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Comparative analysis of tocopherol biosynthesis genes during seed development in the Asian spiderflower, *Cleome viscosa* (Cleomaceae)

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ABSTRACT. *Cleome viscosa* (common names, wild mustard, Asian spiderflower, and tick weed) is an annual oilseed crop plant that has a high vitamin E content, with the most active forms being the tocopherols (α, β, γ and δ). These act as antioxidants in plants and are also useful for human health. The gene expression profiles and partial-length cDNA encoding tocopherol cyclase (Cv*VTE1*), homogentisic acid prenyltransferase (Cv*VTE2*), MPBQ methyltransferase (Cv*VTE3*), gamma-tocopherol methyltransferase (Cv*VTE4*), hydroxyphenyl-pyruvate dioxygenase (Cv*HPPD*) and homogentisate solanesyltransferase (Cv*HST*) genes involved with tocopherol biosynthetic enzymes were determined. Partial cDNA sequences of six genes as 694 bp Cv*VTE1*, 375 bp Cv*VTE2*, 387 bp Cv*VTE3*, 402 bp Cv*VTE*4, 334 bp Cv*HPPD* and 461 bp Cv*HST* showed high identity to their homologs in other higher plants. Expression levels of Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST* genes were analyzed by real-time quantitative PCR (qPCR) at plant nodes L1-L3 in leaves and 1-4 weeks after fruit set (WAF) of seeds. Cv*VTE3* and Cv*HPPD* were predominantly expressed in the leaves, while in seeds Cv*VTE2* was expressed at both 2 and 3 WAF at rates of 231-fold and 224-fold, which coordinated with Cv*VTE1* and Cv*VTE3* expression. Co-expression of Cv*VTE4* and Cv*HPPD* supports high levels of flux toward the synthesis of tocopherols at 4 WAF without any down regulation. We conclude that Cv*VTE2* plays a key role in tocopherol biosynthesis in *C. viscosa* seeds.

Key words: *Cleome viscosa*; Tocopherol; Homogentisic acid prenyltransferase; Vitamin E

INTRODUCTION

Cleome viscosa, or wild mustard, is an annual herb that thrives in humid and warm habitats. Plants are commonly found growing in disturbed sites, gardens, forest undergrowth, wastelands and along roadsides. *C. viscosa* is native to paleotropical areas (Africa, Asia)., but now has been introduced to other parts of the world and is naturalized throughout the tropical and subtropical regions in Asia, Africa, the Americas, and Oceania (Acevedo-Rodriguez and Strong, 2012; Flora of China Editorial Committee, 2015; PIER, 2015; USDA-ARS, 2015).The whole plant, leaves, seeds, and roots of *C. viscosa* are widely used in traditional medicine as a remedy to promote a cooling sensation, with stomachic, laxative, diuretic and anthelmintic properties (Mali, 2010). The seed oil contains palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) (Kumari et al., 2012). Several minerals, vitamin C and vitamin E have been isolated from *C. viscosa*, with vitamin E in relatively high amounts of 0.318 mg/g (Pallai and Nair, 2013).

Vitamin E is a generic term for a group of compounds that are also known as tocochromanols. The general structure of tocochromanols consists of a hydrophilic chromanol head group and a hydrophobic phytyl diphosphate tail that varies according to the type of tocochromanol (Mène-Saffrané and Pellaud, 2017). The phytyl diphosphate precursors of tocopherols, tocotrienols, tocomonoenols and plastochromanol-8 (PC-8) are phytyl diphosphate (PPP), geranylgeranyl diphosphate (GGPP), tetrahydrogeranylgeranyl diphosphate (THGGPP) and solanesyl diphosphate (SPP). Tocopherol is one of the most powerful antioxidants and forms α- (three-methyl group), β- and γ- (two-methyl group) and δ-(one-methyl group) (Mène-Saffrané, 2017). Among the different tocopherol types, α-tocopherol has the highest vitamin E activity at 100% with β-tocopherol 50%, γ-tocopherol 10% and δ-tocopherol 3% (Kamal-Eldin and Appelqvist, 1996).

The initial step of tocopherol synthesis is the condensation of polar aromatic head homogenetisic acid (HGA) precursor from the shikimic acid pathway with phytyl diphosphate (PPP) precursor from chlorophyll degradation into 2-methyl-6-phytylbenzoquinol (MPBQ). The reaction is catalyzed by homogentisate phytyl transferase (*HPT* or *VTE2*). The HGA is produced from 4-hydroxyphenylpyruvate (HPP) by a 4-hydroxyphenylpyruvate dioxygenase (*HPPD*), through which HPP derives from tyrosine degradation. The phytyl diphosphate (PPP) derives from geranylgeranyl diphosphate (GGPP) from the methylerythritol phosphate pathway (MEP). The MPBQ is either a direct precursor of δ-tocopherol or can alternatively be methylated by a methyltransferase (*MPBQ MT* or *VTE3*) to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ). MPBQ and DMPBQ are cyclized by tocopherol cyclase (*TC* or *VTE1*) to form δ-tocopherol and γ-tocopherol, respectively. These isoforms are transformed into β-tocopherol and α-tocopherol by γ-tocopherol methyl transferase (*γ-TMT* or *VTE4*). The tocopherol biosynthetic pathway has been briefly investigated on other plants. Increasing *HPPD* expression level in lettuce (*Lactuca sativa*) plays an essential role in tocopherol biosynthesis, resulting in α-tocopherol accumulation (Ren et al., 2011). *HPPD* and *VTE4* expressions are highly correlated with seed vitamin E accumulation, thus appearing to be a key point in the regulation of metabolic

flux through the tocopherol pathway in oat seed (*Avena sativa*) (Gutierrez-Gonzalez and Garvin, 2016). The *VTE4* gene is the marker-assisted selection to α-tocopherol content in rapeseed (*Brassica napus*) (Endrigkeit et al., 2009).

This study focused on the isolation and temporal and spatial expression profiles of Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST* genes, which are involved the tocopherol biosynthetic enzyme, in *C. viscosa*. Developmental programs comprised a period of three degrees of plant nodes in leaves and four weeks after fruit set (WAF) in seeds.

MATERIAL AND METHODS

Plant materials

Leaves from three developmental stages of *C. viscosa*, designated as L1 (nodes 1-5), L2 (nodes 10-15) and L3 (nodes 20-25), and *C. viscosa* seeds from four different developmental stages, designated as 1 WAF- 4 WAF corresponding to 1-4 weeks after fruit set (WAF) were used for analysis. Leaves and seeds were obtained from natural fields around Naresuan University, in Phitsanulok, Thailand, during fruiting in October. All materials were thoroughly ground in liquid nitrogen and stored at -80°C until required for RNA extraction.

RNA extraction, rDNase treatment and cDNA synthesis

Total RNA was extracted from 100 mg of leaves and seeds from different developmental stages using a modified extraction method adopted from Verwoerd et al. (1989), while extracted RNAs were treated with 1 µl (1U) of DNase I (Thermo Fisher Scientific, USA). The RNA integrity was quantified using a nanodrop spectrophotometer. First-strand cDNA was synthesized using a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with 1 µg of each mRNA template in a 20 µl final volume.

RT-PCR condition

The degenerate primer pairs for amplification were designed based on consensus sequences of seed oil plants (Table 1), with PCR reactions conducted on a Biometra TOne Thermocycler using Taq DNA polymerase (recombinant) (Thermo Fisher Scientific, USA). A 25 µl PCR mixture was prepared containing 30 ng of the cDNA solution, 1X of Taq DNA polymerase buffer (10X Taq Buffer with KCl), 2.5 mM MgCl2, 0.2 mM dNTP mix, 0.2 pmol of forward primer, 0.2 pmol of reverse primer and 1 U (5U/µl) Taq DNA polymerase. The PCR reactions were directed with an initial denaturing step of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52°C for 1 min and 72°C for 45 s and a final extension at 72°C for 10 min and infinite hold at 4°C. Amplified PCR products were separated in a 1% agarose gel using the electrophoresis method at 90 V in 1X TAE buffer. Ethidium bromide was used for DNA fragment visualization under a UV chamber. DNA fragments with expected size were purified from agarose gel using an E.Z.N.A.® Cycle-Pure Kit following the manufacturer's guideline.

Table 1. Degenerate primers used for RT-PCR analyses of *Cleome viscosa*.

Gene	Primer	5'Nucleotide sequence 3'	Tm (°C)
VTE1	*VTE1_F*	AGTTCTTCGAGGGATGGTAT	56.4
	VTE1_R	TGCAGTNARAGCAACTTCTCC	57.4
VTE2	*VTE2_F*	CAATCAGTTGTCTGATGTTGAAAT	58.4
	VTE2_R	GAAGCTCATAAACGCAGTGG	58.4
VTE3	*VTE3_F*	TGCNGAGGATCTYCCTTTTC	56.4
	VTE3_R	ACRTCCTCTTCCTTBGGACC	58.4
VTE4	*VTE4_F*	GGGGAGATCATATGCATCA	55.2
	VTE4_R	CATTGACCAAACAAGATCGAA	55.5
HPPD	*HPPD_F*	GGCGATGTTGTTCTCCGAT	57.3
	HPPD_R	TCTGAATCTGACTCTTCCTCT	57.4
HST	*HST_F*	TTGCTCTTATATGTGGGAATGG	58.4
	HST_R	GCAACTGGAAATCTCTTCATTC	58.4

VTE1: tocopherol cyclase gene; *VTE2*: homogentisic acid prenyltransferase gene; *VTE3*: MPBQ methyltransferase gene; *VTE4*: gamma-tocopherol methyltransferase gene; *HPPD*: hydroxyphenyl pyruvate dioxygenase gene; *HST*: homogentisate solanesyltransferase gene; Tm = melting temperature.

Plasmid cloning

The purified PCR fragments were ligated into the pJET 1.2 /blunt Cloning Vector (Thermo Scientific, USA) following the manufacturer's protocol. The isolated DNA fragments from the positive clone were sequenced using the capillary electrophoresis sequencing (CES) automation system by Macrogen, with the pJET1_2F and pJET1_2R primer pair used for bidirectional sequencing. DNA sequences were deposited in the DDBJ/EMBL/GenBank DNA database under their accession numbers.

Real-time RT-PCR analysis

Real-time PCR mix was prepared using the SensiFAST™ SYBR® No-ROX Kit and conducted on CFX Connect Real-Time PCR. The ACTIN was selected as the reference gene, and the oligonucleotide primers for RT-PCR were designed based on the cloning sequence of each gene (Table 2). The PCR reactions were initialed with a denaturing step of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. The relative expression level was calculated using the derived normalization method.

Table 2. Oligonucleotide primers used for real-time RT-PCR analyses in *Cleome viscosa*.

Gene	Primer	5' Nucleotide sequence 3'	Tm (°C)
VTE1	qVTE1_F	AGGTGCAAGGCCTCCAAGTAA	61.2
	qVTE1_R	CCTGCCATCATCGCAAATATAAC	60.9
VTE2	qVTE2_F	CGTTGGCATCAGGGGAGTAT	60.5
	qVTE2_R	GCCACGAACCAACAATCCAC	60.5
VTE3	qVTE3_F	TGCAGCTGAAGAGGATTGGTC	61.2
	qVTE3_R	TGACACCAGTGACAGAACACC	61.2
VTE4	qVTE4_F	CGAGGAGGCTCTCCGTTTC	61.6
	qVTE4_R	ACACCCCACATCAACCACACT	61.2
HPPD	qHPPD_F	AGCTGATTTCGAGTTCCTCCC	61.2
	qHPPD_R	GAGCAAGCTCCGGGACGTT	61.6
HST	qHST_F	CACGGTACGAGGTTTCCTTCT	61.2
	qHST_R	TGTGATGAAAGCCACAGGTGC	61.2

VTE1: tocopherol cyclase gene; *VTE2*: homogentisic acid prenyltransferase gene; *VTE3*: MPBQ methyltransferase gene; *VTE4*: gamma-tocopherol methyltransferase gene; *HPPD*: hydroxyphenyl pyruvate dioxygenase gene; *HST*: homogentisate solanesyltransferase gene; Tm = melting temperature.

Construction of the phylogenetic tree

The identities of Cleome *VTE1*, *VTE2*, *VTE3*, *VTE4, HPPD* and *HST* genes were confirmed with the nucleotide-nucleotide basic local alignment search tool (BLASTn) using somewhat similar sequences (Blastn) on the NCBI database (Altschul et al., 1990). Partial cDNA sequences were aligned with their homolog genes from other plants using multiple sequence alignment by the Clustal X package (Larkin et al., 2007). To obtain optimal trees, bootstrap analyses were conducted with 1,000 replicates using Mega X based on the maximum-likelihood method (Kumar et al., 2018).

RESULTS

The cDNAs were amplified by the RT-PCR reaction of RNA from leaves and seeds, using six pairs of degenerate primers involved in the tocopherol biosynthetic pathway. The PCR products were completely constructed for six degenerate primer pairs, including *VTE1_FR*, *VTE2_FR*, *VTE3_FR*, *VTE4_FR*, and *HPPD_FR* and *HST_FR*. Committed steps in the tocopherol biosynthetic pathway have been traditionally considered as condensation of HGA and different phytyl diphosphate precursors to form tocopherols by *VTE2* and plastochromanol-8 by *HST*. *VTE2* has been identified in both plants and cyanobacteria (Mène-Saffrané, 2018). Tocochromanols, tocopherols and tocotrienol are exclusively synthesized by photosynthetic organisms. Tocopherols occur ubiquitously in various plant tissues especially in leaves and seeds of most dicots, while tocotrienols are rarely exclusive forms present in the seed endosperm of most monocots such as wheat, rice, and barley (Kamal-Eldin and Appelqvist, 1996).

Partial sequences of *C. viscosa* tocopherol biosynthetic genes were obtained from cloning and named as Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST*. Sequence analysis showed that Cv*VTE1* (MW193603) was a 695 bp (231 aa) that encoded 52.53% of coding sequences (CDS) compared with *Tarenaya hassleriana* (Th) *VTE1*, Cv*VTE2* (MW193601) was a 376 bp (125 aa) (31.89% of CSD compared with Th*VTE2*), Cv*VTE3* (MW193602) was a 387 bp (128 aa) (36.26% of CSD compared with Th*VTE3*), Cv*VTE4* (MW193604) was a 366 bp (121 aa) (36.55% of CSD compared with Th*VTE4*), Cv*HPPD* (MW193600) was a 334 bp (111 aa) (24.47% of CDS compared with Th*HPPD*, and Cv*HST* (MW193605) was a 461 bp (153 aa) (40.02% of CSD compared with CSD of Th*HST*). BLAST analysis on NCBI databases identified single cDNA sequences for each gene. The Cv*VTE1* gene shared high similarity with *VTE1* from *T. hassleriana* (92.21%), *Eutrema salsugineum* (86.77%) and *Thellungiella halophile* (86.77%), while the Cv*VTE2* gene showed high similarity to *VTE2* from *T. hassleriana* (90.13%), *Arabidopsis lyrata* subsp. lyrata (88.24%) and *Camelina sativa* (88.24%). The Cv*VTE3* gene had similarity with *VTE3* from *T. hassleriana* (86.05%), *Raphanus sativus* and *Brassica rapa* (85.79%). The Cv*VTE4* gene shared >75% nucleotide sequence identity with *VTE4* from *T. hassleriana* (82.61%), *E. salsugineum* (79.57%) and *B. napus* (76.50%). The Cv*HPPD* gene shared high similarity with *HPPD* from *T. hassleriana* (88.82%), *E. salsugineum* (84.32%) and *Raphanus raphanistrum* (81.98%). The Cv*HST* gene shared high identity with *HST* from *B. rapa* (91.76%), *B. napus* (91.76%) and *T. hassleriana* (91.58%).

Phylogenetic relationships of *C. viscosa* vitamin E biosynthesis genes with other monocots and dicots were construed for each gene. Maximum likelihood trees could be

divided into two monophyletic clades; monocot and dicot (Figures 1-6). Six genes, Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST* formed a group with dicot species, while all the *C. viscosa* and *T. hassleriana* genes were sister groups with high bootstrap support. *T. hassleriana* was formerly named *Cleome hassleriana* but the genus *Cleome* has undergone recent taxonomic revisions (Iltis and Cochrane, 2007). Results revealed that these genes evolved differently in dicot and monocot species. The date of the monocot-dicot split was confirmed at 140-150 Myr ago using a large chloroplast genome database (Chaw et al., 2004). Moreover, phenotypic differences between species may result from the presence of codons evolving under both neutral and positive selection (Almida et al., 2011).

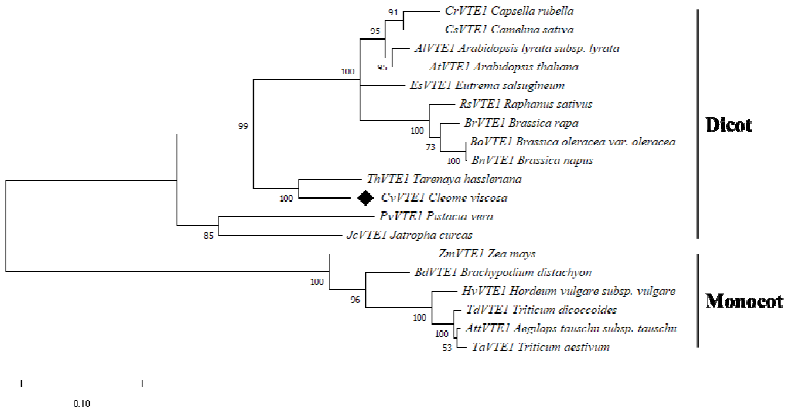

Figure 1. Phylogenetic tree constructed by ML (Tamura-Nei +G +I (TN93 +G +I) Model) using the derived partial cDNA sequences of the tocopherol cyclase gene from *Cleome viscosa* (Cv*VTE1*) with other plants. The bootstrap values are shown at branching points.

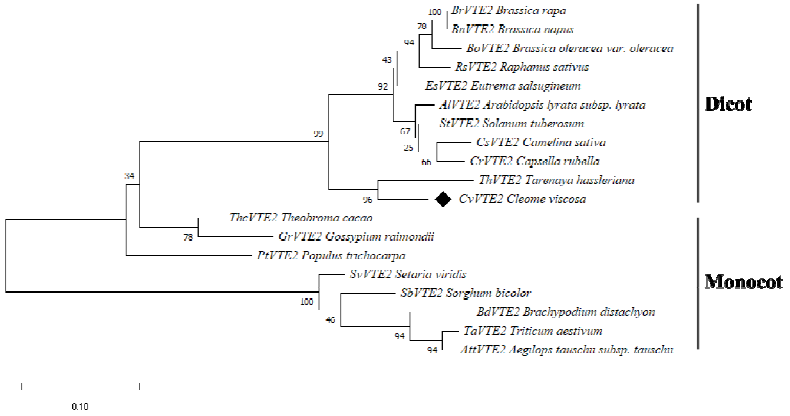

Figure 2. Phylogenetic tree constructed by ML (Tamura-Nei +G (TN92 +G) Model) using the derived partial cDNA sequences of the homogentisic acid prenyltransferase gene from *Cleome viscosa* (Cv*VTE2*) with other plants. The bootstrap values are shown at branching points.

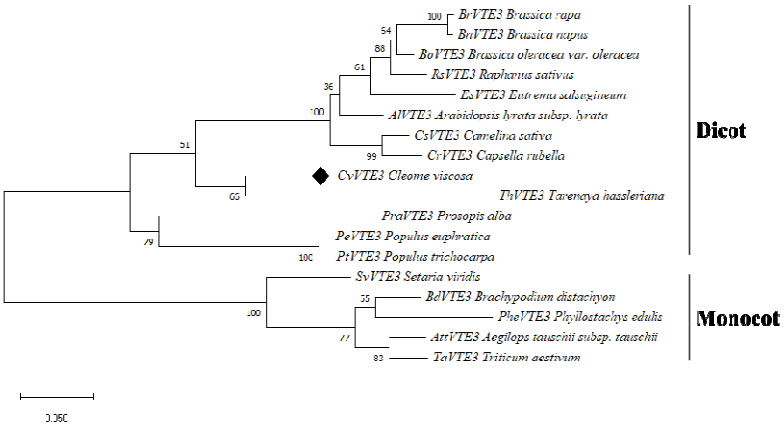

Figure 3. Phylogenetic tree constructed by ML (General Time Reversible +G +I (GTR +G +I) Model) using the derived partial cDNA sequences of MPBQ methyltransferase gene from *Cleome viscosa* (Cv*VTE3*) with other plants. The bootstrap values are shown at branching points.

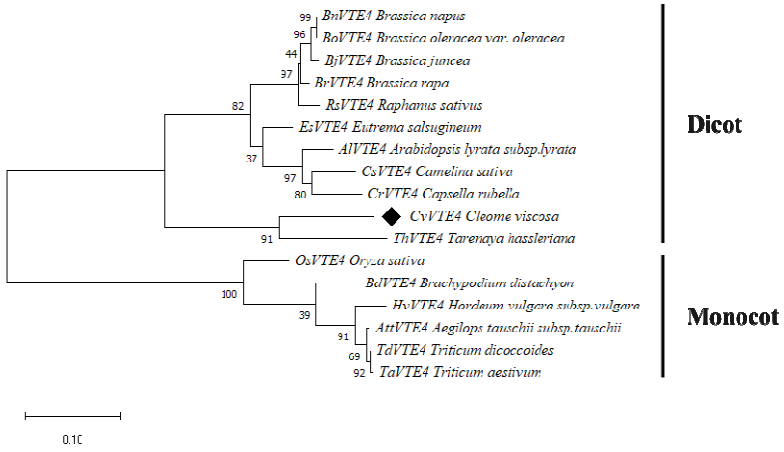

Figure 4. Phylogenetic tree constructed by ML (Tamura 3-parameter +G (T92 +G) Model) using the derived partial cDNA sequences of the gamma-tocopherol methyltransferase gene from *Cleome viscosa* (Cv*VTE4*) with other plants. The bootstrap values are shown at branching points.

Temporal and spatial expressions of the six vitamin E biosynthesis genes (Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST*) in leaves and seeds were investigated using qRT-PCR. The expression levels were different among various tissues. Genes in the biosynthetic pathway expressed in three stages of leaves and of seeds at 1-4 WAF. Relative expression levels of the first stage of each gene were calculated and compared.

Expression levels were different in each gene in the L1 stage of leaves, whereas Cv*VTE3* was the most expressed in the L2 stage (Figure 7). *Arabidopsis VTE3* mutants showed decreased levels of γ- and α-tocopherol in leaves (Sattler et al., 2003). Different expression patterns for Cv*VTE2*, Cv*VTE1* and Cv*HST* were distinguished in the L1 stage

and decreased at L2 and L3. Moreover, expression of the Cv*HST* gene was consistent in every stage. The Cv*HPPD* gene showed high expression levels in the L3 stage. This result agreed with a report on *L. sativa* which presented the highest expression levels of Ls*HPPD* in mature leaves (Ren et al., 2011), while up-regulation of *HPPD* resulted in over-ripening processes (Georgiadou et al., 2015).

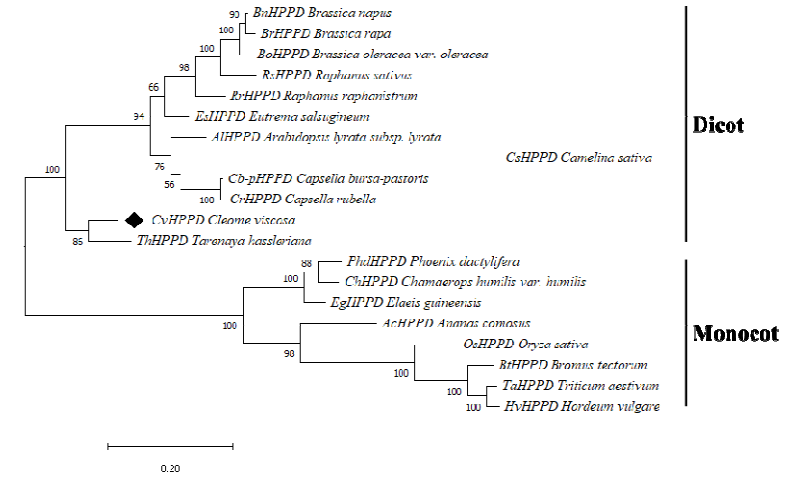

Figure 5. Phylogenetic tree constructed by ML (Tamura 3-parameter +G (T92 +G) Model) using the derived partial cDNA sequences of the hydroxyphenyl pyruvate dioxygenase gene from *Cleome viscosa* (Cv*HPPD*) with other plants. The bootstrap values are shown at branching points.

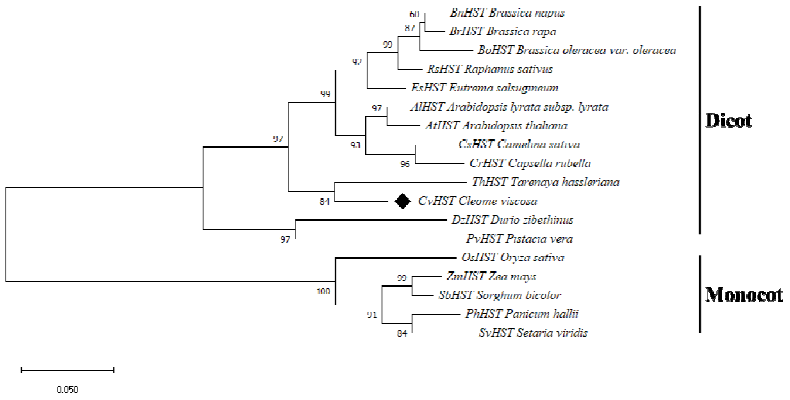

Figure 6. Phylogenetic tree constructed by ML (Tamura 3-parameter +G (T92 +G+I) Model) using the derived partial cDNA sequences of the homogentisate solanesyltransferase gene from *Cleome viscosa* (Cv*HST*) with other plants. The bootstrap values are shown at branching points.

The biosynthesis of tocochromanol is localized in the chloroplasts of leaves and the plastids of seeds. However, seeds are the plant organ that accumulates more distinct vitamin

E forms than other organs and in larger amounts (Dellapenna, 2005). Results for seeds showed three patterns of six gene expressions (Figure 8). The first expression pattern was detected as up-regulation during the middle stage. The Cv*VTE1* gene was up-regulated during 2–4 WAF with the highest expression at 3 WAF, being 28-fold. The highest expressed level of Cv*VTE3* was represented at 3 WAF increasing 4-fold. A similar expression pattern was observed in Cv*VTE2*, which was highest at 2 and 3 WAF, as 231-fold and 224-fold, respectively. Gene expressions were strongly up-regulated during 2-3 WAF, with a significant drop at 4 WAF. The enhancement of tocopherol in seeds depended on co-expression of *VTE2*, *VTE1* and *HPPD* with increases in tocopherol content at 2 to 2.5-fold in *B. napus* seeds (Raclaru et al., 2006). The *VTE2* gene overexpression also showed strongly increased tocopherol content of seed of *B. napus* (Kumar et al., 2005; Raclaru et al., 2006) and soybean (Karunanandaa et al., 2005), as the most efficient enhancement of tocopherol levels in an oilseed. In *Arabidopsis*, *VTE2* overexpression increased seed tocopherols from 40% to 100%, while the activity was not completely limited to *Arabidopsis* seeds (Collakova and DellaPenna, 2003).

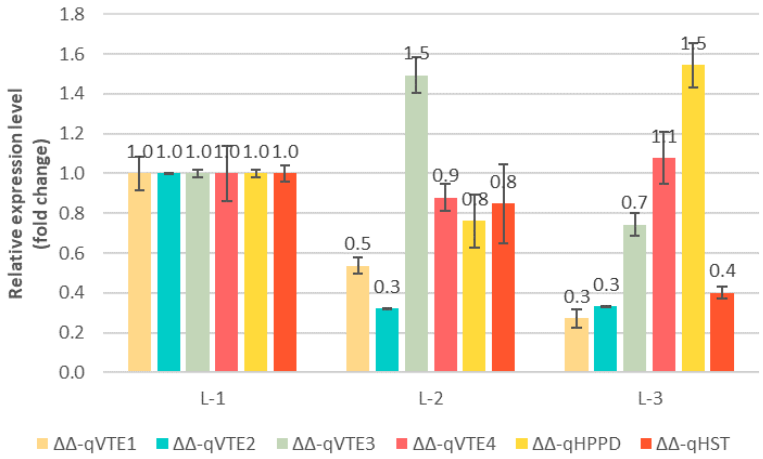

Figure 7. Relative expression level of vitamin E biosynthesis (Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST*) in leaves of *Cleome viscosa*

Another pattern was observed in Cv*VTE4* and Cv*HPPD*, with the highest peak at 4 WAF for 15-fold and 17-fold increases respectively. These genes observed up-regulation after 2 WAF without the breaker stage at 4 WAF. Typically, ripening and senescence relate to various stresses, including a decrease in chlorophyll content for disassembly of photosynthesis and lipid breakdown (Paliyath and Droillard, 1992; Dangl et al., 2000; Alagna et al., 2009). The phytyl-PP co-substrate from chlorophyll degradation increased for tocopherol biosynthesis (Ischebeck et al., 2006; Valentin et al., 2006), and may have an effect on this expresseion pattern at 4 WAF senescence stage. Rajesh et al. (2011) observed that *HPPD* transcript accumulation showed increased levels in mango fruits, followed by an increment in tocopherol levels during senescence. An increase in *HPPD* was also observed in other tissues such as leaves, particularly during senescence. Barley leaves also showed

increased *HPPD* transcript accumulation during senescence (Jon et al., 2002). The relationship between the first step of *HPPD* expression suggests flux to *HGA*, while the last step of *VTE4* is the committed step in tocochromanol synthesis. Both steps encourage limiting tocopherol synthesis. These results concurred with those reported in oat seed. The *HPPD* and *VTE4* enzymes catalyzed the first and last step in the synthesis of tocopherol and thus appear to be key points in regulating metabolic flux through the vitamin E pathway (Gutierrez-Gonzalez and Garvin, 2016). Moreover, increased expression of *VTE4* influenced tocopherol transformation from γ-tocopherol to α-tocopherol in seeds of crop plants including soybean (Van Eenennaam, et al., 2003; Karunanandaa et al., 2005) and *Brassica juncea* (Yusuf and Sarin, 2007). *VTE3* and *VTE4* from *Arabidopsis* were co-expressed in transgenic soybeans, which increased the tocopherol content in seeds (>90%).

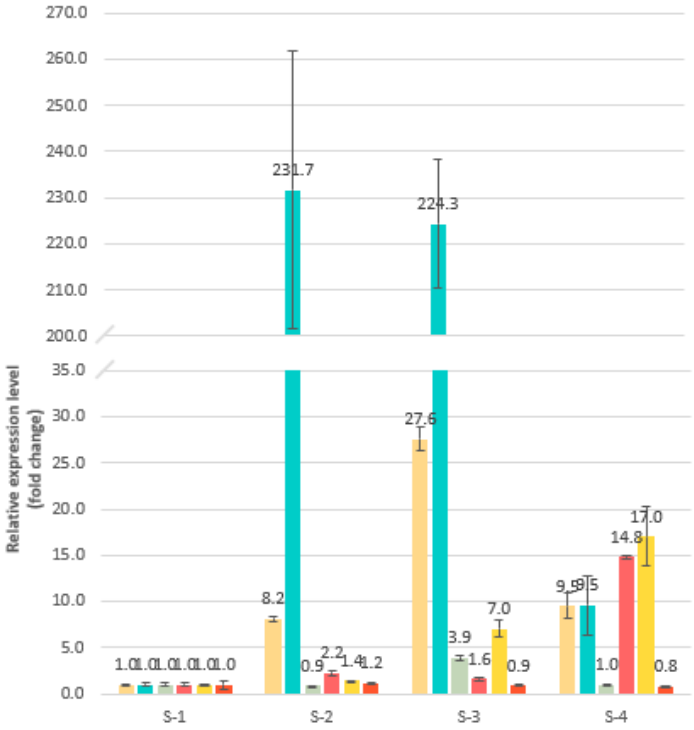

Figure 8. Relative expression level of vitamin E biosynthesis (Cv*VTE1,* Cv*VTE2,* Cv*VTE3,* Cv*VTE4,* Cv*HPPD* and Cv*HST*) in seeds of *Cleome viscosa*

However, the expressed pattern of the Cv*HST* gene was steady and regular, similar to that in the leaves. The committed step of Cv*VTE2* synthesized tocopherols, while Cv*HST* synthesized plastochromanol-8 with different patterns and expression levels. The Cv*VTE2* gene had a stronger expression compared with Cv*HST* for accumulating tocopherols. Interestingly, the most expression profile supported γ- and α- tocopherol and γ- and α-tocomonoenol accumulation during 3-4 WAF in oil seeds of *C. viscosa*.

Moreover, quantitative trail loci (QTL) and allelic variation within tocochromanol genes impact tocochromanol composition and content. *VTE2* and *HPPD* were identified on

chromosome 7 can alter total precursor influx to tocopherol biosynthesis in tomato (Almeida, et al., 2011). Polymorphism of *VTE3* and *HPPD* candidate genes were significantly correlated with tocopherol trait. Therefore, QTL, allelic variation and level of gene expression of tocopherol key genes through the control of accumulation in plants (Fritsche, et al., 2012).

CONCLUSIONS

This was the first study on *C. viscosa* to identify six genes in the biosynthetic pathway of vitamin E including Cv*VTE1*, Cv*VTE2*, Cv*VTE3*, Cv*VTE4*, Cv*HPPD* and Cv*HST*. However, all cleome tocochromanal biosynthesis genes also indicated a high degree of similarity across all species, consistent with the qualification significance of the vitamin E family in plants. Quantitative gene expression analysis demonstrated that Cv*VTE2* had a stronger expression level than Cv*HST*, and it accumulated more tocopherols than plastochromanol-8 by Cv*HST*. Interestingly, Cv*VTE2* played an important role, with highest expressions at 2 WAF and 3 WAF, while Cv*VTE1* and Cv*VTE3* were observed during 2-3 WAF in seeds. Moreover, the expressions of Cv*VTE4* and Cv*HPPD* promoted a situation with a gradual increase that reached a maximum at 4 WAF and approached senescence without any down-regulation. These results supported its role in accumulating γ- and α- tocopherol and γ- and α- tocomonoenol.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Acevedo-Rodríguez P and Strong MT (2012). Catalogue of the Seed Plants of the West Indies. Washington, DC, USA: Smithsonian Institution. 1192. Available at [http://botany.si.edu/Antilles/WestIndies/catalog.htm].

Alagna F, D'Agostino N, Torchia L, Servili M, et al. (2009). Comparative 454 pyrosequencing of transcripts from two olive genotypes during fruit development. *BMC Genet.* 10: 399-413.

Almeida J, Quadrana L, Ası´s R, Setta N, et al. (2011). Genetic dissection of vitamin E biosynthesis in tomato. *J. Exp. Bot.* 62(11): 3781-3798.

Altschul SF, Gish W, Miller W, Myers EW, et al. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.

Chaw SM, Chang CC, Chen HL and Li WH (2004). Dating the monocot-dicot divergence and the origin of core eudicots using whole chloroplast genomes. *J. Mol. Evol.* 58: 1-18.

Collakova E and DellaPenna D (2003). Homogentisate phytyltransferase activity is limiting for tocopherol biosynthesis in *Arabidopsis*. *Plant Physiol.* 131: 632-642.

Dangl JL, Dietrich RA and Thomas H (2000). Senescence and programmed cell death. In: Biochemistry & Molecular Biology of Plants (Buchanan BB, Gruissem W, Jones RL eds.). American Society of Plant Physiologists, Rockville 1044-1100.

Dellapenna D (2005). A decade of progress in understanding vitamin E synthesis in plants. *J. Plant Physiol.* 162: 729-737.

Endrigkeit J, Wang X, Cai D, Zhang C, et al. (2009). Genetic mapping, cloning, and functional characterization of the *BnaX. VTE4* gene encoding a γ-tocopherol methyltransferase from oilseed rape. *Theor. Appl. Genet.* 119: 567-575.

Flora of China Editorial Committee. (2015). Flora of China. St. Louis, Missouri and Cambridge, Massachusetts, USA: Missouri Botanical Garden and Harvard University Herbaria. Available at [http://www.efloras.org/flora_page.aspx?flora_id=2].

Fritsche S, Wang X, Li J, Stich B, Kopisch-Obuch FJ, et al. (2012). A candidate gene-based association study of tocopherol content and composition in rapeseed (*Brassica napus*). *Front Plant Sci.* 26(3): 129.

Georgiadou EC, Ntourou T, Goulas V, Manganaris GA, et al. (2015). Temporal analysis reveals a key role for *VTE5* in vitamin E biosynthesis in olive fruit during on-tree development. *Front. Plant Sci.* 6: 871-881.

Gutierrez-Gonzalez JJ and Garvin F (2016). Subgenome-specific assemble of vitamin E biosynthesis genes and expression patterns during seed development provide insight into the evolution of oat genome. *Plant Biotechnol. J.* 14: 2147-2157.

Iltis HH and Cochrane TS (2007). Studies in the Cleomaceae V: A new genus and ten new combinations for the flora of North America. *Novon.* 17: 447-451.

Ischebeck T, Zbierzak AM, Kanwischer M and Dörmann P (2006). A salvage pathway for phytol metabolism in *Arabidopsis*. *J. Biol. Chem.* 281: 2470-2477.

Jon F, Nina K, Dorothee D and Karin K (2002). The senescence associated gene of barley encoding 4-hydroxyphenylpyruvate dioxygenase is expressed during oxidative stress. *J. Plant Physiol*. 159: 1245-1253.

Kamal-Eldin A and Appelqvist LA (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids.* 31(7): 671-701.

Karunanandaa B, Qi Q, Hao M, Baszis SR, et al. (2005). Metabolically engineered oilseed crops with enhanced seed tocopherol. *Metab. Eng*. 7: 384-400.

Kumar R, Raclaru M, Schu¨ßeler T, Gruber J, et al. (2005). Characterisation of plant tocopherol cyclases and their overexpression in transgenic *Brassica napus* seeds. *FEBS Lett.* 579: 1357-1364.

Kumar S, Stecher G, Li M, Knyaz C, et al. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* 35: 1547-1549.

Kumari R, Tyagi A, Sharma V, Jain VK, et al. (2012). Variability in the accessions from Aravali range assessed for domestication of the Cleomaceae biodiesel plant *Cleome viscosa* Linn. *Indian. J. Nat. Prod. Resour.* 3: 246-255.

Larkin MA, Blackshields G, Brown NP, Chenna R, et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*. 23: 2947-2948.

Mali RG (2010). *Cleome viscosa* (wild mustard): A review on ethnobotany, phytochemistry, and pharmacology. *Pharm. Biol.* 48(1): 105-112.

Mène-Saffrané L (2018). Vitamin E Biosynthesis and Its Regulation in Plants. *Antioxidants*. 7(1): 2-18.

Mène-Saffrané L and Pellaud S (2017). Current strategies for vitamin E biofortification of crops. *Curr. Opin. Biotechnol.* 44: 189-197.

Paliyath G and Droillard MJ (1992). The mechanism of membrane deterioration and disassembly during senescence. *Plant Physiol. Biochem*. 30: 789-812.

Pallai LS and Nair BR (2013). Proximate composition, mineral elements and anti-nutritional factors in *Cleome viscosa* L. and *Cleome burmanni* W. & A. (Cleomaceae). *Int J Pharm Sci.* 5(1): 384-387.

PIER (2015). Pacific Islands Ecosystems at Risk. Honolulu, USA: HEAR, University of Hawaii. Available at [http://www.hear.org/pier/index.html].

Raclaru M, Gruber J, Kumar R, Sadre R, et al. (2006). Increase of the tocochromanol content in transgenic *Brassica napus* seeds by overexpression of key enzymes involved in prenylquinone biosynthesis. *Mol. Breed.* 18: 93-107.

Rajesh KS, Sharique AA, Pravendra Nath and Vidhu AS (2011). Activation of ethylene-responsive p-hydroxyphenylpyruvate dioxygenase leads to increased tocopherol levels during ripening in mango. *J. Exp. Bot.* 62(10): 3375-3385.

Ren W, Zhao L, Zhang L, Wang Y, et al. (2011). Molecular cloning and characterization of 4-4-hydroxyphenyl-pyruvate dioxygenasegene from *Lactuca sativa*. *J. Plant Physiol*. 168: 1076-1053.

Sattler SE, Cahoon EB, Coughlan SJ and DellaPenna D (2003). Characterization of tocopherol cyclases from higher plants and Cyanobacteria. Evolutionary implications for tocopherol synthesis and function. *Plant Physiol.* 132: 2184-2195.

USDA-ARS (2015). Germplasm Resources Information Network (GRIN). Online Database. Beltsville, Maryland, USA: National Germplasm Resources Laboratory. Available at [https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysearch.aspx].

Valentin HE, Lincoln K, Moshiri F, Jensen PK, et al. (2006). The *Arabidopsis* vitamin E pathway gene5-1 mutant reveals a critical role for phytol kinase in seed tocopherol biosynthesis. *Plant Cell*. 18: 212-224.

Van Eenennaam AL, Lincoln K, Durrett TP, Valentin HE, et al. (2003). Engineering vitamin E content: from *Arabidopsis* mutant to soy oil. *Plant Cell.* 15: 3007-3019.

Verwoerd TC, Dekker BM and Hoekema A (1989). A small- scale procedure for the Rapid isolation of plant RNAs. *Nucleic Acids Res.* 17(6): 2362.

Yusuf MA and Sarin NB (2007). Antioxidant value addition in human diets: genetic transformation of *Brassica juncea* with gamma-TMT gene for increased alpha-tocopherol content. *Transgenic Res.* 16: 109-113.