

Cloning and polymorphism analysis of the 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase gene (*VTE3*) in *Arachis hypogaea*, *A. duranensis*, and *A. ipaënsis*

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ABSTRACT. One of the critical enzymes involved in vitamin E biosynthesis in plants is 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (MPBQ MT). The full-length *VTE3* cDNA (designated *rVTE3-1* and -2) encoding MPBQ MT and the full-length DNA of *VTE3* (designated *gVTE3-1* and -2) were isolated from cultivated peanuts (*Arachis hypogaea*). The full-length DNA of *VTE3* (designated *gVTE3-A* and -*B*) was isolated from the wild groundnut species *A. duranensis* (A-genome) and *A. ipaënsis* (B-genome), and polymorphism analysis of *VTE3* was performed. The results demonstrated that *rVTE3-1* and -2 both have a DNA sequence that is 1059 bp long and encodes 351 amino acids; the homology of the 2 amino acid sequences was 98.6%. The *gVTE3-1* of cultivated Fenghua 2 peanut samples was 2710 bp long, with 3 introns located at 44-163, 772-1295, and 1603-2437 bp, and the Fenghua 2 *gVTE3-2* was 2706 bp long, with 3 introns located at

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44-169, 778-1291, and 1599-2433 bp. The homology for gVTE3-1 and -2 across 13 cultivated peanut samples was 99.9 and 100%, respectively. gVTE3-1 and -2 were from A- and B-genome, respectively, with 96.6% homology between the 2 sequences. The present study demonstrated that abundant polymorphisms were present in the VTE3 genes from different genomes. Additionally, polymorphisms were observed in the gVTE3-1 alleles of the 13 cultivars and wild *A. duranensis* species but not in the gVTE3-2 alleles of the 13 cultivars and wild *A. ipaënsis* species.

Key words: Gene cloning; *Arachis* sections; Vitamin E; 2-Methyl-6-phytyl-1,4-benzoquinone methyltransferase

INTRODUCTION

The antioxidant vitamin E plays an important physiological role in plants, animals, and human beings. There are 8 different forms of vitamin E (4 tocopherols: α , β , γ , and δ ; 4 tocotrienols: α , β , γ , and δ), and it occurs mainly in the form of tocopherols in dicotyledonous plants (Rimbach et al., 2002; DellaPenna and Pogson, 2006). Different types of tocopherols have dissimilar activities. Among the 4 tocopherols, α -tocopherol has the highest activity, whereas β -, γ -, and δ -tocopherols achieve only 50, 10, and 3% of the activity level of α -tocopherol, respectively. Given that humans and animals both possess significant levels of proteins with a high affinity for α -tocopherol, α -tocopherol absorption and utilization is more significant than that of β -, γ -, and δ -tocopherols in these organisms (Brigelius-Flohe and Traber, 1999).

Natural vitamin E can only be synthesized by photosynthetic bacteria and green plants, and humans and animals depend solely on their dietary intake for vitamin E (Cheng et al., 2003). Plant tocopherols exist primarily in non-green plant tissues, such as the oil of many crop seeds; however, high-activity α -tocopherol has a much lower proportion of tocopherols when compared to low-activity tocopherols (Grusak and DellaPenna, 1999). As one of the critical enzymes involved in plant vitamin E biosynthesis, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (MPBQ MT) can catalyze MPBQ to generate 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ). MPBQ and DMPBQ, however, are cyclized by tocopherol cyclase to yield δ - and γ -tocopherol, respectively, which are subsequently further converted to β - and α -tocopherol by γ -tocopherol methyltransferase (γ -TMT) (Shintani and DellaPenna, 1998; Porfirova et al., 2002; Shintani et al., 2002).

The *Arabidopsis* genes *VTE3* and *VTE4*, which encode MPBQ MT and γ -TMT, were transferred to soybeans together, and a significant increase of 85% was observed in α -tocopherol expression in soybean seeds (Van Eenennaam et al., 2003). The *Arabidopsis* genes hydroxyphenylpyruvate dioxygenase and *VTE3* were transferred to maize together, and a 3-fold increase in vitamin E expression was observed in corn grain (Naqvi et al., 2011). Peanuts are one of the most important commercial oil crops. Some key enzymes (such as MPBQ MT, tocopherol cyclase, and γ -TMT) can affect vitamin E biosynthesis; thus, they can be used to increase the vitamin E content and proportion of α -tocopherol in peanut-based oil crops by improving expression of the enzyme gene through the transference to peanut.

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Studies on VTE3 of peanut

The genus *Arachis* originated from South America and contains 80 documented species assembled into 9 taxonomic groups according to morphology, geographic distribution, and sexual compatibility (Moretzsohn et al., 2009). The *Arachis* section is the largest, comprising 29 wild diploid species and the tetraploid species *A. hypogaea* and *A. monticola* (Moretzsohn et al., 2009). The cultivated peanut (*A. hypogaea*) is an allotetraploid (2n = 4x = 40) with 2 genome types (A- and B-genome), and thus can be considered to have originated from 2 distinct wild diploid species of *Arachis* (Kochert et al., 1996). To date, most researchers support the hypothesis that the cultivated peanut originated via hybridization of *A. duranensis* (A-genome) and *A. ipaënsis* (B-genome), followed by a rare spontaneous duplication of chromosomes (Halward et al., 1991; Kochert et al., 1996; Fávero et al., 2006; Seijo et al., 2004, 2007). According to its characteristics, the cultivated peanut is divided into the subspecies *A. hypogaea* ssp *fastigiata* and *A. hypogaea* ssp *fastigiata* var. *fastigiata* and *A. hypogaea* and *A. hypogaea* ssp *fastigiata* var. *fastigiata* and *A. hypogaea* and *A. hypogaea* ssp *fastigiata* var. *fastigiata* and *A. hypogaea* and *A. hypogaea* ssp *fastigiata* var. *fusua*). Peanut cultivars are classified into Runner, Virginia, Spanish, and Valencia market types (Hong et al., 2010).

Analysis of random amplified polymorphic DNA and inter-simple sequence repeat markers on peanut cultivars has revealed a low level of polymorphisms and a narrow genetic base, suggesting that variant cultivars share the same wild ancestors (Raina et al., 2001). Studies using amplified fragment length polymorphism (Milla et al., 2005) and fluorescence in situ hybridization (Seijo et al., 2004) methods confirmed that A. hypogaea variant cultivars share the same ancestral origin, and the present study concluded that A. duranensis and A. *ipaënsis* are likely to be the ancestors of contemporary cultivated species. Singh (1988) synthesized amphidiploid peanuts, hybridized them with different cultivars, and studied the cell morphology and chromosome synapsis. Analysis of isozymes in different cultivars (Lu and Pickersgill, 1993) demonstrated that differences exist between the 2 peanut subspecies. Both studies (Singh, 1988; Lu and Pickersgill, 1993) indicated that the 2 cultivated subspecies of peanuts possess distinct and independent evolutionary systems. While most researchers believe that different types of cultivars share similar ancestry, these findings confirm the suggestion put forth by several contemporary researchers that the 2 subspecies have independent evolutionary systems. By comparing homologous genes, the common ancestry of different peanut subspecies can be inferred at the molecular level.

To our knowledge, although VTE3 plays an important role in vitamin E biosynthesis in peanuts, little is known about the conservation and diversity of VTE3 genes in cultivars and wild species. In addition, and most importantly, the wild ancestry of various peanut cultivars is questionable, and different researchers have reached diverse conclusions regarding this issue. In the present study, we expect to reveal the molecular characteristics of VTE3 and investigate peanut VTE3 polymorphism by cloning VTE3 genes from cultivated and wild peanut species. The present study not only provides new evidence for the determination of the ancestral origin of different peanut subspecies, but also lays the foundation for enhancing vitamin E production in plants, especially in commercial peanut crops, using genetic engineering.

MATERIAL AND METHODS

Material

The resources for VTE3 collection and analysis were selected from different plant

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material genotypes, comprising 13 distinct peanut cultivars (Table 1) and 2 wild species, i.e., *A. duranensis* and *A. ipaënsis*.

In silico cloning of peanut VTE3

To predict the full-length cDNA sequence of VTE3 in peanuts, Arabidopsis thaliana VTE3 gene sequences were obtained from the National Center for Biotechnology (NCBI) database (accession No. NM 116206.3) and used as an information probe against the peanut database for expressed sequence tags (dbEST) by the Basic Local Alignment Search Tool (BLAST) analysis to retrieve homologous ESTs, resulting in a single homologous sequence to be recorded and used as the seed sequence (accession No. ES764300.1). Next, the determined seed sequence was used in BLAST against the same dbEST, revealing partially overlapping ESTs, and these sequences were assembled into a longer new sequence. Subsequently, the above mentioned 2 steps were repeated until the new sequence could not be extended further. Finally, with an open reading frame (ORF) finder programming application, the final assembled sequence was established and its encoding amino acid sequence was determined. These results were compared with the corresponding A. thaliana VTE3 nucleotide and amino acid sequences documented previously. Based on the computer-simulated cloned sequence of peanut VTE3, the primer sequences F1 (5'-TTCGTGCTCCTCTCTCATCC-3') and R1 (5'-TGACGTTGGGCAAACTGTAT-3') were designed. These primers were synthesized by Shanghai Biological Engineering Co. Ltd. (China).

DNA extraction of cultivated and wild peanut species

Young leaves were collected from the cultivated and wild peanut species, and subsequent total DNA extraction was performed using the hexadecylmethylammonium bromide method (Rogers and Bendich, 1985).

RNA extraction and cDNA synthesis

Thirty-five days after being pegged into the soil, young peanut pods were collected and preserved by freezing in liquid nitrogen (-70°C) for subsequent RNA extraction. The extraction method used was the TRIzol Reagent Kit (Beijing TransGen Biotech Co. Ltd., China). Using the EasyScript First-Strand cDNA Synthesis Kit (Beijing TransGen Biotech Co. Ltd.), RNA sequences were reverse-transcribed to generate cDNA.

PCR amplification of VTE3 from cultivated and wild peanut species

VTE3 cDNA transcribed from RNA sequences collected from the Fenghua 2, Lanna 1, and Lipudahuasheng cultivars were PCR-amplified using the F1 and R1 primers. The amplification process was as follows: 94°C for 5 min; 94°C for 30 s; 55°C for 30 s; 72°C for 1.5 min; 35 cycles; and 72°C for 10 min. *VTE3* DNA from the 13 cultivars and 2 wild species genomes were PCR-amplified using the same F1 and R1 primers. The amplification protocol was as follows: 94°C for 5 min; 94°C for 30 s; 72°C for 2.5 min; 35 cycles; and 72°C for 10 min.

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PCR product cloning and sequencing

A TIANgelTM Gel Extraction Kit (Beijing TransGen Biotech Co. Ltd.) was used to recycle PCR products and link to the cloning vector pEASY-T1 (Beijing TransGen Biotech Co. Ltd.). The complex was then transferred into *Escherichia coli* DH5 α via overnight culture on the plates and identified by colony PCR. Finally, target fragments were sent to the Beijing Genomics Institute for sequencing. To guarantee experimental reproducibility, between 6 and 10 clones from each species were selected for sequencing.

Analysis of *VTE3* DNA and amino acid sequences in cultivated and wild peanut species

Sequence homology was analyzed by BLAST search and the DNAMAN software. ClustalX and ClustalW were used to align the DNA sequences as well as the amino acid sequences, and the PHYLIP software was used to construct a *VTE3* phylogenetic tree based on this alignment. To investigate *VTE3* in a comprehensive manner, bioinformatic analyses were performed as follows: various physicochemical properties of *VTE3*, including molecular weight and protein theoretical isoelectric point (pI), were assessed by ProtParam (http://www.expasy.ch/tools/protparam.html); transmembrane helices in VTE3 proteins were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/); the VTE3 protein secondary structure was analyzed by the SOPMA software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat. pl?page=/NPSA/npsa_sopma.html); the subcellular localization of VTE3 proteins was predicted by WoLF PSORT (http://wolfpsort.org/); and finally, conserved domains in VTE3 proteins were searched by the CDD software (http://www.ncbi.nlm.nih.gov/Structure/cdd/ wrpsb.cgi).

RESULTS

Cloning of cultivated and wild peanut species VTE3

The full-length of the assembled EST (1396 bp) in peanuts has an ORF (1056 bp) and 71.8% homology with the *A. thaliana VTE3* coding region, and the homology of the corresponding amino acid sequences between the 2 species was approximately 77%. The results of this study therefore suggest that the assembled sequence is highly likely to be peanut *VTE3*.

We obtained 1.2-kb fragments by PCR amplification of reverse-transcribed cDNA with the F1 and R1 primers; the fragments were linked to pEASY-T1 vectors for sequencing. The sequencing results revealed 2 different sequences from the products of 3 cultivars, which were designated as rVTE3-1 and -2 (Table 1). PCR amplification of extracted DNA using primers F1 and R1 produced 2.7-kb fragments that were linked to pEASY-T1 vectors for sequencing. The sequencing results revealed 2 different sequences from the DNA amplification products of 13 cultivars; the sequences were designated as gVTE3-1 and -2 (Table 1). PCR amplification of extracted DNA using primers F1 and R1 revealed that the other 2 sequences were produced from *A. duranensis* and *A. ipaënsis*, respectively, and the sequences were designated as gVTE3-A and -B.

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Subspecies	Varieties	Accessions	DNA sequence symbol	cDNA sequence symbol
fastigiata	var. vulgaris	Fenghua 2	FhgVTE3-1, FhgVTE3-2	FhrVTE3-1, FhrVTE3-2
fastigiata	var. vulgaris	Shanhua 8	Sh8gVTE3-1, Sh8gVTE3-2	-
fastigiata	var. vulgaris	Yuanza 9102	YzgVTE3-1, YzgVTE3-2	-
fastigiata	var. fastigiata	Krapt.st.16	KtgVTE3-1, KtgVTE3-2	-
fastigiata	var. fastigiata	ICG6848	IggVTE3-1, IggVTE3-2	-
fastigiata	irregular type	Shanhua 7	Sh7gVTE3-1, Sh7gVTE3-2	-
fastigiata	irregular type	Shanhua 9	Sh9gVTE3-1, Sh9gVTE3-2	-
hypogaea	var. hypogaea	Lanna 1	LngVTE3-1, LngVTE3-2	LnrVTE3-1, LnrVTE3-2
hypogaea	var. hypogaea	Nongda 818	NdgVTE3-1, NdgVTE3-2	-
hypogaea	var. hypogaea	ICGV86699	IvgVTE3-1, IvgVTE3-2	-
hypogaea	var. hypogaea	E1	ElgVTE3-1, ElgVTE3-2	-
hypogaea	var. hirsuta	Lipudahuasheng	LpgVTE3-1, LpgVTE3-2	LprVTE3-1, LprVTE3-2
hypogaea	var. hirsuta	Rugaoxiyangsheng	RggVTE3-1, RggVTE3-2	-

Fenghua 2 VTE3 nucleotide sequence analysis

The *FhrVTE3-1* and -2 sequences shared 99.0% homology, and the 2 sequences shared 98.7 and 97.7% homology, respectively, with the assembled sequences produced by the *in silico* cloning. Both sequences had ORFs of the same length encoding 351 amino acids; the start and stop codons were located at 59 and 1114 bp, respectively. The sequence near the ATG start codon adhered to the Kozak rule (AXXATGG), and a polyadenylation signal (AATAAA) was located 25 bp downstream from the stop codon (Figure 1). *FhrVTE3-1* and -2 both had an encoding length of 1059 bp, the homology of the 2 sequences was 97.8%, and 8 single nucleotide polymorphism (SNP) sites existed within the sequences. The protein sequences shared 98.6% homology with 5 amino acid differences.

The *FhgVTE3-1* and *-2* sequences shared 96.6% homology. The DNA sequences of *FhgVTE3-1* were 2710 bp long, having 3 introns located at 44-163, 772-1295, and 1603-2437 bp with lengths of 120, 524, and 835 bp, respectively. The DNA sequences of *FhgVTE3-2* were 2706 bp long, having 3 introns located at 44-169, 778-1291, and 1599-2433 bp with lengths of 126, 514, and 835 bp, respectively. In addition, the intron-splicing pattern adhered to the GA-AG rule (Figure 1).

Analysis of the *FhgVTE3-1* and -2 sequences demonstrated that the 3 introns possessed 3, 15, and 18 SNPs (Figure 1), and the full-length sequences had 56 variable sites, of which 44 were SNPs, accounting for 79% of introns.

Analysis of restriction enzyme sites in *FhgVTE3-1* and -2 by DNAMAN indicated that *FhgVTE3-2* possessed 3 restriction enzyme recognition sites (*PacI*, *AftII*, and *BgIII*). In contrast, none of the 3 restriction sites was found in *FhgVTE3-1* (Figure 2).

VTE3 nucleotide sequence analysis in different cultivars

Sequence alignment analysis revealed 100% homology of the *FhrVTE3-1*, *LnrVTE3-1*, and *LprVTE3-1* sequences (Table 1), as well as 100% homology of the *FhrVTE3-2*, *LnrVTE3-2*, and *LprVTE3-2* sequences (Table 1).

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Fh-1 Fh-2	TICOIGCICCICICICACCACCATCITICICACGCCAAAAAGGOTTTTCCCCCCCGTTTCTTAATTTAAT	70 66
Fh-1 Fh-2	TTAATTTAATTTAATTATTATTTTTCTGCTTCAAGTCTTCAACTACACTTCACATTTTCCARTTGATTTC	140 134
Fh-1 Fh-2	ANTTTCAGTCTTTGCAATTTCAG <u>GTTCCTTCTTCAACAANGGCTTCATCAATGTTCAG</u> - <mark>C</mark> CCACTTCAATTT-	198 204
Fh-1 Fh-2	TGGAACCGAAAGCCTCACACTTTTCAGTAGCAAAACCCCCGAACGGTTTGTG@TTCAATGCTTCCAWTTTT 	268 274
Fh-1 Fh-2	CACTETAAAAGAATAGGTTTEAATTETAGCACTACTAATTTEAATTTEAGTAATAAAGETAGGGTEGTE	338 344
Fh-1 Fh-2	ATAA®AGTAATAGGATIGITAGAACCATAGTACCAAAATGTAGTITATCAGCTICTAGGCCAACATCACA	408 414
Fh-1 Fh-2	ACCTAGGTTCATTCAGCACAAAAAAGAAGCATTTTGGTTCTATAGGTTCCTTTCAATTGTGTATGACCAT	478 484
Fh-1 Fh-2	GTCATAAACCCTGGCCATTGGACTGAGGACATGAGAGATGAGGCACTTGAGCCTGCTGACCTAAGTGACA	548 554
Fh-1 Fh-2	GGAACATGATIGTIGTIGATG TEGETGGCGCGCGCGCGCTTCACCACATGGGGATIGTTAAGCATGTGGA	618 624
Fh-1 Fh-2	TGCCAAGAATGTCACCATTCTTGACCAGTCCCCGCACCAGCTCGCCAAGGCCAAGCAGAAGGAGCCCTTG	633 694
Fh-1 Fh-2	AAGGATTGCAAGATTATCGAAGGGGATGCCGAGGA@CTCCCCTTCGAAC@GATTATGCTGACAGATATG	758 764
Fn-1 Fn-2	TOTO TO CTO CACTO CACTO CACO TO CAAGO TTO TAAGO TTTO CACTAGO TTA CONTROL CACTO	828 834
Fh-1 Fh-2	AAAACCACTITICCAC ITA TATI TANGGOGGGGGCCOTITG IT ICAT IT IC IGAAAAAAAAAAAAGGIGACI	898 899
Fh-1 Fh-2	@ТАССБААЛАЛАТСТБАЛАЛАТСАТССТТСТБАТАЛССТА@ТАТТБІСАССТІСТССВАЛАЛАЛАЛАЛАЛА — - А ТТС С А	963 967
Fh-1 Fh-2	AAAAAAMCTTMGCATCTGGGTTATTTTATAAAATTTATMCAAATATTTGAAAACTGAT.CTTTTTTAGTI	1037 1032
Fh-1 Fh-2	AACACTGAAAAGCTTCTATTGTGACGTTTCATCTGCAATTCATATAATTGAATATGAATGA	1107
Fh-1 Fh-2	AGAIDCTATE ATG CATGCCCTTCTTGATGATAAGTATGGATTAGTATGCTATAGT TGGTATTGTTGTTGTTATA	1176
Fh-1 Fh-2	ATGITATCAAGTGATCAATTTATTCTCTTGAAATATTTGAGGTTACTTTATGTTATGATACGAAGTAGCT	1246
Fh-1 Fh-2	TGTC TTAAAGAGGCAATGCTGATGATGTGCTGTTGTTTC TTGATCTTAG <u>CATTGAGTACTGGCCGGATCC</u>	1316 1312
Fh-1 Fh-2	ACAGOGOGOCATCAGOGAAGCATACAGOGTATTGAAACTTGOGOGCAAAGCATGTGTAATTGOTCCCGTC	1386 1382
Fh-1 Fh-2	TACCCAACATTCTGGTTGTCACGATTCTTIGCTGATGTTTGGATGCTTTTCCCCAAGGAGGAAGAGTACA	1456 1452
Fh-1 Fh-2	TIGAGTGGTTTCAAAAGGCTGGATTCAAGGATGTCCAACTAAAACGGATTGGCCCAAAGTGGTATCGTGG	1526 1522
Fh-1 Fh-2	1001010801010 6000006411010800041196001101108001600060100000000000000000	1596 1592
Fh-1 Fh-2	TIGCAG GTA TITIG TCA TC TIGO TT TC TCAATCAATA TC TITIG TCGA TG TT TT TCC G TG TT TT TC TT TG GGG	1666
Fh-1 Fh-2	ATT T TG T TG T TA C T TA AAC TG AGT TG TG G G T T TG TA TG AGT A TC TATCT TG T TG	1736 1732
Fh-1 Fh-2	CCOTTIGIGIAA TRAACA TOOTOTIC TOAT TOATO TOATC TOC TOT TO TOCCC C TCCA IT TO TOOT TAACT	1806 1802
Fh-1 Fh-2	TAATGAATTATCCAAGCATATAAAAGGAGAGACGAATGAGGAAAAAGTAGAATTGGGATTTGAGAGCCA	1876 1872
Fh-1 Fh-2	CTTGGTTGCTCHACAATGACAAAAICCAAGACTHATCTCACTAGATGGAGTTGGTTATTTGAATCAACAC	1945 1942
Fh-1 Fh-2	CATGTOTIGATA TACCITCICCGAAGGTTCATICCBCCTAAABCAAATTCAATGATCTTTTGTA TAAACT	2015 2011
Fh-1 Fh-2	CCCC TAA TTOTO TO TO TO TO TO TAAC OTGAA TGO TOAAGGAGGTTTA TT TTO TO TTO TO TO TTOTTT TG TG	2085
Fh-1		2081
FB-2	A TOTAC TOCTO TITITC TO AGO TTACA OF A TANTA ITO AGO TIC CAOGO TO ACTI TOGIA TITACC CCCAT	2081 2155 2151
Fh-1 Fh-2	АТОТАСТОСТОТТЕТСТВАВСЕТТАСАВТАТЩІТА ТТОАВСЕТЕСАВОВОТОАСТІТОВТА ПТІАСССССАТ —	2081 2155 2151 2225 2221
Fn-1 Fn-2 Fn-1 Fn-2	АТОТАСТОСТО ТТТТСТОАОСТТАСАОТАТЩІТА ТГОАОС ТТССАОООТОАСТІ ТООТА ТТТАСОСССАТ — — — — — — — — — — — — — — — — — — —	2081 2155 2151 2225 2221 2295 2291
Fn-1 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2	АТОТАС ТОСТО ТТІТІ ТОАВСТІАСАОТАТВІТА ТТОАВС ТІС САОВОТОАСТІТОСТА ІТ ТАСССССАТ — — — — — — — — — — — — — — — — — — —	2081 2155 2151 2225 2221 2295 2291 2365 2361
Fh-1 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2	A TOTAC TOCTO TITITC TOAOCTTACAGTATAITA TTGAOC TTC CAOGOTGACTT TOGTA IT TACCCCCAT 	2081 2155 2151 2225 2221 2295 2291 2365 2361 2435 2431
Fn-1 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2	АТОТАС ТОСТО ТТІТІ ТО АВСТІАСАВІ ТАДІТА ПОАВС ПІС САВОВОТО АСПІТОВІ А ПІ ЛОСОССАТ — — — — — — — — — — — — — — — — — — —	2081 2155 2151 2225 2221 2365 2361 2435 2431 2505 2501
Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2 Fh-1 Fh-2	A TOTAC TOCTO TITIC TO AGCTTACAGOTAT MITA TO AGC TIC CAOGO TO ACTITOGTA IT TACCCCCAT AGCAAACCCAATO THE TATOM TO THE ATOM TO THE AGC ATC CAOGO TO ACTITOGTA IT TACCCCCAT TOTTOC CACTOC TATO THE TATOM TO ATAACTA AATTA ACT AGA TITIC CAACCA MICAC MITA AATTITICAATOT TOTTOC CACTOC TATO AAAACATTAT THAATOC TICC TCATCTO TAATA TGATCCATCO ATG AAAAT TOTTOC CACTOC TATO AAAATAT TO TATACT TO TO TCATATA TGATCCATCO ATG AAAAT TOTTAAGO TAAAATG AATAT TGATACT TO TO TAAAAATG TO TO CATTATA TAM TTO TTO TO TAGOTTCATAATAG GAAGTACTAATAACACAAGATACTAATG TO CACHTTO TTTICTATAT TO TTO TO ACCTOC TCATAATAG GAAGAACATTG TGO AGCCTO TTAATACTIC TO TO TTO TTATATO TTO TO ACCTOC TCATAATAG GAAGAACATG TGO AGACCTO TTAATACTIC TO TO TTO TTATATO TTO TO ACCTOC TAATAG GAAGAACATG TGO AGACCTO TTAATACTIC TO TO TTO TTATATO TTO TO ACCTOC TAATAG GAAGAACATG TGO AGACCTO TTAATACTIC TO TO TTO TTATATO TTO TO ACCTOC TAATAG GAAGAACATG TGO AGACCTO TTAATACTIC TO TO TTO TTATATO TO TTO TO ACCTOC TAATAG GAAGAACATG TGO AGACACATG TO TO TTO TO TTO TO TTO TO ACCTOC TAATAG GAAGAACATG TGO AGACACATG TTO TO TTO TTO TO TO TO ACCTOC TAATAG GAAGAACATG TATAACACAAGAACATTATAT TO TO TO TO TO ACCTOC TO TTO TATATAG GAAGAACATG TGO AGACCTO TTAAT ACTOC TO TTO TTO TTAT TO TTO TO TO ACCTOC TAAT AGG GAAGAACATG TO TAATAACACAAGAACTTO TO TTO TTO TTO TTO TTO TTO TTO TO TTO TO	2081 2155 2151 2225 2221 2365 2361 2435 2431 2505 2501 2435 2431
Fn-1 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2 Fn-1 Fn-2	ATGTAC TGCTG TTTTC TO AGCTTACAGGTATA TTG AGC TTC CAOGGTO ACTTTG GA TT TACCCCCAT AGCAAACCCAATTO TTG TA TATATICAACTA AATTA ACTA AATTTTC CAACCAA ACCA ATATATI TTG AACTT AGCAAACCCAATTO TTG TA TATATI TTG AATCA TAACTA AATTTTC CAACCAA ACCA ATTATI TG AATCA TTTTC CAACTA TTTTTC CACCTG CTAGTO AAAACATTATTTA AATOC TTC CTATCT TG TATATATATATATATATATTTC AATCA TTTTAAGGOTA AAATGAATATOGTA ATTCC TOTTO ATAAAATGO TG TO CCATTTATA TAATTTTC TAGCCT TAGOGTTG ATAATGGAAGTACTAATAACCAAGAATACTAATGT TG CACCTTTC TTTTTTTTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TTC TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCTTTG TG TTCT TATTTC TTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGATG TG GAGAAGACCT GTTAATACCT TTG TG TTCT TATTTC TTC TATTTC TTC AGCTTG GTC AAAGGAAGAAGTACTAATAACCAAGAATACTAATG TG CCCTTTG TG TTCT TATTTC TTC TATTTO TTC AGCTTG GTC AAAGGAAGAAGTACTAATAACCAAGAATACTAATG TG CCTTG TG TTCT TATTTC TTC TATTTC TTC AGCTTG GTC AAAGGAAGAAGTACTAATAACCAAGAAGACCT GTTAATACCT TG TG TTCT TATTC TTTT TTC TATTTC TTC TATTTC TTC	2081 2155 2151 2225 2221 2295 2291 2365 2361 2435 2431 2505 2431 2435 2431 2505 2501
28-2 Ph-1 Ph-2 Ph-2 Ph-2 Ph-2 Ph-2 Ph-2 Ph-2 Ph-2	A TOTAC TOCTO THIS TO AGONTACA AGONTATING AGONTACCA AGONTACA AGONT	2081 2155 2151 2225 2221 2365 2361 2435 2431 2505 2501 2435 2431 2505 2501 2505 2501 2575 2571
 FR-1 FR-1 FR-1 FR-2 FR-1 FR-2 FR-1 FR-1 FR-1 FR-1 FR-2 FR-2 FR-2 FR-2 FR-2 FR-2 FR-2 FR-3 FR-4 <li< td=""><td>A TOTAC TOCTO TITUE TO AGENTACAGETAR (TA TTO AGE TITE CAOGO TO ACTITO OT A TT TACC COCAT </td><td>2081 2155 2151 2225 2221 2365 2361 2435 2431 2505 2501 2435 2431 2505 2501 2555 2571 2645 2641</td></li<>	A TOTAC TOCTO TITUE TO AGENTACAGETAR (TA TTO AGE TITE CAOGO TO ACTITO OT A TT TACC COCAT 	2081 2155 2151 2225 2221 2365 2361 2435 2431 2505 2501 2435 2431 2505 2501 2555 2571 2645 2641

Figure 1. Alignment of 2 genomic nucleotide sequences of *VTE3* from *Arachis hypogaea*. Fh-1 = *FhgVTE3-1*; Fh-2 = *FhgVTE3-2*; exons are underlined; (\cdot) = nucleotide deletions; the initiator codon ATG and the stop codon TAA are shaded by dark blocks and the sites of SNP variations by pale gray.

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Α	FhgVTE3-1 FhgVTE3-2	ААGGTTTTTCCCCTCCTTTC <mark>TTAATTTA</mark> ATTTAATTTAATTT	100 94
B	FhgVTE3-1	CCGTTTGTTTCATTTTCTGAAAAAAA <mark>AAAAAG</mark> GTGACTCTACGAAAAAATCTGAAAAATG	920
	FhgVTE3-2	GTGTTTGTTTCATTTTCTGAAAAAAT <mark>CTTAAG</mark> TATAATTGAAAAAGATGAAAAATG	921
С	FhgVTE3-1	TGCAATTCATATAATTGAATATGATGAACTTCTATAA <mark>AGACCT</mark> ATAATGTATGCCCTTCT	1130
	FhgVTE3-2	TGCAATTCATATAATTGAATATAATGAACTTCTATAA <mark>AGATCT</mark> ATTATGTATGCCCTTCT	1125

Figure 2. Alignment of partial genomic nucleotide sequences of *VTE3* from *Arachis hypogaea*. (-) = nucleotide deletions. **A.** The endonuclease recognition site of *PacI* is shaded by dark blocks and the unrecognitive nucleotides are shaded by pale gray; **B.** the endonuclease recognition site of *AfIII* is shaded by dark blocks and the unrecognitive nucleotides are shaded by pale gray; **C.** the endonuclease recognition site of *BgIII* is shaded by dark blocks and the unrecognitive nucleotides are shaded by pale gray; **C.** the endonuclease recognition site of *BgIII* is shaded by dark blocks and the unrecognitive nucleotides by pale gray.

The homology of *gVTE3-1* nucleotide sequences in the 13 cultivars was 99.9%, and polymorphisms were found to exist within the homologous sites. According to the different numbers of A-bases (A's) found within the *gVTE3-1* nucleotide sequences, the 13 cultivars could be classified into 4 categories: category 1 comprised Fenghua 2, Krapt. st. 16, and ICG6848 with 4 A's at the diversity site; category 2 was made up of Yuanza 9102, E1, and Rugaoxiyangsheng with 3 A's; category 3 included Shanhua 7, 8 and 9, Lanna 1, Nongda 818, and Lipudahuasheng, which shared 2 A's; category 1 (Figure 3). The former 3 categories comprised different peanut varieties, and no consistency was found between the varieties. The *gVTE3-2* nucleotide sequences in the 13 cultivated varieties were found to be totally conserved with 100% homology.

FhgVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	990
KtgVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	990
IggVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	990
YzgVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAA	989
ElgVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAA	989
RggVTE3-1	тсадсттстдсаааааааааааааааааааааааааааа	989
Sh7gVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAA	988
Sh8gVTE3-1	тсадсттстдсаааааааааааааааааааааааа стттдсатстдддтт	988
Sh9gVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA CTTTGCATCTGGGTT	988
LngVTE3-1	тсадсттстдсааааааааааааааааааааааа стттдсатстдддтт	988
NdgVTE3-1	тсадсттстдсааааааааааааааааааааааа стттдсатстдддтт	988
LpgVTE3-1	тсадсттстдсааааааааааааааааааааааа стттдсатстдддтт	988
IvgVTE3-1	ТСА GCTTCTGCAAAAAAAAAAAAAAAAA	987

Figure 3. Alignment of partial genomic nucleotide sequences of *VTE3* from 13 accessions (as shown in Table 1) of *Arachis hypogaea*.

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Studies on VTE3 of peanut

Phylogenetic analysis of VTE3 sequences from cultivars and wild species

The *FhgVTE3-1* (Fenghua 2) and -2 (Fenghua 2) and *gVTE3-A* (*A. duranensis*) and -*B* (*A. ipaënsis*) sequences were aligned with ClustalX, and a phylogenetic tree (Figure 4) was constructed by PHYLIP based on the maximum parsimony method. In addition, bootstrap (1000 replicates) was used to assess the tree. The tree revealed that the *FhgVTE3-1* and *gVTE3-A* clusters formed a group and the *FhgVTE3-2* and *gVTE3-B* clusters formed another group. Sequence alignment analysis revealed that the homology of *FhgVTE3-1* and *gVTE3-A* was 98.7% and that of *FhgVTE3-2* and *gVTE3-B* was 100%. Currently, the majority of researchers believe that *A. duranensis* and *A. ipaënsis* are the donors of A-and B-genome in the cultivated peanut. Therefore, the present study inferred that *gVTE3-1* and -2 in cultivated peanuts are from A- and B-genome, respectively.



Figure 4. Phylogenetic tree based on the genomic nucleotide sequences of *FhgVTE3-1* and -2, *gVTE3-A* and -B.

Bioinformatic analyses of protein sequences predicted by peanut VTE3 nucleotide sequences

The amino acid sequences predicted by analysis of the *FhrVTE3-1* and *-2* coding sequences were designated Ah1 and Ah2. Bioinformatics analyses (see Material and Methods) were performed on the 2 proteins. For Ah1, the molecular weight, pI, and grand average of hydropathicity were 39.90 kDa, 9.27, and *-*0.288, respectively. The molecular weight, pI, and grand average of hydropathicity of Ah2 were 39.86 kDa, 9.33, and *-*0.279, respectively. The results indicate that the 2 proteins are hydrophobic.

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Conserved domain analysis showed that Ah1 and Ah2 shared the same conserved domain of *S*-adenosylmethionine-dependent methyltransferases (Figure 5). Secondary structure analysis revealed that Ah1 comprises $31.34\% \alpha$ -helices, 22.51% extended strands, $5.13\% \beta$ -turns, and 41.03% random coils; Ah2 comprises $28.49\% \alpha$ -helices, 23.36% extended strands, $5.13\% \beta$ -turns, and 43.02% random coils. Transmembrane structure analysis using TMHMM determined that the transmembrane region was found at positions 318-340 in both the Ah1 and Ah2 proteins. In addition, analysis of subcellular localization by WoLF PSORT revealed that Ah1 and Ah2 are located in the chloroplast.

Ahl Ah2	MASSMF SGTESL TLFS SK TPNGLCFNASNFHSKRIG FNSS T TNFNFSNKARVRHNSNRIVR TIVPKCSLS	70 70
6	and he still a stadil a foreur active head foreign and	61
Gm	-g-v-ikf.ti-gg-tg-di-giprvs-aatts-k-pnistvv-	01
Hai	l-lpqn-alapKg-knpdikcfsisipks-ligks.fnigaKtimrv-	58
Ha2	l-lpqn-atapkg-knpdlkcrststpks-ligks.rnlgaktpmrv-	58
Ha3	-tly-a-n-aiirgrvaagg-elngrkfplkv-lacgsis-gktlvv-	62
НЪ	l-ln-a-nfmgikg-lg-dgnhfprv-liss-isrtvmn	59
At	l-lnai-fpkgspgl-arsprptllsvtrtstprlsvatrcssss.v-	56
Bn	l-ln-, ai-fp,, kg-pl-ar,, pspplslvsntat-r, lsvatresss-v-	55
Ls	ly-gahtn kgg-dl-g-nl lkm-lygr- onf-s ktlyy-	56
Che .	hann aga ta adamphaslain l centre in la artist	
Cm	-naanp-qiaa-sqiipiiqagsiui	55
Ahl	ASRPTSQPRFIQHKKEAFWFYRFLSIVYDHVINPGHWTEDMRDEALEPADLSDRNMIVVDVGCGTGFTTLG	141
Ah2		141
Gm	sn	132
Hal	vae-dn-dlv	129
Ha2	vae-dn-dlv	129
Ha3	lp-avsl	133
Hb	adhnll	130
At	ssahpd-r	127
Bn	sahpd-rhpd-r	126
Ls	vp-addd	127
Chr	vi-alvsvagoivdak-d-p-lkc	126
Ahl	IVKHVDAKNVTILDQSPHQLAKAKQKEPLKDCKI	212
Ah2		212
Gm	k	203
Hal	ee-vae-rek	200
Ha2	eee-vae-rek	200
Ha3	skkkkkk	204
НЪ	p	201
At		198
Rn		107
Т.	tex	100
~~~		190
Chr	vt-xpeima-pagvt-ipueeee	197
Ahl	YR <mark>VLKLGGKAC</mark> VIGPVYPTFWLSRFFADVWMLFPKEEEYIEWFQKAGFKDVQLKRIGPKWYRGVRRHGLIM	283
Ah2		283
Gm	11	274
Hal	i-kiaiaia	271
Ha2	i-klmeiqiq	271
Ha3	ikikiki	275
Hb		272
At	kn	269
Bn	h	268
Ls	kiki	269
Chr		268
Ahl	GCSVTGVKPASGDSFLQLGFKEEDVEKPVN TFVFLYRFILGALAATWFVLVPIYMWLKDQIVFKGQPI	351
Ah2	lililili	351
Gm	vvv	342
Hal	tvplvs-gvyyv	339
Ha2	tvpla-gvyyv	339
Ha3	gvyyv	343
НЪ	kiapligayyf	340
At	taiid	338
Bn	taiid	337
Ls	vreplaavm-giyyv	337
Chr	kavma-vsg-mntnplslnlita-gfyyfclyi-nl-wnwem	337

**Figure 5.** Alignment of amino acid sequences of 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase in peanuts compared with other plants. Ah = *Arachis hypogaea*; Gm = *Glycine max*; Ha = *Helianthus annuus*; Hb = *Hevea brasiliensis*; At = *Arabidopsis thaliana*; Bn = *Brassica napus*; Ls = *Lactuca sativa*; Chr = *Chlamydomonas reinhardtii.* (-) = same amino acid residues. Conserved domains are shaded by dark blocks.

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# Homology analysis of VTE3 amino acid sequence in peanuts compared with other plants

Comparison of the homologous amino acid sequences of peanut Ah1 and Ah2 with homologous proteins from other plants demonstrated that Ah1 shares 84.9, 81.2, 78.1, 78.1, 77.3, 76.7, and 69.7% homology with MPBQ MT from soybeans, rubber, lettuce, sunflower, *A. thaliana, Brassica napus*, and *Chlamydomonas*, respectively. It also revealed that Ah2 shares 84.6, 81.2, 77.8, 77.6, 76.4, 75.9, and 70.4% homology with the MPBQ MT from the species listed above.

The homologous MPBQ MT proteins from the species mentioned above (i.e., peanuts, soybeans, rubber, lettuce, sunflower, *A. thaliana, B. napus*, and *Chlamydomonas*) were aligned (Figure 5) by PHYLIP in combination with ClustalW and the subsequent phylogenetic tree construction (Figure 6). Figure 5 illustrates the high homology of MPBQ MT across different species, even in distant species such as *Chlamydomonas*, indicating strong conservation of genetic material in MPBQ MT. Figure 6 shows that, among the 11 amino acid sequences from 8 sampled species, *A. thaliana, B. napus*, and *Chlamydomonas* are clustered on 1 branch; sunflower and lettuce are clustered on another branch; peanut and soybean share 1 branch; and rubber is the sole occupant of the final branch. The first branch can be further classified into 2 sub-branches consisting of *A. thaliana* and *B. napus* on one sub-branch and *Chlamydomonas* on the other. This demonstrates that *Chlamydomonas* is much more distantly related to other plants used in this study. The protein molecular tree is consistent with previous studies utilizing classical taxonomy, specifically in the case of peanuts and soybeans belonging to legume groups, sunflowers and lettuces belonging to Asteraceae, and *A. thaliana* and *B. napus* belonging to Cruciferous.



**Figure 6.** Phylogenetic tree of 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase for related species. Chr = *Chlamydomonas reinhardtii*; At = *Arabidopsis thaliana*; Bn = *Brassica napus*; Ha = *Helianthus annuus*; Ls = *Lactuca sativa*; Ah = *Arachis hypogaea*; Gm = *Glycine max*; Hb = *Hevea brasiliensis*.

#### DISCUSSION

This study utilized EST assembly and RT-PCR techniques to obtain 2 peanut *VTE3* sequences, demonstrating that the coding nucleotide sequences share more than 70% homology with *A. thaliana VTE3*. The corresponding predicted amino acid sequences share more than 76% homology with the homologous MPBQ MT from any variety of *Arabidopsis* species, soybeans, rubbers, lettuces, sunflowers, and *B. napus*. All of the proteins had an S-adenosylmethionine-dependent methyltransferase conserved domain in common.

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Bioinformatic analyses demonstrated that peanut MPBQ MT is primarily hydrophobic, is located within the chloroplast, and possesses a distinct transmembrane region. These results are consistent with previous findings studying the related enzyme-producing genes involved in vitamin E synthesis (Arango and Heise, 1998). Therefore, the sequence we obtained in this study is actually the peanut VTE3 sequence, and the corresponding MPBQ MT proteins are highly conserved across different species.

Phylogenetic analysis completed in the present study demonstrated that *FhgVTE3-1* and -2 in Fenghua 2 are from the A- and B-genome, respectively. The 2 sequences were shown to contain 56 variable sites, and these sites were used to design primers that allowed VTE3 genomes from different sources to be distinguished. Analysis of restriction enzyme recognition sites showed that *FhgVTE3-2* has 3 restriction sites corresponding to *PacI*, *AffII*, and *Bg/II*, whereas FhgVTE3-1 cannot be recognized by any of the 3 restriction enzymes. This finding suggests that these restriction sites are likely to have genomic specificity. Differentiation methods utilizing PacI, AfII, and BgIII recognition sites on the VTE3 gene sequences from B-genome sources have thus been shown to give researchers the ability to distinguish between the 2 VTE3 genes in peanut cultivars.

VTE3 sequences were cloned from 2 subspecies of peanut cultivars comprising 13 distinct varieties, producing 2 homologous VTE3 DNA sequences, gVTE3-1 and -2, cloned from each of the sampled varieties. Alignment showed that gVTE3-2 is totally conserved with 100% homology across the 13 varieties, and gVTE3-1 has 99.9% homology. The 0.1% difference can be accounted for by subtle differences in the intron repeat sequence between the various samples. Different molecular markers were used by Raina et al. (2001) and Milla et al. (2005), confirming the conclusions that the genetic base of cultivated peanuts is narrow and that polymorphisms occur only at low levels. Different peanut VTE3 cultivars have been demonstrated to be highly homologous, with the 2 subspecies A. hypogaea ssp fastigiata and A. hypogaea ssp hypogaea being the most likely to share the same diploid wild ancestral species, although validation through further study, including larger cohorts and examination of additional molecular markers is needed to validate these results.

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## **Conflicts of interest**

The authors declare that they have no conflict of interest.

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