (Tecan's Infinite 200, Madison, WI). Radical scavenging was calculated as $(A_c - A_t/A_c) \times 100$, where A_t and A_c represent absorbance readings with and without sample extracts, respectively.

Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC assay is based on the abilities of antioxidants to scavenge the radical cation $ABTS^+$ relative to a standard Trolox curve (*22*). The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was diluted with ethanol and the absorbance read at 734 nm. For the photometric assay, 200 μL of $ABTS^{\bullet+}$ solution and 100 μL of sample were mixed for 45 s, and then the absorbance was measured immediately using a microplate reader. The antioxidant activity of each extract was calculated by determining the decrease in absorbance at different concentrations using the following equation: $(A_c - A_t/A_c) \times 100$, where A_t and A_c represent absorbance readings with and without sample extracts, respectively.

Lipid Peroxidation. Germinating seeds (1−4 days old) were collected from transgenic soybean lines, and lipid peroxidation was determined by estimating malondialdehyde content (*23*). Tissue samples (0.5 g) were macerated in 5 mL of 0.1% trichloroacetic acid, and then the homogenate was centrifuged at 24903*g* for 10 min. The supernatant was separated into 1 mL aliquots, which each received 4 mL of 20% trichloroacetic acid containing 0.5% TBA. The mixture was heated at 95 °C for 30 min and then cooled rapidly on ice. Following centrifugation at 11971*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at

Table 2. Tocopherol (T) and Tocotrienol (T3) Contents (μg/g Dry Weight) in Seeds from Wild-Type Soybean (WT), Nontransgenic Control Plants (Null-Segregant Lines, Null23 and Null24), and Nine Transgenic Lines Expressing *OsHGGT*[a]

construct	line	α-T	β-T	γ-T	γ-T3	δ-T	δ-T3	T	T3	vitamin E
	WT	11.3 ± 0.1	4.2 ± 0.0	98.5 ± 0.9	0.0 ± 0.0	57.8 ± 0.6	0.0 ± 0.0	171.8 ± 1.6	0.0 ± 0.0	171.8 ± 1.6
	Null23	11.4 ± 1.1	4.0 ± 0.2	96.7 ± 0.6	0.0 ± 0.0	59.1 ± 0.4	0.0 ± 0.0	171.2 ± 2.6	0.0 ± 0.0	171.2 ± 2.6
	Null24	12.0 ± 1.0	4.1 ± 0.1	98.7 ± 0.7	0.0 ± 0.0	59.9 ± 0.6	0.0 ± 0.0	174.7 ± 1.5	0.0 ± 0.0	174.7 ± 1.5
Glb-HGGT	B7	11.1 ± 0.4	3.3 ± 0.0	107.5 ± 1.8	5.1 ± 0.2	55.6 ± 1.0	1.8 ± 0.0	177.5 ± 3.3	6.9 ± 0.3	184.4 ± 3.5
	B17	14.6 ± 0.1	4.3 ± 0.2	108.4 ± 1.9	4.6 ± 0.5	61.0 ± 0.7	1.5 ± 0.2	188.3 ± 0.4	6.1 ± 0.7	194.4 ± 1.1
	B20	12.5 ± 0.1	3.3 ± 0.2	133.9 ± 6.7	6.1 ± 1.1	59.6 ± 1.3	1.7 ± 0.1	209.3 ± 7.7	7.8 ± 1.0	217.1 ± 8.6
	B35	13.1 ± 0.1	3.5 ± 0.1	126.1 ± 1.1	5.2 ± 0.1	62.5 ± 0.5	1.8 ± 0.0	205.2 ± 1.9	7.0 ± 0.1	212.2 ± 2.0
35S-HGGT	C3	11.5 ± 0.3	4.1 ± 0.1	101.4 ± 2.2	1.4 ± 0.2	67.5 ± 1.8	0.5 ± 0.0	184.5 ± 4.4	1.9 ± 0.2	186.4 ± 4.2
	C5	13.3 ± 0.3	3.9 ± 0.1	121.0 ± 2.7	1.0 ± 0.0	77.3 ± 1.9	0.5 ± 0.0	215.5 ± 4.8	1.5 ± 0.0	217.0 ± 4.8
	C11	13.4 ± 0.2	4.5 ± 0.1	120.3 ± 1.6	1.1 ± 0.0	77.3 ± 1.2	0.5 ± 0.0	215.5 ± 3.0	1.6 ± 0.0	217.1 ± 3.1
	C18	11.0 ± 0.1	4.4 ± 0.0	105.9 ± 0.9	1.1 ± 0.0	68.9 ± 0.7	0.5 ± 0.0	190.2 ± 1.8	1.6 ± 0.0	191.8 ± 1.8
	C22	12.4 ± 0.2	4.9 ± 0.1	101.8 ± 1.1	1.0 ± 0.0	68.2 ± 0.9	0.5 ± 0.0	187.3 ± 2.3	1.5 ± 0.0	188.8 ± 2.3

[a] Data represent the means of three replicates ±SD. Soybean seeds (3.0 g) were extracted with 140 mLof *n*-hexane/EtOAc (85:15, v/v containing 0.01% BHT) using a Soxtherm automatic extraction unit (Gehardt, Germany). α-T, α-tocopherol; β-T, β-tocopherol; γ-T, γ-tocopherol; γ-T3, γ-tocotrienol; δ-T, δ-tocopherol; δ-T3, δ-tocotrienol; T, sum of tocopherols; T3, Sum of tocotrienols; vitamin E, sum of T and T3.

600 nm. Malondialdehyde concentration was calculated using an extinction coefficient of 157 mM^{-1} cm^{-1}.

Ferrous Oxidation−Xylenol (FOX) Assay. The FOX version 2 assay is used to quantify lipohydroperoxides (LOOHs) in artificial systems and plant tissue extracts. LOOHs oxidize ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions, which bind to the ferric-sensitive dye xylenol orange, yielding an orange to purple complex (color depends upon the abundance of −OOH) that can be measured at 560 nm. The FOX assay for determining lipid peroxides was performed as described previously (*24*). FOX 2 reagent was prepared according to standard methods (*25*) by mixing 1 mM xylenol orange and 2.5 mM ammonium ferrous sulfate in 250 mM H_2SO_4. One volume of this concentrated reagent was added to 9 volumes of HPLC grade methanol containing 4.4 mM BHT to make the working reagent [250 μM ammonium ferrous sulfate, 100 μM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol]. Plant extracts were prepared by shaking 12 seedlings with 4 mm glass beads in 10 mL of ethanol/water (8:2, v/v) containing BHT and 1 mL of 150 mM acetic acid. The mixture was shaken for 5 min in a commercial paint shaker, and then each sample was centrifuged at 3000*g* for 10 min. TPP-treated samples were used to determine the background levels of oxidation that could not be attributed to lipid peroxides. To reduce lipid peroxides, 100 μL aliquots of supernatant were combined with 100 μL of 2.5 mM TPP in methanol. Half of the samples were left untreated. The reducing mixture was stirred and incubated at room temperature for 30 min. FOX reagent (200 μL) was added to each sample, and the absorbance at 560 nm was recorded exactly 10 min after reagent addition using a microplate reader. Hydrogen peroxide values were then expressed as micromolar H_2O_2 equivalents using a standard curve for the 0−100 μM H_2O_2 range.

Fatty Acid Analysis. Fatty acids were analyzed as described previously (*26*). The procedures were as follows: 0.5 g of germinating seed was heated with a reagent mixture [methanol/heptane/benzene/2,2-dimethoxypropane/H_2SO_4, 37:36:20:5:2 (v/v)]. Simultaneous digestion and lipid transmethylation were performed in a single phase at 80 °C, and then samples were cooled at room temperature. Upper phases containing fatty acid methyl esters (FAMEs) were analyzed by capillary GC analysis on an HP 6890 system (HP Co., Wilmingston, NC) equipped with a flame ionization detector. An HP-Innowax capillary (cross-linked polyethylene glycol) column (0.25 μm × 30 m) was used. The initial temperature of 150 °C was raised to a final temperature of 280 °C at a rate of 4 °C/min. Nitrogen was used as the carrier gas at a flow rate of 10 mL/min. During the analysis, inlet and detector temperatures were maintained at 250 and 300 °C, respectively. Standard FAME mix (C14−C22) was obtained from Supelco (Bellefonte, PA).

RESULTS AND DISCUSSION

Generation of Transgenic Soybean Plants. Five putative transgenic soybean plants derived from *Agrobacterium*-mediated transformation were tested for the presence of the transgene using Basta spraying, PCR, and Southern blot analysis (data not shown). Two T0 soybean plants (one containing the *Glb-HGGT* construct and the other containing the *35S-HGGT* construct) were selected and produced T1 progeny. The T3 generation of four homozygous *Glb-HGGT* lines (B7, B17, B20, and B35) and five homozygous *35S-HGGT* lines (C3, C5, C11, C18, and C22) stably expressing the *OsHGGT* gene were selected for further experiments. In addition, two null-segregant lines (Null23 and Null24) were identified and used as controls. Genomic DNA was extracted and digested with *Eco*RI and *Dra*I. Southern blotting was used to determine transgene copy number in the T3 transgenic lines. Four of the *Glb-HGGT* lines contained a single copy of *OsHGGT*, whereas five of the *35S-HGGT* lines had two copies of the transgene (**Figure 2**).

Content and Composition of Tocopherol and Tocotrienol in Seeds. HPLC analysis was used to determine the content and composition of tocochromanols in T4 seeds from transgenic lines overexpressing *OsHGGT*. In addition to the four tocopherol isomers, two new γ- and δ-tocotrienols were identified in transgenic seeds containing the *Glb-HGGT* and *35S-HGGT* constructs, respectively (**Table 2**). No significant differences were observed between the seeds of wild-type and null-segregant lines (Null23, Null24), which produced only tocopherols and contained total vitamin E contents of 171.8 and 171.2−174.7 μg/g, respectively. In contrast, the *Glb-HGGT* transgenic lines showed elevated tocochromanol contents of 184.4−217.1 μg/g (tocopherols, 177.5−209.3 μg/g; and tocotrienols, 6.1−7.8 μg/g). In the B20 line, the vitamin E content was 26.3% higher than in the wild-type. This elevated level was due to the production of γ- and δ-tocotrienol, as well as a 35.9% increase in γ-tocopherol. Increased total vitamin E concentration was also observed in *35S-HGGT* lines (tocopherols, 184.5−215.5 μg/g; and tocotrienols, 1.5−1.9 μg/g). In these transgenic lines, the C11 line accumulated the highest levels of vitamin E (217.1 μg/g), with increased levels of γ-tocopherol (22.1%) and δ-tocopherol (33.7%). When transgenic lines containing the seed-specific rice globulin promoter or the constitutive CaMV 35S promoter were compared, the *Glb-HGGT* lines showed a 2-fold higher tocotrienol content, whereas the *35S-HGGT* lines exhibited a 20% higher γ-tocopherol content. In a previous paper describing the transgenic expression of barley *HGGT* in *Arabidopsis* and corn, the authors observed a considerable accumulation of tocotrienols, mainly as γ-tocotrienol, but little or no change in tocopherol content (*10*). Our results show tocochromanol contents quite different from that experiment.

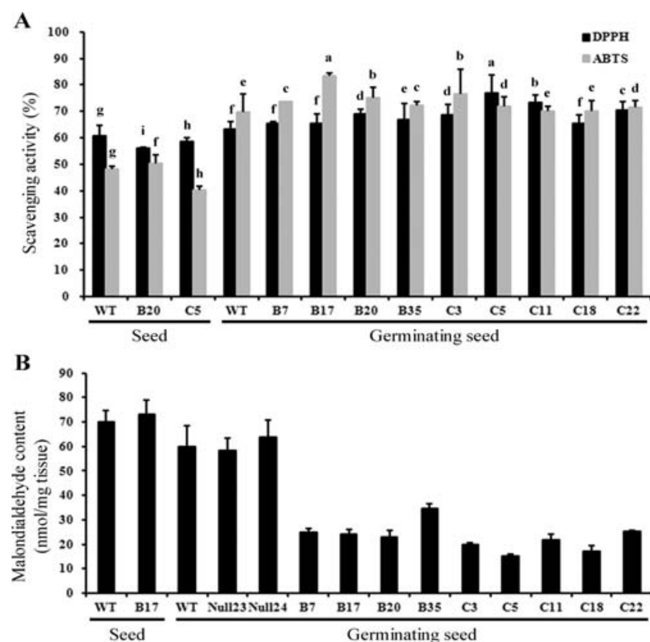

Figure 3. Antioxidant activities of intact and 3-day-old germinating soybean seeds were determined with DPPH and ABTS (**A**) and lipid peroxidation (**B**). Three-day-old germinating seeds of WT, four *Glb-HGGT* lines (B7, B17, B20, B35), and five *35S-HGGT* lines (C3, C5, C11, C18, C22) were compared to intact seeds from WT, B20, B17, and C5. DPPH and ABTS assays were used to evaluate the scavenging activities of WT and transgenic line extracts. DPPH and ABTS assays used 25 and 10 μg/mL extracts, respectively. Differences between WT and transgenic lines were statistically significant (LSD, $p < 0.01$).

Although tocotrienol abundances were much lower in our experiments than in the study described above, significantly increased tocopherol contents were observed in most transgenic lines. Discrepancies between the studies are thought to be due to differences between the constructs used and, in particular, the types of promoters driving expression and the source of the target gene, which was isolated from a different crop species.

Antioxidant Activities in Germinating Soybean Seeds. A variety of in vitro antioxidant assays were used to evaluate antioxidant activity in extracts from transgenic plants producing tocotrienols. The DPPH and ABTS methods are widely used for determining the antioxidant activity of hydrogen-donating and chain-breaking antioxidants (*27*). The DPPH and ABTS assays did not show any significant differences between the radical-scavenging activities of seed extracts from wild-type and transgenic lines (**Figure 3A**). Moreover, statistical analysis indicated that antioxidant activities were slightly higher in wild-type seeds. These findings are unexpected, because previous in vitro assays (*28*) have shown that tissues containing higher levels of tocopherols and tocotrienols exhibit higher antioxidant activities. Until now, few investigations have used in vitro analyses to examine the potency of antioxidants in transgenic plants. However, one study suggested that antioxidant activity increased during the germination of transgenic seeds with enhanced α-tocopherol contents (*12*). Therefore, the effects of seed germination on antioxidant activity were investigated in transgenic soybeans producing tocotrienols. After 3 days of germination, the DPPH and ABTS radical-scavenging activities of seed extracts from transgenic lines were up to 17 and 35.3% higher, respectively, than those of extracts from intact (ungerminated) wild-type seeds (**Figure 3A**). Increased antioxidant activity was also observed in wild-type seeds that had germinated for 3 days, that is, increases of 3.1 and 21.5% in the DPPH and ABTS assays, respectively. When 3-day-old germinating seeds from wild-type and transgenic lines were compared, the transgenic lines showed increases of 2.3−13.9 and 0.2−13.7% in the DPPH and ABTS assays, respectively. These results agree with the previous study, which showed that soybean germination induces modification of certain biologically active components such as saponin, lecithin, and estrogenic compounds, as well as the overall nutritive value of seeds (*29*, *30*).

Enhanced production of oxygen free radicals can result in lipid peroxidation, which may damage membrane integrity, increasing membrane permeability and leading to enzyme inactivation, structural damage of DNA, and cell death (*31*). Free radical-induced lipid peroxidation has been implicated in the pathogenesis of a wide range of human diseases, including atherosclerosis, diabetes, cancer, chronic inflammatory diseases, and neurodegenerative diseases, as well as in the aging process (*32*, *33*). Malondialdehyde (MDA), an end product derived from the breakdown of polyunsaturated fatty acids, is widely used as an indicator of lipid peroxidation (*33*). In this study, the thiobarbituric acid-reactive substances (TBARS) assay was applied to estimate MDA content. Similar to the results obtained from the DPPH and ABTS assays, no significant differences were found between the MDA contents of intact wild-type and transgenic seeds. However, germinating seeds from transgenic lines exhibited dramatically lower MDA contents than intact and germinating wild-type seeds (**Figure 3B**). In particular, the transgenic line C5 exhibited a 4.5-fold lower MDA content than intact wild-type seeds.

To determine which factors are associated with increased antioxidant activities in the DPPH, ABTS, and TBARS assays, the relative contents of tocopherols and tocotrienols were analyzed in germinating seeds (**Table 3**). Although transgenic seed extracts showed increased tocopherol concentrations, that is, up to 29% higher than the wild-type, there were no significant differences in the composition of tocopherol. However, marked changes were observed in the content and composition of tocotrienol. α-Tocotrienol was not detected in intact seeds, but detectable amounts (2.2−3.6 μg/g) were present in germinating seeds of both *Glb-HGGT* and *35S-HGGT* lines (**Table 3** and **Figure 4**). Furthermore, an increased tocotrienol concentration was observed in most transgenic lines after germination for 3 days, and *35S-HGGT* lines showed a large increase in tocotrienol content. In particular, total tocochromanol content increased by up to 33% in transgenic line C5 (136.8 μg/g) compared with the wild-type (102.5 μg/g). However, the total vitamin E content decreased considerably in germinating seeds compared to intact seeds. This decrease was due to a reduction in γ-tocotrienol (78%) and δ-tocotrienol (76%), as well as to differences between the extraction methods described under Materials and Methods (**Tables 2** and **3**). Thus, the increased antioxidant activity of germinating seeds may be associated with de novo synthesis of new tocotrienol isomers, such as α-tocotrienol, as well as with enhanced tocotrienol content.

The antioxidant activity of α-tocotrienol is thought to be more potent than that of α-tocopherol. In comparison to α-tocopherol, α-tocotrienol shows 40−60-fold more antioxidant activity against ferrous iron/ascorbate and ferrous iron/NADPH-induced lipid peroxidation in rat liver microsomal membranes (*3*), and it has greater peroxyl radical scavenging potency than α-tocopherol in liposomes (*34*). In addition, much lower concentrations of α-tocotrienol are required for protection against glutamate-induced cell death (*35*). Therefore, the results presented here agree with previous studies and confirm that α-tocotrienol plays an important role in protecting lipid components from oxidative damage.

Lipid Peroxidation and α-Tocotrienol Content during Seed Germination. To determine when antioxidant activity is greatest

Table 3. Tocopherol (T) and Tocotrienol (T3) Contents (μg/g FW) in 3-Day-Old Germinating Seeds from Wild-Type (WT), Null, and Transgenic Soybean Lines Expressing *OsHGGT*[a]

line	α-T	α-T3	β-T	γ-T	γ-T3	δ-T	δ-T3	T	T3	vitamin E
WT	8.3 ± 1.1	0.0 ± 0.0	3.2 ± 0.6	60.3 ± 5.7	0.0 ± 0.0	30.7 ± 1.4	0.0 ± 0.0	102.5 ± 2.6	0.0 ± 0.0	102.5 ± 2.6
null	6.3 ± 0.2	0.0 ± 0.0	2.5 ± 2.6	60.1 ± 9.2	0.0 ± 0.0	32.2 ± 9.5	0.0 ± 0.0	101.1 ± 2.8	0.0 ± 0.0	101.1 ± 9.8
null	3.8 ± 0.5	0.0 ± 0.0	4.7 ± 4.1	62.8 ± 8.0	0.0 ± 0.0	27.0 ± 5.8	0.0 ± 0.0	98.3 ± 4.7	0.0 ± 0.0	98.3 ± 8.7
B7	10.3 ± 3.0	3.0 ± 0.2	2.9 ± 0.4	59.9 ± 4.1	3.8 ± 0.8	34.2 ± 2.9	2.2 ± 1.2	107.3 ± 5.5	9.0 ± 1.4	116.3 ± 4.2
B17	8.3 ± 2.0	3.5 ± 0.6	2.6 ± 0.1	61.0 ± 4.7	2.9 ± 0.8	31.3 ± 1.9	0.9 ± 0.1	103.2 ± 6.7	7.3 ± 1.3	110.5 ± 8.0
B20	9.4 ± 1.2	3.6 ± 0.7	2.6 ± 1.7	69.9 ± 7.3	3.0 ± 0.4	26.1 ± 4.9	1.0 ± 0.0	108.0 ± 3.5	7.6 ± 1.8	115.6 ± 5.2
B35	9.6 ± 0.2	2.8 ± 1.0	2.7 ± 0.2	72.1 ± 1.7	3.1 ± 0.3	30.9 ± 1.4	1.2 ± 0.0	115.3 ± 3.3	7.1 ± 0.9	122.4 ± 3.3
C3	8.8 ± 0.6	2.4 ± 1.0	2.8 ± 0.6	64.1 ± 5.0	2.8 ± 0.4	31.1 ± 2.7	0.0 ± 0.0	106.8 ± 6.0	5.2 ± 1.2	112.0 ± 5.2
C5	11.4 ± 0.1	3.0 ± 0.9	4.2 ± 0.3	74.2 ± 2.3	2.4 ± 0.7	40.0 ± 1.6	1.6 ± 0.4	129.8 ± 4.1	7.0 ± 0.4	136.8 ± 4.2
C11	10.5 ± 1.0	2.4 ± 1.8	3.7 ± 0.8	71.2 ± 5.1	1.9 ± 0.0	42.2 ± 2.9	0.2 ± 0.4	127.6 ± 4.2	4.5 ± 2.5	132.1 ± 6.7
C18	10.4 ± 0.3	2.2 ± 0.5	3.1 ± 0.1	75.1 ± 4.0	2.2 ± 0.4	43.8 ± 4.4	0.0 ± 0.0	132.4 ± 8.8	4.4 ± 0.8	136.8 ± 8.7
C22	9.3 ± 0.4	2.3 ± 0.3	2.7 ± 0.2	68.6 ± 3.4	3.1 ± 0.5	33.0 ± 2.3	0.0 ± 0.0	113.6 ± 1.2	5.4 ± 1.1	119.0 ± 1.6

[a] Data represent the means of three replicates ± SD. Germinating seeds were ground with liquid nitrogen, and 1.0 g of the powder was extracted with 3 mL of 1% pyrogallol in ethanol (w/v). α-Tc, α-Tocotrienol.

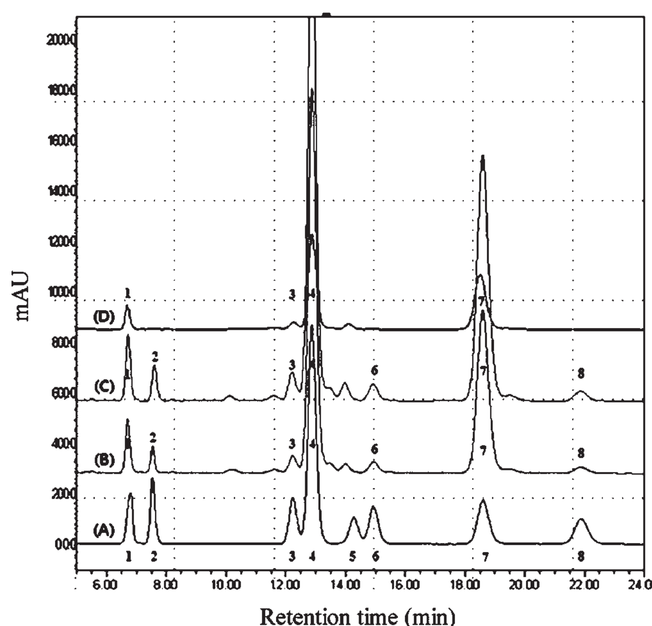

Figure 4. HPLC profiles for tocopherol and tocotrienol isomers in a standard mixture (**A**) and 3-day-old germinating seeds of C5 (**B**), B20 (**C**), and wild-type (**D**). Peaks: 1, α-tocopherol; 2, α-tocotrienol; 3, β-tocopherol; 4, γ-tocopherol; 5, β-tocotrienol; 6, γ-tocotrienol; 7, δ-tocopherol; 8, δ-tocotrienol. In the chromatograms for B20, C5, and WT, the peaks for α-tocotrienol, γ-tocotrienol, and δ-tocotrienol overlap with newly identified peaks.

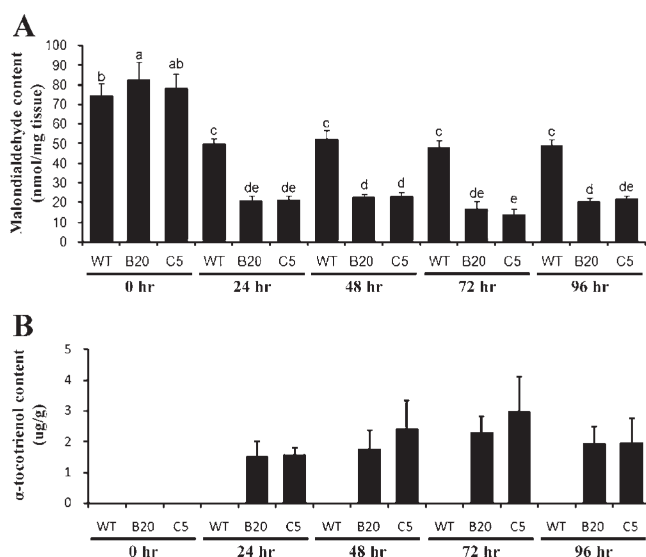

Figure 5. Changes in lipid peroxidation (**A**) and α-tocotrienol content (**B**) during the germination of seeds from WT and transgenic B20 and C5 lines. Data represent the means of three replicates ± SD. Differences were statistically significant (LSD, $p < 0.01$).

in germinating seeds, changes to lipid peroxidation and α-tocotrienol contents were compared in germinating seeds from wild-type soybean and transgenic B20 and C5 lines. In extracts from 1-day-old germinating seeds of B20 and C5, the MDA contents were 2-fold lower than in intact seeds (50.1 nM/mg). After 3 days of germination, the lowest MDA levels were observed in transgenic lines B5 and C5 (16.7 and 13.8 nM/mg, respectively) (**Figure 5A**). However, the MDA content increased slightly in B5 and C5 extracts from 4-day-old germinating seeds. In addition, the α-tocotrienol content and inhibition of lipid peroxidation showed similar patterns during germination, increasing slowly at 1 day, reaching a peak at 3 days, and then decreasing at 4 days (**Figure 5B**). Thus, the inhibition of lipid peroxidation was greatest in 3-day-old germinating seeds, and this activity correlated positively with α-tocotrienol content.

RT-PCR (**Figure 6A**) and real-time PCR (**Figure 6B**) were used to measure *OsHGGT* expression in germinating seeds from the B20 and C5 lines. *OsHGGT* expression was strongly induced during seed germination. However, the B20 line, in which the rice globulin promoter drives expression, showed much higher transcript levels than the C5 line, which has expression directed by a constitutive promoter. In the B20 line, *OsHGGT* expression levels peaked 2 days after germination, whereas maximum levels were reached only at 4 days in the C5 line. In the previous result, the B20 line showed strong expression of *OsHGGT* in vegetative stage (V4) as well as reproductive stages (R1, R5, and R6) (data not shown). These results suggest that the rice globulin promoter may be useful for high-level expression of transgenes in germinating soybean.

Fatty Acid Composition and LOOH Content. As lipid-soluble antioxidants, it is likely that tocochromanols prevent propagation of free radical reactions, thereby protecting polyunsaturated fatty acids and other membrane components from oxidation by free radicals. In this study, the fatty acid composition of germinating seeds was determined to find out whether polyunsaturated fatty acids were affected by the tocotrienols produced during seed

germination (**Figure 7**). In intact seeds and 1 day after germination, seeds from wild-type and transgenic lines showed little difference in the composition of either unsaturated fatty acid (USFA) or saturated fatty acid (SFA) (**Table 4**). However, after 2 days of germination, the USFA contents of transgenic lines B20 and C5 were 2.5 and 3.1% greater than the corresponding wild-type seeds. At 3 days after germination, the USFA contents of B20 and C5 seeds were 6.2 and 8.2% higher than the wild-type, respectively. The USFA contents of B20 and C5 reduced slightly at 4 days of germination. These results appear similar to our findings for lipid peroxidation and α-tocotrienol content.

During the late stage of lipid peroxidation, MDA is formed from polyunsaturated fatty acids containing three or more double bonds. The TBARS determination of MDA content may underestimate the true extent of lipid peroxidation (*24*). Therefore, LOOHs, which are formed as a result of the oxidation of fatty acids during the early stages of lipid peroxidation, can be used as biomarkers to quantify early-stage or acute lipid peroxidation. When LOOH levels were determined using the FOX assay, distinctly lower levels of LOOH were detected in the transgenic B20 and C5 lines than in the wild-type for both intact and germinating seeds. The LOOH abundances in intact seeds of B20 and C5 were 1.9- and 1.6-fold lower than the wild-type (74.9, 84.8, and 143.1 nM/g, respectively). During germination, LOOH levels increased slowly, reaching a peak after 2 days and then

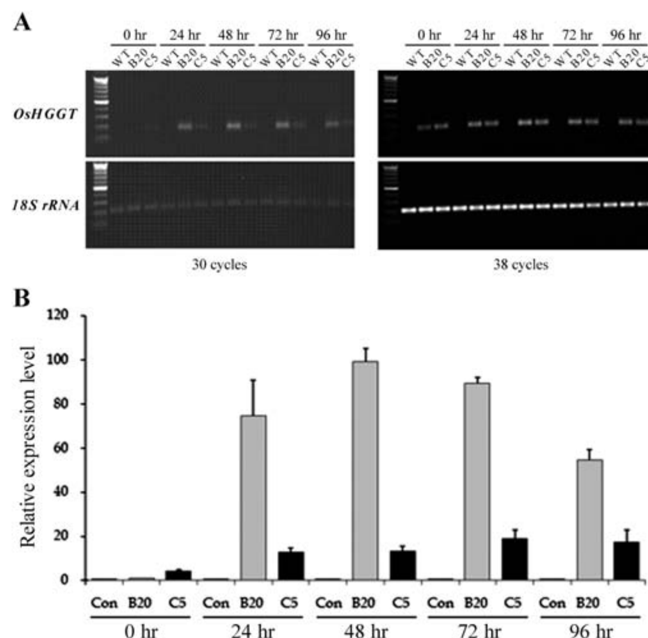

Figure 6. Quantification of *OsHGGT* expression in WT and transgenic B20 (gray bars) and C5 (black bars) lines, during seed germination during using RT-PCR (**A**) and real-time PCR (**B**). *OsHGGT* expression levels were measured by real-time PCR, and relative accumulation was normalized against *18S rRNA*. Data represent the means of three independent RNA preparations from germinating seeds at the time periods indicated.

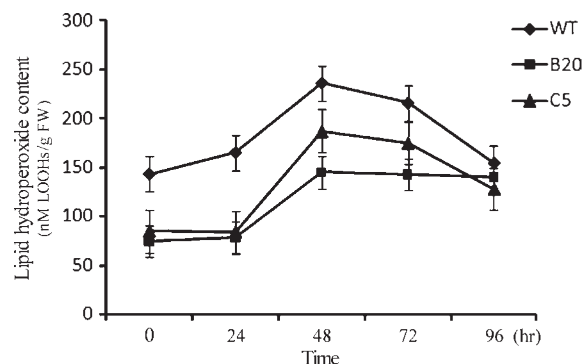

Figure 7. Lipohydroperoxide (LOOH) contents during seed germination in WT and transgenic B20 and C5 lines: (◆) WT; (■) B20; (▲) C5. LOOH levels were measured using the ferrous oxidation−xylenol orange (FOX) assay version 2. Hydrogen peroxide was used to construct a standard curve, and LOOH levels were expressed as nanomoles of LOOH per gram of fresh weight. Data represent means from three independent measurements ± SD.

Table 4. Changes in Fatty Acid Composition in WT and Transgenic B20 and C5 Lines during Seed Germination[a]

		fatty acid composition (%)								
germination period (h)	line	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	SFA	USFA
0	WT	13.2 ± 0.1c[†]	5.1 ± 0.0bc	22.1 ± 0.0a	50.7 ± 0.1bc	7.9 ± 0.0b	0.4 ± 0.0a	0.6 ± 0.0a	19.3 ± 0.1c	80.7 ± 0.1a
0	B20	13.3 ± 0.3c	4.5 ± 0.2c	17.3 ± 0.6ab	55.6 ± 0.4a	8.5 ± 0.4a	0.3 ± 0.2b	0.6 ± 0.0a	18.7 ± 0.2c	81.4 ± 0.2a
0	C5	13.8 ± 0.3c	5.0 ± 0.1bc	18.7 ± 0.6ab	53.2 ± 0.3ab	8.4 ± 0.2a	0.4 ± 0.0a	0.5 ± 0.0b	19.7 ± 0.2c	80.3 ± 0.2a
24	WT	19.8 ± 1.0b	5.7 ± 0.3b	16.1 ± 3.2ab	51.4 ± 2.5ab	7.0 ± 0.3cd			25.5 ± 0.9b	74.5 ± 0.9b
24	B20	20.8 ± 2.6ab	4.9 ± 0.4bc	17.7 ± 6.1ab	49.3 ± 3.2bc	7.3 ± 0.1cd			25.7 ± 2.9b	74.3 ± 2.9b
24	C5	20.3 ± 0.7ab	5.6 ± 0.3bc	13.9 ± 0.6c	53.1 ± 0.9ab	7.0 ± 0.2cd			25.9 ± 0.6b	74.0 ± 0.6b
48	WT	23.2 ± 1.2a	5.4 ± 0.1bc	8.5 ± 3.5d	55.5 ± 2.4a	7.4 ± 0.0c			28.6 ± 1.1ab	71.4 ± 1.1bc
48	B20	21.5 ± 1.0ab	5.2 ± 0.2bc	18.4 ± 4.5ab	48.4 ± 4.3bc	6.4 ± 0.4d			26.7 ± 1.1b	73.2 ± 1.1b
48	C5	21.0 ± 0.3ab	5.3 ± 0.2bc	13.2 ± 0.8c	53.3 ± 1.0ab	7.1 ± 0.1cd			26.3 ± 0.2b	73.6 ± 0.2b
72	WT	22.5 ± 2.4ab	8.2 ± 2.0a	13.5 ± 2.7c	49.4 ± 1.5bc	6.5 ± 0.2d			30.7 ± 3.3a	69.4 ± 3.3c
72	B20	21.2 ± 0.7ab	5.1 ± 0.3bc	13.7 ± 0.5c	52.7 ± 0.2ab	7.3 ± 0.3cd			26.3 ± 0.9b	73.7 ± 0.9b
72	C5	19.6 ± 1.6b	5.4 ± 0.4bc	17.4 ± 4.5ab	50.4 ± 2.7bc	7.3 ± 0.3cd			25.0 ± 2.1b	75.1 ± 2.1b
96	WT	22.5 ± 0.9ab	5.6 ± 0.1bc	12.0 ± 2.4 cd	53.0 ± 1.8ab	6.9 ± 0.4d			28.1 ± 1.0ab	71.9 ± 1.0bc
96	B20	20.6 ± 1.0ab	6.0 ± 1.6b	12.6 ± 1.0c	52.9 ± 1.9ab	7.9 ± 0.4b			26.6 ± 2.0ab	73.4 ± 2.0bc
96	C5	21.7 ± 2.3ab	5.4 ± 0.2bc	15.4 ± 8.2bc	50.3 ± 6.6bc	7.3 ± 0.3cd			27.1 ± 2.4ab	73.0 ± 2.4bc

[a] Data represent mean values (%) from three experiments ± SD. C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; C20:0, arachidic acid; C22:0, docosanoic acid; SFA, saturated fatty acid (sum of palmitic acid, stearic acid, arachidic acid, and docosanoic acid); USFA, unsaturated fatty acid (sum of oleic acid, linoleic acid, and linolenic acid). [†], $p < 0.01$.

decreasing at 3 and 4 days. Significant differences in LOOH levels were observed between wild-type and transgenic lines from 1 to 3 days of germination, but these differences were not found at 4 days. These results strongly support the suggestion that tocotrienols, in particular, α-tocotrienol, perform an important role in reducing lipid peroxidation.

In conclusion, germinating seeds that overexpressed *OsHGGT* showed de novo synthesis of α-tocotrienol, as well as enhanced total tocotrienol contents. Increased tocotrienol levels correlated with significant improvements in antioxidant activity. These findings suggest that antioxidant potential may be enhanced by germination of transgenic seeds.

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