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Formation of 8-hydroxylinalool in tea plant *Camellia sinensis* var. *Assamica* 'Hainan dayezhong'

Ying Zhou [a,*], Wei He [a,b], Yunchuan He [a,b], Qiulin Chen [a,b], Yang Gao [a,b], Jiamei Geng [a,b], Zeng-Rong Zhu [a,b,*]

[a] Hainan Institute, Zhejiang University, Yazhou District, Sanya 572025, China
[b] College of Agriculture and Biotechnology, Zhejiang University, Xihu District, Hangzhou 310030, China

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ABSTRACT

Linalool and its derivatives contribute greatly to tea aroma. Here, 8-hydroxylinalool was found to be one of the major linalool-derived aroma compounds in *Camellia sinensis* var. *assamica* 'Hainan dayezhong', a tea plant grown in Hainan Province, China. Both (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool were detected, and the *E* type was the main compound. Its content fluctuated in different months and was the highest in the buds compared with other tissues. CsCYP76B1 and CsCYP76T1, located in the endoplasmic reticulum, were identified to catalyze the formation of 8-hydroxylinalool from linalool in the tea plant. During withering of black tea manufacturing, the content of both (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool significantly increased. Further study suggested that jasmonate induced gene expression of *CsCYP76B1* and *CsCYP76T1*, and the accumulated precursor linalool may also contribute to 8-hydroxylinalool accumulation. Thus, this study not only reveals 8-hydroxylinalool biosynthesis in tea plants but also sheds light on aroma formation in black tea.

1. Introduction

Tea is one of the most popular non-alcoholic beverages in the world. Its popularity is inseparable from its captivating aroma. Therefore, aroma is an important evaluation factor for tea quality. The aroma compounds in tea mainly come from three biosynthetic pathways: fatty acid derivatives, terpenoid biosynthetic pathway, and phenylpropanoid/benzenoid derivatives. Terpenes, mainly monoterpenes and sesquiterpenes, are the most abundant aroma compounds in tea. Linalool is a ubiquitous and high-content monoterpenoid aroma compound in tea plant. In general, the linalool content of *Camellia sinensis* var. *assamica* is significantly higher than that of *C. sinensis* var. *sinensis* (Zeng et al., 2021). *C. sinensis* var. *sinensis* is a slow-growing shrub with smaller leaves, which are usually the raw materials of green and oolong teas. *C. sinensis* var. *assamica* is quick-growing with larger leaves, which are often processed into black and dark teas. *C. sinensis* var. *sinensis* is hardier than *C. sinensis* var. *assamica* and therefore has a wider distribution (Wei et al., 2018).

In plants, linalool can be oxidatively metabolized to form a series of compounds. For example, hydroxylation at the C(8) position of linalool can form 8-hydroxylinalool. Then the C(8) position is further oxidized to form 8-oxolinalool, which is then oxidized to form lilac alcohol and lilac aldehydes (Boachon et al., 2015). Apart from these linalool-derived compounds, lilac alcohol epoxide, various linalool oxides, 2,6-dimethylocta-3,7-diene-2,6-diol, and hotrienol are found in plants (Ilc, Parage, Boachon, Navrot, & Werck-Reichhart, 2016; Ilc, Werck-Reichhart, & Navrot, 2016; Matich et al., 2007). However, the synthetic pathways of these compounds are mostly unclear. CYP450 oxidase is involved in the catalytic formation of these compounds. Enzymes of the CYP76 family in *Arabidopsis* catalyze a cascade of oxidation reactions (Boachon et al., 2015; Ginglinger et al., 2013; Hofer et al., 2014), forming hydroxylinalool, lilac alcohol, and lilac aldehydes. In *Citrus mangshanensis*, CYP78A7 catalyzes the formation of linalool oxides from linalool (Zhang et al., 2019). Linalool and its derivatives are the main terpenoids in tea, and they greatly contribute to the formation of floral, fruity, sweet, and honey aromas in tea. To date, the most reported linalool-derived aroma compounds in tea are *trans/cis*-linalool oxide (furanoid), *trans/cis*-linalool oxide (pyranoid), 2,6-dimethylocta-3,7-diene-2,6-diol (diendiol I), and hotrienol. 8-Hydroxylinalool is rarely reported in tea, and only one study showed its trace amounts in young leaves (Yamashita et al., 2021). It is widely present in *Vitis vinifera* (Campos-Arguedas et al., 2022), *Actinidia arguta* (Matich et al., 2003), and *Osmanthus fragrans*

* Corresponding authors at: Hainan Institute, Zhejiang University, Yazhou District, Sanya 572025, China (Y. Zhou, Z.R. Zhu).
E-mail addresses: yzhyzb@zju.edu.cn (Y. Zhou), zrzhu@zju.edu.cn (Z.-R. Zhu).

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(Zeng et al., 2015). It has a sweet, citrus-like odor (Elsharif et al., 2015), and it plays an important role in providing aroma precursors (Ilc, Parage, et al., 2016; Ilc, Werck-Reichhart, et al., 2016; Kreck et al., 2003; Matich et al., 2007).

Camellia sinensis var. *assamica* 'Hainan dayezhong' with trichomeless buds is a unique tea germplasm resource in Hainan. It is suitable for making black tea, and wild tea trees are widely distributed in Hainan Island. Because of the lack of research, the quality-related compounds in 'Hainan dayezhong' are unknown, which limits its development and utilization. In addition, its aroma composition, a key factor for quality assessment, has not yet been revealed. We found that linalool and its derivatives were the main terpenoids in the tender leaves of 'Hainan dayezhong'. Among these derivatives, the 8-hydroxylinalool content was higher, which was comparable to the linalool oxide content. In this study, we revealed the synthetic pathway of 8-hydroxylinalool in 'Hainan dayezhong' and the mechanism of its content increase during withering of black tea processing.

2. Materials and methods

2.1. Materials and reagents

Linalool oxide (furanoid, mixture of *trans* and *cis* isomers, ≥97.0%), linalool (97.0%), α-terpineol (≥95.0%), geraniol (98%), nerolidol (98%), polyethylene glycol (PEG) 4000, KCl (≥99.0%), $MgCl_2$ (≥98%), morpholineethanesulfonic acid (MES, ≥99.0%), mannitol (≥98%), methanol (LC–MS grade), and formic acid (LC–MS grade) were purchased from Merck (Darmstadt, Germany). Farnesene (98%) was purchased from Aladdin (Shanghai, China). Linalool oxide (pyranoid, mixture of *trans* and *cis* isomers, >98.0%) and jasmonic acid (JA, >85%) were purchased from TCI (Tokyo, Japan). d5-JA was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). 2,6-Dimethyl-3,7-octadiene-2,6-diol (diendiol I, 96%) was purchased from BioBioPha (Yunnan, China), and (*E*)-8-hydroxylinalool (90%) was synthesized by Xin Yao Biotech Co., Ltd. (Shenzhen, China). The chemical structures and isomers of synthesized 8-hydroxylinalool were confirmed by ^1H and ^{13}C NMR spectrum analysis (Fig. S1) and previous studies (Knapp et al., 1998). ^1H NMR (600 MHz, $CDCl_3$) δ 6.01–5.85 (m, 1H), 5.43 (ddt, J = 8.6, 7.2, 1.4 Hz, 1H), 5.24 (dd, J = 17.3, 1.3 Hz, 1H), 5.09 (dd, J = 10.8, 1.2 Hz, 1H), 4.00 (d, J = 1.4 Hz, 2H), 2.20–2.00 (m, 2H), 1.68 (d, J = 1.4 Hz, 3H), 1.64–1.56 (m, 4H), 1.31 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 13.67 (Me-C2), 22.34 (C4), 27.90 (Me-C6), 41.72 (C5), 68.83 (C1), 73.34 (C6), 111.88 (C8), 125.93 (C3), 135.05 (C2), 144.87 (C7).

2.2. Plant materials and treatments

To assay 8-hydroxylinalool content variation in different months, tea shoots of *C. sinensis* var. *assamica* 'Hainan dayezhong' and *C. sinensis* var. *sinensis* 'Fudingdabaicha' were sampled every month from March to November at the Jianfengling National Forest Park, Ledong, Hainan, China. Then one bud and two leaves were harvested from tea shoots in the lab for internal aroma assay. To assay the aroma content in different tissues, the bud, the first leaf, the second leaf, the third leaf, the fourth leaf and the stem were sampled from tea shoots and frozen in liquid N_2 immediately. Withering treatment: One bud and two leaves were picked from sampled tea shoots of 'Hainan dayezhong' and subjected to withering at room temperature with humidity around 50%. The leaves were spread evenly and thinly on a bamboo sieve in a natural state. Samples were harvested at 0, 1, 4, 8, and 16 h. Jasmonic acid (JA) treatment: The tea shoots were incubated with 2.5 mM JA solution (dissolved in 0.5% ethanol) for 10 h at 25 °C. Tea shoots incubated in 0.5% ethanol (Aladdin, Shanghai, China) were used as a control. One bud and two leaves were harvested after JA treatment. Three replicates were performed for each treatment. All the harvested samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Enzyme activity assay in nicotiana benthamiana

The candidate gene was inserted into the vector pCAMBIA3300-GFP. The construct was then transformed into *Agrobacterium tumefaciens* GV3101. The overnight *Agrobacterium* cultures were sedimented at 3,500 *g* for 15 min. The pellet was resuspended in a solution containing 10 mM $MgCl_2$, 10 mM morpholineethanesulfonic acid (MES, pH 5.6), and 100 μM acetosyringone to OD600 of 0.4. The resuspended bacterial solution was infiltrated from the lower epidermis to the entire leaves of *N. benthamiana* using a needle-free syringe as described previously (Zhou et al., 2015). After infiltration, the tobacco plants were placed in a climate chamber at 23–25 °C with 70% humidity. The light/dark cycle was 16 h/8h. Five days after infiltration, the leaves were fed with 1 mL of 2.5 mM linalool and then stored at −80 °C until analysis. The leaves were grinded using Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) with liquid nitrogen. To analyze 8-hydroxylinalool in tobacco leaves, a 0.4 g finely powdered sample was extracted with 5 mL dichloromethane for 6 h at room temperature (Zhou, Deng, et al., 2020; Zhou, Zeng, et al., 2020). The extracts were then passed through anhydrous sodium sulfate to remove water and concentrated under nitrogen to about 200 μL. Concentrated extracts of 1 μL were subjected to gas chromatography–mass spectrometry (GC–MS) (GCMS-QP2020 NX; Shimadzu, Tokyo, Japan) analysis. A SUPELCOWAXTM 10 column (30 m × 0.25 mm × 0.25 μm; Supelco Inc., Bellefonte, PA, USA) was used. The carrier gas was helium at a velocity of 1.1 mL/min. The GC program was 40 °C for 3 min, increased at 4 °C/min to 240 °C, and maintained at 240 °C for 20 min. 8-hydroxylinalool was identified by comparing with authentic compound.

2.4. Endogenous volatile analysis of tea leaves

The sample tea leaves were grinded using Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) with liquid nitrogen. A sample of 0.4 g finely powdered tea leaves was extracted with 5 mL dichloromethane for 6 h at room temperature according our previous study (Zhou, Deng, et al., 2020; Zhou, Zeng, et al., 2020). 5 nmol ethyl decanoate (50 μL) was added as internal standard. Subsequently, the tea leaf extracts were passed through anhydrous sodium sulfate to remove water and concentrated under nitrogen to about 200 μL. The GC–MS analysis procedure was the same as described above. Using this method, the recovery rates of aroma compounds were 89.71% − 101.39% and the relative standard deviation was 1.79% − 6.44%. Tea aroma compound was identified by comparing with authentic compound. The endogenous aroma content in tea was quantified by the calibration curves of commercial standards. The content of all tea aroma compounds fell within the range of the calibration curve ($R^2 > 0.99$).

2.5. Quantitative real-time PCR analysis

Total RNA was isolated with the Quick RNA Isolation Kit (Huayueyang, Beijing, China). The first strand cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). *Elongation factor-1α* (*CsEF*) was used as an internal reference gene (Zhou et al., 2017). The primers 5′-TGTATGGGCTATTGGGCGAGACTC-3′ and 5′-CCTGGGCACATCCTCCTTCCT-3′ were used for *CsCYP76T1*, 5′-CTACGGCGGCAGAAGGTTCA-3′ and 5′-CCTGACTTGGGTCGGCTAAATCC-3′ were used for *CsCYP76B1*, and 5′-GTGTGGAGAAGAAGGACCCA-3′ and 5′-CGAGGCTAGTGAACAGCAAC-3′ were used for *CsEF*. These primers were synthesized at Qingke Company (Haikou, China). Quantitative real-time PCR was performed on the QuantStudio Q5 system (Applied Biosystems, Waltham, MA, USA) using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China). A melt-curve analysis was performed at the end of each reaction to verify PCR product specificity.

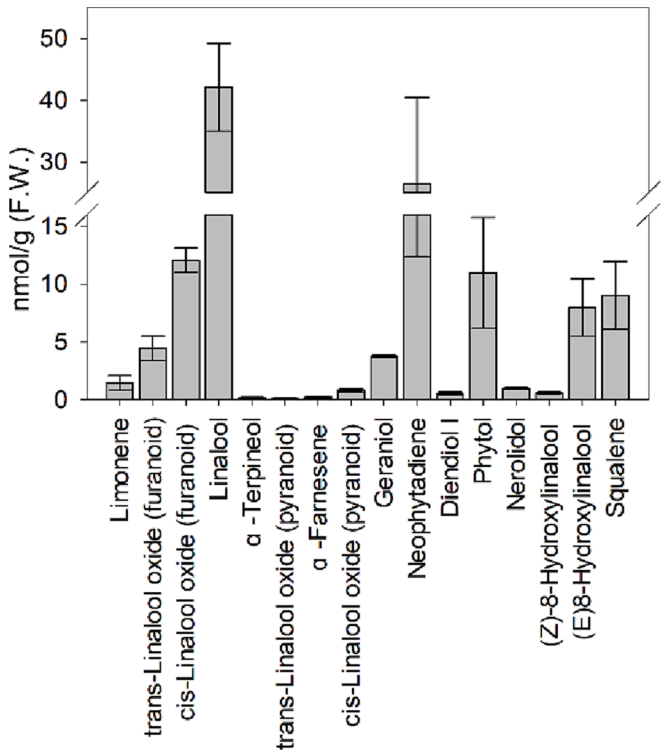

Fig. 1. Distribution of terpenoids in *Camellia sinensis* var. *assamica* 'Hainan dayezhong'. Three biological replicates had been performed. The error bars represented standard deviation.

2.6. Subcellular localization

The candidate gene was inserted into the vector pSAT6-EYFP-N1. To obtain protoplasts, the lower epidermis of *Arabidopsis* leaves was removed by adhesive tapes (Wu et al., 2009). The treated leaves were digested for 3 h at 25 °C in enzyme solution containing 20 mM MES (pH 5.7), 1.5% cellulase R10 (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.4% macerozyme R10 (Yakult Pharmaceutical Industry Co., Ltd.), 0.4 M mannitol, and 20 mM KCl (Yoo et al., 2007). The construct was transformed into protoplasts with PEG 4000 as previously described (Yoo et al., 2007). After incubation at 22 °C for 16 h, the protoplasts were stained with ER-Tracker Red (Beyotime, Shanghai, China) for 30 min at room temperature. The fluorescence intensity was observed under a confocal laser scanning microscope (LSM880, Zeiss, Oberkochen, Germany). Fluorescence was measured at excitation wavelengths of 510 nm for yellow fluorescent protein and 587 nm for ER-Tracker Red.

2.7. JA analysis

JA extraction and analysis were performed according to our previous study (Zhou, Deng, et al., 2020; Zhou, Zeng, et al., 2020). The sample tea leaves were grinded using Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) with liquid nitrogen. A sample of 300 mg finely powdered tea leaves was extracted with 3 mL ethyl acetate. 25 ng d5-JA was added as an internal standard. The mixture was sonicated on ice for 20 min and then centrifuged at 12,000 *g* for 10 min at 4 °C. Subsequently, 2.5 mL of supernatant was transferred to a new tube and evaporated under nitrogen flow. The resulting pellet was redissolved in 200 μL methanol. JA was analyzed using a SCIEX ExionLC/X500R QTOF system (AB SCIEX, Framingham, USA) equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm × 1.7 μm; Waters Corp., Milford, MA, USA). Distilled water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B) were used as mobile phases. The elution gradient was initiated with 30% B for 4 min, and then increased linearly to 65% B over 15 min. The column temperature was 40 °C. Electrospray ionization in the negative mode was used for the ionization of plant hormones. The curtain gas was set at 35 psi. The spray voltage, declustering potential, and collision energy were applied at −4500, −75, and −20 V, respectively. The range of calibration curve was defined on the basis of the amount in tea samples with $R^2 > 0.99$. The content of JA in all samples fell within the range of the calibration curve. Using this method, the recovery rate of JA was 91.53% with the relative standard deviation of 5.27%.

2.8. Statistical method

One-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test was used to test the difference among multiple groups. ANOVA was performed with SPSS, version 19.0. Student's *t*-test performed with Microsoft Excel (Office 2019) was used to analyze the difference between two groups. Different lowercase letters represent significant statistical difference ($P < 0.05$).

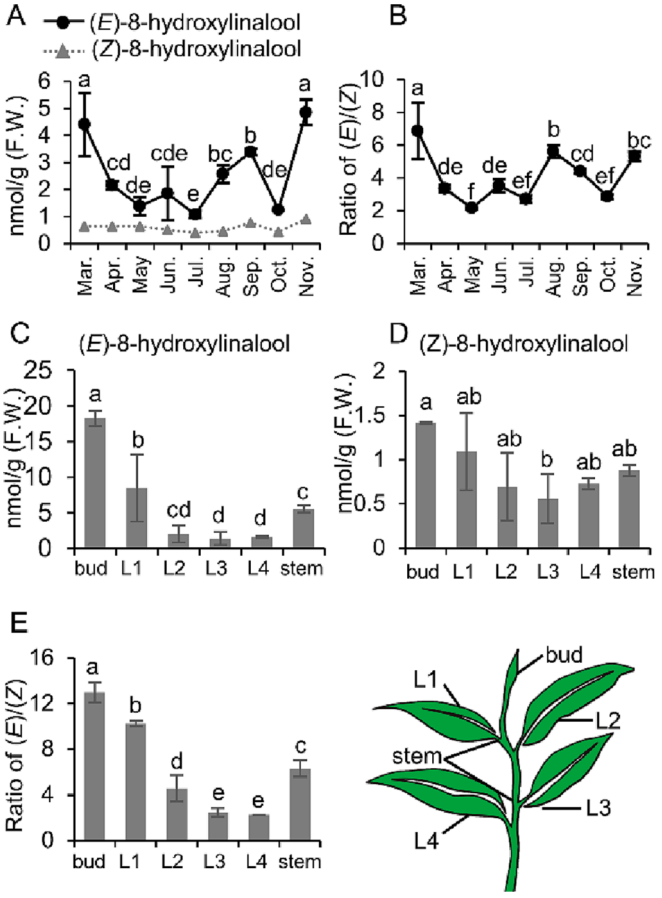

Fig. 2. Profiles of 8-hydroxylinalool in different months and tissues.A, Content of (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool in different months. B, Ratio of (*E*)/(*Z*)-8-hydrolinalool in different months. C, Content of (*E*)-8-hydroxylinalool in different tissues. D, Content of (*Z*)-8-hydroxylinalool in different tissues. E, Ratio of (*E*)/(*Z*)-8-hydrolinalool in different tissues. L1, the first leaf; L2, the second leaf; L3, the third leaf; L4, the fourth leaf. One–Way analysis of variance (ANOVA) followed by Tukey *post-hoc* test was performed with SPSS, version 19.0. Different lowercase letters represent significant statistical difference ($P < 0.05$). Three biological replicates had been performed. The error bars represented standard deviation.

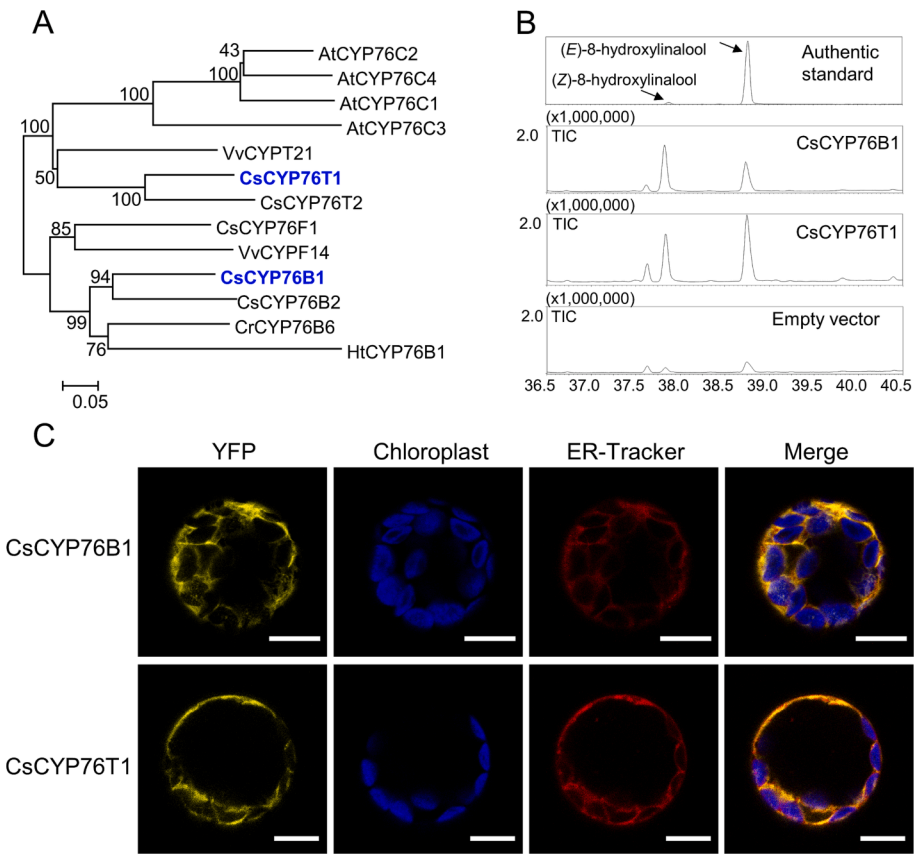

Fig. 3. Characterization of 8-hydroxylinalool synthase CsCYP76B1 and CsCYP76T1. A, Phylogenetic tree analysis of 8-hydroxylinalool in plants. AtCYP76C1 accession number NP_850439.1; AtCYP76C2, accession number NP_182081.1; AtCYP76C3, accession number NP_182082.2; AtCYP76C4, accession number NP_182079.1; VvCYPT21, accession number XP_002276576.1; VvCYPF14, accession number XP_010658029.1; CrCYP76B6, accession number Q8VWZ7.1; HtCYP76B1, accession number O23976.1; CsCYP76B1, accession number OP913222; CsCYP76T1, accession number OP913223; CsCYP76T2, accession number OP913224, CsCYP76B2, accession number OP913225; CsCYP76F1, accession number OP913226. B, Enzyme activity assay by GC–MS. Compared with empty vector, CYP76B1 and CYP76T1 convert linalool to (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool. C, Subcellular localization analysis of CYP76B1 and CYP76T1. CYP76B1 and CYP76T1 were both localized in endoplasmic reticulum.

3. Results and discussion

3.1. Analysis of 8-hydroxylinalool in the tender leaves of C. Sinensis var. Assamica 'Hainan dayezhong'

Most aroma compounds found in one bud and two leaves of 'Hainan dayezhong' belonged to terpenoids, fatty acid derivatives, and phenylpropanoid/benzenoid derivatives. Terpenoids were the main aroma compounds in the fresh leaves of 'Hainan dayezhong' (Fig. 1). Monoterpene aroma compounds included limonene, *trans/cis*-linalool oxide (furanoid), linalool, α-terpineol, *trans/cis*-linalool oxide (pyranoid), geraniol, 2,6-dimethyl-3,7-octadiene-2,6-diol (diendiol I), and (*Z*)/(*E*)-8-hydroxylinalool. Sesquiterpene aroma compounds included α-farnesene and nerolidol. Diterpene aroma compounds included neophytadiene and phytol. Triterpene aroma compounds included squalene. Linalool was the most abundant aroma compound found in the tender leaves of 'Hainan dayezhong', followed by neophytadiene. Compared with *C. sinensis* var. *sinensis* cultivars such as 'Jinxuan', the geraniol content of 'Hainan dayezhong' was low, and it was not a major aroma compound. Diendiol I was detected in all samples harvested from March to November (Fig. S2). This characteristic aroma compound is formed after the attack of the tea green leafhopper *Empoasca onukii*. Its ubiquity in tea leaves may be related to the occurrence of the tea green leafhopper throughout the year in Hainan Province, where the mean annual temperature is about 25 °C.

Among the terpenoids, four linalool derivatives were found, including *trans/cis*-linalool oxide (furanoid), *trans/cis*-linalool oxide (pyranoid), diendiol I, and (*Z*)/(*E*)-8-hydroxylinalool. The linalool content and its derivatives accounted for 52.57% of all terpenoid aroma compounds. Among these, the linalool derivative 8-hydroxylinalool has rarely been reported in tea plants. In 'Hainan dayezhong', 8-hydroxylinalool was found with higher content, which was comparable to the linalool oxide (furanoid) content (Fig. 1). Both *E* and *Z* types of 8-hydroxylinalool were detected, and the *E* type was the main one. We attempted to analyze the chiral configuration of 8-hydroxylinalool in tea leaves. Even after trying a variety of chiral columns, the *R* and *S* isomers of (*E*)-8-hydroxylinalool could not be separated well. However, both *R* and *S* isomers of (*E*)-8-hydroxylinalool were present in tea leaves (Fig. S3). Chiral isomers of (*Z*)-8-hydroxylinalool could be separated, but the absolute configuration needs to be identified (Fig. S3).

Seasonal and tissue variation in aroma compounds is common in tea plants (Kfoury et al., 2019; Ahmed et al., 2019). Therefore, the trend of 8-hydroxylinalool in different months and tissues was studied. The 8-hydroxylinalool content of one bud and two leaves of different months varied greatly (Fig. 2A). Of these months, the highest levels of (*E*)-8-hydroxylinalool were found in March and November. The changes in (*E*)-8-hydroxylinalool and (*Z*)-8-hydroxylinalool in different months were usually consistent (Fig. 2A), but the configuration ratios of *E* and *Z* types fluctuated in different months (Fig. 2B). The 8-hydroxylinalool content also differed greatly in various tissues (Fig. 2C, 2D). The (*E*)-8-hydroxylinalool content of the buds (18.30 nmol/g) was significantly higher than in other tissues, followed by the first leaf (8.50 nmol/g) and stems (5.51 nmol/g). The (*E*)-8-hydroxylinalool content of the second leaf (2.07 nmol/g), third leaf (1.44 nmol/g), and fourth leaf (1.63 nmol/g) was similar. The (*Z*)-8-hydroxylinalool content was also slightly higher in the buds than in other tissues (Fig. 2D). As shown in Fig. 2E, the ratio of (*E*)/(*Z*)-8-hydroxylinalool decreased with leaf position. Yamashita et al. (2021) showed that the 8-hydroxylinalool content was much higher in new stems than in new leaves (one bud and two leaves), which was different from our results. Perhaps the tissue distribution of 8-hydroxylinalool is cultivar-dependent. The linalool content was positively correlated with the (*E*)/(*Z*)-8-hydroxylinalool content of different tissues except for the buds (Fig. S4).

The odor of 8-hydroxylinalool is similar to that of linalool, which is citrus-like, sweet, and floral (Elsharif et al., 2015). 8-Hydroxylinalool is the major linalool derivative in *V. vinifera*. As a major aroma compound,

in addition to the free state, it exists in the form of glycosides in *V. vinifera* (Ilc, Parage, et al., 2016; Ilc, Werck-Reichhart, et al., 2016). However, the glycoside types in tea leaves need further research. The levels of linalool and linalool oxides were negatively correlated with different cultivars (Zeng et al., 2021). Tea leaves of *C. sinensis* var. *assamica* had a high level of linalool and a low level of linalool oxides, whereas tea leaves of *C. sinensis* var. *sinensis* had a low level of linalool and a high level of linalool oxides. We compared the cultivars 'Hainan dayezhong' and 'Fudingdabaicha' (*C. sinensis* var. *sinensis*), which were grown in the same field, and found that the linalool content was positively correlated with the (*E*)/(*Z*)-8-hydroxylinalool content of both tea varieties (Fig. S5). As *C. sinensis* var. *assamica* usually contains a high level of linalool (Zeng et al., 2021), a higher content of 8-hydroxylinalool may exist in *C. sinensis* var. *assamica*.

3.2. *CsCYP76B1 and CsCYP76T1 catalyze the formation of 8-hydroxylinalool*

Proteins encoded by genes belonging to the *CYP76* family catalyze the formation of 8-hydroxylinalool from linalool (Ilc et al., 2017; Hofer et al., 2014). To study the 8-hydroxylinalool synthetic pathway in tea plants, we selected five homologous genes in tea based on the genes involved in 8-hydroxylinalool synthesis in other plants (Fig. 3A) (Hofer et al., 2014; Ilc et al., 2017). The proteins encoded by these genes were expressed in *N. benthamiana*. After feeding with linalool, the enzyme activity of these proteins was analyzed by detecting the aroma compounds in tobacco leaves with GC–MS. Linalool was metabolized by CsCYP76B1 and CsCYP76T1, and 8-hydroxylinalool was detected based on a comparison of retention times and mass spectra with authentic standards (Fig. 3B). No activity was detected with CsCYP76B2, CsCYP76F1, and CsCYP76T2. Moreover, both (*Z*) and (*E*)-8-hydroxylinalool could be formed by CsCYP76B1 and CsCYP76T1. The subcellular localization analysis showed that CsCYP76B1 and CsCYP76T1 were located in the endoplasmic reticulum (ER) (Fig. 3C), which was the same as 8-hydroxylinalool synthase CYP76C1 in *Arabidopsis* (Boachon et al., 2015). Linalool is synthesized in the chloroplasts of tea plants (Zhou, Deng, et al., 2020; Zhou, Zeng, et al., 2020). Chloroplasts are surrounded by ER, which most likely favors the conversion of chloroplast-synthesized linalool by ER-localized CYP450 enzymes.

All known 8-hydroxylinalool synthases belong to the CYP76 family. The results in *V. vinifera* showed that the protein encoded by three genes, *CYP76T21*, *CYP76F14*, and *CYP76F12*, can catalyze the formation of 8-hydroxylinalool from linalool (Ilc et al., 2017). Among these, CYP76T21 and CYP76F14 were involved in the formation of (*E*)-8-hydroxylinalool, and CYP76F12 could simultaneously catalyze the formation of (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool. Proteins encoded by multiple genes of the *CYP76C* and *CYP76B* families in *Arabidopsis* can catalyze the formation of 8-hydroxylinalool, but the configuration of the product has not been confirmed (Hofer et al., 2014). CsCYP76T1 shows the highest homology with CYP76T21 in *V. vinifera*, but the difference is that CsCYP76T1 can simultaneously catalyze the formation of (*Z*) and (*E*)-8-hydroxylinalool. In addition to the C8 position, other positions such as C5, C6, C7, and C9 hydroxylated linalool have been reported in plants (Ginglinger et al., 2013; Ilc, Parage, et al., 2016; Ilc, Werck-Reichhart, et al., 2016). However, CsCYP76B1 and CsCYP76T1 could only catalyze linalool to form 8-hydroxylinalool, and no other hydroxylated linalool was found in the product, which was consistent with only 8-hydroxylinalool found in tea plants. The more reported linalool derivatives in tea are linalool oxides, and 8-hydroxylinalool is rarely reported, which might be related to its trace amounts in many tea plants. Linalool oxide and 8-hydroxylinalool are derived from two pathways with linalool as the precursor, and linalool forms linalool oxide by intermediate 6,7-epoxylinalool (Correddu et al., 2022; Meesters et al., 2007; Raguso & Pichersky, 1999), but it is directly hydroxylated at C8 to form 8-hydroxylinalool. In many tea plants, the enzymes involved in the synthesis of linalool oxide may have a better affinity to the substrate

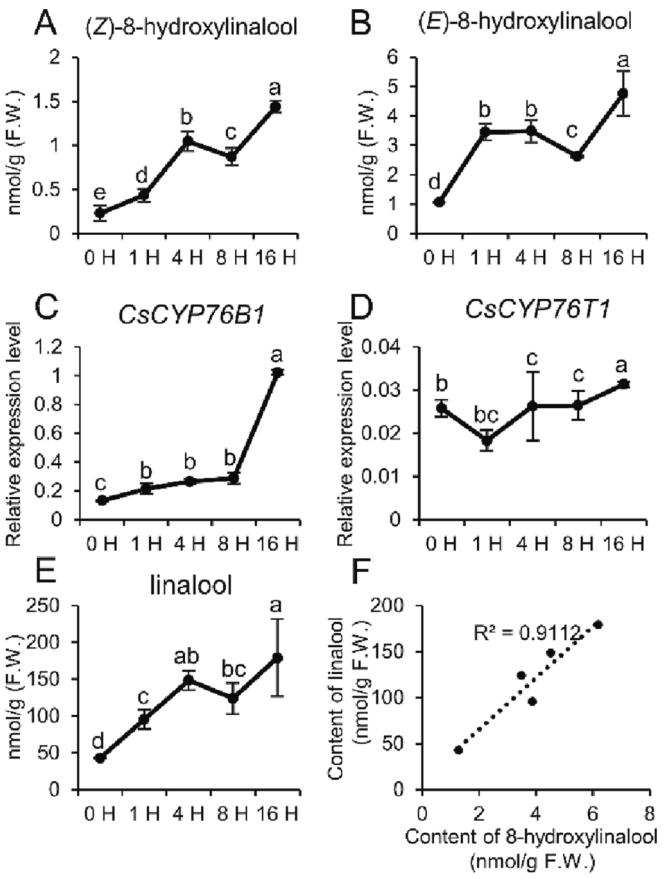

Fig. 4. Dynamic change of 8-hydroxylinalool and gene expression level of *CYP76B1* and *CYP76T1* during tea withering stage.A, Dynamic change of (*Z*)-8-hydroxylinalool during tea withering stage. B, Dynamic change of (*E*)-8-hydroxylinalool during tea withering stage. C, Dynamic change of *CYP76B1* during tea withering stage. D, Dynamic change of *CYP76T1* during tea withering stage. E, Dynamic change of linalool during tea withering stage. F, Correlation analysis between linalool content and 8-hydroxylinalool content. One–Way analysis of variance (ANOVA) followed by Tukey *post-hoc* test was performed with SPSS, version 19.0. Different lowercase letters represent significant statistical difference ($P < 0.05$). Linear regression and R^2 value were calculated by Microsoft Excel (Office 2019). Three biological replicates had been performed. The error bars represented standard deviation.

linalool, thus biasing the metabolic flux toward linalool oxide. Understanding the metabolic flux of linalool in tea plants requires knowledge of the enzymes involved in linalool oxidation.

3.3. *Increased CsCYP79B1 gene expression and accumulated precursor linalool co-induced 8-hydroxylinalool accumulation during withering*

The 8-hydroxylinalool content significantly increased during the withering stage of black tea made of 'Hainan dayezhong'. After withering for 16 h, the content of (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool was about five and three times that of the beginning, respectively (Fig. 4A, 4B). To elucidate the reasons for the increase in the 8-hydroxylinalool content of tea leaves during withering, the expression levels of *CsCYP76B1* and *CsCYP76T1* were analyzed (Fig. 4C, 4D). The *CsCYP76B1* expression level increased during withering (Fig. 4C). Further investigation showed that linalool, the precursor of 8-hydroxylinalool, accumulated during withering (Fig. 4E). In addition, the content of linalool and 8-hydroxylinalool was significantly positively correlated with the withering stage (Fig. 4F). Lewinsohn et al. (2001) showed that enhancing the linalool content by overexpressing linalool synthase genes promoted 8-hydroxylinalool accumulation in tomato,

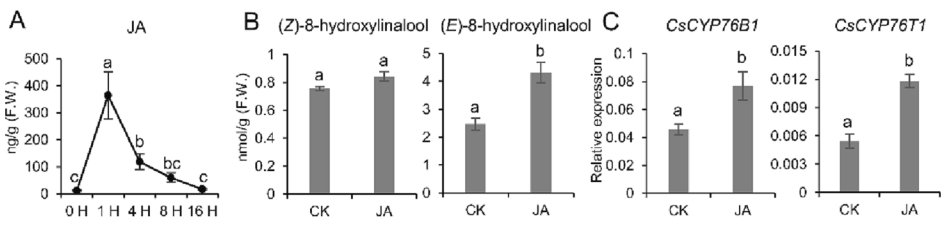

Fig. 5. 8-hydroxylinalool and their synthesis genes were induced by jasmonate.A, Dynamic change of JA during tea withering stage. B, content of (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool were increased after JA treatment. C, gene expression level of *CYP76B1* and *CYP76T1* were stimulated after JA treatment. One–Way analysis of variance (ANOVA) followed by Tukey *post-hoc* test was performed with SPSS, version 19.0 in Fig. 5A. The significance of the difference was assessed by the Student's *t*-test (two-tailed test) in Fig. 5B and 5C. Different lowercase letters represent significant statistical difference ($P < 0.05$). Three biological replicates had been performed. The error bars represented standard deviation.

suggesting that the precursor linalool played a crucial role in 8-hydroxylinalool accumulation. Therefore, both enhanced synthetic gene expression level and increased precursor linalool content might contribute to 8-hydroxylinalool accumulation during the withering stage.

Picking causes mechanical damage to tea leaves. The JA content was measured to further elucidate the reason why the expression levels of 8-hydroxylinalool synthetic genes increased during withering. It was significantly induced during withering, reached the peak in about 1 h, and then decreased (Fig. 5A). JA treatment induced 8-hydroxylinalool accumulation and promoted the expression of *CsCYP76B1* and *CsCYP76T1* in one bud and two leaves (Fig. 5B, 5C). Thus, mechanical damage during picking induced an increase in JA content, which in turn promoted the expression level of 8-hydroxylinalool synthase genes, thereby contributing to the increase in the 8-hydroxylinalool content.

JA can induce volatile terpenoids by regulating the expression of their synthase genes (Kiryu et al., 2018; Martin et al., 2003; Rodriguez-Saona et al., 2001; Taniguchi et al., 2014). Linalool is a well-known JA-induced terpenoid (Rodriguez-Saona et al., 2001; Taniguchi et al., 2014; Martin et al., 2003). The increased JA content was consistent with the accumulated linalool content during the withering stage. JA can also increase the expression of *CYP450* genes, such as *WsCYP76* and *WsCYP749*, in *Withania somnifera* (Rana et al., 2014; Shilpashree et al., 2022) and multiple *CYP76* family genes in *Salvia miltiorrhiza* (Firneno et al., 2022). The increase in the expression level of these genes promotes the synthesis of downstream metabolites. Mechanical injury during manufacturing processes can induce the formation of many aroma compounds in tea plants via *de novo* biosynthesis, such as indole (Zeng et al., 2016), nerolidol (Zhou et al., 2017), and jasmine lactone (Zeng et al., 2018), in which JA plays a crucial role. These mechanical injury-induced aroma compounds are an important source of floral and fruity aromas in tea. Withering, considered vital for black tea quality (Tomlins & Mashingaidze, 1997), is necessary for the balanced taste and flavor of the final product. It not only greatly affects the production of the aroma compounds in black tea (Huang et al., 2022; Li, Hao, et al., 2022; Li, He, et al., 2022; Mahanta & Baruah, 1989) but also facilitates the accumulation of many floral and fruity aroma compounds such as linalool and its oxides (Li, Hao, et al., 2022; Li, He, et al., 2022; Tomlins & Mashingaidze, 1997; Zhou et al., 2022). Because the intact cells still maintain activity during withering, the *de novo* synthesis of aroma compounds caused by enzymatic reactions is the reason for the change in tea aroma during withering. Thus, the increase in 8-hydroxylinalool during withering is the result of enhanced biosynthesis caused by the combination of increased synthetic gene expression and increased precursor linalool content. In addition to the hydrolysis of glycosides during fermentation, the *de novo* synthesis of tea aroma compounds during withering has a great impact on black tea aroma.

4. Conclusion

In the present study, the formation of 8-hydroxylinalool in *C. sinensis* var. *assamica* 'Hainan dayezhong' was studied in detail. Linalool and its

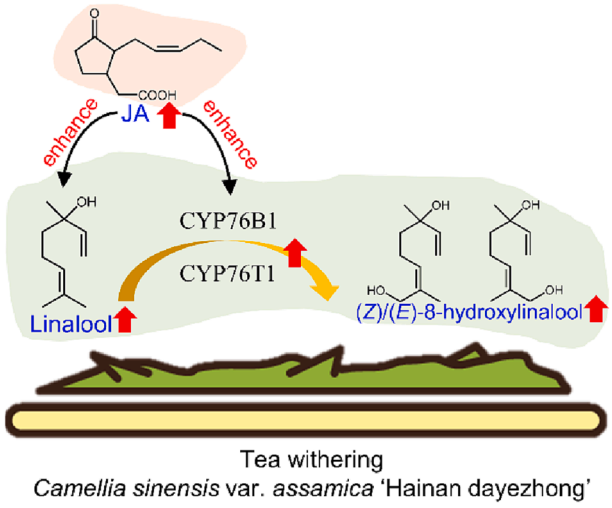

Fig. 6. Accumulated linalool and enhanced synthetic gene expression level contributed to the accumulation of (*Z*)/(*E*)-8-hydrolinalool during black tea withering stage.

derivatives were the major terpenoids in the tender leaves of 'Hainan dayezhong', and 8-hydroxylinalool was one of the main linalool derivatives. Both (*Z*)-8-hydroxylinalool and (*E*)-8-hydroxylinalool were detected with seasonal variation. Among different tissues, the buds contained the highest level of 8-hydroxylinalool. The 8-hydroxylinalool synthase genes *CsCYP73B1* and *CsCYP76T1* in tea plants were identified. *De novo* biosynthesis of aroma compounds during withering contributed to black tea aroma quality. The increased synthetic gene expression level and accumulated precursor linalool content, both of which were induced by JA, enhanced *de novo* 8-hydroxylinalool biosynthesis and stimulated its accumulation during the withering stage of the black tea manufacturing process (Fig. 6).

CRediT authorship contribution statement

Ying Zhou: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Wei He:** Methodology, Investigation. **Yunchuan He:** Investigation. **Qiulin Chen:** Investigation. **Yang Gao:** Investigation. **Jiamei Geng:** Investigation. **Zeng-Rong Zhu:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2023.100173.

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