Article

Function and transcriptional regulation of CsKCS20 in the elongation of very-long-chain fatty acids and wax biosynthesis in Citrus sinensis flavedo

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Abstract

Cuticular wax on plant aerial surfaces plays a vital role in the defense against various stresses, and the genes related to wax metabolism have been well documented in several model plants. However, there is very limited research on the key enzymes and transcription factors (TFs) associated with carbon chain distribution and wax biosynthesis in citrus fruit. In this study, an analysis of wax metabolites indicated that even carbon-chain (C24-C28) metabolites are the dominant wax components in citrus fruit, and a 3-ketoacyl-CoA synthase (KCS) family gene (CsKCS20) plays an important role in the carbon chain distribution during wax biosynthesis in a wax-deficient mutant (MT). Expression of CsKCS20 in yeast indicated that CsKCS20 can catalyze the biosynthesis of C22 and C24 very-long-chain fatty acids (VLCFAs). In addition, transcriptome and sequence analysis indicated that the differential expression of CsKCS20 between the wild-type (WT) and MT fruit can be partly attributed to the regulation of CsMYB96, which was further confirmed by yeast one-hybrid (Y1H) assays, electrophoretic mobility shift assays (EMSAs) and dual luciferase assays. The functions of CsMYB96 and CsKCS20 in wax biosynthesis were further validated by heterologous expression in Arabidopsis. In summary, this study elucidates the important roles of CsKCS20 and CsMYB96 in regulating VLCFA elongation and cuticular wax biosynthesis, which provides new directions for the improvement of citrus fruit wax quality in genetic breeding programs.

Introduction

The plant aerial surface is generally coated with a layer of cuticular wax that is synthesized by epidermal cells and acts as a barrier between the environment and plants [1–3]. Recently, many reports have suggested that the total content of wax and the proportions of individual wax components vary significantly among different species and drive adaption to diverse terrestrial habitats during the evolutionary process [4, 5]. In Arabidopsis, C29 alkanes are the dominant component of wax in various organs, while C31 and C33 alkanes and several triterpenes are the dominant wax metabolites in tomato fruit [1, 6, 7]. As odd-chain alkanes are transformed from the corresponding even-chain very-long-chain fatty acyl-CoAs (VLC acyl-CoAs) and/or aldehydes [8–10], it can be inferred that C30, C32 and C34, which originate from the fatty acid elongation process, are the dominant carbon chain lengths in Arabidopsis and tomato. AtKCS6/CER6 and SICER6 are important proteins in the fatty acid elongase (FAE) complex, and functional analysis has revealed that they are responsible for the biosynthesis of VLCFAs above C24 and C28, respectively [11, 12]. In addition, previous research on the diversity of cuticular wax between Satsuma mandarin and “Newhall” navel orange has demonstrated that wax biosynthesis is synchronous with fruit maturation by transcriptomic and metabolomic profiling [13, 14]. However, different from the findings in Arabidopsis and tomato, research on citrus wax has revealed that C24, C26 and C28 aldehydes are the dominant wax metabolites [13–15], and there has been very limited research on the regulation of the special carbon chain distribution and wax components in citrus fruit.

Owing to its important role in the response to several stresses, the transcriptional regulation of Arabidopsis wax has been extensively studied. MYB96 has been identified as a well-known transcription factor (TF) that participates in cuticular wax biosynthesis, it can directly induce the expression of genes involved in cuticular wax biosynthesis, such as AtKCS1, AtKCS2, AtKCS6 and AtKCR1 [16–18]. In addition, other MYB TFs, such as MYB16 [19],

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of VLC acyl-CoAs above C20 is blocked, we identified a
in this study, by comparing “Newhall” navel orange (WT)
vest quality [2, 25]. In tomato fruit, several TFs, such as
defense against pathogen infection to maintain posthar-
plates. In contrast, the MT fruit surface showed much
scattered distribution of dense platelets and crystalline
findings are mainly focused on the description of wax-
process [24] but also prevents water loss and promotes
defense against fungal pathogens [15, 32, 33].
In citrus, research on TFs involved in fruit cuticular wax biosynthesis is limited.
Citrus is one of the most important fruits worldwide
and has attracted much attention in research illustrating
the regulatory mechanism of fruit quality formation. However, the high heterozygosity, long juvenile phase
and difficulty in transgenic processes have largely hin-
dered the validation of such regulatory mechanisms, and there are only a limited number of reports on the biosynthesis of anthocyanins and carotenoids based on
natural mutants [29–31]. Over the past decade, although
several glossy citrus mutants have been analyzed by
integrated transcriptome and metabolome analysis, the
findings are mainly focused on the description of wax-
deficient phenotypes, influence on plastid membrane
lipids and defense against fungal pathogens [15, 32, 33].
In this study, by comparing “Newhall” navel orange (WT)
with its glossy mutant (MT), in which the elongation
of VLC acyl-CoAs above C20 is blocked, we identified a
wax biosynthesis enzyme, CsKCS20, which can catalyze
the elongation process to form C22 and C24 VLCFAs and plays important roles in carbon chain distribution and cuticular wax biosynthesis in citrus fruit. In addition, CsKCS20 can be directly activated by CsMYB96 (homolog of AtMYB96) to promote the formation of C22 and C24 VLC acyl-CoAs and wax biosynthesis in citrus fruit. Our findings are expected to fill the gap in knowledge regarding the molecular mechanisms underlying the special carbon chain distribution and wax biosynthesis on citrus fruit surfaces.

Results
Wax deficiency in the glossy “Newhall” navel orange mutant increased fruit water loss
The surface of mature MT fruit was much glossier than that of WT fruit (Fig. 1a). To analyze the microstructure of cuticular wax crystals, we evaluated the flavedo of mature WT and MT fruit by scanning electron microscopy (SEM) (Fig. 1b). The WT fruit surface exhibited an amorphous structure of epicuticular wax, with a scattered distribution of dense platelets and crystalline plates. In contrast, the MT fruit surface showed much fewer rugged wax crystals and a smooth wax film. Cuticular wax is very important in plant resistance to drought stress and shelf life maintenance of fresh fruit by reducing nonstomatal water loss [25]. The postharvest water loss of the MT was substantially higher than that of the WT during the whole storage period, particularly at 24 days after storage, with an approximately 2.37-fold change in the MT (18.40 ± 0.38%) relative to that in the WT (7.77 ± 0.47%) (Fig. 1c). However, no significant differences were found between the WT and MT fruit in internal quality, including TSS, TA and maturity index (TSS/TA ratio), indicating that the internal quality was hardly influenced (Fig. 1d, e, and f).

Blocking VLCFA elongation above C20 led to wax deficiency in MT fruit
To further investigate the specific difference in wax components between the MT and WT, fruit cuticular wax was quantified by gas chromatography-flame ionization detection (GC-FID). As shown in Fig. 2a, the total wax decreased by an approximately 8-fold change in MT (142.46 ± 18.17 μg dm⁻²) relative to that in WT (1138.10 ± 46.16 μg dm⁻²). Analyses of individual cuticular wax components revealed that almost all aliphatic compounds were reduced in the MT compared with those in the WT (Fig. 2b). The cuticular wax of WT fruit consisted of chain lengths of 20–34 carbons, among which the even-carbon chains C24, C26 and C28 were dominant over C30 and C32 (Fig. 2c), while the components of cuticular wax above C20 significantly decreased on the MT fruit surface (Fig. 2c). These results demonstrated that the cuticular wax biosynthesis pathways were significantly influenced and that the elongation of VLCFAs above C20 was more seriously blocked in the MT. In addition, since the components of C24, C26 and C28 are dominant in citrus wax and are the early products of VLCFA elongation of C20 metabolites, WT and MT fruits are appropriate materials for studying the molecular mechanism underlying the carbon chain distribution in citrus fruit wax.

ER-localized CsKCS20 played a vital role in the distribution of carbon chains above C20 in citrus fruit wax
Considering that β-ketoacyl-CoA synthase (KCS) is the rate-limiting enzyme in the VLCFA elongation process and determines the carbon chain distribution of wax components, we first analyzed the expression of all 16 KCS family genes based on previous transcriptome data [32] (Supplementary Fig. S1). Nine KCSs were expressed in the mature flavedo of the WT and MT (FPKM >10) (Supplementary Table S1). To further confirm the differential expression of these nine genes between the WT and MT, their flavedo was collected at the mature stage in 2019 and 2020 and then used to analyze the transcriptional levels of these genes by qRT-PCR. As a result, the expression of CsKCS3, CsKCS6, CsKCS10, CsKCS11–3, CsKCS19–1 and CsKCS20 was significantly

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reduced in the MT (Fig. 3a and Supplementary Fig. S2). Although several KCS genes were downregulated, such as CsKCS6, which might influence the biosynthesis of VLCFAs above C24, it is worth noting that CsKCS20, the closest homolog to AtKCS20 and AtKCS2, which is speculated to catalyze the production of C22 and C24 VLCFAs, was the most significantly downregulated in MT. This result is in agreement with the blocked biosynthesis of cuticular wax components above C20. Hence, it could be inferred that downregulation of CsKCS20 might decrease the amount of metabolites longer than C20 in the cuticular wax of the MT.

Moreover, we performed transient overexpression of a CsKCS20-GFP fusion protein in tobacco leaves with the ER membrane marker RFP-HDEL. The results revealed that the CsKCS20 protein was colocalized with the ER membrane marker (Fig. 3b), revealing that CsKCS20 is localized to the ER, which is consistent with its function in catalyzing VLCFA formation on the ER.

CsKCS20 catalyzed the formation of C22 and C24 fatty acids in yeast cells

Due to its low accumulation of C22 and C24 VLCFAs [34], the yeast mutant strain BY4741 Δfah1 was used to investigate the function of KCS20. The CsKCS20 protein was expressed in BY4741 Δfah1 yeast, and the yeast fatty acyl chains were quantified by gas chromatography–mass spectrometry (GC–MS) (Fig. 4a).

As shown in Fig. 4b, the yeast cells expressing CsKCS20 accumulated larger amounts of C22 (0.17 ± 0.01 vs. 0.01 ± 0.01 μg/OD600 unit) and C24 (0.24 ± 0.02 vs. 0.03 ± 0.01 μg/OD600 unit) VLCFAs than those expressing the empty vector. In contrast, no significant differences in C20, C26 and C28 VLCFAs were observed between them.

CsMYB96 specifically bound to the CsKCS20 promoter and significantly activated its expression

Sequencing analysis revealed that there was no difference in the promoter and coding sequences of CsKCS20 between MT and WT (Supplementary Table S2a and b). We therefore hypothesize that CsKCS20 may be regulated by an upstream TF whose expression level is different in the MT and WT. To trace the upstream TF that regulates CsKCS20 expression, we analyzed the promoter of CsKCS20 and the expression patterns of TFs related to wax biosynthesis in the flavedo of mature MT and WT fruit. The results showed that the promoter of CsKCS20 harbored at least four conserved motifs, which
are analogous to the DNA binding sequences of the MYB family (Supplementary Fig. S3). In addition, CsMYB96, an important TF that regulates wax synthesis, was consistently downregulated in the MT from 2018 to 2020 and exhibited a high correlation with the expression pattern of CsKCS20 (Supplementary Fig. S4). Thus, it could be inferred that CsMYB96 may be responsible for the difference in CsKCS20 expression and cuticular wax between the MT and WT.

The subcellular localization assay indicated that the CsMYB96 protein was colocalized with the nuclear marker DAPI (Fig. 5a), which is consistent with the function of CsMYB96 in regulating gene expression in the nucleus. To determine whether CsMYB96 binds to the promoter of CsKCS20, Y1H and EMSAs were conducted. The Y1H results indicated that CsMYB96 could bind to the promoter of CsKCS20 (Fig. 5b). In addition, the EMSA results indicated that purified 6*His-CsMYB96–6*His protein could specifically bind to the 20-bp probe containing the CAGTTA sequence found in the CsKCS20 promoter, while the mutant probe (GGGGGG) hardly affected the binding activity (Supplementary Fig. S5a and Fig. 5c). In addition, three other predicted binding sites were also combined with the purified 6*His-CsMYB96–6*His protein (Supplementary Fig. S5b). To further investigate the regulatory effect of CsMYB96 on CsKCS20 expression, a dual-luciferase assay (LUC) was carried out. Approximately 1.7 kb of the CsKCS20 promoter, including the MYB binding consensus sequence, was fused to the firefly luciferase reporter gene. The LUC/REN ratio was increased by approximately 2.44–5.14-fold relative to the empty SK control, indicating
that CsMYB96 can significantly induce the expression of CsKCS20 (Fig. 5d). In summary, these results demonstrated that CsMYB96 can directly activate the expression of CsKCS20.

**Ectopic expression of CsKCS20 and CsMYB96 increased cuticular wax on Arabidopsis stems**

To further understand the exact function of CsKCS20 and CsMYB96 in cuticular wax biosynthesis, over-expression (OE) of CsKCS20 and CsMYB96 was conducted in Arabidopsis Col-0. Among the transgenic lines, CsKCS20 OE-4, CsKCS20 OE-8, CsMYB96 OE-13 and CsMYB96 OE-29 were selected for further experiments (Supplementary Fig. S6a). The total wax load in the inflorescence stems of CsKCS20 OE-4 and CsKCS20 OE-8 was significantly higher than that of the WT, which could be mainly attributed to the increase in C_{29} alkanes (Fig. 6a and b). An analysis of the cuticular wax of CsMYB96 OE-13 and CsMYB96 OE-29 transgenic lines revealed that the cuticular wax content was 124% and 139% that of the wild type, respectively (Fig. 6a), which was also generally higher than that of the CsKCS20 OE lines. These results could be ascribed to the significant accumulation of most dominant stem cuticular wax components, including C_{26} and C_{28} primary alcohols, C_{29} secondary alcohols, C_{29} alkanes and C_{29} ketones (Fig. 6b).

**Discussion**

Although cuticular wax plays vital roles in the ripening process and postharvest storage, little is known about the enzymes responsible for the carbon chain distribution of cuticular wax components and the related transcriptional regulation in citrus fruit. Here, we demonstrate that CsKCS20 can catalyze the formation of C_{22} and C_{24} VLC acyl-CoAs to participate in the carbon chain distribution in wax components of citrus fruit and is directly induced by the R2R3-MYB transcription factor CsMYB96, a predominant regulator of cuticular wax metabolism.
CsKCS20 regulates the carbon chain distribution in the wax components of citrus fruit

Approximately 450 million years ago, after the evolution of biosynthesis pathways of wax to cover the aerial surface of plants against desiccation and ultraviolet radiation stress, ancestral freshwater algae successfully landed and adapted to diverse terrestrial habitats [4, 5, 35]. During the following evolution from bryophytes to seed plants, the primary carbon chain lengths of wax components developed from C24 and C26 to above C28. Wax components with longer carbon chain lengths (> C28) usually have stronger hydrophobic ability than wax components with shorter carbon chain lengths, which contributes to higher hydrophobicity and moisture retention capacity for adaptation to different habitats [5]. The carbon chain distribution of wax components in the “Newhall” navel orange surface found in this study is consistent with the finding previously reported in other citrus species that C24, C26 and C28 are relatively dominant over C30 and C32 (Fig. 2b and c) [13–15]. These findings are different from those in other plants, such as apple and Arabidopsis, which have abundant C29 and C31 wax components derived from C30 and C32 VLC acyl-CoAs [1, 2]. The phylogenetic tree indicated that Citrus is phylogenetically more ancient than Arabidopsis and is placed on a different evolutionary branch than apple [36]. In addition, the original habitats of citrus were the subtropical and tropical regions of northeastern India, northern Myanmar and southwestern China, whose environmental moisture is higher than that in...
temperate regions of central Asia, the center of origin of domesticated apple [37–40]. These results indicate that the dominant positions of C_{24}, C_{26} and C_{28} wax components in the cuticular wax of citrus fruit might be ascribed to evolution and adaptation to the habitat.

In this study, the formation of wax components above C_{20} on the fruit surface was significantly blocked in the MT, leading to a dramatic reduction in wax derivatives (Figs. 1 and 2). This phenotype provides an opportunity to explore the regulatory mechanism of the specific carbon chain distribution of C_{24}, C_{26} and C_{28} in citrus wax components. It has been reported that cuticular wax components with various carbon chain lengths (C_{20}–C_{34}) are catalyzed by different KCSs to form VLC acyl-CoAs [1, 3]. Therefore, the deficiency in wax components above C_{20} implies that the VLFA elongation process in the MT flavedo is influenced, which may be particularly associated with KCS genes. In this study, qRT–PCR analysis showed that the differentially expressed genes related to VLC acyl-CoA biosynthesis (such as KCS3, KCS6 and KCS20) were highly correlated with the disruption of carbon chain distribution in citrus fruit (Fig. 3a).

Previous studies have shown that KCS6 is responsible for the biosynthesis of VLC acyl-CoAs above C_{24}/C_{28} [11, 24]. Moreover, our sequence analysis indicated that CsKCS20 shares high homology with AtKCS20/2, which is responsible for the biosynthesis of C_{22} and C_{24} VLFA derivatives [41, 42]. In addition, the yeast assay also confirmed that CsKCS20 can induce the accumulation of C_{22} and C_{24} VLFAAs in yeast cells (Fig. 4). The ER localization of CsKCS20 and overexpression of CsKCS20 in Arabidopsis significantly increased the wax content in stems, which further verifies the function of CsKCS20 in wax biosynthesis in citrus fruit (Fig. 3b and 6). It is worth noting that AtKCS2 is the homolog of CsKCS20 and is functionally redundant in C_{22} VLFA elongation and wax biosynthesis in Arabidopsis [41,42]. However, CsKCS2 is hardly expressed in citrus flavedo based on the previous transcriptome data in our group [32], implying that CsKCS20 may play a more dominant role in C_{22} and C_{24} VLFA biosynthesis than CsKCS2 in citrus flavedo. Moreover, the expression of CsKCS20 was also significantly decreased in another wax-deficient mutant ("Ganqi 3"), as indicated by transcriptome analysis [43], which further indicates that CsKCS20 may be one of the most important genes in the formation of cuticular wax in citrus fruit. In addition, previous studies have reported that duplication, sub/neofunctionalization and redundancy of the KCS family occurred in the process of evolution from aquatic plants to terrestrial plants [5, 44]. Therefore, the finding that CsKCS20 in Citrus is a functional ortholog of Arabidopsis AtKCS20 indicates that KCS20 might be highly conserved in the evolution process of land plants and act as a vital regulator in wax formation. Overall, these results indicate that CsKCS20 plays an important role in citrus fruit wax biosynthesis and the specific carbon chain distribution in citrus wax components.

The positive regulation of CsMYB96 on CsKCS20 enriches the regulatory network of citrus cuticular wax

Cuticular wax biosynthesis is crucial for plant development and growth, and several TFs, such as MYB96 [18] and SHN1/2/3 [22, 23], were identified to regulate wax biosynthesis. Here, we found that CsMYB96 is involved in regulating wax biosynthesis by analyzing the cis-acting elements of the CsKCS20 promoter and its coexpression pattern with CsMYB96 (Supplementary Fig. S3 and S4). The results of subcellular localization, Y1H assay and EMSAs demonstrated that CsMYB96 acts as a TF and can directly bind to the promoter of CsKCS20 (Fig. 5a, b, c and Supplementary Fig. S5). A dual luciferase assay further verified that CsMYB96 significantly induced the activity of the CsKCS20 promoter (Fig. 5d). Moreover, stable overexpression of CsMYB96 in Arabidopsis significantly induced the expression of AtKCS20 and increased the wax content in Arabidopsis stems (Fig. 6 and Supplementary Fig. S6). Our results validate the important role of CsMYB96 in regulating fruit wax biosynthesis in citrus, and the regulation of CsKCS20 by CsMYB96 enriches the regulatory network of citrus cuticular wax. Interestingly, the contents of VLC aldehydes in CsKCS20 and CsMYB96 OE transgenic Arabidopsis stems showed no significant accumulation compared with those in the WT (Fig. 6b), although the amount of VLC aldehydes was significantly decreased in MT fruit compared with that in WT fruit (Fig. 2b). Over the past decades, many studies have reported that aliphatic alkanes are the main wax component in Arabidopsis, while aliphatic aldehydes are dominant in the cuticular wax of citrus fruit [1, 2, 11, 14, 15]. Moreover, VLC aldehydes have been proposed as the intermediate products of alkanes in plants [8–11, 45]. Therefore, the molecular mechanism of wax biosynthesis and regulation can be partly distinguished among species, as aldehydes are quickly converted to downstream alkanes in Arabidopsis but accumulate in citrus fruits. Hence, ectopic expression of CsKCS20 and CsMYB96 in Arabidopsis could significantly increase VLC alkanes instead of aldehydes in the stems, and the massive accumulation of VLC aldehydes in citrus fruit needs to be further investigated. In addition, in previous research on Arabidopsis, the MYB96-deficient myb96–1 mutant exhibited a serious decrease in total wax load in leaves and stems, in which a number of wax biosynthetic genes were downregulated, such as AtKCS1, AtKCS2, AtKCS6, and AtKCR1 [18]. In this study, the expression of CsMYB96 was significantly reduced in the MT from 2018 to 2020 (Supplementary Fig. S4), and the expression of several wax biosynthetic genes, including CsKCS20 and CsKCS6, was decreased accordingly (Fig. 3a). Furthermore, the promoter of CsKCS6 (approximately 1.6 kb) also contains at least six binding sites for CsMYB96, which is similar to that of CsKCS20. Therefore, these results imply that CsMYB96 may directly activate several wax biosynthetic genes (such as CsKCS6) in addition to CsKCS20, and the dramatic downregulation
of CsMYB96 can result in the downregulation of CsKCS20, CsKCS6 and other wax biosynthetic genes, which finally leads to the wax-deficient phenotype on the MT fruit surface. To date, many studies have demonstrated that several proteins can interact with MYB96 to participate in wax biosynthesis, such as MYB30-INTERACTING E3 LIgASE 1 (MIEL1) [46]. Both previous transcriptome data in our group and another report by Liu et al. showed that CsMYB16 is continuously downregulated in MT compared with WT, and its homologous gene AtMYB16 is involved in cuticle formation [19, 32, 43]. Overall, to expand the regulatory network in the future, CsMYB96 can act as a bait to explore interacting factors (such as CsMYB16) to more comprehensively analyze wax accumulation to improve citrus fruit wax quality and maintain shelf life.

Point mutations, deletions and retrotransposon insertions are important sources of bud mutants that can be used for breeding valuable fruit crops such as citrus [30, 47] and persimmon [48]. The combination of these mutations with reverse genetics approaches can facilitate the exploration of important genes to improve the fruit quality of perennial fruit crops. In this study, we elucidated the regulatory mechanism of wax biosynthesis in citrus fruit based on CsKCS20 and CsMYB96. However, because of the identical promoters and coding sequences of CsKCS20 and CsMYB96 in the WT and MT (Supplementary Table S2), further work should be focused upstream of CsKCS20 and CsMYB96 to explain the glossy phenotype of MT fruit.

Materials and methods

Plant materials and growth conditions

The spontaneous glossy mutant (MT) of “Newhall” navel orange (WT) (Citrus sinensis Osbeck) was initially found in 2005 in a garden in Anyuan County of Jiangxi Province, China. The mutant phenotype remained stable from 2005 to 2012 after grafting onto different rootstocks.

MT and WT fruits were harvested at commercial maturity in the same garden from five different trees with 20 representative fruit from each tree in 2018, 2019 and 2020. The flavedo was separated with four biological replicates of MT and WT samples (each replicate contained eight fruits), frozen in liquid nitrogen immediately, and stored at −80°C. Other materials were used for cuticular wax extraction and fruit quality analysis [33].

Nicotiana benthamiana and Arabidopsis thaliana were cultivated under 50–75% humidity, 100–120 mol m⁻² sec⁻¹ light, a 16-h/8-h light/dark cycle and 24°C (N. benthamiana) or 20–22°C (Arabidopsis thaliana) [49].

Scan electron microscopy

The epicuticular wax crystal morphology on the flavedo was imaged by a JEOL JSM-6390LV scanning electron microscope (SEM) as described by Wang et al. [14].

Water loss determination

The water loss of WT and MT fruit was measured following the method of Wang et al. [13] under dark conditions and at three-day intervals.

Total soluble solid and titratable acid analysis

Total soluble solids (TSS) and titratable acid (TA) were analyzed according to the instructions of Zhu et al. [30] and Sheng et al. [50], respectively. The TSS/TA ratio represented the maturity index.

Cuticular wax extraction and analysis

Fruit cuticular wax was extracted with chloroform from eight fruits with the method of Wang et al. [14] and analyzed as described by Yang et al. [49]. Four biological replicates were analyzed for WT and MT. An Agilent 6890B gas chromatograph-flame ionization detector (GC-FID) equipped with a TG-1MS capillary column (30 m × 0.25 mm × 0.25 μm) was used to analyze the cuticular wax samples. Quantification of individual compounds was based on the FID peak area relative to that of the internal standard n-tetracosane and qualitative analysis by gas chromatography coupled with DSQ II mass spectrometry under the same conditions with helium as the carrier gas.

The wax of A. thaliana stems was extracted as described by Liu et al. [51]. Samples were quantified and qualified as described above with a 0.5 mL min⁻¹ flow rate of carrier gas.

RNA extraction and quantitative RT–PCR (qRT–PCR)

After the extraction of total RNA from citrus flavedo following the method of Zhu et al. [30], the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) Kit was used to synthesize cDNA. qRT–PCR primers were designed as described in a previous study by Wang et al. [13]. qRT–PCR was carried out as described by Zhu et al. [30], and CsACTIN was used as the internal reference. Primer sequences are shown in Supplementary Table S3.

Phylogenetic analysis

Multiple sequence alignment and a neighbor-joining tree were performed using Clustal X version 2.0 and MEGA 7, respectively [52, 53].

Yeast assay

The coding sequence of CsKCS20 was obtained from cDNA from “Newhall” navel orange and cloned into the p426 yeast expression vector. The fatty acid methyl esters (FAMEs) of the yeast strain were analyzed as described by Yang et al. [34]. Individual FAMEs were determined by the characteristic ion fragments. Quantification was based on each peak area relative to that of the internal standard heptadecanoic acid (C17:0).
Subcellular localization of CsKCS20 and CsMYB96

The coding sequences of CsKCS20 and CsMYB96 without stop codons were obtained from the cDNA of the WT and inserted into the pDONR207 vector and then into the pK7FWG2 vector to obtain 35S::CsKCS20::GFP and 35S::CsMYB96::GFP vectors. Both vectors were transiently transformed into tobacco leaves with RFP-HDEL as an ER marker [54], and DAPI staining was used as the nuclear marker [55]. Confocal laser scanning microscopy (Leica, marker [54], and DAPI staining was used as the nuclear marker [55].

Yeast one-hybrid assay (Y1H)

Y1H assays were carried out following the description by Han et al. [56] with some modifications. The promoter of CsKCS20 was cloned into the pABAi vector. The full-length sequence of CsMYB96 was cloned into the pGADT7 vector (AD).

Recombinant protein purification and electrophoretic mobility shift assay (EMSA)

To obtain the CsMYB96 protein, its coding sequence was amplified and inserted into the pET32a vector. The recombinant protein was purified using the Ni-NTA His-Bind® Resin Kit (product no. 3421974) following the manufacturer’s instructions. EMSA was carried out using the method of Shi et al. [57].

Dual luciferase assay

pGreen II 0029 62-SK was used as the effect vector, and the coding sequence of CsMYB96 was introduced into it. The pGreen II 0800-LUC vector was used as the reporter vector, and the promoter of CsKCS20 was inserted into it. The experiment was conducted as described by Han et al. [56] and Zhang et al. [47]. Tobacco leaves were infected with infiltration with a mixture of Agrobacterium cells containing CsMYB96, the CsKCS20 promoter and P19 (4:1:1, v/v/v).

Transgenic experiments of A. thaliana

The full-length coding sequences of CsKCS20 and CsMYB96 were inserted into the pDONR207 vector through the pDONR207 vector. Agrobacterium carrying the 35S::CsKCS20 and 35S::CsMYB96 constructs was used for Arabidopsis transformation [58]. Homozygous transgenic lines of the T3 generation were used for phenotype analysis.

Statistical analysis

Significant differences were analyzed by paired t-test when they passed the homogeneity test of variance and by unpaired t-test when they did not. The statistical analyses were performed and figures were drawn with GraphPad Prism 8.2.1 software (GraphPad Software Inc.). Data are presented as the mean value ± the standard deviation (SD). P < 0.05 was used to indicate a significant difference and is represented with a single asterisk (*).

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Authors contributions

Y.W. performed the experiments, analyzed the data and wrote the manuscript. J.Z. and K.S. helped Y.W. to carry out some of the experiments. Y.W., X.P.Y. and F.Z. analyzed the data and revised the manuscript. X.P.Y., Z.X.C., Y.Z.H. and R.W.X. provided technical assistance. Y.J.C. designed the research and revised the manuscript.

Data availability

All data in this work are available within the paper and its Supplementary Information files. Supplementary Information accompanies this paper.

Conflicts of interest

We declare that there are no conflicts of interest.

Supplementary data

Supplementary data is available at Horticulture Research online.

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