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Full-Length Transcriptome Analysis of the Genes Involved in Tocopherol Biosynthesis in *Torreya grandis*

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Supporting Information

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ABSTRACT: The seeds of *Torreya grandis* (Cephalotaxaceae) are rich in tocopherols, which are essential components of the human diet as a result of their function in scavenging reactive oxygen and free radicals. Different *T. grandis* cultivars (10 cultivars selected in this study were researched, and their information is shown in Table S1 of the Supporting Information) vary enormously in their tocopherol contents (0.28−11.98 mg/100 g). However, little is known about the molecular basis and regulatory mechanisms of tocopherol biosynthesis in *T. grandis* kernels. Here, we applied single-molecule real-time (SMRT) sequencing to *T. grandis* (X08 cultivar) for the first time and obtained a total of 97 211 full-length transcripts. We proposed the biosynthetic pathway of tocopherol and identified eight full-length transcripts encoding enzymes potentially involved in tocopherol biosynthesis in *T. grandis*. The results of the correlation analysis between the tocopherol content and gene expression level in the 10 selected cultivars and different kernel developmental stages of the X08 cultivar suggested that homogentisate phytyltransferase coding gene (*TgVTE2b*) and γ-tocopherol methyltransferase coding gene (*TgVTE4*) may be key players in tocopherol accumulation in the kernels of *T. grandis*. Subcellular localization assays showed that both TgVTE2b and TgVTE4 were localized to the chloroplast. We also identified candidate regulatory genes similar to *WRI1* and *DGAT1* in *Arabidopsis* that may be involved in the regulation of tocopherol biosynthesis. Our findings provide valuable genetic information for *T. grandis* using full-length transcriptomic analysis, elucidating the candidate genes and key regulatory genes involved in tocopherol biosynthesis. This information will be critical for further molecular-assisted screening and breeding of *T. grandis* genotypes with high tocopherol contents.

KEYWORDS: *Torreya grandis, tocopherol biosynthesis, transcriptional regulation, SMRT sequencing, kernels*

INTRODUCTION

Tocopherols are amphipathic molecules consisting of a polar chromanol headgroup and a lipophilic isoprenoid tail. Tocopherols usually occur in one of four forms, namely, α-, β-, γ-, and δ-tocopherol, differing only in the number and position of the methylation on the chromanol ring. They possesses antioxidant activity and can inhibit membrane lipid peroxidation and scavenge reactive oxygen species.[1,2] In addition, other functions of tocopherols have also been demonstrated in plant metabolism, such as sugar export from the leaves to phloem.[3] In the human diet, tocopherols serve as both lipid-soluble antioxidants and the essential nutrient vitamin E,[4,5] with α-tocopherol having the highest vitamin E activity.[6,7] As a result of this, dietary tocopherols are thought to play an important role in improving immune function and limiting the incidence and progression of several degenerative human diseases, including certain types of cancer, cataracts, neurological disorders, and cardiovascular disease.[8−10]

Tocopherols are synthesized from the condensation of homogentisate (HGA), synthesized through the cytosolic shikimate (SK) pathway, and phytyl diphosphate (PDP), synthesized from the plastidial methylerythritol phosphate (MEP) pathway.[4] HGA is synthesized by *p*-hydroxyphenylpyruvate dioxygenase (HPPD/PDS1).[11] Homogentisate phytyltransferase (HPT1/VTE2) then catalyzes the prenylation of HGA with PDP to form 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ).[12,13] 2,3-Dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) is derived from the methylation of MPBQ by 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (MT/VTE3),[14,15] following which MPBQ and DMPBQ are converted to δ- and γ-tocopherols, respectively, by tocopherol cyclase (TC/VTE1).[16,17] Finally, the methylation of δ- and γ-tocopherols by γ-tocopherol methyltransferase (γ-TMT/VTE4) results in the production of β- and α-tocopherols, respectively.

As a result of the importance of vitamin E in human and plant physiology, the tocopherol biosynthetic pathway has become a focus in plant metabolic engineering. To successfully manipulate the tocopherol content and/or composition of various plant tissues, enzymes with high flux coefficients must be identified in the pathway. This requires the cloning of individual tocopherol biosynthetic enzymes and a detailed understanding of the molecular and biochemical regulation of the individual steps of the pathway. Genes involved in the biosynthetic pathways for tocopherol have been widely cloned and engineered for improved nutritional content in a number of plant species, and their regulatory role in tocopherol

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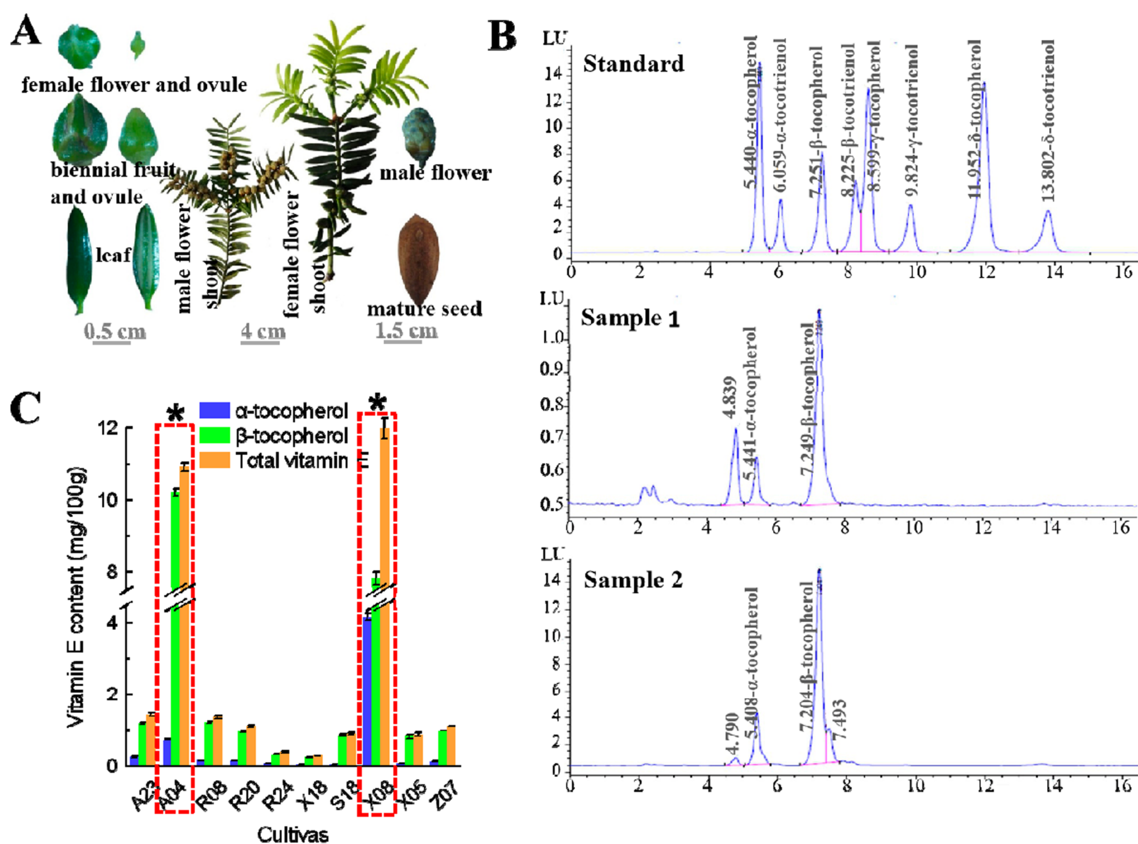

Figure 1. Tissues of *T. grandis*, chromatograms of vitamin E components in the kernel of *T. grandis* and vitamin E contents in the kernels of different *T. grandis* cultivars: (A) Tissues of male and female *T. grandis*, (B) HPLC chromatograms of vitamin E (including α, β, γ, and δ forms of tocopherols and tocotrienols) standards and kernel samples, and (C) detectable vitamin E contents in the kernels of different *T. grandis* cultivars.

synthesis has also been reported.[13,18−22] An increase in the activity of HPPD or HPT1 in transgenic plants resulted in an elevated tocopherol content in the seeds and leaves of *Arabidopsis*.[18,19] It was concluded that flux into tocopherol is predominantly controlled by PDS1 and VTE2, but downstream VTE1 in the tocopherol biosynthetic pathway is not a key factor limiting tocopherol synthesis.[19] However, Kanwischer et al. suggest that VTE1 is strongly induced during oxidative stress and is a major factor limiting tocopherol synthesis in the leaves.[23] In transgenic *Brassica napus*, the co-expression of *PDS1* with *VTE2* and *VTE1* resulted in a significant increase in total tocochromanol levels in the seeds, suggesting that the three enzymes are critical in determining the total tocochromanol content in the seed oil of *B. napus*.[20] In soybean seeds, it was found that TC/VTE1 plays key roles in tocopherol accumulation.[24] These reports suggest that the key enzymes limiting tocopherol synthesis vary in different plants or tissues.

The evergreen and ornamental coniferous gymnosperm *Torreya grandis* (Cephalotaxaceae) is mainly distributed in Southeast China.[25] It has drupe-like fruits with nut seeds that have been used as food for thousands of years in China. The seeds have multiple biological properties, including antioxidative, anti-inflammatory, antiviral, antiatherosclerosis, anti-helminthic, antitussive, carminative, laxative, antifungal, antibacterial, and antitumor, as a result of their rich nutritive content and bioactive components, including alkaloids, flavonoids, tannins, terpenoids, and saponins.[25−29] As a result of the influence of the environment and cultivation management, *T. grandis* is represented by different cultivars and varieties in China and, thus, exhibits different qualities.[30] Chemical investigation revealed that *T. grandis* contains abundant bioactive substances, such as tocopherols; however, different *T. grandis* cultivars vary enormously in their tocopherol content,[31] suggesting that *T. grandis* constitutes a suitable plant material to study the biosynthetic pathway of tocopherols and the molecular regulatory mechanisms of tocopherol biosynthesis. However, the genome of *T. grandis* has not been sequenced, and genetic resources are scarce.

Single-molecule real-time (SMRT) sequencing is a third-generation sequencing platform that is becoming increasingly popular in full-length sequencing applications.[32,33] It overcomes the limitations of short-read sequences by enabling the generation of full-length cDNA sequences without assembly (average of 4−8 kb),[32−34] which dramatically increases the gene discovery and accuracy of alternative splice detection. Despite the higher error rate (up to 15%) observed with SMRT sequencing and relatively lower throughput that might miss some rare transcript isoforms, the third-generation sequencing platform can self-correct via circular-consensus (CCS) reads and/or correct with high-throughput and high-accurate short read data.[35,36] The use of SMRT sequencing then offers access to more complete (i.e., full-length) transcriptome data, as recently demonstrated in *Salvia miltiorrhiza*, *Triticum aestivum*, and *Dendrobium officinale*.[37−39]

In this study, we sequenced the first full-length transcriptome of *T. grandis* using the newest PacBio reagents (P6/C4) on the PacBio RS II platform. We identified candidate genes encoding enzymes potentially involved in the biosynthesis of tocopherols based on previous reports[25,40−42] and

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conducted amino acid sequence alignment and phylogenetic analysis. The key genes regulating tocopherol biosynthesis in *T. grandis* were also identified by Pearson's correlation analysis. Moreover, we also further clarified the subcellular localization of the key genes and identified the regulatory genes potentially involved in tocopherol biosynthesis regulation based on a phylogenetic analysis of the known regulatory genes involved in tocopherol biosynthesis regulation in other plants. Our results will be critical for the further molecular-assisted screening and breeding of high-tocopherol-content genotypes of *T. grandis*.

■ MATERIALS AND METHODS

Plant Materials. Samples for quantitative real-time polymerase chain reaction (qRT-PCR) and tocopherol determination were collected from 10 *T. grandis* cultivars (designated as A23, A04, R08, R20, R24, X18, S18, X08, X05, and Z07, respectively). Information relating to their cultivation is listed in Table S1 of the Supporting Information. Samples for PacBio sequencing were collected from X08 tissues (Figure 1A). After collection, the arils and seed coats were removed and the remaining kernels for RNA extraction were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use.

Oil Extraction and Tocopherol and Tocotrienol Determination. The kernels of *T. grandis* were directly pressed using a household ZYJ901 expeller (Bear Electric Appliance Co., Ltd., Jiangmen, China) at ambient temperature. The pressed oils with fine suspended solids were collected and centrifuged at 10000*g* for 15 min at 4 °C. The supernatant consisted of purified oils. The oils were then placed in brown glass bottles, flushed with nitrogen, and stored in a refrigerator at 5 °C until further analysis. A total of 1 g of the oil was mixed with 10 mL of *n*-hexane, following which the sample was filtered using a 0.45 μm polytetrafluoroethylene filter for high-performance liquid chromatography (HPLC) analysis. Analysis of tocopherols and tocotrienols was performed on an Agilent 1290 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) equipped with a silica column (5 lm, 250 × 4.6 mm inner diameter, Waters Co., Milford, MA, U.S.A.) and a fluorescence detector (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). The column temperature was 30 °C. The mobile phase was 99:1 (v/v) *n*-hexane/isopropanol at a flow rate of 1.2 mL/min. The fluorescence detection was operated with an excitation wavelength of 295 nm and an emission wavelength of 333 nm. Tocopherols and tocotrienols were identified by comparing their retention times to authentic standards and were quantified on the basis of the peak areas compared to the external standards. The results were expressed as milligrams of tocopherol or tocotrienol per 100 g of original kernel oil.

RNA Extraction, cDNA Preparation, and PacBio Sequencing. Total RNA was isolated from different *T. grandis* tissues using the RNAprep Pure Plant Kit (DP441, Tiangen), after which the RNA samples were combined for PacBio sequencing. The isoform sequencing (Iso-Seq) library was prepared according to the Iso-Seq protocol using the Clontech SMARTer PCR cDNA Synthesis Kit and the BluePippin size selection system protocol as described by Pacific Biosciences (P/N100-377-100-05 and P/N100-377-100-04) with the following modifications. The cDNA products of the combined RNA sample were used to construct one SMRTbell library following the manual of the DNA Template Prep Kit 3.0 (Pacific Biosciences, Menlo Park, CA, U.S.A.). The fragmented cDNA was concentrated by AMPure PB beads, and the ends were repaired. Then, blunt hairpin adapters were ligated to the cDNA, and exonucleases were added to remove failed ligation products. SMRTbell templates containing cDNA inserts were purified by AMPure PB beads. The sequencing primers and polymerase were then sequentially annealed to the SMRTbell templates using the DNA/Polymerase Binding Kit P6 v2 (Pacific Biosciences). The MagBead Loading Kit (Pacific Biosciences) was used to load the annealed templates onto a Pacific Biosciences RS II sequencer. The sequencing was performed using seven SMRT cells with the DNA Sequencing Reagent Kit 4.0 v2 (Pacific Biosciences).

Sequence Data Assembly and Annotation. The Iso-Seq protocol (Pacific Biosciences, Menlo Park, CA, U.S.A.) was used to process the sequenced reads to CCS RNA BIOLOGY 5. Effective subreads were obtained using the P_Fetch and P_Filter function (parameters: miniLength, 50; readScore, 0.75; and artifact, −1000) in the SMRT analysis software suite (http://www.pacificbiosciences.com/devnet/). CCS reads were obtained from the P_CCS module using the parameter MinCompletePasses = 2 and MinPredictedAccuracy = 0. After examination for the poly(A) signal and 5′ and 3′ adaptors, only the CCS reads with all three signals were considered as a FLNC read.[43] Unmerged subreads were also examined for the three signals, and those with three signals were incorporated into the final FLNC read set. The full-length transcripts were compared against public protein databases, such as the National Center for Biotechnology Information (NCBI) Nonredundant (Nr) and Nucleotide (Nt) databases, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/kegg2.html), Cluster of Orthologous Groups of proteins (COG), and TrEMBL using BLASTX with an *E* value of $\leq 10^{-5}$. Gene ontologies (GOs) were assigned to each full-length transcript using Blast2GO.

qRT-PCR Analysis. Total RNA was extracted from the kernels as described above. First-strand cDNA was synthesized from 1 μg of total RNA using PrimeScript RT Master Mix (Takara). Gene expression levels were determined by qRT-PCR using ChamQ SYBR qPCR Master Mix (Vazyme) on a C1000 Touch Thermal Cycler (Bio-Rad). The relative expression was normalized to the expression level of the internal control (*Actin*). The primer pairs of each gene are listed in Table S2 of the Supporting Information. The reaction condition was 45 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 20 s. The relative expression was calculated by the formula $2^{-\Delta\Delta C_p}$. The experiment was performed with three biological and technical replications.

Subcellular Localization of the Protein. The coding sequences of signal peptides and genes were cloned and ligated into the binary vector *35S::GFP* (modified from pCAMBIA1300), respecively. The resultant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101. Positive clones were then incubated in Luria-Bertani medium supplemented with 50 mg/L kanamycin at 28 °C until an OD_{600} of 0.6 was reached, and the cells were collected and resuspended with infiltration buffer [10 mM $MgCl_2$, 0.2 mM acetosyringone, and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.6]. The buffer was then injected into the epidermal cells of tobacco leaves (*Nicotiana benthamiana*). Green fluorescent protein (GFP) fluorescence was observed via confocal laser scanning microscopy (LSM710, Karl Zeiss).

Statistical and Sequence Analyses. Statistical analyses were carried out using SPSS, version 16.0 (SPSS, Inc., Chicago, IL, U.S.A.). Correlations among data were calculated by Pearson's correlation coefficients (*r*). Significant differences were determined using Duncan's new multiple range test at $p < 0.05$. Sequences of the full-length transcripts used in this study were confirmed via reverse transcription polymerase chain reaction (PCR) amplification and sequencing. Amino acid sequence alignment was performed using DNAMAN software. For phylogenetic analysis, an unrooted neighbor-joining (NJ) tree was generated with MEGA 7.0. Bootstrap values from 1000 replicates were indicated at each branch.

■ RESULTS AND DISCUSSION

Quantitative Analysis of Tocopherols and Tocotrienols in *T. grandis*. The α, β, γ, and δ forms of tocopherol and tocotrienol are the main vitamin E components in plants. However, different plants or plant tissues vary enormously in their vitamin E content and vitamin E components.[1] Herein, the content of these major vitamin E components in the kernels of different *T. grandis* cultivars was quantified by HPLC. Using authentic standards, we determined that the

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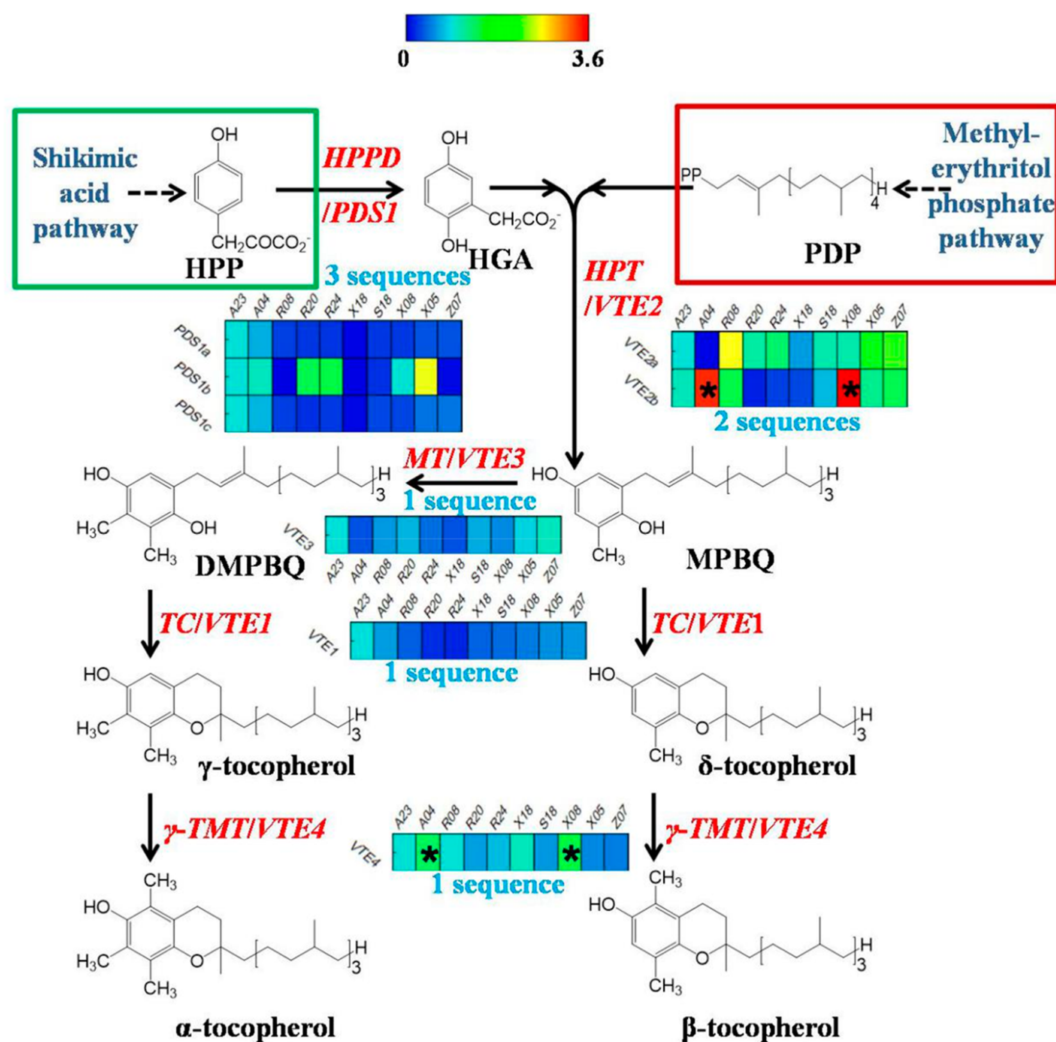

Figure 2. Putative pathways for tocopherol biosynthesis in *T. grandis* and expression levels of the candidate enzyme genes involved in the biosynthesis of tocopherols in different *T. grandis* cultivars. The enzymes found in this study are marked in red. Abbreviations: HPP, *p*-hydroxyphenylpyruvate; HPPD/PDS1, *p*-hydroxyphenylpyruvate dioxygenase; PDP, phytyl diphosphate; HPT/VTE2, homogentisate phytyltransferase; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MT/VTE3, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; TC/VTE1, tocopherol cyclase; and γ-TMT/VTE4, γ-tocopherol methyltransferase. Heatmap depicting the expression profile of the genes. The relative expression ranged from 0 to 3.6.

mature kernels of all of the *T. grandis* cultivars completely lacked tocotrienols and the γ and δ forms of tocopherols (panels B and C of Figure 1). In contrast, the contents of α- and β-tocopherols among the different cultivar mature kernels were varied and abundant, ranging from 0.04 to 4.16 mg/100 g and from 0.23 to 10.20 mg/100 g of oil, respectively (Figure 1C). The total tocopherol content in the *T. grandis* kernel oils ranged from 0.28 to 11.98 mg/100 g. The total tocopherol content in *T. grandis* kernel oils is closed to that in some common edible oils, such as olive, soybean, peanut, corn, sunflower, and canola,[44] and the kernel oils of some *T. grandis* cultivars contained a relatively higher total tocopherol content, indicating that the *T. grandis* kernel oil probably possesses relatively better oxidative stability.

SMRT Sequencing, Similarity Analysis, and Functional Annotation. SMRT sequencing was performed using the PACBIO RS II sequencing platform. A total of 867 864 885 reads were generated. After filtering, the numbers of subreads from the different libraries (1−2, 2−3, and 3−6 kb) were 1 762 438, 2 094 117, and 968 209, respectively (Table S3 of the Supporting Information). After collapse, a total of 97 211 transcripts with a total length of 235 890 123 bp were obtained (Table S4 of the Supporting Information). In comparison to other plants, such as *Salvia miltiorrhiza*[37] and *Triticum aestivum*,[38] for which 223 368 and 197 709 full-length SMRT cDNA reads were respectively generated, *T. grandis* obtained far fewer transcripts from the SMRT sequencing. This might be due to the high proportions of repetitive elements and the numerous pseudo-genes in *T. grandis*.

BLASTx similarity analysis against the Nr NCBI database showed that the *T. grandis* full-length transcripts were similar to a wide range of plant species (Figure S1 of the Supporting Information). Among them, 20 515 (24%) transcripts indicated significant homology with the sequences of *Picea sitchensis*, and 7555 (9%) and 4719 (6%) full-length transcript sequences had high similarity with the sequences of *Amborella trichopoda* and *Nelumbo nucifera*, respectively.

The full-length transcripts were annotated by aligning with those deposited in a variety of protein databases (Nr, Nt, Swiss-Prot, KEGG, COG, GO, and TrEMBL). The best

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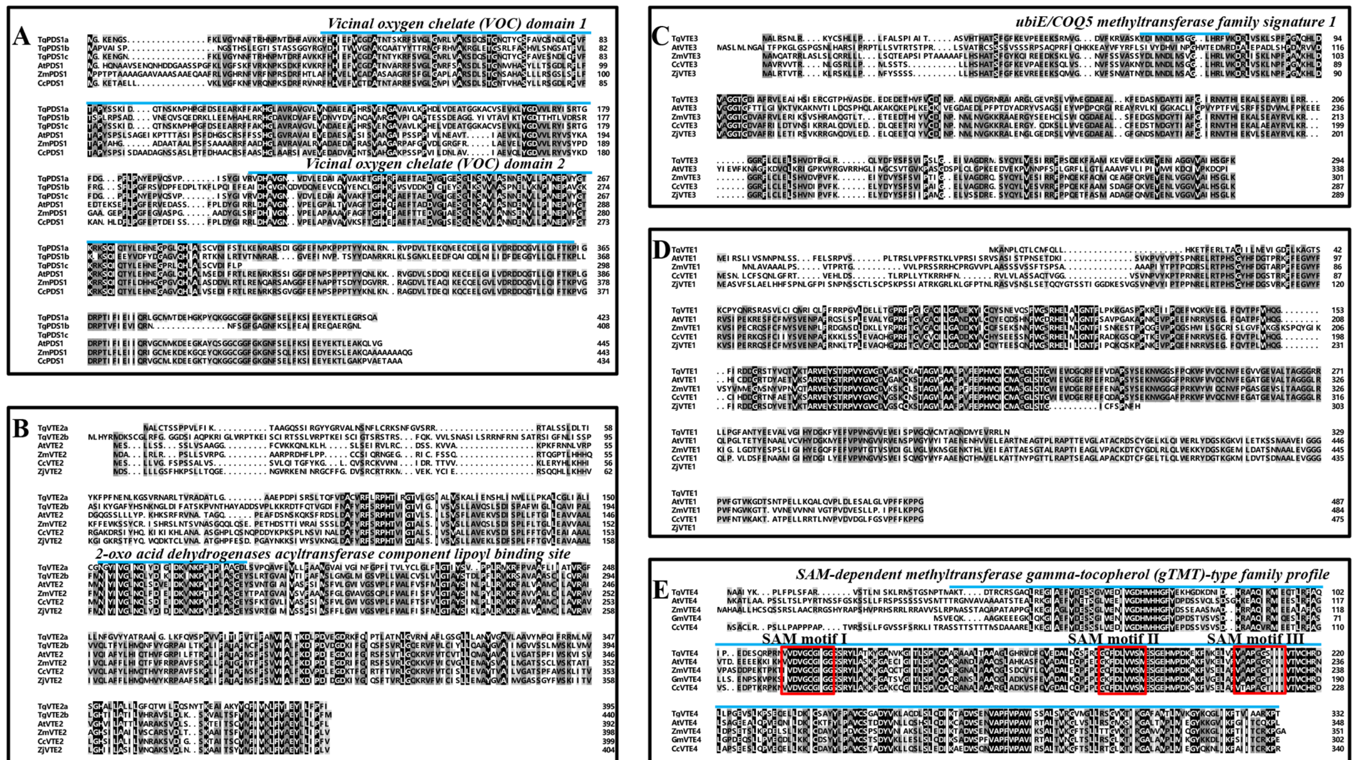

Figure 3. Amino acid sequence alignment of the proteins involved in tocopherol biosynthesis from *T. grandis* and other plants. (A) Alignment of the deduced amino acid sequences of TgPDS1a, TgPDS1b, and TgPDS1c with AtPDS1 (At1g06570), ZmPDS1 (*Zea mays* PDS1, NP_001105782.2), and CcPDS1 (*Citrus clementina* PDS1, XP_006437130.1). The conserved VOC domains are indicated. (B) Alignment of the deduced amino acid sequences of TgVTE2a and TgVTE2b with AtVTE2 (At2g18950), ZmVTE2 (*Z. mays* VTE2, NP_001105877.1), CcVTE2 (*C. clementina* VTE2, XP_024033814.1), and ZjVTE2 (*Ziziphus jujuba* VTE2, XP_024924092.1). The conserved 2-oxo acid dehydrogenases acyltransferase component lipoyl binding site is indicated. (C) Alignment of the deduced amino acid sequences of TgVTE3 with AtVTE3 (At3G63410), ZmVTE3 (*Z. mays* VTE3, XP_008656840.1), CcVTE3 (*C. clementina* VTE3, XP_006453310.1), and ZjVTE3 (*Z. jujuba* VTE3, XP_015885895.1). The conserved ubiE/COQ5 methyltransferase family signature 1 is indicated. (D) Alignment of the deduced amino acid sequences of TgVTE1 with AtVTE1 (At4G32770), ZmVTE1 (*Z. mays* VTE1, PWZ20004.1), CcVTE1 (*C. clementina* VTE1, XP_006450664.1), and ZjVTE1 (*Z. jujuba* VTE1, XP_015870209.1). (E) Alignment of the deduced amino acid sequences of TgVTE4 with AtVTE4 (At1G64970), ZmVTE4 (*Z. mays* VTE4, AGF92707.1), GmVTE4 (*Glycine max* VTE4, NP_001240883.1), and CcVTE4 (*C. clementina* VTE4, XP_006420797.1). The SAM-dependent methyltransferase γ-tocopherol (gTMT)-type family profile and SAM motifs are indicated.

transcript was selected from the matches with an *E* value of less than 10^{-5}. The overall functional annotation is described in Table S5 of the Supporting Information. GO enrichment analysis was carried out to classify the gene functions of the identified full-length transcripts. Among them, the majority of the GO terms (92 946) were assigned to biological process, and 42 094 and 72 869 were assigned to the molecular function and cellular component, respectively (Figure S2A of the Supporting Information). Furthermore, all of the *T. grandis* full-length transcripts were searched against the COG database for functional prediction and classification. Overall, 30 267 full-length transcripts were assigned to the COG classification (Figure S2B of the Supporting Information). The largest group was the cluster for general function prediction (7822).

Identification of Full-Length Transcripts Involved in Tocopherol Biosynthesis from the *T. grandis* Transcriptomes. Because only α- and β-tocopherols were detected in the mature *T. grandis* kernel, we focused on the discovery of genes involved in tocopherol biosynthesis. The precursor of tocopherol synthesis is homogentisate, derived from the shikimate pathway. Through a series of enzymatic reactions, α, β, γ, and δ forms of tocopherols are synthesized.[40] To date, although the biosynthesis pathway of tocopherols has been characterized in other plant species,[45] the pathway in *T. grandis* has not yet been determined. Therefore, we proposed the biosynthesis pathways based on previous reports and the present transcriptome data (Figure 2).

On the basis of the sequence annotation and further manual screening from the NCBI database, we discovered the most likely transcripts encoding known enzymes for tocopherol biosynthesis from our SMRT sequencing data. In this process, three, two, one, one, and one full-length transcripts encoding HPPD/PDS1, HPT1/VTE2, MT/VTE3, TC/VTE1, and γ-TMT/VTE4, respectively, were identified in *T. grandis* (Figure 2).

Characterization of Candidate Genes Encoding Enzymes Involved in Tocopherol Biosynthesis. Using the BLAST and ORFfinder tools in NCBI, the coding regions of PDS1a, PDS1b, PDS1c, VTE2a, VTE2b, VTE3, VTE1, and VTE4 were identified from the sequences of the full-length transcripts. As predicted by PROSITE (https://prosite.expasy.org/prosite.html), each of the TgPDS1a, TgPDS1b, and TgPDS1c protein sequences contain two vicinal oxygen chelate (VOC) domains that are similar to those of AtPDS1, ZmPDS1, and CcPDS1 (Figure 3A). Each of TgVTE2a and TgVTE2b contain a conserved 2-oxo acid dehydrogenase acyltransferase component lipoyl binding site (Figure 3B). An ubiE/COQ5 methyltransferase family signature 1 was

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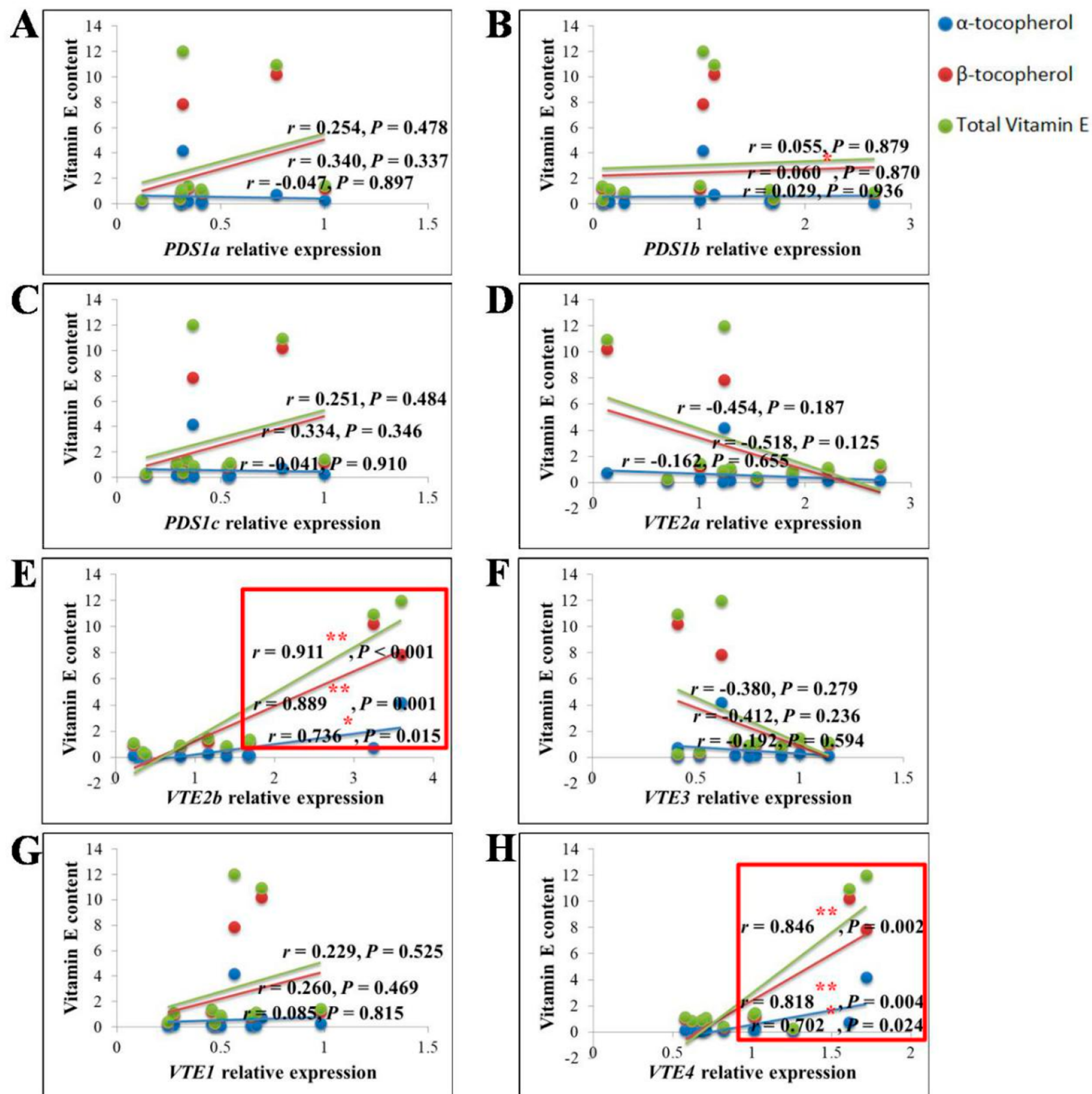

Figure 4. Correlation analysis between vitamin E contents and the expression level of candidate genes involved in the tocopherol biosynthesis pathway in the 10 selected cultivars. *r* represents the Pearson's correlation coefficient.

predicted in TgVTE3 (Figure 3C). The TgVTE1 protein sequence was similar to the AtVTE1, ZmVTE1, CcPDS1, and ZjVTE1 protein sequences (Figure 3D). TgVTE4 contains three highly conserved sterile alpha motifs (SAMs) and shares significant amino acid identity to AtVTE4 (64.7%), ZmVTE4 (72.5%), GmVTE4 (58.1%), and CcVTE4 (59.2%) (Figure 3C). To characterize the evolutionary relationships between the tocopherol synthesis pathway enzymes from *T. grandis* and known tocopherol synthesis pathway enzymes from other plant species, a NJ tree was constructed. As shown in Figure S3 of the Supporting Information, TgPDS1a and TgPDS1c had high homology with PpPDS1, GmPDS1, and AtPDS1, while TgPDS1b had high homology with ZjPDS1. TgVTE2a and TgVTE2b were most closely clustered with OsVTE2 from rice and ZjVTE2 from jujube, respectively. TgVTE3 was most closely clustered with GmVTE3 and ZjVTE3. TgVTE1 was most closely clustered with PaVTE1 and PpVTE1. TgVTE4 had high homology with SbVTE4 and ZmVTE4. Phylogenetic analysis showed that known tocopherol synthesis pathway enzymes from different plants did not cluster into the same clades, and some of them were only loosely related (Figure S3 of the Supporting Information), suggesting that they have deep evolutionary histories and may have distinct roles other than in tocopherol synthesis. There are few reports on the roles of tocopherol synthesis enzymes with regard to other functions. One study found that the overexpression of VTE1 led to increased tolerance against salt stress in rice and drought stress in tobacco plants.[46,47] However, no research on tocopherol synthesis enzymes has been reported in *T. grandis*.

The expression levels of all of the identified full-length transcripts encoding enzymes involved in tocopherol biosynthesis were detected by qRT-PCR in different *T. grandis* cultivar kernels. The qRT-PCR data are displayed as a heatmap (Figure 2). Our data revealed that *PDS1a* and *PDS1c* have similar expression patterns. *PDS1a*, *PDS1c*, *VTE3*, and *VTE1* exhibit only slightly expression changes among the different *T. grandis* cultivars. *PDS1b* and *VTE2a* were most abundantly expressed in the X05 and R08 cultivars, respectively. Interestingly, the highest expression levels of *VTE2b* and *VTE4* were both presented in the A04 and X08 cultivars, in

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which the contents of α- and β-tocopherols were much higher than those of the other cultivars (Figure 1C). To further investigate the correlation between the gene expression level and vitamin E components, Pearson's correlations were calculated. The results are shown in Figure 4, and the gene expression levels are shown in Figure 2 and Figure S4 of the Supporting Information. *VTE2b* expression was positively and significantly correlated with α-tocopherol, β-tocopherol, and total vitamin E contents, with r of 0.911 ($p < 0.001$), r of 0.889 ($p = 0.001$), and r of 0.736 ($p < 0.015$), respectively. In addition, a significant correlation between the *VTE4* expression and vitamin E component was also found in this study, with an r range of 0.702−0.846 ($p < 0.05$). These results suggest that VTE2b and VTE4 are key factors limiting tocopherol synthesis in *T. grandis* kernels, which corroborates previous studies.[19] To further support the proposed thesis, we measured the tocopherol contents of kernels harvested from five kernel developmental stages of the X08 cultivar (Figure S5 of the Supporting Information) and performed the correlation analysis between tocopherol contents and the expression of tocopherol biosynthesis pathway genes in the five kernel developmental stages of the X08 cultivar (Table S6 of the Supporting Information). The results also showed that both *VTE2b* and *VTE4* expressions were positively and significantly correlated with α- and β-tocopherols. In *Arabidopsis*, *VTE2* and *VTE4* are also limiting enzymes for tocopherol synthesis, because the overexpression of *VTE2* in *Arabidopsis* resulted in a significant increase in VTE2-specific activity and total tocopherol content in the leaves and seeds and γ-tocopherol was almost entirely converted to α-tocopherol when *VTE2*-overexpressing plants were crossed with lines constitutively overexpressing *VTE4*.[19] In our study, it is obvious that *VTE1* was not limiting in tocopherol synthesis in *T. grandis*, which is in accordance with the report of Collakova and DellaPenna on *Arabidopsis*.[19] Hwang et al. also found that OsVTE2 plays key roles in tocopherol accumulation in rice.[48] However, it was reported that TC/VTE1 is significant in tocopherol accumulation in soybean seeds.[24] The content, composition, and presence of tocopherols varies widely in different plants or plant tissues. The most abundant form in the leaves is α-tocopherol, whereas the dominant tocopherol form in the seeds is γ-tocopherol.[49,50] Nevertheless, in some crops, for instance, sunflower, olive, safflower, wild *Euphorbia*, or grape, α-tocopherol is the primary tocopherol form in the seeds.[51−55] Therefore, different plants or different plant tissues may have different regulatory mechanisms for tocopherol biosynthesis.

As shown above, the mature *T. grandis* kernels lacked tocotrienols and γ and δ forms of tocopherols but were abundant in α- and β-tocopherols (Figure 1). However, α- and β-tocopherols are derived from γ- and δ-tocopherols via the methylation of γ- and δ-tocopherols using VTE4, respectively.[56] In transgenic *Arabidopsis*, soybean, and lettuce, the increased expression of *VTE4* results in a massive conversion of γ-tocopherol to α-tocopherol.[15,56,57] These data indicate that the low α-tocopherol level in wild-type tissues results from low *VTE4* expression in these specific organs. This conclusion was also supported by the characterization of naturally occurring sunflower mutants.[58] The corresponding mutations greatly reduced or disrupted the expression of the two sunflower *VTE4* paralogues in developing kernels, thus confirming that *VTE4* expression in sunflower seed directly results in α-tocopherol accumulation. Therefore, we hypothesized that *VTE4* expression was upregulated and/or some *VTE4* upstream genes were downregulated during *T. grandis* kernel development, especially during the mature stage, which resulted in γ- and δ-tocopherols being almost entirely converted to α- and β-tocopherols. To examine this hypothesis, we conducted qRT-PCR experiments to detect gene expression during *T. grandis* kernel development of the X08 cultivar. As expected, *VTE4* expression was upregulated (Figure 5H) about 2.8-fold, while the expression of *PDS1b*

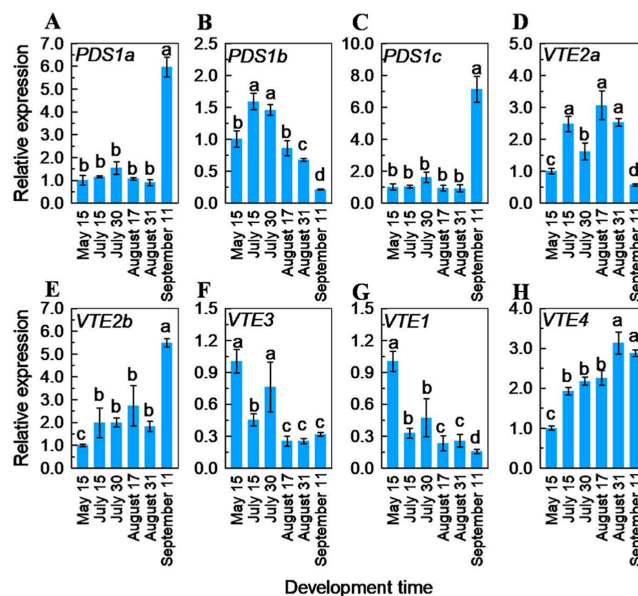

Figure 5. Expression profiles of tocopherol biosynthesis pathway genes in X08 kernels: (A) *PDS1a*, (B) *PDS1b*, (C) *PDS1c*, (D) *VTE2a*, (E) *VTE2b*, (F) *VTE3*, (G) *VTE1*, and (H) *VTE4*. The mRNA levels were quantified by qPCR using the total RNAs from six kernel developmental stages in the X08 *T. grandis* cultivar. Different letters indicate a significant difference in the mRNA level ($p < 0.05$).

(Figure 5B), *VTE1* (Figure 5G), and *VTE3* (Figure 5F) was downregulated by approximately 5.2-, 6.1-, and 3.3-fold, respectively, in the kernels harvested from May 15 to September 11 (Figure 5). During *T. grandis* kernel development, *PDS1a* (Figure 5A), *PDS1c* (Figure 5C), and *VTE2b* (Figure 5E) exhibited slight expressional changes until the mature stage, after which the expression abundance increased significantly and was upregulated approximately 5.9-, 7.2-, and 5.4-fold in the kernels harvested from May 15 to September 11. The expression of *VTE2a* was upregulated from 1.6- to 3.1-fold at the early development stage and was downregulated about 2.0-fold at the mature stage compared to the expression of the kernels harvested on May 15th (Figure 5D). Our study showed that, in the mature stage, γ- and δ-tocopherols were almost entirely converted to α- and β-tocopherols in all non-transgenic *T. grandis* cultivars as a result of the relatively high *VTE4* expression in this stage, suggesting that there is a special regulatory mechanism for tocopherol biosynthesis in *T. grandis*.

The biosynthesis of tocopherols occurs in the plastids of plant cells, and previous studies have identified the plastid localization of tocopherol biosynthesis-related enzymes.[12,14−16,57,59] To further identify and characterize the enzymes involved in tocopherol biosynthesis, we selected key enzymes, VTE2b and VTE4, identified above to gain insight into their localization. The coding sequences for N-terminal 79

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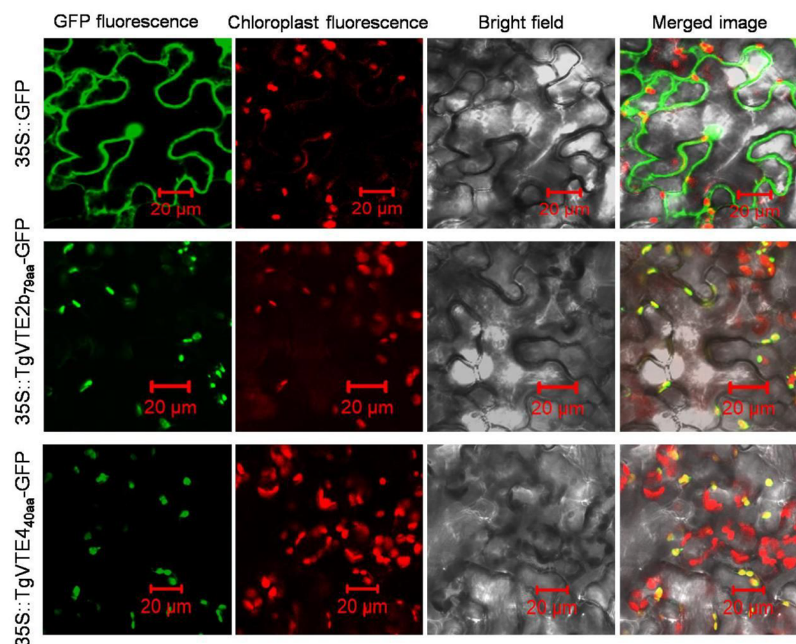

Figure 6. Subcellular localization of the N-terminal amino acid sequences of TgVTE2b and VTE4 containing plastid transit peptides. Subcellular localization studies were conducted with the translational fusions of the N-terminal 79 amino acids of TgVTE2b (upper panels) and the N-terminal 40 amino acids of TgVTE4 (middle panels) with GFP as well as with the GFP control vector (lower panels). The coding sequences for these fusion proteins were transiently expressed in the leaves of *N. benthamiana*. Proteins were localized with confocal fluorescence microscopy. Scale bars = 20 μm.

amino acids from VTE2b and for the N-terminal 40 amino acids from VTE4 were linked to the 5′ end of the GFP coding sequence, respectively. These sequences were chosen in part because they are predicted to encode a plastid transit peptide based on *in silico* analysis using the ChloroP version 1.1 server (http://www.cbs.dtu.dk/services/ChloroP/). Next, we used the fusion constructs under the control of the CaMV 35S promoter for transient expression in *N. benthamiana* leaves and to examine whether the GFP signal is co-localized with the chloroplast autofluorescence. The result showed that the expression of both genes resulted in the co-localization of GFP with chlorophyll fluorescence in the chloroplasts (Figure 6). This result is consistent with the subcellular localization of *Arabidopsis* VTE2 and VTE4.[59−61] In contrast, the GFP protein alone was present in both the cytoplasm and nuclei (Figure 6). These results further suggested that *T. grandis* VTE2b and VTE4, similar to *Arabidopsis* VTE2 and VTE4, function as homogentisate phytyltransferase and γ-tocopherol methyltransferase, respectively.

Identification of the Regulatory Genes Potentially Controlling Tocopherol Biosynthesis. To date, little is known regarding the regulatory genes controlling tocopherol biosynthesis. Although previous studies have attempted to elucidate some regulatory genes controlling tocopherol biosynthesis in several plants through quantitative trait locus (QTL) and genome-wide association (GWA) studies,[58,62−68] few loci controlled the tocopherol composition that have been mapped, while these loci could not control tocopherol synthesis. Thus, the genes regulating tocopherol synthesis remain largely unknown. Recently, the first two regulatory genes, *AtWRI1* (a member of the APETALA2−ethylene-responsive element binding protein family of transcription factors) and *AtDGAT1* (acyl-CoA:diacylglycerol acyltransferase 1), which regulate the synthesis of tocopherols in *Arabidopsis* seeds, were identified. In the *wri1* and *dgat1* mutants, tocopherols, γ-tocomonoenol, and PC-8 were overaccumulated.[69] In the transcriptomic data of *T. grandis*, we identified 7 full-length transcripts encoding the APETALA2−ethylene-responsive element binding protein family of transcription factors and 19 full-length transcripts encoding diacylglycerol acyltransferase. Their phylogenetic relationships are shown in Figure 7. BRD TGR81967 and BRD TGR82194 were most closely clustered with AtWRI1 and AtDGAT1, respectively, suggesting that they might be involved in the regulation of tocopherol biosynthesis in *T. grandis*. We named BRD TGR81967 and BRD TGR82194 as *TgWRI1* and *TgDGAT1*, respectively.

To further identify and characterize the regulatory genes, the expression profiling of *TgWRI1* and *TgDGAT1* in the 10 selected cultivars and different kernel developmental stages of the X08 cultivar was detected (Figure S6 of the Supporting Information). We also analyzed the correlation of the gene expression levels of the two candidate regulatory genes with tocopherol contents and the expression of tocopherol biosynthestic genes (Tables S7−S9 of the Supporting Information). The results showed that the expressions of *TgWRI1* and *TgDGAT1* were positively and significantly correlated with α-tocopherol, β-tocopherol, and the expressions of *TgVTE2b* and *TgVTE4*, respectively. The correlation results further suggested that *TgWRI1* and *TgDGAT1* are regulators involved in tocopherol biosynthesis. In addition, the subcellular localization of TgWRI1 and TgDGAT1 was also investigated (Figure S7 of the Supporting Information). The results showed that TgWRI1 and TgDGAT1 were localized to the nucleus and chloroplasts, respectively. TgWRI1 may be a transcription factor regulating the expressions of *TgVTE2b* and *TgVTE4*. TgDGAT1 may be a regulator of TgWRI1 and TgDGAT1 in chloroplasts. Characterizing the functions of these full-length transcripts and the regulatory mechanism of tocopherol biosynthesis requires further investigations.

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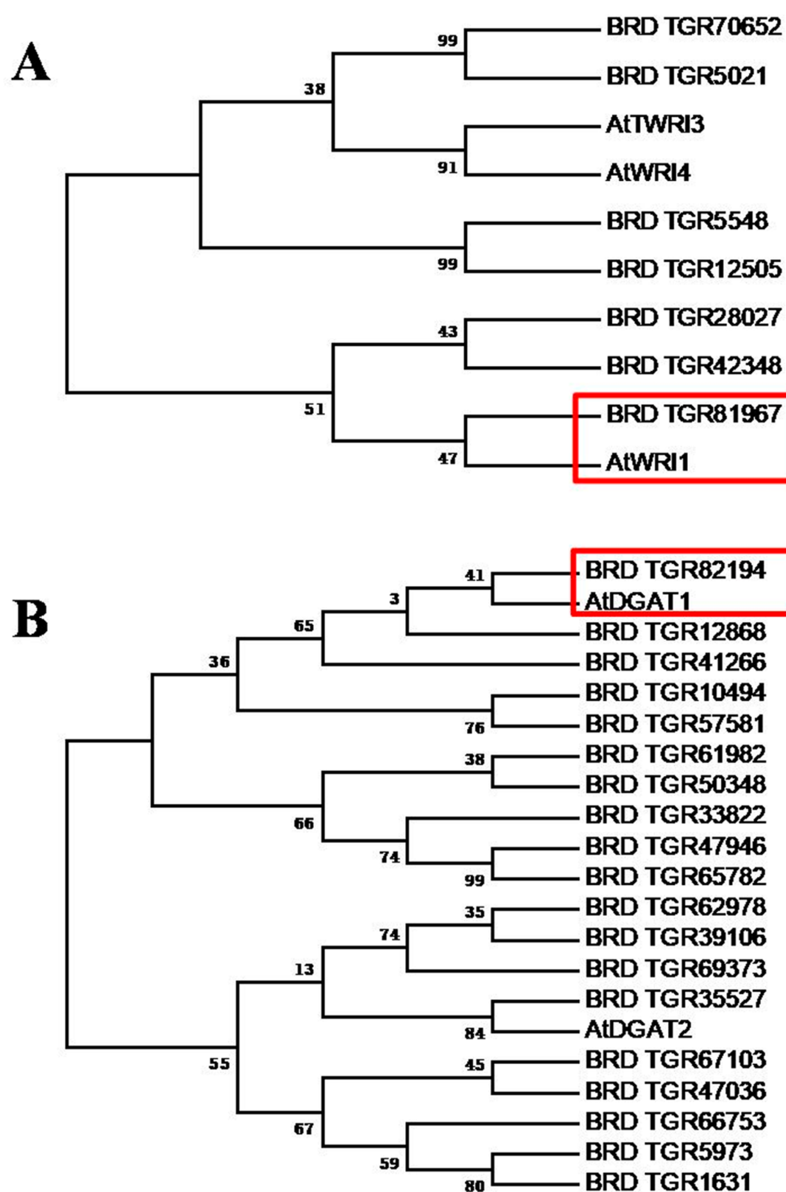

Figure 7. Phylogenetic analysis of the deduced regulatory proteins potentially controlling tocopherol biosynthesis from *T. grandis* and known regulatory proteins controlling tocopherol biosynthesis from *Arabidopsis*. (A) Phylogram of APETALA2−ethylene-responsive element binding protein family of transcription factors from *T. grandis* and *Arabidopsis*. The TAIR accession numbers of *AtWRI1*, *AtWRI3*, and *AtWRI4* are At3G54320, At1G16060, and At1G79700, respectively. (B) Phylogram of Acyl-CoA:diacylglycerol acyltransferase from *T. grandis* and *Arabidopsis*. The TAIR accession numbers of *AtDGAT1* and *AtDGAT2* are At2G19450 and At3G51520, respectively.

In summary, we first carried out full-length transcriptomic analysis of *T. grandis* using SMRT sequencing and obtained a total of 97 211 full-length transcripts, which provide valuable genetic resources for this multifunctional Chinese food. We further proposed the tocopherol biosynthetic pathway in *T. grandis*. Eight full-length transcripts were identified as involved in the tocopherol biosynthesis pathway in the kernels, which encode five protein families, namely, PDS1, VTE2, VTE3, VTE1, and VTE4. We detected the expression levels of the candidate genes, and the results showed that the expression levels of *TgVTE2b* and *TgVTE4* were significantly higher in the kernels of the high-tocopherol cultivars than the low-tocopherol cultivars, indicating that they might have a significant role in tocopherol accumulation in the kernels of *T. grandis*. In addition, the roles of TgVTE2b and TgVTE4 were further supported by their plastid localization. At the mature stage, only α- and β-tocopherols were detected in the *T. grandis* kernels, which differed from the results from other plants, suggesting that a unique regulatory mechanism of tocopherol biosynthesis in *T. grandis* might exist. In addition, the identification and phylogenetic analysis of the regulatory genes potentially involved in tocopherol biosynthesis regulation are of great importance for revealing the molecular basis of tocopherol biosynthesis pathways.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06138.

Species distribution of the top BLAST hits in the Nr database (Figure S1), classification of full-length transcripts in *T. grandis* (Figure S2), phylogenetic trees of

enzyme proteins involved in tocopherol biosynthesis (Figure S3), expression profiles of tocopherol biosynthesis pathway genes in the selected 10 cultivar kernels at the mature stage (Figure S4), tocopherol contents of kernels in different kernel developmental stages of the X08 cultivar (Figure S5), expression profiles of *TgWRI1* and *TgDGAT1* in the seleted 10 cultivar kernels at the mature stage and different kernel developmental stages of the X08 cultivar (Figure S6), subcellular localization of TgWRI1 and TgDGAT1 (Figure S7), cultivation localities of the 10 *T. grandis* cultivars selected in this study (Table S1), primers used in this study (Table S2), summary of the transcriptome sequencing for *T. grandis* (Table S3), summary of the transcripts for *T. grandis* (Table S4), functional annotation of the *T. grandis* transcriptome (Table S5), correlation analysis between tocopherol contents and the expression level of candidate genes involved in the tocopherol biosynthesis pathway in the different kernel developmental stages of the X08 cultivar (Table S6), correlation analysis between tocopherol contents and the expression levels of *TgWRI1* and *TgDGAT1* in the 10 selected cultivars (Table S7), correlation analysis between tocopherol contents and the expression levels of *TgWRI1* and *TgDGAT1* in the different kernel developmental stages of the X08 cultivar (Table S8), and correlation of the two candidate regulatory genes (*TgWRI1* and *TgDGAT1*) with the expression of tocopherol biosynthetic genes (Table S9) (PDF)

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Notes

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ABBREVIATIONS USED

HPPD/PDS1, *p*-hydroxyphenylpyruvate dioxygenase; HPT1/VTE2, homogentisate phytyltransferase; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MT/VTE3, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase; TC/VTE1, tocopherol cyclase; γ-TMT/VTE4, γ-tocopherol methyltransferase

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