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Expression analysis of tocopherol key genes during seed development in *Cleome viscosa* Linn

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ABSTRACT. *Cleome viscosa* (Cv) Linn is an annual oilseed crop plant that has high vitamin E, with the most active form represented as the tocopherols (α, β, γ and δ). These act as antioxidants in plants and are also essential components for human health. The gene expression profiles and partial-length cDNA encoding tocopherol cyclase (CvVTE1), homogentisic acid prenyltransferase (CvVTE2), MPBQ methyl-transferase (CvVTE3), gamma-tocopherol methyltransferase (CvVTE4), hydroxyphenyl-pyruvate dioxygenase (CvHPPD) and homogentisate solanesyltransferase(CvHST) genes involved with tocopherol biosynthetic enzymes were determined. Partial cDNA sequences of six genes as 694 bp CvVTE1, 375 bp CvVET2, 387 bp CvVTE3, 402 bp CvVET4, 334 bp CvHPPD and 461 bp CvHST showed high identity to their homologs in other higher plants. Expression levels of CvVET1, CvVET2, CvVET3, CvVET4, CvHPPD and CvHST genes were analyzed by real-time quantitative PCR (qPCR) in 3 degrees of plant nodes (L1-L3) in leaves and 1-4 weeks after fruit set (WAF) of seeds. Results showed that CvVET3 and CvHPPD were dominantly expressed in the leaves, while the seeds showed that CvVET2 was obviously

expressed in both 2 and 3 WAF at 231-fold and 224-fold, which cooperated with CvVTE1 and CvVTE3 expression. Co-expression of CvVET4 and CvHPPD supported high levels of flux toward the synthesis of tocopherols at 4 WAF without any down-regulation. Results indicated that CvVET2 played a key role in tocopherol biosynthesis in the seed of *Cleome viscosa.*

Keywords: *Cleome viscosa;* Tocophero; Homogentisic acid prenyltransferase; Real-time quantitative PCR

INTRODUCTION

Cleome viscosa Linn 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, and wastelands and along roadsides. The whole plant, leaves, seeds, and roots of *Cleome viscosa* are widely used in traditional medicine as a remedy to promote cooling, 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 *Cleome viscosa*, with vitamin E showing relatively high contained amounts of 0.318 mg/g (Pallai and Nair, 2013).

Vitamin E is a generic term for a group of compounds, which 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), Geranyl Geranyl Diphosphate (GGPP), Tetrahydrogeranylgeranyl Diphosphate (THGGPP) and Solanesyl Diphosphate (SPP), respectively. 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), where 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, encouraging 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 CvVTE1, CvVTE2, CvVTE3, CvVTE4, CvHPPD and CvHST genes, which involved the tocopherol biosynthetic enzyme. Developmental programs in *Cleome viscosa* comprised a period of 3 degrees of plant nodes in leaves and 4 WAF in seeds.

MATERIAL AND METHODS

Plant materials

Leaves and seeds of C. viscosa were used to conduct the following experiment. Leaves from 3 developmental stages, designated as L1 (nodes 1-5), L2 (nodes 10-15) and L3 (nodes 20-25), and seeds from 4 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 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 nano drop spectrophotometer. First-strand cDNA was synthesized using a Thermo Scientific Revert Aid 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 l 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.

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 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

Table 1. Degenerate primers used for RT-PCR analyses.

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

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

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

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 integrity 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 CvVTE1, CvVTE2, CvVTE3, CvVTE4, CvHPPD and CvHST. Sequence analysis showed that CvVTE1 (MW193603) was a 695 bp (231 aa) that encoded 52.53% of coding sequences (CDS) compared with Tarenaya hassleriana (Th) VTE1, CvVTE2 (MW193601) was a 376 bp (125 aa) (31.89% of CSD compared with ThVTE2), CvVTE3 (MW193602) was a 387 bp (128 aa) (36.26% of CSD compared with ThVTE3), CvVTE4 (MW193604) was a 366 bp (121 aa) (36.55% of CSD compared with ThVTE4), CvHPPD (MW193600) was a 334 bp (111 aa) (24.47% of CDS compared with ThHPPD, and CvHST (MW193605) was a 461 bp (153 aa) (40.02% of CSD compared with CSD of ThHST). BLAST analysis on NCBI databases identified single cDNA sequences for each gene. The CvVTE1 gene shared high similarity with VTE1 from T. hassleriana (92.21%), Eutrema salsugineum (86.77%) and Thellungiella halophile (86.77%), while the CvVTE2 gene showed high similarity to VTE2 from T. hassleriana (90.13%), Arabidopsis lyrata subsp. lyrata (88.24%) and Camelina sativa (88.24%). The CvVTE3 gene had similarity with VTE3 from T. hassleriana (90.13%), Raphanus sativus and Brassica rapa (85.79%). The CvVTE4 gene shared >75% nucleotide sequence identity with VTE4 from T. hassleriana (82.61%), E. salsugineum (79.57%) and B. napus (76.50%). The CvHPPD gene shared high similarity with HPPD from T. hassleriana (88.82%), E. salsugineum (84.32%) and Raphanus raphanistrum (81.98%). The CvHST 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 2 monophyletic clades; monocot and dicot. Six genes, CvVTE1, CvVTE2, CvVTE3, CvVTE4, CvHPPD and CvHST 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 140-150mya 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).Temporal and spatial expressions of the six vitamin E biosynthesis genes (CvVTE1, CvVTE2, CvVTE3, CvVTE4, CvHPPD and CvHST) in leaves and seeds were investigated using qRT-PCR. The expression levels were different among various tissues. Genes in the biosynthetic pathway expressed 3 stages of leaves at 1-4 weeks after fruit set (WAF) of seeds. 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 CvVTE3 was the most expressed in the L2 stage. Arabidopsis VTE3 mutants showed decreased levels of γ- and α-tocopherol in leaves (Sattler et al., 2003). Different expression patterns for CvVTE2, CvVTE1 and CvHST were distinguished in the L1 stage and decreased at L2 and L3. Moreover, expression of the CvHST gene was consistent in every stage. The CvHPPD 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 LsHPPD in mature leaves (Ren et al., 2011), while up-regulation of HPPD resulted in over-ripening processes (Georgiadou et al., 2015).

CONCLUSION

This was the first study on C. viscosa to identify six genes in the biosynthetic pathway of vitamin E including CvVTE1, CvVTE2, CvVTE3, CvVTE4, CvHPPD and CvHST. 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 CvVTE2 had a stronger expression level than CvHST, and accumulated more tocopherols than plastochromanol-8 by CvHST. Interestingly, CvVTE2 played an important role, with highest expressions at 2 WAF and 3 WAF, while CvVTE1 and CvVTE3 were observed during the middle stage. Moreover, the expressions of CvVTE4 and CvHPPD 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.

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