Cloning and expression analysis of *Cucumis sativus* L. *CER4* involved in cuticular wax biosynthesis in cucumber

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**ABSTRACT**

Eceriferum (*cer*) genes are a group of genes that have functions in wax biosynthesis, which play important roles in plant epidermal permeability, drought resistance, fertility, and organ fusion. In this study, *CsCER4*, an *AtCER4* homologue, was cloned from cucumber (*Cucumis sativus* L.). The full-length cDNA of *CsCER4* is 1464 bp, encoding a protein with 487 amino acid residues. *CsCER4* was expressed in all analysed tissues, including leaves, male flowers, female flowers, and fruit. Its expression level among fruit development increased and then decreased, and the expression was markedly different between 3413 (glossy type cucumber) and 3401 (waxy type cucumber), suggesting that *CsCER4* has a role in wax biosynthesis.

**ARTICLE HISTORY**

Received 8 January 2018
Accepted 9 July 2018

**KEYWORDS**

Cucumber; wax biosynthesis; *CsCER4*; gene

**Introduction**

Most of the terrestrial plants are covered with a layer of wax on the epidermis [1]. As the aboveground protective layer of plant, wax plays a vital role in protecting the plants from being burnt by ultraviolet radiation, in reducing the adhesion of dust and pathogenic bacteria and in preventing non-stomatal transpiration [2–8]. It is synthesized in epithelial cells and then secreted out onto the epidermis of plants. Wax can be either embedded into cutin (within the matrix of cutin) or not (deposited on the external surface of the polymer). It is made up of long-chain compounds (with a chain length longer than C20) and cyclic compounds. The former mainly include derivatives of very-long-chain fatty acids (VLCFA), such as alcohols, alkanes, esters, aldehydes, and ketones; whereas the latter are composed of aromatic compounds and alicyclic compounds [9]. The chain length of such substances is usually between C20 and C36, mainly C24 and C34 [10,11]. The components of wax differ among species. Even the same species may have dynamic wax content during different periods of growth or in different tissues in the same period of growth [12].

Previous studies have unveiled the synthetic process of wax in *Arabidopsis*, which can be split into three steps. The first step is the complete synthesis of C16 and C18 fatty acids in plastids. Those two fatty acids are the common precursor substances for the synthesis of all lipid substances within plants. In the second step, C16 and C18 fatty acids in the endoplasmic reticulum are further extended to form VLCFAs with a length of C20–C34. From there, C20–C34 acyl-CoAs are divided into alkane pathway and primary alcohol pathway according to their products. The alkane pathway produces aldehydes, VLC alkanes, secondary alcohols, and ketones, whereas the primary alcohol pathway generates primary alcohols and esters [13,14].

According to existing studies, *CER4* in *Arabidopsis* encodes an alcohol-forming fatty acyl-coenzyme A reductase (FAR), which plays a critical role in the synthesis of primary alcohols. Accordingly, exogenous expression of *AtCER4* in *Saccharomyces cerevisiae* promotes the accumulation of C24:0 and C26:0 primary alcohols [15].

Although the mechanism of wax biosynthesis in *Arabidopsis* has been relatively defined, only a small number of genes encoding wax synthesis enzymes from cucumber (*Cucumis sativus* L.) have been cloned and functionally verified. As suggested by previous studies, *CsCER1* and *CsWAX2* play pivotal roles in wax biosynthesis in cucumber. Abnormal expression of *CsCER1* in the *wax2* mutant of *C. sativus* provides dramatic effects on the alkane content [16]. Beyond that, the ectopic expression of *CsWAX2* in the *wax2* mutant of *C. sativus* has been shown to increase the production of both alkane and primary alcohol content [17].
Arabidopsis could partially revert the glossy stem phenotype. Furthermore, altered expression of CsWAX2 also shows changes in cutin biosynthesis, pollen viability, and resistance to biotic stresses [16]. On the other hand, cucumbers overexpressing CsCER1 have specifically increased content of very-long-chain (VLC) alkanes and increased drought tolerance [17]. CsCER1 can be induced by ABA (abscisic acid) and it plays a role in regulating the formation of wax in the pericarp of cucumber [18].

To further define the wax biosynthesis pathway in cucumber, we cloned the CsCER4 gene, analysed its genetic buildup and protein conservation. Its expression patterns in different organs and fruit and at different developmental stages were analysed using real-time polymerase chain reaction (RT-PCR), in order to clarify the role of CsCER4 in wax biosynthesis. The reported results may provide new theoretical ground for investigation into the molecular mechanism of wax formation in cucumber.

Material and methods

Plant material and growth conditions

The test materials were Cucumis sativus L. of high-generation inbreeding line 3401 and 3413 provided by the Greenhouse Vegetables Growth and Development Regulation Beijing Laboratory, China Agricultural University. The cucumber line 3401, which shows waxy fruit phenotype, is much taller than the cucumber line 3413, which displays glossy fruit phenotype. The cucumber seeds were first placed in water at 50–60 °C and then left still after natural cooling. Soaked seeds were then spread into a culture vessel laid with wet gauze for 3 h, and the vessel was put into a black incubator at 28 °C to stay overnight. Sprouting seeds were then sowed into prepared nutrient soil (flowers and plants nutrient soil: vermiculite =2:1), and the incubator was set to have 16 h illumination (25 °C) and 8 h darkness (18 °C). Robust seedlings with two leaves and one stem were transplanted into a solar greenhouse.

Gene cloning

Using the protein sequence of Arabidopsis (AT4G333790), the most homologous gene Csa6G151810 was selected from the cucumber genome database (http://cucurbitgenomics.org/) through sequence alignment, and was named CsCER4. The PCR primers were designed on the basis of the CDS sequence of the aforesaid genes: upstream primer: 5′-ATGGAGTTTCTTGAGAAACAGAGCA-3′; downstream primer: 5′-TCATTGTGACATGTGTTAACAAGT-3′. The RNA of cucumber was extracted with RNA extraction kit purchased from Promega[AQ], and cDNA was synthesized with a reverse transcription kit from ABI. The primers were designed according to the known sequence, and amplification was conducted with cDNA as the template using hi-fi DNA polymerase enzyme. The amplification cycle was as follows: 5 min pre-degeneration at 95 °C, 30 s degeneration at 95 °C, 30 s annealing at 52 °C; 90 s extension at 72 °C, 33 cycles, and 10 min extension at 72 °C (ABI 7500). Afterwards, the PCR product was subjected to gel electrophoresis and extraction, and the extracted product was used for cloning into pMD18-T vector. The cloning product was used to transform Escherichia coli DH5α strain. After PCR confirmation, the positive monoclonal product containing the target fragment was sent to Huada Biological Technology Service Co., Ltd (Shanghai, China) for sequencing verification.

Sequence alignment and phylogenetic analysis

The predicted amino acid sequence of CsCER4 was subjected to BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org) to extract the amino acid sequences of the CER4 protein in different species. ClustalW program in [AQ]MEGA5 software was employed to compare the protein sequences of CsCER4, AtCER4, CmCER4, McCER4, and PaCER4, and MEGA version 5.0 to construct a phylogenetic tree [19]. The neighbour-joining (NJ) method was used.

Gene expression analysis by real-time PCR

Stems, spires, newly blooming male and female flowers of cucumber and cucumber fruit at different growth periods (2 days before flowering, the flowering day, 4 days after flowering, 6 days after flowering and 8 days after flowering) were collected for analysis of the expression of CsCER4. With cDNA as the template, amplification was performed on a 7500 RT-PCR machine through the SYBR® Premix Ex TaqTM fluorescent quantitation kit by Dalian RaKaRa Biology Company. The primers of CsCER4 were 5′-AGCCATGAAGAGACTTGGGGTCTC-3′ and 5′-CAATCCAGCCAGAAAAGGT-3′. The reference gene used was TUA, and the primers were 5′-ACGCTTGTGGTGTTGCTAC-3′ and 5′-GAGAGGGTAAACAGTGAAATC-3′. The PCR amplification process was as follows: 30 s pre-degeneration at
95 °C, 5 s degeneration at 95 °C, 35 s renaturation at 60 °C, 40 cycles. The relative transcript level of the gene was determined according to the 2^−ΔΔCt relative quantitative analysis method.

**Results and discussion**

**Molecular cloning and sequence analysis of CsCER4**

The BLAST analysis in the NCBI database showed some AtCER4 homologs, among which the cucumber-belonging gene was designated as CsCER4. The CDS sequence of CsCER4 was obtained through homology-based cloning. The cDNA sequence of CsCER4 is 1464 bp. The gene structure analysis revealed that CsCER4 has a similar genetic structure to that of AtCER4, which also contains 10 exons and 9 introns (Figure 1(a)). Encoding 487 amino acids, CsCER4 contains a conservative fatty acyl-CoA reductase family domain. Amino acid sequence comparison of CsCER4 with AtCER4, CmCER4, McCER4, and PaCER74 suggests that CsCER4 and CER4 proteins from other species share the structural domain of the fatty acyl-CoA reductase family (Figure 1(b)). CsCER4 is most homologous to McCER4 in protein sequence, with a score of 75.81%, and its homology to CmCER4, PaCER4, and AtCER4 is 74.90, 59.03, and 53.92%, respectively. This shows the highly conservative nature of the amino acid sequence of CsCER4. It can be thus speculated that CsCER4 is a fatty acyl-CoA reductase that has a similar function to that of AtCER4 in Arabidopsis, affecting the biosynthesis of wax.

**Phylogenetic tree analysis**

In order to dissect the evolutionary relationship between CsCER4 and other CER4 proteins, we utilized the protein sequences of 22 CER4s from the genomes...
of cucumber, *Arabidopsis*, *Momordica charantia*, *Cucurbita maxima*, *Theobroma cacao*, etc. to build a phylogenetic tree (Figure 2). The results suggested that the CER4 protein has evolved into two clades in which CsCER4 remains most genetically close to CmCER4 and is on the same clade with AtCER4. It can be thus inferred that CsCER4 is a member of the wax-formation-related CER family.

**Expression pattern of CsCER4**

To analyse the expression of CsCER4 in the fruit development process, we performed quantitative real-time PCR (qRT-PCR) (Figure 3). The results showed that the CsCER4 transcripts increased first and then decreased during the fruit development process. It is noteworthy that the expression level of CsCER4 was higher in the glossy type (3413) than in the waxy (3401 type). The cucumber glossy phenotype is reportedly caused mainly by a decrease in alkanes, which accounted for 46.1% of the total fruit cuticular wax [16]. With VLCFA as the common precursor of alkanes and primary alcohols, reduction of alkane synthesis may result in promotion in primary alcohol production [14,20,21].

Previous studies have demonstrated this inference: in the stem of the *Arabidopsis wax2* mutant, the percentage of alkanes decreased from 52.9 to 20.6%, whereas the percentage of primary alcohols increased from 11.3 to 50.1% [22]. Therefore, we infer that this is why the CsCER4 expression level in 3413 (glossy type) was higher than that in 3401 (waxy type). These results suggest that CsCER4 plays a role during wax formation in cucumber.

The qRT-PCR analysis of the expression pattern of CsCER4 in different tissues of cucumber showed that CsCER4 was expressed in all the organs and tissues, especially high in male flowers, female flowers and fruits, but low in stems and leaves (Figure 4). It is noteworthy that CsCER4 was most highly expressed in male flowers. Previous studies have shown that AtCER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase. The trend in CsCER4 expression was consistent with that in the expression of AtCER4, both of which have highest expression in flowers [15]. Previous studies report that tryphine, deposited on mature pollen grains, contains esters, and other kinds of waxes [23,24]. In cucumber, CsWAX2 is also strongly expressed in pollen. Its abnormal expression affects...
not only the pollen viability, but also the pollen germination and the pollen tube length [16].

**Conclusions**

*CsCER4* belongs to the CER family. As a homologue of *AtCER4*, it possesses the fatty acyl-CoA reductase family domain in the C-terminal region, which catalyses the reduction of fatty acyl-CoA to fatty alcohols. Expression analysis showed that *CsCER4* is expressed in various tissues, including leaves, male flowers, female flowers, and fruit, with especially high expression in the fresh male flowers, where pollen exists in great amounts. *CsCER4* expression appeared to increase first and then decline in the fruit development process. Phenotypically, *CsCER4* expression correlated with wax synthesis in 3413 (glossy type) rather than in 3401 (waxy type). These results demonstrate that *CsCER4* likely plays a role in wax formation, and will facilitate further research on the wax formation mechanisms in cucumber.

**Acknowledgements**

The authors thank the members of Prof. Hou’s Laboratory for helpful discussions and technical assistance.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This study was supported by the Key research and development projects of Shanxi Province [201703D211001], the Natural Science Foundation of Shanxi Province [201701D221155] and the Science and Technology
References

[1] Lee SB, Suh MC. Advances in the understanding of cuticular waxes in *Arabidopsis thaliana* and crop species. Plant Cell Rep. 2015;34(4):557–72.

[2] Fukuda S, Satoh A, Kasahara H, et al. Effects of ultraviolet-B irradiation on the cuticular wax of cucumber (*Cucumis sativus*) cotyledons. J Plant Res. 2008;121:179–189.

[3] Eigenbrode SD, Espelie KE. Effects of plant epicuticular lipids on insect herbivores. Annu Rev Entomol. 1995;40:171–194.

[4] Eigenbrode SD, Rayor L, Chow J, et al. Effects of wax bloom variation in *Brassica oleracea* on foraging by a vespid wasp. J Exp Bot. 2000;51:274.

[5] Kerstiens G. Cuticular water permeability and its physiological significance. J Exp Bot. 1996;47:1813–1832.

[6] Riederer M, Schreiber L. Protecting against water loss: analysis of the barrier properties of plant cuticles. J Exp Bot. 2001;52:2023–2032.

[7] Liu DM, Tang J, Liu ZZ, et al. *Cgl2* plays an essential role in cuticular wax biosynthesis in cabbage (*Brassica oleracea* var. *capitata*). BMC Plant Biol. 2017;17(1):223.

[8] Gölz PG, Müller E, Schmitz K, et al. Chemical composition and surface structures of epicuticular leaf waxes of *Ginkgo biloba*, *Magnolia grandiflora* and *Liriodendron tulipifera*. Z Naturforsch C. 1992;47:516–526.

[9] Buschhaus C, Jetter R. Composition differences between epicuticular and intracuticular wax substrates: how do plants seal their epidermal surfaces? J Exp Bot. 2011;62:841–853.

[10] Kunst L, Samuels A. Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res. 2003;42:51–80.

[11] Kunst L, Samuels L. Plant cuticles shine: advances in wax biosynthesis and export. Curr Opin Plant Biol. 2009;12:721–727.

[12] Jetter R, Kunst L. Plant surface lipid biosynthetic pathways and their utility for metabolic engineering of waxes and hydrocarbon biofuels. Plant J. 2008;54:670–683.

[13] Samuels L, Kunst L, Jetter R. Sealing plant surfaces: cuticular wax formation by epidermal cells. Plant Biol. 2008;59:683–707.

[14] Rowland O, Zheng H, Hepworth SR, et al. *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in *Arabidopsis*. Plant physiol. 2006;142:866–877.

[15] Wang WJ, Liu XW, Gai XS, et al. *Cucumis sativus* *WAX2* plays a pivotal role in wax biosynthesis, influencing pollen fertility and plant biotic and abiotic stress responses. Plant Cell Physiol. 2015;56(7):1339–1354.

[16] Wang WJ, Zhang Y, Xu C, et al. *Cucumis sativus* *ECERIFERUM1* (*CsCER1*), which influences the cuticle properties and drought tolerance of cucumber, plays a key role in VLC alkanes biosynthesis. Plant Mol Biol. 2015;87:219–233.

[17] Liu XF, An JB, Zhang LX, et al. Cloning and expression analysis of *CsCER7*, a relative gene may regulate wax synthesis in cucumber. Acta Horticulturae Sinica. 2014;41(4):661–671.

[18] Tamura K, Peterson D, Peterson N, et al. MEGAS: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–2739.

[19] Takuya T, Kaeko K, Takahisa O, et al. Wax ester synthase/diacylglycerol acyltransferase isoenzymes play a pivotal role in wax ester biosynthesis in *Euglena gracilis*. Sci Rep. 2017;7(1):13504.

[20] Preuss D, Lemieux B, Yen G, et al. A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. Genes Dev. 1993;7:974–985.

[21] Aarts M, Keijzer CJ, Stiekema WJ, et al. Molecular characterization of the *CER1* gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell. 1995;7:2115–2127.